Naturally Occurring Peptides
Recognized by Cytotoxic T Lymphocytes (CTL):
Peptide Abundance as a Determinant of CTL Function

by
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in partial fulfillment of the requirements for
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ABSTRACT

Cytotoxic T lymphocytes (CTL) have attracted much interest because of their involvement in cellular immune reactions against a variety of targets, most notably virus-infected cells, allogeneic cells, and syngeneic tumor cells. By means of clonally distributed receptors (TcR), CTL scan the surfaces of other cells for specific antigens made up of peptides (=8-25 amino acids) and polymorphic membrane proteins encoded by the major histocompatibility complex (MHC). These antigens arise by endogenous processing pathways or by addition of synthetic peptides to intact cells.

In the past 10 years, the central importance of peptides in T cell recognition has been documented by functional studies, structural determination of peptide-MHC complexes, and biochemical characterization of endogenous peptides. In addition to the chemical identity of a peptide, the abundance of specific peptide-MHC complexes on target cell surfaces (“ligand density”) is a critical factor in regulating T cell responses. To investigate the role of ligand density, naturally processed peptides appearing on relevant target cells must be known. Purification, sequencing, and quantitation of natural peptides recognized by CD8+ T cells in three systems has been undertaken: HIV-infected cells lysed by specific CTL from HIV-seropositive donors; allogeneic cells recognized by a mouse CTL clone; and melanoma cells lysed by human tumor infiltrating lymphocytes. In addition, the density of peptide-MHC complexes obtained with synthetic peptides is explored, and the minimal numbers of complexes required for CTL activation are estimated.

Among the conclusions drawn: (1) ligand densities with synthetic peptides vary by several orders of magnitude (<10 to >10,000 per cell), and densities of natural ligands also vary widely; (2) low ligand densities may limit the lysis of HIV-infected target cells by CTL; (3) "self" peptide-MHC antigens can escape immune recognition primarily because of low abundance; (4) most peptide sequences recognized by anti-melanoma CTL are not mutated, making tumor rejection an apparent autoimmune response. Better understanding of ligand density as a determinant of T cell activation may come from further measurements and development of a theory linking ligand density, TcR affinity for peptide-MHC complexes, and T cell function.
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Finally, I wish to thank members of the late night MIT housekeeping staff, who were always quick with a word of encouragement and who at times seemed to be the only ones not under duress.
“If you can dream — and not make dreams your master;
   If you can think — and not make thoughts your aim,
       If you can meet with Triumph and Disaster
       And treat those two impostors just the same...”

– Rudyard Kipling
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Addendum: This thesis was defended on November 23, 1994.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>antigen-presenting cell(s)</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte(s)</td>
</tr>
<tr>
<td>DTH</td>
<td>delayed-type hypersensitivity</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin(s)</td>
</tr>
<tr>
<td>$K_a$</td>
<td>equilibrium association constant</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>$\alpha$-KGDH</td>
<td>2-oxoglutarate dehydrogenase</td>
</tr>
<tr>
<td>MHC-I</td>
<td>class I major histocompatibility complex</td>
</tr>
<tr>
<td>MHC-II</td>
<td>class II major histocompatibility complex</td>
</tr>
<tr>
<td>$\beta_2$m</td>
<td>$\beta_2$-microglobulin</td>
</tr>
<tr>
<td>PTH</td>
<td>phenylthiohydantoin</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>SD$_{50}$</td>
<td>peptide concentration that sensitizes target cells for half-maximal lysis</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen processing</td>
</tr>
<tr>
<td>TcR</td>
<td>T-cell receptor(s)</td>
</tr>
<tr>
<td>TEAAc</td>
<td>triethylamine acetate</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TIL</td>
<td>tumor infiltrating lymphocyte(s)</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
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</table>
INTRODUCTION
It is difficult to imagine a more exciting and dynamic field of research than that of antigen presentation during the past several years, spanning the period from the first glimpses of a role for small peptides in T cell recognition to the present rather detailed understanding of at least certain aspects of this process. Longstanding immunological puzzles that seemed intractable as little as ten years ago have now yielded to explanation in a way that threatens to make them appear obvious to a new student reading about immunology or to those of us who have become conditioned. What is the nature of clonally distributed T-cell receptors and their ligands? How do T cells distinguish the normal from the aberrant on a molecular level? What accounts for the absence of overtly self-reactive T cells in a healthy individual? Why did two quite different families of immunological mediators — T cell receptors and immunoglobulins — evolve in vertebrates? Recent progress has either led to answers, at least in outline form, or promises to do so. There are even hopes and efforts to translate some of this knowledge into clinical application in such areas as vaccine design and autoimmunity. The following is a brief chronicle of some of the defining moments as seen by an erstwhile student of chemistry, followed by a synopsis of the unifying themes and organization of this thesis.

First, some essential concepts and definitions of immunology. One traditional classification of immune reactions distinguishes between the humoral, or fluid-borne (i.e., mainly antibodies), and the cell-mediated. Cell-mediated immunity depends upon the activities of various types of leukocytes, including lymphocytes that exhibit exquisite specificity for antigen. In general, antigen-reactive lymphocytes are named "T" or "B" based on their ontogenetic derivation in the thymus or the bursa of Fabricius (the latter found in birds; in mammals, B cells originate in the bone marrow and fetal liver). B cells produce and secrete antibodies and thereby support humoral immunity. T cells, on the
other hand, are responsible for a variety of cell-mediated immune responses including delayed-type hypersensitivity (DTH), lysis of infected target cells, lysis of tumor cells, graft rejection, some forms of autoimmunity, and inflammation. In all cases the stimulus for a T cell response is the interaction of specific, clonotypic receptors on the T cell with antigenic structures on the surface of another cell (termed a target cell or antigen-presenting cell, APC). This is true for newly developing (i.e., thymic) cells, circulating but immunologically naïve T cells, and fully functional T cells.

T cells are further subdivided based on their surface expression of two glycoproteins now known as CD4 and CD8. CD4-CD8- ("double negative") and CD4+CD8+ ("double positive") thymocytes represent immature precursors of functional T cells; certain double positive thymocytes progress to mature forms and exit the thymus once they express only CD4 or CD8 on their surface. Mature CD4+ T cells generally react to stimulation by secreting soluble factors ("lymphokines") that are essential for B cell responses, macrophage activation, and other processes, leading these cells to be functionally classified as "helper" T cells. More recently, two types of helper T cells — Th1 and Th2 — have been delineated based on their patterns of lymphokine secretion and on whether their ultimate effect is enhancement of antibody production (Th2) or DTH and other cell-mediated responses (Th1). Interestingly, the two groups of cells inhibit each other, and for some parasitic infections there is a clear dichotomy as to which response is beneficial.

Mature T cells belonging to the CD8+ subset react to stimulation by lysing target cells that bear the offending antigen; for this reason they are termed cytotoxic T lymphocytes (CTL). The antigen might be "non-self," as in the case of a virus-infected
target cell or a cell transplanted from another individual or species (allograft or xenograft, respectively), but it could also be “self” if it is present on an autochthonous tumor cell or an autoimmune target (e.g. pancreatic β islet cells in type 1 diabetes). The distinguishing feature common to these instances of CD8+ (as well as CD4+) T cell activation is the appearance on target cell (or APC) surfaces of an unusual antigen: either one that is not encoded in the organism’s genome and hence is bona fide non-self, one that is present but not normally expressed, one that is normally expressed but at a different level, or one that appears to be self in all respects yet becomes a target for T cells as a consequence of other events. Thus “normal vs. aberrant” may be a more appropriate descriptor of what T cells distinguish than “self vs. non-self.” Finally, it should be noted that the classification of CD4+ and CD8+ T cells as helper and cytotoxic cells, respectively, is not absolute: a minority of cytotoxic T cells are in fact CD4+, and CD8+ cells can secrete lymphokines that influence other cells.

**Dual nature of the cell surface antigens recognized by T cells**

As noted above, the ligands for T cells uniformly reside on the surfaces of other cells, clearly distinguishing the universe of T-cell antigens from that of B-cell antigens. B cells, via immunoglobulins (Ig) embedded in their surface membrane (and later secreted as soluble antibodies), collectively respond to virtually any chemical structure: protein, carbohydrate, nucleic acid, lipid, as well as organic molecules that do not occur naturally. These antigens may be on cell surfaces or free in solution, and their recognition is defined entirely by the molecule’s three-dimensional conformation (reviewed by Davies et al., 1990). T cells respond only to proteinaceous cell surface antigens, which furthermore must consist of a noncovalent complex between a small
peptide (see below) and an integral membrane protein encoded by the major histocompatibility complex (MHC).

The MHC is a genetic region originally implicated in studies of tumor transplantation: the rejection of tumors transplanted from one mouse to another was due to "major histocompatibility antigens" present on all cells, both normal and neoplastic, and not to unique tumor antigens (Gorer, 1937; Snell, 1948; reviewed by Klein, 1975). However, the relationship between this histocompatibility role and an immune function for the MHC did not become clear until the early 1970s, when several studies pointed to a requirement for shared MHC specificities between T cells and B cells (or macrophages) in order to elicit helper T cell function (Kindred and Schreffler, 1972; Katz et al., 1973; Rosenthal and Shevach, 1973). Then, in a seminal publication, Zinkernagel and Doherty (1974a) showed unequivocally that virus-specific CTL responses also require shared MHC specificities between the T cell and the infected target cell, a phenomenon known as "MHC restriction." Shearer (1974) and Bevan (1975) independently made analogous observations using CTL specific for trinitrophenyl-modified cell surface proteins or so-called minor histocompatibility antigens, respectively. (Alloresponses, in contrast, involve T cell recognition of unshared MHC products on other cells, as discussed in chapter 3).

Molecular genetic studies of the MHC (also called H-2 in the mouse, HLA in the human) revealed a few dozen highly polymorphic and tightly linked genes clustering into various regions. Genes in the H-2K, D and L regions (mouse) and the HLA-A, -B and -C regions (human) code for ca. 45 kDa membrane-spanning polypeptides that, together with a non-polymorphic, non-MHC-encoded, 12 kDa product termed \( \beta_2 \)-microglobulin (\( \beta_2m \)), make up class I MHC (MHC-I) proteins. Genes in the I-A and I-E regions
(mouse) and the HLA-DR, -DP and -DQ regions (human) code for both α (≈34 kDa) and β (≈28 kDa) polypeptides that noncovalently associate to form heterodimeric class II MHC (MHC-II) proteins. Nearly all cells express MHC-I proteins, albeit at different levels, but MHC-II expression is limited to certain cell types commonly referred to as APC (macrophages, dendritic cells, B cells, and a few others). The significance of the high degree of polymorphism (more than any other known genes) is that each individual within an outbred population is likely to possess different alleles and hence a characteristic set of MHC molecules on the surface of its cells; because MHC expression is codominant, a human or mouse has up to six different MHC-I products (three genetic loci from each parent, constituting a haplotype) and six MHC-II products. Inbred strains of mice have fewer, since they are homozygous at the MHC and therefore express only a single haplotype (H-2^b, H-2^d, etc.).

MHC restriction of CD4^+ T cells has been found to map to MHC-II genes, as a rule, and that of CD8^+ T cells to MHC-I genes. Subsequently, it has been shown that CD4 and CD8 can bind to non-polymorphic domains of MHC-II and MHC-I molecules, respectively, and that CD4 and CD8 are likely to have roles in T cell maturation and activation (reviewed by Janeway, 1992); however, there are also instances in which cloned T cells seem to function independently of CD4 or CD8 (e.g. Cai and Sprent, 1994). As a consequence of this general partitioning between MHC-I and MHC-II, CD4^+ T cells react only with APC, while CD8^+ T cells potentially react with any target cell type (see table 1, p. 35).
Nature of antigen-specific T-cell receptors

Following the discovery of MHC restriction, there ensued a controversy as to whether two distinct receptors on a given T cell were required (one for self MHC, one for the “non-self” component), or whether a single receptor somehow accommodated both entities (vaguely portrayed as “altered self,” in the absence of any real structural model). Much work proved the single receptor hypothesis to be correct, including experiments by Kappler, Marrack and colleagues (1981) in which T cells of different specificity and MHC restriction were fused and the resulting hybridomas recognized only the parental pairings.

The advent of monoclonal antibody technology and of methods for culturing T cells enabled the production of antibodies to specific T-cell receptors (TcR) (Allison et al., 1982; Meuer et al., 1983; Haskins et al., 1983; Kranz et al., 1984a), which quickly led to their molecular characterization. In each case the TcR was a disulfide-linked heterodimer (≈90 kDa). Perhaps surprisingly, the component α and β chains (and later, γ and δ chains for another heterodimeric TcR) displayed many of the properties of Ig molecules: sequence similarities sufficient to lead to the prediction of an Ig-like domain structure for the TcR, and regions of hypervariability that might account for antigen specificity (Novotny et al., 1986; Chothia et al., 1988; Davis and Bjorkman, 1988). The isolation of a large number of cDNA clones for α and β subunits confirmed these features, rooted in a similar overall genetic organization of TcR genes (reviewed by Davis, 1990) and Ig genes (Tonegawa, 1983) (see also Eisen et al., In press; appendix, p. 317). These apparent similarities belie the striking contrast between the nature of the ligands for TcR and for Ig; a more complete understanding of the molecular basis for this contrast will probably have to await the determination of a complete TcR structure by x-ray crystallography.
**MHC-I-restricted antigen presentation to CD8+ CTL**

Viral infections generally elicit both a strong antibody response and a vigorous CTL response, leading in many instances to neutralization of free virions and lysis of virus-infected cells. Yet the past decade has been a powerful reminder that these mechanisms do not always eliminate all viruses. Partly because the reasons for escape from antibody recognition are relatively well-explored, and partly out of disenchantment following failures in antibody-directed approaches against both tumors and HIV, there has been a resurgence of interest in understanding and eliciting CTL responses. CTL have long been said to offer a potentially superior means of protection against viruses because they target the sites of viral replication. Moreover, unlike antibodies, CTL are largely specific for conserved, intracellular viral proteins. For example, influenza virus-specific CTL from both mice (Zweerink et al., 1977) and humans (McMichael and Askonas, 1978) cross-react with target cells infected by different influenza subtypes, because a majority of these CTL recognize the conserved nucleoprotein and matrix protein (Townsend and McMichael, 1985; Yewdell et al., 1985) rather than the highly mutable surface glycoproteins recognized by antibodies (Wiley et al., 1981; Wilson and Cox, 1990). Despite this, efforts to detect conserved proteins like influenza virus nucleoprotein at the cell surface, where they should be accessible to T cells, were unsuccessful.

Further pursuit of this conundrum led Townsend and colleagues (1986) to a conceptual breakthrough when they demonstrated that incubation of uninfected target cells with synthetic peptides mimicking parts of the nucleoprotein rendered the cells susceptible to lysis by virus-specific CD8+ CTL. Incubation with whole nucleoprotein was
ineffective, and only certain peptides from the nucleoprotein sequence were active. It has since become clear that the natural ligand for a TcR (of a CD8+ T cell) is a cell surface complex consisting of a short peptide and an MHC-I protein (peptide-MHC-I); that under normal conditions the peptide is produced intracellularly by limited proteolysis ("processing") of newly synthesized ("endogenous") viral proteins and is transported into a compartment where it can bind to a newly formed MHC-I protein; and that this physiological pathway can be circumvented by adding to whole cells a synthetic peptide that binds directly to a population of cell surface MHC-I molecules that are free of endogenous peptides.

In retrospect, Peter Doherty probably spoke for many when he remarked: "I felt pretty stupid that I hadn't thought of the peptide, but that's the way science is." (J. NIH Res., 1991, 3:72). As it turns out, Townsend's experiments worked even though he was not using the "correct" peptides, in the sense that they did not precisely mimic those made within cells and therefore those which had stimulated the production of his virus-specific CTL. But by the time that was clear, no one cared much, for a new period of progress in understanding T-cell ligands was in full bloom.

At around the same time, studies of antigen recognition by MHC-II-restricted CD4+ T cells had culminated in the measurement of direct binding between synthetic peptides (from ovalbumin or lysozyme) and purified MHC-II molecules (Babbitt et al., 1985; Buus et al., 1986). Evidence that proteins required "processing" (e.g., denaturation) in order to serve as effective antigens for CD4+ T cells had been obtained earlier (Ziegler and Unanue, 1982; Shimonkevitz et al., 1983), but proof of a direct interaction between antigen and the restricting MHC-II (or Ia) molecule had been a
missing link of sorts. This, together with the identification of a single antigen-specific receptor on T cells, finally made possible a molecular interpretation of previous work on so-called immune response (Ir) genes encoded by the MHC (Levine et al., 1963; McDevitt and Sela, 1965). In short, proteins had to be fragmented before they could interact with MHC-II molecules to create antigenic determinants recognized by T cells; failure of a protein to undergo fragmentation into peptides capable of binding to the available MHC-II molecules could account for MHC-linked immune unresponsiveness to that protein (Schwartz, 1985; Unanue and Allen, 1987).

This sudden progress toward understanding antigen recognition by CD4+ T cells did not immediately impact the field of CD8+ T cells, perhaps because these two types of responses still seemed so qualitatively different. CD4+ T cells were known to interact only with MHC-II+ macrophages and other APC, which internalize extracellular ("exogenous") antigens (such as ovalbumin and lysozyme), process them in some undefined way in a low pH endosomal compartment, and then present them at the APC surface. CD8+ T cells, on the other hand, respond to a completely different set of antigens: those synthesized within a cell (endogenous), as exemplified by virus-encoded products. This was convincingly shown by Morrison et al. (1986) using MHC-I- and MHC-II-restricted influenza virus-specific CTL. But by this time, with publication of the first results implicating peptides in both MHC-I- and MHC-II-restricted recognition, as well as data indicating that the variable regions of receptors on MHC-I- and MHC-II-restricted T cells are encoded by the same pool of genes (Kronenberg et al., 1986), the commonalities between CD4+ and CD8+ T cells in terms of antigen recognition were becoming apparent.
The view from above

Then a landmark publication added a new dimension to the field and offered tantalizing clues of things to come: the first structural determination of an MHC protein, the human MHC-I molecule HLA-A2, by x-ray crystallography (Bjorkman et al., 1987a). The structure consisted of four domains of \( \approx 90 \) amino acids, three derived from the \( \alpha \) chain (\( \alpha_1, \alpha_2, \) and \( \alpha_3 \)) and one comprising \( \beta_2m \). While the membrane proximal \( \alpha_3 \) and \( \beta_2m \) resembled Ig domains, \( \alpha_1 \) and \( \alpha_2 \) paired to form a novel structure: two long \( \alpha \)-helices overlying an 8-stranded antiparallel \( \beta \)-sheet, with an exposed "groove" between the two \( \alpha \)-helices. This groove was proposed to be a binding site for peptides (Bjorkman et al., 1987b), based on (1) earlier sequence and functional data, (2) the overall dimensions of the groove (\( \approx 25 \times 10 \times 11 \, \text{Å} \), enough to accommodate a peptide of between 8 and 20 amino acids), and (3) an additional unexpected and dramatic finding: between the two \( \alpha \)-helices was a continuous region of electron density not attributable to the HLA-A2 sequence and believed to represent one or more unknown peptides that had co-crystallized with the protein. A second crystal structure also solved by Wiley and colleagues (Garrett et al., 1989), that of HLA-Aw68, displayed comparable architectural features and similarly harbored unassignable electron density in the proposed binding site, but this time the electron density was distinguishable from that seen with HLA-A2, suggesting a different composition of putative peptide occupant(s).

Once the structure of an MHC-I molecule strongly suggested a capacity for peptide binding, and synthetic peptides had been shown to sensitize target cells for lysis by MHC-I-restricted CTL, several groups attempted to demonstrate peptide binding by purified, soluble MHC-I molecules. All essentially failed (e.g. Chen and Parham, 1989;
Tsomides and Eisen, 1990). Data from assays based on solid-phase immobilization of peptides (Bouillot et al., 1989; Chen et al., 1990; Frelinger et al., 1990) or MHC-I molecules (Kane et al., 1989a) were obtained, but their interpretation was uncertain. In light of $K_a$ values of $\approx 10^6 \text{ M}^{-1}$ that had been measured for peptide binding to purified MHC-II molecules by conventional equilibrium dialysis and gel filtration assays, this discrepancy was puzzling at first.

Eventually, two clear explanations emerged. First, the synthetic peptides used in binding assays were usually too long (e.g. a 15-mer in Chen and Parham, 1989), because they were selected on the basis of cytotoxicity assays. In these assays the peptides added to target cells need not match precisely the natural ligands they mimic; they can be substantially longer, for example, and then undergo trimming by proteases in the assay medium or on cell surfaces to reach the optimal length for binding to an MHC-I protein. However, at this time there was no precise information about the lengths or sequences of naturally processed peptides in peptide-MHC complexes. Second, the preparations of affinity-purified HLA molecules used for \textit{in vitro} binding assays did not have sufficient sites available for peptide binding because these sites were already occluded by endogenous peptides. Of course, this is exactly what the crystal structures had intimated, but a fuller understanding of why peptides bind to cell surface MHC-I molecules in cytotoxicity assays and not to isolated MHC-I proteins was not achieved until Ploegh and colleagues showed that stable MHC-I molecules require peptide as an integral partner, and that peptides added to whole cells bind to a minority population of functionally “empty” MHC-I molecules that are unstable at 37°C in the absence of added peptide (Ljunggren et al., 1990; Schumacher et al., 1990).
So many peptides, so few MHC...

A major dilemma – one which had been a cause for skepticism in accepting the likelihood of a physical interaction between antigen and MHC molecule – still remained unsolved. How could the small number of distinct MHC products in a given individual possibly bind in a specific manner to the many thousands of different antigens that must be presented to T cells? The tremendous variability of antibody and TcR sequences enabled these molecules to cope with a complex antigenic universe, but MHC molecules clearly had to be using a different strategy.

Already some clues were available, notably from the crystal structures (Bjorkman et al., 1987a; Garrett et al., 1989), from T-cell assays showing that MHC proteins vary in their abilities to present specific peptides (e.g. Carreno et al., 1990), and from a study by Jardetzky et al. (1990) in which only one or a few peptide side chains appeared to be important for binding to an MHC-II molecule. Efforts had been made to discern common features among the peptides recognized by MHC-restricted T cells (Rothbard and Taylor, 1988; Margalit et al., 1987; Stille et al., 1987; Claverie et al., 1988), but in retrospect the conclusions drawn were quite limited. Gradually, from the accrual of data obtained using large numbers of synthetic peptides and T cells, or perhaps from a few more crystal structures, a complete picture of the peptide–MHC-I interaction would have been pieced together. However, Rammensee and colleagues found a remarkable shortcut.

Having shown that tightly bound endogenous peptides could be separated from MHC-I molecules by treatment with trifluoroacetic acid (TFA) (Rötzschke et al., 1990a), much in the same way that Buus et al. (1988) had shown for MHC-II molecules using
acetic acid, Falk et al. (1991a) eluted peptides from isolated MHC-I molecules by treatment with TFA and performed Edman degradation on the resulting complex mixture ("pool sequencing"). While each amino acid residue was found at virtually every cycle during the Edman procedure, several key facts about naturally processed peptides emerged from this study: (1) for a given MHC-I protein (such as HLA-A2), the eluted peptides contain a predominance of one or two amino acids at certain key "anchor" positions (e.g. leucine or methionine at position two, and valine or leucine at position nine); (2) amino acid yields dropped precipitously after 9 cycles, suggesting that most bound peptides are nonamers (octamers for certain MHC-I molecules); and (3) the "motifs" characterizing peptide length and anchor positions are distinctive from one MHC-I protein to another.

The definition of motifs provided an appealing model to explain how a single MHC-I molecule can bind to a highly degenerate yet restricted set of peptides: a great number of peptide sequences of the proper length can bind to a given MHC-I molecule, provided that certain positions have the correct anchor residues, and different MHC-I molecules have distinctive requirements for these peptide anchor positions. (A sampling of motifs is given in the table on p. 233). Even with two positions fixed within a nonameric peptide, a total of over $10^9$ sequences can still satisfy the motif for a particular MHC-I protein (although not all of these peptides will bind to the MHC-I protein).

This biochemical discovery of shared properties among endogenous MHC-I-bound peptides fit very nicely with emerging crystallographic data from the laboratories of Wiley and Wilson. Peptide-MHC-I complexes containing just a single peptide rather than a complex mixture were obtained and crystallized, revealing important structural
similarities and differences among peptides binding to the same MHC-I molecule (Fremont et al., 1992; Zhang et al., 1992; Silver et al., 1992; Madden et al., 1993). “Pockets” within the MHC-I binding site were shown to accommodate peptide side chains at the positions described as anchors (Garrett et al., 1989; Matsumura et al., 1992a; Young et al., 1994). Peptides longer than 9 residues could fit by “bulging out” in the middle, with both peptide termini substantially buried in the binding site (Guo et al., 1992). Soon, the dual requirements for specificity and permissiveness in peptide–MHC-I interactions were reconciled by a new paradigm for immunologic recognition, and now it is difficult to imagine that it could be otherwise.

These motifs have also been useful for the rapid identification of candidate sequences recognized by T cells when the protein of origin is known (Rötzschke et al., 1991a; Pamer et al., 1991). However, this approach is not infallible, for several reasons: (1) some MHC-binding peptides do not conform to the expected motif and therefore will be missed (e.g. Udaka et al., 1992; Corr et al., 1992); (2) synthetic peptides that are found to be active need not always correspond to naturally processed peptides, even when optimized for activity, since heteroclitic reactions can occur for TcR (e.g. Bodmer et al., 1988) as well as for antibodies (Day, 1990, chapter 12); and (3) nonphysiological cross-reactions can be observed when target cells are sensitized with high doses of synthetic peptides, due to artificially high peptide–MHC densities (see below). Despite these caveats, several individual naturally processed peptides recognized by T cells have turned out to match precisely the peptides predicted from motifs (van Bleek and Nathenson, 1990; Rötzschke et al., 1990b; Tsomides et al., 1994), leading to a common assumption that this will often be the case.
Corresponding findings of peptide motifs and pockets within MHC peptide-binding sites were later made for MHC-II molecules, although in this case the situation proved more complex. Naturally processed peptides binding to MHC-II molecules include sets of nested peptides sharing a core sequence but having different N- and C-termini (Demotz et al., 1989; Chicz et al., 1992; Nelson et al., 1992). These peptides tend to be longer than those eluted from MHC-I proteins, ranging from about 12 to 25 residues (Rudensky et al., 1991a; Hunt et al., 1992b). Again, this information correlated nicely with results from crystallography. The structure of an MHC-II molecule complexed with endogenous peptides (Brown et al., 1993) or with a single viral peptide (Stern et al., 1994) revealed two major differences from MHC-I: (1) the MHC-II peptide-binding groove allows bound peptides to extend out at both ends, rather than having their termini tucked into the binding site, thereby explaining the length heterogeneity among naturally occurring MHC-II-associated peptides; and (2) many of the peptide–MHC-II contacts involve the peptide backbone rather than specific peptide side chains, implying different mechanisms for degenerate peptide binding by MHC-I and MHC-II proteins (Stern and Wiley, 1994; see p. 35 and 36).

Origin of the peptide•MHC-I complexes recognized by CD8+ T cells

The generation of endogenous peptide•MHC-I complexes by intracellular events collectively termed antigen processing is not considered in detail here (reviewed by Monaco, 1992; Yewdell and Bennink, 1992; Germain and Margulies, 1993; Heemels and Ploegh, 1995). In essence, CD8+ T cells react with peptide•MHC-I complexes that form within a target cell’s endoplasmic reticulum (ER) as newly synthesized MHC-I molecules (α chains and β2m) assemble. The peptides to be loaded onto MHC-I molecules are
generated by limited proteolysis in the cytosol and translocated into the ER by an ATP-dependent peptide transporter made up of MHC-encoded subunits named TAP-1 and TAP-2 (Spies and DeMars, 1991; Powis et al., 1991; Attaya et al., 1992), or in some cases via TAP-independent pathways (Anderson et al., 1991; Henderson et al., 1992; Zweerink et al., 1993; Hammond et al., 1993; Zhou et al., 1993). Once inside the ER, peptides may or may not be subject to further proteolysis (Falk et al., 1990) before binding to nascent MHC-I molecules which are then exported to the cell surface as mature peptide-MHC-I complexes.

Given several thousand intracellular proteins potentially available for degradation in any given nucleated cell, and many-fold higher numbers of peptides therefore theoretically available for transport into the ER and subsequent binding to MHC-I molecules, it is apparent that competition among peptides must be a significant feature of antigen presentation. It may be that only the most tightly-binding peptides compete effectively for MHC-I binding sites, explaining the slow dissociation rates implied by endogenous peptide persistence throughout the purification and crystallization of MHC-I molecules. Certainly peptide selectivity exists at the levels of MHC-I binding (Falk et al., 1991a; Schumacher et al., 1991) and TAP-mediated translocation into the ER (Shepherd et al., 1993; Neefjes et al., 1993; Androlewicz et al., 1993), and perhaps also at the level of proteolysis (Goldberg and Rock, 1992; Driscoll et al., 1993; Gaczynska et al., 1993), but much remains to be clarified about the generation of MHC-I-binding peptides in vivo.

What is clear is that the peptide-MHC-I complexes ultimately arriving at a cell’s surface represent a sampling of that cell’s contents, with some peptides present at relatively high copy numbers, e.g. several hundred or more identical peptide-MHC-I
complexes per cell (van Bleek and Nathenson, 1990; Falk et al., 1991b; Udaka et al., 1992, 1993), and many more relatively scarce, e.g. between 1 and 100 complexes per cell (Hunt et al., 1992a). Most natural MHC-I-binding peptides arise from normal self proteins and as such are not recognized efficiently by mature T cells that have been negatively selected for self-reactivity in the thymus. However, the same complexes can trigger reactions from T cells of an MHC-different individual (alloreactions). For a peptide from a foreign source such as a virus or an aberrantly-expressed protein as may occur in a transformed cell, specific CD8+ T cells are expected to respond to the peptide-MHC-I complex provided its cell surface density is sufficient and there is a TcR that binds to the complex with favorable affinity and kinetics (see below).

Assays for exogenous peptide binding to MHC-I molecules

A variety of binding assays for the peptide–MHC-I reaction have been established using either intact cells (Christinck et al., 1991; Benjamin et al., 1991; Luescher et al., 1991; Tsomides et al., 1991), purified MHC-I molecules that appear to be free of endogenous peptides when expressed in Drosophila cells (Matsumura et al., 1992b; Saito et al., 1993) or other transfected cells (Boyd et al., 1992; Ojcius et al., 1993; Fahnestock et al., 1994), or other approaches (Cerundolo et al., 1991; Silver et al., 1991; Parker et al., 1992a, 1994; Ruppert et al., 1993; Khilko et al., 1993; Olsen et al., 1994; Sette et al., 1994b). Each of these experimental systems suffers from certain limitations, a common one being that the binding of synthetic peptides to fully formed MHC-I molecules may not accurately mirror events in the ER, where this reaction ordinarily takes place. Nevertheless, the measured $K_a$ values and other data seem to reflect the specificities otherwise observed in peptide–MHC-I reactions, and some of the binding assays have
proved useful in predicting potential peptide immunogenicities (Feltkamp et al., 1993; Celis et al., 1994).

A further reason for measuring equilibrium binding constants for peptide–MHC reactions is to calculate by means of the Karush equation (Karush, 1970) the number of peptide–MHC complexes per target cell required to trigger the activity of a given T-cell clone. Specifically:

\[ r = \frac{K_a \cdot c \cdot n}{(1 + K_a \cdot c)} \]

where \( r \) is the number of peptide–MHC complexes per target cell at a free peptide concentration \( c \) that sensitizes target cells for lysis by a given T cell, \( K_a \) is the equilibrium association constant for the peptide–MHC reaction, and \( n \) is the number of MHC binding sites per target cell accessible to extracellular peptide. Because the peptide–MHC reaction never reaches true equilibrium, and because the peptide is subject to proteolytic degradation during whole cell binding assays, the minimum value of \( r \) that can be calculated using this equation (using for \( c \) the peptide concentration that leads to half-maximal lysis) represents an upper limit to the number of peptide–MHC complexes required for T cell activation. As discussed by Kageyama et al. (1995; see p. 62), and in contrast to earlier studies reporting values of a few hundred (Harding and Unanue, 1990; Demotz et al., 1990; Vitiello et al., 1990; Christinck et al., 1991), the minimum number of complexes was recently found to vary over several orders of magnitude depending on the particular T cell, and could be as low as ten or fewer in optimal combinations.
What determines the efficacy of CTL-mediated target cell lysis?

From the vastly improved resolution in our view of the antigenic ligand for T cells, can we derive any understanding of what is most important in influencing the strength of a T cell response? For CD8+ CTL, the response is lysis of target cells bearing suitable peptide-MHC-I complexes. At least the following factors may be critical to the outcome: (1) affinity of the TcR for its ligand; (2) off-rate of the TcR–peptide-MHC reaction (it is assumed that the off-rate of the peptide–MHC reaction is not limiting for a T cell response, although there are some interesting reports to the contrary (Fairchild et al., 1993; Nelson et al., 1994); it is also assumed that TcR on-rates are not limiting in physiological responses); (3) number or density of peptide-MHC complexes on the target cell; (4) number or density of TcR on the T cell (possibly more important during T cell development than for mature T cells); (5) coreceptor and accessory molecule interactions between the T cell and its target (reviewed by Springer, 1990); and (6) lytic pathways of the CTL (e.g. Eisen, 1990, chapter 19).

T-cell receptors do not exhibit the somatic hypermutation that underlies the generation of high-affinity antibodies. It was therefore suggested early on (Eisen, 1986) that high affinities are unlikely for TcR, and moreover that while high affinities may be useful for antibodies because they allow lower concentrations of antibody to accomplish a particular amount of binding, the affinities of membrane-associated T-cell receptors need only surpass a threshold value required to activate cell function, beyond which higher values might increase the risk of deleterious cross-reactions. The first reported measurements of TcR affinities in the $K_a$ range of $=10^4$-$10^5$ M$^{-1}$ (Matsui et al., 1991; Weber et al., 1992) appeared to reinforce that view. However, further measurements
have uncovered much higher values, up to $=1.5 \times 10^7$ (6$\times$10$^6$ at 37°C) (Sykulev et al., 1994; see p. 145), prompting a reevaluation (Eisen et al., In press; see appendix, p. 317). It now seems that TcR intrinsic affinities for their natural peptide-MHC ligands will vary widely (e.g. $=10^3$-10$^7$ M$^{-1}$), although probably not up to the very high values exhibited by some antibodies for antigens (10$^8$-10$^{11}$ M$^{-1}$). Thus it becomes manifestly relevant to evaluate the functional importance of differences in TcR affinity in vitro and in vivo.

It is often assumed, implicitly or explicitly, that “high-affinity T cells” (i.e., high affinity of the TcR for its peptide-MHC ligand) are better effector cells than “low-affinity T cells.” Is this really true? Very little data are available so far to address the question. For example, it would be of interest to determine whether there is an inverse correlation between the density of peptide-MHC ligands on the surfaces of target cells and the required affinity of a CTL’s TcR in order to effect lysis of the cells. In a recent study (Sykulev et al., 1994), ligand density was manipulated by using related synthetic peptides to sensitize target cells for lysis by a clone of CTL, and the intrinsic affinity of the CTL’s TcR against each peptide-MHC-I ligand was measured; the result was a general correlation between TcR affinity and the peptide concentrations required to sensitize target cells for half-maximal lysis (SD$\_50$). However, the use of SD$\_50$ as a marker for ligand density is limited because of variations in peptide-MHC-I binding affinities (e.g. Kageyama et al., 1995). In particular, a synthetic peptide with relatively low affinity for a restricting MHC-I molecule may need to be added to target cells at a significantly higher concentration (SD$\_50$ value) in order to match the ligand density of a different peptide, regardless of the ability of a CTL’s TcR to recognize each peptide-MHC complex. That TcR affinity alone is not a sufficient predictor of T cell function is also apparent from a comparison of two established CTL clones: although their TcR differ $=10$-fold in affinity
for the respective peptide-MHC ligands, the two CTL are equally effective in lysing target cells pulsed with synthetic peptide (i.e., same SD$_{50}$ values) (Sykulev et al., 1994; see p. 145).

Density of the ligand for antigen-specific receptors on T cells

The principal factor in determining T cell efficacy to be considered in what follows is the cell surface density of peptide-MHC-I complexes. Ligand density undoubtedly influences the probability of target cell lysis by specific CTL, as is commonly demonstrated by titration curves with synthetic peptides in cytotoxicity assays. The role of ligand density in CTL reactions when using synthetic peptides has been explored (e.g. Alexander et al., 1991; Christinck et al., 1991). Whether the densities of endogenous peptide-MHC-I complexes are often limiting in vivo is less clear, and is difficult to investigate. In one study, HIV-infected human target cells were lysed very poorly in vitro by HIV-specific CTL, even though these CTL were capable of lysing uninfected target cells pulsed with as little as 1 pg/ml of a synthetic HIV peptide (Tsomides et al., 1991, 1994). Isolation of the relevant endogenous HIV peptide from infected cells revealed a maximum of only $\approx 12$ peptide-MHC-I complexes per cell, suggesting that low peptide abundance might be responsible for poor killing of infected cells – even by what would have to be considered a highly efficient clone of CTL.

In the second example, a peptide recognized by an alloreactive (H-2$^b$ anti-H-2$^d$) mouse T-cell clone was found: (1) to be indigenous in H-2$^b$ mice (“self”) (Udaka et al., 1992; see p. 121); (2) to bind a self MHC-I product (K$^b$) almost as well as the allogeneic MHC-I product (L$^d$) (Kageyama et al., 1995; see p. 62); and (3) to elicit cytolytic activity
from the same alloreactive T-cell clone in the context of either L$^d$ or K$^b$, but with a marked difference in the peptide concentrations required (Dutz et al., 1994; see p. 136). Since H-2$^b$ mice (as well as H-2$^b$ mice transgenic for the TcR of this alloreactive clone (Sha et al., 1988a)) show no signs of autoreactivity, these studies indicate that lack of recognition by specific T cells of a self peptide-MHC-I complex \textit{in vivo} is due to its low cell surface density, not to its absence or to the absence of potentially autoreactive T cells; as a corollary, increased expression of the complex might well trigger an autoimmune response (Dutz et al., 1994).

It is worth noting that for reasons of convenience and economy, most studies of T cell specificity have relied on the use of synthetic peptides. Because peptides can bind to functionally empty MHC-I molecules on appropriate target cells, and because many longer peptides undergo degradation during T-cell assays, this approach has been successful in leading to the identification of antigens recognized by T cells specific for various types of targets (infected, tumor, allogeneic, xenogeneic, etc.). Often, very high concentrations of a synthetic peptide are used, e.g. 10-100 μM. At such concentrations, peptides with even modest affinities for the restricting MHC-I molecules (K$_a$ values of 10$^6$-10$^8$ M$^{-1}$) will occupy over 90% of available MHC-I binding sites at equilibrium, resulting in orders of magnitude more specific peptide-MHC-I complexes than the few hundred per cell that have been measured for several naturally processed peptides. For example, the HIV peptide mentioned above will form up to 10,000 peptide-MHC-I complexes per target cell when added at micromolar concentrations, far higher than its natural density; other synthetic HIV peptides can readily sensitize target cells for lysis in T-cell assays even though they are not detectable in infected cells (Tsomides et al., 1994). At artificially high ligand densities, misleading cross-reactions have also been described.
in other viral (Milligan et al., 1990; Speiser et al., 1992) and self (Schild et al., 1990; Dutz et al., 1994) systems. Thus exclusive reliance on synthetic peptides sharply limits any possibility for extrapolation to CTL–target cell interactions as they might occur in vivo.

More to the point here, it is not even possible to begin a study of the physiological role of ligand density until the naturally processed peptides in a given system are known. In practice, this can be a formidable task precisely because of the limited abundances of many naturally processed peptides. The strategies that have been devised for the identification of natural T-cell ligands can be divided into four categories (so far).

The “easiest” case is that of a peptide derived from a protein of known sequence, such as a viral protein. A relatively small number of cells expressing the antigen of interest (ca. 10^8-10^9) are lysed with TFA to extract MHC-bound peptides as originally shown by Rötzschke et al. (1990a), and a peptide fraction from this lysate is subjected to fractionation by HPLC. Individual HPLC fractions are screened in a suitable T-cell assay (i.e., using a target cell expressing the restricting MHC-I protein but not the peptide, and ideally with a high density of peptide-free MHC binding sites). The retention time of each active HPLC fraction is then compared with those of synthetic peptide candidates based, for example, on the motif for natural peptides associated with the restricting MHC-I molecule. The method becomes much more discriminating when several different HPLC conditions (columns, solvents, and gradients) are used to establish a chromatographic profile of the unknown peptide for comparison with all synthetic peptide candidates.

The earliest identifications of naturally processed peptides all used this approach (van Bleek and Nathenson, 1990; Rötzschke et al., 1990b; Rötzschke et al., 1991a;
Pamer et al., 1991; del Val et al., 1991), which exploits the far greater sensitivity of T cells than that of chemical analysis when it comes to peptide detection. Specifically, it is not uncommon to demonstrate T cell activity at a peptide concentration of 1 pg/ml; in an assay volume of 200 µl, this corresponds to the detection of some 200 attomoles of a typical nonameric peptide, several orders of magnitude less than what is required for chemical sequencing. Van Bleek and Nathenson (1990) employed a radiolabeling technique to help establish the sequence of a naturally processed viral peptide, but still relied on chromatographic comparison with synthetic peptides in order to define this peptide. Some of these early results presaged the discovery by pool sequencing that most endogenous MHC-I-binding peptides are nonamers (see table 2, p. 38).

The second strategy permits the identification of a completely unknown T-cell ligand, for instance one expressed on a tumor cell or an allogeneic target cell. In this case, it becomes necessary to isolate enough material for chemical sequence analysis. For a relatively scarce peptide, say 100 copies per cell, this means starting with nearly $10^{12}$ cells in order to isolate just 15 pmoles of peptide for Edman sequencing, assuming 10% overall yield. The first successful application of this method was the sequencing by Udaka et al. (1992, 1993) of two naturally processed peptides recognized by a clone of alloreactive T cells. Using microcapillary HPLC coupled directly to a mass spectrometer for peptide detection and sequencing, Hunt and colleagues (1992a) improved sensitivity by at least one to two orders of magnitude and ultimately determined the sequence of a melanoma peptide recognized by specific T cells from five patients (Cox et al., 1994). An important advantage of mass spectrometry over Edman degradation is the ability to sequence peptides from mixtures. Storkus et al. (1993) found a way to reduce the total number of cells needed: they used a novel, non-cytotoxic elution procedure to remove
MHC-bound peptides from cultured cells, then allowed the cells to recover and repopulate their cell surface peptide-MHC-I complexes.

Each of the first two strategies can be applied either with or without purification of MHC molecules. Extraction of whole cells with TFA is expedient but leads to a much more heterogeneous mixture for HPLC fractionation, of particular concern when the goal is purification of an active peptide to homogeneity. Purification of MHC protein followed by TFA extraction may result in lower yields of peptide, particularly for rapidly dissociating peptide-MHC complexes, but is necessary in order to establish that a given peptide is associated with MHC molecules and is not strictly cytosolic.

A third strategy is the identification of naturally processed peptides based on their abundance and not on their recognition by established T cells. For example, bound peptides are eluted from purified MHC-I molecules by treatment with TFA, the peptides are fractionated by HPLC, and peaks that appear prominent by ultraviolet absorbance are subjected to Edman sequencing. Jardetzky et al. (1991) first sequenced eleven nonameric peptides associated with HLA-B27 by this approach, and several of these matched known sequences of abundant cytosolic or nuclear proteins. By mass spectrometry, Hunt et al. (1992a) sequenced several nonamers associated with HLA-A2, and also discovered in TAP-deficient cells a family of natural peptides that are derived from signal sequences of normal cellular proteins (Henderson et al., 1992); Wei and Cresswell (1992) simultaneously reported similar findings by Edman sequencing. Several groups isolated MHC-II molecules and their associated peptides (Rudensky et al., 1991a; Chicz et al., 1992; Nelson et al., 1992; Hunt et al., 1992b; Riberdy et al., 1992; Kropshofer et al., 1992), and these differ significantly from MHC-I-bound peptides in terms of their
proteins of origin, lengths, and relationships to one another. Some salient features are highlighted in tables 1 and 2 on p. 35 and 38.

A fourth way of identifying natural T-cell ligands is completely independent of their abundance in target cells. Pioneered by Boon and colleagues (De Plaen et al., 1988; Boon et al., 1994), this molecular genetic approach involves transfecting cDNA from a target cell of interest (e.g. a tumor cell) into an antigen^- cell and screening large numbers of transfectants for their ability to stimulate the relevant T cells. Ultimately the gene encoding an antigen recognized by these T cells can be sequenced. This technique has been used to identify the first precursors of T-cell antigens on tumor target cells (van der Bruggen et al., 1991; Traversari et al., 1992; Brichard et al., 1993; Kawakami et al., 1994a, b; Coulie et al., 1994). However, candidate peptides based on the gene sequence must be synthesized and tested for activity, and in order to determine whether an active peptide corresponds to a naturally processed one, and whether it is present on tumor cells at a sufficient density to elicit a T cell response, it must be analyzed as described above. This has been done for a mouse tumor-specific peptide (Wallny et al., 1992a) but not for any of the human tumor antigens so far described (see chapter 4).

Having identified a naturally processed peptide, it is possible to quantitate its level of abundance either directly, if Edman sequencing has been employed, or indirectly by constructing a standard curve for a T-cell assay with the corresponding synthetic peptide. A sample of natural material from a modest number of cells will suffice to provide a signal in the T-cell assay that can be correlated with the standard curve. Then, the greater challenge is to interpret this quantitative information further.
TABLE 1. Comparison between peptide binding to MHC-I and MHC-II molecules.

<table>
<thead>
<tr>
<th></th>
<th>MHC-I</th>
<th>MHC-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distribution</td>
<td>All nucleated cells</td>
<td>Specialized APC</td>
</tr>
<tr>
<td>Domain structure</td>
<td>$\alpha_1$, $\alpha_2$, $\alpha_3 + \beta_2m$</td>
<td>$\alpha_1$, $\alpha_2 + \beta_1$, $\beta_2$</td>
</tr>
<tr>
<td>Accessory molecule</td>
<td>CD8</td>
<td>CD4</td>
</tr>
<tr>
<td>Typical T cell response</td>
<td>Cytolytic activity</td>
<td>B cell help, DTH</td>
</tr>
<tr>
<td>Origin of most bound peptides</td>
<td>Cytosolic (endogenous)</td>
<td>Extracellular/membr. proteins</td>
</tr>
<tr>
<td>Length of bound peptides</td>
<td>Usually 8-9 residues</td>
<td>Variable, =12-25 residues</td>
</tr>
<tr>
<td>Pockets in MHC groove</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Peptide N- and C-termini</td>
<td>Buried in groove</td>
<td>May extend outside groove</td>
</tr>
<tr>
<td>Many critical contacts</td>
<td>Peptide side chains</td>
<td>Peptide backbone</td>
</tr>
<tr>
<td>Equilibrium constants ($K_a$)</td>
<td>$=10^4$-$10^9$ M$^{-1}$ measured</td>
<td>$=10^6$-$10^8$ M$^{-1}$ measured</td>
</tr>
<tr>
<td>Peptide–MHC kinetics</td>
<td>Slow ($\geq$30 min)</td>
<td>Slow ($\geq$30 min); 2 modes</td>
</tr>
</tbody>
</table>


Comparison of MHC-I (HLA-Aw68) and MHC-II (HLA-DR1) structures. Ribbon diagrams show a peptide (blue) binding above the large β-sheet and between the α-helical regions of the MHC protein (green and yellow). Although the domain organizations of HLA-Aw68 and HLA-DR1 differ, their three dimensional structures are very similar.

Cut-away of the molecular surface of the peptide-binding site of HLA-A2, with CPK model of HIV peptide ILKEPVHG, shown in side view.

*Figures from Stern and Wiley (1994) kindly provided by L. Stern.*
Strategies for the identification of naturally occurring peptides associated with MHC molecules. Starting with whole organs, tissues, or cells, two possible pathways consist of preparing crude lysates in TFA (left) or first isolating MHC molecules and then extracting bound peptides with TFA (right). In the first case, ultrafiltrates of whole cell homogenates are fractionated, the fractions are tested for sensitizing activity in a T-cell assay, and each active fraction is subjected to repeated rounds of HPLC until suitable for sequencing by Edman degradation and/or mass spectrometry. In the second case, peptides eluted from MHC molecules can be fractionated and assayed to identify T-cell ligands as above, peptides can be sequenced based on relatively abundance by ultraviolet absorbance, or the crude peptide mixture can be sequenced directly without HPLC fractionation to establish motifs for peptides associated with a particular MHC product.
### TABLE 2. Examples of naturally processed peptides associated with MHC molecules.$$

<table>
<thead>
<tr>
<th>MHC</th>
<th>Peptide origin</th>
<th>Peptide sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-2K$^d$</td>
<td>Influenza NP 147-155</td>
<td>TYQRTRALV</td>
<td>Rötzschke et al., 1990b</td>
</tr>
<tr>
<td>H-2D$^b$</td>
<td>Influenza NP 366-374</td>
<td>ASNENMETM</td>
<td>Rötzschke et al., 1990b</td>
</tr>
<tr>
<td>H-2K$^b$</td>
<td>Vesicular stomatitis virus NP 52-59</td>
<td>RGYVYQGL</td>
<td>Nathenson, 1990</td>
</tr>
<tr>
<td>H-2K$^b$</td>
<td>Ovalbumin 257-264</td>
<td>SIINFEKL</td>
<td>Rötzschke et al., 1991a</td>
</tr>
<tr>
<td>H-2K$^d$</td>
<td><em>L. monocytogenes</em> listeriolysin 91-99</td>
<td>GYKDGYEYI</td>
<td>Pamer et al., 1991</td>
</tr>
<tr>
<td>H-2L$^d$</td>
<td>Mouse α-ketoglutarate dehydrogenase</td>
<td>LSPFPFDL</td>
<td>Udaka et al., 1992</td>
</tr>
<tr>
<td>H-2L$^d$</td>
<td>Mouse α-ketoglutarate dehydrogenase</td>
<td>VAITRIEQLS</td>
<td>Udaka et al., 1993</td>
</tr>
<tr>
<td>HLA-A2</td>
<td>HIV type 1 reverse transcriptase 476-484</td>
<td>ILKEPYNHG</td>
<td>Tsomides et al., 1994</td>
</tr>
<tr>
<td>HLA-A2</td>
<td>HIV type 1 gag 77-85</td>
<td>SLNVTATS</td>
<td>Tsomides et al., 1994</td>
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<tr>
<td>Class II</td>
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<tr>
<td>I-A$^b$</td>
<td>Mouse leukemia virus envelope 145-157</td>
<td>HNEGFYCCPGHPR</td>
<td>Rudensky et al., 1991a</td>
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<tr>
<td></td>
<td></td>
<td>145-158</td>
<td></td>
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<tr>
<td>I-E α chain</td>
<td>56-73</td>
<td>ASFEAQGalANIAVDKA</td>
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<td>Invar. chain</td>
<td>39-53</td>
<td>KPVZQMRMATPLLMR</td>
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</tr>
<tr>
<td>I-E$^b$</td>
<td>Mouse leukemia virus envelope 454-467</td>
<td>SPSVYHYQFERRAK</td>
<td>Rudensky et al., 1991a</td>
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<tr>
<td></td>
<td></td>
<td>454-468</td>
<td></td>
</tr>
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<td></td>
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<td>454-469</td>
<td></td>
</tr>
<tr>
<td>I-A$^k$</td>
<td>Hen lysozyme 48-60</td>
<td>DGSTDYGILQNS</td>
<td>Nelson et al., 1992</td>
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<td></td>
<td></td>
<td>48-61</td>
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<td>52-64</td>
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<td>KMRRMATPLLMQALP</td>
<td>Chicz et al., 1992</td>
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§ adapted from Tsomides and Eisen (1993a).
† only MHC-I-binding natural peptides that are recognized by established T-cell clones are shown; sequences identified on the basis of high abundance are not included.
* for brevity, invariant chain residues 106-117 are represented by an ellipsis (...).
**Thesis organization**

The work summarized in this thesis concerns the naturally processed peptides recognized by established CD8+ CTL in three distinct systems using virus-infected, allogeneic, and tumor target cells. Within each system, a better understanding should ultimately include answers to the following:

- How many different naturally processed peptides are recognized by a single T cell?
- What is the sequence of the active peptide (or peptides)?
- If more than one, what is the chemical relationship among these peptides, and what does that reveal about physiological antigen processing pathways?
- Is the naturally processed peptide also the most active synthetic peptide (if known)?
- What is the abundance of the naturally processed peptide(s)?
- How does the abundance relate to activation of specific T cells?
- What are the roles of TcR affinity and kinetics in determining T cell activation?
- What is the protein of origin for the naturally processed peptides?
- How general are the results for each class of T cells?

Chapter 1 relates several binding assays using synthetic peptides and MHC-I molecules that were either affinity-purified prior to the addition of peptide, affinity-purified after the addition of peptide to cells, or used in situ on intact cells to measure the binding of peptides. By this last approach, it became possible not only to measure equilibrium association constants ($K_a$ values) for the peptide–MHC-I reaction, but also to estimate the number of peptide•MHC-I complexes required to activate various CTL.
To carry out these studies, peptides were radiolabeled using $^3$H or $^{125}$I (or $^{131}$I). Internal substitution with $^3$H does not cause any structural perturbations, so $^3$H was used as a trace label. Much greater specific radioactivities could be achieved with $^{125}$I (or $^{131}$I), but iodine significantly alters peptide structure and function, so its validity as a reporter group was questioned. In particular, conventional iodination procedures typically result in <1% labeling, and labeled molecules were shown here to have strikingly different biological activities than unlabeled molecules; consequently, new procedures were developed to obtain stoichiometrically labeled and therefore chemically homogeneous iodopeptides. This work is described in chapter 1 and in a detailed method included in the appendix (see p. 282).

Chapters 2, 3 and 4 describe three systems in which naturally processed peptides were studied. In chapter 2, the viral peptides generated within HIV-infected target cells and recognized by two A2-restricted CTL clones from HIV-seropositive individuals were identified. The abundances of these peptides in infected target cells were measured, and a potential link between peptide abundances and target cell lysis by CTL was considered. Chapter 3 includes several studies, all centered on the discovery of natural peptide sequences that sensitize target cells for lysis by an alloreactive (H-2$^b$ anti-H-2$^d$) CTL clone. Chapter 4 summarizes efforts to identify the melanoma peptide recognized by a line of A2-restricted tumor infiltrating lymphocytes, and the importance of this information in the context of other recent studies on A2-restricted anti-melanoma T cells.

Relevant publications in which the author was deeply involved are included in each chapter. In addition, each chapter begins with a short overview to connect the different studies and describe continuing work.
CHAPTER 1

Peptide binding to affinity-purified MHC-I molecules and to MHC-I molecules on intact cells
Overview

Peptide-MHC-I complexes on the surfaces of target cells can arise in one of two ways. Normally, antigenic peptides are generated cytosolically and translocated into the ER, where they come into contact with newly synthesized MHC-I proteins (α chain and β2m). Heterotrimeric complexes (α•β2m•peptide) then assemble for constitutive transport to the cell surface. (It has also been suggested that further peptide proteolysis may occur within the ER.) Alternatively, synthetic peptides added to the extracellular medium can bind directly to mature (but presumably peptide-free) MHC-I molecules at the cell surface. The existence of this second pathway originally permitted the discovery that short peptides are recognized by T cells; it is not known to have any relevance to physiological processes, although it could play a role in peptide immunization (Aichele, 1990; Kast, 1991; Zhou, 1992), in the induction of immune tolerance by antigen administration (Khoury et al., 1993), and in the phenomenon of “cross-priming” (whereby mice immunized with cells bearing an antigen on non-self MHC molecules sometimes develop CTL that recognize the antigen with self MHC).

In studying antigen recognition by T cells, it is of interest to understand the densities of peptide-MHC-I complexes achieved by both pathways. To characterize the complexes formed in the normal (endogenous) pathway, peptides furnished by intracellular processing mechanisms must first be identified. Chapters 2, 3 and 4 are concerned with naturally processed peptides and their abundances on virus-infected, allogeneic, and tumor target cells. In this chapter, ligand densities resulting from the sensitization of target cells with synthetic peptides are explored by means of a direct binding assay and the use of uniformly labeled peptides. The study of peptide–MHC binding using exogenous peptides offers experimental accessibility, allows peptides that
are not produced naturally to be investigated, and provides information about the number of peptide-MHC complexes required for T cell activation.

At the outset of these studies, it was not yet clear how synthetic peptides interact with MHC-I products on cells; no in vitro binding assays had yet been established. Using standard methods (equilibrium dialysis, gel filtration) that had recently been applied to peptide-MHC-II binding systems, we attempted to measure the binding of synthetic peptides to MHC-I proteins purified by either papain digestion/ion exchange/gel filtration or detergent solubilization/immunoaffinity chromatography. The human MHC-I protein HLA-A2 (A2) was chosen on the basis of (1) the availability of specific reagents for use in its affinity purification; (2) its known crystal structure; (3) its prevalence in the population (e.g. ≈40-50% of Caucasians); and (4) information about peptides thought to bind A2 because they are recognized by A2-restricted CTL.

The first peptide, from influenza virus matrix protein (KGILGFVFTLTVPESR, Gotch et al., 1987), was radiolabeled by acylation with 125I-hydroxyphenyl propionate ester (Bolton and Hunter, 1973). The Nα- and Nε-derivatives were separated by reverse phase HPLC and identified by hydrolysis and amino acid analysis (after blocking free amino groups with dinitrofluorobenzene). Upon equilibrium dialysis of the labeled peptides against purified HLA-A2, HLA-B7, or a control protein (ovalbumin), no binding was found. By gel filtration, the peptide and MHC protein were resolved completely, except for a trace peak attributable to aggregation of the hydrophobic peptide. To eliminate the possibility that radiolabeling interfered with the binding activity of the peptide, a new synthesis was performed manually using 3H-leucine as a precursor and standard t-boc solid phase chemistry procedures (Stewart and Young,
1984). Still no binding could be detected. Given the sensitivity of the assays, it was concluded that the equilibrium association constant was $\leq 10^4 \text{ M}^{-1}$ or that A2 peptide-binding sites were not available (or both).

A second peptide recognized by A2-restricted CTL from HIV-seropositive patients (PLTEEALELAENREILKEPVHGVY, Walker et al., 1989) was internally labeled with $^3\text{H}$ at several positions and also failed to bind purified A2 in vitro. A whole cell binding assay was developed, in which $^{125}\text{I}$-labeled peptides were incubated with A2+ lymphoblastoid target cells, A2 was isolated by immunoaffinity purification, and the extent of peptide binding was quantitated. Using a shorter version of the HIV peptide that was still capable of sensitizing target cells for lysis by specific CTL (REILKEPVHGVY), no radioactivity was found to be associated with purified A2. However, examination of the cell supernatant by HPLC revealed that the radiolabel had shifted, i.e., the chemically homogeneous iodopeptide added to cells was no longer the major radioactive peak after a 2 hour incubation at 37°C. By a combination of amino acid analysis, sequencing, and HPLC, the product was shown to be iodotyrosine; either extracellular medium (containing 10% serum) or isolated cell membranes could effect virtually complete removal of this C-terminal residue from the peptide.

Extracellular “processing” proved to be the pivotal clue in understanding peptide–MHC-I binding in our system. A series of synthetic peptides lacking C-terminal tyrosine was screened for activity by cytotoxicity assays, and one nonamer (ILKEPVHGV, termed IV9) was at least as active as any known antigenic peptide: 1-10 pg/ml could sensitize target cells for 50% maximal lysis (this concentration was called the SD$_{50}$ value), less than one-millionth what was required for the longer peptide
(SD$_{50}$ >1 μg/ml). To measure binding between IV9 and A2, the peptide was radioiodinated on histidine by carrying out the oxidative reaction at a higher pH than usual (Wolff and Covelli, 1969), and the mono- and diiodinated products were resolved by HPLC and characterized by Edman sequencing. Labeled peptide was found to occupy 0.35% of affinity-purified A2 molecules after a 2 hour incubation with whole cells; no peptide binding was observed to HLA-B7 or MHC-II molecules on the same cells. Furthermore, it was possible to show by Edman sequencing that the same antigenic peptide added to target cells could be recovered intact from peptide–MHC-I complexes, thereby establishing unambiguously the identity of a peptide eliciting T cell activity and permitting direct measurement of the dissociation rate for a peptide–MHC-I complex (Tsomides and Eisen, 1991; see p. 50).

As an indication of extracellular processing, the longer peptide (REILKEPVHGVY), when iodinated on both histidine and tyrosine and added to cells, was recovered from purified A2 as IV9 (see figure, below). (An alternative explanation for this result, that IV9 was a minor contaminant of the longer peptide and was selected by A2 because of its tight binding (Schumacher et al., 1991), was unlikely because the radiolabeled peptide had been subjected to several successive HPLC steps.) Finally, the report by Falk et al. (1991a) of motifs for endogenous peptides associated with MHC-I molecules, including A2 (leucine or methionine at position two and valine or leucine at position nine), independently predicted that IV9 would be an A2-binding peptide. Despite its herculean biological potency, peptide IV9 still exhibited no binding to affinity-purified A2 by equilibrium dialysis, strongly suggesting that due to occupancy by unknown endogenous peptides, isolated A2 molecules are simply not competent to bind added peptides under the conditions used.
Peptides recovered from affinity-purified HLA-A2 differ from the peptide 12-mers added to cells

\[
\begin{align*}
I_1\text{-RY12} & \quad R \quad E \quad I \quad L \quad K \quad E \quad P \quad V \quad H \quad G \quad V \quad Y^* \\
I_2\text{-RY12} & \quad R \quad E \quad I \quad L \quad K \quad E \quad P \quad V \quad H \quad G \quad V \quad Y^{**} \\
I_3\text{-RY12} & \quad R \quad E \quad I \quad L \quad K \quad E \quad P \quad V \quad H^* \quad G \quad V \quad Y^{**} \\
I_4\text{-RY12} & \quad R \quad E \quad I \quad L \quad K \quad E \quad P \quad V \quad H^{**} \quad G \quad V \quad Y^{**} \\
I_1\text{-IV9} & \quad I \quad L \quad K \quad E \quad P \quad V \quad H^* \quad G \quad V \\
I_2\text{-IV9} & \quad I \quad L \quad K \quad E \quad P \quad V \quad H^{**} \quad G \quad V \\
\end{align*}
\]

* side chain stoichiometrically labeled with iodine atom
More recently, a quantitative whole cell binding assay was developed to explore more readily the thermodynamics and kinetics of the peptide-MHC-I reaction for a variety of different peptides, including unlabeled ones. Stoichiometrically labeled indicator peptides were found to bind specifically to cells bearing the appropriate MHC-I molecules, and by Scatchard analysis the equilibrium association constants as well as the number of accessible peptide-binding sites per cell could be determined. By inhibition analysis, the $K_a$ values for unlabeled peptides were also measured, and the use of non-radioactive $^{127}$I-labeled peptides confirmed the equivalence between $K_a$ values calculated by means of inhibition and those measured directly with $^{125}$I-labeled peptides. The $K_a$ values ranged from $\approx 10^9$ M$^{-1}$ to $\approx 10^4$ M$^{-1}$ (lower limit of detection). The time constant for peptide-MHC-I association (the time needed to reach 63% of equilibrium binding) and the half-life of cell surface complexes were both found to be several hours using an $^{125}$I-labeled peptide (Kageyama et al., 1995; see p. 62).

The law of mass action has been used to measure the fractional occupancy of antibodies in their reactions with antigens (Karush, 1970). We applied this equation (see Introduction, p. 26) to the peptide-MHC-I reaction, using values for $K_a$ and $n$ (the number of specific peptide-binding sites per cell) obtained by Scatchard plot analysis (Scatchard, 1949), and free peptide concentrations corresponding to the SD$_{50}$ values measured in cytotoxicity assays with CTL against HIV-infected, ovalbumin-transfected, or allogeneic (Ld$^+$) target cells. The principal findings were: (1) an extremely wide range in the average number of peptide-MHC-I complexes per target cell required to elicit T cell activity (almost four orders of magnitude); and (2) in optimal settings, much lower numbers than those previously described, e.g. 1-10 peptide-MHC-I complexes per cell.
The implications of T cell recognition of target cells expressing 10 or fewer peptide-MHC-I complexes are intriguing. (1) It suggests that the surveillance function of CTL can be performed when cells (infected, transformed, or otherwise aberrant) express very little specific ligand, at least by certain CTL. (Whether cloned T cells really mirror the activities of CTL in vivo remains an important question.) At the single cell level, it seems almost incredible that a CTL can consistently ferret out such a small number of complexes, let alone undergo signal transduction and activation as a result of ligation of the corresponding number of TcR. (2) The universe of T-cell antigens presented at the cell surface is larger than previously thought, since peptides present at <100 copies per cell (at least half of the total (Hunt et al., 1992a)) can be recognized. If only peptides present at several hundred copies were known to T cells, then a typical target cell expressing \( \approx 10^5 \) MHC-I molecules (per allele) would only be able to present up to \( \approx 1000 \) different peptide species, even though the potential number of nonamers expressed by a cell is enormously greater. (3) The biochemical isolation of naturally processed peptides can be excruciatingly demanding, since at 1 copy per cell (to take the extreme case), 15 pmol of peptide (a reasonable amount for Edman sequencing) would require starting with 15 pmol of cells (nearly \( 10^{13}! \)) – actually more to compensate for obligatory losses during the purification.

In order to confirm the validity of our calculations that such low numbers of peptide-MHC-I complexes can trigger T cell function, we have been developing a system in which a radiolabeled peptide (I\(_1\)-QL9-Y5, QLSPY*PFDL) is added to target cells at its SD\(_{50}\) concentration (10\(^{-11}\) M), and specific peptide-MHC-I complexes are then measured directly. Since the peptide is stoichiometrically labeled with carrier-free
$^{125}$I (Tsomides and Eisen 1993b; Schumacher and Tsomides, 1995), its specific radioactivity ($3.5 \times 10^{18}$ cpm/mol) allows the detection of just a few molecules per cell when using $\approx 10^7$ cells. The low numbers of peptide-MHC-I complexes that are detected generally agree with calculations based on $K_a$ and $n$ as described above, but are in the process of refinement as of this writing (Joo, Tsomides and Eisen).
An optimal viral peptide recognized by CD8+ T cells binds very tightly to the restricting class I major histocompatibility complex protein on intact cells but not to the purified class I protein

ABSTRACT CD8+ cytotoxic T lymphocytes recognize cell surface complexes formed by class I major histocompatibility complex (MHC-I) glycoproteins and antigenic peptides. We have identified a peptide nonamer (termed IV9) derived from the human immunodeficiency virus that is over a millionfold more active (at subpicomolar concentrations) than peptide analogues longer or shorter by one or two amino acid residues. Although IV9 does not detectably bind to isolated MHC-I molecules as measured by equilibrium dialysis, we quantitated the dissociation kinetics of purified peptide/MHC-I complexes isolated by affinity chromatography and found these complexes to be exceedingly stable (1/2 = 200–600 hr).

Antigenic peptides arising from endogenously synthesized proteins (as during viral infection) associate with the two component chains of class I major histocompatibility complex (MHC-I) proteins (α and β2-microglobulin) in the endoplasmic reticulum to form heterotrimeric complexes which are transported to the cell surface (reviewed in ref. 1). Exogenous (chemically synthesized) peptides offered to MHC-I-expressing cells can also result in complexes that are recognized by cytotoxic T lymphocytes (CTL) (2). Exogenous peptides have been used extensively to map the specificity of CTL clones and may also represent a physiological pathway for antigen presentation (3). Yet the specificity and mechanism by which exogenous peptides form complexes with MHC-I and the stability of these complexes under physiological conditions are not known; in addition, the nature of the active peptide species in synthetic preparations has been called into question (4). To understand the dynamics of binding between MHC-I and exogenous peptides, we have carried out studies with either purified MHC-I molecules or intact MHC-I-expressing cells. Our findings lend support to the idea that a small proportion of MHC-I molecules at the cell surface are competent to bind exogenous peptides (presumably because they lack previously bound peptides of endogenous origin (5, 6)) but that purified MHC-I molecules do not bind exogenous peptides because of tightly bound preexisting peptides. Heterotrimeric peptide/MHC-I complexes that form on intact cells are shown to be effectively irreversible at 37°C.

MATERIALS AND METHODS

Peptide Synthesis and Purification. Peptides were synthesized either manually by conventional solid-phase methods using tBoc chemistry or on an Applied Biosystems 430A synthesizer and were analyzed by HPLC and amino acid analysis. Some peptides (including RY12 and IV9) were purified by C18 reverse-phase HPLC (Vydac 218TP104) in aqueous trifluoroacetic acid with a 1%/min (or shallower) CH3CN gradient. All peptide concentrations were measured by micro BCA (bicinchoninic acid) assay (Pierce). Iodinations were performed by using Na121I and a 4- to 5-fold molar excess of Na121I over peptide to achieve stoichiometric incorporation by the IodoBead method (Pierce). For histidine residue iodination, a higher pH (8.2 vs. 7.0) was used to favor imidazole ring proton dissociation (7). Iodinated peptide species were separated by HPLC for cytotoxicity assays (Fig. 1B). Radioactivity of the HPLC eluate was monitored by using an on-line radioisotope detector (Beckman 170).

Cytotoxicity Assay. CTL assays were performed in duplicate by incubating peptide dilutions and 31Cr-labeled YJ lymphoblastoid target cells (HLA-A2-, -B7+) for 1 hr at 37°C and then adding CTL clone 68A62 (8) at a CTL-to-target ratio of 4–6:1. After 4 hr at 37°C cell supernatants were assayed for 31Cr release. Total 31Cr release was determined by detergent lysis, and percent specific lysis was calculated as 31Cr release due to peptide – spontaneous release)/(total release – spontaneous release) × 100. Peptide toxicities were checked by adding medium in place of CTL and were always <3% specific lysis. Active peptides were each tested three to five times. With reproducible results. A2-restriction of the CTL clone was reconfirmed by its inability to lyse RPMI 7666 target cells (HLA-A2-, -B7+) plus peptide (not shown).

HLA Purification. JY cells (105–106) were pelleted and lysed in hypotonic medium at 4°C as described (9); all subsequent steps were performed at 4°C in the presence of 100 μM phenylmethylsulfonyl fluoride, 200 μM diithiothreitol, and 0.02% NaBH4. Membranes were collected by centrifugation for 60 min at 105,000 × g, solubilized in 4% Brij 58 (Pierce), and passed over six affinity columns arranged in series as follows (10): Sepharose only, mouse pooled immunoglobulin-Sepharose (binds Fc receptors and other membrane proteins), PA2,1-Sepharose (anti-HLA-A2), B7,1-Sepharose (anti-HLA-B7), W6/32-Sepharose (anti-MHC-I), and 9.49-Sepharose (anti-MHC-II). The affinity columns

Abbreviations: CTL, cytotoxic T lymphocyte(s); MHC-I, class I major histocompatibility complex; MHC-II, class II major histocompatibility complex; HIV, human immunodeficiency virus; RT, reverse transcriptase.
were prepared by staphylococcal protein A purification of each monoclonal antibody, peridate oxidation of the carbohydrate moiety in the Fc domains, and covalent coupling to hydrazide-modified Sepharose. After being washed with at least 30 column volumes of buffers, each column was eluted with 50 mM diethylamino-HCl, pH 11/0.2% Brij 58 into 2-ml fractions containing 1 M Tris-HCl, pH 8. HLA purity was assessed by SDS/PAGE, and yields (as well as purity) were further determined by quantitative amino acid analysis.

HLA-Bound Peptide Elution. Purified HLA-A2 and HLA-B7 were concentrated to remove unbound peptides by ultrafiltration at 5000 x g (Centricon C10) and then denatured with 0.5% trifluoroacetic acid (11) for 60 min at 37°C; the released peptides were collected by ultrafiltration at 5000 x g and subjected to HPLC fractionation.

Equilibrium Dialysis. A 100-μl sample of ~10 μM affinity-purified HLA-A2, HLA-B7, or buffer only (0.1 M Tris-HCl, pH 8/0.2% Brij 58/0.02% NaN3) was separated from 100 μl of 3 μM 125I-labeled IV9 or 125I-labeled RY12 (~3 x 10^6 cpm) by a 12,000-14,000 molecular weight cutoff membrane (Spectra-Por), and the samples were rocked gently (60 rpm) at 37°C. Each sample was prepared in triplicate. After 5 days, 50 μl from each compartment of each sample was assayed for 125I. Peptide was shown to equilibrate across the membrane within 12 hr under these conditions.

RESULTS AND DISCUSSION

Identification of the Optimal Peptide. We studied peptide binding by the human MHC-I molecule HLA-A2 because its crystal structure has been solved (12) and an A2-restricted CTL clone against the human immunodeficiency virus (HIV) reverse transcriptase (RT) has been described (8). This clone recognizes a 25-mer synthetic peptide from RT (8). We found that successive truncations of the N terminus to 17, 15, or 12-residue peptides had no effect on target cell sensitization, whereas further truncation to a 9-mer greatly reduced the sensitizing activity (Fig. 1). To quantitate specific binding between the shortest active peptide and A2, we radioiodinated the 12-mer, which contains one histidine and one tyrosine residue, and separated the mono-, di-, and tetraiodinated products from the unlabeled 12-mer by reverse-phase HPLC. This alternative to conventional trace iodination procedures (which label <1% of the iodocitrate) was considered necessary to ascertain the biological activity of each labeled product (Fig. 1B) and to quantitate MHC binding by a chemically homogeneous peptide.

Stoichiometrically monoiodinated 12-mer (termed I1-RY12) was incubated for 2 hr at 37°C with JY cells [expressing 7 x 10^7 A2 molecules per cell, determined by using Fab fragments of the anti-A2 antibody PA2.1 (13)]. The cells were lysed, their membranes were harvested and solubilized with detergent, and their MHC-I molecules (A2 and B7) were purified by affinity chromatography (9, 10), with A2 yields of 50–80% in different experiments. However, no iodopeptide was found in association with purified A2 (Fig. 2A), although the specific radioactivity of I1-RY12 and the amount of A2 recovered provided sufficient sensitivity to detect as few as 50 complexes per cell. Unexpectedly, HPLC analysis of the radioactivity recovered from these cells revealed a shift in retention time of the radiolabel (Fig. 2B and C), leading us to suspect processing of I1-RY12 to a shorter active peptide lacking the C-terminal iodotyrosine and thus accounting for failure of the iodopeptide to copurify with A2. By a combination of amino acid analysis, sequencing, and HPLC, we confirmed the identity of the recovered radioactive material as iodotyrosine. Serum-containing medium alone did not cleave iodotyrosine from I1-RY12, but both isolated membrane and cytosolic fractions of JY cells did, suggesting the existence of a cell-associated activity that processes this peptide.

To define the active peptide that may be generated from I1-RY12, we tested the biological activity of all possible 8- and 9-mers contained within I1-RY12 that exclude the C-terminal

![Fig. 1](image-url)

**Fig. 1.** Ability of various HIV RT peptides to sensitize cells for CTL lysis. (A) Representative peptide titrations: peptides are identified by the symbols next to their names in B. (B) Peptide sequences and sensitizing doses.

1All peptides are named by their first and last residue and length.
2SD50 is peptide concentration giving 50% of maximal specific lysis, which in all cases amounted to 75–90%: "none" indicates no sensitization at a peptide concentration of 100 μg/ml. SD50 values are rounded to the nearest order of magnitude.
3Stoichiometric iodination of histidine or tyrosine residues by one (●) or two (**) iodine (I) atoms.
tyrosine (Fig. 1). Recent work using virus-infected target cells has shown peptides of this length to be the "natural" species recognized by other CTL (14, 15). The 9-mer IL-KEPVHG (termed IV9) sensitized target cells at a concentration less than one-millionth that needed for RY12 (<1 pg/ml vs. 1 μg/ml). Other peptides, including both longer and shorter relatives of IV9, had intermediate activities (Fig. 1). The remarkable potency of IV9 at picomolar concentrations, together with the widely held view that the binding sites of MHC-I molecules are already largely occupied by diverse peptides of endogenous origin (12), suggested high-affinity binding between IV9 and the relatively few cell surface A2 molecules that may be accessible for binding to exogenous peptide under physiological conditions (6).

Isolation of Peptide/MHC-I Complexes. To characterize these complexes directly from cells, we iodinated IV9 on its histidine residue (7) with 125I (and an excess of 127I) and resolved the unlabelled, mono- and diiodinated species by HPLC (see Fig. 4, below). Purified monoiodinated IV9 (1-IV9) at 1 μg/ml (SD50 = 10 pg/ml) was incubated with JY cells for 2 hr at 37°C, and the MHC proteins (A2, B7, and class II) were affinity-purified from cell membranes as before. A large peak of radioactivity, over 70-fold above background, was coeluted with A2 from the affinity column, whereas no radioactivity copurified with B7 or with MHC-II (Fig. 3). To determine whether this radioactivity was still complexed with A2 after elution from the affinity column, we separated low molecular weight material by ultrafiltration through a 10,000 molecular weight cutoff membrane (Centricon). We found over 99% of the total radioactive material still bound to A2. However, when the complexes were denatured with trifluoroacetic acid for 60 min, all of the radioactivity was released as low molecular weight material that was eluted from an HPLC column at the precise retention time of 1-IV9. This material was coeluted with A2 and was highly enriched at a retention time of 38 min (Fig. 4A). The material copurifying with A2 from the HPLC column was identified as iodotyrosine through all affinity columns. This peak was identified as iodotyrosine after elution from the anti-A2 column is shown; eluates from the other columns also contained no cpm above background (not shown).

Five separate experiments yielded closely similar results. HPLC column at the precise retention time of 1-IV9 on a highly resolving gradient (Fig. 4). This material sensitized JY cells for 85% specific lysis by the anti-RT CTL clone, whereas material from other HPLC fractions or from the corresponding fraction of B7 peptides had no sensitizing activity (Fig. 4). Direct Edman sequencing of the sensitizing HPLC fraction confirmed the IV9 sequence. Thus the A2-bound radioactive peak was identical to 1-IV9, the input material, and the binding of this peptide was shown to be highly specific for A2 (at least 1000-fold greater than binding to B7 or MHC-II).

Given the specific radioactivity of IV9 and the yield of A2, the number of IV9/A2 complexes present on peptide-pulsed cells after 2 hr amounts to 0.35% of the total A2. Since a 2-hr pulse also sensitizes JY cells for a maximal CTL response, the roughly 2500 IV9/A2 complexes per cell obtained by using exogenous IV9 can elicit CTL activity. The minimal number of complexes is undoubtedly lower because (i) a saturating concentration of IV9 was used and (ii) incubations as short as a few minutes also sensitize JY cells (data not shown). A threshold number of 200–300 complexes has previously been determined for MHC-II-restricted T cells (16), and a similar number was recently published for MHC-I-restricted T cells (17).

The number of complexes isolated could represent the number of peptide-free A2 molecules that are available at the cell surface for binding to exogenous peptide (18). However, in view of evidence that peptide-free MHC-I molecules are conformationally unstable unless "rescued" by peptide (or low temperature) (6, 19), binding is likely to be a dynamic process dependent on the continual arrival at the target cell surface of newly exported A2 (20). Assuming a net export of 7 x 105 molecules (one cell's worth for JY) per 12-hr doubling time, we estimate that A2 molecules reach the cell surface at a rate of approximately 1000 per min, or 120,000 during a 2-hr pulse. If all the molecules that are competent to bind exogenous peptide are included in this population and their rate of appearance (rather than the on-rate) limits peptide binding, then the IV9/A2 complexes detected here represent about 2% of the A2 molecules synthesized in 2 hr. Consistent with this interpretation, the amount of IV9 specifically copurified
Various times, assayed for radioactivity, and returned to the incubation mixture to maintain constant volume and peptide concentrations. Fig. 5 shows that after 120 hr at 37°C, 37% and 24% of bound peptide had dissociated in the presence and absence of excess nonradioactive peptide, respectively. Since the latter figure could reflect denaturation of the protein, the difference of 13% dissociation after 120 hr was used to calculate an off-rate constant on the order of 3 × 10⁻⁷ sec⁻¹ and a half-time of ≈600 hr, far greater than the lifetime of the cells. Even at the other extreme of 37% peptide dissociation, the off-rate constant would be 1 × 10⁻⁶ sec⁻¹, for a half-time of ≈200 hr. These unusual values indicate that binding is effectively irreversible at 37°C; they argue against peptide exchange in this system and support the view that sensitization of target cells for CTL lysis occurs by way of peptide-free A2 molecules (unless some endogenous peptides are more loosely bound than IV9, a prospect contrary to expectation but difficult to distinguish from peptide-free molecules per se). Long half-times were also derived in a recent study in which peptides were added to dilute cell lysates at 4°C (21).

Equilibrium Dialysis. Given such a low off-rate constant, one can readily calculate a range of possible intrinsic association constants by assuming representative values for the on-rate constant. Using an on-rate constant of ≈10⁶ M⁻¹ sec⁻¹ that is (i) representative for many protein-ligand interactions and (ii) supported by the rapid kinetics of target cell sensitization, we estimate the intrinsic association constant could be as high as 3 × 10¹⁵ M⁻¹. On the other hand, the on-rate constant of 1 M⁻¹ sec⁻¹ established for some peptide/MHC-II systems (whose binding parameters are known to differ from class I [22, 23]) would lead to a calculated association constant of 3 × 10⁶ M⁻¹, also high enough to predict detectable binding by equilibrium dialysis. Yet, when we carried out equilibrium dialysis with IV9 and purified A2 for 5 days at 37°C we detected no binding. Thus, 3 × 10⁶ cpm of ¹²⁵I-IV9 distributed into compartments containing A2, B7, or buffer to an equal extent (49.9%, 49.4%, or 49.3% of total cpm, respectively) under conditions sensitive enough to detect an association constant ≈10⁶ M⁻¹. This result contrasts with readily detectable peptide/MHC-II binding by equilibrium dialysis (22, 23) and is likely due to a high (perhaps even stoichiometric) occupancy of peptide-binding sites in purified A2 by unknown peptides (12, 24). Peptide-free A2 molecules are either unstable to conventional purification or else too few in number to detect. Consequently, the lack of detectable binding by equilibrium dialysis also reinforces our measurement of a very low off-rate constant, since no peptide exchange occurred during this period.

Physiologically, peptide/MHC-I binding presumably never reaches chemical equilibrium due to the dynamic

![Fig. 4. Monoiodinated peptide IV9 added to JY cells and the peptide subsequently recovered from the cells' A2 are indistinguishable.](image)

![Fig. 5. Dissociation of isolated ¹²⁵I-IV9/A2 complexes at 37°C in the presence (△) or absence (●) of a 3000-fold molar excess of ¹²⁵I-IV9.](image)
nature of binding with cells. Nevertheless, the low measured off-rate constant and implied moderately to extremely strong binding argue against an earlier impression of markedly low affinities based on (i) the ability of A2 to interact with many structurally different peptides in T-cell assays (25) and (ii) our prior failure to detect binding between purified A2 and peptides by equilibrium dialysis (26).

Exogenous peptide binding to murine MHC-I is influenced by β2-microglobulin (β2m) in some cases (27, 28). We found that the CTL response to target cells pulsed with different RY12 concentrations was reproducibly unaffected by adding exogenous β2m at 10–20 μg/ml or by performing CTL assays in serum-free (and consequently β2m-free) medium, indicating that β2m exchange is not a primary determinant of peptide binding in this system.

In contrast to our findings with IV9, two recent studies involving exogenous peptides (both 12-mers) and MHC-I immunoprecipitated from intact cells detected binding only by peptides that differed from the peptides that had been added to cells (4, 20). In one case the MHC-bound peptide was identified as a 9-mer present as a trace contaminant of the synthetic 12-mer (the C-terminal 9-mer, consistent with premature peptide termination during solid-phase synthesis (4)). Because the radiolabeled tyrosine residue in this 12-mer was identified as a 9-mer present as a trace contaminant of the mixture of total peptides (unlike our 12-mer RY12), the bound 9-mer was very likely that each mechanism may find further support in different systems. The present work identifying IV9 as an optimal sensitizing peptide [as active as any peptide so far identified positions (30)]. Using the two most prominent consensus patterns and are not absolute requirements for A2-binding peptide. Our study provides independent experimental confirmation of this prediction. Moreover, our extensive CTL structure–function data on peptides related to IV9 (Fig. 1) suggest that the observed motifs are indeed conserved patterns and are not absolute requirements for sensitizing activity. For example, two peptides lacking C-terminal valine (IG8 and LG7) are as active as RY12 (SDso 0.15 and 0.16, respectively), although much less active than IV9. Whether these sensitizing activity. For example, two peptides lacking C-ter-molal valine (IG8 and LG7) are as active as RY12 (SDso 0.15 and 0.16, respectively), although much less active than IV9. Whether these peptides contain an iodinatable tyrosine or histidine residue, the first question can be addressed directly. It is also important to determine whether peptide IV9 arises from the endogenous MHC-I pathway after HIV infection of A2+ (or A2−) cells.
Stoichiometric Labeling of Peptides by Iodination on Tyrosyl or Histidyl Residues

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Radioiodination with 125I or 131I is a favored technique for labeling biologically active peptides or proteins because of high specific radioactivities and convenience in counting γ-emissions. Previous studies used trace labeling, in which fewer than 1% of the molecules are iodinated. We describe procedures for obtaining stoichiometrically iodinated and therefore chemically homogeneous peptides with specific activities exceeding 10^9 cpm/Mg (= 10^8 Ci/mmol). By analyzing the pH dependence of iodination on tyrosyl and histidyl residues, we show that the method described can be applied to many short peptides and optimized for labeling on tyrosine and/or histidine. The power of reverse-phase HPLC is exploited to resolve multiple products substituted with different molar equivalents of iodine from each other and from unlabeled peptide. Specific radioactivity ratios can be used to identify the products, as confirmed by Edman sequence analysis under conditions that separate iodinated tyrosine and histidine derivatives from all other amino acids. We also show that the biological activities of iodinated and unlabeled peptides can differ by several orders of magnitude in a T cell assay and demonstrate the usefulness of stoichiometric labeling to overcome ambiguities inherent in studying biological activities with trace-labeled peptides.

In studies aimed at analyzing the interaction between a bioactive peptide and its target molecule or cell, it is frequently desirable to label the peptide for sensitive and easy quantitation. Radioiodination with 125I or 131I offers numerous benefits, most notably high specific radioactivities and convenience in carrying out the reaction and detecting the products. The original development of radioiodination procedures concerned the labeling of proteins rather than peptides and as such placed great emphasis on minimizing protein damage by trace labeling with carrier-free iodine (1,2) or acylating with radioiodinated acid ester (3) or imidoester (4) compounds. Trace labeled proteins could be shown to retain biological activities equivalent to the unlabeled protein (1,3) and have since found widespread application in radioimmunoassays and hormone-receptor studies, for example.

The radioiodination of short peptides devoid of stable tertiary structure is not restricted by the problem of denaturation during exposure to oxidative conditions, although some amino acid side chains (Cys, Met, Trp) can be affected. However, labeling of a short peptide is likely to cause significant perturbations in the physical and biological properties of the peptide. Thus, iodine is similar in size to a phenyl group and is markedly hydrophobic (see below); moreover, the pKₐ of the phenolic hydroxyl group of tyrosyl residues is substantially reduced by each substitution with iodine (5). Consequently strategies based on trace radioiodination of peptides carry a very real risk that the labeled and unlabeled peptides will behave differently, and that failure to quantitate these differences will lead to false inferences about an unlabeled peptide based on detection of only the radiolabeled form.

One solution to this problem is the use of internally radiolabeled peptides. We have incorporated 3H-labeled amino acids into the solid-phase synthesis of 17- to 25-residue peptides by synthesizing tBoc-protected derivatives (6). While this method has the distinct advantages of not perturbing peptide structure and of a very long radioisotopic half-life (12.26 years), the specific radioactivities we achieve are at least 1000-fold lower than those obtained with radioiodinated derivatives, and counting β-emissions from 3H is a destructive process, unlike 125I or 131I counting. Moreover, virtually any peptide can be radioiodinated quickly and on a small scale (<1 mg), without the need to prepare 3H-labeled...
Thus it can be seen that even with highly efficient methionine or approximately 200 nmol tyrosyl residues (on nmol) is chemically or enzymatically oxidized to react with one batch of a peptide. Thus the most common methods employed for radiolabeling peptides are based on reactions with $^{125}$I or $^{131}$I.

Typically, 0.1-10 mCi of carrier-free $^{125}$I (60 pmol-6 nmol) is chemically or enzymatically oxidized to react with up to 500 μg of a protein or peptide. For a protein of molecular mass 100 kDa, 500 μg is equal to 5 nmol protein or approximately 200 nmol tyrosyl residues (on average). For a peptide of M, 1000, 500 μg is 500 nmol. Thus it can be seen that even with highly efficient methods for the incorporation of $^{125}$I, these conditions will lead to labeling of only up to 1% of the iodinatable sites, i.e., trace labeling. A typical yield of 10 μCi radiiodine per microgram of protein (M, 100,000) represents the labeling of less than one atom iodine per protein molecule (having about 40 tyrosyl residues on average). For short peptides, <1% of the molecules are labeled in most applications (<15 μCi/μg).

**Materials and Methods**

**Peptides**

All peptides were synthesized at the MIT Biopolymers Laboratory on an Applied Biosystems 430A using conventional TBOc chemistry followed by HF cleavage. Crude peptides were subjected to amino acid analysis, with results always within 10% of expected values. Peptide purification was performed by C18 reverse-phase HPLC (Beckman C18 or Vydac C4 10 × 250 mm, 5-μm particles) on a Beckman HPLC system (Model 110B pumps, 165 variable wavelength detector, 170 radioisotope detector, 427 integrator). The standard gradient went from 5% B to 85% B between 10 and 60 min after an initial isocratic period, with A = 0.1% trifluoroacetic acid (TFA) in H$_2$O and B = 0.085% TFA in acetonitrile. Detection was based on absorbance at 220 and 280 nm as well as radioisotope detection. Peptide sequencing was performed by Edman degradation on an Applied Biosystems 477A using an extended gradient (42 min per cycle) for the detection of iodinated amino acid derivatives. Monoiodotyrosine (Aldrich) and diiodotyrosine (Sigma) standards were applied to the sequencer in order to determine their retention times.

**Iodination and Removal of Free Iodine**

All steps were carried out at room temperature. For 1 mg peptide, 4 Iodo-Beads (Pierce) were washed twice with 0.1 M sodium phosphate, pH 7.0 (for iodination on tyrosine) or pH 8.2 (for iodination on histidine or both histidine and tyrosine) and added to 0.3 ml of the same buffer in a microcentrifuge tube. Na$^{125}$I (2-40 mCi; NEN, Amersham, or ICN) was mixed with 30-50 μl 0.1 M Na$^{125}$I (Mallinkrodt) and added to the Iodo-Beads for 5 min in a glove box equipped with a charcoal filter. A color change to yellow/brown indicated active Iodo-Beads. Peptide dissolved in 0.5 ml H$_2$O or phosphate buffer was added to the oxidized iodide for 30 min (Tyr labeling) or 45 min (His labeling), with occasional agitation. The mixture was transferred by syringe to a C18 Sep Pak cartridge (Waters) prepared according to manufacturer's instructions with methanol and H$_2$O followed by 20 ml 0.1% TFA (HPLC solvent A). After the Sep Pak was loaded and washed with an additional 20 ml solvent A to remove free iodide, two fractions were eluted with 4 ml 50% solvent B/50% solvent A and 4 ml 100% solvent B (0.085% TFA in acetonitrile). These fractions were removed from the glove box for drying in a SpeedVac Concentrator (Savant).

**Purification and Specific Radioactivities**

The fractions, containing unlabeled peptide and iodinated products, were resuspended in 1 ml solvent A, filtered through 0.45 μm, and injected on a Beckman HPLC system equipped with a C18 reverse-phase column (Vydac or Beckman) and an on-line radioisotope detector. Each peak was collected, dried in a SpeedVac Concentrator, and taken up in water to determine peptide concentration by Micro BCA Assay (Pierce). The standard curve was generated from manufacturer-supplied BSA or from unlabeled peptide that had been quantified by amino acid analysis. An aliquot from the BCA assay (usually 10 μl, or 1%) was counted in a gamma counter (Packard) for calculation of the specific radioactivities; the counting efficiency of this instrument was determined to be 70%.

**T Cell Assay**

T cell assays were performed as previously described (7). In brief, duplicate peptide dilutions were added to $^{51}$Cr-labeled JY lymphoblastoid target cells for 60 min at 37°C in a 5% CO$_2$ incubator. The peptide-specific T cell was then added at a T cell-to-target ratio of 5:1 for 4 h at 37°C, and cell supernatants were assayed for $^{51}$Cr release. Percentage specific lysis is given by [$^{51}$Cr release due to peptide – spontaneous release]/(total release in detergent – spontaneous release) × 100.

**Results and Discussion**

To achieve stoichiometric labeling of peptides, we combine the desired quantity of $^{125}$I with a 3- to 5-fold
molar excess over peptide of nonradioactive iodide \((^{127}I)\). Alternatively, \(^{131}I\) can be used, with a half-life of 8 vs 60 days for \(^{125}I\) and higher energy \(\gamma\)-emissions (with correspondingly greater demands for effective shielding). This moderate molar excess of total iodide over peptide enhances the rate of reaction and results in iodination of \(\approx 70\%\) of the starting material (see below, Fig. 5). For a given quantity of \(^{131}I\) (e.g., 20 mCi), increasing the amount of \(^{127}I\) proportionately diminishes the specific radioactivities of the products. For instance, a 4-fold molar excess of \(^{127}I\) over peptide results in the incorporation of at most 25% of the \(^{125}I\) added (5 mCi); a 10-fold excess would limit radioisotope incorporation to 10% (2 mCi). To oxidize the iodide we utilize Iodo-Beads, nonporous polystyrene beads derivatized with N-chloro-benzenesulfonamide (equivalent to immobilized chloramine \(T\)—see Ref. (8) and Pierce literature). The mixture of \(^{127}I\) + \(^{125}I\) is added to several Iodo-Beads for 5 min at room temperature (activation step according to the manufacturer's recommended protocol), followed by the peptide for 30 to 45 min. During the initial activation step volatile iodine is generated, as evidenced by the development of a yellow/brown color, necessitating appropriate safety precautions: we perform all reactions in a capped microcentrifuge tube handled within a glove box equipped with a charcoal filter. After the reaction is complete, unreacted iodide is separated from peptide by passing the mixture over a C18 cartridge that retains peptide (Fig. 1). This departure from the conventional use of ion exchange or gel filtration chromatography to remove unbound iodide—techniques more appropriate for proteins than for short peptides—produces highly efficient separations for all peptides tested.

After elution of the C18 cartridge with acetonitrile, the radiolabeled material no longer poses a volatilization hazard and may be transferred out of the glove box or other contained area used to handle Na\(^{125}I\) and Na\(^{127}I\). The eluted material is a mixture of unlabeled peptide and various iodinated products (mono-, di-, triiodinated, etc.), all of which are well-resolved by reverse-phase HPLC (Fig. 2 and 3). Retention times of the unlabeled and labeled peptides on reverse-phase HPLC invariably increase with the addition of each successive iodine atom, reflecting the hydrophobicity of iodine. The integral ratios of specific radioactivities among the products conveniently serve to identify mono-, di-, triiodinated, etc. peptides (Table 1). A typical specific radioactivity obtained under these conditions, \(10^5\) cpm/ug, corresponds to \(\approx 10\) Ci/mmol for a peptide of \(M_\text{r}\) 1500 and represents labeling with 0.006 mol \(^{125}I\) (and 0.994 mol \(^{127}I\)) per mole peptide. Each chemically homogeneous product can be assayed for \(\alpha\)2a biological activity, thereby ascertaining the effects of substitution with iodine (Fig. 4 and see below).

Besides tyrosyl residues, which can incorporate up to two iodine atoms per phenolic side chain, histidyl residues can also be iodinated in one or two positions, particularly at higher pH's (9). We successfully labeled peptides stochiometrically on tyrosine only (Fig. 2, Table

![FIG. 1. Separation of free iodine from peptide following radiolabeling procedure. Two milligrams of a 12-residue peptide (termed RY12, sequence REILKEPVHGVY) was radioiodinated with a mixture of 2 mCi \(^{125}I\) and 10 \(\mu\)mol \(^{127}I\) in the presence of 8 Iodo-Beads. After 30 min the total volume of 1 ml was loaded on a C18 Step Pak, washed with 20 ml HPLC solvent A, and eluted with 4 ml 50% B and 4 ml 100% B; 1-ml fractions were collected and 5 ul was removed for \(\gamma\)-counting. Total cpm recovered appear to be less than 2 mCi due to nonlinear \(\gamma\)-counting in fractions containing \(>10^6\) cpm.](image)

![FIG. 2. Reverse-phase HPLC separation of iodinated and uniodinated peptide containing tyrosine. Two milligrams of the 8-residue peptide Y8 (sequence SIINYEKL) was iodinated with 20 mCi \(^{125}I\) and 5 \(\mu\)mol \(^{127}I\) at pH 7.0 using 6 Iodo-Beads, injected on a Beckman C18 semi-preparative column, and eluted with an acetonitrile gradient of \(17^\circ\)/min at a flow rate of 3 ml/min. Peaks are described in Table 1.](image)
FIG. 3. Reverse-phase HPLC separation of iodinated and uniodinated peptide containing both tyrosine and histidine. Two milligrams of the 12-residue peptide RY12 (sequence REILKEPVHGVY) was iodinated with 20 mCi $^{127}$I and 10 μmol $^{127}$I at pH 8.2 using $^8$Iodo-Beads, injected on a Vydac C18 analytical column, and eluted with an acetonitrile gradient of 1%/min at a flow rate of 1 ml/min. Peaks are described in Table 1.

1), histidine only (Fig. 4), or both tyrosine and histidine (Fig. 3, Table 1). In the latter case four major products are obtained from reverse-phase HPLC fractionation in addition to unreacted starting material. Specific activity ratios indicate that these products are mono-, di-, tri-, and tetraiodinated species, respectively; in some cases there is a second minor diiodinated derivative eluting before the first.

TABLE 1

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Retention time (min)</th>
<th>Absorbance 220 nm (%)</th>
<th>Specific activity (cpm/μg)</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 2B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>39.2</td>
<td>11</td>
<td>0</td>
<td>$^{127}$I$_{-}$Y$_8$</td>
</tr>
<tr>
<td>2</td>
<td>43.0</td>
<td>34</td>
<td>$1.0 \times 10^{7}$</td>
<td>$^{127}$I$_{-}$Y$_8$</td>
</tr>
<tr>
<td>3</td>
<td>46.1</td>
<td>55</td>
<td>$2.0 \times 10^{7}$</td>
<td>$^{127}$I$_{-}$Y$_8$</td>
</tr>
<tr>
<td>Fig. 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>34.7</td>
<td>13</td>
<td>0</td>
<td>$^{127}$I$_{-}$RY12</td>
</tr>
<tr>
<td>2</td>
<td>37.9</td>
<td>18</td>
<td>$0.5 \times 10^{7}$</td>
<td>$^{127}$I$_{-}$RY12</td>
</tr>
<tr>
<td>3</td>
<td>40.4</td>
<td>20</td>
<td>$1.0 \times 10^{7}$</td>
<td>$^{127}$I$_{-}$RY12</td>
</tr>
<tr>
<td>4</td>
<td>41.1</td>
<td>17</td>
<td>$1.4 \times 10^{7}$</td>
<td>$^{127}$I$_{-}$RY12</td>
</tr>
<tr>
<td>5</td>
<td>43.5</td>
<td>32</td>
<td>$2.1 \times 10^{7}$</td>
<td>$^{127}$I$_{-}$RY12</td>
</tr>
</tbody>
</table>

Note. The peptides labeled as described in the legends to Figs. 2 and 3 were subjected to specific radioactivity determination in order to identify their degree of substitution with iodine.

To define the products precisely, we iodinated a 9-residue peptide containing one tyrosyl residue and one histidyl residue (LY9) with $^{127}$I only and subjected each HPLC-purified major peak to sequence analysis by Edman degradation. The results are summarized in Table 2. By means of a modified gradient (extended by 11 min per cycle), PTH derivatives of iodinated tyrosine and histidine can be detected on the sequencer as follows: monoiodotyr elutes between Val and Trp, diiodotyr elutes after norleu, and monoidohis elutes between Ala and Arg. Thus we could unambiguously determine that mono- and diiodinated peptides are labeled only on tyrosine, triiodinated peptide contains diiodotyrosine and monoidohistidine, and tetraiodinated peptide contains diiodotyrosine and diiodohistidine (see Table 2). In addition, we found a smaller peak of diiodinated peptide containing monoidohistidine and monoidotyrosine between the mono- and diiodotyrosine-labeled species (Fig. 5, unlabeled peak between $^{127}$I$_{-}$LY9 and $^{127}$I$_{-}$LY9). This order of products is in agreement with expectations based on increasing hydrophobicities with iodine substitution and the greater reactivity of tyrosyl than histidyl residues in short peptides where tertiary structure (and therefore reactive site accessibility) is not an issue.

When we investigated the pH dependence of iodination on tyrosyl and histidyl residues, we found pronounced effects on the overall efficiency of the reaction and on the distribution of iodinated products (Fig. 5). For example, 94% of the LY9 peptide was iodinated at pH 6.0 and only 71% at pH 9.0; at pH 6.0 the major product was diiodotyrosine-labeled (80% yield), whereas
at pH 9.0 the major product was diiodohistidine-labeled (40% yield). Because it is often desirable to generate various purified iodinated peptide derivatives (i.e., one or two iodines per tyrosyl residue and one or two iodines per histidyl residue), we routinely use pH 6.0 or 7.0 to obtain mostly tyrosine-labeled products and pH 8.2 to promote iodination on histidine or both tyrosine and histidine. At each pH iodination can occur on both tyrosine and histidine, but the ratios are markedly different (see Fig. 5). The deliberate use of reaction conditions that lead to a complex mixture of products is practical because of the power of reverse-phase HPLC in efficiently separating these products.

The peptides described above all lack tryptophan, methionine, and cysteine, residues that are sensitive to oxidation (also among the least abundant residues in many proteins). Difficulties arose when Trp was present in the sequence, presumably because of oxidative damage to the indole during iodination, yielding a complex mixture of peaks that were not further characterized. In one 9-residue methionine-containing peptide, the expected mono- and diiodotyrosine-labeled products were obtained, but each resolved into a doublet of peaks on reverse-phase HPLC (not shown); the earlier-eluting, smaller peak of each doublet was assumed to represent a methionine sulfoxide derivative, as these are known to elute earlier than their reduced counterparts. All of the products were well-separated by HPLC, allowing application of this labeling method to methionine-containing peptides.

When we iodinated a peptide containing two tyrosyl residues at positions 3 and 5, seven of the eight possible products were isolated by reverse-phase HPLC: one monoiiodinated (I₁-Y₃), three diiodinated (I₂-Y₃, I₂-Y₅, I₁-Y₃/I₁-Y₅), two triiodinated (I₂-Y₃/I₁-Y₅, I₁-Y₃/I₂-Y₅) and one tetraiodinated (I₄-Y₃). Thus even short peptides with two tyrosyl residues can be subjected to iodination with the expectation of obtaining homogenous labeled derivatives by HPLC.

Our original purpose in obtaining stoichiometrically iodinated peptides was to analyze their interactions with cell surface class I MHC glycoproteins (MHC-I). Certain peptides combine non-covalently with MHC-I proteins to form target structures recognized by cytotoxic T cells. We previously identified a 9-residue peptide (termed IV₉, sequence ILKEPVHG) from the human immunodeficiency virus reverse transcriptase that binds specifically and tightly to HLA-A2, an MHC-I protein present on cell surfaces in 30–45% of Caucasians (7). Complexes of IV₉ and HLA-A2 are recognized by HIV-specific T cells obtained from an HIV-infected individual. Thus, addition of IV₉ to HLA-A₂⁺ cells, followed by incubation with these T cells, results in lysis of the HLA-A₂⁺ cells (cytotoxicity). Because we were interested in carrying out in vitro binding studies with radiiodinated peptides and HLA-A₂ (6), we used this T cell assay to assess the effects of peptide labeling on biological activity.

As shown in Fig. 4, IV₉ and its mono- and diiodohistidine derivatives (IV₉, I₁-IV₉, I₂-IV₉) each have markedly different biological activities in the T cell assay. The peptide concentrations required to sensitize HLA-A₂⁺ cells for 50% specific lysis were 1, 10, or 1000 pg/ml for IV₉ having 0, 1, or 2 iodine atoms per peptide molecule. The 1000-fold difference between unlabeled and diiodinated IV₉ clearly demonstrates the need for caution in extrapolating results observed with trace labeled preparations to the unlabeled peptide.

It is important to note that stoichiometric iodination does not avert altering peptide structure and therefore activity as a result of labeling; only an internal radiolabel (such as ³²H, ¹¹C, or ¹³¹I) can be expected to accomplish this. However, by providing chemically homogenous products, stoichiometric iodination does permit
unambiguous quantitative analysis of the effects of labeling (Fig. 4). Stoichiometrically iodinated peptides can also provide valuable information about unlabeled peptides. For instance, the equilibrium constant for the binding of $^{125}$I-IV9 to HLA-A2 on intact cells is readily determined by means of a cell surface binding assay (not shown). The equilibrium constant for the binding of unlabeled IV9 (or any other nonradioactive peptide) to HLA-A2 can then be measured by using such a peptide as a competitive inhibitor to block the binding of $^{125}$I-IV9 to HLA-A2. From the concentration of competitive inhibitor needed to effect a given level of inhibition (e.g., 50%), the equilibrium constant for the binding of any nonradioactive inhibitor to HLA-A2 can be calculated (10). Using IV9 as an inhibitor, it was found that unlabeled IV9 binds less well than $^{125}$I-IV9 to HLA-A2, even though unlabeled IV9 is far more active than $^{125}$I-IV9 in the T cell assay (Fig. 4). These results, to be described elsewhere in detail, can provide critical insights into the three-way interaction between peptide, MHC-I protein, and T cell receptor. Thus, stoichiometrically iodinated peptides are useful tools for investigating the biological activities of unlabeled peptides, even though they may differ considerably. Indeed, it is precisely because of major differences in the activities of labeled and unlabeled peptides that the trace labeled peptides commonly used in binding and other studies may result in significant ambiguities. Such ambiguities can be avoided by stoichiometric iodination procedures that lead to chemically homogeneous products.

**SUMMARY**

The stoichiometric labeling procedures described have been applied to several short peptides (8–25 residues) containing tyrosyl and/or histidyl residues. In each case the identification of products after reverse-phase HPLC separation was facilitated by the integral specific radioactivity ratios. Characterization of the iodinated products was carried out by Edman sequencing, confirming the following order of elution for peptides containing both tyrosine and histidine: unlabeled, monoiodotyrosine labeled, diiodotyrosine labeled, monoiodohistidine and diiodotyrosine labeled (i.e., triiodinated), and diiodohistidine and diiodotyrosine labeled (i.e., tetraiodinated). The pH dependence of the iodination reaction is such that tyrosine is the favored site at pH 6.0–7.0, but an increasing proportion of iodination occurs on histidine at pH 8.0–9.0, although there is also more unreacted starting material (Fig. 5). The described procedures should be useful in any peptide labeling ap-

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![Absorbance (220 nm)](image)

**FIG. 5.** pH dependence of iodination at tyrosyl or histidyl residues. One milligram of the 9-residue peptide LY9 (sequence LKEPVHGVYI) was iodinated with 5 nmoles $^{125}$I at pH 6.0, 7.0, 8.0, or 9.0 using 6 Iodo-Beads. The products were resolved on a Vydac C1 semipreparative column and collected. Chromatographic results are shown for the pH 7.0 reaction only. $^{125}$I-LY9 was the same as starting material by retention time. All products were characterized by Edman sequencing as described in the text and in the footnote to Table 2. Integration was performed using peak areas for the five peaks identified, with the resulting yields shown as a function of reaction pH.

![Absorbance (220 nm)](image)

**FIG. 6.** Reverse-phase HPLC separation of iodinated products from a peptide containing two tyrosine. Three milligrams of the 9-residue peptide RGYVYQEL was iodinated with 30 nmoles $^{125}$I at pH 6.0 using 12 Iodo-Beads, injected on a Vydac C4 semipreparative column, and eluted with an acetonitrile gradient of 1%/min at a flow rate of 1 ml/min. Peaks were collected, dried, and characterized by Edman sequencing, with the results shown in Table 3.
STOICHIOMETRIC PEPTIDE IODINATION

TABLE 3

Sequence Determination of Products from the iodination of Peptide RGYVYQEL

<table>
<thead>
<tr>
<th>Edman cycle</th>
<th>Residue</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
<th>Peak 4</th>
<th>Peak 5</th>
<th>Peak 6</th>
<th>Peak 7</th>
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<td>7</td>
<td>51</td>
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<td>23</td>
<td>82</td>
</tr>
</tbody>
</table>

Note. Each peak obtained from the HPLC separation carried out as described in the legend to Fig. 6 was subjected to Edman degradation. Results are given as pmol of the indicated residue (as its PTH derivative); mono- and diiodotyrosine were identified on the basis of the retention times of standards applied to the sequencer as described in the footnote to Table 2.

plication where a chemically homogeneous product is desired in order to eliminate the ambiguities inherent in trace labeling methods. Furthermore, stoichiometrically labeled peptides may be useful in probing the biological activities of unlabeled peptides.

ACKNOWLEDGMENTS

We are grateful to Mitch Galanek, Don Haes, and others at the MIT Radiation Protection Office for all their help in facilitating and overseeing radioiodinations. We thank Richard F. Cook and the MIT Biopolymers Laboratory for expert peptide sequencing, including mapping of iodotyrosines. We acknowledge grant support from the National Cancer Institute (Grants CA09255, CA14051, and R35-CA42504).

REFERENCES

Variations in the Number of Peptide-MHC Class I Complexes Required to Activate Cytotoxic T Cell Responses1,2

Shigeki Kageyama,3 Theodore J. Tsomides, Yuri Sykulev, and Herman N. Eisen4

Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

We determined equilibrium constants for the binding of 16 peptides (based on four T cell epitopes) to three MHC class I proteins (A2, Kb, and Ld) on intact cells and estimated the number of accessible peptide-binding sites on these cells. From these results, and the concentrations of peptides required to sensitize target cells for lysis by CD8+ CTL, we conclude that the critical number of peptide-MHC complexes required per target cell for the activation of CTL responses varies with different combinations of peptide-MHC complexes and CTL clones from several thousand complexes to fewer than ten per target cell. The Journal of Immunology, 1995, 154: 567-576.

Under natural conditions the peptide-MHC complexes on a target cell that are recognized by a CD8+ CTL are formed from peptides produced by limited proteolysis of the target cell's proteins. These peptides bind to nascent class I MHC (MHC-I) molecules in the endoplasmic reticulum, forming complexes that are translocated to the cell surface (1). The same complexes, termed pep-MHC3 after Williams and Beyers (2), can also be formed with synthetic peptides that are added to the extracellular medium and that bind to a subset of cell surface MHC-I proteins having functionally empty peptide-binding sites, either because they lack peptide adducts or contain loosely bound peptides that exchange readily with the added peptide (3). This mode of addition of extracellular peptides to cell surface MHC-I proteins is widely used to form antigenic pep-MHC complexes on target cells to 1) identify the naturally occurring peptides of pep-MHC complexes that are recognized by Ag-specific TCR; 2) evaluate the effects of amino acid substitutions on peptide interactions with MHC-I proteins and on pep-MHC interactions with TCR; and 3) identify peptides that are capable of bringing about positive or negative selection of developing thymocytes. Synthetic peptides can also be used, as shown previously (4-7) and as emphasized here, to estimate the number of pep-MHC complexes per target cell required to trigger T cell responses. The minimal number is of particular interest because it sets an upper limit on both the number of ligated TCR molecules that are necessary for activating a T cell and on the complexity of the universe of T cell Ags displayed on the surface of any given target cell.

To evaluate pep-MHC interactions, some previous studies measured the binding of peptides to soluble MHC proteins, either isolated from target cells or produced by insect or bacterial cells by cDNA methods (8-23). Other studies measured the binding of peptides to MHC proteins in their natural form on intact cells (4, 24, 25). We have here adopted the latter approach as we were interested in establishing the equilibrium binding constants for several sets of closely related peptides to several MHC-I proteins under conditions that are similar to those used for sensitizing target cells for lysis by CTL. Accordingly, we measured the binding of peptides to MHC-I molecules on intact cells and related the results to the activities of the peptides in cytotoxicity assays.

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1 This paper is dedicated to Professor Fred Karush, who died July 2, 1994. Some of his classic contributions to our understanding of the principles underlying the antibody-antigen reaction are used here as a basis for analyzing the reactions of peptides with MHC proteins.

2 This work was supported by NIH Research Grants R35-CA42504, CA60686, and AI34247, Cancer Center Core Grant CA14051, and Training Grant CA09255 from the National Cancer Institute. S.K. was supported by the Fuji Photo Film Corp., Asaka Research Laboratory, Japan.

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5 Abbreviations used in this paper: pep-MHC: peptide-MHC; MHC-I, MHC class I molecules; SD50, peptide concentration required for half-maximal target cell lysis; β2m, β2-microglobulin.

0022-1767/95/$5.02 00
We analyzed three systems in which the representative peptide and the interacting MHC-I protein are: 1) the nonapeptide ILKEPVHGV, termed IV9 (12), from reverse transcriptase of human immunodeficiency virus type 1 and HLA-A2; 2) the octapeptide SIINFEKL, termed pO8 (26, 27) from hen egg OVA and K^b; and 3) the octapeptide LSPFPFDL, originally termed p2CL (28) and later p2Ca (29), from murine a-ketoglutarate dehydrogenase and L^d.

In the course of measuring the equilibrium constants we also determined how many cell surface MHC-I molecules per target cell are accessible to added synthetic peptides. The latter values and the equilibrium constants, together with the peptide concentrations required to sensitize target cells for half-maximal lysis by CTL, provide a means for estimating the number of pep-MHC complexes per target cell required to trigger a CTL's cytotoxic response. We show here that this number, previously reported to be 100 to 400 per cell (4-7), can vary several thousandfold for different combinations of peptides, MHC molecules, and CTL clones. We estimate that, with the most effective combinations, fewer than 10 complexes per target cell may be sufficient.

Materials and Methods

Peptide synthesis and purification

Peptides were synthesized on an Applied Biosystems 430A with standard t-Boc chemistry and were analyzed by reverse phase HPLC and amino acid analysis (Biopolymers Laboratory, Massachusetts Institute of Technology). The C-terminal amide of peptide IV9 was synthesized with a benzhydrylamine resin. All peptides were cleaved by hydrogen fluoride. Some peptides (IV9, p2Ca, pCMV) were purified by HPLC on a Beckman system 324 with a 4.6 x 250 mm C18 reverse phase column (Vydac 218TP104) and a gradient of 1% to 10% trifluoroacetic acid in H,O and B = 0.085% trifluoroacetic acid in acetonitrile. Peptide concentrations were measured by quantitative amino acid analysis used as a standard.

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Radioiodinated peptides

These peptides were prepared as described (30). To achieve stoichiometric labeling, Na^211 and Na^212 were generally mixed to give a four-to-fivefold molar excess of total iodide over peptide. For efficient labeling of peptides containing either a single tyrosine or a single histidine residue, the reaction was performed at pH 7.0 or pH 8.0, respectively. Typically, 1 mg of peptide was iodinated with 20 mCi ^211 or six Iodobeads (Pierce) for 30 min. The two products (mono- and di-iodinated) and remaining unlabelled peptide were resolved by HPLC with a 10 x 250 mm C4 reverse phase column (Vydac 214TP110) and a gradient of 1% B/min. Peaks were monitored with a UV detector set at 220 and 280 nm (Beckman 165) connected in series with a radioisotope detector (Beckman 170). Specific radioactivities of the chemically homogeneous product ranged from 1 x 10^10 to 2.5 x 10^12 dpm/mmol, indicating ~1% labeling with ^211 and 99% labeling with ^212; although these products contain a mixture of the two isotopes, they are referred to as ^211-IV9, ^212-IV9, etc. For stoichiometrically labeled, nonradioactive (cold iodinated) peptides, only ^211 was used in the iodination reaction, to give products ^211-IV9, ^212-IV9, etc. Cold iodinated peptide sequences, including iodinated residues, were confirmed by Edman sequence analysis as described (30).

Cell lines

The human cell lines used were the EBV-transformed B cell line JY (homogeneous for HLA-A2, HLA-B7), the T cell line Jurkat (HLA-A2, A25, B7, B41), Jurkat cells transfected with the gene for HLA-A2 (clone 31), the B cell line RPMI7666 (HLA-A3, A29, B7, B12), the TxB hybrid Ag-processing mutant line T2 (HLA-A2, B5), and T2 cells transfected with the genes for L^d or K^b (T2-L^d and T2-K^b). The murine cell line P815, an H-2^d mastocytoma, was also used for some L^d binding experiments. All cells were maintained in K medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 10 mM Hepes, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 μg/ml BME). Neomycin-resistant J22, T2-L^d, and T2-K^b cells were additionally supplemented with 250 μg/ml G418 (Life Technologies, Gaithersburg, MD).

The CTL used were the murine H-2^d anti-H-2^b alloreactive clone 2C (33), the murine K^b-restricted, OVA-specific H-2^b clone 4G3 (34), and the human HLA-A2-restricted, HIV-1 reverse transcriptase-specific clone 09A02 (35). CTL clones were maintained as previously described.

Direct assay to measure binding of radioiodinated peptides

Cells were harvested, washed with cold PBS, and resuspended at 10^6 cells/ml in various media: K medium (for conditions a and e, listed in Table I, below), K medium plus 0.02% NaNO_3 and 50 mM 2-deoxyglucose (conditions b and f), or K medium with 0.1% BSA instead of 10% FCS, plus the protease inhibitors aprotonin (2 µg/ml), PMSF (100 µg/ml), EDTA (5 mM), and indomethacin (20 µg/ml) (conditions c and d). A total of 2.5 x 10^6 cells were then incubated in a total volume of 500 Ĵl microtube with HPLC-purified radioiodinated peptide at various concentrations in the presence or absence of a 500-fold molar excess of an unlabeled peptide known to bind to the same MHC-I protein. Specific binding was the difference between the total radioiodinated peptide bound and the amount bound nonspecifically (i.e., bound in the presence of excess unlabeled peptide). To measure equilibrium binding constants, reaction mixtures were incubated with peptide for 7 h at 23°C (conditions a, b, and c), 2 h at 37°C (condition d), or 16 h at 4°C (conditions e and f). Incubation times were chosen on the basis of preliminary trials showing no further binding over longer periods (data not shown).

After incubation, tubes were centrifuged for 20 s. The pellet cells were then washed rapidly twice, each time with a 20-x centrifugation, and transferred to tubes containing 400 Ĵl of oil (84% silicon oil, d = 1.050, and 16% paraffin oil, d = 0.838, v/v) to separate free and cell surface-bound peptide by centrifugation. In view of the half-time (t_1/2) for dissociation of representative peptides (see below), dissociation of bound peptides during the washes was negligible. The cell pellet, supernatant, and both supernatants from the initial washes were assayed in a Packard gamma counter by counting each tube for 10 min and subtracting background (~40 cpm) from each count. Equilibrium constants for specifically bound radioiodinated peptides were determined from Scatchard plots of bound and free peptide (36). Linear regression was applied to derive a best fitting line with negative slope equal to the association constant (K_a) and x-intercept equal to the number of accessible MHC-I peptide-binding sites per cell (n). Titrations were conducted in duplicate. For those experiments performed two to four times, SD were on average 34% of the mean value for K_a and 30% of the mean value for n (Table I).
The above with a ~I-radioiodinated peptide at (including T2-Ld cells or unlabeled from supernatants by titrations were conducted in duplicate. For reduced to half its level in the absence of competing unlabeled peptide. concentration of constant of the radiolabeled peptide (Fig. 1). Titrations were conducted in the absence or presence of a 400- to 5000-fold molar excess of the same peptide in non-iodinated) unlabeled peptides were present to measure the binding of unlabeled peptide) amounted to <10% of the total ~I2-I-IV9 peptide bound to JY cells (homozygous for HLA-A2 and HLA-B7). Significant binding of this peptide was also evident on Jurkat cells that express a transfected A2 gene (IA2 cells) but not on untransfected Jurkat cells (A2") or RPMI7666 cells (HLA-A2", HLA-B7").

Previous studies showed that the recognition of a mouse cytomegalovirus peptide (YPHFMPMTNL, termed pMCMV) by virus-specific CD8" CTL was restricted by Ld (38). This peptide blocked a CD8" CTL response to a K"-restricted peptide (pOV8), indicating that it also can bind to K" (27). ~I2-I-pMCMV was therefore used as an indicator peptide to measure the binding of unlabeled peptides to K" as well as to Ld on T2-K" and T2-Ld cells, respectively (see below). As shown in Figure 1B, ~I2-I-pMCMV bound well to T2-K" cells but not to untransfected T2 cells (K"), and the binding to T2-K" cells was blocked extensively (~90%) by peptides previously shown to bind K" (pOV8, p2Ca (39), and pMCMV). Specificity for Ld is evident in Figure 1C, in which the same indicator peptide (~I2-I-pMCMV) bound to T2-Ld cells but not to untransfected T2 cells. Its binding to T2-Ld cells was strongly inhibited by unlabeled pMCMV but only partially inhibited by p2Ca, which binds less well than pMCMV to Ld (see Fig. 3, below). Specificity was also demonstrated with peptide pOV8, as this peptide, recognized by K"-restricted CTL, blocked the radioiodinated indicator peptide's binding to T2-K" cells but not to T2-Ld cells (cf. Figs. 1, B and C).

**Equilibrium constants for indicator (~I2-I-labeled) peptides and the numbers of peptide-binding sites per cell**

The results of incubating a fixed number of A2", K"+, or Ld" cells (JY, T2-K", or T2-Ld") with various concentrations of indicator peptides are shown as Scatchard plots in

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**Competition assay to measure binding of unlabeled peptides**

To determine equilibrium constants for the binding of unlabeled peptides (including ~I2-I-labeled (cold iodinated) peptides), cells were incubated as above with a ~I2-I-radioiodinated peptide at a concentration equal to its dissociation constant and with unlabeled peptide at varying concentrations. At the end of the incubation period the centrifuged cell pellets and supernatants were counted in a gamma counter as (residual binding in the presence of excess unlabeled peptides).

**Cytotoxicity assay**

Peptides were incubated in duplicate with ~I3-Cr-labeled target cells (2 x 10^6 cells/well) for 30 to 60 min at 37°C in 96-well round bottom microtiter plates before adding CTL at a CTL:target cell ratio of 5:1. Assays were performed in K medium except for peptides p2Ca-SL9 and p2Ca-A5 (Table III), in which 0.1% BSA replaced 10% FCS. After additional incubation for 4 h at 37°C, plates were centrifuged at 200 x g for 5 min, and 100-mu aliquots from each supernatant were used to determine percent specific lysis according to the formula: 100 x (13Cr release into supernatant - spontaneous release)/(total release in detergent - spontaneous release). Spontaneous release values varied from 5 to 20% of total release. All peptides were devoid of toxicity when incubated with target cells in the absence of CTL.

**Results**

**Specificity**

To distinguish between specific and nonspecific binding of radiolabeled peptides to intact cells, the binding reactions were performed in the absence or presence of a 400- to 1000-fold molar excess of the same peptide in non-iodinated form or another peptide known to bind to the same MHC-I protein. As shown in Figure 1A, nonspecific binding (residual binding in the presence of excess unlabeled peptide) amounted to <10% of the total ~I2-I-IV9 peptide bound to JY cells (homozygous for HLA-A2 and HLA-B7). Significant binding of this peptide was also evident on Jurkat cells that express a transfected A2 gene (IA2 cells) but not on untransfected Jurkat cells (A2") or RPMI7666 cells (HLA-A2", HLA-B7").
TABLE 1. Equilibrium constants and numbers of peptide-accessible MHC-I binding sites on A2, A2', and Ld cells under various conditions

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Cell</th>
<th>$K_a$ (nM$^{-1}$)</th>
<th>Binding Sites/Cell ($\times 10^4$)</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{125}$l-T2-IV9</td>
<td>JY</td>
<td>$4.7 \pm 2.6 \times 10^7$</td>
<td>26 ± 7</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$2.6 \times 10^7$</td>
<td>37</td>
<td>c</td>
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<tr>
<td></td>
<td></td>
<td>$1.9 \pm 0.8 \times 10^6$</td>
<td>8.5 ± 3.5</td>
<td>e</td>
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<tr>
<td></td>
<td></td>
<td>$1.5 \times 10^6$</td>
<td>15</td>
<td>f</td>
</tr>
<tr>
<td>$^{125}$l-pMCMV</td>
<td>T2-Kb</td>
<td>$7.7 \pm 1.1 \times 10^6$</td>
<td>38 ± 4</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$7.9 \times 10^6$</td>
<td>24</td>
<td>b</td>
</tr>
<tr>
<td>$^{125}$l-pMCMV</td>
<td>T2-Ld</td>
<td>$1.9 \pm 0.3 \times 10^6$</td>
<td>39 ± 20</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$2.9 \times 10^6$</td>
<td>14</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$5.6 \times 10^5$</td>
<td>5.8</td>
<td>d</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$2.9 \times 10^5$</td>
<td>18</td>
<td>e</td>
</tr>
<tr>
<td></td>
<td>P815</td>
<td>$5.9 \pm 2.5 \times 10^4$</td>
<td>13 ± 3</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1.4 \times 10^4$</td>
<td>4.3</td>
<td>b</td>
</tr>
</tbody>
</table>

*Values without a SD are based on duplicate titrations performed once.
*a, 7-h incubation at 23°C in K medium (see Materials and Methods): b, same as a, plus 0.02% NaN$_3$ and 50 mM 2-deoxyglucose; c, same as a, plus protease inhibitors and 0.1% BSA instead of 10% FCS; d, 2 h at 37°C in K medium with protease inhibitors and 0.1% BSA; e, overnight at 4°C in K medium; f, same as e, plus 0.02% NaN$_3$ and 50 mM 2-deoxyglucose.

Figure 2. From the linearity of these plots, a single equilibrium constant was determined for each of the pep-MHC-I combinations (Table 1). The numbers ($n$) of MHC-I sites per cell available for binding to peptides are given by extrapolation to the x-axis. By replotting the data according to Klotz (bound peptide per cell vs log free peptide concentration) (40), the values of $n$ found by extrapolation in Figure 2 were verified (not shown).

There are $7 \times 10^6$ A2 molecules on the surface of JY cells, as measured with an $^{125}$I-labeled F(ab) fragment of mAb PA2.1 (12). Figure 2A shows that in 7 h at 23°C, about 30,000 of these molecules can bind to exogenous peptide ($^{125}$I-T2-IV9). As is now well established, the binding sites of the remaining A2 molecules are occupied by tightly bound (slowly dissociating) peptides (12, 41, 42). T2 cells lack the TAP-1 and TAP-2 peptide transporters that translocate cytosolically generated peptides into the endoplasmic reticulum where they can load newly synthesized MHC-I proteins (43). Accordingly, in the absence of added peptides, T2-Kb and T2-Ld cells have low levels of stable (peptide-loaded) K$^b$ or L$^d$ at the cell surface (44, 45). But in the presence of increasing concentrations of an extracellular peptide that can bind to the relevant MHC-I molecule, up to 40,000 cell surface K$^b$ or 60,000 cell surface L$^d$ molecules can be peptide loaded in 7 h at 23°C (Fig. 2, B and C). All of these new pep-MHC complexes consist, presumably, of MHC-I molecules associated with a single peptide (the one added to the medium).

Equilibrium constants for unlabeled peptides

Representative titrations of three unlabeled peptides, conducted to determine their effectiveness as inhibitors of the binding of indicator peptide $^{125}$I-pMCMV to T2-Ld cells, are illustrated in Figure 3. The order of effectiveness was $^{125}$I-pMCMV > pMCMV > p2Ca. ($^{125}$I-pMCMV is identical to the indicator peptide except that $^{125}$I was used in place of $^{125}$I + $^{125}$I to prepare the monoiodotyrosine
The studied (A2, T2-Ld cells, were 4 × 10^8 M^−1, 8 constants, calculated from the concentrations of unlabeled derivative of K^+, and peptide giving half-maximal binding of another only to a small extent, e.g., within two- to three- ing (see Figure 4. After correction for nonspecific bind-

![Graph showing binding kinetics](image)

**FIGURE 4.** Rate of binding of radiolabeled peptide to Ld. A total of 2.5 × 10^6 T2-Ld cells were incubated for the indicated times at 23°C in 500 µl of 0.1% BSA-containing medium with 62 nM [125I]-pMCMV. The inset shows radiolabeled peptide binding to cells in the absence (A) or presence (B) of a large excess (>100-fold) of unlabeled pMCMV. The difference at each time point corresponds to specific cell-bound [125I]-pMCMV (C). The kinetics of binding were analyzed according to Pt = α(1 - e^−τ), where Pt is the concentration of MHC-bound peptide at time t, α is the maximum concentration of bound peptide (at the free peptide concentration used), and τ is the time constant for the pep-MHC reaction (found to be 4.3 h).

### Table II. Equilibrium constants for peptide binding to HLA-A2, K^0, and Ld^1

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>MHC-I</th>
<th>K_eq (M^−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV9</td>
<td>ILKEPVHG</td>
<td>A2</td>
<td>4.3 ± 2.1 × 10^8</td>
</tr>
<tr>
<td>LV9</td>
<td>ILKEPVHG</td>
<td>A2</td>
<td>5.5 × 10^8</td>
</tr>
<tr>
<td>IC8</td>
<td>ILKEPVHG</td>
<td>&lt;10^4</td>
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</tr>
<tr>
<td>IY10</td>
<td>ILKEPVGY</td>
<td>&lt;10^4</td>
<td></td>
</tr>
<tr>
<td>EV10</td>
<td>ILKEPVHG</td>
<td>4.8 × 10^8</td>
<td></td>
</tr>
<tr>
<td>IV9(DNP)</td>
<td>ILKEPVHG</td>
<td>2.4 ± 0.4 × 10^8</td>
<td></td>
</tr>
<tr>
<td>IV9(NH_2)</td>
<td>ILKEPVHG</td>
<td>2.0 ± 10^7</td>
<td></td>
</tr>
<tr>
<td>I1-α,pMCMV</td>
<td>YSPFNPWL</td>
<td>2.9 ± 2.0 × 10^8</td>
<td></td>
</tr>
<tr>
<td>pCMV</td>
<td>YPFNPWL</td>
<td>3.1 ± 1.2 × 10^8</td>
<td></td>
</tr>
<tr>
<td>p2Ca</td>
<td>LSPPFDL</td>
<td>1.0 ± 0.4 × 10^8</td>
<td></td>
</tr>
<tr>
<td>pO8V</td>
<td>SIIFFEL</td>
<td>1.5 ± 0.7 × 10^8</td>
<td></td>
</tr>
<tr>
<td>pO8V(DNP)</td>
<td>SIIFFEL</td>
<td>1.9 ± 10^8</td>
<td></td>
</tr>
<tr>
<td>Y1-α,pMCMV</td>
<td>YSPFNPWL</td>
<td>3.5 ± 1.9 × 10^8</td>
<td></td>
</tr>
<tr>
<td>pCMV</td>
<td>YPFNPWL</td>
<td>3.3 × 10^8</td>
<td></td>
</tr>
<tr>
<td>p2Ca</td>
<td>LSPPFDL</td>
<td>7.9 ± 5.3 × 10^7</td>
<td></td>
</tr>
<tr>
<td>p2Ca-Y4</td>
<td>LSPPFDL</td>
<td>1.8 ± 0.1 × 10^8</td>
<td></td>
</tr>
<tr>
<td>p2Ca-Y4</td>
<td>LSPPFDL</td>
<td>3.7 ± 0.6 × 10^8</td>
<td></td>
</tr>
<tr>
<td>p2Ca-Y4</td>
<td>LSPPFDL</td>
<td>1.3 ± 0.3 × 10^8</td>
<td></td>
</tr>
<tr>
<td>p2Ca-Y4</td>
<td>LSPPFDL</td>
<td>5.0 ± 10^8</td>
<td></td>
</tr>
</tbody>
</table>

* Values without a SD are based on duplicate titrations performed once.
* Condition c. (see Table 1); all other K_eq obtained by using condition b.
* Lysine modified by N-DNP.
* Valine modified by carboxyl amide.
* Tyraine monomonomodinated with 1^+.
* PB15 cells; all other K_eq obtained by using T2-Ld cells.

### Determination of the equilibrium constant from kinetics of the pep-MHC reaction

The rate at which [125I]-pMCMV binds to T2-Ld cells is shown in Figure 4. After correction for nonspecific binding (see inset, Fig. 4), the concentrations of bound peptide at various times were fit to the equation: \( P_t = \alpha (1 - e^{-\tau}) \), where \( P_t \) is the concentration of MHC-bound peptide at time \( t \), \( \alpha \) is the maximal concentration of peptide bound (at a free peptide concentration \( c \), taken to be the total peptide concentration since \( c >> P_0 \)), and \( \tau \) is the time constant of the pep-MHC reaction (the time needed to reach 63% of the equilibrium value). From the best fit, \( \alpha \) (as \( \tau = 60 \mu M \)) and \( \tau \) were determined. Using \( \alpha \) and the total number of accessible sites (\( n \)) derived from the Scatchard plot in Figure 2C, we calculated the equilibrium binding constant (\( K_e \)) for the following reaction: \( K_e = \alpha (cn - c) \). The resulting \( K_e \) was 1.3 × 10^8 M^−1, agreed with the values obtained for the same peptide in equilibrium binding measurements by Scatchard plot analysis (\( 1.9 \times 10^8 M^{-1} \), Table I, and \( 3.5 \times 10^8 M^{-1} \), Table II).

The rate constant of association (\( k_{+1} \)) for the pep-MHC reaction shown in Figure 4, \( 1.8 \times 10^9 M^{-1} s^{-1} \), was obtained from \( \alpha = e^{Mrk_{+1}} \) (46), where \( \alpha \), \( c \), and \( \tau \) are as defined above and \( M \) (the molar concentration of accessible peptide-binding sites) is calculated from \( n \) (the number of such sites), the number of cells, and the reaction volume. From this value and the equilibrium binding constant for the reaction, we determined the rate constant of dissociation (\( k_{-1} \)) for [125I]-pMCMV-Ld complex to be \( 1.4 \times 10^{-5} s^{-1} \), corresponding to a half-life (\( t_{1/2} \)) of 14 h at 23°C. We also measured \( k_{-1} \) for this complex at 37°C directly from the actual rate of dissociation of bound peptide from intact cells (data not shown); the \( t_{1/2} \) value found, ~3 h, was similar to those found by others for the binding of some nonamers to D^8 (11) or L^d (16), although shorter.
than the t_{1/2} found for dissociation of 125I-IV9 from affinity-purified HLA-A2 (12).

**Discussion**

The number of pep-MHC complexes per target cell required to activate a cytotoxic response sets a limit on the number of pep-MHC complexes per target cell, i.e., from approximately 1 to 20,000 complexes at equilibrium when the peptide is at its SDso concentration. These findings are consistent with observations that different CTL raised against the same peptide require markedly different concentrations of that peptide to sensitize a given target cell for the same degree of lysis (49).

Given the contrast between the wide-ranging estimates in Table III and those reported previously, it is necessary to consider the sources of error in our calculation of r (see Discussion). The SDso values are from References 12 (IV9, l2-lY9), 57 (pOV8), 39 (p2Ca-Kb), 28 (p2Ca-Ia), and 51 (p2Ca-A5, p2Ca-SL9) and from the present study (IV9(DNP), p2Ca-Y4, and p2Ca-SL9). Kc values for p2Ca-A5 and p2Ca-SL9 are also from Reference 51.

### Table III. MHC-I binding sites on target cells occupied by peptide at a free peptide concentration (c) that leads to half-maximal lysis of the target cells (SDso)

<table>
<thead>
<tr>
<th>Synthetic Peptide</th>
<th>MHC-I</th>
<th>Kc (M⁻¹)</th>
<th>SDso (M)</th>
<th>c/n</th>
<th>r/c</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV9</td>
<td>A2</td>
<td>5 × 10⁶</td>
<td>5 × 10⁻¹²</td>
<td>&lt;0.0001</td>
<td>&lt;1</td>
</tr>
<tr>
<td>l2-lY9</td>
<td>Ia</td>
<td>5 × 10⁷</td>
<td>1 × 10⁻⁸</td>
<td>0.05</td>
<td>750</td>
</tr>
<tr>
<td>(IV9(DNP))</td>
<td></td>
<td>1 × 10⁻⁷</td>
<td>1 × 10⁻³</td>
<td>0.67</td>
<td>10,000</td>
</tr>
<tr>
<td>pOV8</td>
<td>Kb</td>
<td>1.5 × 10⁶</td>
<td>3 × 10⁻¹²</td>
<td>0.00045</td>
<td>9</td>
</tr>
<tr>
<td>p2Ca</td>
<td>Ld</td>
<td>1 × 10⁹</td>
<td>1 × 10⁻⁸</td>
<td>0.09</td>
<td>1,800</td>
</tr>
<tr>
<td>p2Ca-Y4</td>
<td></td>
<td>7 × 10⁸</td>
<td>1 × 10⁻⁸</td>
<td>0.0007</td>
<td>21</td>
</tr>
<tr>
<td>p2Ca-A5</td>
<td></td>
<td>7 × 10⁹</td>
<td>1 × 10⁻⁸</td>
<td>0.0005</td>
<td>15</td>
</tr>
<tr>
<td>p2Ca-SL9</td>
<td></td>
<td>5 × 10⁵</td>
<td>2 × 10⁻⁸</td>
<td>0.09</td>
<td>2,700</td>
</tr>
<tr>
<td>p2Ca-SL9</td>
<td></td>
<td>4 × 10⁷</td>
<td>1 × 10⁻⁸</td>
<td>0.29</td>
<td>8,700</td>
</tr>
</tbody>
</table>

a The numbers of A2, Kb, and Ld molecules measured on IV9, T2-Kb, and T2-Ld cells at 23°C were 30,000, 40,000, and 60,000, respectively. The corresponding numbers at 37°C are taken to be one-half of these values for purposes of computing r (see Discussion). The SDso values are from References 12 (IV9, l2-lY9), 57 (pOV8), 39 (p2Ca-Kb), 28 (p2Ca-Ia), and 51 (p2Ca-A5, p2Ca-SL9) and from the present study (IV9(DNP), p2Ca-Y4, and p2Ca-SL9). Kc values for p2Ca-A5 and p2Ca-SL9 are also from Reference 51.

b Fraction of available MHC-I sites occupied by peptide.

c peptide at a free peptide concentration (c) that leads to half-maximal lysis of the target cell (SDso).

d Synthetic peptides recognized by T cells.
and $1 \times 10^6$ M$^{-1}$, respectively, whereas using soluble L$eta$
Bruunmark et al. found $4 \times 10^6$ M$^{-1}$ (51). The basis for this
difference is not clear but may relate to the different $\beta_2$-m
subunits associated with L$eta$ heavy chains in the different as-
say systems (human $\beta_2$-m in T2-L$^{a}$ cells and mouse $\beta_2$-m in
P815 cells and in the soluble L$^{b}$ preparations).

Another source of uncertainty is the free peptide con-
centration, $c$. Peptide degradation by proteases during cy-
totoxicity assays is known to occur and may even be es-
tential to generate active (MHC-binding) peptides when
longer precursors are added to target cells (14, 29, 52–55).

We evaluated the degradation of peptide $^{125}$I-$\gamma$9 by in-
cubating the peptide for 4 h in K medium and measuring
its recovery by reverse phase HPLC, under conditions that
resolve the shorter, less active fragments from the intact
peptide. We obtained yields of $\approx50\%$ (data not shown).
Thus, $c$ could be in error by a factor of $\approx2$. However, such
an error would not affect our conclusion of a broad range
of $r/n$ values and would only lead to a calculation of fewer,
not more, complexes per target cell required to trigger T
cell responses.

Table III is based on equilibrium constants that were
measured at room temperature ($\approx23^\circ$C) and on $SD_{50}$
values that were determined in cytotoxicity assays performed
at $37^\circ$C. To estimate $r/n$ at $37^\circ$C, the binding of peptide
$\gamma$1-pMCMV to T2-L$^{d}$ cells was also measured at this
temperature. As shown in Figure 2D, pep-MHC binding at
$37^\circ$C could be represented by a linear Scatchard plot, giving
a single equilibrium association constant that differed
only little (three- to fourfold) from that measured at $23^\circ$C
(cf. Fig. 2, C and D). However, the extrapolated value for
$n$ was $\approx10$-fold lower at $37^\circ$C than at $23^\circ$C. Values of
$n$ are determined by several processes, including 1) the rate
of synthesis and appearance of peptide-accessible MHC-I
molecules at the cell surface; 2) their rate of decay; and 3)
the rate at which peptides bind to and stabilize these mol-
cules. Measurements of $n$ are thus subject to more un-
certainty than those of $K_d$ or $c$. The binding assay at $37^\circ$C
shown in Figure 2D was performed in the presence of protease inhibitors to block peptide degradation, including
0.1 M iodoacetamide, which hinders the production of cel-
lar ATP and may reduce the rate of protein synthesis,
including new MHC molecules; this probably contributed
at an underestimate for $n$ at $37^\circ$C. On the basis in part
of ongoing studies, we chose a value of $n$ for T2-L$^{d}$ cells at
$37^\circ$C for purposes of calculating $r$ that is approximately
one-half of the number found at $23^\circ$C (Table III). In sup-
port of this estimate, a previous study found $\approx2500$
pep-A2 complexes per cell when JY cells were incubated
with peptide $\gamma$1-$\gamma$9 at $37^\circ$C, or $\approx15,000$ complexes after
recognition for affinity purification yields of 10 to 25% (12);
this is about one-half of the number we measured at $23^\circ$C
(see Fig. 2A). Notwithstanding these uncertainties in the
actual values of $n$ at $37^\circ$C, it is clear that at this temper-
ature there is still an extremely wide variation in $r/n$ and $r$
values, reflecting mostly the wide variation in $SD_{50}$ values.

Consider, for example, the lysis by 2C CTL of T2-L$^{a}$
target cells sensitized with peptides p2Ca (LSPFPFDL),
p2Ca-Y4 (LSPYPYFDL), p2Ca-A5 (LSPFAFDL), or p2Ca-
SL9 (SPFPFDLL). Inasmuch as $K_d$ values at $23^\circ$C differ
little from those at $37^\circ$C (Fig. 2, C and D), the $SD_{50}$ values
found for these peptides mean that in standard cytotoxicity
assays the corresponding $r/n$ values vary from 0.0007 to
0.29 (Table III). If $n$ at $37^\circ$C is half that at $23^\circ$C, the
respective $r$ values for the four peptides are in the ratio
1:0.75:135:435, i.e., 20, 15, 2700, and 8700 pep-MHC
complexes per cell (or twice these values if $n$ is approxi-
mately the same at 37 and 23$^\circ$C). Thus, in contrast to
earlier findings that suggested that 100 to 400 complexes
per cell were generally required, the present findings sug-
gest that the number can vary over a range of many thou-
sandfold. It is possible that, broadly speaking, few pep-
MHC complexes are required when the affinity of the TCR
that recognizes them is high and that a large number of
complexes is needed when they and the corresponding
TCR interact with low affinity.

In Figure 5, pep-MHC equilibrium constants ($K_d$) are
plotted against peptide activities in standardized cyto-
toxic assays, expressed as the peptide concentrations re-
quired to sensitize JY target cells for half-maximal lysis by
the A2-restricted CTL clone 68A62 ($SD_{50}$). The absence
of a simple linear correlation is obvious, i.e., the best
MHC-binding peptides are not always those that elicit T
cell activity at the lowest concentrations. For example,
peptide $^{125}$I-$\gamma$9 (two iodine atoms incorporated on his-
idine at position 7) binds approximately
1000 times better
than $\gamma$9 to A2 molecules, yet has an $SD_{50}$ that is a
1000fold higher. This result can be reconciled with the crystal
structure of the $\gamma$9-A2 complex (56), since the iodine
substitutions appear to be at a position that would interfere
with recognition of $\gamma$9-A2 complexes by the 68A62
TCR. Furthermore, such data are consistent with the
recently reported variations that are observed in the affinities
of a given TCR for related pep-MHC ligands (51) or of different
TCR for their respective pep-MHC ligands (57).
Minimal concentration of peptide that renders target cells susceptible to lysis by CTL

We estimate that for the loading of cell surface MHC-I molecules by extracellular peptide in cytotoxicity assays, the minimal effective concentration of free peptide cannot be lower than $10^{-13}$ to $10^{-14}$ M. This follows because the minimum concentration is given by $m/(K_p n)$, where $m$ is the number of pep-MHC complexes per target cell (taken here as one, the smallest number theoretically possible), $K_p$ is the equilibrium constant for the pep-MHC reaction, and $n$ is the number of peptide-binding sites per cell. Because $K_p$ values of $10^9$ M$^{-1}$ and $n$ values of $10^5$ to $10^6$ per target cell represent upper limits for the measurements so far made, it may be expected that in conventional titrations of peptides (e.g., Fig. 5), reports of target cell sensitization with peptide at a free concentration of $<10^{-13}$ M would mean that either the pep-MHC equilibrium constant or the number of accessible MHC-I-binding sites are extraordinarily high, or the minimum peptide concentrations are in error.

The antigenic universe as seen by T cells

A target cell displays on its surface up to several hundred thousand MHC-I molecules complexed with an array of peptides representing diverse intracellular proteins, including products encoded by pathogens such as viruses (3) and products that arise from aberrant protein expression in tumor cells (58). Some pep-MHC complexes are particularly abundant (42, 59–63), whereas others are extremely scarce (26, 28, 29, 64–66), complicating efforts to isolate and sequence individual peptides recognized by T cells of interest (antiviral, antitumor, etc.). If a given MHC-I allele product (e.g., HLA-A2) is present at $\sim10^5$ copies per target cell (in the absence of transformation by EBV, which greatly increases this number (67)), and, as commonly supposed, a minimum of 100 pep-MHC complexes per cell are needed for T cell activation, then a maximum of 1000 different peptides can be presented effectively at the cell surface in association with A2 (or any other given MHC product). This would mean that many intracellular proteins are not represented at the cell surface, at least at levels sufficient for T cell recognition. However, the present study indicates that lower numbers of complexes can indeed be recognized for optimal combinations of peptide, MHC molecule, and T cells. Thus the complexity of the universe of peptide Ags effectively displayed by a target cell for immunosurveillance by T cells could be considerably greater than 1000. This result also implies that rare peptide epitopes, present at say 1 to 10 complexes per target cell, will be all the more difficult to obtain in amounts sufficient for direct chemical sequencing.

Acknowledgments

We thank Dr. Linda Sherman for Jurkat cells transfected with HLA-A2; Dr. Peter Cresswell for cell lines T2, T2-K3, and T2-L5; Richard F. Cook and the MIT Biopolymers Laboratory for peptide synthesis and analysis; Mimi Rasmussen and Carol McKinley for valuable technical assistance; and Ann Hicks for help in preparation of the manuscript.

References


NUMBERS OF PEPTIDE-MHC COMPLEXES RECOGNIZED BY T CELLS


Evidence That a Single Peptide-MHC Complex on a Target Cell Can Elicit a Cytolytic T Cell Response

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Summary

Using a chemically homogeneous radiolabeled peptide of high specific activity (\(^{125}\text{I}_{-}\text{QLSPYPFDL}, 3.5 \times 10^{18} \text{cpm per mole}\)) we show that at a peptide concentration (5 pM) causing half-maximal lysis of target cells by a cytotoxic T lymphocyte (CTL) clone that recognizes the peptide in association with L\(^d\), a class I MHC protein, only 3 peptide molecules on average are bound by L\(^d\) per target cell. From the distribution of L\(^d\) on the target cells we suggest that a single peptide-MHC complex per target cell can trigger activation of the T cell cytolytic response.
Introduction

In the initial antigen-specific event that leads to T cell activation, antigen-specific receptors on T cells (TcR) react with peptide-MHC complexes (pep-MHC) on antigen presenting cells or target cells. As shown in innumerable in vitro assays in which synthetic peptides are incubated with target cells, the intensity of the T cell response depends upon peptide concentration, which, in turn, affects the density of the corresponding pep-MHC complexes ("epitopes") on target cells. While the number of these complexes per cell ("epitope density") appears to be one of the critical determinants of the outcome of T cell–target cell encounters (e.g., Ashton-Rickardt et al., 1994; Sykulev et al., 1994a; Tsomides et al., 1995), measuring them is laborious and only a few values have been reported. In some studies, using radiolabeled peptides and immunoprecipitating the appropriate MHC protein, the minimum epitope density found to trigger antigen-specific T cell proliferation and lymphokine production (Harding and Unanue, 1990; Demotz et al., 1990) or a T cell cytolytic response (Christinck et al., 1991) was around 100 to 400 complexes per antigen presenting cell.

Another approach for estimating epitope densities has taken advantage of mutant target cells with a defect in the peptide transporter that results in cell surface class I MHC molecules largely devoid of stably bound peptides (Heemels and Ploegh, 1995). Because of this defect, widely different epitope densities can be established on these cells by incubating them with synthetic peptides at various concentrations, and the average number of pep-MHC complexes formed per target cell at steady state can then be estimated from: (1) the free peptide concentration; (2) the equilibrium constant for the peptide-MHC reaction; and (3) the total number of accessible ("functionally empty") MHC binding sites per cell. Although this approach can be considered to yield only approximate values, it suggested that the epitope densities required for half-maximal cytolytic responses by cytotoxic T lymphocytes (CTL) varied from several thousand pep-MHC complexes per target cell to fewer than ten with different combinations of MHC proteins, peptides, and CTL (Kageyama et al., 1995). In addition, two independent models for T cell–target cell interactions have proposed that activation of a T cell can be initiated by 3-5 pep-MHC complexes (Brower et al., 1994) or by fewer than 10 complexes per target cell (Sykulev et al., 1995).

The low values are of interest because, if correct, they focus attention on a critical question concerning the physiologic activation of T cells: "whether T cell triggering involves cross-linking TcR molecules, or whether activation occurs by perturbation of a TcR-multimolecular complex by a single MHC-peptide" (Williams and Beyers, 1992). Accordingly, we have sought to determine if the low values previously suggested by indirect approaches and reliance on models can be verified by measuring directly the number of peptide molecules on antigen presenting cells under conditions where these cells elicit a half-maximal cytolytic response by CD8+ T cells. For this purpose we made use of a chemically homogeneous radioiodinated peptide of exceptionally high specific radioactivity. Prepared with large amounts of carrier-free $^{125}$I and purified by HPLC, the peptide had the same specific radioactivity as carrier-free $^{125}$I (3.5x10$^{18}$ cpm/mole). With this peptide we found that an average of 3 pep-MHC per target cell could elicit a half-maximal cytolytic T cell response. This finding and the distribution of L$^d$ on the target cells suggest that the cytolytic response of some T cells may be elicited by a target cell that bears a single pep-MHC complex.
Materials and Methods

Cells

The CD8+ cytotoxic T cell clone 2C was maintained as described (Kranz et al., 1984). The human hybridoma T2 transfected with the Ld gene (Alexander et al., 1989) was cultured in K medium (RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM β-mercaptoethanol) in the presence of 320 µg/ml G418.

Peptides

Except for the influenza virus matrix peptide GILGFVFTL, which was kindly provided by Drs. Marlène Bouvier and Don Wiley (Harvard University, Cambridge, MA) (Bouvier and Wiley, 1994), all other peptides were synthesized by solid phase t-Boc chemistry in the M.I.T. Biopolymers Laboratory and purified by reverse phase HPLC. The monoidinated, nonradioactive form of peptide QLSPYPFDL, termed $^{127}$I-QL9-Y5, was prepared in the same way, using t-Boc-protected monoidinated tyrosine (Peninsula, Inc., Belmont, CA). Because the N-terminal glutamine rearranges to cyclized pyrrolidone carboxylate, the peptide exists in two forms (separable by HPLC). Both were recognized by 2C cells in association with Ld. The preparations used here were predominantly in cyclized form. All peptide concentrations were measured by quantitative amino acid analyses.

Stoichiometric iodination of peptide QLSPYPFDL (QL9-Y5) with $^{125}$I

The monoidinated, radioactive form of peptide QL9-Y5 was prepared by iodination with carrier-free Na$^{125}$I (Du Pont NEN) as described (Tsomides and Eisen, 1993; Schumacher and Tsomides, 1995). Briefly, in a typical reaction, 200 µg of purified peptide QL9-Y5 was reacted with 30 mCi Na$^{125}$I using 2 Iodo-Beads (Pierce) for 30 min at pH 6.0. After unbound iodide was removed from the peptide mixture using a Sep-Pak C18 cartridge (Waters) in a glove box, the labeled peptide products and unreacted QL9-Y5 were resolved from one another on a 4.6 x 250 mm reverse phase C18 HPLC column (Vydac) with a 1%/min acetonitrile gradient, using on-line radioisotope detection (Beckman model 170). Individual radiolabeled products were dried, resuspended in H2O, and aliquots were counted in a γ-counter (Packard) with a known counting efficiency of 75% to determine the peptides' specific radioactivities. The specific radioactivity of monoidinated $^{125}$I-QL9-Y5 was the same as that of carrier-free $^{125}$I (3.5x10$^{18}$ cpn/mol). The composition of this peptide was further confirmed by Edman degradation after HPLC purification (Tsomides and Eisen, 1993).

Antibodies

Mouse monoclonal antibodies PA2.1 (IgG1, anti-HLA-A2 (Brodsky et al., 1979)) and 30-5-7 (IgG2a, anti-Ld (Ozato et al., 1980; Lie et al., 1990)) were isolated from supernatants of hybridoma cell cultures using protein A affinity chromatography.
Cytotoxicity assay

50 µl of peptides at various concentrations in phosphate buffered saline were added to 5x10³ ⁵¹Cr-labeled target cells (T2-Lᵈ) and 1.5-2.5x10⁴ CTL (2C) in 150 µl of K medium in round-bottom wells of microtiter plates. After brief centrifugation (300 g, 5 min) the plates were incubated in a CO₂ incubator for 4 hr at 37°C. Percent specific lysis was calculated from the average of duplicates as ((⁵¹Cr experimentally released – spontaneous release)/(total release in 0.1% NP40 – spontaneous release)) x 100.

Flow cytometry

To measure peptide-dependent stabilization of A² and Lᵈ molecules on T2-Lᵈ cells, the cells were incubated with various peptide concentrations at 37°C for 6-12 hr. Expression of cell surface A² and Lᵈ was detected with antibodies PA2.1 and 30-5-7, respectively, followed by staining the cell-bound antibodies with FITC-labeled F(ab')₂ fragments of goat antibodies to mouse immunoglobulins. The levels of expression of the MHC proteins were measured by mean fluorescence on a linear scale.

Kinetic analysis of the reaction between I₁-QL9-Y5 and Lᵈ on T2-Lᵈ cells

Sixteen tubes, each containing 5x10⁷ cells in 5 ml K medium, were incubated with influenza virus matrix peptide GL9 at 1-2 µM for 1 hr at 37°C. I₁-QL9-Y5 was then introduced into all tubes at 10⁻¹⁰ M. To correct for nonspecific binding of the ¹²⁵I-peptide to the cells, peptide YPHFMPTNL (pMCMV, Reddehase et al., 1989) was added to half the tubes at a final concentration of 1-2x10⁻⁶ M; this concentration is in great excess over what is required to saturate the Lᵈ binding sites, since the equilibrium binding constant for the pMCMV–Lᵈ reaction is ≈2x10⁹ M⁻¹ (Sykulev et al., 1994a). Tubes were slowly rotated on a stirring wheel at 37°C, and after each hr duplicate tubes containing the labeled peptide only (to measure total binding) or both the labeled peptide and the Lᵈ-blocking unlabeled peptide were centrifuged at 4°C. After removing supernatants, which were counted to measure the free concentration of ¹²⁵I-peptide, the cell pellets were washed twice with ice cold K medium, resuspended in 2-3 ml of ice cold K medium, and layered on 2 ml of silicone-paraffin oil mixture (see above) and centrifuged at 4°C. After freezing the centrifuge tubes, the tips were cut off to measure cell-bound ¹²⁵I-peptide. Dissociation of the labeled cell-bound peptide during manipulations at 4°C was shown to be negligible (Vturina et al., in preparation).

To determine the rate constant of dissociation (k⁻¹) of I₁-QL9-Y5 from Lᵈ, 5x10⁵ T2-Lᵈ cells were preincubated in a total volume of 200 µl with 5 µM brefeldin A (BFA) for 40 min at 37°C to block delivery of newly produced MHC molecules to the cell surface. Peptide ¹²⁷I₁-QL9-Y5 was then added at a final concentration of 4x10⁻⁴ M and incubation was continued at 37°C. After the cell suspension was subjected to rapid centrifugation, the supernatant was promptly removed and the cells were washed twice with ice cold K medium and resuspended in 2 ml of silicone-paraffin oil mixture (see above) and centrifuged at 4°C. After freezing the centrifuge tubes, the tips were cut off to measure cell-bound ¹²⁵I-peptide. Dissociation of the labeled cell-bound peptide during manipulations at 4°C was shown to be negligible (Vturina et al., in preparation).

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Direct measurement of I₁-QL9-Y5:Ld complexes on T2-Ld cells

The number of I₁-QL9-Y5:Ld complexes on T2-Ld cells was measured directly with cells that were incubated at 37°C for 3 hr (see equilibrium time, Fig. 2) with ¹²⁵I₁-QL9-Y5 at 3 different concentrations: 5x10⁻¹¹, 2.3x10⁻¹¹, and 5x10⁻¹² M. To 4 tubes, each prepared with 5x10⁷ cells as above, peptide ¹²⁵I₁-QL9-Y5 was added to achieve one of the indicated concentrations in the presence or absence of unlabeled pMCMV at a concentration in great excess over what was required to saturate Ld. All tubes contained peptide GL9 at 1-2 µM to saturate peptide binding sites on A2 (which is present on T2-Ld cells). After 3 hr cell-bound and unbound ¹²⁵I-peptide were separated and measured as above. The number of Ld-bound peptide molecules per cell was determined from: (1) the difference between total cell-bound cpm measured in the absence and presence of the Ld-saturating concentration of peptide pMCMV; (2) the specific radioactivity of ¹²⁵I₁-QL9-Y5 (3.5x10¹⁸ cpm/mole); and (3) the number of cells in the sample. The amount of specifically cell-bound ¹²⁵I-peptide was the same when the GL9-saturated cells were incubated for 1 hr at 37°C before the addition of ¹²⁵I₁-QL9-Y5 and when the ¹²⁵I-peptide and pMCMV were added at the same time.

The distribution of Ld molecules on T2-Ld target cells

T2-Ld target cells were labeled with the PKH26 dye according to the manufacturer's protocol (Zynaxis Cell Science, Inc., Malvern, PA), and they were then labeled in the standard way with ⁵¹Cr. 5x10³ double-labeled target cells were incubated with various concentrations of ¹²⁷I₁-QL9-Y5 and 1.5x10⁴ 2C cells in a total volume of 200 µl in round-bottom 96-well plates for 4 hr at 37°C. Percent specific lysis was determined by counting ⁵¹Cr in the supernatants as described above. The presence of PKH26 on the target cells had no effect on their specific lysis by CTL 2C. In parallel, T2-Ld targets tagged with PKH26 and not ⁵¹Cr-labeled were incubated under precisely the same conditions at the peptide concentrations required for 20 to 80% of maximal lysis, i.e., 1x10⁻¹² to 3x10⁻¹¹ M, using 24 identical wells for each of the peptide concentrations tested. Two control 24-well sets of the target cells were also examined at the same time: the first set contained only target cells and are referred to below as the "initial" cells; the second set contained the target cells and CTL 2C but no added peptide. At the end of the incubation period (4 hr) cells from each 24-well set were pooled, immediately chilled on ice, and stained with 30-5-7 anti-Ld antibody as described above. After adding propidium iodide (PI) to detect dead cells, all samples were analysed by flow cytometry on the FACStarPlus Flow Cytometer. About 90% of the initial target cells were PI⁻, whether or not they had been incubated alone or with CTL 2C in the absence of the peptide. However, in the presence of the peptide the fraction of surviving target cells (PKH26⁺PI⁻) was reduced and the decrease was greater as the peptide concentration increased.

The levels of Ld on the initial and surviving T2-Ld target cell populations (PKH26⁺PI⁻) were compared by flow cytometry by measuring FITC fluorescence intensity of the antibody-stained cells on a linear scale. The fluorescence of these cells fell within channels 20 and 520, and this region was divided into 25 equal intervals. Numbered from 1 to 25, the intervals provided a relative measure of the cell surface level of Ld. The distributions of Ld on both the initial and surviving target cell
populations were displayed by plotting the percent of all PKH26+PI-FITC+ cells for a given population in each of the numbered (n) intervals. To determined the Ld distribution on the lysed target cells, we calculated the percent of lysed cells in each interval ($P_n^k$) from:

$$P_n^k = \frac{P_n^{\text{int}} - (1 - f_k) P_n^s}{f_k},$$

where $P_n^{\text{int}}$ and $P_n^s$ are the percentages of "initial" and "survived" target cells, respectively, in the nth interval, and $f_k$ is the fraction of specifically "lysed" cells determined from released $^{51}$Cr. Values of $P_n^k$ were plotted as a function of the interval number n. Relative weighted average values (means) of Ld expression ($\bar{n}$) in all 3 populations of target cells (initial, survived, and lysed) were calculated as:

$$\bar{n} = \frac{\sum_{n=1}^{25} P_n \cdot n}{100}.$$
Results

Peptide concentration required for half-maximal cytolysis

CTL clone 2C recognizes pep-MHC complexes formed by the class I MHC molecule Ld and one of several naturally processed peptides whose sequences derive from \( \alpha \)-ketoglutarate dehydrogenase (Udaka et al., 1993), including p2Ca (LSPFPFDL, Udaka et al., 1992) and QL9 (QLSPFPFDL); the latter is the most potent Ld-sensitizing peptide known for CTL 2C, having an SD\(_{50}\) value (concentration required to elicit half-maximal lysis) of 5 pM (Sykulev et al., 1994b), or about 100-fold less than that of peptide p2Ca. To estimate the number of Ld-bound peptide molecules per target cell required to activate 2C cells, we first determined the free peptide concentration that results in half-maximal lysis using peptide I1-QL9-Y5, a monoiodinated analog of QL9 available in chemically equivalent nonradioactive (\( ^{127}I \)) and radioactive (\( ^{125}I \)) forms. As shown in Fig. 1, the SD\(_{50}\) value of I1-QL9-Y5 was about equal to that of peptide QL9, or 5 pM.

Specificity of I1-QL9-Y5 binding to Ld on T2-Ld cells

The T2-Ld target cells used in these studies are of human origin and express HLA-A2. Since the sequence of I1-QL9-Y5 happens to fit the A2 consensus motif (it has leucine at positions 2 and 9), and this peptide can bind to A2 as well as Ld (Table 1), it was necessary to include in the reaction mixture, along with I1-QL9-Y5 and T2-Ld cells, a peptide that could saturate the peptide binding sites of A2 without occupying the peptide binding sites of Ld. This requirement was met by the influenza virus peptide GILGFVFTL (Bednarek et al., 1991; Morrison et al., 1992), termed GL9. As shown in Table 1, on T2-Ld cells that were incubated with GL9 the level of cell surface A2 increased markedly, but the level of Ld was unchanged. Moreover, GL9 at 1 \( \mu \)M, a concentration in great excess over what was required to saturate A2, did not interfere at all with the Ld-dependent lysis of T2-Ld cells by CTL 2C in the presence of peptide p2Ca (data not shown). Thus, by including GL9 at 1 \( \mu \)M in all assays, the binding of I1-QL9-Y5 to A2 could be prevented without interfering with its binding to Ld.

Kinetics of I1-QL9-Y5 – Ld reaction

The rate of binding of I1-QL9-Y5 to Ld on T2-Ld cells was examined to determine whether this peptide–MHC reaction can approach steady state during standard CTL assay conditions (4 hr). Visual inspection of Fig. 2 shows that the number of peptide molecules bound specifically to T2-Ld cells reached steady state in about 2 hr. When fit to the equation \( P_t = \alpha (1 - e^{-t/\tau}) \), where \( P_t \) is the number of peptide molecules bound specifically per T2-Ld cell at time \( t \), the kinetic data of Fig. 2A yielded \( \alpha \), the total number of Ld-bound peptide molecules per cell at steady state at the free concentration shown (1x10^{-10} M), and also \( \tau \), the time constant, i.e., the time required to reach 63% of the steady state value. This time (\( \tau \)) was around 50 min. \( \tau \) becomes longer as the peptide concentration decreases, but it cannot be longer than the reciprocal of the rate constant of dissociation (\( k_r \)) for the peptide–Ld reaction (see Eq. 5a from Sykulev et al., 1994a).

To measure \( k_r \) we made use of an anti-Ld antibody (30-5-7) that reacts
specifically with Ld if the binding site is occupied by peptide (Lie et al., 1990): when the peptide dissociates, the resulting empty Ld molecule undergoes rapid denaturation at 37°C. Thus, by loading Ld on T2-Ld cells under conditions where brefeldin A blocked the delivery of newly synthesized Ld to the cell surface, the time course of Ld–peptide dissociation could be monitored by flow cytometry using antibody 30-5-7 (Vturina et al., in preparation; see Materials and Methods). From the results shown in Fig. 2B, the value of \( k_{-1} \) was found to be 3.3x10^{-4} sec^{-1}, yielding an upper limit for \( \tau \) of ≈50 min. Thus, even at the lowest peptide concentration used in this study (5 pM, see below) the I\(_1\)-QL9-Y5 – Ld reaction approached steady state.

**Direct measurement of epitope densities**

To measure the binding of I\(_1\)-QL9-Y5 directly to Ld on target cells, peptide QL9-Y5 was radiolabeled with carrier-free \( ^{125}\text{I} \) and incubated at various concentrations with 5x10\(^7\) T2-Ld cells in a total volume of 5 ml. Free peptide concentrations and the numbers of specifically bound peptide molecules per cell were determined directly from the peptide's specific radioactivity, 3.5x10\(^{18}\) cpm/mole. At 3 free peptide concentrations, 5x10\(^{-11}\), 2.3x10\(^{-11}\), and 5x10\(^{-12}\) M, the directly measured average epitope density values were 50, 40, and 3 pep-MHC complexes per cell, respectively.

In view of the implications of these values, it is necessary to consider possible sources of error. One potential error is that \( ^{125}\text{I} \)-peptide dissociated from Ld when the cells were washed to remove free peptide. This possibility was ruled out by measuring the stability of the peptide-Ld complexes by flow cytometry. The cells were washed at 4°C in ≈10 min (see Materials and Methods), but at this temperature no dissociation could be detected over many hr (data not shown). Another source of error arises from cell counts: we estimate this error to be at most ± 20%. Hence, at the lowest peptide concentration (5x10\(^{-12}\) M), the epitope density value in this system was 3 ± 1 pep-MHC complexes per target cell. With this average number the frequencies in the target cell population of cells having 0, 1, 2, 3, 4 and 5 pep-MHC complexes per cell would be 0.05, 0.15, 0.22, 0.22, 0.17, and 0.10, respectively, assuming the Poisson distribution to be applicable. In that event, from the sum of these frequencies (0.91) the titration shown in Fig. 1 implies that when half the target cells were lysed, most of the lysed cells would have had fewer than 5 complexes per cell and only about 10-20% of them would had more than 5 per cell.

**Is there selective lysis of T2-Ld target cells expressing higher than the average number of pep-MHC complexes per cell?**

Alternatively, it could be argued that the distribution of Ld molecules on T2-Ld cells is extremely asymmetric, especially as Ld on these cells is derived from a transfected gene. In that event only those target cells with Ld levels much above the average might be preferentially lysed. To test this possibility we compared the distribution of Ld on the initial target cells, before they were introduced into a CTL assay, with those that survived after 4 hr when half had been lysed. As shown in Fig. 3, the Ld distribution was virtually the same on the initial and surviving target cells. By subtraction it was clear that the lysed target cells also had a similar distribution.
Discussion

With a high specific activity radioiodinated peptide it was possible to establish directly that with an average of only 3 pep-MHC complexes per target cell, a CD8+ T cell clone (2C) could be stimulated to make a half-maximal cytolytic response. Although the levels of Ld expressed on the target cells had a broad distribution, it was clear (Fig. 3) that there was essentially no preferential lysis of cells having higher than the average number of Ld molecules. Since the probability of a peptide's binding to Ld is proportional to the total number of Ld molecules per cell and to the number of accessible Ld binding sites per cell, the distribution of I1-QL9-Y5-Ld complexes on the target cells should match the Ld distribution shown in Fig. 3, with the average number of 3 cognate pep-MHC complexes per cell corresponding to the position of the weighted average for total Ld (9th subset) in this figure. The number of complexes on the lysed cells would thus extend from a low of 1 per cell (3rd subset at the extreme left) to a high of about 8 (at the extreme right). As seen in the bottom panel of Fig. 3, 67% of the lysed target cells had 3 or more pep-MHC complexes per cell, but 33% of the lysed cells had fewer than 3 and 12% had only 1-2 complexes per cell. Given this extremely low number and the likelihood that pep-MHC complexes are randomly distributed over the target cell surface, it is likely that a single complex per target cell can render a cell subject to specific lysis in the 2C system.

Of the directly measured target cell epitope densities required to trigger T cell activity, the value reported here is the lowest by far. Two parameters that determine epitope density requirements are the intrinsic affinity of the TcR-pep-MHC reaction and the stability of the TcR-pep-MHC bond (bond lifetime). As was recently suggested on the basis of the law of mass action (Sykulev et al., 1995), target cells with epitope densities of 1-10 pep-MHC complexes per cell can elicit half-maximal cytolytic responses if the CTL's TcR bind these complexes with an intrinsic affinity of ≈10^6 M^-1 or higher. Elsewhere we show that the TcR on 2C cells does indeed have such an affinity for the I1-QL9-Y5-Ld complex (Sykulev et al., in preparation).

How long would a single TcR-pep-MHC bond have to persist to elicit a T cell cytolytic response? We previously found that the lifetime of the bond formed by the 2C TcR and the QLSPFPFDL-Ld (QL9-Ld) complex is ≈1 min under physiological conditions (intact T cells, 37°C) (Sykulev et al., 1994b). Since peptide I1-QL9-Y5 behaves very similarly to the QL9 peptide (i.e., it also sensitizes target cells for half-maximal lysis at 5 pM (Fig. 1) and the I1-QL9-Y5-Ld complex is bound by the 2C TcR with high intrinsic affinity), it is likely that under physiological conditions the lifetime of the 2C TcR–I1-QL9-Y5-Ld bond is also around 1 min. For other systems having lower intrinsic TcR affinities and forming shorter-lived TcR-pep-MHC bonds, higher epitope densities and a larger number of bonds are probably required.

How can the present findings be reconciled with many previous studies suggesting that T cell activation requires multivalent ligands to bring about aggregation or "cross-linking" of TcR molecules on the T cell surface (e.g., Symer et al., 1992)? In considering the apparent disparity between these findings and ours, we note, first, that the term "activation" covers a multitude of responses. Some are rapid (minutes to hours) and do not require transcription of silent genes, while others are slow (requiring a day or more) and depend upon activation of gene expression. The cytolytic response
is relatively fast (typical assay conditions are 4 hr) and is unaffected by treating the T cells with inhibitors of transcription or protein synthesis (e.g., Zychlinsky et al., 1991). That it differs from slow responses, such as cell proliferation or cytokine production, is evident in "split anergy," whereby an antigen presenting cell lacking costimulatory components was found to render a CD8+ cytotoxic T cell clone partially anergic, i.e., the T cells retained their ability to lyse target cells but proved unable subsequently to proliferate (a slow response) in response to an effective antigen presenting cell (Otten and Germain, 1991). Studies in this laboratory have also shown that CTL can be stimulated under conditions where their cytolytic function is preserved but their proliferative activity is depressed (Dutz et al., 1992).

A single reactive pep-MHC on a target cell amounts to a monovalent ligand. In accord with our evidence that such a ligand might trigger a T cell cytolytic response, it has been reported that an increase in T cell intracellular Ca\(^{2+}\) concentration, an extremely rapid response, can be elicited almost as well by the Fab fragment of an anti-CD3 antibody as by the intact bivalent antibody (Oettgen et al., 1985). It has also been shown that monovalent Fab fragments from the same anti-CD3 antibody were able to stimulate IL2 secretion by both syngeneic and allogeneic MHC class II-restricted T cell clones (Tamura and Nariuchi, 1992). Whether a single pep-MHC on a target cell can be demonstrated directly to elicit such slow responses is not clear. From the Brower et al. model (1994) it was deduced that 3-5 pep-MHC complexes could stimulate a T cell to produce \(\gamma\)-INF. However, in that model, class I MHC was adsorbed on plastic to which relatively long peptides were added (10-15 amino acids in length); whether cytokine production can be elicited by a similar number of pep-class I MHC complexes in a more physiological setting remains to be seen.

Although antigen-induced cross-linking and oligomerization of TcR molecules on the T cell surface are often considered as though they are the same, a distinction between them emerges from the evidence that a single pep-MHC complex on a target cell can stimulate a T cell. While a single complex, acting as a monovalent ligand, cannot literally cross-link T cell receptors, it could conceivably bring about their oligomerization. That TcR molecules have a propensity to form dimers is suggested by the recent crystallographic study by Fields et al. (1995), and it can be imagined that monovalent pep-MHC ligation of a TcR could enhance this tendency, perhaps by inducing a conformational change in the TcR (e.g., Rojo and Janeway, 1988). In an analogous situation conformational changes of immunoglobulin resulting from antigen binding have been demonstrated (e.g., Stanfield et al., 1993).

While a single pep-MHC can react with only a single TcR at any instant, over time it can engage many of them, reacting repetitively with the same TcR molecule or serially with many different TcR molecules as recently emphasized by Valitutti et al. (1995). The number of engagements associated with a given T cell response depends, in part, on the lifetime of the TcR–pep-MHC bond. The few dissociation rate constants reported so far indicate that the lifetimes of these bonds range from about 1 to 100 sec (Sykulev et al., 1994a; Sykulev et al., 1994b; Matsui et al., 1994; Corr et al., 1994), but how long the bonds have to persist in order to elicit particular responses remains to be determined.
Acknowledgments

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Figure legends

Figure 1. Specific lysis of $^{51}$Cr-labeled T2-L$^d$ target cells in the presence of various concentrations of peptides p2Ca (LSPFPFDL), QL9 (QLSPFPFDL), and $^{127}$I$_1$-QL9-Y5, the monoiodinated form of QL9-Y5 (QLSPYPFDL).

Figure 2. Kinetics of binding of monoiodinated peptide $^{125}$I$_1$-QL9-Y5 to L$^d$ on T2-L$^d$ cells at 37°C. A. The rate of binding of $^{125}$I$_1$-QL9-Y5 at a free peptide concentration of $10^{-10}$ M. The increase over time in specific binding of the peptide to L$^d$ is shown by the filled circles (●). The solid line represents the best fit of the experimental points to the theoretical curve described by the single exponential equation for first order kinetics (see Results for details). Insert: Change in cell-bound radioactivity (cpm) over time in the presence (○) and absence (■) of a large (10,000-fold molar) excess of the L$^d$-binding mouse cytomegalovirus peptide pMCMV. The difference represents peptide specifically bound to L$^d$. B. Rate of dissociation of $^{127}$I$_1$-QL9-Y5 from L$^d$. Dissociation was monitored by the loss of peptide-stabilized L$^d$ from the surface of brefeldin A-treated T2-L$^d$ cells, measured by the disappearance of FITC fluorescence from antibody-stained cells (see Materials and Methods). The experimental points (■) were fitted to $\ln (F_t/F_0) = -k_{-1} \cdot t$, where $F_0$ and $F_t$ are the mean fluorescence values on a linear scale at time zero and time t, respectively. The slope of a plot of ln ($F_t/F_0$) versus t yielded $k_{-1}$.

Figure 3. Distribution of L$^d$ and I$_1$-QL9-Y5-L$^d$ complexes on T2-L$^d$ target cells. Percent of the target cells with different levels of L$^d$ expression is shown for the target cell populations that were incubated for 4 hr at 37°C without the CTL and peptide (the "initial" population) or with the CTL and the peptide at 5 pM (the "survived" population). The L$^d$ distribution on the "lysed" cells was determined by difference (see Materials and Methods). To determine if the distribution shown (upper panel) changed over 4 hr, target cells expressing low or high L$^d$ levels were sorted, incubated at 37°C for 4 hr and examined again (with and without re-staining) by flow cytometry. No change in distribution in either subset was seen (data not shown).
Sykulev et al. Figure 1
Sykulev et al. Figure 2
Sykulev et al. Figure 3
Table 1. Peptide binding to HLA-A2 and L^d proteins on T2-L^d cells measured by enhanced surface expression of these MHC proteins.\( ^a \)

<table>
<thead>
<tr>
<th>Peptide concentration (μg/ml)</th>
<th>GILGFVFTL</th>
<th>QLSPY(I1)PFDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HLA-A2(^b)</td>
<td>L(^d)(^c)</td>
</tr>
<tr>
<td>0</td>
<td>136.4</td>
<td>30</td>
</tr>
<tr>
<td>0.016</td>
<td>171</td>
<td>26</td>
</tr>
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<tr>
<td>0.4</td>
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</tr>
<tr>
<td>2.0</td>
<td>229</td>
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</tr>
</tbody>
</table>

\(^a\) Mean fluorescence values (linear scale) were determined after 8.5 hr incubation of T2-L\(^d\) cells with the indicated peptides at the concentrations shown.

\(^b\) Measured using mouse monoclonal antibody PA2.1 (IgG\(^1\), specific for HLA-A2) and FITC-labeled anti-mouse immunoglobulin.

\(^c\) Measured using mouse monoclonal antibody 30-5-7 (IgG\(^2a\), specific for the α\(_1\) and α\(_2\) domains of L\(^d\)) and FITC-labeled anti-mouse immunoglobulin.

\(^d\) Not done.
References


CHAPTER 2

Naturally processed peptides recognized by HIV-specific CTL
Overview

The results in the previous chapter showing peptide IV9 to be highly active in cytotoxicity assays, to bind reasonably well to A2 in appropriate assay systems, and to form small numbers of peptide-MHC-I complexes when added to target cells at its SD₅₀ concentration did not provide any information about whether this peptide is actually a product of antigen processing; that is, can it be detected in HIV-infected target cells? An answer to this question is a prerequisite for measuring ligand density and attempting to understand the role of IV9 (or other peptides) in CTL-mediated lysis of infected target cells.

To approach this problem, we first established new cell lines stably expressing HIV type 1 (in collaboration with Rick Young and Anna Aldovini). This was necessary because adding infectious HIV to cell cultures usually only results in a fraction of cells becoming infected, and these tend to be overgrown by uninfected cells unless fresh virus is continually supplied. Our strategy was transfection of various human cell lines with cloned proviral DNA linked to an antibiotic resistance gene (neomycin or hygromycin). Subsequent selection and maintenance in the presence of antibiotic ensured a uniformly HIV-expressing population of cells suitable for use as targets in cytotoxicity assays. Five of the cell lines have been deposited with the NIH AIDS Research and Reference Reagent Program for future access by other investigators.

The question of ligand density in HIV-infected cells seemed particularly compelling because of the nature of this infection: as a chronic infection evidently compatible with long-term cell viability and proliferation, it is distinguished from many
previously studied viral infections (e.g. influenza, vaccinia, Sendai, and vesicular stomatitis viruses (VSV)), in which host cell protein synthesis shifts toward virus-encoded products and eventually the infected cells die. Thus, unlike products of lytic viruses expressed at high levels (e.g. 5-10% of MHC-I sites occupied by a single VSV peptide (van Bleek and Nathenson, 1990)), HIV-derived products must compete with the full complement of normal host cell peptides for presentation by a limited number of MHC-I molecules to CD8+ T cells.

Using two CTL reactive against HIV-infected cells (one specific for reverse transcriptase (RT) and one for gag), we identified a single naturally processed peptide recognized by each T cell and determined the absolute abundances of these peptides. The methodologies, results, and conclusions are given in Tsomides et al., 1994, reproduced on the following pages. In the end, we found only up to =12 molecules of peptide IV9 per HIV-infected Jurkat-A2 cell (vs. =400 molecules of gag peptide), probably accounting for the poor lysis of these target cells by RT-specific CTL (vs. excellent killing of the same targets by gag-specific CTL). Notably, these two CTL both recognize uninfected Jurkat-A2 cells pulsed with picomolar concentrations of synthetic peptides (including peptides not found in extracts of infected cells), illustrating that such target cell sensitization assays must be interpreted carefully.

Human CTL clones are notoriously difficult to maintain in culture for extended periods, making longitudinal studies of a particular clone problematic. To circumvent this predicament, we set out to derive mouse T cell clones specific for HIV and restricted by A2, with the expectation that mouse CTL would generally be more amenable to long-term cell culture through the use of known interleukins and other
growth conditions. We immunized A2 transgenic mice (provided by Hidde Ploegh) with peptide IV9 in complete Freund's adjuvant (details to be elaborated elsewhere) and eventually obtained several A2-restricted, IV9-specific mouse CTL clones. The sensitivity of two of these clones (reflected by their SD50 values) is indicated in the graph below and compared with that of a human CTL clone having the same specificity. Of the many issues raised by these preliminary results, one of immediate interest was whether the mouse CTL could recognize and lyse infected target cells expressing endogenous levels of antigen; as shown below, good cytolysis was observed using the human TxB hybridoma cell line T1 as a target. (The density of naturally processed IV9 on T1 cells is estimated to be greater than that on Jurkat-A2 cells by indirect measures but has not yet been determined because of technical problems in expanding infected T1 cells to the required numbers.)
Human CTL Clone 68A62  Mouse CTL Clones

IV9 concentration (g/ml)

CTL clone 2082-2-30

CTL:target cell ratio
Naturally Processed Viral Peptides Recognized by Cytotoxic T Lymphocytes on Cells Chronically Infected by Human Immunodeficiency Virus Type 1

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Summary

We have established long-term cultures of several cell lines stably and uniformly expressing human immunodeficiency virus type 1 (HIV-1) in order to (a) identify naturally processed HIV-1 peptides recognized by cytotoxic T lymphocytes (CTL) from HIV-1-seropositive individuals and (b) consider the hypothesis that naturally occurring epitope densities on HIV-infected cells may limit their lysis by CTL. Each of two A2-restricted CD8+ CTL specific for HIV-1 gag or reverse transcriptase (RT) recognized a single naturally processed HIV-1 peptide in trifluoroacetic acid (TFA) extracts of infected cells: gag 77-85 (SLYNTVATL) or RT 476-484 (ILKEPVHGV). Both processed peptides match the synthetic peptides that are optimally active in cytotoxicity assays and have the consensus motif described for A2-associated peptides. Their abundances were 400 and =12 molecules per infected Jurkat-A2 cell, respectively. Other synthetic HIV-1 peptides active at subnanomolar concentrations were not present in infected cells. Except for the antigen processing mutant line T2, HIV-infected HLA-A2+ cell lines were specifically lysed by both A2-restricted CTL, although infected Jurkat-A2 cells were lysed more poorly by RT-specific CTL than by gag-specific CTL, suggesting that low cell surface density of a natural peptide may limit the effectiveness of some HIV-specific CTL despite their vigorous activity against synthetic peptide-treated target cells.

MHC-restricted CTL play a central role in immune responses against many viruses by destroying virus-infected cells (1). Unlike antibodies, CD8+ CTL recognize conserved sequences from intracellular viral proteins in addition to sequences from virus-encoded cell surface glycoproteins (2). CD8+ CTL specific for diverse HIV products (e.g., gag, pol, nef, env) appear within 1–2 wk of infection, and the importance of these CTL is suggested by their unusually high frequencies: they can often be detected in freshly isolated PBMC without the in vitro antigenic stimulation usually required to demonstrate CTL activity in other viral infections (3–14).

It has been proposed that the CD8+ CTL response to HIV is one of the main factors contributing to a long asymptomatic period in infected individuals (15, 16). Recent studies documenting HIV-specific T cells in HIV-exposed but uninfected individuals further support a role for these cells in controlling infection (17–19). Yet CTL usually do not succeed in eradicating HIV (20, 21). Two possible mechanisms for this failure are viral mutation leading to escape from CTL recognition (22–25) and altered lymphokine production secondary to depletion of CD4+ T cells. Another possibility is that the density of many HIV epitopes (i.e., particular peptide-MHC complexes) on infected cells is too low to trigger effective lysis of these cells by mature CD8+ CTL in vivo.

In considering this third possibility, it is important to distinguish between acute infection by viruses such as influenza or vaccinia, in which viral genes are overexpressed at the expense of host cell protein synthesis, and chronic infection by HIV, in which cells continue to express their normal complement of self proteins (as evidenced by their ability to grow and proliferate for long periods; see below). In the latter case, HIV-derived peptides must compete with a much larger excess of host cell peptides for presentation by class I MHC (MHC-I)1 proteins to CD8+ T cells. Can a sufficient

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1 Abbreviations used in this paper: MHC-I, class I MHC; RT, reverse transcriptase; SD50, peptide concentration that sensitizes target cells for half-maximal lysis; TFA, trifluoroacetic acid; VSV, vesicular stomatitis virus.
number of HIV peptides succeed in occupying MHC-I binding sites in order to render infected cells good targets for destruction by CTLs. The presence of a vigorous and specific CTL response in HIV infection implies that the density of HIV epitopes is sufficient to stimulate the differentiation of CD8+ precursor T cells into mature CTLs (at least on those cells involved in CTL induction). However, it remains unclear whether the number of specific peptide-MHC-I complexes on most HIV-infected cells (i.e., CD4+ T cells, macrophages) is adequate for efficient killing of these cells by mature CD8+ CTLs.

The density of an HIV epitope on infected cells cannot be determined without knowing the precise identity of the HIV peptide involved. Prior work characterizing HIV epitopes has relied on vaccinia recombinants (26) and synthetic peptides up to 25 amino acids in length (27, 28). However, the peptides produced by cellular antigen processing pathways may or may not be identical to the synthetic epitopes produced by vaccinia recombinants or peptides produced by cellular processing pathways may or may not be identical to the synthetic epitopes used in target cell sensitization assays (29), and so the exact identities of HIV epitopes in infected cells are not known. To reveal both the identities and abundances of HIV peptides presented endogenously by MHC-I molecules, as well as other properties such as their MHC binding affinities and immunogenicities, we have established an assay system based on several newly developed cell lines that stably and uniformly express HIV-1 and CTLs obtained from HIV-seropositive individuals.

Materials and Methods

**Cells.** The following human cell lines were used for transfection and infections (MHC-I molecules expressed by each cell are in parentheses): Jurkat (HLA-A9, A25, B7, B41); Jurkat stably transfected with the gene for HLA-A2 and here designated J2A (kindly supplied by Dr. Linda Sherman, The Scripps Research Institute, La Jolla, CA) (30); H9 (HLA-A1, Bw6, Cw6); MOI4; the Txb hybridoma T1 (HLA-A2, B5); the antigen processing mutant T2 derived from T1 (both kindly provided by Dr. Peter Cresswell, Yale University, New Haven, CT) (31); the EBV-transformed B cell line JY (HLA-A2, B7, Cw7); and the promonocytic cell line U937. All cell lines were grown in "K" medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 10 mM Hepes, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL 2-ME). CTLs were derived from PBMC obtained from asymptomatic HIV-1-seropositive subjects and maintained as described (32, 33).

**Transfection with Chimeric HIV-1 DNA.** The establishment of cell lines stably expressing HIV-1 proviral DNA was achieved by transfection with plasmid vectors R7neo and R7hyg, modifications and here designated R7neo (26). Transfection with plasmid R7neo (at least on those cells involved in CTL induction). However, it remains unclear whether the number of specific peptide-MHC-I complexes on most HIV-infected cells (i.e., CD4+ T cells, macrophages) is adequate for efficient killing of these cells by mature CD8+ CTLs.

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aqueous TFA on ice (and additional TFA if needed to bring the pH below 2), and douseing with 50 strokes. The material was transferred to a Centriprep 10 ultrafiltration device (Amicon Corp., Beverly, MA) and subjected to centrifugation at 2,600 g for several hours at 4°C (37). The ultrafiltrate (molecular mass <10 kDa) was removed (>12 ml), an equal volume of 1% TFA was added to the retentate, and centrifugation was repeated twice in order to maximize the recovery of extracted peptides. Pooled filtrates were dried by lyophilization and redissolved in 0.1% TFA for fractionation by HPLC using the conditions described above for either the gag or the RT peptides. 40 fractions were collected at 1 min intervals in presiliconized microfuge tubes, dried and resuspended in H2O with vortexing.

Expression of HIV-1. Several human cell lines were chosen for transfection with HIV-1 proviral DNA linked to a drug resistance gene, including T cells (Jurkat, JAI, H9, and MOLT-4), a TxB hybrid (T1) and its derivative antigen processing mutant cell line (T2), an EBV-transformed B cell line (JY), and a promonocytic cell line (U937). 2d after transfection of each line by electroporation with plasmid R7neo or R7hyg (35), cell culture supernatants were shown to contain HIV-1 p24 antigen by ELISA. Selection was initiated either at this time or 3 wk later in order to permit infection of untransfected cells with virus produced by successfully transfected cells in the same culture; such infected (as opposed to transfected) cells also acquired the drug resistance gene. For convenience, HIV-expressing cell lines are referred to as HIV-infected even though they may include both transfected and infected cells. After several weeks during which fresh medium was supplied two to three times per week, all eight cell cultures were found to be 100% HIV+ by p24 immunofluorescence (Fig. 1).

Results

Establishment of Long-term Cell Lines Stably and Uniformly Expressing HIV-1. Several human cell lines were chosen for transfection with HIV-1 proviral DNA linked to a drug resistance gene, including T cells (Jurkat, JAI, H9, and MOLT-4), a TxB hybrid (T1) and its derivative antigen processing mutant cell line (T2), an EBV-transformed B cell line (JY), and a promonocytic cell line (U937). 2d after transfection of each line by electroporation with plasmid R7neo or R7hyg (35), cell culture supernatants were shown to contain HIV-1 p24 antigen by ELISA. Selection was initiated either at this time or 3 wk later in order to permit infection of untransfected cells with virus produced by successfully transfected cells in the same culture; such infected (as opposed to transfected) cells also acquired the drug resistance gene. For convenience, HIV-expressing cell lines are referred to as HIV-infected even though they may include both transfected and infected cells. After several weeks during which fresh medium was supplied two to three times per week, all eight cell cultures were found to be 100% HIV+ by p24 immunofluorescence (Fig. 1). JA2/R7hyg (JA2 cells transfected with plasmid R7hyg) and H9/R7neo were readily expanded to 10 liters over a period of several weeks by maintaining the cells in log phase. They have remained HIV+ for over 1.5 yr, showing that HIV expression is compatible with long-term cell viability and proliferative activity, at least under special circumstances. H9/R7hyg, Jurkat/R7hyg, and T1/R7hyg were also maintained and expanded for several months, whereas MOLT-4/R7hyg, JY/R7hyg, and U937/R7hyg grew more slowly, possibly reflecting their greater susceptibility to cytopathic effects of the virus and the gradual emergence of variants resistant to these effects. Only T2/R7hyg suffered steadily declining viability, and these cells did not survive beyond a few months despite evidence of successful transfection and initial selection of an HIV+ population. In light of CD4-dependent syncytia formation as a mechanism for HIV-mediated cytopathicity (39), the high CD4 levels in T2 cells (70-80% higher than T1 and U937, the next highest) were considered a possible explanation for the singular failure of these cells to survive infection. However, when T2 cells were first sorted by FACSort® (Becton Dickinson & Co.) for a CD4low subset (stable for at least 1 mo at a level 30-40% higher than T1) and then transfected, the resulting T2/R7hyg was still only a short-term cell line. Nevertheless, these cells were uniformly HIV+ and could be tested as targets for lysis by specific CTL along with the other stably transfected lines.

Identification of the Optimal Synthetic Peptides Recognized by Two HIV-specific CTL. Two sets of peptides based on previously described gag (33, Johnson, R. P., unpublished data) and RT (32, 38) sequences were synthesized and tested for their ability to sensitize uninfected A2+ target cells for lysis by the A2-restricted CTL 115Ip and the A2-restricted RT-specific clone 68A62. CTL line 115Ip readily lysed the A2+ HIV-infected target cells JA2/R7hyg, JY/R7hyg, and T1/R7hyg but not their untransfected counterparts (Fig. 2, A-C). CTL clone 68A62 specifically lysed target cells T1/R7hyg, JY/R7hyg, and JA2/R7hyg (to a lesser extent), but JA2/R7hyg was hardly lysed more than uninfected JA2 cells (Fig. 2, E-G). The fourth available A2+ HIV-infected target cell, T2/R7hyg, is a cell line lacking genes for the peptide transporter subunits TAP-1 and TAP-2; it was not lysed by CTL 115Ip despite high levels of p24 expression (Fig. 2 D). Consistent with this result, previous studies showed that T2 cells infected with influenza virus, vaccinia virus, or vesicular stomatitis virus (VSV) failed to present known viral epitopes to appropriate CD8+ CTL (40-43); however, these same cells were shown to present epitopes from Sendai virus (45), HIV-1 env (44), and minigene expression vectors encoding certain viral peptides (45), apparently via a TAP-independent pathway for loading MHCI molecules with cytosolic peptides. Failure to detect lysis of T2/R7hyg cells under conditions where T1/R7hyg and other HIV+ cells were efficiently lysed suggests that the TAP pathway is required for normal presentation of the gag peptide recognized by CTL 115Ip.

Peptide A2 clone (SDso = 1 pg/ml) and gag-SL9 consistently had the lowest SDso (30 pg/ml). The closely related 9-residue peptides gag-LY9 and gag-R710 were 10,000-fold less active than gag-SL9, although at micromolar concentrations and gag-SL9 consistently had the lowest SDso (30 pg/ml). The closely related 9-residue peptides gag-LY9 and gag-R710 were 10,000-fold less active than gag-SL9, although at micromolar concentrations and gag-SL9 consistently had the lowest SDso (30 pg/ml).
Figure 1. Lymphoblast cell lines transfected with HIV-1 DNA stably and uniformly express viral antigen while continuing to proliferate. Cells were transfected with plasmid R7hyg, grown in the presence of hygromycin B for at least 1 mo, and stained with anti-p24 monoclonal antibody and FITC-labeled secondary antibody as described in Materials and Methods. For each cell line, a single field is visualized by fluorescence (left) and phase contrast (right) microscopy. Cell lines shown are JA2/R7hyg (A and B), H9/R7hyg (C and D), T1/R7hyg (E and F), and JY/R7hyg (G and H). For H9/R7hyg cells, HIV-induced syncytia can be seen. Untransfected control cells exhibited no fluorescence under the staining conditions used.

Figure 2. HIV-expressing cell lines are susceptible to lysis by HIV-specific CTL. Cytotoxicity assays were carried out using the gag-specific CTL line 115Ip (A-D) or the RT-specific CTL clone 68A62 (E-G) and the 3HCr-labeled target cells indicated at the top of each panel. Lysis of T2 cells by CTL clone 68A62 was not tested. Target cells were either untransfected (O) or had been transfected with plasmid R7hyg at least 1 mo before and were uniformly positive for HIV expression by p24 immunofluorescence (S). In addition, the BW62-restricted gag-specific CTL lines 35C18 and 35C44, obtained by stimulating PBMC from an HIV-1-seropositive individual with the 23-residue peptide p17/2 (33), efficiently lysed H9/R7hyg cells but not untransfected H9 cells (not shown).

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Identification of Two HIV-1 Peptides Produced by Endogenous Antigen Processing in Infected Cells. To identify the naturally processed HIV-1 peptides present in HIV-infected cells, JA2/R7hyg cells were expanded to several liters, and MHC-bound peptides were isolated by TFA extraction, ultrafiltration, and reverse phase HPLC fractionation. When the HPLC fractions were added to uninfected A2 target cells in cytotoxicity assays, only a single HPLC fraction had sensitizing activity for each of the two A2-restricted HIV-specific CTL examined: fraction 41 for CTL 115Ip (Fig. 4A) and fraction 32 for CTL 68A62 (Fig. 5A).

To identify the active peptides in these fractions, their retention times were compared with those of all the synthetic peptides that could be recognized by the same CTL. Under an appropriate set of HPLC conditions (column, solvents, gradient, etc.), peptides differing even slightly in length or sequence can be resolved and therefore differentiated based on characteristic retention times. Thus, for all the active synthetic gag peptides tested using CTL 115Ip (including all possible 8-, 9-, and 10-mers contained within a longer active synthetic peptide), only gag-SL9 eluted in fraction 41 under the same HPLC conditions used for the JA2/R7hyg cell extract.
Figure 5. HIV-expressing cell line JA2/R7hyg produces a single peptide, RF-IV9, for recognition by CTL 68A62. (A) 10⁹ JA2/R7hyg cells were lysed in 1% TFA and prepared as described in Fig. 4 A, except that HPLC fractionation was performed using a gradient of 1% acetonitrile per min. Fractions were added to ³⁵S-labeled JY target cells and tested for toxicity (A) and for lysis by CTL 68A62 (B). (B) HPLC retention times of the synthetic peptides that can be recognized by CTL 68A62 are shown under the same chromatographic conditions used in A.

The Abundance of Two Naturally Processed HIV-1 Peptides. To measure the abundance of the naturally processed peptide gag-SL9, 10⁹ JA2/R7hyg cells were lysed and treated as described above, and the active HPLC fraction was titrated in a cytotoxicity assay using CTL 115Ip. As shown in Fig. 6 A, the amount of peptide recovered from 10⁷ or 10⁸ JA2/R7hyg cells gave 19 or 50% specific lysis, respectively. These values, compared with the standard curve from a cytotoxicity assay carried out under identical conditions with synthetic gag-SL9, correspond to a recovery of ≈100 peptide molecules per JA2/R7hyg cell. To estimate the overall efficiency of peptide recovery, a "spiking" experiment was performed in which 100 pg synthetic gag-SL9 was added to 10⁹ untransfected JA2 cells. Subsequent extraction and fractionation...
steps were identical to the treatment of JA2/R7hyg cells, culminating in cytotoxicity assay of HPLC fractions using CTL 115lp (Fig. 6 B). From the percent specific lysis due to either 5 or 20% of the sample in fraction 42 (5 pg or 20 pg if the overall yield was 100%), comparison to a standard curve for synthetic gag-SL9 led to an estimated 25% overall yield (e.g., 20% of the sample gave 35% specific lysis, corresponding to 5 pg synthetic gag-SL9 on a standard curve generated in the same assay; standard curve not shown). Similarly, for synthetic gag-RL10 added to uninfected JA2 cells, the overall yield was 30%. Since even a very tightly A2-binding peptide (radiolabeled RT-IV9) completely dissociated from A2 after 60 min of exposure to 1% TFA (38), the calculated yield likely reflects all steps in the isolation of naturally processed gag-SL9, including the release of peptide from preexisting peptide-MHC complexes.

The abundance of naturally processed peptide RT-IV9 was established similarly by comparison with a standard curve of synthetic RT-IV9. The amount of peptide recovered from 2 x 10⁹ JA2/R7hyg cells gave the same degree of lysis as 6 x 10⁵ molecules of synthetic RT-IV9 when added to uninfected A2* target cells (i.e., a synthetic peptide concentration of 5 x 10⁻¹² M in a total volume of 200 μl; not shown). Therefore only =3 molecules of RT-IV9 per HIV-infected cell were recovered, for a calculated abundance of =12 molecules per infected cell (assuming a similar overall yield to gag-SL9 and gag-RL10).

Expression of an HLA-A2–binding HIV-1 Peptide Occurs Independently of HLA-A2. Given numerous findings by Remmers et al. (46) that peptides recognized by CTL accumulate to detectable levels only in cells expressing the restricting MHC molecules, we were surprised to find a strong peak of activity corresponding to gag-SL9 in TFA extracts of the A2* infected cell line H9/R7/neo (Fig. 7). However, it remains unknown whether gag-SL9 binds to a different HLA molecule expressed by H9 cells, e.g., HLA-A1, Bw62, or Cw6.

Discussion

The reasons for incomplete elimination of HIV-infected cells by CTL in vivo are not yet understood (20, 21). One possibility is that high mutation rates in viral proteins could impair recognition and lysis of infected cells by virus-specific CTL (22), much as mutations in viral surface glycoproteins can result in escape from antibody recognition (47). To date, evidence both for and against the emergence of CTL escape mutants in HIV infection has been published (23, 24, 48, 49). Another mechanism for diminished CTL activity in HIV infection may be altered lymphokine production by "helper" T cells as a result of steadily declining CD4+ T cell counts and aberrant CD4+ T cell function in infected individuals. However, some individuals with low CD4+ T cell levels retain vigorous CTL activity (50); furthermore, intact CD8+ CTL function in CD4-deficient "knockout" mice argues against an absolute dependence of CTL on CD4+ T cells (51). Here we raise a third possibility: CTL of HIV-infected individuals may be incompletely effective in eradicating the virus because of a paucity of viral epitopes expressed on infected cells.

When cells are infected by lytic viruses (including influenza, vaccinia, Sendai, and VSV), host cell protein synthesis shifts toward virus-encoded products at the expense of endogenous ("self") protein synthesis. This overproduction of viral proteins gives rise to relatively high levels of viral peptides, which in turn form high cell surface densities of peptide–MHC-I complexes and facilitate lysis of the infected cells by virus-specific CTL. Accordingly, previous studies of naturally processed viral peptides seen by T cells have made use of lytically infected cells (52–54). However, infection by HIV (or by certain other viruses, such as EBV and CMV) can result in chronic expression of viral proteins for months or years, along with the full complement of normal self proteins necessary for cell viability and function. In these cases, a relatively small number of viral proteins (i.e., =10 for HIV) must compete with all the proteins normally expressed in a cell (perhaps 5,000–10,000) for presentation by a limited number of MHC-I proteins to CD8+ T cells. Can HIV-derived peptides compete with such a vast excess of self peptides and occupy sufficient numbers of MHC-I binding sites to result in effective killing of HIV-infected cells by CTL?

Previous studies of peptides recognized by HIV-specific T cells have relied on vaccinia virus recombinants containing HIV genes or gene segments and synthetic peptides corresponding to HIV sequences (27, 28). While these studies have led to the delineation of numerous T cell epitopes, oper-
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In contrast, the amount of peptide recovered from JA2/R7hyg even at a CTL/target cell ratio of a method of establishing infection tends to result in cultures Edman degradation or mass spectrometry. what is usually required for direct chemical sequencing by cells is typically in the low or subfemtomole range, well below urally processed peptide(s) to be identified and quantitated. under well-defined HPLC conditions is highly reproducible, assay (e.g., Fig. 7). Establishment of the infected B cell line JY/R7hyg, CD4+ by flow cytometry, was also made possible by this technique of transfection and direct selection. Other HIV-infected A2-negative cell lines have previously been established for various studies, including derivatives of A3.01, H9, HeLa-T4+, and U937 cells (56).

The RT-specific CTL clone 68A62 lysed cell lines T1/R7hyg and JY/R7hyg reasonably well, but hardly lysed JA2/R7hyg even at a CTL/target cell ratio of 50:1. Since JA2/R7hyg was lysed well by CTL 115Ip, these results suggested that levels of the naturally processed peptide(s) seen by 68A62 may be low on JA2/R7hyg cells. To address this issue directly, we applied techniques described by Rammensee et al. (46) to determine the precise identity and abundance of this naturally processed peptide(s). Briefly, HIV-infected target cells were treated with TPA, the extracted peptides were fractionated by reverse phase HPLC, and individual fractions were added to uninfected A2+ cells for cytotoxicity assay. Because the naturally processed peptides from, say, 109 cells can be loaded onto 106 A2+ target cells in a cytotoxicity assay, the sensitivity of peptide detection is amplified by as much as 10,000-fold (or even more if more cells are extracted, subject to solubility and toxicity limitations). Another advantage of this assay system is that both A2+ and A2− infected cells can be examined for the presence of a particular peptide, since A2+ target cells are used in the final assay (e.g., Fig. 7). Because the retention time of a given peptide under well-defined HPLC conditions is highly reproducible, comparison with synthetic peptide standards allows the naturally processed peptide(s) to be identified and quantitated. (In contrast, the amount of peptide recovered from 108−109 cells is typically in the low or subfemtomole range, well below what is usually required for direct chemical sequencing by Edman degradation or mass spectrometry.) By these methods it was established that the single naturally processed peptides recognized by CTL 115Ip and 68A62 were gag-SL9 and RT-IV9, respectively (Figs. 3–5). Both peptides matched the optimally active synthetic peptides recognized by these CTL, and both conform to the A2 consensus motif (57).

The abundance of naturally processed gag-SL9 was found to be ~400 molecules per infected cell by measuring the recovery of this peptide from JA2/R7hyg cells (~100 molecules/cell) and the efficiency of the isolation procedure (25%) (Fig. 6). Assuming that half the losses are associated with the HPLC step (50% yield), repeated rounds of HPLC fractionation will result in much more severe losses. For example, the purification of two overlapping peptides from the enzyme α-ketoglutarate dehydrogenase that are recognized by an allosreactive CTL clone was accomplished by five (58) or six (59) successive HPLC rounds before sequencing; if each round was associated with a 50% yield, the cumulative yield would have been only 1–3%. In this scenario, the natural abundance of these two peptides is far greater than that of the HIV-1 gag peptide reported here: since ~400 (58) or 7,000 (59) peptide molecules per allogeneic cell were actually recovered, their abundances are likely to be as high as 12,000 and 400,000 molecules per cell, allowing for the expected yields. Such high abundances of naturally processed peptides in the allosreactive system may result from high levels of their precursor, a normal cell protein, and probably account for the appearance of a family of peptides rather than the single peptide seen by two HIV-specific (as well as other virus-specific [53–54]) CTL.

The extent of lysis by CTL 115Ip (as much as 80% specific lysis of JY/R7hyg target cells) indicates that HIV-1 peptides can compete with the myriad self peptides generated in the same cells for binding to MHC-1 proteins and transport to the cell surface. In fact, the levels of gag peptide (~400 molecules per infected cell) were not much lower than the recoveries of two influenza peptides from influenza virus–infected cells (220–540 molecules/cell, uncorrected for extraction efficiencies [60]), despite the significantly different nature of the two infections (lytic vs. chronic). For VSV-infected cells, however, van Bloek and Nathenson reported that 5–10% of MHC-bound peptides were of viral origin, and that these were dominated by a single octamer, implying a significantly higher abundance in this system (52). A naturally processed Listeria monocytogenes peptide epitope similarly was present at >7,000 copies per infected cell (61). But in cells transfected with the ovalbumin gene and recognized by ovalbumin-specific CTL, only ~100 molecules of a sensitizing ovalbumin peptide were recovered (no correction for extraction efficiency) (62).

In contrast to CTL 115Ip, CTL 68A62 exhibited lower levels of activity against HIV-infected target cells, and lysed JA2/R7hyg only poorly even though the optimally active peptide RT-IV9 was shown to be present in these same cells. This result can be explained by the relatively low abundance of RT-IV9 on JA2/R7hyg cells (~12 molecules per cell), consistent with the 10–20-fold lower expression of RT than gag protein in HIV-infected cells due to a ribosomal frameshift required for RT expression (63). Since growth of JA2/R7hyg cells under selective pressure for HIV expression might have
resulted in higher than normal levels of HIV-1 peptides. RT-IV9 could be even more sparse on naturally infected cells. 

T1/R7hyg cells, which are lysed better than JA2/R7hyg cells by CTL 68A62, probably have a higher epitope density, as suggested by higher levels of p24 antigen (not shown) and by the fact that uninfected T1 and JA2 cells are similarly sensitive to exogenous RT-IV9 (SD90 = 10–20 pg/ml). Thus, in spite of the extreme sensitivity of KT1/R7hyg to synthetic RT-IV9 (Fig. 3B) and the demonstration of RT-IV9 in JA2/R7hyg cell extracts (Fig. 5), CTL 68A62 lysed JA2/R7hyg cells inefficiently (relative to their lysis by CTL 115Ip, Fig. 2), emphasizing the critical role of epitope density in lysis of these infected target cells and raising a serious question as to the likely effectiveness of this CTL clone in vivo.

In this context it is worth noting that when synthetic peptides are added to target cells in cytotoxicity assays, very high peptide concentrations are often used, e.g., 10–100 μM. At such concentrations, peptides with even modest affinities for the restricting MHC molecule (K° = 10¹⁰–10¹⁴ M⁻¹) will occupy a large fraction of available MHC binding sites at equilibrium (53, 54, 62, 64). For example, CYJ cells have ~10,000 A2 molecules available for binding to exogenous peptides (38), so peptide RT-IV9 (equilibrium constant for binding to A2 = 10⁶ M⁻¹) will occupy >99% of these sites. Thus, for efficient lysis of target cells, it is still possible that CTL bearing high-affinity receptors may lyse infected target cells having very low epitope densities (Kageyama et al., manuscript submitted for publication; and Sykulev et al. [64a]); however, no information is currently available regarding T cell receptor affinities for HIV-specific CTL. The outcomes of adoptive therapy trials using CTL in HIV-infected humans (65, 66) may therefore depend on T cell receptor affinities as well as on the numbers and specificities of the CTL used. The most effective CTL against HIV-infected cells ultimately may be those that can recognize the most abundant naturally processed HIV epitopes; using the stably infected cell lines described here, it should be possible to identify and quantitate these epitopes.

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References


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CHAPTER 3

Naturally processed peptides recognized by an alloreactive CTL clone
Overview

Alloreactions, in which T cells of a given haplotype respond to target cells of the same species expressing one or more different MHC products ("allogeneic"), have long been of interest both because of their importance in graft rejection and because of an arresting puzzle: the frequency of alloreactive T cells is at least 100 times greater than that of T cells specific for a conventional foreign antigen, e.g. one encoded by a virus (Fischer Lindahl and Wilson, 1977; Teh et al., 1977). Two contrasting models were proposed to account for this phenomenon. In one, the large number of alloreactive T cells was thought to reflect recognition of a multitude of specific antigens (not yet known to be peptides) in combination with MHC products (Matzinger and Bevan, 1977). In the other model, a high cell surface density of allogeneic MHC molecules (ca. $10^5$ per target cell, vs. many fewer for a conventional foreign antigen) permits T cells with low affinity receptors to respond directly to MHC molecules independently of any specific foreign antigens (Bevan, 1984).

At the outset of the work described in this chapter, one goal was to establish whether the alloreaction mediated by a well-characterized CD8+ T-cell clone (termed 2C; Kranz et al., 1984a, b; Saito et al., 1984a, b; Sha et al., 1988a, b) is dependent upon peptides (as components of peptide-MHC-I complexes) or whether MHC-I molecules devoid of peptides can trigger allore cognition. Studies in which HPLC fractions from extracts of allogeneic cells were capable of sensitizing target cells for allore cognition (Heath et al., 1989, 1991; Rötzschke et al., 1991b; Heath and Sherman, 1991) had provided evidence for the first view, but no specific peptide sequences were known to be involved in MHC-I-restricted alloreactions.
CTL clone 2C, derived from an H-2\textsuperscript{b} mouse immunized with H-2\textsuperscript{d} cells, was known to lyse L\textsuperscript{d+} target cells such as the mastocytoma cell line P815 (Kranz et al., 1984a). Screening other L\textsuperscript{d+} cells as targets for 2C led to the identification of certain cell lines killed only poorly by 2C but killed well by other peptide-specific, L\textsuperscript{d} restricted CTL (in the presence of appropriate peptides). Combined with HPLC fractionation of L\textsuperscript{d+} cells (either whole cell extracts or extracts of affinity-purified L\textsuperscript{d} molecules), a scheme was now available for purification of the peptide(s) responsible for allore cognition by CTL clone 2C (Udaka et al., 1992; see figure, p. 37). Tissues (spleen, thymus, and later liver and others (Wu et al., 1995; see p. 150)) proved to be better sources of peptide than cultured cells, and ultimately the sequence of an octameric peptide was obtained from 263 H-2\textsuperscript{d} mouse spleens by purifying the peptide to homogeneity through 5 rounds of HPLC under different conditions. Approximately 5-7 pmoles of purified peptide were isolated, just enough for sequence determination by a combination of Edman degradation and mass spectrometry (Udaka et al., 1992). An ambiguity at the C-terminus, either leucine or isoleucine, could not be resolved by these analytical methods, so two peptides were synthesized: p2CL (LSPFPFDL) and p2CI (LSPFPFDI). Peptide p2CL (later renamed p2Ca) was chosen as the naturally occurring sequence because: (1) its HPLC retention time agreed with the biological material (p2CI eluted \(\approx 1\) min earlier); and (2) it was more active in cytotoxicity assays than p2CI.

Although still formally possible that p2Ca confers upon L\textsuperscript{d} a conformation which enables it to be recognized by the 2C TcR without any direct contact between TcR and peptide, an interpretation eagerly promoted by one reviewer of the Udaka et al. manuscript, the weight of crystallographic evidence from three-dimensional structures
of MHC-I molecules containing mixtures of peptides (Bjorkman et al., 1987a; Garrett et al., 1989) or single peptides (Fremont et al., 1992; Zhang et al., 1992; Silver et al., 1992; Madden et al., 1993), together with the observation that other strongly Ld-binding peptides do not elicit recognition by the 2C TcR, strongly favors the view that p2Ca is an integral part of the ligand recognized by 2C. Structural determination of p2Ca-Ld complexes in association with the 2C TcR should definitively put this question to rest.

To our surprise, not one but three distinct peaks of sensitizing activity for lysis by 2C were evident in HPLC fractions of extracted tissues or cells. Identification of the first peptide was followed by isolation (through 6 rounds of HPLC) and characterization of a second 16-residue peptide containing the entire sequence of the first (p2Cb, VATIREQLSPFPFDL; Udaka et al., 1993). The protein of origin for p2Ca and p2Cb was revealed by noting 87% sequence identity between p2Cb and human α-ketoglutarate dehydrogenase (α-KGDH) and then cloning and sequencing part of the corresponding mouse gene to confirm an exact match with this protein (thereby also verifying the C-terminal leucine) (Udaka et al., 1993). The unusual length of p2Cb relative to other MHC-I-binding peptides and its sequence overlap with p2Ca immediately prompted two questions: (1) was recognition of p2Cb by 2C cells due to a direct interaction between the peptide and Ld, to degradation of p2Cb (e.g. to p2Ca) during cytotoxicity assays, or to contamination of p2Cb by small amounts of other peptides? and (2) does p2Cb represent a physiological precursor of p2Ca, thereby providing an opportunity to shed light on endogenous antigen processing pathways? The first issue is explored in Udaka et al. (1993; see p. 132-133). The second remains an active area of investigation, as described briefly here.
One of the major proteolytic activities in cytosol is a 700 kDa multisubunit complex termed the proteasome (or 20S proteasome for its sedimentation coefficient) (Arrigo et al., 1988; reviewed by Orlowski, 1990; Tanaka et al., 1992; Goldberg, 1992). An even larger complex responsible for degrading ubiquitin-conjugated proteins, the 26S proteasome, contains the 20S proteasome (Driscoll and Goldberg, 1990). Two of the proteasome subunits were found to be identical to previously identified (Monaco and McDevitt, 1982) MHC-encoded products designated LMP-2 and LMP-7 (for low molecular mass polypeptide) (Brown et al., 1991; Glynne et al., 1991; Ortiz-Navarrete et al., 1991). Because of their location within the MHC and their induction by interferon-γ (along with class I and II MHC genes and TAP genes), LMP-2 and LMP-7 were seized upon as candidate genes encoding components of the endogenous antigen processing pathway. These hopes suffered when mutant cell lines with a large MHC deletion encompassing both LMP and TAP genes regained antigen presenting capability upon transfection with TAP (but not LMP) genes (Momburg et al., 1992; Arnold et al., 1992; Yewdell et al., 1994; Zhou et al., 1994). On the other hand, studies using purified proteasomes (Driscoll et al., 1993; Gaczynska et al., 1993), mice with disrupted LMP genes (Fehling et al., 1994; Van Kaer et al., 1994), or proteasome inhibitors (Rock et al., 1994) have shown that the substrate specificity of proteasomes is dependent upon LMP-2/LMP-7 and that MHC-I expression and/or antigen presentation are altered in the absence of LMP genes.

With a few recent exceptions (Boes et al., 1994; Dick et al., 1994), previous studies carried out using purified 20S proteasomes have relied on amino acids or very short peptides (up to three residues) coupled to fluorescent labels. Inasmuch as endogenous precursors of antigenic peptides must be longer than octamers or nonamers,
we sought to exploit the isolation of a naturally occurring 16-mer (p2Cb) by testing whether it could be converted in vitro to p2Ca or other L\textsuperscript{d}-binding peptides. Naomi Fukusen purified 20S and 26S proteasomes according to Tanaka et al. (1986) and Driscoll and Goldberg (1990), and we exposed highly-purified peptide p2Cb to these proteasomes and analyzed the products by HPLC and mass spectrometry (with Ioannis Papayannopoulos and Klaus Biemann, M.I.T. Department of Chemistry). We found progressive exoproteolytic trimming at the N-terminus to nine residues, as well as evidence for a single endoproteolytic cleavage between leucine and serine to give an inactive 9-mer (VAITRIEQL) and an active but not apparently naturally occurring 7-mer (SPFPFDL) (unpublished data). The significance of these data is still under consideration.

A parallel study addresses the same question by means of the human TxB hybridoma cell line T1, its derivative mutant line T2 lacking LMP and TAP genes, and a transfectant of T2 expressing TAP-1 and TAP-2 that was made available to us by Frank Momburg (German Cancer Research Center, Heidelberg) (Momburg et al., 1992). A cDNA clone for L\textsuperscript{d} was obtained from Peter Cresswell (Yale University) and transfected into T1 and T2/TAP; T2 cells transfected with the same L\textsuperscript{d} gene were already available from P. Cresswell. Using these three cell lines – T1-L\textsuperscript{d}, T2-L\textsuperscript{d}, and T2/TAP-L\textsuperscript{d} – we could determine whether or not the steady-state levels of p2Ca and p2Cb depend upon TAP and LMP genes. These experiments are still in progress; however, the preliminary data shown below, as well as cytotoxicity assays in which T2-L\textsuperscript{d} and T2/TAP-L\textsuperscript{d} cells are lysed by 2C cells to exactly the same extent, suggest that p2Ca and p2Cb are present in all three cell lines, although probably at different absolute and relative levels.
The third peak of sensitizing activity for 2C cells in HPLC fractions of mouse tissue extracts ("peak c") has resisted vigorous efforts toward sequence determination and at present remains enigmatic, except that it is known to be recognized by the same 2C TcR on the basis of inhibition by a monoclonal antibody specific for this TcR (antibody 1B2, Kranz et al., 1984b). However, a fourth peak of activity found only in liver extracts contains a peptide that has recently been purified and sequenced (Wu et al., 1995): it is an octamer with a single phenylalanine-to-tyrosine difference from p2Ca (LSPYPFDL, hence p2Ca-Y4). This naturally occurring peptide was proposed to arise from post-translational modification by liver microsomal phenylalanine hydroxylase (Guroff et al., 1967) of phenylalanine at position four in peptide p2Ca. In view of the human autoimmune disease primary biliary cirrhosis, in which the major targets of autoantibodies are α-KGDH and the related pyruvate and branched chain α-ketoacid dehydrogenases (Burroughs et al., 1992), and recent findings by Connolly (1994) that peptide p2Ca is immunodominant in certain alloreactions, it seems warranted to explore whether individuals with this autoimmune disease have CTL specific for peptides derived from α-KGDH (or the other two dehydrogenases). The possible generation of antigenic peptides by post-translational modification could represent one pathway by which normal self-tolerance mechanisms (i.e., negative selection of T cells in the thymus) are thwarted, although not by any means the only such pathway.

A fifth peptide potentially involved in 2C allore cognition (QLSPFPFDL, termed QL9) is especially interesting for several reasons. (1) This peptide, originally detected as a product of the reaction between purified p2Cb and isolated 20S proteasomes, is more active than any of the naturally occurring peptides recognized by 2C that have been identified (i.e., p2Ca, p2Cb, and p2Ca-Y4). The SD50 for peptide QL9 is ca.
10^{-12} \text{ M}, comparable to the most active synthetic peptides that have been described in other systems (Bodmer et al., 1988; Reddehase et al., 1989; Rötzschke et al., 1990b, 1991a; Falk et al., 1991b; Schulz et al., 1991; Tsomides et al., 1991, 1994; Wallny et al., 1992a; Cox et al., 1994). (2) By virtue of its N-terminal glutamine residue, peptide QL9 can exist in two distinct forms – one with a free amino terminus and one with cyclized pyrrolidone carboxylate at the N-terminus (see below) – and both forms are recognized well by 2C. In fact, the cyclized version of QL9 has an approximately 5- to 10-fold lower SD_{50} than the linear form (unpublished data). (3) Whether or not QL9 is a naturally processed peptide remains unknown, despite much effort, but is possible because cyclized QL9 serendipitously co-migrates with p2Cb on HPLC and linear QL9 co-migrates with p2Ca; thus QL9 may have evaded detection during our original HPLC mapping of the peptides recognized by 2C. (4) the affinity of the 2C TcR for QL9•L^{d} complexes is the highest so far measured for any T cell (=1.5 \times 10^{7} \text{ M}^{-1}, \text{ Sykulev et al., 1994; see below}).

Efforts to detect QL9 in tissue extracts have exploited the absence of a free amino group in the cyclized form of this peptide. Thus, after a three day incubation at 37°C in 1% TFA to achieve quantitative cyclization, extracts of liver (or other tissues) are chemically modified with amino-reactive reagents (e.g. isothiocyanate groups attached to glass beads or dinitrofluorobenzene); only N-terminal cyclized, lysine-free
peptides are unaltered by these protocols. Despite our attempts, it remains uncertain whether QL9 is a naturally processed peptide or simply an interesting synthetic peptide involved in a heteroclitic T cell cross-reaction; current data suggest that if it is present, QL9 is much less abundant than the other naturally processed peptides recognized by 2C.

A list of some of the peptides recognized by 2C and their properties is provided in the table on p. 120. To date, no other naturally processed peptides recognized by alloreactive CD8+ T cells are known to have been sequenced.

A second surprise in the original study by Udaka et al. (1992) was the presence of p2Ca and p2Cb (as well as peak c) in tissues of other haplotype mice besides H-2d (i.e., H-2b and H-2k). This became more understandable once it was established that the protein source of p2Ca and p2Cb is a ubiquitous cellular enzyme essential for intermediary metabolism (Udaka et al., 1993). Since clone 2C originated in an H-2b mouse, the presence of p2Ca in this haplotype raised the question of whether p2Ca can be recognized by the 2C TcR in association with a self MHC-I protein (i.e., K\textsuperscript{b} or D\textsuperscript{b}). Dutz et al. (1994) showed that peptide p2Ca is in fact recognized by 2C cells in association with K\textsuperscript{b} (but not D\textsuperscript{b}), although at 1000-fold higher peptide concentrations than required for L\textsuperscript{d}-restricted recognition, and Kageyama et al. (1995) showed that the affinity of p2Ca for K\textsuperscript{b} was only \approx 10-fold lower than its affinity for L\textsuperscript{d}.

Since there is no apparent autoimmune reaction against p2Ca in normal H-2b mice or in H-2b mice transgenic for the 2C TcR (Sha et al., 1988a), p2Ca•K\textsuperscript{b} complexes, although present in these mice, apparently do not achieve a sufficiently high density to elicit a response from 2C cells. Indeed, calculations suggest that \approx 90-fold
more p2Ca•K\textsuperscript{b} than p2Ca•L\textsuperscript{d} complexes are needed to activate 2C, while the amounts of peptide recovered from H-2\textsuperscript{b} and H-2\textsuperscript{d} tissues are about the same. Commensurate with these findings, Sykulev et al. (1994) measured an affinity of the 2C TcR for p2Ca•K\textsuperscript{b} that is about 700-fold lower than its affinity for p2Ca•L\textsuperscript{d} (3\times10\textsuperscript{3} M\textsuperscript{-1} vs. 2\times10\textsuperscript{6} M\textsuperscript{-1}). However, weak TcR reactions are hypothesized for the positive selection of immature thymocytes, and the possibility that p2Ca might positively select T cells bearing the 2C TcR remains open, particularly in light of recent work in other systems (Ashton-Rickardt et al., 1993, 1994) and because positive selection of 2C TcR\textsuperscript{+} cells has been shown to depend upon K\textsuperscript{b} (not D\textsuperscript{b}) and to involve one or more peptides (Sha et al., 1990).

Like p2Ca, the liver-specific peptide p2Ca-Y4 binds to K\textsuperscript{b}, apparently with a K\textsubscript{a} of \approx 10\textsuperscript{5} M\textsuperscript{-1} (Wu et al., 1995; see p. 150). However, p2Ca-Y4•K\textsuperscript{b} complexes are not recognized by the 2C TcR in cytotoxicity assays, illustrating that a low-affinity T cell reaction (between the 2C TcR and p2Ca•K\textsuperscript{b}) nevertheless exhibits striking specificity (p2Ca and p2Ca-Y4 differ by only a single oxygen atom). This observation supports the idea of an affinity threshold for eliciting T cell responsiveness, and suggests it may be \approx 10\textsuperscript{3} M\textsuperscript{-1}, given a high enough ligand density (Wu et al., 1995).

The affinity of the 2C TcR for the QL9•L\textsuperscript{d} complex, in contrast, is the highest so far measured for any TcR (\approx 1.5\times10\textsuperscript{7} M\textsuperscript{-1}, Sykulev et al., 1994; see p. 145). As elaborated by Eisen et al. (In press; see appendix, p. 317), this relatively high value may be related to the fact that the reaction between the 2C TcR and QL9•L\textsuperscript{d} is an alloreaction; specifically, since T cells are not exposed to complexes of peptides and allogeneic MHC proteins in the thymus during their development, there is no opportunity for cells with TcR having high affinities for such complexes to undergo
negative selection. Some of these cells may thus be positively selected (by low-affinity interactions with self peptide-MHC complexes) and emerge from the thymus with the potential for high-affinity alloreactions. This, together with the fact that polyclonal alloreponses probably involve many different peptide-MHC ligands, may account for the intensity and high frequency of alloreactions (Matzinger and Bevan, 1977; Fischer Lindahl and Wilson, 1977).

In addition to its strong reaction with QL9-Ld complexes (alloreaction) and a presumably weak reaction with one or more peptides in association with K\textsuperscript{b} (positive selection), it is likely that CTL clone 2C can recognize a specific foreign peptide (or peptides) in association with a self MHC-I protein, i.e., its “normal” peptide-MHC-I ligand. An experiment was undertaken to search for such a putative foreign peptide, using two libraries of peptides developed by Schumacher et al. (1992) and made available by Hidde Ploegh. These synthetic peptide libraries contain mixtures of 8-mers or 9-mers, respectively, based on the sequences of known K\textsuperscript{b}-binding peptides; their overall complexity was limited to 432 (8-mers) or 864 (9-mers). Each library was fractionated by reverse phase HPLC, and the fractions were screened in a cytotoxicity assay using 2C CTL and T2-K\textsuperscript{b} target cells. No fractions contained sensitizing activity, indicating that these libraries probably do not contain the postulated foreign peptide recognized by 2C cells as part of a self MHC-I (K\textsuperscript{b})-restricted response. It remains possible that a more extensive peptide library would harbor such a peptide.

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**Table.** Some peptides recognized by CTL clone 2C and their properties.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>MHC-I</th>
<th>$K_a$ for peptide–MHC reaction (M$^{-1}$)§</th>
<th>$K_a$ for 2C TcR–peptide–MHC reaction (M$^{-1}$)†</th>
<th>SD$50$ value (M)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>p2Ca</td>
<td>LSPFPFDL</td>
<td>L$^d$</td>
<td>5–10 x 10$^5$</td>
<td>2 x 10$^6$</td>
<td>10$^{-9}$ - 10$^{-10}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K$^b$</td>
<td>1 x 10$^5$</td>
<td>3 x 10$^3$</td>
<td>10$^{-6}$</td>
</tr>
<tr>
<td>p2Ca-Y4</td>
<td>LSPYPFDL</td>
<td>L$^d$</td>
<td>5 x 10$^5$</td>
<td></td>
<td>10$^{-9}$ - 10$^{-10}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K$^b$</td>
<td>1 x 10$^5$</td>
<td></td>
<td>no activity</td>
</tr>
<tr>
<td>p2Ca-Y6</td>
<td>LSPFPYDL</td>
<td>L$^d$</td>
<td></td>
<td></td>
<td>10$^{-9}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K$^b$</td>
<td></td>
<td></td>
<td>10$^{-7}$</td>
</tr>
<tr>
<td>p2Cb</td>
<td>VAITRIEQLSPFPFDL</td>
<td>L$^d$</td>
<td></td>
<td></td>
<td>10$^{-7}$ - 10$^{-8}$</td>
</tr>
<tr>
<td>QL9</td>
<td>QLSPFPFDL</td>
<td>L$^d$</td>
<td>2 x 10$^8$</td>
<td>1.5 x 10$^7$</td>
<td>5 x 10$^{-12}$</td>
</tr>
<tr>
<td>QL9-Y5</td>
<td>QLSPYPFDL</td>
<td>L$^d$</td>
<td></td>
<td></td>
<td>5 x 10$^{-11}$</td>
</tr>
<tr>
<td>I$_1$-QL9-Y5</td>
<td>QLSPY*PFDFL</td>
<td>L$^d$</td>
<td>2 x 10$^7$</td>
<td></td>
<td>5 x 10$^{-12}$</td>
</tr>
</tbody>
</table>

Values from Kageyama et al., 1995; Sykulev et al., 1994; and unpublished data.

Peptides in **boldface** are known naturally processed peptides.

§ at 37°C; † at room temperature.

* denotes stoichiometric labeling of tyrosine with $^{127}$I or $^{125}$I.

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![Graph 1](https://via.placeholder.com/150)

**T2-L$^d$**

peptide concentration (g/ml)

![Graph 2](https://via.placeholder.com/150)

**T2-K$^b$**

peptide concentration (g/ml)

120
A Naturally Occurring Peptide Recognized by Alloreactive CD8\(^+\) Cytotoxic T Lymphocytes in Association with a Class I MHC Protein

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Summary

The antigenic structures that initiate T cell responses to foreign (allogeneic) cells have long attracted considerable interest. We have purified and sequenced a peptide from mouse spleen that is recognized in association with the class I MHC protein H-2L\(^a\) by 2C, an alloreactive CD8\(^+\) T cell clone. The peptide (LSPFPFDL) greatly enhances the susceptibility of L\(^a\) cells to lysis by 2C, and this activity is completely blocked by a clonotypic antibody against the 2C T cell receptor. Thus, this study characterizes the naturally occurring peptide moiety of an MHC-I/peptide complex recognized by alloreactive CD8\(^+\) T cells. The peptide, which occurs in the thymus of MHC-disparate mice, can be used to study T cell development in mice expressing transgenes for the 2C T cell receptor.

Introduction

Several lines of evidence suggest that in its lifetime, a CD8\(^+\) T cell recognizes via the same receptor a variety of different antigenic structures, each consisting of a class I MHC protein (MHC-I) in association with a peptide. Thus, mature CD8\(^+\) T cells can function as cytotoxic T lymphocytes (CTL), which characteristically react with peptides of foreign (e.g., viral) origin in association with syngeneic ("self") MHC-I, i.e., foreign–self complexes. Moreover, the high frequency of CD8\(^+\) T cells that recognize any given allogeneic MHC-I (\(\sim 1:100\); Erard et al., 1985), taken together with the large number of different class I MHC alleles, indicate that many (probably most) CD8\(^+\) T cells can also recognize an allogeneic MHC-I (Bevan, 1977); and from considerable indirect evidence, it appears that the structures seen by most alloreactive CTL are formed by unknown peptides of allogeneic cells in association with the allogeneic cells’ MHC-I, i.e., what might be termed foreign–foreign complexes (Heath et al., 1989, 1991; Rożtsczek et al., 1991). In addition, during their development in the thymus, precursors of CD8\(^+\) T cells appear to respond selectively to unknown thymic peptides in association with syngeneic MHC, i.e., self–self complexes (Sha et al., 1990; Nikooz-Zugic and Bevan, 1990). The number of different peptide–MHC complexes that can be recognized (foreign–self, foreign–foreign, self–self) suggests that the specificity of a single T cell receptor (TcR) may be substantially degenerate.

To learn more about the diversity of antigenic structures seen by an individual TcR, we are studying the structures recognized by 2C cells, a clone of alloreactive CD8\(^+\) CTL. These cells are particularly useful for this purpose for several reasons. First, a clonotypic monoclonal antibody (1B2) to the well-characterized TcR of this clone provides a specific reagent with which to determine unambiguously whether a particular antigenic complex is recognized by this TcR (Kranz et al., 1984a). Secondly, genes for this TcR’s \(\alpha\) and \(\beta\) chain variable domains have been cloned and sequenced and used to generate transgenic mouse strains (Saito et al., 1984a, 1984b; Sha et al., 1988a). In transgenic mice expressing certain MHC-I, T cells that bear the 2C TcR are deleted in the thymus ("negative selection"), whereas in transgenic mice expressing certain other MHC-I, these T cells are stimulated to proliferate, mature, and populate peripheral tissues ("positive selection"); Sha et al., 1988b, 1990)

The 2C clone was originally derived from an H-2\(^b\) mouse (BALB.B strain) that had been inoculated with H-2\(^a\) cells (Sha et al., 1984a, 1984b; Kranz et al., 1984a). 2C cells are specific for L\(^a\) in the sense that they normally lyse only L\(^a\)-bearing target cells, and this activity is blocked by some antibodies to L\(^a\). In the present study, we have succeeded in purifying and determining the sequence of a naturally occurring peptide octamer recognized by 2C cells in association with L\(^a\). When synthesized, this peptide, whose sequence is LSPFPFDL, greatly enhanced the susceptibility of L\(^a\)-bearing target cells to lysis by 2C cells, and this activity was completely blocked by the clonotypic antibody to the TcR of 2C cells. Hence, this study characterizes the peptide moiety of a peptide–MHC-I complex that is recognized by alloreactive CD8\(^+\) T cells.

Results

Selection of a Target Cell for Screening Assays

The design of a cytotoxicity assay to screen for peptides that are recognized by 2C cells in association with L\(^a\) depended upon the identification of a target cell that expressed L\(^a\) but was not significantly susceptible to lysis by 2C until reconstituted with the appropriate peptide(s).

Table 1 lists the target cells that were considered. P815 and A20 cells were rejected because they were lysed very well by 2C without addition of exogenous peptides. T1.1.1 (L cells transfected with the L\(^a\) gene) were promising because they were killed poorly by 2C cells despite expressing reasonable levels of L\(^a\), but they were rejected because they were also killed poorly by an unrelated L\(^a\)-restricted CTL clone (8A5) in the presence of the synthetic peptide recognized by this clone (T2H), perhaps because T1.1.1 cells have low levels of the surface adhesion molecule ICAM (Table 1). The Sp2/0 myeloma cell line (H-2\(^d\) haplotype), which expresses low levels of L\(^a\) and other class I
MHC proteins (K\textsuperscript{\alpha}, D\textsuperscript{\alpha}, and L\textsuperscript{\alpha} were 42%, 25%, and 2%, respectively, of the corresponding levels on P815 cells), was killed poorly. After Sp2/0 was transfected with the L\textsuperscript{\alpha} gene under heterologous enhancer (immunoglobulin heavy chain enhancer, IgH) and promoter (metallothionein IIa) elements, one of the transfected clones, B2, expressed high levels of L\textsuperscript{\alpha} (100% of P815 cells) and yet was still killed poorly by 2C cells (about 15% specific \textsuperscript{51}Cr-release under the assay conditions employed). However, B2 cells could be lysed nonspecifically in the presence of concanavalin A by an unrelated CTL (4G3 cells, restricted by Kb, not L\textsuperscript{\alpha}), and they were fully competent as specific target cells for another L\textsuperscript{\alpha}-restricted CTL (clone 8A5 in the presence of the known cognate peptide T2H (see Table 2) was present at a concentration of 100 nM.

Several High Pressure Liquid Chromatography Fractions Sensitize B2 Target Cells for Lysis by 2C CTL

The peptide fraction (molecular mass < 10 kd) from acid-denatured homogenates of BALB/c thymus or spleen was subjected to high pressure liquid chromatography (HPLC) on a C18 reverse phase column, and the resulting fractions were tested for sensitizing activity using B2 as target cells and 2C as CTL in a \textsuperscript{51}Cr-release assay. As illustrated in Figure 1A, three active fractions, a, b, and c, were consistently observed with a thymus extract. The combined active fractions were pooled and rechromatographed on the same C18 column using a much shallower and thus more highly resolving gradient (0.067% B per min, gradient program 8). As shown for a thymus extract in Figure 1B, cytotoxic activity was again seen in three fractions (a, b, and c), and the broad peak at fraction a occasionally split into two discernible peaks.

The same activity profile was seen in extracts from BALB/c spleen or thymus, and also from P815 cells. Fractions a, b, and c from thymus eluted with the same retention times as the corresponding fractions from spleen (Figure 2) when samples were run on an analytical scale. However, fraction d elicited a stronger CTL response from the spleen preparations, and fraction c tended to give a higher response from thymus preparations. Fraction a

from thymus was used for additional characterization of the biological properties of the active components, and the corresponding fraction from spleen was subjected to further purification for sequence analysis.

Table 1. Selection of a Target Cell Line for the Cytotoxicity Screening Assay

<table>
<thead>
<tr>
<th>Flow Cytometric Measurement of*</th>
<th>Cell Diameter</th>
<th>sCr-Release Due to CTL*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell</td>
<td>L\textsuperscript{\alpha}</td>
<td>ICAM</td>
</tr>
<tr>
<td>P815</td>
<td>(100)</td>
<td>(100)</td>
</tr>
<tr>
<td>A20</td>
<td>53</td>
<td>121</td>
</tr>
<tr>
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<td>481</td>
</tr>
<tr>
<td>Sp2/0</td>
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<td>692</td>
</tr>
<tr>
<td>T1.1,1</td>
<td>90</td>
<td>12</td>
</tr>
<tr>
<td>L</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>

* Values are given as percent of the indicated protein (L\textsuperscript{\alpha}, ICAM) in P815 cells (100%), assuming linearity between fluorescence intensity and the cell surface density of each protein. Relative cell diameter is based on forward low angle light scattering.

Values separated by a comma are percent specific \textsuperscript{51}Cr-release from target cells tested at two CTL:target cell ratios, 1.25:1 and 2.5:1. All values are averages of duplicates. In assays with CTL clone 8A5, the tumor-specific peptide T2H (see Table 2) was present at a concentration of 100 nM.

Figure 1. HPLC Fractionation and Cytotoxicity Assay of Peptides Derived from BALB/c Thymus

Low molecular mass components (<10 kd) from homogenates of thymus were fractionated by reverse phase chromatography on a C18 column (Vydac). Fractions were collected at 1 min intervals (at a flow rate of 1.0 ml/min) and subjected to the standard cytotoxicity assay using \textsuperscript{51}Cr-labeled B2 cells as targets and 2C cells as CTL (CTL:target cell ratio = 3:1). Solid line, absorbance at 280 nm; line with filled circles, percent specific lysis by 2C, line with open circles, percent specific lysis in toxicity controls consisting of B2 target cells incubated for 4 hr with each fraction in the absence of 2C CTL (A) The initial chromatographic separation, using a gradient of 0.25% B per min (program 7). (B) Rechromatography of pooled fractions a, b, and c on the same C18 column, using a shallow gradient of 0.067% B per min (program 8).
Peptide Recognized by Alloreactive T Cell Clone

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Figure 2. HPLC Fractionation and Cytotoxicity Assay of Peptides Derived from BALB/c Spleen

Low molecular mass components (<10 kd) from homogenates of spleen were fractionated by reverse phase chromatography on a C18 column (0.067% B per min, program B). Fractions were collected at 1 mm intervals and assayed for cytotoxicity as in Figure 1.

Some Characteristics of Active HPLC Fractions

Pronase Sensitivity

Fractions a, b, and c (Figure 1B) lost their activity in cytotoxicity assays after they were incubated with pronase immobilized on agarose beads, but not after they were incubated with agarose beads lacking pronase (data not shown). Hence, the active components in these fractions appeared to be peptidic.

L$^d$ Restriction

To determine whether the recognition of peptides in the three active fractions by 2C cells was L$^d$ restricted, various cell lines were used as targets. Each of the active fractions enhanced lysis of B2 target cells might be due solely to an ability to increase the density of L$^d$ on target cells, various peptides known to associate with L$^d$ were tested on RLA-E2 cells. These cells were produced by transfecting the L$^d$ gene into RMA-S cells (see Experimental Procedures). After RLA-E2 cells were incubated overnight at 37°C with each of the three L$^d$-restricted peptides (5 µM) (see Table 2), they were analyzed by flow cytometry with antibody 30-5-7S (specific for the α1 and α2 domains of L$^d$; Hansen et al., 1988) to measure the cell surface density of L$^d$, and they were also tested as targets for 2C CTL. Surface expression of L$^d$ increased from 7% (in absence of the added peptides) to 17%, 23%, and 10% (of the mean log fluorescence level in P815 cells), but did not result in enhanced cytotoxicity by 2C CTL (data not shown). This observation and the fact that the active peptides in HPLC fractions a, b, and c from BALB/c thymus and spleen were doubtless present at only nanomolar or subnanomolar concentrations (see below) indicate that these peptides were recognized as components of antigenic complexes with L$^d$, rather than indirectly increasing cytotoxicity because of enhanced cell surface expression of L$^d$.

Recovery of Active Peptides from Affinity-Purified L$^d$ Molecules

The active peptides in spleen and thymus homogenates could have been derived from the cytosol or from cytosolic organelles, or they might have been bound by L$^d$ molecules before the homogenates were acid treated. To distinguish between these possibilities, we compared an acid extract of BALB/c thymus with acid-denatured L$^d$ that had at high concentrations (5 µM) to determine whether they interfered with the sensitizing activity of the peptides in the active HPLC fractions. As shown in Table 2, the activities of fractions a, b, and c were each inhibited by three peptides (MCMVH, MCMVR, T2H) that are either known to be recognized in association with L$^d$ or that competitively inhibit the activity of L$^d$-associated peptides. But they were not inhibited by two peptides (T2R, OVA2) having no known association with L$^d$.

Effect of Peptides on L$^d$ Expression

To determine whether the activity of HPLC fractions that enhanced lysis of B2 target cells might be due solely to an ability to increase the density of L$^d$ on target cells, various peptides known to associate with L$^d$ were tested on RLA-E2 cells. These cells were produced by transfecting the L$^d$ gene into RMA-S cells (see Experimental Procedures). After RLA-E2 cells were incubated overnight at 37°C with each of the three L$^d$-restricted peptides (5 µM) (see Table 2), they were analyzed by flow cytometry with antibody 30-5-7S (specific for the α1 and α2 domains of L$^d$; Hansen et al., 1988) to measure the cell surface density of L$^d$, and they were also tested as targets for 2C CTL. Surface expression of L$^d$ increased from 7% (in absence of the added peptides) to 17%, 23%, and 10% (of the mean log fluorescence level in P815 cells), but did not result in enhanced cytotoxicity by 2C CTL (data not shown). This observation and the fact that the active peptides in HPLC fractions a, b, and c from BALB/c thymus and spleen were doubtless present at only nanomolar or subnanomolar concentrations (see below) indicate that these peptides were recognized as components of antigenic complexes with L$^d$, rather than indirectly increasing cytotoxicity because of enhanced cell surface expression of L$^d$.

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Table 2. Inhibitory Effects of Synthetic Peptides on the B2-Sensitizing Activities of Peptides in HPLC Fractions a, b, and c

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Percent Inhibition of T2H-Dependent Lysis by CTL 8A5</th>
<th>Percent Inhibition of B2-Sensitizing Activity of HPLC Fraction</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>MCMVH</td>
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</tr>
<tr>
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<td>YPRFMPTNL</td>
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<td>70</td>
</tr>
<tr>
<td>T2H</td>
<td>ISTQNRADLVA</td>
<td>&lt;10</td>
<td>&lt;10</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>SSSNMEER</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For all assays, the inhibiting peptide was present at 5 µM. Peptides MCMVR and T2R are analogs of MCMVH and T2H, respectively, with a histidine to arginine substitution at the position shown. T2H is recognized by L$^d$ by CTL clone 8A5 (see also Lurquin et al., 1989). The ability of T2H at a concentration of 35 nM to sensitize L$^d$-bearing target cells for lysis by 8A5 cells was inhibited by MCMVH or MCMVR but not by T2R or the ovalbumin peptide OVA2. Thus, MCMVH and MCMVR as well as T2H appear to associate with L$^d$, whereas T2R and OVA2 do not.

*See Carbone and Bevan, 1990.
been purified from P815 cells. L4 molecules were affinity purified with monoclonal antibody 30-5-7S from P815 cell lysates; for control purposes D4 molecules were affinity purified from the same lysates with monoclonal antibody 34-5-8S (Ozato et al., 1982). The isolated L4 and D4 proteins were then treated with trifluoroacetic acid (TFA), subjected to ultrafiltration, and the filtrates were fractionated by reverse phase HPLC as before (Figure 1).

As shown in Figure 3, three active fractions were derived from purified L4, and none from purified D4. The first two active fractions very likely correspond to fraction c of the thymus extract, since, as noted earlier, this fraction from thymus samples tended to split into two peaks when a smaller amount of material was loaded. Fraction c from purified L4 was shown by rechromatography on an analytical scale to correspond to fraction c of the thymus extract. However, fraction b in spleen and thymus extracts, eluting at 53–54 min, was not detected in the acid extract of purified L4.

Direct Sequence Analysis of an Active Peptide

Due to the limited availability of material from thymus and P815 cells, a fraction of the spleen was used for further study. Fraction a was isolated from 26.7 g of spleen (from 263 BALB/c mice) by successive rounds of reverse phase chromatography on a C18 column using two different gradients as shown in Figure 1. It was then purified further by three successive rounds of reverse phase chromatography on a C8 column at pH 8.5, using the cytotoxicity assay after each round to identify an active peak for the next round. Finally the active fraction from the third C8 chromatography, representing an estimated 4.5 g of spleen, was subjected to Edman degradation. The analysis revealed 3–5 pmol of peptide, which was just sufficient to determine unambiguously the first six residues as LSPFFP; the seventh residue was questionably aspartic acid. An aliquot, estimated from Edman degradation to be about 2 pmol, was found by mass spectrometry by Drs. I. Papanayopoulos and K. Biemann (Department of Chemistry, Massachusetts Institute of Technology) to have a mass of 934.4. Based on this mass and the Edman data, the peptide was deduced to be an octamer with the sequence LSPFFPD plus a C-terminal leucine or isoleucine (indistinguishable by mass). Accordingly, two peptides were synthesized, one with leucine and the other with isoleucine at the C-terminus (termed p2CL and p2CI, respectively).

Both synthetic peptides were active in the cytotoxicity assay with 2C cells, but p2CL was 20 times more active than p2CI (Figure 4). Within the accuracy provided by collecting fractions at 1 min intervals, synthetic p2CL had the same retention time as the naturally occurring peptide in fraction a on HPLC gradient program 8 (Figure 2). Under the same HPLC conditions, p2CI eluted 1 min earlier than p2CL. Unlike reports with some other longer synthetic peptides (Rötzschke et al., 1990; Schumacher et al., 1991), when p2CL was subjected to HPLC, and multiple fractions were tested in the cytotoxicity assay, activity was found only in the fraction with the retention time of fraction a and not in other fractions, even when the column was deliberately overloaded. We conclude that p2CL corresponds to the active peptide in fraction a from homogenates of BALB/c spleen or thymus or P815 cells, as well
Peptide Recognized by Alloreactive T Cell Clone

30 with synthetic contains the ratio was were 2C tity of the receptor that recognizes the clarified. Therefore, to establish unambiguously the iden-

0-0 the other (or more) different receptors on these cells, a unique ap3 receptor (TcR) of these cells, one

1984b). When a cDNA library from 2C cells was originally studied to characterize the genes that encode the antigen-specific receptor (TcR) of these cells, one α chain, one β chain, and one γ chain cDNA were sequenced (Saito et al., 1984a, 1984b). Later, a second β chain cDNA was sequenced (Sha et al., 1988a). While there were thus potentially two (or more) different receptors on these cells, a unique αβ receptor was subsequently identified by a clonotypic antibody (1B2) (Kranz et al., 1984d). However, the possibility that 2C cells also have on their surface another TcR, using the other β chain, perhaps in the form of recently described β-β receptors (Goverman et al., 1990; Kishi et al., 1991; Punt et al., 1991; van Moerwijk et al., 1991), has not been clarified. Therefore, to establish unambiguously the iden-

Figure 5. The F(ab)2 Fragment of 1B2 (the Clonotypic Monoclonal Antibody to the TcR of 2C Cells) Blocks Peptide (p2CL)-Dependent Lysis of B2 Cells as Well as Lysis of P815 Cells (Including the Increase in Lysis of P815 Cells Due to p2CL) 2C cells were incubated with the 1B2 F(ab)2 fragment at 150 µg/ml for 30 min at 37°C before they were added to p2CL and target cells (either B2 or P815). The final concentrations of 1B2 F(ab)2 fragment and p2CL were 38 µg/ml and ~15 ng/ml, respectively, and the CTL:target cell ratio was 3:1.

as from affinity-purified Lf from P815 cells. A protein that contains the p2CL sequence has so far not been found in protein sequence data banks.

The peptide in fraction a from a thymus was compared with synthetic p2CL in a 4Cr-release assay. As shown in Figure 4, p2CL at about 350 pg/ml sensitized B2 target cells for half-maximal lysis. Slightly more lytic activity was elicited from fraction a isolated from 0.60 g of thymus (Figure 4). Since this amount of thymus corresponds to approximately 6 x 10^9 thymocytes, we recovered only about 100–200 molecules of the naturally occurring peptide per cell, assuming the peptide is present in all thymocytes. The peptide's actual abundance is unclear because the efficiencies of the extraction and purification procedures, as well as the extent to which the peptide is present in all or just a subset of thymus cells, are all uncertain at present.

The T Cell Receptor That Recognizes Peptide p2CL in Association with Lf When a cDNA library from 2C cells was originally studied to characterize the genes that encode the antigen-specific receptor (TcR) of these cells, one α chain, one β chain, and one γ chain cDNA were sequenced (Saito et al., 1984a, 1984b). Later, a second β chain cDNA was sequenced (Sha et al., 1988a). While there were thus potentially two (or more) different receptors on these cells, a unique αβ receptor was subsequently identified by a clonotypic antibody (1B2) (Kranz et al., 1984d). However, the possibility that 2C cells also have on their surface another TcR, using the other β chain, perhaps in the form of recently described β-β receptors (Goverman et al., 1990; Kishi et al., 1991; Punt et al., 1991; van Moerwijk et al., 1991), has not been clarified. Therefore, to establish unambiguously the iden-

by 2C cells is blocked by antibody 1B2. As shown in Figure 5, the F(ab)2 fragment of 1B2 completely blocked the lysis of p2CL-sensitized B2 and P815 target cells. (It was necessary to use the F(ab)2 fragment of this antibody because Fc receptors are present on B2 and P815 cells and can give rise to peptide- and MHC-independent lysis due to the 1B2 Fc domain, a process termed "redirected lysis" or "antibody-dependent cell-mediated" cell lysis.) The striking effect of the 1B2 F(ab)2 fragment clearly established that the antigenic structure formed by p2CL-Lf is recognized by the same 2C TcR that is expressed on CD8+ T cells of transgenic mice produced by Sha et al. (1988b). The β chain of this receptor is characterized further by its reactivity with monoclonal antibody F23.1 (Sha et al., 1988b, 1990), which is specific for β chain variable do-

Discussion

The historic role of the allograft reaction in the discovery of the MHC, and its importance for analyses of MHC-dependent processes underline ongoing efforts to understand the antigenic structures that are recognized by allo-

reactive T cells. A major question is whether the epitopes seen by alloreactive CD8+ T cells reside in allogeneic class I MHC proteins themselves, regardless of their associated peptides (Bevan, 1984; Kaye and Janeway, 1984; Portoles et al., 1989; Müllbacher et al., 1991), or in peptide-class I MHC complexes where unique combinations of particular peptides with particular MHC proteins determine each epi-
tope (Matzinger and Bevan, 1977; Kourilsky and Claverie, 1988). This paper provides an answer for 2C cells, an extensively studied alloreactive CD8+ T cell clone: we show that the well-characterized TcR of this clone recog-
nizes a particular peptide octamer (p2CL) in association with Lf. This finding underscores recent studies (Heath et al., 1991; Rötzschke et al., 1991) indicating that most alloreactive CTL see complexes of MHC-I and unknown peptides. In the case of an alloreactive CD4+ T cell clone (Panina-Bordignon et al., 1991), the ligand was shown to be a class II MHC protein (MHC-II) in association with a processed form of serum albumin. However, it remains possible that some alloreactive CTL might recognize pep-
tide-independent epitopes on allogeneic MHC-I (Müll-
bacher et al., 1991).

In establishing the sequence of p2CL, our results demon-
strate why the peptides associated with MHC-I proteins that are recognized by other alloreactive T cells have been so difficult to characterize. Thus, in starting with spleens from 263 mice, we ended up with just enough material of sufficient purity for sequence determination of a short peptide (molecular weight ~1000). We estimate that our yield of purified peptide (3–5 pmol) amounted to only about 400 molecules per splenic lymphocyte. In contrast to our results, three earlier studies that established the complete sequence of natural peptide adducts of class I MHC pro-
teins were all dependent on special circumstances. In two cases (Van Bleek and Nathenson, 1990; Falk et al., 1991a), the peptides were of viral origin, and information
Strains Are Recognized

Figure subjected to cytotoxicity assay as in Figure 1B

Low molecular weight material from spleens of BALB/c (H-2b), BALB.K, BALB.K (H-2k), and BALB.B (H-2d) mice was prepared, fractionated, and subjected to cytotoxicity assay as in Figure 1B.

about candidate sequences from synthetic peptides having optimal sensitizing activity on target cells provided powerful guidelines. No such guidelines are available to help characterize the peptides recognized by alloreactive cells. In other cases (Falk et al., 1991b; Jardetzky et al., 1991; Rudensky et al., 1991a, 1991b), the natural peptide adducts were sequenced not because of demonstrated biological activity but on the basis of their high abundance in purified MHC-I or MHC-II preparations. By establishing the sequence of a naturally occurring octapeptide that is recognized by alloreactive CD8+ cells, our results reinforce the emerging generalization that CD8+ T cells see octamers or nonamers in association with class I MHC proteins (for minireviews see Röttschke and Falk, 1991; Tsomides and Eisen, 1991).

It has been reported (Röttschke et al., 1991) that naturally occurring peptides associated with one allelic form of a class I MHC protein are usually not present on cells expressing other allelic variants of that protein. However, we have found that acid extracts of tissue homogenates of spleen or thymus from several MHC-disparate mouse strains (BALB/c [H-2k], BALB.B [H-2d], and BALB.K [H-2k]) have HPLC activity profiles that are essentially indistinguishable (Figure 6). The slight amount of variability seen can be attributed to discrete rather than continuous sampling (1 min fractions) and to extreme heterogeneity in the starting mixtures loaded onto the HPLC column (i.e., tissue homogenates).

Based on our finding that some of the active peptides in spleen and thymus homogenates from H-2d mice can also be recovered from affinity-purified L6, it is reasonable to suggest that the active components in corresponding homogenates from the other mouse strains are likewise associated with their class I MHC proteins. For instance, our results with BALB.B tissues suggest that p2CL, shown here to be an L6 adduct, may bind to K6 or D6. Yet in transgenic H-2d mice expressing the 2C TcR, thymocytes bearing this receptor are not subjected to negative selection (Sha et al., 1988b). Hence, we propose that although p2CL is present in the thymus of H-2d mice, it is either bound too weakly by K6 or D6 to form a sufficient number of stable complexes per cell, or such complexes are formed but are not bound strongly enough by the 2C TcR to result in negative selection. In the latter case, it is even conceivable that p2CL is involved in positive selection of T cells expressing the 2C TcR in transgenic H-2d mice (Sha et al., 1990). To distinguish between these alternatives, we are measuring the binding of p2CL to K6 or D6 on intact H-2d cells, using a recently described procedure (Tsomides et al., 1991).

The presence of p2CL in tissues of MHC-disparate mice raises a question about the designation of peptides as "foreign" or "self." For 2C cells, whose haplotype is H-2d, the p2CL molecules they see on H-2k target cells (in association with L32) arise in these allogeneic target cells and are thus clearly foreign. However, if the same peptide is present in H-2d cells but is invisible to TCRs of H-2d mice, including the TCR of 2C cells, it is not seen as a self peptide. Thus, whether a peptide behaves as self or nonself depends not only upon its presence or absence but upon its capacity to form complexes with self or nonself MHC proteins that are capable of being recognized by particular TcRs.

Recently, Falk et al. established by direct sequence analysis consensus motifs for the mixtures of peptides associated with several class I MHC proteins (K6, D6, K6, and HLA-A2.1) (Falk et al., 1991b). For L6, however, they detected only a single major residue, which was proline at the second position from the N-terminus. This finding agrees with the sequences of two L6-associated peptides of viral origin (Table 3). However, p2CL, the peptide seen by 2C cells in association with L6, has proline at positions 3 and 5. Since p2CL corresponds to one of several active peaks, it is possible that other active HPLC fractions contain related sequences differing from p2CL by one or two amino acid residues at the N- or C-terminus. For instance, it is possible that an L6-associated peptide in fractions b and c (Figure 1) lacks the N-terminal leucine of p2CL and has perhaps an additional one or two residues at the C-terminus and would thus have proline at position 2, in accord with the proposed motif. The recent discovery that three peptides bound to the MHC-I protein HLA-A2 share the same sequence but vary in length (9, 10, or 12 residues) offers a precedent for finding such a family of closely related peptide sequences bound to MHC-I (Henderson et al., 1992). Nevertheless, the biological activity of p2CL indicates that proline at position 2 is not essential for binding to L6 (Another L6-restricted peptide [T2H, see Table 3] also lacks proline at this position; Lurquin et al., 1989).

Given the fact that 2C recognizes at least three chroomatographically distinguishable peptides in association
with L\(^4\), the question can be raised as to whether the ligand directly in contact with the 2C TcR consists of both L\(^2\) and peptide or L\(^4\) alone. In the second case, the role of peptide would be to confer a specific conformation on the L\(^4\) molecule. While the latter model still represents a formal possibility, the considerable weight of high resolution crystallographic evidence strongly favors the view that peptide forms an important part of the actual ligand seen by a TcR and that different peptides do not result in different MHC conformations. Thus Wiley et al. have shown that the three-dimensional structures of class I MHC molecules are virtually identical whether they bind a single peptide or a heterogeneous mixture of peptides (Bjorkman et al., 1987; Garrett et al., 1989; D. C. Wiley, personal communication), and recent studies by Wilson and colleagues have shown that the three-dimensional structures of K\(^\alpha\) with three different bound peptides are remarkably similar (I. A. Wilson, personal communication). Since in our case the L\(^4\) molecule is not recognized by 2C when an irrelevant peptide known to bind L\(^4\) is added (Table 2), we conclude that the correct peptide (p2CL) forms part of the complex recognized by 2C. The existence of three peaks of activity in no way implies that peptide is not involved directly in recognition by 2C. The alternative possibility, that p2CL exerts its effect via specific conformational changes on L\(^4\), remains theoretical at this point and ultimately can be addressed conclusively only by structural studies with p2CL, L\(^4\), and the 2C TcR.

We are currently accumulating additional material to determine the sequences of active peptides in the other fractions of spleen and thymus homogenates and from affinity-purified L\(^4\). We have determined that all of these peptides must be recognized by the same TcR that sees p2CL, because antibody 182 (Fab\(^4\) fragment) blocks the susceptibility of B2 cells sensitized by these peptides to lysis by 2C cells (as shown in Figure 5 for p2CL). Whether the active fractions represent peptides that differ from p2CL by 1 or 2 aa at the N- or C-terminus, as suggested above, or have the same sequence and represent isomers (e.g., proline cis-trans isomers), or have entirely different sequences remains to be determined. Differences in the sequences may help shed light on the mechanisms by which MHC-associated peptides are generated. The apparent presence of p2CL (or a closely related peptide) in the thymus of H-2\(^\text{re}\) mice suggests that this peptide is likely to provide special opportunities for investigating the development of immature T cells in the thymus and the regulation of mature CD8\(^\text{T}\) cells in peripheral tissues of 2C transgenic H-2\(^\text{re}\) mice, as well as in conventional H-2\(^\text{d}\) mice.

### Table 3. Synthetic Peptides Recognized with L\(^4\) by Cytotoxic T Lymphocytes

<table>
<thead>
<tr>
<th>Peptide</th>
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<tr>
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<td>endogenous</td>
<td>8</td>
<td>\text{pp}</td>
<td>present study</td>
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### Experimental Procedures

#### Cell Lines

The 2C CTL clone originated in the spleen of a BALB/c mouse (H-2\(^\text{d}\)) that had been injected with P815 cells (from strain DBA/2, H-2\(^\text{d}\)) and BALB/c spleen cells (H-2\(^\text{b}\)) (Kraus et al., 1987). The antigenic determinant I\(^\text{A}\) was raised in this laboratory against P911 cells, a nontumorigenic variant of P815 cells, as described (Maryanski et al., 1982). The anti-ovalbumin clone 403 was established in this laboratory by Dr. C. Nagler-Anderson as described by Carbone and Seven (1990). All CTL were stimulated weekly in culture with the appropriate irradiated tumor target cells in K medium (RPMI supplemented with 10 mM HEPES, 2 mM glutamine, 100 U/ml penicillin, and 100 \u00b5g/ml streptomycin, plus 10% fetal calf serum) with the addition of rat concanavalin A supernatant containing about 20 U/ml IL-2. Tumor cell lines RMA and RMA-S and the L\(^4\)-transfected L cell line T1.1.1 were generous gifts from Drs. J. Monaco and J. Seidman, respectively (Ljunggren et al., 1990; Evans et al., 1982).

#### Cytotoxicity Assay

To measure lysis of target cells, 1 \times 10\(^6\) \text{Cr}\(^{51}\)-labeled target cells were incubated with various numbers of CTL in 200 \(\mu\)l of K medium in round-bottom microtiter wells. Peptides or peptide-containing samples in 50 \(\mu\)l of Dulbecco's modified Eagle's medium were added to 50 \(\mu\)l of target cells suspended in K medium, and after 30 min at 37\(^\circ\)C, CTL at a CTL:target cell ratio of 3:1 were added. After brief centrifugation, the cells were incubated for 4 hr at 37\(^\circ\)C, and the amount of \text{Cr} released into the extracellular medium was determined. The percent specific lysis was calculated as 100 \times \((\text{Cr} released - spontaneous release))/(total release in detergent - spontaneous release). Toxicity controls consisted of peptide plus \text{Cr}\(^{51}\)-labeled target cells without addition of 2C CTL.

#### Gene Construction and Transfection

The B2 cell line was established by introducing a genomic form of the L\(^4\) gene, a generous gift from Drs. M. Zuniga and L. Hood (Moore et al., 1982), into the Sp2/0 cell line using the expression vector pHER. Developed in this laboratory, pHER is pSV2neo modified to carry the metallothionein promoter, an immunoglobulin heavy chain enhancer, and the poly(A) signal from an immunoglobulin \(\kappa\) gene. The construct will be described elsewhere. To subclone the L\(^4\) gene under regulatory elements of vector origin, an Xhol linker (NEB \# 1030, New England Biolab, Beverly, MA) was introduced at a BamHI site, 15 bp 5' of the initiation codon after eliminating the BamHI site with S1 nuclease. At the 3' end, a BamHI linker (NEB \# 0212) was introduced at a Pvull site 6 bp downstream of the stop codon. The resulting plasmid, pHERmpl\(^4\), was linearized at its HindIII site for transfection. The RMA-S transfec- tant, RL\(^{E2}\), was generated by introducing the L\(^4\) gene under its own regulatory elements: a 12 bp HindIII fragment containing the genomic L\(^4\) gene was excised from the plasmid 27-5-27 (Moore et al., 1982) and coinfected with pSV2neo linearized at the BamHI site.

Transfections were performed by electroporation with the Cell Porter (BRL). Approximately 50 \(\mu\)g of linearized DNA with or without 20 \(\mu\)g of linearized pSV2neo was mixed with 2 \(\times\) 10\(^5\) cells in 700 \(\mu\)l of Dulbecco's modified Eagle's medium and charged at 750 V, 800 \(\mu\)F. After 24 hr at 37\(^\circ\)C, G418 (Gibco) was added to a final concentration of 1 mg/ml. L\(^4\) expression was assessed with the conformation-dependent monoclonal antibody 30-5-7S (a generous gift of Dr. David Sachs). Before FACs analyses were carried out, RMA-S transfecants were incubated overnight with an L\(^4\)-binding peptide derived from
et,
Affigel coupled to following columns connected in series: unmodified Affigel-hydrazide, through the carbohydrate moiety in their 0.5% cavitation, and the resulting membrane fraction was solubilized in Tsomides modification of previously described methods (Mescher Subsequent steps were the same as for tissue extracts. Dounce homogenizer starting with cells at 2 x 10^6 per ml in 0.7% TFA, Beverly, MA). Filtrates were dried 31,000 were then sonicated for 2 min at 40°C (Model W-225R, Ultrasonics, (Brinkman Instruments, Westbury, NY). Homogenization was carried out for 2 min while chilling the sample on ice, and the homogenates were then sonicated for 2 min at 4°C (Model W-225R, Ultrasonics, Inc.) and kept on ice for an additional 30 min. After centrifugation at 31,000 x g for 30 min, the supernatants were subjected to ultrafiltration through a Centricon 10 membrane (molecular mass cut-off 10 kd, Amicon, Beverly, MA). Filtrates were dried by Speed Vac and redissolved in 0.1% TFA for reverse phase HPLC fractionation. Acquisition of P815 cells was carried out with homogenates prepared in a Dounce homogenizer starting with cells at 2 x 10^6 per ml in 0.7% TFA. Subsequent steps were the same as for tissue extracts.

Affinity Purification of L* and D* L* and D* proteins were affinity purified from P815 cells by a slight modification of previously described methods (Messer et al., 1983; Tsonides et al., 1991). Briefly, 2 x 10^6 P815 cells were lysed by N-caviton, and the resulting membrane fraction was solubilized in 0.5% NP40, 20 mM Tris–HCl (pH 8.0), 1 mM phenylmethylsulfonyl fluoride. To prepare columns for affinity chromatography, polyonal mouse Ig or monoclonal antibodies 30-5-7S (anti-L*) or 34-5-8S (anti-D*) were coupled to Affigel-hydrazide (Bio-Rad, Richmond, CA) through the carbohydrate moiety in their Fc domain after periodate oxidation. The solubilized membrane fraction was loaded onto the following columns connected in series: unmodified Affigel-hydrazide, Affigel coupled to polyclonal mouse Ig, Affigel coupled to 30-5-7S, and Affigel coupled to 34-5-8S, in that order. After washing the columns with 125 mM triethylamine acetate (TEA(Ac) (pH 7.5) and 0.3% NP40, the bound material was eluted with 125 mM TEA(Ac) (pH 11.0). An aliquot of the eluate was tested for purity by SDS–polyacrylamide gel electrophoresis. The remaining material was haphazardized and redissolved in 0.7% TFA for ultrafiltration through a Centricon 10 membrane to separate bound peptides. HPLC Fractionation of Tissue and Cell Extracts Ultrafiltrates of acid extracts from tissues or cells were loaded on a C18 reverse phase column (218TP104, Vydac) in batches of up to 30 mg total peptide per injection. For larger scale purification, a Beckman C18 semipreparative column was used. HPLC was performed on a Beckman system equipped with a 451A controller, two 110A pumps, and a 150 absorbance detector (280 nm) connected in series to an Applied Biosystems 757 absorbance detector (220 nm). Chromatograms from the two detectors were recorded on Beckman 427 and Hewlett Packard HP3394A integrators. Solvent A was 0.1% TFA, and solvent B was acetonitrile containing 0.085% TFA. The gradient used for the first round of chromatography was from 5% to 30% B over 25 min (1% B per min) followed by a shallower gradient of 0.25% B per min over 40 min (gradient program 7). Fractions (1.0 ml) collected at 1 min intervals were assayed for their ability to sensitize B2 cells for lysis by 2C CTL. The active fractions were pooled and subjected to a second round of chromatography on the same C18 column but with a shallower gradient of 0.067% B per min (gradient program 8). Active fractions from the second gradient were subjected separately to further HPLC purification on a pH-stable C8 column at pH 8.5 (Vydac 225TP104). For this column, solvent A was 125 mM TEA(Ac) (pH 8.5), and solvent B consisted of 15% buffer A and 85% acetonitrile containing 77 mM TEA(Ac). For chromatography at pH 8.5, the gradient was 0.1% B per min, starting at 32% B, for 70 min. The fractions with sensitizing activity were identified by cytotoxicity assay, and the pooled active peak was rechromatographed twice on the same pH-stable column isocratically at 31% B and 24.4% B, successively, using the cytotoxicity assay after each chromatography to identify the active fraction(s).

Mass Spectrometry

The active fraction from the final chromatography under isocratic conditions (see above) was analyzed by Dr. Ioannis Papayannopoulos and Professor Klaus Biamann by fast atom bombardment mass spectrometry to determine the peptide(s) molecular weight. Identical values were obtained using either glycerol or N-benzylalcohol as the matrix for ionization.

Peptide Sequencing and Synthesis

Purified peptides were sequenced by Edman degradation on an Applied Biosystems instrument, model 477. Synthetic peptides were prepared by standard Merrifield solid phase methods using F-Moc chemistry on an Applied Biosystems 430A at the Massachusetts Institute of Technology Biopolymers Laboratory. Peptide concentrations were usually measured by micro BCA Assay (Pierce). However, the concentrations of p2Cl and p2Cl were determined by quantitative amino acid analyses.

Acknowledgments

We are grateful to R. Cook and the Massachusetts Institute of Technology Biopolymers Laboratory for expert peptide sequencing and synthesis, Drs. I. Papayannopoulos and K. Biamann for careful mass spectrometry, Dr. P. Matsudaira for useful discussions and advice on peptide purification and sequencing, and Stewart Conner for flow cytometry analyses. We thank DRS. P. Walden and H.-G. Rammensee (Max Planck Institut, Tübingen, Germany) for the use of their laboratory and HPLC at the last stage of this work during a period of language training for K. U. supported by the Alexander von Humboldt Foundation. We also thank Ann Hicks for excellent assistance in preparation of the manuscript and Mimi Rasmussen for help with Fab experiments. This work was supported by a Research Grant (R35-CA42504), a Cancer Center Core Grant (CA14051), and a Training Grant (CA09255) from the National Cancer Institute, National Institutes of Health. The M. T. Biopolymers Laboratory and Cell Sorter Facility are supported in part by the Howard Hughes Medical Institute.

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References

Peptide Recognized by Alloreactive T Cell Clone


A ubiquitous protein is the source of naturally occurring peptides that are recognized by a CD8\(^+\) T-cell clone

(T-cell receptor/major histocompatibility complex/alloreactivity/2-oxoglutarate dehydrogenase/proteasomes)

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**ABSTRACT** We previously isolated from mouse spleen an octapeptide (LSPFPFDL) that in association with the class I major histocompatibility complex protein L\(^d\) is recognized by the T-cell receptor of an alloreactive CD8\(^+\) T-cell clone (2C). Guided by an assay dependent upon the same 2C T-cell receptor, we have now isolated from the same source another naturally occurring peptide. The second peptide (VAITRIEQLSPFPFDL) includes the entire octapeptide sequence and preliminary evidence suggests that it may be a natural precursor of the octapeptide. On finding extensive sequence homology between the 16-mer and part of human 2-oxoglutarate dehydrogenase, we determined the cDNA sequence. In accordance with the deduced amino acid sequence matches precisely the two naturally occurring peptides, indicating their origin by cellular processing of this ubiquitous self protein.

CD8\(^+\) T lymphocytes recognize short peptides displayed on the surface of cells (termed target cells) in association with proteins of the class I major histocompatibility complex (MHC-I) (1). The peptides appear to be derived by proteolytic cleavage of intracellular proteins. When they arise from viral or other microbial proteins in infected cells or from tumor-specific proteins in transformed cells, the resulting cell surface peptide-MHC-I complexes frequently elicit CD8\(^+\) cytotoxic T lymphocytes (CTLs) capable of destroying the infected or transformed cells. Peptide-MHC-I complexes in which the peptides are derived from normal self proteins are also important. When immature CD8\(^+\) CD8\(^+\) T cells recognize such self peptides in association with MHC-I proteins on thymic, thymocytes that would otherwise develop into mature potentially autoreactive T cells are clonally deleted. Self peptides are also likely to be involved in the positive selection of immature thymocytes. In addition, peptides derived from normal proteins are recognized in association with MHC-I proteins by many of the mature CD8\(^+\) T cells that react specifically with cells from individuals of the same species that have different MHC proteins; these "alloreactive" T cells are largely responsible for the rejection of normal organ allografts (skin, kidney, heart, etc.).

One way to learn about the normal proteins that are effectively represented as peptide-MHC-I complexes is to characterize the peptides of normal target cells that are recognized by alloreactive CD8\(^+\) CTLs and from their sequences to identify the proteins from which these peptides arise. In this context we have been studying a CD8\(^+\) T-cell clone termed 2C. A typical alloreactive CTL clone, 2C arose in a mouse having the H-2\(^d\) haplotype (BALB.B) in response to injections of allogeneic cells having the H-2\(^d\) haplotype (BALB/c and DBA/2), and it is specifically reactive with one of the MHC-I proteins (i.e., L\(^d\)) from H-2\(^d\) cells (2). We have shown (3) that when trifluoroacetic acid (TFA) homogenates of mouse spleen or thymus were subjected to ultrafiltration (Mr < 10,000) and the filtrates were fractionated by reverse-phase HPLC (RP-HPLC), three distinct peptide-containing fractions could sensitize L\(^d\) target cells to lysis by 2C cells. From the earliest eluting fraction (fraction a), we purified a single active octapeptide, previously called p2Ca and herein termed p2Ca, and established its sequence (LSPFPFDL). However, a search of protein sequence data bases with this octamer failed to identify a matching protein. Furthermore, the relationship among the three (or more) different peptides that are present in normal tissue extracts and that can be seen by 2C cells remained unknown. In the present study we isolated a single active peptide from the second sensitizing RP-HPLC fraction (fraction b), determined its sequence and relationship to p2Ca, and identified the protein source of both peptides.

**MATERIALS AND METHODS**

Cell Lines. The 2C CTL clone (2) was grown as described in K medium (RPMI 1640 medium supplemented with 10 mM Hepes, 2 mM glutamine, penicillin at 100 units/ml, streptomycin at 100 μg/ml, and 10% (vol/vol) heat-inactivated fetal calf serum). B2 cells, used as target cells in cytotoxicity assays and as a source of proteasomes (see Fig. 3, below), are murine myeloma Sp2/0 cells (BALB/c origin) transfected with the L\(^d\) gene as described (3). RL\(^E\)-B2 cells, used in L\(^d\)-binding assays (see below), are RMA-S cells transfected with the L\(^d\) gene (3). T2-L\(^d\) cells, used as target cells in some cytotoxicity assays, are the human antigen-processing mutant cell line T2 (4) transfected with the L\(^d\) gene; they were kindly provided by P. Cresswell (Yale University). All L\(^d\) transfectants were maintained in K medium with G418 (GIBCO) at concentrations between 200 and 500 μg/ml.

Cytotoxicity Assay. To screen HPLC fractions for peptides that can sensitize B2 cells to lysis by 2C CTLs, the fractions were dried (SpeedVac; Savant), taken up in Dulbecco's modified Eagle's medium, and tested in cytotoxicity assays as described (3). Percent specific lysis of target cells was calculated from released \(^{31}\)Cr as 100 × (experimental release − spontaneous release)/total release in detergent − spontaneous release). Toxicity controls consisted of peptide plus \(^{31}\)Cr-labeled target cells (2C CTLs omitted).

Purification of Naturally Occurring Peptides. Spleens from BALB/c Kh mice (Max-Planck-Institut, Tübingen) were homogenized in 0.7% TFA (3). After the homogenates were sonicated for 2 min and centrifuged at 31,000 × g for 30 min at 4°C, supernatants were subjected to ultrafiltration through 0.7% TFA (3).

Abbreviations: MHC, major histocompatibility complex; OGDH, 2-oxoglutarate dehydrogenase; CTL, cytotoxic T lymphocyte; TFA, trifluoroacetic acid; RP, reverse phase.

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RESULTS AND DISCUSSION

Isolation and Characterization of Peptide p2Cb. Fractionation of ultrafiltrates of BALB/c mouse spleen or thymus homogenates by RP-HPLC was found (3) to consistently yield three fractions (a-c) that were capable of sensitizing L<sup>4</sup> target cells (B2) to lysis by 2C CTLs. To isolate the active peptide from fraction b, we subjected pooled ultrafiltrates from 471 BALB/c mouse spleens to successive rounds of RP-HPLC fractionation using different chromatographic conditions. After six rounds, the active fraction from what was originally termed b (Fig. 1) was analyzed. The Edman procedure yielded the sequence VAITRIEQLSPFFP, which was not complete because the purified peptide was found by mass spectrometry to have a molecular mass of 1846 Da. From these results and the known sequence of the active peptide in fraction a (p2Ca, LSPPF-PFDL), we concluded that the complete sequence of the active peptide in fraction b (hereafter termed p2Cb) was VAITRIEQLSPFFPFDL (mass, 1846 Da). To verify this conclusion a peptide having the 16-residue sequence was synthesized and tested: it had the same RP-HPLC retention as the standard.

A229; a Centricon-10 membrane (Amicon) and to successive rounds of fractionation by RP-HPLC. Active fractions corresponding to peak b were rechromatographed until only a single peak was detected by UV absorbance. For the first three rounds, solvent A was 0.1% TFA in H<sub>2</sub>O and solvent B was 0.1% TFA in acetonitrile, the column was a SuperPac pep-S (Pharmacia), and gradients of 0.1%, 0.067%, and 0.067% solvent B per min were used, respectively. For the final three rounds, solvent A was 125 mM triethylamine acetate in acetonitrile, the column was a Vydac pH-stable C<sub>4</sub> column (219TP54), and the gradient was 0.1% solvent B per min. Fractions were collected at either 1-min or 0.5-min intervals.

Peptide Sequencing and Synthesis. From the active fraction of the final HPLC run of p2Cb (see Fig. 1), 8% was analyzed by Edman degradation on an Applied Biosystems model 477. Another aliquot was analyzed by matrix-assisted laser desorption time-of-flight mass spectrometry by Ioannis Papayannopoulos and Klaus Biemann (Department of Chemistry, Massachusetts Institute of Technology). Peptide concentrations were synthesized on an Applied Biosystems model 430A using t-butoxycarbonyl chemistry (Biopolymers Laboratory, Massachusetts Institute of Technology). Peptide concentrations were generally determined by MicroBCA Assay (Pierce). Because the concentration of peptide p2Ca was underestimated by MicroBCA when manufacturer-supplied bovine serum albumin was used as the standard (T.J.T., unpublished observation), p2Ca was quantitated by 6 M HCl hydrolysis and amino acid analysis or by MicroBCA using amino acid analyzers as the standard.

Purification of Synthetic p2Cb. After 2 hr at 60°C, synthetic p2Cb was purified by three rounds of RP-HPLC on a Beckman model 334, subjecting fractions after each round to cytotoxicity assay as described above and selecting the major peak, containing the active peptide, for the next round. Round one was on a Vydac C<sub>4</sub> semipreparative column (214TP310) using a gradient of 1% solvent B per min (solvent A = 0.1% TFA in H<sub>2</sub>O; solvent B = 0.085% TFA in acetonitrile) and a flow rate of 3 ml/min. The second and third rounds were on a Vydac diphenyl analytical column (219TP54) and a Vydac C<sub>4</sub> analytical column (218TP104), respectively, using a gradient of 0.067% solvent B per min and a flow rate of 1 ml/min. The Mouse 2-Oxoglutarate Dehydrogenase (OGDH) Gene. From total RNA isolated by acid phenol extraction of Sp2/0 cells, single-stranded cDNA was synthesized using the PerScript preamplification system (BRL). cDNA from 0.35 µg of RNA was used to amplify by PCR a part of the OGDH sequence in 50 µl by using 10 pmol of the following two oligonucleotide primers at 55°C for annealing: 5'-GCTATATGACTGCAATTGGGTGTTCTTGTGC-3' and 5'-ATAGTAGCTTTGGT-GTTCTTGTGC-3'. The primers correspond to nt 2524–2545 and 2938–2959 of human OGDH (5). The amplified DNA was cloned into pUC18 at the Smal site and the nucleotide sequences of three individual clones were determined by the dideoxynucleotide method using Sequenase version 2.0 (United States Biochemical). The sequence was confirmed by independent PCR amplification, cloning, and sequencing.

FIG. 1. Chromatogram and cytotoxicity assay from the final step in the purification of peptide p2Cb from mouse spleen. Solid line, A<sub>229</sub>; dotted line, acetonitrile gradient; •, percent specific lysis by 2C due to fractions collected at 0.5-min intervals. Baseline drift in the chromatogram arises from triethylamine acetate.

FIG. 2. Degradation of peptide p2Cb in medium used for cytotoxicity assays. After four rounds of RP-HPLC purification, 2 µg of synthetic p2Cb was incubated with assay medium for 2 hr and subjected to ultrafiltration and further chromatography on a C<sub>18</sub> column using a gradient of 0.067% solvent B per min. Percent specific lysis due to fractions from p2Cb incubated with assay medium (e) and fractions from p2Cb incubated with medium that had been heated to 100°C for 10 min (a) are shown.
rounds of RP-HPLC on different columns, its effective sensitizing activity decreased by ~20-fold (SDDp = 100 ng/ml).

That intact p2Cb may not bind to L^4 to a significant extent was suggested by our earlier finding that fractions a and b do not fractionate at L^4 (3), if so, the sensitizing activity of naturally occurring p2Cb could result from its being cleaved in serum-containing medium during the course of cytotoxicity assays. To test this possibility, purified synthetic p2Cb freed of contaminating sensitizing components (see above) was incubated for 2 hr in the serum-containing medium used for these assays. RP-HPLC analysis of TFA ultrafiltrates of the incubation mixture revealed several new peaks of biological activity eluting earlier than p2Cb (Fig. 2). If, however, the assay medium was first heated in a boiling water bath for 10 min, followed by addition of p2Cb for 2 hr at 37°C, RP-HPLC fractionation of the mixture and CTL assay of each fraction showed that the only peak of activity corresponded to authentic p2Cb (Fig. 2). Thus, it is likely that during the standard 4-hr cytotoxicity assay both naturally occurring and synthetic p2Cb are cleaved to some extent by serum peptidases, generating smaller fragments responsible for some or possibly all of the apparent sensitizing activity of the 16-mer.

It is possible, nevertheless, that intact p2Cb binds weakly to L^4 because we found that this peptide enhanced cell surface expression of L^4 on RL-E2 cells (RMA-S cells that lack the peptide transport gene TAP-2 and had been transfected with the L^4 gene (3), thereby providing a sensitive L^4-binding assay (10, 11)). After RL-E2 cells were kept overnight at 26°C and then incubated with p2Cb at 10^{-3} M for 30 min at 37°C, increased surface expression of L^4 was measured by flow cytometry using monoclonal antibody 30-5-S-7 (12) and a fluorescence isothiocyanate-labeled secondary antibody [210 vs. 180 (background) mean log fluorescence units]; though definite, this effect was much less pronounced than that due to the octapeptide p2Ca or the L^4-binding nonapeptide YHFMAPFTNL from measles virus (13), which increased L^4 expression in the same assay to 260 or 310 mean log fluorescence units, respectively.

To determine whether p2Cb-L^4 complexes might be recognized by 2C cells, we measured the level of intracellular Ca^{2+} ([Ca^{2+}]_i) in indo-1-loaded 2C cells 5-6 min after they were mixed (and briefly centrifuged) with RL-E2 cells that had been pulsed with p2Cb or p2Ca at concentrations ranging from 10^{-7} to 10^{-10} M; as in the fluorescence assay above, RL-E2 cells were maintained at 26°C overnight before adding peptides. With both peptides, concentration-dependent increases in [Ca^{2+}]_i were seen, but 15- to 30-fold more p2Cb than p2Ca was required to elicit the same effect. Moreover, the maximal increase elicited by p2Cb was lower than that elicited by p2Ca (~7-fold vs. 10-fold above the background [Ca^{2+}]_i level of 200 μM). Because the increase in [Ca^{2+}]_i was seen after only 5 min and the assay was carried out in medium that contained 1% bovine serum albumin in place of fetal calf serum, the confounding effect of proteolytic fragmentation was minimized. Although these findings indicate that p2Cb binds weakly to L^4 and that its complexes with L^4 may be recognized to some extent by 2C cells, we believe that the naturally occurring peptide’s activity in the cytotoxicity assays that led to its detection and isolation was due largely to its cleavage into smaller active fragments (see Fig. 2).

Although peptidases present in fetal calf serum, on cell surfaces, or released from dead cells introduce uncertainty about the precise sequences of peptides that elicit T-cell responses in cytotoxicity assays, this ambiguity is advanta-

- **Peptide p2Cb as a Possible Precursor of p2Ca.** Longer peptides that may be intermediates in the processing pathway from intracellular protein to the octamers and nonamers commonly bound to MHC-I molecules have not yet been isolated (14). Comparison of the sequences of p2Ca (LSPPPFPDL) and p2Cb (VAITRIEQSQFPPFDL) suggested that p2Cb might be such an intermediate. To determine whether proteasomes isolated from mouse cells can cleave p2Cb to give p2Ca, synthetic p2Cb freed of contaminants (see above) was incubated with purified proteasomes, and RP-HPLC fractions from TFA ultrafiltrates of the incubation mixture were tested for sensitizing activity. After a 2-hr incubation, we observed two new peaks of activity (Fig. 3). One, eluting at 50-51 min in Fig. 3, had the same retention time as p2Ca using a shallow (and, therefore, highly resolving) gradient on two different HPLC columns (C18 and diphényl), but its yield was too low to establish its mass or sequence. Thus, we cannot conclude definitively that p2Cb was cleaved to yield p2Ca; however, notwithstanding arguments against an obligatory role for MHC-linked proteasome subunits in antigen processing (16, 17), these results provide a basis for further extended activity detection of longer peptides (such as p2Cb) that might otherwise go undiscovered.

Crystallographic studies of peptides bound to MHC-I proteins suggest that the peptide N and C terminal are buried in the MHC-I binding groove so that the binding of peptides longer than 8 or 9 residues is unlikely unless accommodated by bulging of the peptide’s misdirection away from the MHC-I molecule. A bulge of 8 amino acids in the p2Cb misdirection seems extreme, and it is possible that L^4 is more permissive than other MHC-I proteins and allows several amino acids at the N terminus of this longer peptide (e.g., VAITRIEQ), to extend beyond the binding groove.

**Amount of p2Cb Recovered.** From the Edman analysis of naturally occurring p2Cb, we estimate that the 471 spleens originally extracted had a minimum of 500 pmol of p2Cb (Fig. 1), assuming no loss of peptide during purification and 100% yield of the N-terminal amino acid as a phenylthiohydantoin derivative. This value corresponds to a recovery of ~7000 molecules of p2Cb per cell, assuming 10^6 nucleated cells per mouse spleen, or ~20 times more than we estimated for the p2Ca octamer (3).

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The cleavage of this normal intracellular protein.

Identifications of the Protein from Which p2Ca and p2Cb Are Derived. A search of protein sequence data bases revealed an unmistakable similarity between the sequences of p2Ca and OGDH (also called \( \alpha \)-ketoglutarate dehydrogenase) from yeast (19), *Escherichia coli* (20), and other bacteria (21). The sequence of the human homolog, recently determined from cloned cDNA (5), was even more similar: fourteen out of 16 amino acids in p2Ca were identical (87.5%). Peptide fragments from the homologous pig enzyme (5) also had very similar sequences (Fig. 4).

If OGDH was the source of peptides p2Ca and p2Cb, the mouse homolog (whose sequence was not in the data base) should precisely match the sequences of the two peptides. Accordingly, we determined a partial sequence of mouse OGDH by the PCR, using mouse cDNA and 5' and 3' deoxynucleotide primers that were selected on the basis of the human OGDH sequence. Fig. 4 shows the partial DNA sequence of mouse OGDH and the deduced amino acid sequence aligned with the corresponding sequences of human, pig, and yeast OGDH. From the complete match between p2Ca, p2Cb, and mouse OGDH, it is highly likely that the two natural peptides are derived from proteolytic cleavage of this normal intracellular protein.

**Concluding Remarks.** Previous analyses of peptide-containing HPLC fractions from tissue or cell extracts found that most alloreactive CTL clones respond to only one of many fractions, presumably the one containing a singular peptide recognized by that clone in association with a particular allogeneic MHC-I protein (22-25). However, a few clones (\( \approx 20\% \) of those examined) responded to two or three fractions (23). That several fractions can elicit a response by a single CTL clone has been puzzling: are the peptides in these fractions related or totally unrelated? The present study answers this question for CTL clone 2C, for here we show that two peptides detected by the same T-cell receptor on these cells share an octapeptide sequence and differ only in length. The sequence of the longer peptide led to the identification of the protein source of these peptides, OGDH, an essential participant in the tricarboxylic acid cycle and present in all cells. Since this mitochondrial protein is ubiquitous and the two peptides are produced from it in cells of all three MHC haplotypes tested (H-2\( \text{a} \), H-2\( \text{b} \), and H-2\( \text{k} \)), this system may prove useful for analyzing mechanisms of self tolerance.

Neither mass spectrometry nor Edman degradation enabled us previously to determine unambiguously whether the C-terminus of p2Ca was Leu or Ile. Therefore, we relied on cytotoxicity assays and RP-HPLC chromatography to compare two synthetic peptides with either Leu or Ile at the C terminus and to conclude that naturally occurring p2Ca terminated with Leu (3). Confirmation of this conclusion by the sequence of OGDH (Fig 4, boxed area) testifies to the remarkable ability of CTL assays and RP-HPLC, particularly

![Graph](https://example.com/graph.png)

**Fig. 4.** Partial sequence of mouse OGDH and comparison with sequences of human, pig, and yeast homologs. The cDNA sequence of mouse OGDH is shown in the top line. Flanks on the human OGDH cDNA sequence (italic type and boxed) used for PCR. The deduced amino acid sequence of mouse OGDH is compared with the corresponding amino acid sequences of OGDH from humans (5), pigs (5), and yeast (19). Dash, same amino acid as mouse OGDH; no mark, amino acid not determined (pig only); boxed amino acids, sequences corresponding to peptides p2Ca and p2Cb. Note the deduced amino acid at position 68 is incorrectly shown as L (Leu); it should be H (His).
in combination, to discriminate between a structural difference as minor as the position of a single methyl group in Leu vs. Ile.

Naturally occurring longer peptides like p2Cb that may be precursors of MHC-I-binding shorter peptides, to our knowledge, have not previously been described in virus-infected or normal cells (7, 8, 26-28). The reason may be that most attempts to identify the naturally occurring peptides recognized by T cells have relied on affinity purification of MHC-I proteins as a preliminary step; hence, only peptides that bind very stably to MHC-I proteins (primarily octamers and nonamers) are isolated by such approaches (14). By isolating peptides from whole tissue homogenates, as in this study, longer putative precursor peptides are more likely to be detected, providing they are active in T-cell assays, an activity that may depend upon their being cleaved into smaller fragments directly responsible for sensitizing activity in T-cell assays. Longer precursor peptides probably also have to accumulate to relatively high levels in tissue or cell homogenates, as is the case with p2Cb, to compensate for the likelihood that they have lower sensitizing activities than shorter precursors.

Precursors of MHC-I-binding proteins (primarily octamers and nonamers) are probably commonplace in cells. Their sequence, abundance, and intracellular compartmentalization may help illuminate the processing pathways for peptide presentation by MHC-I proteins.

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A CYTOTOXIC T LYMPHOCYTE CLONE CAN RECOGNIZE THE SAME NATURALLY OCCURRING SELF PEPTIDE IN ASSOCIATION WITH A SELF AND A NONSELF CLASS I MHC PROTEIN

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Abstract—The alloreactive CD8+ cytotoxic T lymphocyte (CTL) clone 2C was previously shown to recognize complexes made up of the class I MHC (MHC-I) molecule Ld and an octapeptide (LSPFPFDL, termed p2Ca) isolated from tissues of H-2d mice. Because peptide p2Ca has also been found in BALB.B (H-2b) mice, the strain from which clone 2C originated, the question arises as to whether these T cells can recognize peptide p2Ca in association with a self MHC protein of the H-2b haplotype. Here we show that 2C CTL do indeed recognize peptide p2Ca in association with K" on the surface of H-2b cells or on transfected cells expressing K"; but that an approximately 1000-fold higher concentration of this peptide is required to sensitize K" than Ld" target cells for lysis by 2C cells. However, the peptide's binding to K" was not much weaker than to Ld", with only an approximately 10-fold difference in the respective equilibrium constants. These results predict that the T cell receptor (TcR) of clone 2C has a much lower intrinsic affinity for p2Ca-K" complexes than for p2Ca-Ld" complexes, and they provide some quantitative limits on the requirements for triggering T cell-mediated autoimmune reactivity.

Key words: alloreactivity, class I MHC, natural peptide, autoimmunity, positive selection.

INTRODUCTION

A previous study led to the biochemical isolation and sequence determination of a naturally occurring peptide that, in association with Ld, forms a ligand for the antigen-specific T cell receptor (TcR) of the alloreactive (H-2b anti-H-2d) CD8+ cytotoxic T cell clone 2C (Udaka et al., 1992). This peptide, an octamer termed p2Ca (LSPFPFDL), derives from α-ketoglutarate dehydrogenase, which is present in all cells as it is an essential component of the Krebs cycle (Udaka et al., 1993). In cytotoxicity assays, target cells bearing p2Ca-Ld" complexes are specifically and effectively lysed by 2C cells. In mice that are transgenic for the α and β genes of the TcR of 2C cells, thymocytes that express this receptor (2C-TcR) are eliminated if the mice express Ld", suggesting that under natural conditions recognition of the p2Ca-Ld" complex by immature thymocytes causes their "negative selection". If, however, these transgenic mice express K" and not Ld", T cells expressing the 2C-TcR are positively selected, leading to their abundance in the periphery, where they account for ≈50% of all circulating T cells in H-2b" mice transgenic for the 2C-TcR (Sha et al., 1988b). Inasmuch as peptide p2Ca is also detectable in the thymus of H-2b" mice (Udaka et al., 1992), we have asked here whether this naturally occurring octapeptide can bind to K" (or Dd"), and if so, whether the p2Ca-MHC complexes that are formed can be recognized by the 2C-TcR.

Our results show that the peptide binds to K" and not to Dd", and that the binding to K" is not much weaker than to Ld". Yet, an approximately 100-fold higher concentration of p2Ca is required to sensitize K" target cells than to Ld" target cells for 50% maximal lysis by 2C cells. This great disparity leads to the prediction that the 2C-TcR has a much lower intrinsic affinity for p2Ca-K" complexes than for p2Ca-Ld" complexes, and helps to explain why the presence of p2Ca-K" complexes does not result in overt autoimmune reactions in H-2b" mice transgenic for the 2C-TcR. Finally, the results support the hypothesis that peptide p2Ca may be one of the naturally occurring peptides involved in the positive selection of T cells with the 2C-TcR in H-2b" 2C-TcR transgenic mice.

MATERIALS AND METHODS

Cell lines and cytotoxic T lymphocyte (CTL) clones

B2 cells are Sp2/0 myeloma cells (H-2d"), transfected with the Ld" gene (Udaka et al., 1992). T2 cells, a human TxB hybridoma, are defective in the presentation of
endogenous peptides by class I MHC (MHC-I) proteins (Alexander et al., 1989); these cells and T2-Ld, T2-Kb, and T2-D* (T2 cells transfected with the a chain genes for Ld, Kb, or D*, respectively) were all gifts from Dr P. Cresswell. RMA-S cells (H-2b), also defective in the ability to present endogenous peptides with MHC-I proteins (Kärre et al., 1986; Townsend et al., 1989), were a gift from Dr J. Monaco. All of these cells, as well as EL4 cells (H-2d thymoma) and P815 cells (H-2d mastocytoma), were grown in RPMI supplemented with 10% fetal calf serum, 10mM HEPES, 2 mM glutamine, 50 μg/ml streptomycin, and 50 μg/ml β-mercaptoethanol (“K medium”). The medium used to grow transfected cells was supplemented with 270 μg/ml G418.

CTL clone 2C was derived and maintained in culture as described (Kranz et al., 1984). The anti-PR8 CTL line, derived from a C57/Bl/6 mouse (Hosken and Bevan, 1992), recognizes an influenza virus nucleoprotein peptide (NP366, see Table 1) in association with Db. It was selected as described (Kranz et al., 1992), was stoichiometrically labeled with a mixture of 121I+127I and isolated as its monoiodotyrosoyl derivative 121I-pMCMV (termed 121I-pMCMV for simplicity) (Tsomides and Eisen, 1993a). To measure the equilibrium constants for the binding of this indicator peptide to Ld or Kb, intact T2-Ld or T2-Kb cells were incubated with various concentrations of the peptide for several hours at 25°C in the presence of NaN3 and 2-deoxyglucose, washed twice, and centrifuged through oil to separate bound from free peptide. To correct for the nonspecific binding of peptide to the cells, titrations were carried out in the presence of a 500-fold or greater molar excess of an unlabeled peptide known to bind strongly to Ld (pMCMV) or to Kb (pOV8, see Table 1). Scatchard plots of the resulting specific binding data for 121I-pMCMV (bound/free vs bound peptide) were linear and yielded single equilibrium binding (association) constants (Ks values): for Ld the Ks value was 4 x 10^7 M^-1 and for Kb it was 3 x 10^6 M^-1.

To measure the Ks values for p2Ca and Ld or Kb, different concentrations of p2Ca were mixed with 121I-pMCMV and incubated with T2-Ld or T2-Kb cells, and the bound and free 121I-pMCMV were separated as described above. Since both the labeled and unlabeled peptides were substantially in excess over the number of MHC-I molecules on T2-Kb or T2-Ld cells, the Chen–Prusoff equation (Chen and Prusoff, 1973) could be applied to calculate the Ks value for the binding of p2Ca to Ld or Kb. Each Ks value reported is the average of three or more experiments; independent determinations generally agreed to within a factor of two.

### Peptides

The peptides used or referred to in this study are described in Table 1. They were synthesized at the M.I.T. Biopolymers Laboratory by solid phase tBoc chemistry using an Applied Biosystems 430A and HF cleavage and were purified by reverse phase HPLC; their amino acid compositions were confirmed by amino acid analysis. Stoichiometric iodination of peptides with 121I+127I followed by reverse phase HPLC purification of each product was performed as described (Tsomides and Eisen, 1993a).

#### Peptide binding to MHC-I proteins

The method used to measure equilibrium constants for the interaction of p2Ca with Ld or Kb is described elsewhere in detail (S. Kageyama et al., submitted). Briefly, the binding of p2Ca was measured by its inhibition of the specific binding of a uniformly iodinated “indicator” peptide (I1-pMCMV) to Ld or Kb on intact cells (T2-Ld or T2-Kb, respectively). pMCMV, which can bind to both Ld (Reddehase et al., 1989) and Kb (Dutz et al., 1992), was stoichiometrically labeled with a mixture of 121I+127I and isolated as its monoiodotyrosoyl derivative 121I-pMCMV (termed 121I-pMCMV for simplicity) (Tsomides and Eisen, 1993a). To measure the equilibrium constants for the binding of this indicator peptide to Ld or Kb, intact T2-Ld or T2-Kb cells were incubated with various concentrations of the peptide for several hours at 25°C in the presence of NaN3 and 2-deoxyglucose, washed twice, and centrifuged through oil to separate bound from free peptide. To correct for the nonspecific binding of peptide to the cells, titrations were carried out in the presence of a 500-fold or greater molar excess of an unlabeled peptide known to bind strongly to Ld (pMCMV) or to Kb (pOV8, see Table 1). Scatchard plots of the resulting specific binding data for 121I-pMCMV (bound/free vs bound peptide) were linear and yielded single equilibrium binding (association) constants (Ks values): for Ld the Ks value was 4 x 10^7 M^-1 and for Kb it was 3 x 10^6 M^-1.

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### Table 1. Peptides used or referred to in the present study

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence*</th>
<th>Origin</th>
<th>MHC restriction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>p2Ca</td>
<td>LSPFFDFL</td>
<td>x-ketogluatrate dehydrogenase</td>
<td>Ld; Kb</td>
<td>Udaka et al., 1992; 1993; present study</td>
</tr>
<tr>
<td>pMCMV</td>
<td>YPHFMPTNL</td>
<td>Murine cytomegalovirus pp89</td>
<td>Ld; Kb</td>
<td>Reddehase et al., 1989; Dutz et al., 1992</td>
</tr>
<tr>
<td>pOV8</td>
<td>SIINFEKL</td>
<td>Ovalbumin</td>
<td>Kb</td>
<td>Rötzschke et al., 1991b</td>
</tr>
<tr>
<td>SV5</td>
<td>RGYYVQGL</td>
<td>Vesicular stomatitis virus nucleoprotein</td>
<td>Kb</td>
<td>van Bleek and Nathenson, 1990</td>
</tr>
<tr>
<td>SV9</td>
<td>FAPGNYPAL</td>
<td>Sendai virus nucleoprotein</td>
<td>Kb</td>
<td>Schumacher et al., 1991</td>
</tr>
<tr>
<td>NP366</td>
<td>ASNENMETM</td>
<td>Influenza virus nucleoprotein</td>
<td>D*</td>
<td>Townsend et al., 1986</td>
</tr>
<tr>
<td>E1A234</td>
<td>SGGSNTTPEEI</td>
<td>Adenovirus E1A</td>
<td>D*</td>
<td>Kast et al., 1989</td>
</tr>
</tbody>
</table>

*aAmino acid residues in bold face refer to presumptive anchor residues for Kb and D* (Falk et al., 1991). For Ld, the presumptive anchor residues are proline at position 2 and leucine at the C-terminus (Corr et al., 1992).*
A CTL clone recognizes a self peptide with self MHC

**Fig. 1.** Peptide p2Ca binds to K\(^{b}\) and the p2Ca-K\(^{b}\) complex is recognized by 2C cells. \(^{51}\)Cr-labeled target cells expressing different cell surface MHC-I proteins were compared for their ability to be sensitized by peptide p2Ca for lysis by 2C CTL. Arrows point to S\(_{90}\) values, i.e. peptide concentrations at which 50% of maximal lysis is observed: \(\approx 1\) ng/ml for B2 and T2-L\(^{d}\) and \(\approx 1\) \(\mu\)g/ml for EL4 and T2-K\(^{b}\). (a) Comparison of B2 cells (Sp2/0 cells transfected with the L\(^{d}\) gene) and EL4 cells (H-2\(^{b}\) haplotype and thus expressing K\(^{b}\) and D\(^{b}\)). (b) Comparison of T2 cells singly transfected with the \(\alpha\) chain genes for L\(^{d}\), K\(^{b}\), or D\(^{b}\) termed respectively, T2-L\(^{d}\), T2-K\(^{b}\), and T2-D\(^{b}\).

**Cytotoxicity assay**

\(^{51}\)Cr-labeled target cells (10\(^{4}\)) were incubated with CTL in the presence or absence of various peptides or with reconstituted HPLC fractions in a total volume of 200 \(\mu\)l K medium in round-bottom 96-well microtiter plates. After 4 hr at 37\(^{\circ}\)C, the plates were centrifuged at 200g for 5 min and 100 \(\mu\)l supernatant from each well was analysed for \(^{51}\)Cr. Percent specific lysis was calculated from the average of duplicate samples as 100 \(\times\) \((^{51}\)Cr released - spontaneous release)/(total release in detergent - spontaneous release). Spontaneous release values varied from 5-20% of total release. Toxicities of synthetic peptides and reconstituted HPLC fractions were tested by omitting CTL from the assay, and were negligible unless otherwise noted (specific lysis over background <5%).

**Peptide competition assay**

For some cytotoxicity assays, two peptides (the cognate peptide and a competing peptide) were added to assess the degree of competition for the restricting MHC-I protein. When the CTL were 2C cells (Fig. 1), the cognate peptide p2Ca was added at the concentration required to sensitive L\(^{d+}\) or K\(^{b+}\) target cells for half-maximal lysis [i.e. the S\(_{90}\) value (Tsomides et al., 1991)]. In experiments with anti-PR8 CTL, the cognate peptide NP366 was added to EL4 target cells at 10 pg/ml. the S\(_{90}\) for this CTL-target cell–peptide combination (Fig. 2). After incubating \(^{51}\)Cr-labeled target cells and CTL with the cognate peptide plus various concentrations of competing peptide for 4 hr at 37\(^{\circ}\)C, the supernatants were assayed for \(^{51}\)Cr release as described above. The potency of the competing peptide was expressed as an IC\(_{50}\) value: the concentration of inhibitor that blocked 50% of the target cell lysis observed in the presence of cognate peptide only.

**Flow cytometric analysis of MHC-I expression**

To measure the cell surface density of L\(^{d}\) or K\(^{b}\) on T2-L\(^{d}\), T2-K\(^{b}\), and RMA-S cells, the cells were incubated (5 \(\times\) 10\(^{5}\) cells per ml K medium) at 37\(^{\circ}\)C with or without various concentrations of peptide for 2.5–8 hr. The cells were then washed in phosphate buffered saline (PBS) and incubated for 20 min at 4\(^{\circ}\)C with affinity-purified anti-MHC-I ("primary") antibody (5 \(\mu\)g/ml) in PBS with 5% fetal calf serum and 0.02% NaN\(_{3}\). The cells were washed and incubated for 20 min with 15 \(\mu\)g/ml FITC-labeled goat anti-rat Ig when the primary antibody was M1/42 [a rat IgG\(_{2}\) monoclonal antibody that binds to all murine MHC-I proteins except D\(^{b}\) (Kane et al., 1989)] or with 15 \(\mu\)g/ml FITC-labeled F(ab')\(_{2}\) of goat anti-mouse Ig when the primary antibody was Y3 (anti-K\(^{b}\) monoclonal antibody (Hämmerling et al., 1982)) or 28-14-8 [anti-L\(^{d}\) monoclonal antibody (Ozato et al., 1980)]. We observed that M1/42 failed to bind murine MHC-I \(\alpha\) chains in association with human \(\beta\)-microglobulin on transfected T2 cells (data not shown); hence for these cells we used Y3 as the primary antibody. Live cells (10\(^{5}\)) were gated by forward and perpendicular light scatter and scored for fluorescence in an EPICS-C flow cytometer. The fluorescence values (in arbitrary units) are representative of at least two independent experiments.
RESULTS AND DISCUSSION

Peptide p2Ca is recognized by 2C cells in association with Ld or Kb

Previous studies established that the naturally occurring peptide p2Ca can associate with Ld (Udaka et al., 1992). To determine whether p2Ca can associate with MHC-I proteins of the H-2b haplotype and whether the resulting complexes are recognized by 2C cells, we compared the p2Ca-dependent lysis of EL4 cells (H-2b haplotype) with that of Ld-expressing B2 cells (Fig. 1a), using 2C cells as CTL. Both target cells were killed but lysis of EL4 cells was very limited and required an approximately 1000-fold higher concentration of peptide to achieve 50% maximal lysis. To learn whether the restricting MHC-I protein on EL4 cells was Kb or Dd, we examined the 2C-mediated lysis of T2 cells that had been transfected with the z chain gene for one of the murine MHC-I proteins Ld, Kb, or Dd. p2Ca was recognized on T2-Ld and T2-Kb target cells, but not on T2-Dd (Fig. 1b). As in the comparison of EL4 (H-2b) with B2 (H-2d) target cells (Fig. 1a), the concentration of peptide required to sensitize target cells for 50% maximal lysis (the SD50 value, Tsomides et al., 1991) was about 1000 times higher for Kb-expressing than for Ld-expressing T2 target cells. The much greater reactivity of T2-Kb than EL4 target cells can be attributed to the defective loading of endogenous peptides onto newly synthesized MHC-I protein in T2 cells, resulting in a relatively high cell surface density of Kb molecules that are accessible for occupancy by exogenous peptide (Hosken and Bevan, 1990).

Peptide p2Ca does not bind to Dd

Some earlier studies have suggested that peptide-MHC-I binding is relatively promiscuous: a peptide that binds to one MHC-I protein may bind to many of them (Bouillot et al., 1989; Frelinger et al., 1990; Chen et al., 1990). Accordingly, we asked whether the absence of reactivity of 2C cells toward T2-Dd incubated with p2Ca (Fig. 1b) arose from failure of p2Ca to bind to Dd or from failure of the 2C-TcR to recognize p2Ca-Dd complexes. To distinguish between these possibilities, we assessed the inhibitory effect of p2Ca on the lysis of T2-Dd target cells sensitized with an influenza virus peptide (NP366) by an anti-influenza CTL line (anti-PR8) that is known to recognize peptide NP366 in association with Dd (Hosken and Bevan, 1992). As shown in Fig. 2, lysis of T2-Dd cells incubated with NP366 at its SD50 concentration (10 pg/ml) was not inhibited by p2Ca at 10 µg/ml, i.e. at a 10-fold excess of p2Ca over the influenza peptide. As a control, a known Dd-binding peptide (E1A234, see Table 1) readily inhibited lysis by the anti-influenza CTL line (Fig. 2b). Thus, while p2Ca can clearly associate with either Ld or Kb (Fig. 1b and see below), it does not bind to a significant extent to Dd.

Sensitization of Kb+ target cells by p2Ca is due to the peptide itself and not to trace amounts of a contaminating peptide

Since concentrations of peptides far too low to be detected by UV absorbance can sensitize target cells for lysis in cytotoxicity assays (Rötschke et al., 1990; Schumacher et al., 1991; Tsomides and Eisen, 1993b), it was necessary to determine whether the sensitization of Kb+ target cells by high concentrations of p2Ca was actually due to this peptide or to unknown trace contaminants, which are commonly present in synthetic peptide preparations. As noted in Fig. 3, under two different and discriminating reverse phase HPLC fractionation conditions, the peaks for p2Ca and for T2-Kb sensitizing activity were coincident, demonstrating that p2Ca and not a minor contaminating peptide is recognized in association with Kb by 2C cells.

The binding affinities of p2Ca for Ld and Kb

The requirement for a much higher concentration of p2Ca to sensitize Kb+ than Ld+ target cells (Fig. 1) could be due to weaker binding of the peptide to Kb than to Ld. This was suggested by the observation that p2Ca was not fully inhibitable by the Kb-binding peptide p2Ca at concentrations up to 10 µg/ml (Fig. 2), which was not the case for Dd. The p2Ca-Db complex was not appreciably inhibited by p2Ca at high concentrations, which is consistent with the previous observation that p2Ca can associate with either Ld or Kb but not with Dd.
A CTL clone recognizes a self peptide with self MHC

The mutant cell lines RMA-S (H-2k), T2-Kb, and T2-Ld have greatly reduced levels of surface MHC-I due to defects in the intracellular loading of these proteins with endogenous peptides, and these levels can be specifically enhanced by incubating the cells with peptides that bind to the relevant MHC-I protein (Townsend et al., 1989; Hosken and Bevan, 1990). Therefore, we compared the ability of p2Ca to enhance the surface expression of Ld or Kb with that of other peptides whose equilibrium constants for binding to Ld or Kb have been determined elsewhere. As shown in Fig. 4A, an approximately 330-fold higher concentration of p2Ca than of pOV8 was required to bring about an equivalent increase in the surface expression of Kb on RMA-S cells (i.e., to the same mean fluorescence value). For T2-Kb cells an approximately 1000-fold higher concentration of p2Ca than of pOV8 was required to achieve the same increase in Kb expression (data not shown). These large differences

**Fig. 3.** Peptide p2Ca, and not a contaminant of the synthetic peptide preparation, is responsible for the sensitization of Kb-expressing target cells. Twenty μg p2Ca was injected into a Beckman 334 HPLC system equipped with a C18 reverse phase column (Vydac 218TP104). Fractions were eluted using a solvent B gradient of 0.25% TFA in water and solvent B = 0.085% TFA in acetonitrile. The eluate was monitored by absorbance at 220 nm using an Applied Biosystems 757 detector. Each 1 ml fraction was dried, taken up in 70 μl K medium, and analyzed in duplicate (20 μl/well) in a 4 hr lysis assay using 5Cr-labeled T2-Kb as target cells and 2C as CTL (●); possible toxicities associated with the fractions were also tested by omission of the CTL (○). A second HPLC fractionation method using a C4 reverse phase column with 5 μm particles (Beckman) and a shallower gradient (0.167% TFA = 0.007 pg/ml for p2Ca (shown by vertical arrows). (B) T2-Ld cells were incubated with 12I-pMCMV (filled squares) or p2Ca (△) for 8 hr and then stained with the anti-Ld mAb 28-14-8 and FITC-labeled goat anti-mouse Ig F(ab')2. The fluorescence of live cells (gated by low angle and 90° scattered light) was measured by flow cytometry. Fluorescence values were converted to an arbitrary linear scale from 0 (background) to 100 (maximum). The concentration of peptide required to elicit 50% of the maximal response was ≈0.006 μg/ml for pOV8 (●) or p2Ca (△) for 8 hr and then stained with the anti-Kb mAb Y3 followed by FITC-labeled goat anti-mouse Ig F(ab')2. The fluorescence of live cells (gated by low angle and 90° scattered light) was measured by flow cytometry. Fluorescence values were converted to an arbitrary linear scale from 0 (background) to 100 (maximum). The concentration of peptide required to elicit 50% of the maximal response was ≈0.007 μg/ml for 12I-pMCMV and ≈4 μg/ml for p2Ca (shown by vertical arrows).

**Fig. 4.** Effect of peptides on cell surface expression of Kb and Ld in the antigen processing mutant cells RMA-S and T2-Ld, respectively. (A) RMA-S cells were incubated at 37°C with the indicated concentrations of pOV8 (●) or p2Ca (△) for 8 hr and then stained with the anti-Kb mAb Y3 followed by FITC-labeled goat anti-mouse Ig F(ab')2. The fluorescence of live cells (gated by low angle and 90° scattered light) was measured by flow cytometry. Fluorescence values were converted to an arbitrary linear scale from 0 (background) to 100 (maximum). The concentration of peptide required to elicit 50% of the maximal response was ≈0.006 μg/ml for pOV8 (●) or p2Ca (△) for 8 hr and then stained with the anti-Ld mAb 28-14-8 and FITC-labeled goat anti-mouse Ig F(ab')2. The fluorescence of live cells (gated by low angle and 90° scattered light) was measured by flow cytometry. Fluorescence values were converted to an arbitrary linear scale from 0 (background) to 100 (maximum). The concentration of peptide required to elicit 50% of the maximal response was ≈0.007 μg/ml for 12I-pMCMV and ≈4 μg/ml for p2Ca (shown by vertical arrows).

In view of these relatively low association constants (particularly for the p2Ca-Kb interaction), we evaluated the binding of p2Ca to Ld and Kb by an independent approach: comparison by flow cytometry of the ability of p2Ca to enhance the surface expression of these MHC-I molecules on mutant cells. The mutant cell lines RMA-S (H-2k), T2-Kb, and T2-Ld have greatly reduced levels of surface MHC-I due to defects in the intracellular loading of these proteins with endogenous peptides, and these levels can be specifically enhanced by incubating the cells with peptides that bind to the relevant MHC-I protein (Townsend et al., 1989; Hosken and Bevan, 1990). Therefore, we compared the ability of p2Ca to enhance the surface expression of Ld or Kb with that of other peptides whose equilibrium constants for binding to Ld or Kb have been determined elsewhere. As shown in Fig. 4A, an approximately 330-fold higher concentration of p2Ca than of pOV8 was required to bring about an equivalent increase in the surface expression of Kb on RMA-S cells (i.e., to the same mean fluorescence value). For T2-Kb cells an approximately 1000-fold higher concentration of p2Ca than of pOV8 was required to achieve the same increase in Kb expression (data not shown). These large differences

**Fig. 3.** Peptide p2Ca, and not a contaminant of the synthetic peptide preparation, is responsible for the sensitization of Kb-expressing target cells. Twenty μg p2Ca was injected into a Beckman 334 HPLC system equipped with a C18 reverse phase column (Vydac 218TP104). Fractions were eluted using a solvent B gradient of 0.25% B/min (- - -) and a flow rate of 1 ml/min, with solvent A = 0.1% trifluorocetic acid (TFA) in water and solvent B = 0.085% TFA in acetonitrile. The eluate was monitored by absorbance at 220 nm using an Applied Biosystems 757 detector. Each 1 ml fraction was dried, taken up in 70 μl K medium, and analyzed in duplicate (20 μl/well) in a 4 hr lysis assay using 5Cr-labeled T2-Kb as target cells and 2C as CTL (●); possible toxicities associated with the fractions were also tested by omission of the CTL (○). A second HPLC fractionation method using a C4 reverse phase column with 5 μm particles (Beckman) and a shallower gradient (0.167% TFA = 0.007 pg/ml for p2Ca (shown by vertical arrows). (B) T2-Ld cells were incubated with 12I-pMCMV (filled squares) or p2Ca (△) for 8 hr and then stained with the anti-Ld mAb 28-14-8 and FITC-labeled goat anti-mouse Ig F(ab')2. The fluorescence of live cells (gated by low angle and 90° scattered light) was measured by flow cytometry. Fluorescence values were converted to an arbitrary linear scale from 0 (background) to 100 (maximum). The concentration of peptide required to elicit 50% of the maximal response was ≈0.006 μg/ml for pOV8 (●) or p2Ca (△) for 8 hr and then stained with the anti-Kb mAb Y3 followed by FITC-labeled goat anti-mouse Ig F(ab')2. The fluorescence of live cells (gated by low angle and 90° scattered light) was measured by flow cytometry. Fluorescence values were converted to an arbitrary linear scale from 0 (background) to 100 (maximum). The concentration of peptide required to elicit 50% of the maximal response was ≈0.007 μg/ml for 12I-pMCMV and ≈4 μg/ml for p2Ca (shown by vertical arrows).

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(330-1000-fold) are in approximate agreement with the large difference in equilibrium constants \(K_a\) for the binding of pOV8 and p2Ca to K\(^b\); by two different methods (Matsumura et al., 1992; Kageyama et al., submitted), the \(K_a\) for pOV8 binding to K\(^b\) was 1.5 \times 10^4 \text{M}^{-1} and 1 \times 10^5 \text{M}^{-1}, respectively, whereas the \(K_a\) for p2Ca binding to K\(^b\) was 1 \times 10^5 \text{M}^{-1}, 1000-fold lower.

A similar analysis using T2-L\(^d\) cells, shown in Fig. 4B, revealed that about a 500-fold higher concentration of p2Ca than of \(1_{-1}\)-pMCMV was required to elicit an equivalent increase in L\(^d\) expression. This difference also agrees with the approximately 400-fold difference in equilibrium constants observed using the competition binding assay to measure the binding of these peptides to intact T2-L\(^d\) cells: the \(K_a\) for p2Ca-L\(^d\) binding to L\(^d\) was 4 \times 10^5 \text{M}^{-1}, while for p2Ca binding to L\(^d\) the \(K_a\) was 1 \times 10^6 \text{M}^{-1} \text{(see above).}\) The convenience of flow cytometry as an assay for peptide binding to MHC-I molecules on intact cells has led to its widespread use; it is therefore noteworthy that differences in equilibrium constants for peptides binding to the same MHC-I molecule (although not the actual values of these constants) are fairly well predicted by differences in peptide concentrations required to elicit an equivalent increase in MHC-I expression.

Since the affinity of K\(^b\) for p2Ca is only about 10-fold lower than the affinity of L\(^d\) for this peptide (see above), the 1000-fold difference in peptide concentrations required to sensitize T2-L\(^d\) or T2-K\(^b\) target cells for maximal lysis by 2C cells (Fig. 1b) suggests that the affinity of the 2C-TcR for the p2Ca-K\(^b\) complex is very much lower than for the p2Ca-L\(^d\) complex. This expectation has been verified recently by the finding that these affinity values are 3 \times 10^3 \text{M}^{-1} (2C-TcR for p2Ca-K\(^b\)) and 2 \times 10^5 \text{M}^{-1} (2C-TcR for p2Ca-L\(^d\)), respectively (Sykulev et al., 1994). Thus the difference in sensitizing activities of peptide p2Ca using target cells with two different MHC restriction elements is consistent with the combined effects of different peptide-MHC-I affinities and different TcR affinities for the respective peptide-MHC-I complexes (p2Ca-L\(^d\) and p2Ca-K\(^b\)). It should be noted that use of transfectants of the same target cell in each case (i.e. T2-L\(^d\) and T2-K\(^b\)) minimizes other potential differences between target cells (e.g. accessory molecule differences). Moreover, we have determined that the number of surface L\(^d\) and K\(^b\) molecules accessible for peptide binding on these cells is approximately the same (6000 and 4000 per cell, respectively, Kageyama et al., submitted).

The amino acid sequence of p2Ca is compatible with its binding to K\(^b\) and not to D\(^b\).

Naturally occurring peptides that associate with a given MHC-I protein are typically eight or nine amino acids in length and vary in composition at all positions, except one or two “anchor” positions where only one or a few amino acids occur with high frequency (Falk et al., 1991). For K\(^b\)-binding octapeptides, Tyr and Phe are predominant at position 5 (position 6 for nonamer peptides), and Leu is the C-terminal residue (position 8 or 9) (see Table 1). In peptide p2Ca, Leu at position 8 fits this motif but Phe occurs at positions 4 and 6, not at 5. However, the presence in this peptide of proline residues at positions 3 and 5 could have the effect of constraining the peptide backbone in such a way that Phe-4 or Phe-6 takes the place of Phe-5 in promoting interaction with K\(^b\). This imperfect match may explain the relatively weak binding of p2Ca to K\(^b\).

The sequence of p2Ca is also in accord with its failure to bind to D\(^b\): Asn at position 5, an anchor residue for peptide binding to this MHC-I protein, is absent from p2Ca, which has no Asn anywhere in its sequence. Interestingly, p2Ca also does not satisfy the consensus motif for L\(^d\) [proline at position 2 and leucine at position 9 (Falk et al., 1991; Cort et al., 1992)]; nevertheless, this peptide binds well enough to L\(^d\) \((K_a \approx 1 \times 10^5 \text{M}^{-1})\), demonstrating that not all MHC-I-binding peptides must conform strictly to consensus motifs.

CONCLUDING REMARKS

Naturally processed peptides recognized by CTL in association with cell surface MHC-I proteins on target cells have been identified for several anti-viral CTL (Rötzschke et al., 1990; van Bleek and Nathenson, 1990; Del Val et al., 1991; Tsomides et al., submitted). For each of these CTL, as well as for an anti-ovalbumin clone (Rötzschke et al., 1991b), a single peptide-MHC-I complex has been identified. Similarly, when HPLC fractions from allogeneic cells or tissues have been tested for their ability to sensitize target cells for lysis by alloreactive MHC-I-restricted CTL, sensitizing activity in a single fraction has generally been found (Heath et al., 1991; Rötzschke et al., 1991a). All of these observations suggest that the antigen-specific TcR of a CTL clone recognizes to a significant extent only one peptide-MHC-I complex, regardless of whether the MHC restricting element is syngeneic with the CTL (for anti-viral CTL) or allogeneic (for alloreactive CTL).

However, ongoing experience with CTL clone 2C, extended in the present study, shows that this clone’s TcR can recognize a variety of peptide-MHC-I complexes (Udaka et al., 1992, 1993; Wu et al., in preparation), albeit with diverse affinities (Sykulev et al., 1994).

2C clone 2C arose in a BALB.B (H-2\(^b\)) mouse that had been immunized with H-2\(^b\) cells, and this clone recognizes L\(^d\) (Kranz et al., 1984) in association with several naturally processed peptides, at least two of which represent overlapping sequences derived from the ubiquitous enzyme 3-ketoglutarate dehydrogenase (Udaka et al., 1992, 1993). One of these peptides, p2Ca, is now shown to be recognized by the same TcR in association with K\(^b\). What makes recognition of the p2Ca-K\(^b\) complex of particular interest is that Sha et al. (1988a, b) have shown that it is K\(^b\), and not D\(^b\), that is required for positive selection of maturing CD8\(^+\) thymocytes in mice transgenic for the 2C-TcR. Furthermore, from the effects of various K\(^b\) mutations, and the positions of these mutations in the peptide binding...
A CTL clone recognizes a self peptide with self MHC

groove, they deduced that a self peptide in association with K\textsuperscript{a} drives positive selection of 2C-TcR\textsuperscript{*} thymocytes in 2C-TcR transgenic mice (Sha et al., 1990). Because peptide p2Ca has been detected in the thymus and other tissues of H-2\textsuperscript{b} mice (Udaka et al., 1992), and is shown here to bind to K\textsuperscript{a} (and not to D\textsuperscript{b}) and to form complexes that are recognized weakly by the 2C-TcR, this octapeptide is an interesting candidate for a positively selecting peptide in these mice. However, recent findings (Ashton-Rickardt et al., 1993) suggest that it is not one but several, and perhaps many, peptides in the thymus that, in association with the restricting MHC-I protein, cause positive selection of CD8\textsuperscript{+} T cells having a given TcR. It will therefore be of interest to determine whether p2Ca in K\textsuperscript{a}+ mice transgenic for the 2C-TcR is only one of many peptides that can cause positive selection of CD8\textsuperscript{+} cells having the 2C-TcR or whether, in view of the properties described here, p2Ca has a special role in positive selection of these T cells in these mice.

Peptide p2Ca is derived intracellularly from a protein, \(\alpha\)-ketoglutarate dehydrogenase, that is present in all cells (Udaka et al., 1993). Not surprisingly, the peptide is present in spleen and thymus of H-2\textsuperscript{b} (as well as H-2\textsuperscript{a} and H-2\textsuperscript{d}) mice: it has more recently been found in many other tissues as well (Wu et al., in preparation) and is doubtless also widely distributed in the 2C-TcR transgenic mice. Since almost all CD8\textsuperscript{+} T cells in these H-2\textsuperscript{b} mice express the 2C-TcR, the question arises as to why no evidence for T cell autoreactivity has been observed in these transgenic animals. One reason, suggested by the findings in this study, is that the minimal number of p2Ca-K\textsuperscript{a} complexes per target cell required for activating effector function of 2C CTL (cytolysis) is probably much higher than what is found normally on cells in vivo; and it is clearly higher than that required for similar activity in response to p2Ca-L\textsuperscript{d} complexes.

Some indication of these numbers can be gleaned from the equilibrium constants for the binding of peptide p2Ca to L\textsuperscript{d} and K\textsuperscript{a} and the number (n) of available MHC-I binding sites per target cell under conditions where cells are subject to lysis by 2C in cytotoxicity assays. As described elsewhere for T2-K\textsuperscript{d} and T2-L\textsuperscript{d} cells under these conditions, the values of n at 37\textdegree C are \(\approx 4000\) and \(\approx 6000\) per cell, respectively (Kageyama et al., submitted). Taking the free peptide concentration to be the \(S_{0.5}\) value, or peptide concentration required for half-maximal lysis in cytotoxicity assays, we estimate that a 60-fold higher target cell surface density of p2Ca-K\textsuperscript{a} than of p2Ca-L\textsuperscript{d} is required to trigger cytolytic activity of 2C cells. Nevertheless, the amount of peptide p2Ca recovered from H-2\textsuperscript{d} and H-2\textsuperscript{a} tissues was approximately the same (Udaka et al., 1992). Accordingly, although direct extrapolation from these in vitro considerations to in vivo conditions is fraught with many uncertainties, we believe it likely that the absence of overt autoreactivity in H-2\textsuperscript{a} 2C-TcR transgenic mice is due to the low affinity of the 2C-TcR for p2Ca-K\textsuperscript{a} complexes relative to the naturally occurring cell surface density of these complexes, and not to the complete absence of potentially autoreactive T cells or their natural ligands. According to this model, if the expression of p2Ca-K\textsuperscript{a} complexes were to increase substantially in these mice, e.g. by a large increase in expression of cell surface K\textsuperscript{a} or from an excess production of the protein from which p2Ca is derived (or both), an autoreactive response might well ensue. The significance of similar numerical considerations for assessing a possible role of p2Ca-K\textsuperscript{b} complexes in positive selection of 2C-TcR\textsuperscript{*} thymocytes is now also subject to direct experimental analysis using appropriate assay systems, such as cultured thymus organ grafts from mice genetically deficient in antigen processing (Ashton-Rickardt et al., 1993) and transgenic for the 2C-TcR.

In assuming that at least some of the p2Ca extracted from Kb\textsuperscript{+} tissues is indeed associated with K\textsuperscript{a}, we have relied upon three considerations: (1) in H-2\textsuperscript{a} cells, p2Ca was recovered from immunoprecipitates of L\textsuperscript{d} (but not D\textsuperscript{b}) (Udaka et al., 1992), indicating that this peptide gains access to the intracellular compartment where peptide binding to MHC-I takes place (i.e. endoplasmic reticulum); (2) the mechanism for generating antigenic peptides and translocating them into the endoplasmic reticulum is conserved among inbred mouse strains of different haplotypes (e.g. BALB/c, BALB.B); and (3) our evidence that p2Ca can be bound by K\textsuperscript{a}, albeit with a somewhat lower affinity than by L\textsuperscript{d}. Thus it is highly likely that at least some p2Ca is associated with K\textsuperscript{a} in H-2\textsuperscript{a} tissues.

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High-affinity reactions between antigen-specific T-cell receptors and peptides associated with allogeneic and syngeneic major histocompatibility complex class I proteins

(Alloreactivity/antigen recognition/self-nonself discrimination)

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ABSTRACT We report here that the intrinsic affinities of the antigen-specific T-cell receptors (TCR) of two unrelated CD8+ T-cell clones for their respective peptide-major histocompatibility complex (MHC) ligands are higher than the values generally thought to prevail for TCR. The TCR of one clone (2C) binds an allogeneic class I MHC protein (Ld) in association with an α-ketoglutarate dehydrogenase nonapeptide (QLSPFPFDL; termed QL9) with an intrinsic affinity (intrinsic equilibrium association constant) of 1-2 x 10^4 M^-1. The TCR of the other clone (4G3) binds a syngeneic class I MHC protein (Kb) in association with an ovalbumin octapeptide (SIINFEKL; termed pOV8) with an intrinsic affinity of 1.5 x 10^6 M^-1. A comparison of the two clones, combined with current views of T-cell repertoire selection in the thymus, leads us to propose that TCR affinities are generally likely to be higher for allogeneic MHC-peptide complexes than for syngeneic MHC-peptide complexes.

Because the somatic hypermutation that underlies the generation of high-affinity antibodies is not evident in T cells, it has long been surmised that the affinities of antigen-specific T-cell receptors (TCR) for their natural ligands, now known to be peptide-major histocompatibility complex (MHC) complexes, are likely to be low and in the narrow range (10^4 to 10^5 M^-1) exhibited by antibodies made early in immune responses, before the onset of somatic hypermutation. Previously, however, we showed (1) that one of the two murine T-cell clones studied here (clone 2C) has a relatively high intrinsic affinity (2 x 10^6 M^-1) for its ligand: Ld, a class I MHC protein, in association with an octapeptide (SIINFEKL; termed pOV8) that one of the two murine T-cell clones studied here (clone 2C) has a relatively high intrinsic affinity (2 x 10^6 M^-1) for its ligand: Ld, a class I MHC protein, in association with an octapeptide (SIINFEKL; termed pOV8) with an intrinsic affinity of 1.5 x 10^6 M^-1. A comparison of the two clones, combined with current views of T-cell repertoire selection in the thymus, leads us to propose that TCR affinities are generally likely to be higher for allogeneic MHC-peptide complexes than for syngeneic MHC-peptide complexes.

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peptide binding sites. Soluble peptide–MHC complexes (50 μg/mL) were radiolabeled with 2.2 mCi of carrier-free Na125I (using Iodo-Beads, Pierce; 1 Ci = 37 GBq) in 0.05 M sodium phosphate (pH 8.2) to promote substitution of histidine residues in the six-histidine-residue extension at the C-terminal end of both Ld and Kβ.

Equilibrium Binding of Peptide–MHC Complexes to TCR on Intact T Cells. Two approaches were used to measure the intrinsic equilibrium association constants (intrinsic affinities) for the reaction of soluble peptide–MHC molecules with the TCR on T cells. One depended upon the inhibition of the binding of 125I-labeled Fab' fragments of an anti-receptor antibody to intact T cells. As shown in Fig. 1, sensitization of L4+ target cells (T2-Ld) for lysis by 2C cells was achieved with 50–100 times less QL9 than the octapeptide p2Ca, which was previously the most active known Ld-restricted peptide for 2C cells (2, 3). QL9 was also 1000–10,000 times more potent than two other nonamers, SPPFPDLLSL9 and LSPPFPDLLL9, whose sequences also overlap p2Ca in α-KGDH.

Affinity of the TCR on 2C Cells for the QL9–Ld Complex, Measured in Competition with an Anti-TCR Antibody Fragment. Because QL9 was so active in sensitizing target cells for lysis (Fig. 1), it seemed possible that the QL9–Ld complex might bind with higher affinity than the p2Ca–Ld complex to the 2C TCR. Accordingly, we compared the inhibitory effects of soluble QL9–Ld and soluble p2Ca–Ld on the binding of the 125I-labeled Fab' fragment of antibody 1B2 to intact 2C cells. As shown in Fig. 2, the QL9–Ld complex was a far more potent inhibitor. From the inhibition data, the intrinsic affinity of this TCR for QL9–Ld was calculated to be 1 × 10^8 M⁻¹ (Table 1).

RESULTS

Sensitization of L4+ Target Cells by Various Peptides from α-KGDH. As shown in Fig. 1, sensitization of L4+ target cells (T2-Ld) for lysis by 2C cells was achieved with 50–100 times less QL9 than the octapeptide p2Ca, which was previously the most active known Ld-restricted peptide for 2C cells (2, 3). QL9 was also 1000–10,000 times more potent than two other nonamers, SPPFPDLLSL9 and LSPPFPDLLL9, whose sequences also overlap p2Ca in α-KGDH.

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![Fig. 1. Specific lysis of transfected T2 target cells by two CD8+ CTL clones. (Upper) Lysis of T2-Ld cells by 2C cells in presence of various concentrations of p2Ca or three nonpeptides (QL9, SL9, and L9) having overlapping sequences in murine α-KGDH (3). (Lower) Lysis of T2-Kβ cells by 4G3 cells in presence of various concentrations of the ovalbumin peptide p0V8.](image-url)
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- **Fig. 2.** Binding of soluble QL9-Ld to the TCR on 2C cells measured by inhibition of the binding of 125I-labeled Fab' fragments of an anti-2C TCR clonotypic antibody (1B2). B/Bo is the ratio of the amount of the TCR-bound 125I-labeled Fab' in the absence (Bo) and presence (B) of soluble peptide-MHC complexes at the free concentrations shown (abscissa). The dashed line represents the best fit of experimental points to the theoretical binding curve (for details, see ref. 1). Binding of two other soluble peptide-Fab' complexes to the 2C TCR has been described (1) and is shown here for comparison: a refers to the p2Ca-Ld complex and c refers to the control complex formed by Ld with a mouse cytomegalovirus peptide (YPHFMPFTNL).

**Direct Binding of the QL9-Ld Complex to Intact 2C Cells.** To confirm the high binding constant for the reaction of QL9-Ld with the 2C TCR, we measured the direct binding over time of this complex (trace-labeled with 125I) to intact 2C cells, omitting the competitive anti-receptor antibody (1B2) fragment. As shown in Fig. 3A, the specificity of the direct binding was evident from the inhibitory effect of the clonotypic antibody 1B2 or of an excess of unlabeled QL9-Ld complex (Fig. 3A Inset). From the best fit of the kinetic binding data to Eq. 1, it was possible to determine both (i) the maximal number of specifically bound QL9-Ld complexes per cell at equilibrium (the term a, corrected for the number of cells and the volume of the reaction mixture) and (ii) the time constant \( \tau \) (the time required to reach 63% of the equilibrium value); \( \tau = 3.9 \) min (Table 1) and \( \alpha \) (at a free peptide–MHC concentration of 4 nM) was 10,500 ± 2100 bound complexes per cell. Inasmuch as we had previously determined (and confirmed below, see Fig. 3B) that there are close to 10⁶ TCR molecules per 2C cell, the equilibrium constant was calculated to be \( 2 \times 10^{10} \text{M}^{-1} \), in agreement with the value determined in the competition assay of Fig. 2. In this calculation, the equilibrium association constant is given by \( \alpha/(c_n - c_a) \), where \( n \) is the number of TCR molecules per cell. Since the number of TCR molecules per 2C cell varied in different experiments (1) from 80,000 to 110,000 (100,000 average), the calculated equilibrium constant was accurate to within a factor of 2.1

This binding constant was further confirmed by measuring the number of peptide–MHC complexes bound per 2C cell at equilibrium with various free concentrations of 125I-labeled QL9-Ld. These results, shown in the Scatchard plot in Fig. 3B, yielded an equilibrium constant of \( 1.9 \pm 0.2 \times 10^{10} \text{M}^{-1} \). Extrapolation of the plot in Fig. 3B to infinite free concentration of the peptide–MHC complex yielded a limiting value of 93,000 ± 5600 binding sites per 2C cell, also in agreement with the total number of TCR molecules per cell previously found with the Fab' fragment of the 1B2 antibody (1). Thus, by three different approaches, the affinity of the 2C TCR for the QL9-Ld complex was found to be 1–2 × 10⁶ M⁻¹ at 23°C (Table 1) or 5–10 times higher than the previously reported value for this receptor’s affinity for the p2Ca-Ld complex (1).

All of the affinity and kinetic measurements described above and previously were carried out at room temperature.

**Table 1.** Equilibrium and rate constants for the reactions of TCR of two unrelated T-cell clones (2C and 4G3) with peptide–MHC complexes

<table>
<thead>
<tr>
<th>Binding parameter</th>
<th>pOV9-K⁺</th>
<th>4G3 TCR</th>
<th>QL9-Ld</th>
<th>2C TCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_a ) M⁻¹</td>
<td>1.5 × 10⁶</td>
<td>1.5 × 10⁷</td>
<td>6 × 10⁹</td>
<td></td>
</tr>
<tr>
<td>( K_{M+1} ), M⁻¹sec⁻¹</td>
<td>2.2 × 10⁶</td>
<td>5.3 × 10⁴</td>
<td>9 × 10⁶</td>
<td></td>
</tr>
<tr>
<td>( \tau ), min</td>
<td>0.9</td>
<td>3.9</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>( h_{C+1} ), min</td>
<td>2.1 × 10⁻²</td>
<td>3.1 × 10⁻¹</td>
<td>1.5 × 10⁻²</td>
<td></td>
</tr>
<tr>
<td>( h_{C} ) min</td>
<td>0.5</td>
<td>3.7</td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>

Rate constants of association, \( K_{M+1} \), were calculated from \( K_{M+1} = \alpha/(c_n - c_a) \), where \( K_a \) is the total concentration of TCR, \( M \) is the free concentration of peptide–MHC complexes, \( \alpha \) is the concentration of the receptor-bound peptide–MHC complexes at equilibrium, and \( c_a \) is the time constant. Dissociation rate constants, \( K_{M+1} \), and the corresponding half-lives \( h_{C} \) were calculated from the equilibrium dissociation rate constants and association rate constants \( K_{M+1} = \alpha/(c_n - c_a) \) or measured directly for QL9-Ld by monitoring the rate of dissociation of bound 125I-labeled QL9-Ld from 2C TCR (data are not shown). The binding parameters shown are average values derived from two or more measurements, by using different methods to measure the binding of QL9-Ld to the 2C TCR. Although the accuracy of the measurements made with 4G3 cells was lower as a result of the higher background (Fig. 4A) and lower affinity than was seen with 2C cells, the results of independent titrations agreed to within a factor of 2.
TCR-peptide-MHC reactions to the physiologic responses of T cells, it is of particular interest to analyze the reactions at 37°C. Using both the direct binding assay shown in Fig. 2 and the indirect competition assay of Fig. 2, we found that in comparison with the binding at room temperature, the equilibrium constant at 37°C was only slightly lower (6 × 10⁶ M⁻¹ vs. 1.5 × 10⁷ M⁻¹) but the association and dissociation rates were much faster (Table 1).

Direct Binding of pOV8-K<sup>0</sup> Complex to TCR on Intact 4G3 Cells. To compare the alloreaction between the 2C cell (H-2<sup>b</sup>) TCR and the QL9-L<sup>0</sup> complex with a syngeneic reaction (in which the MHC of the peptide–MHC complex and on the CTL are the same), we determined the intrinsic affinity of the TCR of clone 4G3 (6). A comparison between these two CD8<sup>+</sup> clones is particularly appropriate because about the same concentration (∼3 pM) of QL9 and pOV8, their respective optimally active peptides, was required for half-maximal lysis of the appropriate T2 target cells (T2-L<sup>0</sup> and T2-L<sup>0</sup>, respectively) by 2C and 4G3 cells (see Fig. 1). Both peptides, moreover, are bound equally well by their respective restricting class I MHC proteins: for the binding of pOV8 to K<sup>0</sup> (14) and QL9 to L<sup>0</sup> the equilibrium constants are 1.2–2 × 10⁸ M⁻¹ (data not shown).

Since anti-receptor antibodies to the TCR of 4G3 cells were not available, we measured the direct binding of 125I-labeled pOV8-K<sup>0</sup> to these cells (Fig. 4A). It was necessary to use a relatively high concentration of this labeled peptide–MHC complex (57–112 nM) because the 4G3 TCR’s affinity for it was not high (see below). As a result, nonspecific binding was greater than in the corresponding titration of the 2C TCR (cf. Figs. 3 and 4). Nevertheless, TCR-bound peptide–MHC complexes (determined as the difference between peptide–MHC binding in the absence and presence of a large excess of unlabeled pOV8–K<sup>0</sup>) accumulated over time. The best fit of experimental values for the time-dependent specific binding of 125I-labeled pOV8–K<sup>0</sup> to the 4G3 TCR using Eq. 1 yielded a value for α (the amount of peptide–MHC bound at equilibrium) of 4700 ± 500 complexes per cell. From α, the total number of TCR per cell (10⁷, see below), and the free peptide–MHC concentration (57 nM), the intrinsic equilibrium association constant for the 4G3 TCR reaction with pOV8–K<sup>0</sup> was calculated to be 1 × 10⁹ M⁻¹.

In separate experiments with various free concentrations of this peptide–MHC complex, the number of specifically bound pOV8–K<sup>0</sup> complexes per cell at equilibrium were also measured. The results, presented as the Scatchard plot in Fig. 4B, yielded essentially the same equilibrium constant (1.7 ± 1.1 × 10⁸ M⁻¹). The Scatchard plot extrapolated to 140,000 ± 50,000 TCR molecules per 4G3 cell (mean ± SEM), similar to the number of TCR molecules per 2C cell (1). The association and dissociation rate constants for the pOV8–K<sup>0</sup> reaction with the 4G3 TCR (see Table 1) were also similar to those reported previously for the reaction of the p2Ca–L<sup>0</sup> complex with the 2C TCR (1).

**DISCUSSION**

This study resulted in three principal findings. One concerns the magnitude of the affinities of the TCR on two clones of widely different specificities for their respective peptide–MHC ligands. The previous finding that one of these clones (2C) has a high-affinity TCR (ref. 1, and extended here) might be ascribed to chance. But the results with the second clone (4G3) suggest that TCR affinities of this magnitude are not rare. The earlier reports of CD4<sup>+</sup> T cells with TCR affinities in the 10<sup>9</sup> to 10<sup>10</sup> M⁻¹ range (15, 16) for class II MHC–peptide complexes seemed initially to be in accord with the view that in the absence of somatic hypermutation in T cells, TCR affinities for their natural ligands would generally be low. It is doubtful that the difference between these earlier reports and the present findings reflect a systematic difference between CD4<sup>+</sup> and CD8<sup>+</sup> T cells or between peptide–MHC complexes involving class I and class II proteins. Instead, it is likely that the difference simply reflects a very wide range in TCR affinities for their natural ligands. If so, it will be important to evaluate the functional efficacy in vivo and in vitro of T cells whose TCR differ widely in intrinsic affinity.

That TCR intrinsic affinity alone is not a sufficient predictor of T-cell activity is already evident from the finding here that although their TCR differ 10-fold in intrinsic affinity for their optimal peptide–MHC ligands, the two CTL clones we examined require the same concentrations of their respective peptides to sensitize target cells for lysis (see Fig. 1). This difference could be due to a greater number of TCR molecules per cell on 4G3 than on cells (see above) or, perhaps, to greater numbers of CD8 or other accessory proteins involved in target cell recognition and lysis.

The second finding of interest arises from the difference between the MHC proteins that restrict recognition of the α-KGDH peptide and the ovalbumin peptide. Normally, the restricting MHC molecules on target cells are syngeneic (self or synMHC) with respect to the MHC proteins on T cells, but in alloreactions they are allogeneic (alloMHC). Previously, the affinities of TCR of three CD8<sup>+</sup> T cells that were specific for synMHC class II proteins (and associated peptides) were in the range 10⁴ to 10⁵ M⁻¹ (15, 16). The higher affinity (1.5 × 10⁹ M⁻¹) for the 4G3 TCR reflect a systematic difference between the two T-cell clones.
fected cells

plexes can escape this "negative selection." Left to serve as high-affinity TCR for alloMHC or peptide-alloMHC complexes are not present in the thymus. Developing T cells having synMHC are eliminated for peptide-alloMHC complexes, and for peptide-synMHC complexes. A comparison of only two clones would not ordinarily be sufficient to support a generalization. However, the comparison becomes more arresting in light of the relatively high epitope levels that seem to have been used to elicit the CD4+ T-cell clones previously studied (15, 16).

The still higher affinity (1.5 x 10^7 M^-1) for the alloreactive 4G3 (both kinetic and equilibrium binding), leaving no doubt about their validity. This direct method opens the way to relate systematically, with minimal extrapolation, the kinetics and affinity of TCR reactions with peptide-MHC complexes to the activities of viable T cells against target cells or antigen-presenting cells.

We are grateful to Dr. Kirsten Fischer Lindahl for a critical and useful review of this paper and for useful comments. We are also grateful to Richard F. Cook and the Massachusetts Institute of Technology Biopolymers Laboratory for peptide syntheses and amino acid analyses and to Dr. Peter Cresswell for generous gifts of T2, T2-L4, and T2-K8 cells. We thank Carol McKinley and Mimi Rasmussen for excellent technical support and Ann Hicks for valuable secretarial assistance. We also acknowledge with gratitude Dr. Cathryn Nagler-Anderson's original development of the 4G3 T-cell clone described in ref. 8. This work was supported by research grants CA 60666 and AI 34247, a Cancer Center grant (CA 14051), and a Training Grant (CA 09255), all from the National Institutes of Health awarded to The Scripps Research Institute.

Tissue Distribution of Natural Peptides Derived from a Ubiquitous Dehydrogenase, Including a Novel Liver-Specific Peptide That Demonstrates the Pronounced Specificity of Low Affinity T Cell Reactions

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Department of Biology and Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139

The peptides recognized by CD8+ CTL normally arise by proteolysis of intracellular proteins. To learn whether these peptides are generated similarly in diverse cell types, we examined the variety and abundance of naturally processed peptides that derive from a ubiquitous enzyme, α-ketoglutarate dehydrogenase, and are recognized in association with the class I MHC protein, Ld, by a CTL clone (2C). A characteristic set of three peptides was found in diverse tissues, but their abundance varied greatly, apparently unrelated to differences in class I MHC expression, e.g., they were surprisingly abundant in liver. We also found in liver a fourth naturally processed peptide (p2Ca-Y4, LSPYPFDL) that differs by one oxygen atom from a previously characterized natural peptide (p2Ca, LSPFPPFDL). CTL discrimination between these peptides in association with the same class I MHC protein, Kb, demonstrates the striking specificity that can be exhibited by low affinity T cell reactions. The Journal of Immunology, 1995, 154: 4495–4502.

The natural ligands of the Ag-specific receptors (TCR) on CD8+ T cells are cell surface complexes formed by peptides, usually 8 to 10 amino acids in length, and class I MHC proteins (1, 2). Under normal circumstances, these peptides arise from proteolytic fragmentation of cytosolic proteins in target cells and are transferred by transporter molecules into the cells’ endoplasmic reticulum, where they bind (possibly with further proteolytic trimming) to nascent MHC-I proteins, forming the peptide/MHC complexes that are subsequently displayed at the cell surface (3, 4). Although this “antigen processing pathway” is well supported in broad outlines by a growing body of evidence, the detailed mechanisms are obscure, and much of what is known is based largely on studies of a limited variety of cells, principally lymphoid cells and transformed cultured cell lines. However, under natural conditions, target cells (e.g., virus-infected cells or tumor cells or normal cells in allografts) are extremely diverse and can be found in virtually any tissue or organ. Whether cellular proteins are processed in the same way and to the same extent in widely divergent cell types is, therefore, a matter of some interest.

An opportunity to explore this issue is provided by the recent identification of the E1 subunit of the α-ketoglutarate dehydrogenase (α-KGDH) complex as the source of several naturally processed peptides that are recognized in association with the MHC-I protein Ia by a CD8+ CTL clone (termed 2C) (5). Since α-KGDH is essential for cell survival and is thus present in all cells, we have examined HPLC fractions of homogenates from various tissues for the presence and levels of α-KGDH-derived peptides recognized by CTL clone 2C. This approach, in contrast to simply using diverse cell types as targets in in vitro cytotoxicity assays, permits quantitative estimates of peptide abundance and makes any tissue accessible for analysis, regardless of its suitability as a target in vitro. Our results show that the pattern of the processed peptides previously found in lymphoid cells (5, 6) is largely conserved in all tissues examined, but with pronounced variations in abundance, i.e., they are surprisingly abundant in liver, less...
abundant in spleen, kidney, lung, and heart, exceedingly sparse in skeletal muscle, and undetectable in brain. In the course of analyzing these tissues, we discovered another peptide that is recognized by 2C cells in association with L<sup>1</sup>, and we established its sequence. Found only in liver, this new octapeptide extends the number of naturally processed peptides with closely related or overlapping sequences that are recognized by the Ag-specific receptor of 2C cells. It also provides a striking example of the high specificity of low affinity T cell reactions.

Materials and Methods

**Cells and mice**

Sp2/0 cells (H-2<sup>d</sup>) are non-Ig-secreting mouse hybridomas formed by fusing myeloma cells (P3X63Ag8) with BALB/c spleen B cells. Previous transfection of Sp2/0 cells with the L<sup>1</sup> gene resulted in a high L<sup>1</sup>expressing clone called B2 cells (6). T<sub>2</sub>-L<sup>1</sup>, T<sub>2</sub>-K<sup>1</sup>, and T<sub>2</sub>-D<sup>1</sup> cells (T2 cells transfectaced with the a-chain genes for L<sup>1</sup>, K<sup>1</sup>, or D<sup>1</sup>, respectively) were generous gifts of Dr. Peter Cresswell (7). CTL clone 2C was derived and maintained as described (8). The cloned mAbs 1B2 is specific for the TCR a<sup>3</sup> of CTL clone 2C (9, 10). Swiss Webster mouse cells transfected with the a-chain genes for Sp2/0 were used at 15 to 30 wk of age.

**HPLC fractionation of low molecular mass material from tissues and cells**

Tissues were homogenized in a polytron homogenizer (Brinkmann Instruments, Westbury, NY) for about 2 min on ice after adding 3 to 5 ml of cold 1% trichloroacetic acid (TFA)/g wet weight of tissue, followed by sonication for 2 min on ice (Model W-225R, Ultrasonics, Inc.). After standing overnight at 4°C, homogenates were centrifuged at 126,000 x g for 1 h at 4°C in a TL-100 centrifuge (Beckman Instruments, Palo Alto, CA). The supernatants were subjected to ultrafiltration through a Centricon-10 rotor, and stored at 4°C. The precipitated protein was redissolved in 0.1% TFA at approximately 5 mg of protein/ml as indicated. The proteins were then pooled and used for further analysis.

**Cytotoxicity assay**

For cytotoxicity assays, part of each HPLC fraction was added in duplicate to the wells of a round bottom 96-well plate. 51Cr-labeled target cells were added in a final volume of 0.2 ml of K medium/well. To control for the wells of a round bottom 96-well plate. 5iCr-labeled target cells were added in a final volume of 0.2 ml of K medium/well. To control for possible toxicity of each HPLC fraction, 2C<sup>a14-8</sup> cells and 10<sup>5</sup> cells/ml, and incubated for 1 h at 37°C, followed by centrifugation and the supernatants were subjected to ultrafiltration through a Centricon-10 rotor, and assayed for 9<sup>LD</sup>1 Cr release. Spontaneous release values varied from 8 to 15% of total 9<sup>LD</sup>1 Cr release.

**α-KGDH activity**

α-KGDH activity was determined essentially as described previously (11). Various mouse organs were removed, kept on ice during dissection, and stored at -80°C until used. Frozen material was then weighed and washed twice with 50 mM 3-[N-morpholino]propanesulfonic acid, pH 7.0 containing 2.7 mM EDTA, 0.1 mM DTT, 3% (v/v) Triton X-100, 1 mM PMSF, 1 mM benzamidine hydrochloride, and 0.05% (v/v) silicone antifoam (12). Washed tissues were homogenized for 2 min on ice at 0.3 g/ml of this buffer in a polytron homogenizer, clarified by centrifugation at 200,000 x g using a TLA-100.3 rotor, and assayed for α-KGDH activity at 30°C (11) using potassium α-ketoglutarate as substrate in place of potassium pyruvate. Kidneys were extracted in the same buffer but with 3% (v/v) FCS and 1 mM tosyl-lysine chloromethyl ketone added to minimize proteolysis as described (13).

**Synthetic peptides**

Synthetic peptides used in this study were prepared in the MIT Biopolymers Laboratory using solid phase t-Boc chemistry (Model 430A, Applied Biosystems, Foster City, CA) and hydrofluoric acid cleavage. Peptides were purified by reverse-phase HPLC, their concentrations determined by microbicinchoninic acid assay using as a standard a synthetic peptide solution whose concentration was determined by quantitative amino acid analysis.

Results

After one round of HPLC of TFA extracts derived from ~1 g tissue (wet weight), several active fractions were observed for most of the tissues shown in Figure 1, but their levels of specific lysis were relatively low (e.g., 7–20% using 0.2 g tissue equivalents/well, data not shown). To amplify the signals and reduce toxicity effects, 2 g of each tissue were homogenized, and fractions corresponding to 46 to 57 min from the first HPLC were pooled and subjected to a second round of HPLC using a substantially shallower (and thus more highly resolving) gradient (0.067% B/min vs 0.25% B/min). Cytotoxicity activities of fractions corresponding to 0.5 g tissue equivalents/well from the second round revealed three distinct peaks of activity for most tissues, shown in Figure 1, as in earlier studies with spleen and thymus (6). The slight differences in retention time from the results of Udaka et al. may be attributed to the use of a different C18 column in the present study. Nevertheless, from the profiles of spleen-derived peptides, which provide a common reference point, we determined that peaks marked a, b, and c in Figure 1 correspond to those given the same designations previously (6). The two peptides identified as responsible for some or all of the activity in peaks a and b, respectively, are p2Ca (LSPFPFDL, formerly called p2CL) (6) and p2Cb (VAITIREQLSPPFDL) (5); the active peptide in peak c has yet to be characterized.
FIGURE 1. Cytotoxicity assays of HPLC fractions from acid extracts of BALB/c tissues. TFA extracts of low molecular mass material (<10 kDa) from homogenates of brain, muscle, heart, kidney, lung, spleen, and liver were fractionated by reverse-phase HPLC using a gradient of 0.25% B/min. For each tissue, 10 fractions containing all of the active material were pooled and rechromatographed using a shallower gradient (0.067% B/min) on the same C18 column. After the second round of HPLC, aliquots of each fraction equivalent to 0.5 g wet weight of tissue/well were added to 96-well plates for a 4-h cytotoxicity assay using 2C as CTL and "3Cr-labeled T2-Ld target cells at a ratio of 5:1. A, percent specific lysis by 2C cells; O, percent specific lysis in toxicity controls (no CTL). UV absorbance profiles are not shown. The lower right panel shows a standard cytotoxicity assay in which synthetic peptide p2Ca is titrated in a 96-well plate and subjected to identical assay conditions as the HPLC fractions.

In six of the seven tissues shown in Figure 1, the same peaks were seen, albeit at markedly different levels. In brain only, no activity was observed. The titration curve shown in the lower right panel of Figure 1 was established using synthetic peptide p2Ca and otherwise the same assay conditions as for the HPLC fractions in the other panels, affording an opportunity to estimate the amounts of p2Ca in various tissues. These varied more than 100-fold, from about 4 ng/g liver to about 0.05 ng/g skeletal muscle to undetectable levels in brain (Table I). To address the possibility that peptide p2Ca was produced in brain but not detected in our assay because it was subject to unusually rapid destruction, anomalous behavior on HPLC, or unusually poor recovery from brain for some other unknown reason, we added a known amount of synthetic p2Ca to brain before homogenization. As shown in Figure 2, the added peptide was recovered from this "spiked" sample with a yield of about 25%, closely comparable with yields obtained from similarly spiked samples using other tissues (15). Hence, peptide p2Ca is not produced in brain at levels that can be detected using standard protocols.

The cytolytic responses of 2C cells to peptides in homogenates from various tissues were much more pronounced after the second round of HPLC (Fig. 1) than after the first, probably because the mass of peptides that compete for binding to Ld on the target cells was much

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Peptide p2Ca</th>
<th>α-KGDH Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>4.0</td>
<td>313 ± 3</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.4</td>
<td>66 ± 4</td>
</tr>
<tr>
<td>Lung</td>
<td>0.2</td>
<td>104 ± 5</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.2</td>
<td>76 ± 4</td>
</tr>
<tr>
<td>Cardiac muscle</td>
<td>0.1</td>
<td>106 ± 9</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>0.05</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>Brain</td>
<td>&lt;0.04</td>
<td>41 ± 1</td>
</tr>
</tbody>
</table>

*ng/g wet tissue, not corrected for approximately 25% overall yield of acid extraction, ultrafiltration, and HPLC fractionation steps.

**U/g tissue, where 1 U is the amount of enzyme required to produce 2 mmol of ferrocyanide (Fe(CN)6)4−/h at 30°C using ferrocyanide (Fe(CN)6)3− as electron acceptor (11).
greater in the first round. Residual competing peptides in the second round could have affected the accuracy of our estimates of peptide abundance. However, the error was probably small, because at least 70% of the total UV absorbance was removed by the first HPLC round. Moreover, in control experiments, the amounts of synthetic peptide recovered from HPLC runs were not appreciably different when peptides were introduced alone or were "spiked" into homogenates (as in Fig. 2).

The tissues examined in Figure 1 differ considerably in their expression of MHC-I proteins (16). Detailed studies by Rammensee et al. (2) emphasized that the presence of several peptides in tissue homogenates was dependent on the expression of particular MHC-I proteins; therefore, we compared homogenates from Sp2/0 hybridoma cells, which have barely detectable levels of Ld (Fig. 3 and Ref. 6) and B2 cells (a clone of Ld gene-transfected Sp2/0 cells). As shown in Figure 3, B2 cells express over 30-fold higher levels of Ld than Sp2/0, yet their TFA homogenates contain indistinguishable HPLC profiles of the peptides recognized in association with Ld by 2C CTL.

To determine whether wide tissue variation in the levels of α-KGDH-derived peptides reflected differences in the amounts of α-KGDH E1 subunit (the source of the peptides recognized by 2C CTL) (5), we measured the enzyme's activity in various tissues as an indicator of its abundance. As shown in Table I, α-KGDH activity was highest in liver and lowest in skeletal muscle, paralleling the differences in p2Ca levels in these tissues. However, the correlation was limited, because enzyme activity was only seven- to eightfold higher, and the p2Ca level was about 100-fold higher in liver than in skeletal muscle. The deviation from a linear correlation was even more apparent in brain: although α-KGDH activity in brain was about 40% of that in liver, no α-KGDH-derived peptides were detected in brain homogenates (0.5 g tissue equivalents/well) under standard assay conditions.

The HPLC activity profile from liver, shown in the lower left panel of Figure 1, revealed that, in addition to peaks a, b, and c common to most tissues, a fourth peak of activity with an earlier retention time was observed in liver extracts from both BALB/c and Swiss Webster mice (but not C57Bl/6 mice). Designated a', the active component in this peak was isolated for sequence determination starting from 180 g of mouse liver (Pel-Freeze). The livers were homogenized and subjected to ultrafiltration, followed by seven successive rounds of HPLC fractionation under different conditions as described in Table II. After each HPLC round, fractions were tested in the standard CTL assay, and the active peak (starting with a' in the first round, Fig. 1) was subjected to a subsequent round, until the activity was deemed sufficiently pure for sequence determination. About 90% of the active fraction from the seventh round was analyzed by Edman degradation, and the remainder was analyzed by mass spectrometry (Drs. Ioannis Papayannopoulos and Klaus Biemann, MIT, Department of Chemistry). The Edman procedure resulted in an N-terminal sequence of seven residues, LSPYPFDL, from the molecular mass of 951.4, it was inferred that the complete sequence was LSPYPFDFX, where X = leucine or isoleucine. To distinguish between these possibilities for the C-terminus, the corresponding synthetic peptides were compared in a CTL assay. As shown in Figure 4, the peptide with X = leucine was 20-fold more active than the one with X = isoleucine. Based on this finding and the match in HPLC retention time between synthetic peptide LSPYPFDL and the naturally occurring peptide in peak a' (Fig. 1), we concluded that the active peptide in peak a' is LSPYPFDDL. Because this sequence differs from that of

![Fluorescence intensity vs. HPLC fraction number](image)
Table II. HPLC conditions used to purify peptide p2Ca-Y4 from mouse liver

<table>
<thead>
<tr>
<th>Round</th>
<th>Column*</th>
<th>Gradient</th>
<th>Duration</th>
<th>Flow Rate</th>
<th>Active Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C18 22 mm (Beckman)</td>
<td>1.0</td>
<td>50</td>
<td>3</td>
<td>38</td>
</tr>
<tr>
<td>2</td>
<td>C18 (218TP104)</td>
<td>0.25</td>
<td>40</td>
<td>1</td>
<td>40-41</td>
</tr>
<tr>
<td>3</td>
<td>C8 pH stable (228TP104)</td>
<td>0.1</td>
<td>55</td>
<td>1</td>
<td>34-35</td>
</tr>
<tr>
<td>4</td>
<td>Phenyl (219TP54)</td>
<td>0.2</td>
<td>50</td>
<td>1</td>
<td>46</td>
</tr>
<tr>
<td>5</td>
<td>C18 (218TP104)</td>
<td>0.0375</td>
<td>80</td>
<td>1</td>
<td>45</td>
</tr>
<tr>
<td>6</td>
<td>C4 (214TP54)</td>
<td>0.067</td>
<td>60</td>
<td>1</td>
<td>38</td>
</tr>
<tr>
<td>7</td>
<td>C18 (218TP104)</td>
<td>0.27</td>
<td>60</td>
<td>1</td>
<td>43-44</td>
</tr>
</tbody>
</table>

* For HPLC rounds 1, 2, 4, 5, and 6 the solvents were A = 0.1% TFA in distilled H2O and B = 0.085% TFA in acetonitrile; for round 3, solvent A = 0.1% TFA in distilled H2O and solvent B = 0.1% TFA in isopropanol.

FIGURE 4. Sensitizing activities of synthetic peptides p2Ca (LSPFPFDL), p2Ca-Y4 (LSPYPFDL), and p2CI-Y4 (LSPYPFDI). Titrations of peptides p2CI-Y4 (●), p2Ca (□), and p2Ca-Y4 (○) were added to 51Cr-labeled T2-Ld target cells and 2C CTL in a 4-h cytotoxicity assay. Arrows on abscissa point to SD50 values (peptide concentrations required for half-maximal lysis): ~50-100 pg/ml for p2Ca and p2Ca-Y4 and ~1 ng/ml for p2CI-Y4. Decreased lysis at the highest peptide concentrations may be due to fratricidal lysis of the CTL (29).

FIGURE 5. Synthetic peptides p2Ca and p2Ca-Y4 are recognized differently by 2C CTL in association with different MHC-I molecules. Target cells were 51Cr-labeled T2-Ld, T2-Kb, and T2-Db, and CTL were 2C at a ratio of 5:1. ●, peptide p2Ca-Y4; ○, peptide p2Ca.

peptide p2Ca only by a substitution of tyrosine for phenylalanine at position 4, the active peptide in peak a' is referred to as p2Ca-Y4.

Based on an earlier discovery that peptide p2Ca can be recognized by 2C cells not only in association with Ld (an allogeneic MHC-I) but also in association with Kb (a syngeneic MHC-I) (14), peptides p2Ca and p2Ca-Y4 were each compared for their ability to sensitize T2-Ld, T2-Kb, and T2-Db target cells for lysis by 2C cells. As shown in Figure 5, the two peptides were virtually indistinguishable with Ld+ target cells (as well as Db+ target cells, which were not lysed in the presence of either peptide), whereas with Kb+ target cells, only peptide p2Ca was active.

Although they differed markedly in their ability to sensitize T2-Kb cells for lysis, the two peptides were bound indistinguishably by Kb; as shown in Figure 6, they were equally effective in enhancing surface expression of Kb (and also of Ld) on T2-Kb (or T2-Ld) cells, indicating that the phenolic hydroxyl group of p2Ca-Y4 does not interfere with the peptide's binding to either Kb or Ld. Peptide p2Ca is bound less well to Kb than to Ld, as reflected by their respective equilibrium association constants (14, 17); evidently, the same is true of p2Ca-Y4, as indicated by closely similar behavior of the two peptides in Figure 6.

Using the clonotypic anti-receptor Ab 1B2 (9), we determined that both p2Ca and p2Ca-Y4 reacted with the same TCR on 2C cells, i.e., at 50 μg/ml, the F(ab')2 fragment of 1B2 inhibited by 90% the lysis of T2-Ld cells sensitized with either of these peptides (data not shown). Moreover, the same TCR that recognizes p2Ca-Ld complexes can also recognize p2Ca/Kb (14). Thus, despite the fact that peptides p2Ca and p2Ca-Y4 were bound equally well by Kb and differed only by a single oxygen atom, the
This study of the normal tissue distribution of naturally processed peptides that derive from the E1 subunit of α-KGDH and that are recognized with L^d by a CD8^+ CTL clone (2C) reveals striking variation in the abundance of these peptides in various tissues. Highest in liver (~4 ng/g) and undetectable in brain (<0.04 ng/g), they are present at relatively high and approximately equal levels in spleen, kidney, and lung (and also in thymus (6)) and only at very low levels in skeletal muscle. Despite such quantitative variation, the peptides were detected as a conserved pattern of three HPLC peaks in homogenates from all positive tissues and in roughly constant relative amounts, suggesting that the same peptide-generating machinery functions in these diverse tissues (albeit perhaps at very different levels).

Why are the levels of these peptides so high in liver and so low in brain? In a previous study, Griem et al. (18) examined the normal tissue distribution of peptides derived from several minor histocompatibility Ags and recognized by CD8^+ CTL clones in association with K^b or D^b. Though the sequences of the peptides they studied were unknown, and the protein sources of these peptides were not identified, their results and ours are somewhat similar in that they found relatively high levels in spleen, thymus, and lung and undetectable amounts in brain and cardiac and skeletal muscle. They did not, however, see exceptionally high levels in liver. Moreover, they found considerable evidence in support of the view that differences in the abundance of naturally processed MHC-I-associated peptides in various tissues are determined by tissue differences in the levels of expression of the relevant MHC-I proteins.

For several reasons, tissue differences in MHC-I expression do not seem to provide an explanation for the variation in amounts of α-KGDH-derived peptides seen here. One reason is the approximately equal abundance of these peptides in Sp2/0 and B2 cells (Fig. 3), despite barely detectable levels of L^d on Sp2/0 cells and at least 30-fold higher levels on B2 cells (Sp2/0 transfected with the L^d gene) (6). Another reason is the extremely high abundance of these peptides in liver, far greater than can be accounted for by L^d-bound peptide alone. Approximately 650 pmol of p2Ca were isolated from 180 g of Swiss Webster mouse livers after six rounds of HPLC (not shown), more than 15 times the yield from mouse spleens after the same number of HPLC steps (6); assuming 50% recovery/HPLC round and 10^5 cells/g liver, this suggests the presence of more than 10^7 p2Ca molecules/cell on average in liver. Although MHC-I proteins are readily detected in some liver cells (e.g., bile duct epithelium and Kupffer cells), they have been variously reported as detectable or absent in hepatic parenchymal cells and are, in any event, not present in these cells at a high level (16, 19). Still another reason is the earlier finding of equal abundance of these peptides in spleens of the three MHC-congenic mouse strains BALB/c, BALB.B, and BALB.K (having H-2^d, H-2^b, and H-2^k haplotypes, respectively) (6). It is unlikely that each of the active peptides can bind equally well to MHC-I proteins from these three haplotypes. For example, peptide p2Ca is known to bind to K^b with fivefold lower affinity than to L^d and does not bind detectably to D^b (14, 17).

It is not clear why the peptides studied by Griem et al. (18) differ from those studied here in their dependence on MHC-I expression. An explanation may lie in the relatively weak binding of peptide p2Ca to L^d (K_a ~ 1 x 10^5 M^-1) (17) and the even weaker binding of peptide p2Cb to L^d (5). It may be that the peptides examined by Griem et al. (18) bind much more strongly to the relevant MHC-I proteins and that, in general, the dependence of peptide abundance in a tissue on MHC-I expression is prominent when the peptide/MHC interaction is strong.

It is also possible that the extremely high abundance of α-KGDH-derived peptides in liver stems from a high level...
of the α-KGDH complex in this organ. However, a comparison of this enzyme’s activity in different organs suggests that differences in the tissue abundance of α-KGDH peptides also cannot be attributed to differences in the amount of the parent protein (Table I), e.g., the peptide is 40-fold more abundant in liver than in heart, but there is only a threefold more enzyme in liver. More striking is the disparity between liver and brain. These tissues differ four- to eightfold in α-KGDH activity but at least 100-fold in abundance of the α-KGDH-derived peptides. Griez et al. found that liver contains an additional peptide whose sequence differs from the p2Ca octapeptide only by a phenylalanine-to-tyrosine substitution at position 4 (p2Ca-Y4). The origin of peptide p2Ca-Y4 is unknown. It might arise from an isozyme for α-KGDH complex that is expressed only in liver. A more intriguing possibility is that p2Ca-Y4 might arise from posttranslational hydroxylation by liver microsomal phenylalanine hydroxylase (23) of the corresponding phenylalanine residue in peptide p2Ca. This latter possibility is consistent with our inability to detect in liver an analog of the naturally occurring 16-residue peptide p2Cb (5) with the corresponding phenylalanine-to-tyrosine substitution, which we might have expected if p2Ca-Y4 arises from an isozyme for α-KGDH.

It is commonly assumed that high levels of specificity in immune reactions are associated with high affinity interactions. The results obtained here with p2Ca and the new peptide, p2Ca-Y4, provide an example of the exquisite specificity that can be exhibited by a low affinity reaction. The TCR on 2C cells was found previously to bind the p2Ca/Kb complex with an intrinsic affinity so low as to be barely measurable (3 × 10^3 M^-1) (24), and yet 2C cells were found to lyse Kb+ target cells specifically, i.e., when T2-Kb cells were sensitized with p2Ca but not with other Kb-binding peptides, even though the latter bound with greater than 1000-fold higher affinity than p2Ca to Kb (14, 17). A more striking example of TCR specificity is seen here with the failure of peptide p2Ca-Y4 to sensitize Kb+ target cells for lysis by 2C cells. Octapeptides p2Ca and p2Ca-Y4 differ by a single oxygen atom, and although they bind equally well to Kb (Fig. 6), only p2Ca can elicit specific lysis of T2-Kb target cells by 2C CTL (Fig. 5). To account for this fine discrimination, we suggest that the affinity threshold for eliciting a cytotoxic T cell response may be approximately 1 × 10^3 M^-1. The affinity of the 2C TCR for p2Ca/Kb would thus fall just above this threshold and for p2Ca-Y4/Kb probably just below; hence, lysis of T2-Kb target cells is observed when their surface Kb molecules are loaded with p2Ca but not when they are loaded with p2Ca-Y4 or with other peptides, no matter how strongly these bind to Kb.

The view that T cells are highly specific derives from the commonplace observation that their responses to peptides on APC or target cells can discriminate sharply between closely related peptides. However, these observations have not distinguished between two distinctly different reasons for such T cell discrimination: 1) differences in peptide binding to target cell MHC proteins and 2) differences in the binding of the resulting peptide-MHC complexes by TCR on T cells. In the present study, this distinction is clearly made in Figures 5 and 6, and thus our results provide unambiguous evidence that a TCR can discriminate between two peptide-MHC complexes that differ by only a single oxygen atom. Moreover, this distinction is made by a TCR whose affinity for the recognized peptide-MHC complex is only 3 × 10^3 M^-1, at the lower edge of what can be measured.

A structural basis for the proposed difference in 2C TCR affinities for p2Ca/Kb vs p2Ca-Y4/Kb complexes is suggested by x-ray crystallographic studies of Kb/peptide complexes by Fremont et al. (25). For a Kb-bound octapeptide from vesicular stomatitis virus (RGYVYQGL), the side chain of valine at position 4 points toward the solvent (i.e., away from the Kb peptide binding site). Since Fremont et al. found very similar conformations of two Kb-bound peptides that differ extensively in sequence, and notwithstanding contrasting results for HLA-A2-bound peptides (26), it may be reasonable to suppose that the same orientation applies to the phenylalanine and tyrosine residues at position 4 of p2Ca and p2Ca-Y4 when bound to Kb. A lower affinity interaction of the 2C TCR with p2Ca-Y4/Kb than with p2Ca/Kb would then be understandable because the phenolic hydroxyl group of the
former could interfere with TCR binding due to either steric or charge effects.

Finally, we wish to call attention to a possible connection between the unusual abundance of α-KGDH peptides in liver and the human autoimmune disease, primary biliary cirrhosis (27). The principal autoantibodies in this disease are specific for various subunits of three homologous mitochondrial dehydrogenases specific for pyruvate, α-ketoglutarate, or branched chain α-ketocids. Inasmuch as p2Ca is not only abundant in liver but is also immunodominant in certain alloreactions (28), it seems warranted to consider whether individuals with this disease have CD8+ CTL that are specific for peptides derived from α-KGDH or from the structurally and functionally similar pyruvate dehydrogenase or branched chain α-keto acid dehydrogenase complexes. Moreover, if p2Ca-Y4 is in fact generated by post-translational hydroxylation of p2Ca in liver, this peptide (as well as other peptides that might be modified on phenylalanine or tryptophan by microsomal hydroxylase) (23) might circumvent normal thymic self-tolerance mechanisms (i.e., negative selection of T cells) and thus provide opportunities for autoimmune reactions.

Acknowledgments

We thank Drs. Ioannis Papayannopoulos and Klaus Biemann for analysis of p2Ca-Y4 by mass spectrometry, Richard F. Cook and the MIT Biopolymers Laboratory for peptide synthesis and Edman degradations, and Dr. Peter Cresswell for T2 cells and their transfectants. We gratefully acknowledge Ann Hicks on the occasion of her retirement after many years of dedicated secretarial service and general support to this laboratory.

References

CHAPTER 4

Naturally processed peptides recognized by melanoma-specific CTL
Overview

The idea of understanding – and ultimately learning to augment – immune responses against cancer cells has excited immunologists for many decades. Evidence that tumor cells express specific antigens capable of eliciting vigorous immune responses originally came from studies of chemically induced tumors in MHC syngeneic mice (Gross, 1943; Foley, 1953; Prehn and Main, 1957; Klein et al., 1960; Old et al., 1962). Tumors transplanted from one mouse to another were rejected if the recipient was first immunized with tumor cells that had been irradiated or were subsequently removed surgically; moreover, the immunity provided by a tumor was often specific for that tumor and not others, even from the same mouse, leading to the concept of tumor-specific rejection antigens. However, concerns were raised that spontaneous tumors, often nonimmunogenic, might lack such tumor rejection antigens (Hewitt et al., 1976).

T cells specific for tumor rejection antigens were obtained, but early efforts to elucidate the nature of these antigens failed because they did not elicit an antibody response and therefore could not be isolated by immunoprecipitation. Boon and colleagues provided the first detailed molecular information about tumor antigens recognized by T cells, using mutagenized variants of the mouse mastocytoma P815 that could induce T cell responses, sometimes against the original tumor (reviewed by Boon et al., 1994). Their approach was genetic: DNA from one of the immunogenic P815 variants (termed tum- because they did not form tumors) was transfected into P815 cells, and transfectants were screened for their ability to stimulate T cells. Eventually three genes encoding tum- antigens were cloned, and each had a previously unknown sequence differing by a single point mutation from the homologous tum+ sequence (De Plaen et al., 1988; Szikora et al., 1990; Sibille et al., 1990). In light of emerging information that
T cells recognize short peptides in association with MHC molecules (Townsend et al., 1986), the mutated peptides presumably responsible for tum− antigens were quickly identified (Lurquin et al., 1989), and one was later shown to correspond to the naturally processed peptide obtained from tum− cells (Wallny et al., 1992a) (see table, p. 168).

When a similar approach was used to define tumor rejection antigens on P815 cells (i.e., transfection of P815 DNA into antigen-loss variants selected in vitro by anti-P815 T cells), the first gene identified had a sequence identical to that of normal cells (Van den Eynde et al., 1991). In other words, unlike the tum− antigens, a rejection antigen on P815 cells is not mutated relative to the same sequence in normal mouse tissues. Why, then, was the immune rejection response against this antigen not accompanied by autoimmune manifestations? The explanation turned out to be lack of expression (or low expression) of this gene in normal tissues, including mast cell lines that do not produce tumors. Nevertheless, this study brought up the possibility that other tumor antigens would be normal sequences, although many investigators continued to expect that most anti-tumor T cells would recognize tumor-specific sequences derived from mutated genes (such as oncogene or tumor suppressor gene products).

Quite distinct from this genetic technique, a direct biochemical approach to the identification of tumor antigens had emerged from studies on minor histocompatibility antigens (Rötzschke et al., 1990a) and virus-encoded antigens (van Bleek and Nathenson, 1990; Rötzschke et al., 1990b). Treatment of tumor extracts (or purified MHC molecules from tumor cells) with TFA separates peptides from MHC molecules, and fractionation of the resulting peptide mixture by HPLC partially resolves the endogenous peptides. HPLC fractions are screened in cytotoxicity assays using
appropriate target cells and anti-tumor CTL, and the peptide(s) in an active fraction can be enriched by further rounds of chromatography using distinctive conditions (column, solvents, and gradient) (see figure on p. 37). By this general strategy, the first naturally processed peptides recognized by anti-viral or alloreactive T cells were isolated and sequenced (see table 2, p. 38), and several groups set out to match this success for tumor peptides.

Much of the work on human tumor antigens has focused on metastatic melanoma. This tumor is readily adapted to long-term culture, and in a majority of cases tumor-specific T cells can be obtained by incubating autologous blood cells with melanoma cells (Boon et al., 1994) or by culturing melanoma cell suspensions in high concentrations of the lymphokine interleukin-2 to select for outgrowth of tumor infiltrating lymphocytes (TIL) (Itoh et al., 1986). Such TIL were shown by Rosenberg et al. (1988; Topalian et al., 1989) to mediate dramatic regression of tumor masses in some patients when expanded \textit{ex vivo} and reinoculated. Another incentive to study melanoma came from findings that several melanoma antigens are shared by a majority of tumors from different patients and are recognized by T cells restricted by HLA-A2, the most common MHC-I protein among human populations afflicted by melanoma (i.e. \approx40-50\% of Caucasians) (Darrow et al., 1989; Wölfel et al., 1989, 1993; Hom et al., 1991; Kawakami et al., 1992; Viret et al., 1993). Multiple antigens have also been detected on renal cell and ovarian carcinomas (Ioannides et al., 1991; Peoples et al., 1993).

Using the biochemical methods described above, it was possible to demonstrate several peaks of activity in HPLC fractions from melanoma tumor extracts (Slingluff et al., 1993; Storkus et al., 1993); since the T cells used had not been cloned, the
existence of up to ≈6 active species probably indicated multiple specificities and not recognition of different peptides by the same TcR. It quickly became apparent that sequence determination of these peptides was no minor challenge. The absence of any prior knowledge of their sequences distinguished this problem from that of viral peptide identification, which always had been guided by the use of synthetic peptides. Although many naturally processed peptides could be isolated and sequenced on the basis of their abundance (e.g. the most prominent HPLC peaks by ultraviolet absorbance), the only precedent for isolating and sequencing a T cell antigen of unknown origin was the alloreactive 2C system (Udaka et al., 1992).

Two melanoma peptides recognized by A2-restricted T cells have been identified by direct chemical sequencing. The first report by Cox et al. (1994) made use of the improved sensitivity of mass spectrometry over Edman degradation for peptide sequencing. Nevertheless, \(4 \times 10^{10}\) melanoma cells were used for extraction and HPLC fractionation. The resulting peptide sequence (see table, p. 168) matched exactly that of gp100, a protein specific to melanocytes and melanoma. The original abundance of the peptide could not be established because of losses incurred during purification, but the actual amount of peptide recovered was estimated at ≈0.2 peptide molecules per melanoma cell! Significantly, CTL from five different A2\(^+\) patients could recognize this peptide. A second peptide was isolated from melanoma cells by a novel procedure using a citrate/phosphate buffer, pH 3.3, instead of TFA to remove peptides (Storkus et al., 1993); since cells were not lysed under these conditions, continued culture led to replenishment of MHC-bound peptides by endogenous pathways, and these peptides were harvested daily for several days to amplify yields by ≈10-fold and permit sequencing (see table, p. 168).
A third report of direct sequencing of a tumor peptide (from an established mouse lung carcinoma) appears to be unusual in that the peptide is one of the most abundant MHC-I-associated peptides of the tumor; the peptide sequence matched the protein connexin 37 except for a C→Q mutation, implying substitution at all three positions of a single codon (Mandelboim et al., 1994).

Before these successful applications of the biochemical approach, Boon’s method of DNA transfection into antigen-loss variants selected by CTL had also produced the first sequences of genes encoding human melanoma antigens (van der Bruggen et al., 1991). The MAGE (for melanoma antigen) genes represent a family of 12 closely related, previously unknown sequences that are identical in tumors and in normal tissues. These genes are expressed in tumors of many histological types but not in normal tissues, with the exception of testis and placenta (De Smet et al., 1994). To identify the peptide encoded by MAGE-1 that is recognized by HLA-A1-restricted T cells, several synthetic peptides were screened for activity, and one 9-mer was recognized efficiently (SD50 = 5 nM, Traversari et al., 1992). Subsequently, other MAGE genes were similarly found to encode peptides recognized by anti-melanoma T cells (see table, p. 168).

Moving to A2-restricted CTL and improving the transfection approach by using COS cells (in which vectors containing the SV40 origin of replication multiply episomally to very high copy numbers), the Boon group identified two cDNA clones coding for melanoma antigens: one proved to be the gene for tyrosinase (Brichard et al., 1993), an enzyme in the pathway of melanin biosynthesis, and the other was a new sequence they called Melan-A (Coulie et al., 1994). Both genes are non-mutated and
expressed widely in melanomas as well as in normal melanocytes, leading to their
description as differentiation antigens. By synthetic peptide screening, two active
tyrosinase peptides were identified (Wölfel et al., 1994b).

In parallel, Rosenberg and colleagues had also used a gene transfection approach
to clone the gene for a melanoma antigen recognized by A2-restricted TIL, which they
called MART-1 (for melanoma antigen recognized by T cells, Kawakami et al., 1994a).
Their gene proved to have exactly the same sequence as Melan-A. Kawakami et al.
(1994b) quickly identified an active 9-residue peptide from the MART-1/Melan-A
sequence. A third differentiation antigen, gp100, was encoded by another gene isolated
by Kawakami et al. (1994c) using T cells and by Bakker et al. (1994) using monoclonal
antibodies. When Kawakami et al. (1994a, c) examined a panel of normal tissues for
the expression of MART-1 and gp100, they found that in addition to most melanomas
and melanocyte cell lines, these differentiation antigens are expressed in retinal tissue.

A major advantage of the genetic strategy for tumor antigen identification is that
it is unhampered by low abundance of the final peptide product. However, the naturally
processed peptide can only be discerned by testing various synthetic peptides, which is
an imperfect art. For example, reliance on consensus motifs for MHC-bound peptides
may fail to reveal certain active peptides. Once an active peptide is identified, it is not
certain to correspond to a naturally processed peptide, although this is often assumed to
be the case. Furthermore, no quantitative information about the peptide’s natural
abundance can be garnered. These limitations can be overcome by chromatographic
and quantitative comparisons between synthetic peptides and a sample of natural
material from a tumor cell extract (e.g. $10^9$ cells). This has not yet been done for the
tyrosinase, MART-1/Melan-A, or gp100 synthetic peptides found to be active by the Boon and Rosenberg groups, so their identification as naturally processed peptides must remain provisional.

A peptide sequenced from tumor cell extracts by Cox et al. (1994) corresponds to one of the tyrosinase peptides synthesized by Wölfl et al. (1994b), except for a single N→D difference; whether this represents post-translational deamidation, artefactual deamidation during the purification, tyrosinase polymorphism, or a different protein of origin remains unknown.

This sudden surge of information, mostly within the past year (see table, p. 168), has brought a few surprises and raised some concerns about future immunization plans. First of all, virtually all of the peptides identified (either biochemically or by synthesizing peptides based on a gene sequence) have non-mutated sequences. Why should normal sequences be immunogenic? And what prevents autoimmune reactions from occurring against other tissues expressing the same antigens?

The apparent breach of self tolerance could have several explanations, each hypothetical, and serves to highlight the fact that tolerance is not an all-or-none phenomenon. (1) A normal peptide might be overexpressed on tumor cells, resulting in its recognition by T cells that do not react against tissues expressing lower levels of peptide. (2) Several of the peptides identified so far are said to have low binding affinities for A2, and it is intriguing to speculate that this property may be related to their failure to induce a more sturdy self-tolerance. (3) The local inflammatory reaction to a tumor may result in paracrine effects on lymphocytes that elicit activities not
otherwise observed. The importance of lymphokines such as interleukin-2, interleukin-4, and GM-CSF in anti-tumor responses has been convincingly shown. (4) It has been proposed that CTL against these normal sequences may have low-affinity TcR, because cells with high-affinity TcR would have been deleted or anergized. (5) It should be noted that screening tissues by Northern blot analysis does not necessarily reflect the presence of peptide-MHC complexes at the cell surface; perhaps some of these antigens are tumor-specific at the peptide level, although reports that certain anti-melanoma CTL can lyse normal melanocytes in vitro (Anichini et al., 1993; Bakker et al., 1994) suggest otherwise. It may be useful to measure the abundance of the MART-1/Melan-A and gp100 peptides in extracts of retinal tissue (something not yet done even for the tumors themselves). Another worthwhile experiment may be to test whether individuals without tumors have CTL specific for peptides from tyrosinase, MART-1/Melan-A, or gp100.

A second generalization that can be drawn from the table on p. 168 is that a multiplicity of shared melanoma antigens exists, and their number is likely to increase. Most of the Rosenberg group TIL lines recognize a single MART-1 peptide, but the peptide independently isolated by Storkus et al. is a different 9-mer from MART-1. Both the Rosenberg and Hunt/Engelhard groups identified gp100 peptides as melanoma antigens, but they also found two different sequences from the same protein to represent shared antigens. The Boon lab first implicated tyrosinase as a tumor antigen; more recently, Topalian et al. (1994) discovered that this protein can also stimulate CD4+ TIL. Given these overlapping yet contrasting results, it appears that several families of melanoma antigens may be important – and so far only A1- and A2-restricted responses have been analyzed in any detail.
What are the prospects for using this new information in melanoma (or other cancer) immunotherapy? Boon’s early results with tum− variants provide hope that even poorly immunogenic tumors can be rejected by an immune response following immunization, and he recently outlined a variety of immunotherapeutic modalities under consideration (Boon, 1993). There are plenty of reasons for pessimism: tumor escape by antigen loss, tumor escape by MHC downregulation, and so on. These issues must be investigated. But the greater concern is for safety, given that many of the candidate immunogens are normal self sequences. Will vaccines based on MART-1/Melan-A or gp100 cause ophthalmologic problems? What about MAGE-1 in testis? There is evidence that melanoma patients with local depigmentation reactions (vitiligo) have prolonged survival, suggesting that immune responses against melanocyte differentiation antigens on normal tissues are indeed relevant. Also, melanoma (but not renal carcinoma) patients treated with interleukin-2 tended to develop vitiligo. Until the basis for T cell reactivity against these normal sequences is better understood, it may be hazardous to proceed with immunization; on the other hand, for some patients with metastatic melanoma the risk may be worth taking.
Table. Peptides recognized by anti-tumor T cells.§

<table>
<thead>
<tr>
<th>T cells†</th>
<th>MHC restriction</th>
<th>sensitizing peptide (SD₀₅ value)</th>
<th>parent protein or gene</th>
<th>normal or mutated</th>
<th>tissue distribution</th>
<th>shown to be naturally occurring</th>
<th>ref.</th>
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<tr>
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<td>K&lt;sup&gt;b&lt;/sup&gt;</td>
<td>FEQNTAQP (&lt;10 nM)</td>
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<td>connexin 37</td>
<td>mutated</td>
<td>Lewis lung carcinoma melanomas, other tumors, normal testis</td>
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<td>MLLAVLYCL (&lt;10 nM)</td>
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<td>LN</td>
<td>A2</td>
<td>YLEPGPVTA (1-10 pM)</td>
<td>Pmel 17 (gp100)</td>
<td>normal</td>
<td></td>
<td>yes</td>
<td>(12)*</td>
</tr>
<tr>
<td>TIL</td>
<td>A2</td>
<td>LLDGTATLRL (0.1-1 μM)</td>
<td>gp100</td>
<td>normal</td>
<td></td>
<td>no</td>
<td>(13)*</td>
</tr>
</tbody>
</table>

§ excluding viral peptides and oncogene-encoded peptides used to generate T cells.
† PBL, peripheral blood lymphocytes; TIL, tumor infiltrating lymphocytes; LN, draining lymph node cells.
* (1) De Plaen et al., 1988; Lurquin et al., 1989; Alexander-Miller et al., 1994; (2) Szikora et al., 1990; (3) Sibille et al., 1990; Wallny et al., 1992a; (4) Mandelboim et al., 1994; (5) van der Bruggen et al., 1991; Traversari et al., 1992; De Smet et al., 1994; (6) van der Bruggen et al., 1994a; (7) Gaugler et al., 1994; (8) van der Bruggen et al., 1994b; (9) Brichard et al., 1993; Wölfel et al., 1994b; (10) Kawakami et al., 1994a, b; Coulie et al., 1994; (11) Castelli et al., 1995; (12) Cox et al., 1994; (13) Bakker et al., 1994; Kawakami et al., 1994c.
‡ Cox et al. (1994) reported the isolation of peptide YMDGTMSQV (N→D at position 3).
Results and discussion

Ed Reilly established two long-term TIL lines from solid melanoma samples (MEL660 and MEL663) provided by Steve Rosenberg (Reilly and Antognetti, 1991), and in early 1991 we set out to characterize the melanoma peptide(s) recognized by one of these lines (TIL660) in association with HLA-A2. We soon demonstrated a single prominent peak of activity in HPLC fractions of melanoma peptides extracted from immunoaffinity-purified A2 molecules (below); the shoulder eluting slightly ahead of the main peak was a reproducible finding.
Numerous trials were performed to optimize the purification scheme for the active peptide in these melanoma extracts. Ultimately, initial purification of A2 molecules followed by TFA extraction and fractionation was deemed advantageous because: (1) the mixture of peptides was substantially less complex than that obtained from extraction of whole cells or purified cell membranes with TFA; and (2) problems due to nonspecific toxicity of HPLC fractions were severe when using the whole cell extraction method. The potential disadvantage was reduced yields of the active peptide, particularly if it proved to have a rapid dissociation rate from its complex with A2. A large-scale purification of A2 from $=10^{11}$ melanoma cells was performed, and the active peptide was successively enriched through four sets of HPLC conditions using different columns, solvents, and gradients (details to be given elsewhere). After each step a satisfactory TIL assay result was obtained using 1% or less of the total sample.

After the fourth HPLC step, three fractions eliciting recognition by TIL660 were submitted for mass spectrometric analysis. Two candidate masses were consistent with the observed profiles of activity, i.e. highest in the central fraction and lower in the two flanking fractions: $(M+H)^+ = 906$ and 1007. However, the purified material was insufficient for fragmentation and sequence determination by mass spectrometry.

Another scale-up to $4\times10^{11}$ melanoma cells was undertaken. The active peptide was purified but once again proved insufficient for sequencing. Meanwhile, problems with the TIL line began to develop. Human CD8$^+$ T cells are notoriously difficult to maintain for long periods, and TIL660 was starting to behave inconsistently; occasionally there was no lytic activity against the autologous melanoma cell, which was used for in vitro stimulation of TIL660 and which was ordinarily lysed quite well (50-90%).
Thereafter several results were published from other laboratories, and it became possible to evaluate whether the melanoma antigen recognized by TIL660 was the same as that recognized by other anti-melanoma T cells. We showed that most A2+ melanomas were recognized by TIL660, indicating reactivity against a shared melanoma antigen (see table, below). Thierry Boon made available to us the A2+ melanoma line NA8, which is not a good target for most A2-restricted T cells, as well as NA8 cells transfected with the gene for tyrosinase or Melan-A (the latter is called gene Aa). As shown below, TIL660 specifically lysed NA8 cells transfected with Aa but not untransfected NA8 cells (or NA8 cells transfected with the gene for tyrosinase).

**Lysis by TIL660**

![Graph showing lysis by TIL660](image)

<table>
<thead>
<tr>
<th>CTL:target cell ratio</th>
<th>MEL660</th>
<th>NA8-Aa</th>
<th>NA8</th>
</tr>
</thead>
<tbody>
<tr>
<td>5:1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25:1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50:1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table. Cytotoxicity assays using TIL660.

<table>
<thead>
<tr>
<th>target cell</th>
<th>A2 status (FACS level)</th>
<th>Percent specific lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50:1</td>
</tr>
<tr>
<td>MEL660</td>
<td>A2⁺</td>
<td>82.8</td>
</tr>
<tr>
<td>F0-1/neo</td>
<td>A2⁻ (2)</td>
<td>1.7</td>
</tr>
<tr>
<td>F0-1/A2</td>
<td>A2⁺ (56)</td>
<td>56.3</td>
</tr>
<tr>
<td>SK-MEL-5</td>
<td>A2⁺</td>
<td>59.3</td>
</tr>
<tr>
<td>SK-MEL-28</td>
<td>A2⁻</td>
<td>11.6</td>
</tr>
<tr>
<td>MEL660</td>
<td>A2⁺ (178)</td>
<td>50</td>
</tr>
<tr>
<td>SK-MEL-30</td>
<td>A2⁺ (289)</td>
<td>25</td>
</tr>
<tr>
<td>SK-MEL-75</td>
<td>A2⁺ (95)</td>
<td>19</td>
</tr>
<tr>
<td>SK-MEL-37</td>
<td>A2⁺ (171)</td>
<td>14</td>
</tr>
<tr>
<td>SK-MEL-119</td>
<td>A2⁺ (112)</td>
<td>8</td>
</tr>
<tr>
<td>SK-MEL-133</td>
<td>A2⁻ (1)</td>
<td>1</td>
</tr>
<tr>
<td>SK-MEL-64</td>
<td>A2⁻ (2)</td>
<td>1</td>
</tr>
<tr>
<td>SK-MEL-113</td>
<td>A2⁻ (1)</td>
<td>1</td>
</tr>
<tr>
<td>WM88</td>
<td>A2⁻ (2)</td>
<td>1</td>
</tr>
<tr>
<td>MEL660</td>
<td>A2⁺</td>
<td>92</td>
</tr>
<tr>
<td>NA8</td>
<td>A2⁺</td>
<td>24</td>
</tr>
<tr>
<td>NA8-tyrosinase</td>
<td>A2⁺</td>
<td>21.2</td>
</tr>
<tr>
<td>NA8-Aa</td>
<td>A2⁺</td>
<td>57.1</td>
</tr>
<tr>
<td>Malme-3M</td>
<td>A2⁺</td>
<td>72.6</td>
</tr>
<tr>
<td>HT-144</td>
<td>A2⁻</td>
<td>19.8</td>
</tr>
<tr>
<td>SK-MEL-28</td>
<td>A2⁻</td>
<td>3.1</td>
</tr>
</tbody>
</table>
Next a series of synthetic peptides from the MART-1/Melan-A sequence was tested for activity. To our surprise, none was recognized efficiently by TIL660, including the two different peptides recognized by T cells from the Rosenberg and Storkus laboratories (see table, p. 168). Moreover, by HPLC retention characteristics, the active peptide expressed by MEL660 cells appears to differ from both peptides recognized by the other T cells. If confirmed, this result further supports the idea of significant heterogeneity in T cell recognition of MART-1/Melan-A. Ongoing work is directed toward chromatographic comparisons between the naturally processed MEL660 peptide and the peptides identified as melanoma antigens in other laboratories, as well as synthesis and testing of other MART-1/Melan-A peptides.

Addendum: A manuscript on the melanoma project was prepared and submitted for publication in 1996 after this thesis was defended (see p. 178).
T-cell antigens in cancer

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Department of Biology and Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139

The possibility that immune responses to distinctive antigens on cancer cells might aid in the treatment or prevention of cancer has intrigued immunologists since the early 1900s. Interest in this possibility waned when it became apparent that the regularly seen rejection of tumors transplanted from one rodent to another was due to the recipient's immune response to antigens (later termed histocompatibility antigens) present on all cells, both normal and neoplastic, and not to unique tumor antigens. Only after the development of inbred mouse strains, in which all members of one tumor is often specific for that of the same strain is usually rejected if the existence of distinctive tumor antigens compatibility antigens and accept normal tissue grafts from one another, was the The possibility that immune responses to tumors greatly resembles the rejection of normal organ allografts, which is characterized by a sufficient number of MHC-I molecules of each of the major T-cell subsets defined on the basis of CD4 or CD8 expression, CD4+ T cells recognize peptide fragments derived from endocytosed proteins in association with class I MHC molecules on the cell surface (13). The other major T-cell subset, and the one of particular interest in relation to tumor antigens, is CD8+ T cells. These cells recognize short peptides that arise within any cell by proteolysis of cellular proteins (14) and are then transported to the cell surface in association with class I MHC (MHC-I) molecules (15). Since a nucleated cell typically expresses ~106 molecules of each of ~6 different MHC-I proteins, there are perhaps 109 peptide-MHC-I complexes on the surface of any given cell representing the several thousand different proteins being synthesized in that cell. Normally, an individual's CD8+ T cells do not respond to complexes of self-peptides and self-MHC-I; however, if a foreign peptide is added to a viral protein, a mutated protein, or even an overexpressed normal protein occupies a sufficient number of MHC-I molecules [reported to be ~200 per cell (16)], CD8+ T cells are activated to lyse the affected cell. Thus, CD8+ T cells perform an immune surveillance function, effectively monitoring the contents of virtually all cells by detecting peptides that are sampled by MHC-I molecules and displayed at the cell surface. The rules governing exactly which cellular proteins are represented by cell surface peptide-MHC-I complexes are only beginning to emerge, as a result of studies from various laboratories over the past 3 years.

For example, direct biochemical isolation of peptides that are present in cells as peptide-MHC-I complexes (17-26). These peptides are derived from abundant cellular proteins as diverse as histones, heat shock proteins, elongation factors, metabolic enzymes, and other MHC molecules. Using a mass spectrometric approach, Hunt and colleagues (27) showed that MHC-associated peptides isolated from cells differ markedly in amount; some are relatively abundant, whereas many more are quite rare, perhaps only a few copies per cell. Importantly, HLA-specific peptides so far identified by chemical sequencing have been obtained by virtue of their high abundance—for instance, the largest peaks from an HPLC profile—and not because they are known to be recognized by a particular clone of T cells (28). The task of purifying and identifying an antigenic peptide (or peptides) defined on the basis of its recognition by a given T cell (such as a TIL) is substantially more difficult, mostly because such peptides may be exceedingly rare. In fact, most of the handful of known naturally occurring T-cell antigens are from acute intracellular (e.g., viral) infections (17, 18, 30) or from a transfected gene (31), each of which may well result in higher-than-average cellular levels of the antigenic peptide. Furthermore, in these cases the sequence of the protein of origin is usually already known, and synthetic peptides can be used to guide identification of the naturally occurring antigen. Without such prior information, in order to isolate a known peptide for sequence determination nearly 10^32 cells must be used to obtain just 10 pmol of peptide (generally considered a minimum amount for complete sequence determination), assuming 10% overall recovery and an abundance of 100 peptide molecules per cell. In practice, yields for multistep purification procedures are likely to be significantly lower, with correspondingly greater numbers of cells required. When cultured cells such as tumor cells serve as the source of an antigenic peptide, very large scale cultures are therefore required. In one instance in which a T-cell antigen was isolated without any prior knowledge of its protein of origin [later seen to be a cellular enzyme (26)], <7 pmol was re-
covered after starting with 263 mouse spleens (25).

Although the strictly biochemical approach has not yet resulted in any definite sequences for tumor-specific antigens recognized (41) by T cells, several groups have clearly demonstrated the existence of such peptide antigens in tumor cell extracts (32-34; T. J. T., E. B. Reilly, and H. N. L., unpublished data). Moreover, the finding that some of them can be recognized in tumor cell lines (e.g., A2* nonmelanocytes) for a shared melanoma antigen recognized by TILs or by antisera against melanomas expressing the MHC-I molecule (or introduced into the tumor cells by gene transfection). For example, melanomas expressing the MHC-I molecule HLA-A2 share several peptide antigens recognized by TILs or by antisera against melanomas obtained from peripheral blood (35–40). Because A2 is the most common human MHC-I protein (present in ~50% of Caucasians, for example), knowledge of these particular shared antigen(s) might give rise to improved diagnostic or therapeutic procedures against melanoma in this subset of the population.

Pioneering studies by Boon and colleagues (41) have established an alternative approach to the identification of tumor-specific antigens recognized by cytolytic T cells. This genetic approach, applicable in principle to other peptide antigens as well (e.g., from infectious agents, a minor histocompatibility antigens, and targets of autoimmune reactions), has the advantage of being independent of naturally occurring peptide abundances. Starting with a cDNA library from a tumor cell (e.g., melanoma), several hundred pools of plasmids are transfected into COS cells (along with the appropriate gene) or into melanoma loss tumor cell variants. Transfected cells are then screened for expression of the tumor-specific antigen by their ability to stimulate anti-tumor T-cell clones, and the procedure is repeated. Using similar techniques, Kawakami and coworkers (42) identified a family of melanoma antigen (MAGE) genes that are expressed by human melanomas and some other tumors, but not by normal tissues. Also by this approach, the melanocyte-specific gene for tyrosinase was found to encode an antigen recognized by peripheral blood anti-tumor T cells in association with HLA-A2. Part of the answer lies in knowing something about the level at which a given peptide is expressed in target cells. If only one or a few molecules per cell are expressed, it is unlikely that very many T cells can be efficient in recognizing and lyzing these targets. Again, such questions of abundance can be effectively addressed by the biochemical approach to antigenic peptide characterization (49). Thus, the two techniques are seen to be complementary: the genetic approach permits genes for tumor antigens to be identified, irrespective of the levels of peptide expression, and the biochemical approach establishes whether a presumably tumor-specific peptide encoded on a shared sequence matches the naturally occurring one and allows for measurement of its abundance.

What is the likelihood that the antigenic peptide (or peptides) encoded by MART-1 is the same as one of the shared
A2* melanoma antigens described in several other laboratories? Eventually this question can be addressed in the respective laboratories by comparing HPLC retention times of the naturally occurring antigen isolated from various melanoma cells with that of the active synthetic peptide yet to be identified from MART-1. Alternatively, reactions between melanomas and anti-tumor T cells exchanged among different laboratories can be measured, although this is a lower resolution approach than HPLC. Kawakami et al. have already determined that their TILs do not recognize MAGE-1 or tyrosinase antigens. If different laboratories use different methodologies and different individuals’ T cells ultimately arrive at the same tumor antigens, there will be more ground for hope for future clinical applications.

Both the biochemical and genetic approaches to antigen peptide identification are costly and time consuming, and the practical difficulty of maintaining most human T-cell clones in long-term culture (particularly CDS+ T cells) imposes a further constraint on human studies. Nevertheless, the remarkable potential of this information in cancer, infectious diseases, and autoimmune disorders ensures that much future work will be directed toward discovering the relevant peptide sequences. The greatest coming challenge may be in learning how to make best use of this information.

Anti-Melanoma Cytotoxic T Lymphocytes (CTL) Recognize Numerous Antigenic Peptides Having "Self" Sequences: Autoimmune Nature of the Anti-Melanoma CTL Response

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running title: MELANOMA PEPTIDES RECOGNIZED BY T CELLS

key words: melanoma/tumor-infiltrating lymphocytes/natural peptides/HPLC/immunotherapy

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3 The abbreviations used are: MHC-I, class I major histocompatibility complex; CTL, cytotoxic T lymphocyte(s); TIL, tumor-infiltrating lymphocyte(s); PBL, peripheral blood lymphocytes; LN, draining lymph node cells; TAL, tumor-associated lymphocytes from metastatic effusions or ascitic fluid; mAb, monoclonal antibody; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid.
ABSTRACT

A line of tumor-infiltrating lymphocytes (660TIL) derived by culturing an HLA-A2+ melanoma (660MEL) in the presence of IL-2 specifically lysed most A2+ melanoma cell lines. We immunoprecipitated A2 from >10^{11} 660MEL cells, extracted naturally processed peptides, fractionated them by HPLC, screened the fractions for recognition by 660TIL, and found a single predominant and a minor peak of activity. Although too little was recovered of the major 660MEL peptide to establish its sequence (due to low abundance), HPLC fingerprinting showed that it did not correspond to any of the known A2-associated melanoma peptides recognized by T cells, including peptides from tyrosinase, MART-1/Melan-A, gp100, and MAGE-3. The major 660MEL antigenic peptide appears to be derived from MART-1/Melan-A but is neither AAGIGILTV nor ILTVILGVL nor any other MART-1/Melan-A peptide containing the A2 consensus motif. Thus we define a new melanoma peptide recognized by A2-restricted TIL and present a chromatographic approach to screening synthetic peptides that may be naturally processed tumor antigens. The multiplicity of melanoma peptides recognized by CD8+ T cells, most of which are non-mutated (including the 660MEL peptide), suggests the existence of unknown mechanisms, perhaps similar to those operating in autoimmune disorders, whereby T cells that recognize normal “self” sequences become activated.
INTRODUCTION

Among human cancers, melanoma has attracted great interest for studying immune responses to distinctive tumor antigens, largely because both antibodies and lymphocytes reactive against melanoma cells are commonly found in patients (1, 2). In the 1980s, cytotoxic T lymphocytes (CTL) reactive against autologous melanomas were isolated from patients’ peripheral blood, lymph nodes, and tumor tissues (3-12). Some of these CTL were shown to recognize allogeneic as well as autologous melanomas, provided the melanomas used as target cells expressed the same class I major histocompatibility complex (MHC-I) molecule, implying the existence of key antigens shared among melanomas from different patients (13-17). Together with the discovery that T cell antigens consist of short peptides non-covalently bound to MHC-I molecules (18), the stage was set for attempts to identify the peptides present on melanoma cell surfaces and recognized by melanoma-reactive T cells. As of the early 1990s, no such peptides had been identified (19); since then there has been an explosion of information regarding the identities of T cell antigens on melanoma cells and a few other cancers (see below, Table 2). Currently, trials are in progress to assess whether peptide-induced, tumor-specific immune responses can have significant effects on tumor progression and on survival (20-23).

Our laboratory has been interested in the identification of naturally processed peptide antigens recognized by specific CTL in a variety of systems, including HIV infection, allogeneic responses, and melanoma (24). We wish to understand the reasons why in certain instances (such as influenza virus infection (25)), T cells are effective in ridding the host of unwanted target cells, while in other cases (notably HIV infection and many cancers), abundant and vigorous T cell responses can readily be demonstrated but apparently are insufficient to eradicate target cells. Some factors thought to limit T cell–target cell reactions include: poor MHC expression, absence of appropriate accessory molecules, antigen escape mutations, and low T cell receptor affinity for peptide•MHC ligands. Recently we explored the possibility that a critical and under-appreciated parameter in regulating target cell lysis by CTL in vivo is the density of specific antigens (i.e., naturally processed peptide•MHC-I complexes) on target cell surfaces (24, 26). Perhaps the number of peptide•MHC-I complexes per target cell is often below the threshold required for efficient lysis in vivo by existing CTL specific for that antigen. Because CTL activity is usually studied in vitro using somewhat arbitrary concentrations of synthetic peptides and high numbers of T cells in standardized cytotoxicity assays, information about the density of a naturally processed peptide antigen and how it compares to the density of ligand required for T cell lysis in vivo is virtually nonexistent. Thus a first step in addressing the potential role of ligand density in T cell reactions is to determine the identities and natural abundances of various peptide antigens recognized by T cells.

We describe here our efforts to characterize the peptide(s) involved in recognition and lysis of a melanoma cell line, 660MEL, by an autologous CD8+ tumor-infiltrating lymphocyte (TIL) line (660TIL), using biochemical methods similar to those which had led to the successful identification of naturally processed viral (27, 28) and allogeneic (29-31) peptides. Despite numerous attempts, we were unable to determine a particular peptide sequence. However, we found that: (i) 660TIL recognizes an antigen present on most HLA-A2+ melanomas; (ii) the A2-restricted anti-melanoma response...
of 660TIL is mediated by a single predominant peptide and a second minor peptide; (iii) the natural abundance of the predominant peptide is likely to be extremely low (<50 copies per melanoma cell); (iv) by defining a series of HPLC conditions for elution of the major 660MEL peptide, a "chromatographic signature" of the peptide was obtained that could be compared with those of other known melanoma peptides recognized by A2-restricted T cells; (v) the major 660MEL peptide is different from all other published antigenic melanoma peptides by chromatographic criteria, further indicating the substantial diversity of A2-restricted anti-melanoma T cell responses; (vi) the major 660MEL peptide appears to be encoded by the MART-1/Melan-A gene product (32, 33) but differs from known MART-1/Melan-A antigenic peptides (34, 35); (vii) the major 660MEL peptide lacks the consensus motif for A2-associated natural peptides (36); and (viii) like most known peptides recognized by anti-tumor T cells, the major 660MEL peptide seems to be a "self" rather than a mutated peptide, drawing attention to the autoimmune nature of CTL responses to melanoma cells.
MATERIALS AND METHODS

Cell Lines

Cell line 660TIL was established from cryopreserved fresh 660MEL tumor samples provided by Dr. Steven A. Rosenberg and was maintained as previously described (37, 38) using serum-free AIM V medium (GIBCO BRL), 1000 U/ml rIL-2 (gift of Hoffman-LaRoche), 500 U/ml penicillin, 50 μg/ml streptomycin, 250 μg/ml amphotericin B, and 2 mM L-glutamine. 660TIL was stimulated with autologous 660MEL cells at intervals of 2-3 weeks and retained anti-tumor CTL activity in standardized cytotoxicity assays for at least several months (>50% specific lysis at a CTL:target cell ratio of 50:1), after which cryopreserved 660TIL cells were thawed and maintained in the same way. The 660TIL phenotype was CD3+CD8+HLA-A2+, and lysis of 660MEL by 660TIL was blocked by mAbs OKT3 and OKT8, mAbs against ICAM and LFA-1, mAbs PA2.6, BB7.2, and PA2.1 against A2, but not by mAb BB7.1 against HLA-B7 (38).

Melanoma cell line 660MEL was established from cryopreserved fresh tumor and was maintained in monolayer culture using DME supplemented with 10% heat-inactivated fetal bovine serum (FBS), 500 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 10 mM HEPES, pH 7.3. 660MEL cells were adapted to growth in 850 cm² roller bottles for large scale culture and were harvested by treatment with 5 mM EDTA, pH 8.0, followed by centrifugation. Melanoma cell lines NA8, NA8-Aa, and NA8-tyrosinase (from Drs. Aline Van Pel and Thierry Boon (39)), F0-1/neo and F0-1/A2 (from Dr. Richard A. Zeff (40)), SK-MEL-30, SK-MEL-37, SK-MEL-64, SK-MEL-75, SK-MEL-113, SK-MEL-119, SK-MEL-133, and WM88 (from Drs. Alan Buckler and Nic Dracopoli), and SK-MEL-5, SK-MEL-28, HT-144, and Malme-3M (purchased from ATCC) were maintained similarly to 660MEL, with the addition of 1 mg/ml G418 (GIBCO BRL) for transfected cell lines NA8-Aa, NA8-tyrosinase, F0-1/neo, and F0-1/A2. JY target cells are A2+ EBV-transformed B cells, and T2 are A2+ antigen processing mutant TxB cell hybrids (from Dr. Peter Cresswell).

Peptide Synthesis and Chromatography

Peptides were synthesized by standard t-Boc chemistry on an Applied Biosystems Model 430A, analyzed by reverse phase HPLC and amino acid analysis, and found to be >90% pure. Peptide concentrations were generally measured by Micro BCA Assay (Pierce) and ranged from 10-100% of the concentrations calculated from dry weights. Several peptides derived from the MART-1 sequence were kindly provided by Drs. Yutaka Kawakami and Steven A. Rosenberg (34). HPLC was performed on a Beckman Model 334 equipped with an Applied Biosystems 757 variable wavelength absorbance detector. A characteristic HPLC profile ("chromatographic signature") was determined for each synthetic peptide by recording the retention time of 1 μg peptide under three or four rigidly defined HPLC conditions (column, solvents, gradient, flow rate). HPLC conditions were as follows: (A) C18 column (Vydac 218TP104), solvent A = 0.1% TFA (Pierce) in H₂O, solvent B = 0.085% TFA in acetonitrile (J.T. Baker) ("TFA system"), gradient = 1.0% B/min, flow rate = 1.0 ml/min; (B) phenyl column (Vydac 219TP54), TFA system, gradient = 0.5% B/min, flow rate = 1.0 ml/min; (C) C4 column (Vydac 214TP54), TFA system,
gradient = 0.25% B/min, flow rate = 1.0 ml/min; and (D) pH stable polymeric column (ymc 2x250 mm), solvent A = 125 mM triethylamine acetate (Applied Biosystems) in H2O, pH 7.1, solvent B = 75 mM triethylamine acetate in acetonitrile, gradient = 0.15% B/min, flow rate = 0.25 ml/min. HPLC retention times of synthetic peptides were confirmed by repeat injections and were reproducible to within 1 min for a particular peptide and chromatographic condition when using the same batch of solvents.

Isolation and TFA Elution of Peptide•HLA-A2 Complexes

Peptide•HLA-A2 complexes were harvested from cultured tumor cells as previously described (41-43). Essentially, batches of 10⁹-10¹⁰ melanoma cells were pelleted and lysed in hypotonic medium at 4°C; all subsequent steps were performed at 4°C in the presence of 100 μM phenylmethanesulfonyl fluoride, 200 μM dithiothreitol, and 0.02% NaN₃. Membranes were collected by ultracentrifugation for 60 min at 105,000 x g, solubilized in 3-4% Brij 58 (Pierce), and passed over a set of four immunoaffinity columns arranged in series as follows: Sepharose only, pooled mouse γ-globulin coupled to Sepharose, mAb PA2.1 (anti-A2) coupled to Sepharose, and mAb W6/32 (anti-MHC-I) coupled to Sepharose. The affinity columns were prepared by staphylococcal protein A purification of each mAb, periodate oxidation of the carbohydrate moiety in the Fc domains, and covalent coupling to hydrazide-modified Sepharose (Pierce). After being washed with at least 30 column volumes of buffers, each column was eluted with 50 mM diethylamine•HCl, pH 11, 0.2% Brij 58 into 2-ml fractions containing 0.1 volume of 1M Tris•HCl, pH 8. A2 purity was assessed by SDS-PAGE, and yields (as well as purity) were further determined by quantitative amino acid analysis. Overall yields of A2 were generally ~50% (assuming 5 nmol A2 per 10¹⁰ 660MEL cells, which is based on flow cytometric comparison with JY cells). In later experiments, immunoprecipitation of peptide•A2 complexes was performed using soluble PA2.1 and protein A or protein G coupled to Sepharose (Pharmacia); yields of A2 were comparable to those from immunoaffinity chromatography, while scale-up was greatly facilitated.

Purified A2 (from the PA2.1 column or batch immunoprecipitation) and non-A2 HLA molecules (from the distal W6/32 column) were concentrated by ultrafiltration at 5000 x g (Centricon-10 membrane, molecular mass cutoff ~10 kd, Amicon) and denatured twice with 0.5% trifluoroacetic acid (44) for 60 min at 37°C. The released peptides were collected by ultrafiltration and dried by SpeedVac (Savant) evaporation or lyophilization prior to HPLC fractionation. For some experiments, a small aliquot of ¹²⁵I-labeled peptide ILKEPVHG (which binds tightly to A2 on cell surfaces (45)) was added initially to the melanoma cells as a convenient tracer in order to ensure complete TFA elution of endogenous peptides from purified peptide•A2 complexes (43).

Extraction of Naturally Processed Peptides from Tumor Cells

In addition to large scale purification of peptide•A2 complexes as described above, several other methods were tested in trials for the most efficient isolation of antigenic peptides from cultured melanoma cells (summarized in Figure 1, below). Cell pellets were extracted directly, without HLA purification, by adding 5-15 ml of cold 1% TFA in H₂O per 10⁹ cells (plus additional TFA if needed to bring the pH below 2.0), dousing, and sonicating as described (29). The denatured material was transferred to a
Centriprep-10 ultrafiltration device (molecular mass cutoff \( \approx 10\, \text{kd} \), Amicon) and subjected to centrifugation at 2600 \( \times \) g for several hours at 4°C (46). The ultrafiltrate was removed, an equal volume of 1% TFA was added to the retentate, and centrifugation was repeated in order to maximize the recovery of extracted peptides. Pooled filtrates were dried by lyophilization and re-dissolved in 0.1% TFA for fractionation by HPLC. In another trial, extraction with triethylamine, pH 10, or acetonitrile was performed after TFA extraction in order to determine whether one of these solvents would lead to a higher yield of an unusual peptide sequence (this was not the case). In other variations, melanoma cell membranes were prepared and then extracted with TFA (no HLA purification), or whole cells were solubilized in detergent for HLA purification (no membrane isolation). HPLC conditions for the fractionation of naturally processed peptide extracts obtained by any of the above approaches were identical to conditions A, B, C, and D as described under Peptide Synthesis and Chromatography.

Cytotoxicity Assays

Cultured melanoma cells to be tested directly as targets for lysis by 660TIL were labeled with \( ^{51}\text{Cr} \) for 1 h at 37°C, washed three times with FBS-containing medium, and placed in wells of a 96-well round-bottom plate at 5 \( \times \) 10^{3} cells/well. For synthetic peptide testing or for screening HPLC fractions of naturally processed peptide extracts, the samples were first distributed in 96-well plates, dried overnight, and 5 \( \times \) 10^{3} \( ^{51}\text{Cr} \)-labeled JY or T2 target cells (both A2+) were added for 1 h at 37°C. 660TIL were then added at appropriate ratios of CTL:target cells (50:1 unless otherwise noted) for 4 h at 37°C. Percent specific lysis was calculated from the average of duplicates as 100 \( \times \) (\( ^{51}\text{Cr} \) release into supernatant – spontaneous release)/(total release in detergent – spontaneous release). In a few assays, mAb MA2.1 and \( \beta_{2m} \) were added to T2 cells in an effort to increase the sensitivity of peptide detection as reported previously (35, 47, 48), but no increase in specific lysis values was noted. All synthetic peptides and HPLC fractions were also tested for nonspecific lysis of target cells in the absence of CTL (“toxicity”), and none was found to be toxic.

Flow Cytometry

Cultured melanoma cells were checked to confirm A2 status and to compare mean A2 levels. After cells were washed and resuspended in PBS containing 5% FBS and 0.01% NaN\(_{3}\), 5 \( \times \) 10^{5} cells in 200 \( \mu \)l were placed in wells of a 96-well V-bottom plate, stained with 1 \( \mu \)g of the anti-A2 mAb PA2.1 for 20 min on ice, washed twice with the same buffer, incubated with 5 \( \mu \)g of FITC-conjugated F(ab\('\))\(_{2}\) fragment of goat anti-mouse IgG (Pierce) for 20 min on ice, washed twice, and analyzed with an EPICS C cytometer (Coulter) or by fluorescence microscopy. Reported mean fluorescence values are on a linear (not logarithmic) scale.
RESULTS

660TIL Recognizes a Majority of A2+ Melanoma Cell Lines

The cytotoxic activity of 660TIL against a variety of A2+ and A2- melanoma cell lines was tested, with autologous 660MEL included in each assay as a positive control (Table 1). Of eight different A2+ lines, seven were lysed to an appreciable extent by 660TIL. Susceptibility to lysis did not correlate linearly with mean levels of A2 expression; for example, SK-MEL-30 had over a five times higher mean level of A2 than F0-1/A2 but was lysed more poorly. Of seven A2- cell lines, one was lysed inefficiently by 660TIL. Based on these data and evidence for limited oligoclonality of TIL after many months in culture (49-52), 660TIL appears to recognize one or more antigens shared by a majority of A2+ melanomas.

A Single Melanoma Peptide Mediates Most of the A2-Restricted Response of 660TIL

To determine the structure of the peptide antigen(s) responsible for A2-restricted lysis of melanomas by 660TIL, we examined endogenous peptide•A2 complexes from large scale cultures of 660MEL cells. First, we performed numerous trials using 10^9-10^10 melanoma cells and different purification strategies as outlined in Figure 1 (e.g., direct extraction of cells with TFA or immunoaffinity purification of A2 followed by TFA extraction (53)). The optimal method for isolation and fractionation of antigenic peptides in this system proved to be hypotonic cell lysis, isolation and detergent solubilization of membranes, A2 affinity purification, and TFA elution of peptides from purified peptide•A2 complexes (pathway 3). For large scale work (≥10^11 cells), A2 affinity purification by batch immunoprecipitation with mAb PA2.1 (IgG1 subclass) and protein G-Sepharose was more efficient and convenient than column chromatography.

Figure 2 shows highly efficient lysis by 660TIL of JY target cells pulsed with HPLC fractions of A2-bound peptides from 10^10 melanoma cells (2.5 x 10^9 cell equivalents per well). Fractions 44, 45, and 46 each gave rise to >90% specific target cell lysis and likely represent a single active peptide eluting over 2-3 min, because in comparable experiments (e.g., Figure 3A) this activity was largely contained within a single fraction (no. 44 or 45), and further chromatography of the active fraction(s) under different chromatographic conditions did not resolve separate peaks of activity (e.g., Figure 3B-D). Under a defined set of HPLC conditions (column, solvents, gradient, and flow rate), peptides differing even slightly in length or sequence can be resolved and therefore differentiated based on characteristic retention times. HPLC conditions B-D were especially resolving, such that peptides differing by a single residue and even isomeric peptides could routinely be distinguished (refs. 26, 29, 31). Thus, the presence of more than one active peptide in fractions 44-46 would be expected to result in separate peaks of activity upon re-chromatography (Figure 3B-D).

In addition to the peptide eluting in fractions 44-46 under HPLC condition A (hereafter referred to as the major 660MEL peptide), a second shoulder of activity was reproducibly observed in fraction 41 or 42 (e.g., Figures 2 and 3A). This activity was assumed to represent a second minor peptide present on 660MEL cells. Further
purification and characterization of this minor peptide were not undertaken in view of its comparatively marginal biologic activity. The finding of more than two peaks of sensitizing activity among similarly derived HPLC fractions in other studies may be attributable to a greater degree of polyclonality in the T cell populations used (54-56).

**Abundance of the Naturally Processed 660MEL Peptide is Quite Low**

The natural abundance of the major 660MEL peptide is important for understanding the role of this peptide in recognition of melanoma cells by 660TIL. In particular, the question of whether the cell surface density of peptide•A2 complexes is a limiting factor in melanoma lysis cannot be examined without knowledge of what the naturally processed antigenic structures are and of their abundances (24). From a practical standpoint, natural abundance is also of interest in planning purification strategies.

After four rounds of HPLC (Figure 3), the major 660MEL peptide was judged to be highly purified based on up to 50-fold enrichment at each round. Accordingly, the sample was subjected to mass spectrometric analysis but a sequence could not be derived due to insufficient material. However, we can offer some estimates of natural abundance. We assume an initial peptide yield of 50% from melanoma cell extraction and a yield of 50% per HPLC round, values in line with previous studies (26, 31, 57, 58). Based on the ability to detect <1 pmol of known peptide standards by mass spectrometry, the starting amount of peptide would then be <50 molecules per melanoma cell. An independent estimate of peptide abundance may be calculated by assuming an SD50 value of the unknown 660MEL peptide (concentration at which 50% of maximal lysis occurs (43)). If the SD50 is 10 pM (comparable to other known naturally processed peptides in assays similar to those used here (26, 28, 35, 43, 57-59)), and 1% of the total sample was used in a cytotoxicity assay to give 50% of maximal lysis (i.e., 4 x 10⁹ cell equivalents per 200 μl well), then fewer than ten molecules of peptide were recovered per melanoma cell. While only approximate, these estimates indicate that the natural abundance of the major 660MEL peptide is likely to be extremely low.

**The Major 660MEL Peptide is Not One of the Known Melanoma T Cell Antigens**

At least ten melanoma peptides recognized by A2-restricted T cells (PBL, TIL, or LN) have recently been described (Table 2). Eight of these were identified by a gene cloning approach pioneered by Boon and colleagues (60), and two were isolated biochemically from cultured melanoma cells. All ten peptides are encoded by the tyrosinase, MART-1/Melan-A, gp100/Pmel17, or MAGE-3 genes and have normal, i.e., non-mutated sequences. Tyrosinase, MART-1/Melan-A, and gp100 are expressed by normal melanocytes as well as melanomas, while MAGE-3 is one of an extended family of genes (MAGE, GAGE, BAGE (61-63)) expressed by many different tumors but not by normal tissues (except testis and placenta).

In order to determine whether the major 660MEL peptide recognized by our 660TIL line was one of the known melanoma antigens recognized by other A2-restricted T cells, we employed two distinct strategies. First, we synthesized the known melanoma peptides and tested them for sensitizing activity in cytotoxicity assays with
660TIL. All peptides tested negative; the MART-1/Melan-A peptide AAGIGILTV (34) consistently exhibited slight activity over background (10-23% lysis at the extremely high peptide concentration of 100 µM and less at lower concentrations), but this was far below the activity obtained with HPLC-fractionated natural extracts of the 660MEL peptide (up to 100% specific lysis, see Table 3 and Figures 2 and 3).

To definitively rule out the known melanoma peptides, we devised a second strategy in which each synthetic peptide was characterized chromatographically with the HPLC conditions used to purify the 660MEL peptide (Figure 3). By exploiting the considerable resolving power of serial rounds of HPLC, any given peptide could be excluded from consideration if its chromatographic signature did not match that of the naturally processed 660MEL peptide. A precise match would be strong evidence of a correctly identified sequence. As shown in Table 3, none of the known melanoma peptides tested had the highly reproducible (±1 min) chromatographic signature of the major 660MEL peptide. MART-1/Melan-A peptide AAGIGILTV differed in retention time by 7-8 min from the natural extract under HPLC condition C, effectively ruling out this peptide as the one we had purified. Interestingly, peptide AAGIGILTV eluted in fraction 43 under HPLC condition A, very close to the minor peak of activity we consistently observed in fractions 41-42 of 660MEL extracts (Figures 2 and 3A), and in view of the slight sensitizing activity demonstrated by peptide AAGIGILTV in 660TIL assays, it is possible that the smaller peak of natural activity is in fact due to this peptide (also see below, Figure 4).

660TIL Recognizes a Product of the MART-1/Melan-A Gene

The MART-1/Melan-A gene was independently identified in two laboratories as the source of melanoma peptides recognized by both TIL and PBL (32, 33) and was subsequently found to encode a peptide (AAGIGILTV) recognized in vitro by nine of ten A2-restricted TIL obtained from ten melanoma tumors, including TIL derived independently by Kawakami et al. from the 660MEL tumor (34). To determine whether our 660TIL line recognized a product of the MART-1/Melan-A gene, we relied on cytotoxicity assays using as targets an A2+ melanoma cell line (termed NA8) that is not recognized by 660TIL or NA8 transfected with the Melan-A gene (termed NA8-Aa) (39). NA8 cells transfected with the tyrosinase gene were also tested. Figure 4 shows that our 660TIL recognizes a product generated only in Melan-A-transfected NA8 cells. Specific lysis of NA8-Aa was somewhat less than lysis of autologous 660MEL cells in repeated assays. We could not determine whether recognition of NA8-Aa by 660TIL was due to peptide AAGIGILTV (potentially the minor sensitizing 660MEL peptide seen in HPLC fractions 41-42) or to a different, unknown MART-1/Melan-A peptide (i.e., the major 660MEL peptide present in fractions 44-45). If the former is true, then the major 660MEL peptide might not be derived from MART-1/Melan-A, although we consider this possibility remote because of the very weak activity of the minor peptide in HPLC fractions 41-42 and the high levels of lysis of Melan-A-transfected NA8 cells (Figure 4).

The Major 660MEL Peptide Does Not Possess the A2 Consensus Motif

On the basis of Table 3, the major 660MEL peptide was neither of the two MART-1/Melan-A peptides known to be recognized by A2-restricted anti-tumor T
cells. And since the other logical candidates for the 660MEL peptide were short peptides derived from the 118 amino acid sequence encoded by MART-1/Melan-A, we tested all of the MART-1/Melan-A peptides that contained the empiric consensus motif for natural peptides associated with A2, i.e., nine (or ten) amino acid residues with alanine, isoleucine, leucine, or valine at positions two and nine (or ten) (34, 36). We employed 660TIL cytotoxicity assays and/or the chromatographic signature approach to evaluate these synthetic peptides. None of them matched the major 660MEL peptide (Table 4 and Figure 5), in accord with known limitations in the ability of consensus motifs to predict all naturally processed antigenic peptides (36).

Because of the slight activity of peptide AAGIGILTV in 660TIL assays (Table 3), we wondered whether the 660MEL peptide could be a closely related MART-1/Melan-A peptide of unusual length, as some longer A2-binding peptides are known to occur naturally (64). Also, longer peptides can undergo partial proteolysis in cytotoxicity assays and thereby reveal sensitizing activities due to shorter degradation products (28, 30). We tested a number of synthetic peptides ranging in length from nine to 15 amino acids and flanking the known antigenic sequences from MART-1/Melan-A (Table 4 and Figure 5); none was biologically active. Finally, we synthesized and tested two peptides from the MAGE-1 sequence that contained the A2 consensus motif, as well as two peptides from MART-1/Melan-A that had molecular masses of 1301 and 1007, respectively, because mass spectrometric sequencing efforts had tentatively suggested the presence of peptides of these sizes in our purified natural extracts. None of these synthetic peptides matched the naturally processed 660MEL peptide by cytotoxicity assay or by HPLC fingerprinting (Table 4).
DISCUSSION

Anti-melanoma T cells obtained from patients’ tumor tissues, peripheral blood, or lymph nodes specifically recognize shared melanoma antigens that consist of short peptides bound to MHC-I proteins. Ten different peptide sequences from four proteins (tyrosinase, MART-1/Melan-A, gp100, and MAGE-3) have recently been identified that are recognized by anti-melanoma T cells in association with HLA-A2 (Table 2). In this study, we analyze the naturally processed peptides recognized by a line of tumor-infiltrating lymphocytes (660TIL) obtained from an A2+ melanoma (660MEL). We show, through the use of distinctive chromatographic signatures of naturally processed and synthetic peptides, that the major antigenic peptide isolated from 660MEL cells differs from the peptides recognized by other A2-restricted anti-melanoma T cells (Table 3).

Indeed the considerable diversity of melanoma peptides recognized by A2-restricted T cells is further emphasized by the finding that among the peptides not recognized by our 660TIL line are peptide sequences from MART-1/Melan-A and gp100 that are recognized by an A2-restricted 660TIL line derived independently by Kawakami et al. from the same melanoma tumor (660MEL) (34, 65). That a single tumor mass can present multiple antigens to A2-restricted T cells has been shown previously (54-56). In contrast, virus-infected cells often present one predominant peptide to anti-viral T cells (27, 28, 66, 67).

Several groups have recently noted the existence of A2-restricted anti-melanoma CTL that recognize products other than tyrosinase, MART-1/Melan-A, gp100, and MAGE-3 (33, 68, 69). Still other genes (MAGE, GAGE, BAGE, p15, MUM-1, gp75) encode products recognized by anti-melanoma T cells restricted by HLA-A1, A24, A31, B44, Cw6, and Cw16 (Table 2 and ref. 70). The peptides so far known to be recognized by anti-melanoma CTL derive from at least two different sources: (i) melanocyte differentiation antigens such as tyrosinase, MART-1/Melan-A, gp100, and gp75 that are expressed in melanomas and normal melanocytes (and also in retina (32)) but not in other tissues tested; and (ii) tumor-specific antigens from the MAGE, GAGE, and BAGE families (61-63) that are expressed in melanomas as well as other tumors and in normal testis but not in normal melanocytes. Altogether, anti-tumor T cells from individuals with melanoma recognize a remarkable array of peptides from a surprisingly large number of melanoma-associated proteins (Table 2). An unexpectedly large number of tumor antigens recognized by host antibodies has also recently been described (71).

A second striking feature emerging about melanoma antigens recognized by T cells is that they predominantly have normal “self” (i.e., non-mutated) sequences. The peptide studied here is also likely to be non-mutated, since 660TIL specifically lysed A2+ target cells (NA8) transfected with the Melan-A gene, which has no known mutation (Figure 4). The multiplicity of antigenic peptides that are derived from non-mutated proteins in melanoma cells and recognized by anti-melanoma T cells stands in contrast to the decades-old expectation that tumor-associated protein antigens in general are aberrations — i.e., mutated proteins or proteins normally found primarily in the fetal state (so-called oncofetal antigens, e.g. ref. 72). As noted previously (e.g. refs. 73, 74), the “self” nature of antigens recognized by anti-melanoma T cells suggests an analogy
with autoimmune T cell responses.

How good is this analogy? Self-reactive T cells responsible for autoimmune disorders such as diabetes have been analyzed using the concept of checkpoints, with disease states requiring the progression of self-reactive T cells through several stages (75). Normally, T cells that can recognize self antigens (e.g., insulin-producing pancreatic β-islet cells (75), myelin basic protein and other central nervous system components (76)) can be isolated from healthy individuals without detectable immunopathology (77). Recent studies similarly indicate the existence of MART-1/Melan-A-specific T cells in healthy individuals who have no evidence of autoreactivity against normal melanocytes (such as vitiligo) (78, 79). When self-reactive T cells are activated to the next stage, they extensively infiltrate antigenic sites but do not cause target cell destruction. This situation, exemplified by lymphocytic infiltration in the pancreatic β-islets of male NOD mice and in young mice in a T cell receptor transgenic mouse model (75), may be analogous to the presence of anti-melanoma T cells in melanoma tumors that continue to grow. Finally, in full-blown autoimmune disease the infiltrating T cells bring about destruction of the target organ. This stage, pathological in the case of autoimmune disease, is desired when the target “self” organ is a tumor; however, apart from the successes of Rosenberg et al. in using tumor-infiltrating lymphocytes for immunotherapy in patients with metastatic melanoma (80), this stage seems not to be achieved in most clinically detectable human melanomas.

A difficulty in studying the ability of anti-melanoma T cells to mediate tumor cell destruction is the inescapably artificial nature of in vitro cytotoxicity assays, in which CTL stimulated with high doses of IL-2 are added in great excess over target cells (e.g., 50:1) in a U-bottom well for several hours. While useful as assays, these conditions probably bear little resemblance to what obtains in a typical tumor, particularly when synthetic peptides are also added to the assays at massively supra-physiological levels: e.g., at 10 μM a peptide having a modest association constant of 10^6 M^-1 would occupy over 90% of available MHC-I binding sites under steady state conditions (45), resulting in a far greater number of peptide•MHC-I complexes than achieved by most naturally processed peptides (81). One potential clue to the in vivo activity of anti-melanoma T cells may come from the presence or absence of local depigmentation reactions (vitiligo), at least for melanocyte differentiation antigens such as tyrosinase, MART-1/Melan-A, and gp100. Some anti-melanoma CTL can lyse normal melanocytes in vitro (82, 83), and there is evidence (i) that melanoma (but not renal carcinoma) patients treated with interleukin-2 tend to develop vitiligo and (ii) that melanoma patients with vitiligo have prolonged survival (1, 65). These observations suggest that antigen-specific T cell responses can be stimulated to destroy normal melanocytes, and that these T cells might also be stimulated to kill melanoma tumor cells. Whether this T cell activation is mediated by the expression of particular costimulatory molecules on target cells, by altered production of cytokines with potent paracrine activities, by increased levels of specific antigens, or by other influences is currently an area of intense investigation.

Although the activation of diverse anti-melanoma T cells might account for the rare “spontaneous” regression for which melanoma is known (2), more often these cells do not prevent tumor growth. While there are many plausible contributing factors to
inadequacy of a CD8+ T cell response (e.g., absence of appropriate adhesion or co-stimulatory molecules, decreased MHC-I expression by tumor cells, low T cell receptor affinity for peptide•MHC-I complexes), we propose that a critical parameter in regulating tumor cell lysis \textit{in vivo} could be the low abundance of naturally processed peptide•MHC-I complexes (24). Little information is available to address this possibility, since only two of the peptides listed in Table 2 that are recognized by human anti-tumor T cells were purified directly from tumor samples. Based on our experience, the study of Cox et al. (58), and the failure of other laboratories to report the biochemical isolation of tumor peptides, we suspect that the densities of tumor peptide•MHC-I complexes may in general be quite low. An apparent exception is found in the mouse Lewis lung carcinoma, where a naturally processed tumor antigen is one of the more abundant MHC-I-associated peptides (and is also mutated) (84).

Cells infected by lytic viruses such as influenza, vaccinia, Sendai, or VSV produce large numbers of viral peptide•MHC-I complexes and may be lysed efficiently both \textit{in vitro} and \textit{in vivo} by specific CTL. Tumor cells, however, might resemble cells chronically infected by persistent viruses such as EBV or HIV, in which low viral protein expression results in low densities of peptide•MHC-I complexes at the cell surface, perhaps accounting for ineffectual destruction of these target cells. We recently showed that chronically HIV-infected cells contained an average of \approx 12 and \approx 400 copies of two different peptides recognized by human HIV-specific CTL clones, and the infected cells were lysed much less efficiently by CTL specific for the peptide expressed at the lower level (26).

Our original goal – isolation of sufficient quantities of the 660MEL antigenic peptide for sequence determination – proved elusive for several reasons: (i) the quantity of naturally processed antigenic peptide on 660MEL cells appears to have been extremely low (<50 copies per cell, see above), forcing us to undertake ever larger scale cell cultures and HLA purifications (up to 4 \times 10^{11} melanoma cells) in order to recover an amount of peptide adequate for sequencing efforts; (ii) the longer times required for peptide•A2 isolation in large scale experiments (several hours) may have permitted dissociation and loss of the natural 660MEL peptide, depending upon the (unknown) dissociation kinetics of this particular peptide•A2 complex; and (iii) eventual difficulties in sustaining the 660TIL line after prolonged culture.

These obstacles, especially low tumor peptide abundance, may also have limited peptide sequencing programs in other laboratories; only three successful biochemical isolations of tumor antigens have been reported (35, 58, 84). The majority of peptides recognized by anti-tumor T cells have been defined by gene cloning methods that permit identification and sequencing of the genes encoding tumor antigens irrespective of how low the abundance of the corresponding natural peptides might be (32, 33, 60). However, these approaches serve to identify a gene, not an antigenic peptide. In order to determine what peptide sequences are recognized, the deduced gene products must be scanned for candidate sequences, usually based on empiric consensus motifs (36). Synthetic peptides are then tested in cytotoxicity assays, often requiring high concentrations (up to 100 \mu M, see Table 2) to demonstrate sensitizing activity, unlike the few known naturally processed tumor peptides, which are active at pM concentrations. Wallny et al. took a useful further step for one of the (mouse) tumor peptides that had been defined at the synthetic peptide level and verified its presence in
tumor extracts by HPLC criteria (59); to our knowledge this has not yet been done for any of the human tumor peptides. Until such comparisons are made, and genetically defined peptides are demonstrated to be on the surface of tumor cells, their exact identities and hence their natural abundances will remain unknown.

At least four laboratories have assayed HPLC fractions of extracts obtained from peptide•A2 complexes on melanoma cells and found discrete peaks of sensitizing activity (refs. 54-56 and this study). Inasmuch as these peaks all represent naturally processed peptides, it should be possible to determine how many of the currently defined melanoma antigens (Table 2) are present on tumor cell surfaces in quantities sufficient to elicit a vigorous CTL response. Toward this end, we developed an approach to antigen identification based on defining the distinctive chromatographic signature of a naturally processed peptide for comparison with the signatures of various candidate synthetic peptides. Clearly, more than one HPLC condition is necessary in order to achieve high discrimination (Tables 3 and 4). In principle, tumor specimens and various normal tissues (e.g., melanocytes, retinal cells) as well as cultured tumor cell lines should all be amenable to screening for the presence of known antigenic peptides by this approach.
ACKNOWLEDGMENTS

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FIGURE LEGENDS

Fig. 1. Strategies used for the isolation of naturally processed melanoma peptide(s) recognized by TIL. Cultured melanoma cells were pelleted and either extracted directly with TFA (pathway 1), solubilized in detergent for immunoaffinity purification of A2 molecules and TFA elution of A2-bound peptides (pathway 2), or lysed hypotonically for isolation of cell membranes followed by extraction with TFA or detergent solubilization, A2 purification, and TFA elution (pathway 3). Ultrafiltrates from TFA extracts were fractionated by HPLC, and each fraction was tested for sensitizing activity in a cytotoxicity assay with A2+ target cells and TIL effector cells. Active fractions were subjected to repeated rounds of HPLC purification under different conditions until suitable for analysis by mass spectrometry.

Fig. 2. Cytotoxicity assay of peptides bound to HLA-A2 on melanoma cells. 10^10 660MEL cells were lysed hypotonically, membranes were isolated by ultracentrifugation and solubilized with detergent, and solubilized membranes were passed over a set of four immunoaffinity columns as described in Materials and Methods. Each column was eluted and the protein fractions were concentrated and treated with TFA to separate bound peptides. Peptides extracted from purified A2 (PA2.1 column), purified non-A2 HLA molecules (W6/32 column), and nonspecific proteins (mouse γ-globulin column) were separately fractionated under HPLC condition A. Fractions were collected at 1 min intervals and tested for sensitizing activity in a standardized cytotoxicity assay using 660TIL and JY target cells at a CTL:target cell ratio of 50:1. The shoulder peak at fraction 42 was observed consistently in different experiments; the small peak at fraction 33 was not always seen. Fractions derived from the W6/32 and mouse γ-globulin columns lacked significant activity (not shown).

Fig. 3. Purification of major melanoma antigen recognized by 660TIL. A2-bound peptides were obtained from 4 x 10^11 cultured 660MEL cells and fractionated by HPLC as described in Fig. 2, except that immunoprecipitation was performed instead of affinity chromatography. Active fractions were subjected to re-chromatography under HPLC conditions A, B, C, and D as described in Materials and Methods, for a total of four serial rounds of chromatography. HPLC fractions were collected at 0.5 or 1.0 min intervals and 1-2% of each fraction was tested in standardized cytotoxicity assays using 660TIL and JY or T2 target cells. Ultraviolet absorbance profiles for each round of chromatography were generally unrevealing, since they did not predict the retention times of biologically active peptides, and are not shown.

Fig. 4. Cytotoxicity assay of A2+ melanoma cell lines 660MEL, NA8, NA8 transfected with the tyrosinase gene (NA8-tyrosinase), and NA8 transfected with the Melan-A gene (NA8-Aa). 660TIL were used as CTL in a 4 h assay.

Fig. 5. Synthesis and screening of peptides flanking known antigenic sequences from MART-1/Melan-A. The indicated synthetic peptides were tested for sensitizing activity in standardized cytotoxicity assays using 660TIL and JY target cells at a CTL:target cell ratio of 50:1. Peptides were tested at final concentrations of 100 μg/ml (shown) and 10 μg/ml. As a positive control, 660MEL target cells were included in each assay and gave specific lysis values >50-60%. Peptides enclosed in boxes are known melanoma peptides recognized by A2-restricted TIL in other laboratories (34, 35). ND, not done.
Tsomides et al. Figure 1
Tsomides et al. Figure 2
Tsomides et al. Figure 3
Tsomides et al. Figure 4
Tsomides et al. Figure 5
**Table 1.** Cytotoxicity assays using 660TIL.

<table>
<thead>
<tr>
<th>Target cell</th>
<th>HLA-A2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent specific lysis&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>50:1</td>
<td>10:1</td>
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<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>660MEL</td>
<td>+ (178)</td>
<td>82.8</td>
</tr>
<tr>
<td>SK-MEL-5</td>
<td>+ (109)</td>
<td>59.3</td>
</tr>
<tr>
<td>F0-1/neo</td>
<td>- (2)</td>
<td>1.7</td>
</tr>
<tr>
<td>F0-1/A2</td>
<td>+ (56)</td>
<td>56.3</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>660MEL</td>
<td>+ (178)</td>
<td>50</td>
</tr>
<tr>
<td>SK-MEL-30</td>
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<tr>
<td>SK-MEL-113</td>
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</tr>
<tr>
<td>WM88</td>
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<tr>
<td>Experiment 3</td>
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</table>
| 660MEL      | + (178)            | 92                  | 72                   | 32
| Malme-3M    | +                  | 72.6                | 40.2                 | 16.3
| HT-144      | -                  | 19.8                | 13.9                 | 4.6
| SK-MEL-28   | -                  | 3.1                 | 1.9                  | 0

<sup>a</sup> Numbers in parentheses are mean fluorescence values in flow cytometric analyses using mAb PA2.1 and FITC-conjugated F(ab')<sub>2</sub> fragment of goat anti-mouse IgG.

<sup>b</sup> Percent specific lysis in cytotoxicity assays at the indicated CTL:target cell ratios.
Table 2. Peptides recognized by CD8+ anti-tumor T cells.\(^a\)

<table>
<thead>
<tr>
<th>CTL</th>
<th>MHC restriction</th>
<th>Peptide(^b)</th>
<th>SD(_{50}) value(^c)</th>
<th>Parent protein or gene</th>
<th>Normal or mutated</th>
<th>Tissue distribution</th>
<th>Shown to be naturally processed(^d)</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L(^d)</td>
<td>TQNHRLALDL</td>
<td>1 nM</td>
<td>P91A</td>
<td>mutated</td>
<td>mutagenized mastocytoma</td>
<td>no</td>
<td>85-87</td>
</tr>
<tr>
<td></td>
<td>L(^d)</td>
<td>LPYLGWLVF</td>
<td>5 nM</td>
<td>P1A</td>
<td>normal</td>
<td>&quot;</td>
<td>yes</td>
<td>88-90</td>
</tr>
<tr>
<td></td>
<td>D(^d)</td>
<td>GPPHSNNFYG</td>
<td>8 nM</td>
<td>P35B</td>
<td>mutated</td>
<td>&quot;</td>
<td>yes</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>K(^d)</td>
<td>KYQAVVTTL</td>
<td>25 pM</td>
<td>P198</td>
<td>mutated</td>
<td>&quot;</td>
<td>yes</td>
<td>84, 92</td>
</tr>
<tr>
<td></td>
<td>K(^b)</td>
<td>FEQNTAQQP</td>
<td>=10 nM</td>
<td>connexin 37</td>
<td>mutated</td>
<td>Lewis lung carcinoma</td>
<td>normal melanomas, other tumors, normal testis</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>PBL</td>
<td>A1 EADPTGHSY</td>
<td>5 nM</td>
<td>MAGE-1</td>
<td>normal</td>
<td>&quot;</td>
<td>yes</td>
<td>94-96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A2 EVDPGHLY</td>
<td>=100 nM</td>
<td>MAGE-3</td>
<td>normal</td>
<td>&quot;</td>
<td>no</td>
<td>22, 97-98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A2 FLWGFRAVL</td>
<td>1 nM</td>
<td>MAGE-3</td>
<td>normal</td>
<td>&quot;</td>
<td>no</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A2 MLLAVLYCL</td>
<td>=10 nM</td>
<td>tyrosinase</td>
<td>normal</td>
<td>melanomas, melanocytes</td>
<td>yes</td>
<td>39, 48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A2 YMNGTMSQV</td>
<td>=100 nM</td>
<td>tyrosinase</td>
<td>normal</td>
<td>melanomas, melanocytes</td>
<td>possibly yes</td>
<td>39, 48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TIL/</td>
<td></td>
<td></td>
<td>normal</td>
<td>melanomas, melanocytes, retinal cells</td>
<td>no</td>
<td>32-34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PBL A2</td>
<td>A2 IILTVGVL</td>
<td>=10 pM</td>
<td>MART-1/ Melan-A</td>
<td>normal</td>
<td>yes</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TIL A2</td>
<td>LLDGTATRL</td>
<td>0.1-1 (\mu M)</td>
<td>gp100/Pmel17</td>
<td>normal</td>
<td>yes</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A2 KTWGQYQV</td>
<td>1-10 nM</td>
<td>gp100</td>
<td>normal</td>
<td>&quot;</td>
<td>no</td>
<td>83, 100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A2 (TTIDQVPFSV)</td>
<td>=10 nM</td>
<td>gp100</td>
<td>normal</td>
<td>&quot;</td>
<td>no</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A2 VLYRYSFSV</td>
<td>0.1-1 (\mu M)</td>
<td>gp100</td>
<td>normal</td>
<td>&quot;</td>
<td>no</td>
<td>65, 102</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PBL A2</td>
<td>ACDPHSGHVF</td>
<td>=10 nM</td>
<td>CDK4</td>
<td>mutated</td>
<td>no</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TAL A2</td>
<td>IISAAGVGIL</td>
<td>100 (\mu M)</td>
<td>HER2/neu</td>
<td>normal</td>
<td>no</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TIL A2</td>
<td>KIGSGALFL</td>
<td>100 (\mu M)</td>
<td>HER2/neu</td>
<td>normal</td>
<td>no</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A2 AFLPWHRLF(L)</td>
<td>0.1-1 (nM)</td>
<td>tyrosinase</td>
<td>normal</td>
<td>melanomas, normal tissues melanomas, melanocytes</td>
<td>no</td>
<td>107-108</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B44 SEIWDDDF</td>
<td>0.4 (nM)</td>
<td>tyrosinase</td>
<td>normal</td>
<td>autochthonous melanoma melanomas, other tumors, normal testis</td>
<td>no</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PBL B44</td>
<td>EEKLQVVF</td>
<td>0.4 (nM)</td>
<td>MUM-1</td>
<td>mutated</td>
<td>no</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cw6 YRPRPRRY</td>
<td>100 (nM)</td>
<td>GAGE-1,2</td>
<td>normal</td>
<td>&quot;</td>
<td>no</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cw16 SAYGEPRKL</td>
<td>25 (nM)</td>
<td>MAGE-1</td>
<td>normal</td>
<td>&quot;</td>
<td>no</td>
<td>63</td>
</tr>
</tbody>
</table>

\(^a\) Excluding several viral peptides and oncogene-related peptides that were used to stimulate T cells \textit{in vitro}.

\(^b\) Underlined residues are mutated. Residues in parentheses indicate that both the 9mer and 10mer are recognized to a similar extent by T cells.

\(^c\) SD\(_{50}\) values (43) are approximate synthetic peptide concentrations at which 50% of maximal lysis occurs in cytotoxicity assays. Methods vary with regard to incubation of target cells with peptide throughout the assay or pulsing target cells with peptide prior to adding CTL.

\(^d\) A peptide is considered to be naturally occurring when it is isolated from natural sources or when the HPLC profile of the synthetic peptide is shown to match that of an active natural fraction.

\(^e\) Cox et al. (58) reported the isolation of peptide YMNGTMSQV (N→D at position 3); whether this represents post-translational cellular deamidation, artefactual deamidation during the purification, or a different genetic origin remains unknown.
Table 3. Comparison of the major 660MEL peptide and other melanoma peptides known to be recognized by A2-restricted T cells: 660TIL sensitizing activity and HPLC profiles.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Parent protein or gene</th>
<th>Percent specific lysis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HPLC retention time (min)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>660MEL peptide</td>
<td>unknown</td>
<td>50-100</td>
<td>44-45</td>
</tr>
<tr>
<td>MLLAVLYCL</td>
<td>tyrosinase</td>
<td>2</td>
<td>57</td>
</tr>
<tr>
<td>YMNGTMSQV</td>
<td>tyrosinase</td>
<td>2</td>
<td>34</td>
</tr>
<tr>
<td>AAGIGILTV</td>
<td>MART-1/ Melan-A</td>
<td>10-23</td>
<td>43</td>
</tr>
<tr>
<td>ILTVILGVL</td>
<td>MART-1/ Melan-A</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>YLEPGPVTA</td>
<td>gp100/Pmel17</td>
<td>6-9</td>
<td>35</td>
</tr>
<tr>
<td>LLDGTATLRL</td>
<td>gp100</td>
<td>5-7</td>
<td>43</td>
</tr>
<tr>
<td>KTWGQYWQV</td>
<td>gp100</td>
<td>3</td>
<td>43</td>
</tr>
<tr>
<td>FLWGPRALV</td>
<td>MAGE-3</td>
<td>4</td>
<td>48</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percent specific lysis in cytotoxicity assays with 660TIL and JY or T2 target cells at a CTL:target cell ratio of 50:1. Peptides were tested at final concentrations of 100 μg/ml (shown) and 10 μg/ml. In each assay a positive control using 660MEL target cells gave at least 50% specific lysis. Some assays were performed using mAb MA2.1 and exogenous β2m as described (35, 47, 48), without significantly different results.

<sup>b</sup> HPLC retention times are shown under three different sets of chromatographic conditions (column, solvents, gradient, flow rate) as described in Materials and Methods. ND, not done.
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Parent protein or gene</th>
<th>Reason for testing peptide</th>
<th>Percent specific lysis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HPLC retention time (min)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>660MEL peptide</td>
<td>unknown</td>
<td>50-100</td>
<td>44-45</td>
<td>49-50</td>
</tr>
<tr>
<td>EAAGIGILTV</td>
<td>MART-1</td>
<td>A2 motif&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17</td>
<td>43 40 42</td>
</tr>
<tr>
<td>AAGIGILTVI</td>
<td>MART-1</td>
<td>A2 motif&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
<td>48 40 56</td>
</tr>
<tr>
<td>PVVPNAPPA</td>
<td>MART-1</td>
<td>A2 motif&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3</td>
<td>ND ND ND ND</td>
</tr>
<tr>
<td>NAPPAYEKL</td>
<td>MART-1</td>
<td>A2 motif&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6</td>
<td>35 30 31</td>
</tr>
<tr>
<td>ALMDKSLHV</td>
<td>MART-1</td>
<td>A2 motif&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6-12</td>
<td>37 31 35</td>
</tr>
<tr>
<td>RALMDKSLHV</td>
<td>MART-1</td>
<td>A2 motif&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
<td>ND ND ND ND</td>
</tr>
<tr>
<td>SLHVGTQCA</td>
<td>MART-1</td>
<td>A2 motif&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8</td>
<td>ND ND ND ND</td>
</tr>
<tr>
<td>SLHVGTQCAL</td>
<td>MART-1</td>
<td>A2 motif&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3-17</td>
<td>42 41 44</td>
</tr>
<tr>
<td>SLQEKNCEPV</td>
<td>MART-1</td>
<td>A2 motif&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0-10</td>
<td>34 35 32</td>
</tr>
<tr>
<td>APPAYEKLSAEQ</td>
<td>MART-1</td>
<td>mass spec ion 1301&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7</td>
<td>33 30 32</td>
</tr>
<tr>
<td>HSYTTAEEA</td>
<td>MART-1</td>
<td>mass spec ion 1007&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10</td>
<td>27 9 9</td>
</tr>
<tr>
<td>ILESLFRAV</td>
<td>MAGE-1</td>
<td>A2 motif&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0-2</td>
<td>ND ND ND ND</td>
</tr>
<tr>
<td>QLVFGIEVV</td>
<td>MAGE-1</td>
<td>A2 motif&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0-2</td>
<td>ND ND ND ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Same as Table 3, footnote a.
<sup>b</sup> Same as Table 3, footnote b.
<sup>c</sup> The consensus motif for A2-binding peptides (36) was interpreted broadly to include A, I, L, or V residues at positions 2 and 9 of 9mers or at positions 2 and 10 of 10mers.
<sup>d</sup> See text for details.
REFERENCES


encoding MART1 or gp100 for cancer therapy. J. Immunol., 156: 700-710, 1996.


CONCLUDING REMARKS
A leading immunologist, in discussing molecular interactions, once noted: “It’s not a matter of yes or no, but of more or less.” In that same spirit, this thesis is written. T cell recognition of antigens depends not only on their presence, but on their levels of abundance on target cell surfaces. This is likely to be true for antigens recognized by developing T cells as they mature in the thymus as well as antigens that trigger the activities of mature T cells. Another parameter likely to be critical to the disposition of a T cell response is the affinity of a T cell’s antigen-specific receptor for particular ligands. The interplay between these two factors—ligand density and TcR affinity—represents a fascinating area for future theory as well as practical investigation (e.g. see Eisen et al., In press; appendix, p. 317).

Intuitively, it seems reasonable (though not by any means obligatory) that a low-affinity TcR (on a mature cell) will respond only if the ligand is present at a relatively high density, while a high-affinity TcR may require less ligand for a comparable response. Yet why should this be so? The function of a TcR is to detect peptide•MHC complexes on other cells and to transduce signals into the cytosol, where intracellular mediators can take over. What could underlie the presumptive advantage of a high-affinity receptor, or conversely, how might a high density of ligand somehow compensate for a low-affinity interaction? The general problem is not specific to T cells, although they have their special features, and it may be instructive to borrow a page from pharmacology (e.g. Bowman and Rand, 1980, chapter 39).

There are two main theories for the relationship between drug–receptor interactions and the resulting responses. (1) “Occupation theory” holds that response is a function of the occupation of receptors by an agonist. Assuming that ligation of each
receptor elicits an all-or-none stimulus, and that these stimuli summate in a linear fashion to produce some effect, then the amount of effect is proportional to receptor occupancy, which can be calculated from the law of mass action (i.e., response $\propto$ fraction of receptors occupied = $K_a c / (1 + K_a c)$). Attempting to apply this to a TcR reaction with peptide-MHC complexes, the “concentration” of free ligand (c) becomes the number of specific peptide-MHC complexes per target cell, and the equilibrium association constant $K_a$ is the intrinsic affinity of a TcR for this ligand. Inasmuch as fractional occupancy is deemed critical, the actual number of receptors does not show up here; however, derivation of the above equation assumes that this number is very low relative to the number of ligands (peptide-MHC complexes), and furthermore that the “concentration” of free ligand is not appreciably altered by TcR binding, both very poor assumptions here. A general formulation avoids these assumptions and is mathematically more complex (e.g. “ligand depletion,” Hulme and Birdsall, 1992).

(2) “Rate theory” holds that response is a function of the rate of occupation of receptors by an agonist. As originally expounded for drug–receptor interactions by Paton (1961), the basic assumptions are that the stimulus provided by an agonist ligand is proportional to its rate of association with a receptor and that each combination between ligand and receptor provides a quantum of stimulation. This leads to the intriguing conclusion that at equilibrium (rate of association = rate of dissociation), and given a high concentration of ligand, the maximal response is proportional to the rate constant for dissociation, i.e. drugs that dissociate rapidly will tend to be potent agonists while drugs that dissociate slowly from their complex will produce small or negligible responses (Bowman and Rand, 1980, chapter 39).
(3) A third theory taking shape in the T cell field also holds the dissociation rate to be critical, but predicts that stronger responses will correlate with low rather than high off-rates for the TcR–peptide•MHC reaction.

Clearly the T cell response is a particularly difficult endpoint to model. Unlike the response of a receptor-bearing cell to soluble drug molecules, T cells respond to ligands on the surfaces of other cells, and these ligands may often be limiting to the overall response. Moreover, the ligand itself (1) might be heterogeneous, consisting of different peptide•MHC combinations with different affinities for the same TcR; (2) might be unstable, suffering dissociation (loss of peptide) over time; and (3) is manipulated experimentally by adding synthetic peptides to cells, thereby creating an additional reaction between peptide and functionally empty MHC molecules that has its own kinetic and thermodynamic parameters. Perhaps as a result of these complexities, and given the small number of measurements that have been made of either ligand density or TcR affinity, no general quantitative theory for T cell–target cell interactions has yet been promulgated.

It is certainly not the goal of this work to advance such a model. However, an argument is made that the density of peptide•MHC complexes on target cells is likely to be a critical factor in any rigorous understanding of T cell responses, and that determination of natural ligand densities is not possible until the identities of processed peptides forming endogenous peptide•MHC complexes are known (see also Tsomides, 1996; appendix, p. 301). To this end, the major findings of the work described in chapters 1-4 can be summarized as follows.
• Synthetic peptides can be used to measure binding parameters of the peptide-MHC-I reaction, but only with whole cells or specially engineered “empty” MHC-I molecules, not with affinity-purified MHC-I molecules that inevitably contain bound peptides.

• The number of peptide-MHC-I complexes required for T cell activation (r) may be calculated by measuring $K_a$ for the peptide-MHC-I reaction, the number of available MHC-I binding sites per cell (n), and the peptide concentration required for a CTL response (c); in optimal combinations $r = K_a \cdot c \cdot n / (1 + K_a \cdot c)$ may be <10.

• A method for producing and purifying stoichiometrically labeled peptides for the above studies has been developed and optimized; when carrier-free $^{125}$I is used, maximum specific radioactivities are $3.5 \times 10^{18}$ cpm/mol (monoiido derivative) or $7 \times 10^{18}$ cpm/mol (diiodo derivative).

• The most active synthetic peptide recognized by an HIV-specific human CTL clone is a nonamer (RT 476-484, ILKEPVHG, termed IV9) that effectively sensitizes target cells for lysis at a free concentration of 1-5 pM; an iodinated derivative of this peptide binds specifically to a small portion of cell surface HLA-A2 molecules on JY cells ($\leq 0.35\%$) and dissociates very slowly from these complexes ($t_{1/2} \geq 200$ hr).

• The same peptide (IV9) was shown to be present endogenously in chronically and uniformly HIV-infected target cells that were specially generated for this study; a maximum of $\approx 12$ IV9*A2 complexes per cell are present on HIV-infected Jurkat target cells, which are only poorly recognized by CTL in in vitro cytotoxicity assays.
• A different peptide (gag 77-85, SLYNTVATL, termed SL9) is also produced in HIV-infected target cells, but at higher levels (≈400 molecules per cell) and with higher levels of accompanying lysis by a gag-specific CTL clone.

• None of the other synthetic RT or gag peptides that are active in cytotoxicity assays (some at ≤100 pM) could be detected in extracts of HIV-infected cells, ruling out their physiological relevance.

• Several naturally processed peptides were discovered to form the allogeneic complexes (in association with H-2L^d) that are recognized by a mouse CD8^+ T cell clone (2C); two of these were purified to near-homogeneity from tissue extracts and sequenced by Edman degradation and mass spectrometry (LSPFPFDL, p2Ca; and VAITRIEQLSPFPFDL, p2Cb); both come from the ubiquitous cellular protein α-ketoglutarate dehydrogenase and appear to be highly abundant in mouse tissues of all three haplotypes examined; p2Ca but not p2Cb co-purifies with L^d molecules.

• In the 2C system, antigen processing is largely conserved across eight different tissues, although quantitative variations seem likely because differences in the amounts of peptides recovered far exceed tissue differences in α-KGDH or L^d levels.

• A third peptide recognized by 2C cells was detected only in liver, and its sequence contains a single F→Y modification at position 4 that was speculated to arise post-translationally (but could also represent an isozyme).
• A fourth synthetic peptide (QLSPFPFDL, QL9) is recognized by 2C cells at much lower concentrations than the three known naturally processed peptides, but its presence in tissues or cells remains uncertain; the TcR of 2C cells recognizes this fourth peptide with an intrinsic affinity higher than any others so far measured (Ka = 1.5x10^7 M^-1).

• Two of the naturally processed peptides (p2Ca and p2Ca-Y4) bind equivalently to H-2K^b, a self MHC-I molecule, but only one is recognized by the TcR of 2C cells, illustrating that TcR reactions can be highly specific even when the affinities involved are very low (ca. 3x10^3 M^-1 for the TcR reaction with p2Ca•K^b complexes).

• Despite the likely physiological presence of p2Ca•K^b “self” complexes in H-2^b mice (including mice transgenic for the 2C TcR), autoimmune responses mediated by the 2C TcR are not observed, suggesting that ligand density falls below a critical threshold for eliciting such responses; on the other hand, these observations are consistent with the possibility that p2Ca might participate in the positive selection of 2C TcR^+ cells.

• A human melanoma peptide recognized by HLA-A2-restricted tumor infiltrating lymphocytes was purified, but insufficient amounts were obtained for sequencing; provisional conclusions about the identity of this peptide are based on its chromatographic profile compared with those of known melanoma peptides; low abundance of the melanoma peptide and its likely non-mutated sequence have interesting consequences for immune recognition of tumor antigens.
A primary motivation for these studies has been the pursuit of knowledge and understanding. In a larger scheme, however, the issues addressed here take on considerable practical importance. One of the driving forces behind efforts to identify naturally processed peptides is the hope that a new generation of vaccines can be developed using synthetic peptides (or perhaps DNA encoding immunogenic peptide sequences (Ulmer et al., 1993; Raz et al., 1994)). Several groups have reported that T cell responses can be obtained in mice by immunization with peptides in various formats (± adjuvants, lipid moieties, “helper” components, etc.) (Deres et al., 1989; Aichele et al., 1990; Kast et al., 1991; Gao et al., 1991; Zhou et al., 1992; Feltkamp et al., 1993; Tjoa and Kranz, 1994). In some cases, the resulting responses are protective against subsequent challenges with otherwise lethal doses of a virus or tumor. Recently, clinical trials of synthetic peptide “vaccines” in human melanoma patients have been undertaken (this represents a therapeutic mode more than vaccination in the true sense). A related goal is the blockade of autoimmune T cell reactions through the use of specific peptides, especially those discovered to have antagonist properties when they interact with TcR (Sloan-Lancaster et al., 1993; Racioppi et al., 1993; Jameson et al., 1993; Sette et al., 1994a).

Thus it is hoped that immunization with synthetic peptides will permit T cell responses to be accentuated or attenuated, depending on the clinical setting. For peptide vaccines to be successful, they probably will have to satisfy at least the following criteria: (1) they must elicit many functional CTL whose TcR have relatively high affinities for specific peptide-MHC-I ligands (it has been suggested but not shown that lower doses of peptide may result in higher affinity TcR); (2) the peptides recognized
by these CTL must be naturally processed and presented by target cells \emph{in vivo}; and (3) the natural peptides recognized must be present at levels of abundance sufficient to trigger potent cytolytic activities from the induced CTL. This last issue may be especially critical for chronic infections (e.g. by HIV) and for tumors, since only a tiny minority of the peptide•MHC-I complexes on target cell surfaces are expected to contain relevant antigens; in contrast, when cells are infected by lytic viruses such as influenza, vaccinia or VSV, protein synthesis (and presumably antigenic peptide generation) are dominated by the virus. Hence it may bear considering that the nature of a T cell response elicited by peptide vaccination will be determined not only by the particular sequences used, but also by the amount of ligand delivered and the amount expressed naturally: “...more or less.”

***
APPENDIX 1

Related publications
Minireview

Antigenic Structures Recognized by Cytotoxic T Lymphocytes
Theodore J. Tsanides and Herman N. Eisen
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The antigenic structures recognized by T lymphocytes differ fundamentally from those recognized by B lymphocytes. B cells, through antibody molecules embedded in their cell surface membrane, collectively recognize an enormous diversity of antigens in solution or on cell surfaces, including native and unfolded proteins, polysaccharides, nucleic acids, steroids, and small organic molecules. The antigen-specific receptors on T cells are very similar to antibodies structurally and in the genetic basis for their diversity. Nevertheless, the antigenic structures recognized by T cells consist almost exclusively of peptides associated with integral membrane glycoproteins known as MHC proteins (because they are encoded in a genetic region called the major histocompatibility complex). Thus T cells recognize protein antigens in a fragmented (or “processed”) form on the surface of other cells expressing appropriate MHC molecules (a phenomenon termed MHC “restriction” of the T cell response (1)).

The MHC genetic region (also called HLA in the human and H-2 in the mouse) contains many highly polymorphic, tightly linked genes, and a particular set of alleles defines a haplotype. Any given individual in a population has an array of MHC molecules on the surface of its cells (one haplotype from each parent, codominantly expressed) which serves as a signature for that individual (or for the genetically uniform mice or rats of inbred strains). The essence of MHC restriction is that T cells normally recognize antigens on cells having the same haplotypes as the T cells themselves but not on cells of different haplotypes. (However, in the context of organ transplantation between individuals of different haplotypes, nonself MHC molecules are targets for a T cell-mediated rejection response. It was this “allogeneic” reaction which led to the discovery of the MHC and to the origin of the term histocompatibility.)

Mature T cells can be divided into two groups based on their cell surface expression of either CD8 or CD4 glycoproteins. Each group interacts with a different set of MHC molecules, called class I or class II, respectively. MHC molecules of both classes are noncovalently associated heterodimers on the cell surface, but class I is composed of a membrane-spanning 45-kDa α (or heavy) chain and a soluble, nonpolymorphic 12-kDa subunit (β2-microglobulin or light chain), whereas class II consists of two transmembrane chains, α (35 kDa) and β (29 kDa). Only certain “antigen-presenting” cells (APC) express MHC-II (macrophages, dendritic cells, B cells, and a few others). When CD4+ T cells recognize class II-expressing APC that have reacted with appropriate antigens, they usually respond by secreting lymphokines that influence lymphocytes and other cell types; hence these T cells are designated helper T cells. However, most nucleated cells express MHC-I, so CD8+ T cells can potentially respond to virtually all cell types. In the presence of a suitable antigen, class I-expressing target cells are specifically destroyed by CD8+ T cells; hence the latter are termed killer cells or cytotoxic T lymphocytes (CTL).

The division between class I and class II proteins is important not only for the different T cell responses provoked, but because they define different pathways through which peptides are presented to T cells. In particular, extracellular proteins (also termed exogenous) are taken up by APC, partially digested in a low pH endosomal compartment, and the resulting peptides bound by MHC-II for transport to the cell surface where they can stimulate CD4+ T cells. In contrast, antigens synthesized within a cell (endogenous), such as viral proteins, tumor antigens, or so-called minor histocompatibility antigens, are degraded intracellularly to peptides and associate with MHC-I for transport to the cell surface where they flag the cell for recognition and lysis by CD8+ CTL. The separation between the two antigen-processing pathways (Table I) is not absolute, but it offers a useful framework for probing the cell biology and biochemistry of each pathway and for evaluating exceptions. This minireview will concentrate on the class I pathway and in particular on the molecular interactions between antigen and MHC-I prerequisite to recognition by CD8+ CTL.

CD8+ CTL Recognize Peptides Derived from Endogenous Antigens

Viral infection elicits a vigorous CTL response leading to the specific lysis of infected cells. Experimental studies with influenza virus have revealed important features of the antiviral CTL response (3). Whereas anti-influenza antibodies recognize subtype-specific, virally encoded glycoproteins expressed on the surface of infected cells, CTL from both mice (4) and humans (5) cross-react with target cells infected by different influenza virus subtypes. This cross-reactivity was explained by the subsequent finding that most anti-influenza CTL are specific for conserved, intracellular viral proteins undetectable at the cell surface (e.g. nucleoprotein, matrix protein). Thus, cells transfected with the nucleoprotein gene served as targets for influenza-specific CTL despite the absence of cell surface nucleoprotein, giving rise to the suggestion that the antigen is converted into fragments (peptides) which are transported to the cell surface for CTL recognition (6). This model was confirmed by experiments showing that incubation of cells with synthetic peptides mimicking parts of the nucleoprotein rendered the cells susceptible to lysis by CTL (7). Incubation with whole nucleoprotein was ineffective, and only certain peptides from the nucleoprotein sequence were active. Presumably such peptides, substituting for peptides naturally produced from endogenously synthesized antigen, somehow associate directly with MHC-I molecules to form the complex recognized by CTL.

Subsequent studies with mouse and human CTL by many workers have identified at least 40-50 different synthetic peptides recognized in association with various MHC-I molecules. In addition to virally derived peptides, peptides corresponding to sequences from other endogenously synthesized proteins are recognized specifically by CTL (8), as well as peptides corresponding to mutated sites responsible for tumor
immunogenicity in mouse cell lines (9). Thus many tumor-specific antigens (and minor histocompatibility antigens) are probably peptide fragments recognized at the cell surface by MHC-restricted CTL, explaining the historical failure of antibodies to detect these antigens.

**Evidence for Peptide-MHC-I Complexes**

A dramatic and compelling image of how a peptide-MHC-I complex might look was provided by the 3.5 Å x-ray crystallographic structure of a human MHC-I molecule, HLA-A2 (10, 11). The structure consists of four domains of approximately 90 amino acid residues each, derived from the α-chain (α1, α2, and α3) and one comprising β2-microglobulin (transmembrane and cytosolic portions of the α-chain were removed by papain digestion early in the purification). The membrane proximal α1 and β2-microglobulin domains have tertiary structures resembling immunoglobulin folds, consistent with their known amino acid sequences. The α1 and α2 domains each have a nearly identical but novel structure containing four antiparallel β-strands and a long α-helix, such that when paired those domains form a single platform of eight β-strands topped by two α-helices. A long groove overlying the β-sheet and between the two α-helices was proposed to be the peptide binding site based on several considerations. 1) Most of the polymorphic residues of HLA cluster in or near the proposed binding site, accounting for the ability of different MHC molecules to interact with different peptides. 2) Residues known to be critical for T cell recognition through different MHC-I molecules; 4) reconstitution of peptide-free MHC-I with a known, homogeneous peptide for x-ray crystallography; and 5) elucidation of the physiological pathway for generation of peptide-MHC-I complexes by genetic and cell biological approaches.

Examination of several dozen peptides which elicit MHC-restricted T cell responses (both class I and class II) led to the formulation of predictive schemes based on common structural elements (16-19). The resulting algorithms often reflect some form of recurrent hydrophobicity (20). While several predicted synthetic peptides were biologically active in T cell assays, other peptides found by random screening to have similar activities did not fit the expected patterns. The relatively small (but growing) data base of available peptide sequences may have limited this approach, especially considering the distinctions in peptide binding by different MHC proteins. Furthermore, in most cases it is not known which residues of a given peptide are actually required for MHC binding, which interact primarily with a T cell receptor, which serve a role other than for specific binding, and which are superfluous or artifactual due to their synthetic origin.

Until recently the relationship between synthetic and naturally occurring peptides which bind MHC-I was largely uncertain, probably because characterization of natural peptides is challenging from a technical standpoint in view of their likely heterogeneity and consequent low abundance. Gel filtration fractionation of cell extracts followed by assay of the fractions for T cell recognition first gave clues as to natural peptide size (21, 22) and later led to the identification of two viral peptides (nonamers) whose sequences are contained within known biologically active synthetic peptides (23). A third viral peptide recovered directly from immunoprecipitated MHC-I molecules similarly represents a portion (octamer) of a known synthetic peptide (24). Thus in all likelihood natural peptides are relatively small peptides with no particular chemical modifications or structural themes yet evident.

**What Peptides Bind to MHC-I?**

Efforts to understand the structural basis for binding between peptides and MHC-I proteins have taken several directions, including: 1) tabulation of synthetic peptides recognized by MHC-restricted T cells in order to discern common features or motifs and develop predictive binding algorithms; 2) structural characterization of naturally occurring peptides recognized by MHC-restricted T cells; 3) in vitro binding studies using synthetic peptides and purified MHC-I molecules; 4) reconstitution of peptide-free MHC-I with a known, homogeneous peptide for x-ray crystallography; and 5) elucidation of the physiological pathway for generation of peptide-MHC-I complexes by genetic and cell biological approaches.
binding to the appropriate purified MHC molecules by either gel filtration (30) or equilibrium dialysis (31). Substantial binding of detergent-solubilized MHC-I to peptides immobilized on plastic surfaces can be detected, but this binding appears largely indiscriminate with respect to MHC specificity, and it is unclear whether the solid phase environment favors binding by particular peptides due to physical properties other than intrinsic binding affinities (32-34). A similar report, showing more specificity, involved the addition of peptides to immobilized MHC-I to evoke a T cell response (35). It may be that the low binding observed between peptides and MHC-I in solution accurately reflects an intrinsically low affinity. If the association constants are as low as 10^3 M^-1 (consistent with available data), cell surface class I molecules will be 1% saturated at equilibrium given a peptide concentration of 10^-7 M to induce half-maximal CTL lysis, and this might result in sufficient MHC-I complexes (300-500/target cell) for CTL recognition. However, this scenario is by no means the only plausible one. If the x-ray structures of HLA are taken to include tightly bound peptides, their dissociation rates must be extraordinarily slow, since purification procedures take at least 7-10 days. Therefore the purified HLA used in binding assays may similarly be occupied by unknown peptides which reduce the concentration of available binding sites. If such prior complexes exist in a large (perhaps even near-stoichiometric) proportion, failure to detect binding of labeled peptides by equilibrium dialysis is understandable and constitutes further support for exceptionally slow dissociation rates, since otherwise some exchange would be expected to occur. Assuming cell surface MHC-I is also tightly complexed with unknown peptides, sensitization of target cells for CTL lysis may involve binding to a small proportion of peptide-free MHC-I sites on the cell, again yielding relatively few antigenic complexes per cell. Whether all purified MHC-I molecules harbor unknown peptides is not yet known and in fact cannot be determined by x-ray crystallography, as only a small proportion of purified MHC-I (5%) crystallizes.2

Where Do Peptide-MHC-I Complexes Form?

Recent insights into the assembly of peptide-MHC-I complexes derive from studies of a mutant mouse cell line, RMA-S, having what appears to be a defect in the generation or transport of endogenous peptides into the endoplasmic reticulum (ER) (30). Ordinarily, nascent class I α-chains and β2m associate into heterodimers in the ER and are transported to the cell surface within 30-60 min of their biosynthesis (37, 38). In RMA-S cells, α-chains and β2m are synthesized normally but largely fail to assemble, so that cell surface MHC-I expression is only about 5% of wild-type levels under standard culture conditions (37°C). When infected with influenza virus, RMA-S cells are not recognized by class I-restricted CTL, but when incubated with relatively high concentrations of suitable synthetic peptides from the virus, they become good targets for CTLs, and, surprisingly, express 2-5-fold more MHC-I on their surface (39).

These findings were explained by hypothesizing that synthetic peptides are taken up by RMA-S, somehow gain access to nascent α-chains and β2m in the ER, and promote the proper folding, assembly, and transport of the cell surface peptide-MHC-I complexes. The model implies that cell surface expression of MHC-I normally depends on the presence of peptides in the ER to help α and β2m associate, and that RMA-S lacks this function. Subsequently, however, increased surface expression of MHC-I was fortuitously demonstrated by incubating RMA-S at reduced temperatures (23-31°C), and these MHC-I molecules were unstable at 37°C unless an appropriate synthetic peptide was added (40). This enhanced MHC-I expression in the presence of peptides might also be explained by a stabilization of molecules already at the cell surface (but not otherwise detected because they are conformationally altered and/or short-lived). Such labile and evidently peptide-free MHC-I molecules could either reach the cell surface without peptides or could release bound peptides via dissociation (41).

Further evidence that added peptides can interact with MHC-I at the cell surface comes from studies using inhibitors of protein export. Treatment of non-mutant cells with the compound brefeldin A (which blocks transport from the ER to the Golgi complex) or the viral product E19 (which specifically binds to and retains MHC intracellularly) prevents the presentation of endogenous (e.g. viral) antigens but does not interfere with the presentation of synthetic peptides to CTL (42-44). Similarly, if cells are "fixed" using glutaraldehyde (which stops protein synthesis and turnover), presentation of endogenous antigen but not of added peptide is abrogated (45). On the other hand, the increased MHC-I detected on RMA-S cells in the presence of added peptide could result from either peptide-induced stabilization of cell surface MHC-I or from association of peptide with MHC-I intracellularly, much like endogenously arising peptides.

Whether the effect of exogenous peptides on MHC-I takes place at the cell surface or in the ER (or perhaps both, depending upon peptide concentration), the properties of RMA-S and of a comparable human mutant cell line (46, 47) indicate that most stable MHC-I molecules at 37°C include peptide as an integral partner with α-chain and β2m. Moreover, β2m can also stabilize MHC-I structure, as shown by an increased association between peptides and MHC-I when free β2m is added to cells under limiting conditions of peptide (48).

In addition, in vitro reconstitution of MHC-I from separated α-chains and β2m is more efficient in the presence of either excess β2m (49) or specific peptides (50); similar effects are obtained using detergent lysates from RMA-S cells (51). Finally, distinct MHC-I molecules are known to differ in their
requirements for βm to form stable cell surface structures (52, 53), and they may differ in their requirements for peptide as well (54).

How Are Peptides Generated in Vivo?

To elicit an effective class I-restricted CTL response, an endogenous antigen must be degraded into peptide fragments which can interact with MHC-I (α-chains and βm) to form functional complexes. Since MHC-I is co-translationally inserted into the ER (55), and intracellular antigens which do not enter the ER are recognized by CTL, a cytotoxic proteolytic apparatus as well as a mechanism for peptide transport into the ER must be involved. It is not clear whether peptide fragments are generated by known or as yet unknown proteolytic systems, or how peptides are translocated into the ER, although recently a member of the protein family designated “ABC transporters” (for ATP-binding cassette) (56) has been implicated in the RSA-S mutation (57).

An alternative hypothesis provides for proteolysis within the ER, and to the state of the expected finding that mouse cells identical at all genes but MHC-I possess different peptide profiles, MHC molecules themselves may be involved in the generation or selection of presented peptides (58). For instance, MHC-I could shield bound peptides from degradation or serve as a template for proteolysis by other unknown proteins in the ER. Finally, an unusual pathway proposed for the generation of peptides avoids proteolysis altogether by theorizing the direct transcription and translation of short subgenic regions called “peptons” (59).

From Heterodimer to Heterotrimer

However they come about, it is increasingly clear that stable MHC-I molecules are most often heterotrimers consisting of α-chain, βm, and a short peptide of variable sequence. Association of MHC-I peptide and MHC-I appears not to arise from the equilibrium binding of two stable entities, according to the classical lock-and-key paradigm, but rather as the end point of a complex assembly process involving three chains. The natural order of assembly of the heterotrimer and the factors which govern its stability remain open to question, but once formed, loss of either soluble component (peptide or βm) can soon lead to collapse of the overall structure, although cell surface βm is known to exchange with βm in the medium (60). Given the strong similarity between the x-ray structures of βm as a monomer (61) and as part of HLA complexes, and the lack of direct contact between βm and bound peptides, it appears that conformational changes which accompany heterotrimer formation primarily involve α-chain and peptide (Fig. 1). Direct physical studies may have to await the more readily availability of large quantities of MHC-I protein, but in the meantime the cell biology of this system remains an extremely active and fruitful area for investigation.

REFERENCES

Identification of Naturally Occurring Peptides Associated with MHC Molecules

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Background

Nucleated cells of vertebrate organisms display on their surfaces a complex array of structures that sample the cells' contents and that can be recognized by MHC-restricted T cells [1]. What is the nature of these antigenic structures? Townsend and McMichael [2] and others [3] showed that cytotoxic T lymphocytes (CTL) responding to influenza virus infections in humans and mice are largely specific for conserved, intracellular viral proteins (e.g. nucleoprotein, matrix protein) undetectable at the cell surface. This finding could be explained by fragmentation of the intracellular proteins and transport of the resulting fragments (peptides) to the cell surface along with class I MHC (MHC-I) molecules [4]. Synthetic peptides mimicking parts of these proteins effectively bypassed the requirement for intracellular generation of peptide fragments from intact proteins ('antigen processing'), and target cells incubated with appropriate synthetic peptides from viral protein sequences were rendered susceptible to lysis by antiviral CD8+ CTL [5]. In this chapter we focus on some of the many subsequent advances in the study of MHC-I-restricted T cell responses, on the nature of peptides binding to MHC-I proteins, and on the identification of naturally occurring peptides associated with MHC-I molecules; we consider to a lesser extent class II MHC (MHC-II)-restricted responses by CD4+ T cells, which are more extensively treated elsewhere in this volume.

Synthetic Peptides Are Active in T Cell Assays

The availability of well-established chemistries for solid phase peptide synthesis [6] and the relative convenience of cytotoxicity assays [7] has per-
mitted the identification of numerous epitopes recognized by CTL clones and cell lines with a variety of specificities, including viral [8], mycobacterial [9], parasitic [10, 11], and tumor [12, 13] antigens, as well as normal self proteins [14, 15]. In some instances T cell specificities were initially mapped by infecting target cells with recombinant vaccinia viruses in order to determine which protein was recognized [16], followed by testing a panel of synthetic peptides from that protein for their ability to sensitize target cells for lysis by CTL. Boon and co-workers [17] used a genetic approach in which mutant cells with altered tumorigenicity were analyzed to identify tumor-specific sequences recognized by T cells.

The principle that synthetic peptides bind directly to MHC glycoproteins was firmly established by the measurement of micromolar dissociation constants for MHC-II molecules using in vitro assays such as equilibrium dialysis or gel filtration [18, 19]. Analogous data demonstrating specific peptide binding to MHC-I molecules proved more elusive [20–22], but approaches in which peptides were added to MHC-I molecules that were solubilized in detergent-containing solutions [23–25], immobilized on plastic surfaces [26], or present on intact cell surfaces [27–31] made it abundantly clear that peptide-MHC-I complexes form the antigenic structures recognized by CD8+ T cells [32].

The choice of peptides for synthesis and testing as candidate T cell epitopes was initially addressed in one of two ways. An overlapping library of peptides [33] spanning a protein sequence or even an entire genome (e.g. the human immunodeficiency virus (HIV) genome) could be screened in MHC binding [34] or cytotoxicity [35, 36] assays to identify any active peptides. For reasons of economy and precedent, such libraries often consisted of relatively long peptides (i.e. 20- to 25-mers). Alternatively, efforts to discern shared patterns among known T cell epitopes were applied toward the development of predictive algorithms [37–41] that could inform the choice of peptides to be synthesized [42, 43]. By these procedures it became apparent that only one or a few peptides from a given protein often accounted for the bulk of a polyclonal CTL response to that protein [44–49].

Both approaches to epitope identification, however, were conceived in the absence of any knowledge about the naturally occurring peptides recognized by T cells. Even in those cases where sets of overlapping synthetic peptides from within a longer peptide sequence were titrated in order to define the precise length and sequence of the optimally active peptide for a given T cell clone [30, 47, 48, 50, 51], there was no indication as to whether the naturally occurring peptide matched precisely the optimally active
synthetic one. Although considerable information was gathered by screening synthetic peptides from various sources in target cell sensitization assays, the relationship between those peptides and the actual peptide epitopes generated within cells by the antigen processing machinery remained obscure until more recently.

Properties of Naturally Occurring Peptides

Bjorkman and co-workers [52–54] provided the first clues regarding naturally occurring peptides associated with MHC molecules when they solved the crystal structure of the human MHC-I molecule HLA-A2. A deep groove appearing to be the foreign antigen binding site of the MHC molecule harbored a continuous region of electron density thought to represent one or more peptides tightly bound to HLA-A2. The dimensions of the groove were consistent with its occupancy by octameric peptides if fully extended, or longer peptides (up to 20 residues) if coiled into a helix or some other conformation. A second crystal structure, that of HLA-Aw68, had essentially the same overall architecture as HLA-A2, but the unassignable electron density associated with HLA-Aw68 was distinguishable from that of HLA-A2 and was similarly resistant to sequence determination [55]. These features supported the view that each MHC molecule co-purified with a complex and yet distinctive mixture of self peptides 8–20 residues in length. The apparent stoichiometric presence of naturally occurring peptides in the binding groove of crystallized MHC molecules suggested that these peptides were bound with high affinities, since they did not dissociate during the several days required for MHC purification: furthermore, stably occupied peptide binding sites helped to explain the difficulty in measuring binding constants in vitro by adding synthetic peptides to purified MHC-I molecules [56].

Falk et al. [57] performed an imaginative and pathbreaking experiment that shed considerable light on the nature of the peptide mixture present in the binding groove of MHC-I molecules. Having shown that tightly bound peptides could be separated from MHC-I molecules by treatment with trifluoroacetic acid (TFA) [58], much in the same way that Buus et al. [59] had shown for MHC-II molecules using acetic acid, Rammensee and co-workers [57, 58] eluted peptides from isolated MHC-I molecules by treatment with TFA and performed Edman degradation on the complex mixture. At each cycle of the Edman procedure every possible amino acid derivative was detected, but at certain cycles only one or a few amino acids predominated: these were designated ‘anchor’ positions in the eluted peptides and...
were thought to represent conserved features required for strong association with a particular MHC-I molecule. For instance, the peptides eluted from HLA-A2 had a preponderance of leucine at position 2 and valine or leucine at position 9, while peptides associated with the mouse MHC-I protein H-2Kb largely contained phenylalanine or tyrosine at position 5 and leucine at position 8. Moreover, the precipitous drop in repetitive yields of the Edman procedure after the eighth or ninth cycle (depending on the MHC-I molecule) strongly suggested that most tightly bound peptides were octamers or nonamers [57]. These parameters — length and anchor residues — defined a consensus motif for the natural peptides eluted from each MHC-I molecule.

**Peptide-MHC Binding Is Both Specific and Degenerate**

The definition of motifs provided an appealing means to reconcile the dual requirements for specificity and degeneracy in peptide-MHC interactions: MHC proteins differ in their binding specificities for synthetic peptides [60–62], and yet thousands of peptide antigens must be presentable to T cells by only a handful of different MHC-I molecules present in a given individual (up to six in humans, as few as two in some inbred mice). This seeming paradox could now be resolved by two principles: a great number of peptide sequences of the proper length can bind to a given MHC molecule, provided that certain positions have the correct anchor residues, and different MHC molecules have distinctive requirements at their respective anchor positions (table 1). Even with two positions fixed within an octameric peptide, a total of over $6 \times 10^7$ sequences can still fulfill the consensus motif for a particular MHC-I protein, more than enough to satisfy the degeneracy requirement (although not all of these peptides will actually bind to the MHC protein [63]).

The structural basis for this model of peptide binding was provided by the description of ‘pockets’ in the binding grooves of crystallized MHC-I molecules [54, 55, 64, 65]. While essentially similar in dimensions and overall shape, at high levels of resolution each MHC protein’s binding groove was defined by characteristic ridges and depressions referred to as pockets. The detailed intermolecular contacts between bound peptide and MHC molecule were most vividly revealed by the crystal structures of MHC-I molecules containing a single peptide [65–68]. These homogeneous peptide-MHC-I complexes were obtained by taking advantage of the finding that MHC molecules secreted by *Drosophila* cells transfected with MHC genes were devoid of endogenous peptides, and that these ‘empty’ molecules could be stoichiometrically occupied by an added peptide [69, 70]. Alternatively,
Table 1. Motifs for peptides associated with MHC-I proteins

<table>
<thead>
<tr>
<th>MHC-I protein</th>
<th>Peptide length</th>
<th>Consensus motif</th>
<th>References</th>
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<tr>
<td>H-2K&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9</td>
<td>-Y/F-----L/V</td>
<td>57, 74</td>
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<tr>
<td>H-2D&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9</td>
<td>----N-M/I/L</td>
<td>57</td>
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<tr>
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<td>8</td>
<td>----F/Y-L/M</td>
<td>57</td>
</tr>
<tr>
<td>H-2L&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9/10</td>
<td>-P----(-)L/F/M</td>
<td>57, 76&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HLA-A2.1</td>
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<td>-L------------</td>
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<td>9</td>
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<td>----H-L/I/F</td>
<td>147</td>
</tr>
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</table>

Only the two or three most prominent residues at each anchor position are listed. See references for additional residues.

<sup>a</sup>Soluble, secreted analog of H-2L<sup>d</sup>.

free MHC-I heavy and light chains (α and β<sub>2m</sub>) were reconstituted in vitro in the presence of synthetic peptides to form stoichiometric peptide-MHC complexes [67, 71, 72]. The pockets in these monopeptide complexes were found to accommodate the side chains of residues at the peptide's anchor positions.

The importance of anchor positions in peptide-MHC interactions was further demonstrated by the synthesis and testing of peptides having the prescribed anchor residues for binding to a particular MHC molecule and a residue considered nonspecific (often alanine) at all other positions. For an MHC-II binding peptide in which systematic substitutions had been made, only one position was found critical for binding [73]. A similar approach has been used in studies aimed at defining or verifying various MHC-I binding motifs [74–77].

**Why Are Synthetic Peptides Active in T Cell Assays?**

Since MHC-I molecules were ultimately shown to bind octamers and nonamers (with certain exceptions, see below), what accounted for the earlier results obtained using much longer peptides (e.g. 25-mers) in target cell
Identification of MHC-Associated Peptides

Fig. 1. An ostensibly pure synthetic peptide contains trace contaminants that can sensitize target cells for lysis by CD8+ cytotoxic T lymphocytes. A synthetic peptide [115] synthesized by standard t-Boc chemistry on an Applied Biosystems 430A was fractionated on a C18 reverse-phase HPLC column as described, using a gradient of 1% acetonitrile/min [100] (a). One-minute fractions were collected, dried by SpeedVac, and subjected to a 4-hour cytotoxicity assay using 51Cr-labeled B2 target cells and 2C CTL [100] (b).

sensitization or MHC binding assays? At least four possible explanations have been proposed, of which three are clearly documented. First, it is well known that synthetic peptides contain trace peptide contaminants, for instance products arising from failed couplings, rearrangements, or other side reactions during synthesis [6]. Since some peptide epitopes can be active in T cell assays at concentrations of < 1 pg/ml [30, 50, 78–81], the biological activities of many longer peptides probably result from shorter contaminants present in synthetic preparations (fig. 1 and [82]). Even after several rounds of HPLC purification, activities due to contaminating peptides may still not be completely removed [unpubl. observation], underscoring the need for careful analysis before attributing sensitizing activities to ostensibly pure synthetic peptides. In fact, some investigators have exploited the presence of active contaminants in identifying naturally processed peptides [79] (see below).
Secondly, cytotoxicity assays are generally carried out in medium containing fetal calf serum, which has been shown to possess proteolytic activities [83–85]. Thus, longer peptides can be degraded to octamers or nonamers directly responsible for sensitizing activity during the course of a cytotoxicity assay (typically several hours). A third possibility, raised to explain findings obtained with a mutant cell line, was that peptides added to target cells could be internalized by the cells, where they would play a role in the intracellular assembly and transport of MHC-I molecules [86]. Presumably this might allow for intracellular degradation of the added peptides to shorter, more active products. However, the original observations could also be explained without the need for any internalization process [23].

Finally, in the case of HLA-Aw68, it was shown that peptides longer than nonamers (10 or 11 residues) can bind directly to the MHC molecule by retaining anchor residues near their N- and C-termini but bulging out somewhat from the middle of the groove [87]. The ability of peptides longer than nonamers to bind directly to MHC-I molecules may be limited to special instances [88, 89] or may represent a general feature. In any event, when a synthetic peptide is used to sensitize target cells for lysis by CTL, the precise identity of the active (MHC binding) peptide is very often unknown.

In retrospect, it is noteworthy that the peptides first used to elucidate the nature of T cell epitopes (e.g. a 14-mer from influenza virus nucleoprotein [5]) turned out not to be equivalent to the naturally occurring epitopes (e.g. a nonamer within the 14-mer [79]). Moreover, sensitization of target cells with synthetic peptides of any length is effective only because although most cell surface MHC molecules harbor tightly bound endogenous peptides, a proportion of MHC-I molecules arrives at the cell surface in a receptive state for binding to exogenous peptides, i.e. peptide-free or containing rapidly dissociable peptides [24, 90–92].

**Detection of Naturally Occurring Peptide Epitopes**

The next step in learning about naturally occurring peptide epitopes required knowledge of their individual sequences, abundances, and parent proteins. However, the extensive heterogeneity of natural peptides associated with MHC molecules created a formidable problem for their biochemical characterization: any particular peptide representing a T cell epitope was likely to be present at a very low abundance, e.g. < 0.1% of the restricting MHC molecule. Since roughly $10^5$ molecules of each MHC protein are expressed on the surface of a normal cell, fewer than 100 such peptide molecules would then be present per cell (0.1% of $10^5$), and this number is
Identification of MHC-Associated Peptides

apparently still sufficient to trigger T cell activation [27, 93–95]. The isolation of as little as 15 pmol of such a peptide, an amount typically required for sequence determination by Edman degradation, would therefore require about $10^{11}$ cells, assuming 100% overall recovery. For this reason the precise identification of individual naturally occurring T cell epitopes has not come easily or quickly. However, because of the extraordinary sensitivity of T cells, some of which can detect $<1$ pg/ml of a specific sensitizing peptide, it was possible to first demonstrate the presence (but not establish the sequence) of naturally occurring peptides representing CTL epitopes by lysing only $10^8$–$10^9$ cells, fractionating the mixtures by HPLC, and subjecting the resulting fractions to cytotoxicity assay using appropriate target cells [58, 96, 97].

Strategies for the Characterization of Naturally Occurring Peptides Associated with MHC Molecules

Prediction of Naturally Occurring Peptide Epitopes

Several strategies have been used in efforts to determine the sequences of naturally occurring peptides associated with MHC molecules. One approach rests on predicting and testing candidate peptides. First, the sequence of a protein antigen is examined for peptides containing the correct anchor residues and of the appropriate length for binding to a relevant MHC-I protein (table 1). These peptides are synthesized and tested in cytotoxicity assays, obviating the need to analyze a more extensive peptide library or to rely on fortuitous activities of longer peptides. Bevan and co-workers [98] showed that 1 of 11 nonamers selected from the sequence of *Listeria monocytogenes* listeriolysin (59 kD) based on the consensus motif for H-2K$^d$ was recognized by specific H-2K$^d$-restricted CTL, and that the CD8+ T cell response to this peptide inhibited *L. monocytogenes* replication in H-2d mice [99]. In another instance, the HLA-A2 consensus motif was used to predict a nonamer within the sequence of an active 25-mer as the epitope recognized by an HLA-A2-restricted CTL clone [57], and the same nonamer was found independently of knowledge of the A2 motif to be the most active sensitizing peptide recognized by this CTL clone [30].

Possible Problems in the Prediction of Naturally Occurring Peptides

Although convenient and clearly superior to first-generation schemes for predicting T cell epitopes, this approach has some drawbacks. First, empirical MHC binding motifs are inherently imperfect in that they represent
composite data for which there are individual exceptions (as implied by the actual Edman data, in which nonmotif residues are present at the anchor positions at reduced but measurable frequencies). Indeed, such motifs cannot be absolute requirements for MHC-I binding, because some peptides lacking anchor residues still possess sensitizing activities and therefore must bind with a certain (possibly low) affinity to the restricting MHC-I molecule [e.g. 30]. Consequently, some T cell epitopes may be either missed altogether or misidentified by motif-based predictions. For example, although H-2Ld binding peptides are predicted to have proline at position two [57, 76], at least two epitopes efficiently recognized by H-2Ld-restricted CTL do not contain proline at this position. One has no proline [12] and would presumably have been overlooked by relying on the H-2Ld motif; the other, which was isolated from natural sources and is known to bind well to H-2Ld, has proline at position three [100]. Moreover, when the corresponding peptide that would have been predicted by the H-2Ld motif was synthesized and tested (i.e., same length but with proline at position two instead of three), it also had comparable biological activity [unpubl. observation]. Similarly, van Bleek and Nathenson [101] detected natural peptides associated with H-2Kb that did not possess the anchor residues for binding to H-2Kb.

A related point is that sensitization of target cells by a synthetic peptide does not imply that the same peptide is present naturally on target cells (e.g. virus-infected cells for a viral peptide). Assuming that sensitizing activity is due to the synthetic peptide itself, and not to a more potent contaminant or degradation product, the natural epitope may still have a different sequence [102, 103], or, more likely, differ in length from the sensitizing one. It is not at all surprising that a peptide capable of sensitizing target cells when added exogenously may not be generated intracellularly or transported into exocytic compartments for binding to MHC molecules (at least in sufficient quantities to activate T cells — see below). Finally, it is important to note that empirically derived consensus motifs for naturally occurring peptides (table 1) reflect the influences of antigen processing and transport events as well as binding to MHC molecules, and as such are almost certainly more restrictive than motifs based only on MHC binding properties.

One common assumption is that the optimally active peptide in a given response corresponds to the natural epitope: however, heteroclitic antibodies that bind more strongly to a cross-reacting antigen than to the immunogen are well known [104], and in at least one report of a heteroclitic CTL response, a synthetic mutant peptide was recognized at a 1.000-fold lower concentration than the wild-type sequence [78]. Furthermore, cytotoxicity
Identification of MHC-Associated Peptides

Table 2. Naturally occurring peptides associated with MHC proteins

<table>
<thead>
<tr>
<th>MHC protein</th>
<th>Peptide origin</th>
<th>Peptide sequence</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell epitopes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-2K&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Influenza NP 147</td>
<td>TYQRTRALV</td>
<td>79</td>
</tr>
<tr>
<td>H-2D&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Influenza NP 366</td>
<td>ASNENMETM</td>
<td>79</td>
</tr>
<tr>
<td>H-2K&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Vesicular stomatitis virus NP 52</td>
<td>RGYVYQGL</td>
<td>49</td>
</tr>
<tr>
<td>H-2K&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Ovalbumin 257</td>
<td>SIINFEKL</td>
<td>81</td>
</tr>
<tr>
<td>H-2K&lt;sup&gt;b&lt;/sup&gt;</td>
<td>L. monocytogenes listeriolysin 91</td>
<td>GYKDGNYI</td>
<td>98</td>
</tr>
<tr>
<td>H-2L&lt;sup&gt;d&lt;/sup&gt;</td>
<td>MCMV pp89 168</td>
<td>YPHFMPTNL</td>
<td>108&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>H-2L&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Mouse α-ketoglutarate dehydrogenase P815 tumor variant</td>
<td>LSPFPFDL</td>
<td>100, 115</td>
</tr>
<tr>
<td>HLA-A2.1</td>
<td>Influenza MP 58</td>
<td>GILGFVFTL</td>
<td>148</td>
</tr>
<tr>
<td>I-A&lt;sup&gt;k&lt;/sup&gt;</td>
<td>Hen egg lysozyme</td>
<td>(5 sequences)</td>
<td>142</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Abundant peptides</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>H-2K&lt;sup&gt;d&lt;/sup&gt;</td>
<td>P815 cells</td>
<td>SYFPEITHI</td>
<td>57</td>
</tr>
<tr>
<td>HLA-B27</td>
<td>LG-2 cells</td>
<td>(11 sequences)</td>
<td>131</td>
</tr>
<tr>
<td>HLA-A2.1</td>
<td>transfected C1R cells</td>
<td>(8 sequences)</td>
<td>132</td>
</tr>
<tr>
<td>HLA-A2.1</td>
<td>T2 cells</td>
<td>(4 sequences)</td>
<td>88</td>
</tr>
<tr>
<td>HLA-A2.1</td>
<td>T2 cells</td>
<td>(3 sequences)</td>
<td>89</td>
</tr>
<tr>
<td>HLA-Aw68</td>
<td>lymphoblastoid cells</td>
<td>(6 sequences)</td>
<td>87</td>
</tr>
<tr>
<td>HLA-B53</td>
<td>transfected C1R cells</td>
<td>YPAEITLYW</td>
<td>145</td>
</tr>
<tr>
<td>H-2L&lt;sup&gt;a&lt;/sup&gt;</td>
<td>transfected L cells</td>
<td>(8 sequences)</td>
<td>76</td>
</tr>
<tr>
<td>I-A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>LB27.2 cells</td>
<td>(6 sequences)</td>
<td>136</td>
</tr>
<tr>
<td>I-E&lt;sup&gt;b&lt;/sup&gt;</td>
<td>LB27.2 cells</td>
<td>(6 sequences)</td>
<td>136</td>
</tr>
<tr>
<td>I-A&lt;sup&gt;d&lt;/sup&gt;</td>
<td>A20-1.11 cells</td>
<td>(9 sequences)</td>
<td>149</td>
</tr>
<tr>
<td>I-A&lt;sup&gt;k&lt;/sup&gt;</td>
<td>transfected cells</td>
<td>(3 sequences)</td>
<td>142</td>
</tr>
<tr>
<td>HLA-DR1</td>
<td>WT-100 cells</td>
<td>16-mer</td>
<td>150</td>
</tr>
<tr>
<td>HLA-DR1</td>
<td>LG-2 cells</td>
<td>(20 sequences)</td>
<td>137</td>
</tr>
<tr>
<td>HLA-DR3</td>
<td>transfected T2 cells</td>
<td>(3 sequences)</td>
<td>139</td>
</tr>
</tbody>
</table>

Peptides defined by target cell sensitization assays but not shown to be natural products are not included.

<sup>a</sup> Epitope characterized from cells infected with recombinant pp89-vaccinia virus.

assays reflect two processes: peptide binding to MHC molecules and recognition of the resulting complexes by T cell receptors. Therefore, it is conceivable that due to constraints imposed by the T cell receptor, the best MHC binding peptide (e.g. predicted from a motif) may not be the most potent sensitizing peptide: in such a case either peptide (or neither one) could correspond to the naturally occurring epitope. Nevertheless, with the rapid accumulation of data validating the ability of motifs to predict optimally
active peptides in individual instances [30, 51, 82, 105], and with the finding that natural epitopes often conform to these motifs (table 2), it is likely that many investigators will favor the prediction-based approach.

Another limitation of this approach is its failure to provide quantitative information concerning the abundances and distribution of naturally occurring T cell epitopes. When a synthetic peptide is used in order to sensitize target cells for lysis by CTL, the number of peptide-MHC complexes formed per cell (epitope density) is somewhat arbitrarily manipulated by varying the concentration of added peptide. High epitope densities increase the probability that target cells will be lysed by a T cell with the appropriate receptor specificity, as is commonly demonstrated by peptide titration curves [e.g. 95]. It is likely that the density of a naturally occurring peptide on target cell surfaces - such as a viral epitope on virus-infected cells - will similarly affect the ability of target cells to elicit T cell responses. Braciale and co-workers [106] observed unexpected cross-reactions using influenza virus subtype-specific CTL and targets sensitized with high doses of synthetic peptides, and they suggested that these nonphysiologic cross-reactions required much higher epitope densities than could possibly be achieved by endogenous antigen processing. Another study demonstrated T cells reactive with normal self peptides when nonphysiologic concentrations of protein digests were used to sensitize target cells [107]. Thus, in addition to epitope identity, epitope density is a critical parameter in efficient recognition of naturally occurring peptide-MHC complexes by CTL. However, natural epitope densities cannot be determined by means of target cell sensitization experiments using only synthetic peptides. This limitation is relevant to practical efforts to manipulate T cell responses, either by blocking autoreactive T cells or by immunizing for specific T cell responses.

The practical significance of the abundances of naturally occurring peptides is illustrated by the following finding: an HIV-specific CTL clone exhibits poor killing of HIV-infected target cells that naturally produce a nonameric MHC-I binding peptide from HIV reverse transcriptase [unpubl. observation], even though the same clone efficiently lyses uninfected target cells in the presence of < 1 pg/ml of the same synthetic peptide [30]. It remains to be seen whether low epitope densities account for poor CTL responses against HIV-infected target cells in this instance or more generally, or whether increased numbers of specific HIV peptide-MHC-I complexes could bring about a more effective CTL response. Clearly, however, CTL that are specific for epitopes present only sparsely on infected cells are likely to have little or no effect on the course of natural HIV infection. Interestingly, a
distinguishing feature of this and other chronic, long-term infections is that normal cellular protein synthesis is not inhibited or arrested as it is during acute infections with viruses like vaccinia, influenza, or vesicular stomatitis virus. Consequently, naturally processed peptides in chronic viral infections must compete with a great excess of endogenous self peptides for binding to MHC-I molecules, and virus-specific complexes may be deficient as a result.

One way to address the limitations described above for motif-based prediction of T cell epitopes is to compare the chromatographic behavior of a putative synthetic epitope with that of the corresponding naturally occurring peptide obtained from cell or tissue extracts [49, 79, 81, 98, 108, 109]. Depending upon the resolving power of the chromatographic conditions used, this approach can indirectly establish identity (or nonidentity) between the synthetic and natural peptides. Higher resolution might be attained by determining through mass spectrometry the molecular masses of peptides present in an active fraction from a natural source and checking for agreement with the masses of one or more candidate T cell epitopes.

Identification of Natural Peptides That Are Relatively Abundant

The logical extension of such procedures represents a second category of approaches to the identification of naturally occurring peptides: their purification and direct sequence analysis from natural sources, i.e., cells or tissues (fig. 2). This strategy can be applied to either of two quite different objectives: definition of T cell epitopes, or characterization of the most abundant peptides associated with MHC proteins. For the latter goal, the basic technique generally consists of isolating a particular MHC molecule by immunoprecipitation or immunoaffinity chromatography, eluting the bound peptides by treatment with TFA [58, 97] or acetic acid [49], and fractionating the peptide mixture by reverse-phase HPLC. At this stage the HPLC chromatogram may reveal prominent peaks by ultraviolet absorbance that are reasonably well resolved from other peaks, and fractions containing these peaks can be collected and sequenced by Edman degradation and/or mass spectrometry.

For sequencing by Edman degradation, the sample must contain one major peptide. Therefore, more than one round of chromatography may be required, using different conditions (column, mobile phase, gradient) in order to alter selectivity at each round. One drawback of Edman sequencing is a characteristic drop in repetitive yields near the C-terminus due to washout of the shortened peptide from the sample support; hence more material is required for a complete sequence than for just the N-terminal
Fig. 2. Strategies for the identification of naturally occurring peptides associated with MHC molecules. Starting with whole organs, tissues, or cells, two possible strategies consist of preparing crude lysates in TFA (pathway 1) or first isolating MHC molecules and then extracting bound peptides with TFA (pathway 2). In pathway 1, ultrafiltrates of whole cell homogenates are fractionated, the fractions are tested for sensitizing activity in a cytotoxicity assay, and each active fraction is subjected to repeated rounds of purification until suitable for sequencing by Edman degradation and/or mass spectrometry. In pathway 2, peptides eluted from MHC molecules can be fractionated and assayed to identify T cell epitopes just as for pathway 1 (pathway 2a). Alternatively, peptides appearing to be relatively abundant in the HPLC fractionation step by ultraviolet absorbance may be sequenced directly (pathway 2b), or the crude peptide mixture can be sequenced without HPLC fractionation in order to establish consensus motifs for association with a particular MHC (pathway 2c). Pathways 2b and 2c, which do not rely on a T cell assay, are applicable to the study of peptides associated with either MHC-I or MHC-II proteins.

residues (generally a few picomoles). One way to circumvent this problem is to subject an aliquot of a natural peptide (e.g. 10%) to mass spectrometry in order to determine its mass, commit the remainder to Edman degradation, and use the mass to deduce the identities of any residues missing from the
C-terminus. This strategy was successfully applied to a natural sample containing a total of <7 pmol [100]. Other possible problems with sequencing by Edman degradation are a potential failure to detect N-terminal glutamine due to cyclization to pyrrolidone carboxylic acid (enhanced at low pH) and an absolute failure to detect peptides modified ("blocked") by covalent modification at their N-terminus; while no such peptides have yet been isolated from MHC-I or MHC-II molecules, N-formylated peptides are presented by the mouse nonclassical MHC molecule Hmt [110,111], and it is conceivable that a heavy emphasis on Edman sequencing has introduced a bias against the identification of such peptides from "classical" MHC molecules.

Sequencing by tandem mass spectrometry – in which a peptide is ionized, fragmented, and the masses of the fragments analyzed – eliminates many of these problems: the sensitivity can be greater than sequencing by Edman degradation, all residues are potentially identified (although methods that fragment primarily at amide bonds cannot distinguish between leucine and isoleucine), and, perhaps most usefully of all, the peptide need not be completely pure [112]. However, the availability of state-of-the-art instrumentation and expertise remains limited, while automated Edman sequencing at the 10 pmol level is performed in many laboratories and institutional core facilities [113].

Identification of Natural Peptides That Are Recognized by T Cells

The identification of naturally occurring T cell epitopes is a very different problem from that of sequencing the most prominent peptides associated with MHC proteins irrespective of their recognition by specific T cells. In both cases the initial approaches may be similar, but the identification of epitopes ultimately depends on T cell assays that can detect peptides present at attomole levels (<1 pg/ml, typically in a volume of 200 µl), i.e., far below the level of detection of ultraviolet absorbance or even radiochemical methods. Thus, peptides constituting T cell epitopes may be extremely minor components of the total peptide mixture eluted from MHC molecules (<0.1%), requiring a scale-up of several orders of magnitude in order to accumulate enough material for sequencing. Although challenging for this reason, the definition of T cell epitopes by direct isolation and sequencing is ultimately unambiguous. Provides quantitative information about a peptide's natural abundance and distribution, and is of great interest for understanding and attempting to manipulate T cell responses (see below).
To Purify or Not to Purify the MHC Protein

Isolation of a T cell epitope for structural characterization and quantitation may be pursued with [49] or without [79] first purifying the restricting MHC molecule by immunoprecipitation or immunoaffinity chromatography. The chief advantage of MHC purification is that the association of a peptide with a particular MHC molecule is proved if the peptide is isolated from that molecule, and the amount of peptide that is physiologically relevant (i.e., accessible to T cells) can be estimated. From a procedural viewpoint, peptide purification is also simplified if only MHC-bound peptides are subjected to HPLC fractionation. However, there are some disadvantages: weakly bound peptides may be lost during the purification steps; protein yields of <100% will diminish the amount of peptide recovered; and the process can be laborious, especially for very large numbers of cells (>10^11 may be needed for relatively rare epitopes). Moreover, the reagents and conditions for efficient immunoprecipitation or immunoaffinity purification at the requisite scale have not been established for many MHC proteins.

An alternative strategy is acid treatment of tissues, whole cells, or membranes, followed directly by reverse-phase HPLC of the low molecular weight fraction from such a crude extract (obtained by gel filtration or, more conveniently, membrane ultrafiltration [114]). This technique bypasses MHC purification and theoretically leads to higher peptide recoveries, since peptide molecules that dissociate from peptide-MHC complexes as well as peptides that are never MHC-bound will all be fractionated and tested. However, this same feature is also a drawback: there is no assurance that the peptides recovered by such an approach are physiologically presented to T cells, since they may not be associated with MHC proteins. In one study, three natural peptides present in a whole cell extract could sensitize target cells for lysis by a particular CTL clone; two of these peptides co-purified with the relevant MHC molecule (L^d), while the third turned out to be a longer precursor peptide that probably does not bind directly to L^d [100, 115]. The other main disadvantage of whole cell extraction stems from the substantially greater complexity of the mixture subjected to fractionation and testing. Many rounds of HPLC may be required in order to purify a single peptide from a whole cell extract, and the presence of cellular components having nonspecific toxicity in T cell assays can confound the testing of certain HPLC fractions [unpubl. observation]. Nevertheless, both strategies—MHC purification and whole cell extraction—have been applied successfully, and an optimal approach may be to compare yields from both
Identification of MHC-Associated Peptides

peptide procedures on a relatively small scale (e.g. $10^9$ cells) before committing to large-scale peptide purification; in the process the association of a peptide epitope with a given MHC protein will be established.

Examples of Naturally Occurring Peptides Associated with MHC Molecules

The naturally occurring peptides identified so far fall into two major categories: those peptides that are recognized by specific T cells and are thus known epitopes, and those peptides that have been examined because of their abundance in association with MHC molecules irrespective of possible T cell recognition. Within each category, both MHC-I and MHC-II molecules have been studied (table 2). As discussed above, naturally occurring peptide epitopes are potentially present at extremely low levels relative to the most abundant MHC-bound peptides, and their characterization may therefore be difficult. However, this need not always be the case: during acute viral infections, for instance, virus-related peptides may be produced in abundance and may be represented in MHC complexes at greater levels than other endogenous peptides.

Known Naturally Occurring Peptide Epitopes

Van Bleek and Nathenson [49] took advantage of this expectation and immunoprecipitated H-2Kb and H-2Db molecules from $1.3 \times 10^9$ thymoma cells that had been infected with vesicular stomatitis virus (VSV) for 7 h and biosynthetically labeled with $[^3H]$-amino acids for the last 4.5 of these 7 h. MHC-bound peptides were eluted and fractionated by HPLC, and a naturally occurring viral epitope specifically associated with H-2Kb was identified by comparison of its HPLC retention time with those of synthetic analogs and detection of $[^3H]$-amino acids at certain positions in the natural peptide. Even though 5–10% of the peptides eluted from H-2Kb were of viral origin, and one octamer was the dominant viral peptide, probably less than 10 pmol of this peptide was recovered, emphasizing the quantitative demands of isolating natural peptides even under relatively favorable conditions.

Rötzschke and co-workers [79] reported the isolation of two naturally processed influenza virus epitopes. These epitopes, recognized by H-2Dk- and H-2Kd-restricted CTL, respectively, had previously been mapped to influenza nucleoprotein residues 366–379 (14-mer) [5] and 147–158 (12-mer)
Their approach was TFA extraction of $8 \times 10^8$ influenza-infected cells, separation of a low molecular weight fraction by gel filtration, and reverse-phase HPLC, followed by cytotoxicity assay of the HPLC fractions. Extracts from infected H-2b and H-2d cells each gave a unique, unmistakable peak of sensitizing activity. In each case the natural peptide co-eluted with a contaminant fortuitously present in the longer synthetic peptide preparation. The synthetic contaminants co-eluting with the natural peptides were identified by mass spectrometry or by synthesis and HPLC of a shorter peptide: both were nonamers. The same group also identified a naturally occurring peptide in a cell that had been transfected with the ovalbumin gene and that was lysed by an ovalbumin-specific H-2Kb-restricted CTL clone. They used the sequence of a known sensitizing 19-mer from ovalbumin [117] and the H-2Kb consensus motif [57] to predict a candidate octamer, which was then synthesized and shown to co-elute with the naturally occurring peptide [81].

The first nonviral peptide recognized by CD8+ T cells to be directly isolated and sequenced from natural sources was the peptide ligand of an alloreactive CTL clone [100]. Starting with 263 spleens (26.7 g), the active peptide from one of three sensitizing HPLC fractions was purified by five rounds of HPLC, using T cells to detect the peptide after each round. By a combination of Edman degradation and mass spectrometry (see above), the octameric sequence of this epitope was determined. The protein origin of this natural peptide was initially unknown, but later work has shown that the peptide derives from a ubiquitous enzyme essential for intermediary metabolism: moreover, the second of three sensitizing activities present in the original spleen extracts has also been purified and sequenced; it is a longer precursor of the octamer that does not co-purify with the relevant MHC-I molecule and is probably cytosolic [115]. Interestingly, the latter precursor peptide was present at $\approx 100$-fold higher levels than the MHC binding octamer [115]. These findings directly verified a longstanding hypothesis that alloreactive T cells recognize specific antigens associated with MHC proteins much like other T cells [118], and that these antigens are endogenously produced peptides arising from normal self proteins, at least in the case of alloreactive CD8+ T cells [119]. In a different study, alloreactive CD4+ T cells were found to recognize processed forms of serum albumin in association with MHC-II molecules [120], in accordance with the general model that CD4+ T cells respond primarily to exogenous antigens [121].

Previous work had identified HPLC fractions from other tissue extracts that contained unknown peptide epitopes recognized by alloreactive CTL
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[96, 122–124] or seen as minor histocompatibility antigens [58, 125]. In many cases these fractions correlated with the presence or absence of particular MHC-I molecules, leading to a model which implicates MHC-I molecules themselves in the generation and/or accumulation of naturally occurring peptides [97, 124, 126]. Thus, the MHC-type of a cell appears to influence the pool of natural peptides that are detectable in extracts from that cell. This effect appears not to be universal, since the octameric peptide recognized by the alloreactive CTL clone described above was found at comparable levels in spleens from mice of all three H-2 haplotypes examined [100].

Quantitative Aspects of Naturally Occurring Peptide Epitopes

In order to determine the abundances of naturally occurring peptide epitopes on target cells, the titration curves of a synthetic peptide and the same peptide from a natural source can be compared by cytotoxicity assay [79–81, 100]. A similar quantitative approach was used to compare the abundances of a naturally occurring minor histocompatibility peptide in different tissues [127]. Ideally, recovery of the naturally occurring peptide should be adjusted for the combined overall yields of the peptide extraction and fractionation procedures; however, these yields are often unknown, in which case the resulting estimate represents a lower limit for the abundance of a natural peptide on target cells. Alternatively, the natural source for a given peptide (such as a tissue or cell extract) can be supplemented with a measured amount of an indicator peptide (such as a radioactively labeled peptide or a DNP modified peptide), and the recovery of the indicator peptide then provides an estimate of overall losses. In certain cases an indicator peptide tightly bound to an MHC-I protein may be available [30], and adding these complexes to a crude natural source will control for the efficiency of eluting peptides from MHC-I molecules as well as for the subsequent fractionation steps.

Rammensee and co-workers [80] found that influenza virus-infected cells each expressed a few hundred molecules of a naturally processed nucleoprotein peptide; cells transfected with the ovalbumin gene expressed at least ≈100 copies of the natural T cell epitope from that protein [81], and a similar number was determined for a peptide representing the mutagen-induced tumor antigen recognized by a specific CTL clone [109]. We estimated a recovery of 100–200 molecules per thymus cell of the naturally occurring peptide recognized by an alloreactive CTL clone [100]. These values agree with previous estimates of the minimal numbers of peptide-MHC complexes required to activate T cells [27, 93, 94].
Naturally Occurring Peptide Epitopes Recognized on Tumor Cells

Synthetic peptides mimicking natural epitopes expressed on the surfaces of tumor cells in association with MHC proteins have been shown to constitute effective targets for CTL responses [12, 13, 48]. Several laboratories are engaged in efforts to isolate and sequence naturally occurring tumor-specific peptides, ascertain their origins and abundances, and attempt to apply this information to the design of therapeutic approaches [128]. Using a genetic approach, Boon and co-workers [129] were able to isolate and sequence genes encoding tumor-specific antigens by transfecting DNA from human melanoma lines into antigen-loss variants no longer lysed by specific CTL; from one such tumor-specific antigen the predicted epitope, a nonamer, was synthesized and found to sensitize HLA-A1+ target cells for lysis by melanoma-specific CTL [130]. A similarly identified mouse tumor-specific peptide was further shown to be the likely naturally occurring epitope on tumor cells [109]. Other groups are presently engaged in characterization of tumor antigens by approaches analogous to those described above, i.e., direct biochemical isolation and sequencing.

Naturally Occurring Peptides Isolated by Virtue of Their Relative Abundance

The second broad category of naturally occurring peptides consists of those found in association with MHC molecules independently of any known T cells. Since these tend to be abundant peptides, it is likely that T cells specific for them (and thus potentially autoreactive) would be negatively selected during T cell development, although the same peptides may be recognized by alloreactive T cells that have matured in an MHC-different individual. Rammensee and co-workers [57] reported the sequence of a prominent nonamer eluted from H-2K^d molecules, and calculated that this peptide occupies \(\sim 5\%\) of the H-2K^d molecules on P815 cells. This unusually high occupancy may indicate that even fewer natural peptides can be presented at a level required to elicit T cell responses \((\geq 100 / \text{cell})\) than would be the case if all naturally occurring peptides were equally abundant. Jardetzky et al. [131] eluted self peptides from purified HLA-B27 and obtained both a consensus motif (table 1) and individual sequences after HPLC fractionation of the mixture. Of eleven nonamers identified, seven matched to known protein sequences, and these were all abundant cytosolic or nuclear proteins, e.g., histones, ribosomal proteins, and heat shock proteins, suggesting that naturally occurring peptides associated with MHC proteins may be biased toward
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representing highly expressed endogenous proteins. Each of these natural peptides occupied \(<0.5\%\) of the HLA-B27 molecules [131]. A summary of these and other more recent results is provided in table 2.

Hunt et al. [132] recently developed a novel and exceptionally sensitive approach to the identification of naturally occurring peptides. After immunoprecipitation and acid treatment of HLA-A2.1 molecules, the eluted peptides were loaded onto a microcapillary HPLC column interfaced with the electrospray ion source of a triple quadrupole mass spectrometer. Mass spectra were recorded every 2 s during elution of the HPLC column, permitting an assessment of the evident heterogeneity in natural peptide abundances: 50\% of the total ion current was due to \(\approx200\) peptides, of which one-third represented two-thirds of the signal; the remaining 50\% was presumably due to peptides present at very low levels. Of the approximately 200 peptides detected, 10\% were present at 150–600 fmol/10^8 cells (1–4\% occupancy of the HLA-A2.1), and 90\% were present at 30–150 fmol (0.2–1\% occupancy). By subjecting selected relatively abundant ions to collision-activated dissociation, eight sequences were derived, all of which were nonamers, and four of which could be matched to abundant cytosolic or nuclear proteins [132].

Applying the same technique to HLA-A2.1 recovered from the antigen processing mutant cell line T2 [133, 134], Hunt and co-workers [88] discovered a second family of natural peptides that are derived from signal sequences of normal cellular proteins. These peptides, some of which were longer than nonamers, could also be detected in acid extracts of HLA-A2.1 from nonmutant cells, albeit at lower levels. In studying the same mutant T2 cells, Wei and Cresswell [89] independently isolated and sequenced a small number of abundant and unusually long peptides that could be eluted from HLA-A2.1, and at least two of these were identical to those found by Hunt et al. [88]. Whether signal sequence-derived peptides form appreciable numbers of complexes with MHC-I molecules other than HLA-A2.1 remains an open question [135].

Naturally occurring peptides have also been eluted from MHC-II molecules, and the results, described in more detail elsewhere in this volume, contrast in several ways with those obtained from MHC-I systems. In brief, endogenous peptides associated with MHC-II molecules generally consisted of sets of nested peptides sharing a core sequence but varying in length from 13 to 25 residues [136–139]. The proteins of origin for MHC-II-associated peptides are secreted (e.g. serum albumin) or transmembrane proteins, including the MHC-II-associated invariant chain, consistent with the func-
tion of MHC-II proteins in presenting exogenous antigens [121]. In agreement with these findings, the binding groove of a crystallized MHC-II molecule differed from that of MHC-I molecules in such a way as to allow the N- and C-termini of bound peptides to extend out of the MHC-II groove, thereby accommodating different length peptides [140].

**Concluding Remarks**

The ability of T cells, especially cytotoxic CD8+ T cells (CTL), to destroy virus-infected cells and cancer cells is leading to intensive efforts to develop peptide-based vaccines aimed at stimulating the production of these cells for the prevention of infection or for the treatment of chronic infection (e.g. HIV) or established tumors. For such vaccines to be effective, they must be able to elicit the production of many cytolytically active CTL that satisfy the following criteria: their antigen-specific T cell receptors have high affinities for specific peptide-MHC ligands; the peptide epitopes for these CTL are present as natural ligands on target cells in vivo; and the peptide epitopes must exist at levels of abundance sufficient to trigger potent cytolytic responses (currently thought to be at least \(100\) per target cell, although this number may vary for different T cells, especially as a result of differences in T cell receptor affinities).

What determines the abundances of naturally occurring epitopes on target cells? One factor is likely to be the competition among an enormous number of different peptides within a given cell for binding to the cell’s limited number of MHC-I molecules. A typical cell probably produces about 10,000 different proteins, each of which could potentially be represented by a large number of octameric or nonameric sequences. However, since only a handful of different MHC-I molecules are expressed per cell, each at a copy number of \(10^4\), only up to about 1,000 different peptides could theoretically be displayed (per MHC-I) at an abundance that exceeds the threshold for triggering a T cell response. The actual number of peptide epitopes may be lower if many MHC-I molecules are occupied by peptides present at levels of abundance that are physiologically inconsequential. as is suggested by some data [132]. Hence it is likely that many of the proteins made by a cell are not represented on the cell’s surface by a sufficient number of peptide epitopes to trigger a potent T cell response.

For many viruses, this situation does not hinder the efficacy of antiviral CTL responses because in infected cells the expression of many host genes is shut
down and most of the proteins made are virally encoded. But with some viruses, e.g. HIV, infected cells can grow and proliferate over a long period, and therefore presumably continue to produce the cell's full complement of self proteins in addition to a small number of viral proteins. In cancer, likewise, only a very small proportion of the different proteins made by transformed cells may represent aberrant cancer-specific proteins. Under these circumstances it cannot be taken for granted that peptides derived from a viral gene or a cancer-specific gene will be displayed on the affected cell's surface at a level that is physiologically significant. For this and other reasons it is important to establish not only the identities but also the abundances of naturally occurring virus- or cancer-specific peptide epitopes obtained from infected or transformed cells.

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Sample Preparation for HPLC by Centricon® Ultrafiltration

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ABSTRACT

Peptides and other bioactive materials can be purified from complex biological sources by reverse-phase high-performance liquid chromatography (RP-HPLC), provided the mixture is suitably prepared before injection onto an HPLC system. Ultrafiltration offers a convenient and rapid sample preparation technique with numerous advantages over alternative methods such as conventional gel filtration chromatography. We demonstrate the use of ultrafiltration as an HPLC sample preparation step in the purification of peptides bound to class I major histocompatibility complex (MHC-I) membrane proteins. When ultrafiltration was performed with a Centricon®-10 ultrafiltration device, peptides were efficiently separated from the α (45 kDa) and β2-m (12 kDa) chains of MHC-I proteins and could be subjected to HPLC without further treatment. Furthermore, even samples as crude as whole cell lysates or supernatants could be prepared for HPLC in a single ultrafiltration step, affording a remarkably straightforward route to the purification of biologically important peptides.

INTRODUCTION

The isolation of highly purified peptides or other biomolecules from complex starting materials (e.g., cell supernatants, cell lysates) can be achieved in a surprisingly small number of steps, thereby improving yields and reducing the time required. The excellent resolving power of reverse-phase high-performance liquid chromatography (RP-HPLC) allows even the most closely related molecules to be fractionated under a suitable set of conditions (solvents, gradient, column, flow rate and temperature). Yet, before a sample from a crude biological source can be injected onto an RP-HPLC system with the expectation of efficient chromatographic separation, the sample must be prepared so that it is 1) completely soluble in the HPLC solvents to be used; 2) free of dust or other particulates that can clog HPLC tubing; and 3) free of large hydrophobic protein molecules that irreversibly bind to reverse-phase columns and deteriorate chromatographic performance. Through the use of Centricon® ultrafiltration, all of these conditions can be satisfied conveniently and economically, leading to a general strategy for rapid purification of peptides from complex mixtures.

An area of recent intense interest is the purification of peptides recognized by cytotoxic T lymphocytes, the cells that mediate immunological reactions against viruses, tumors and transplants, for example. These peptides are arrayed on cell surfaces in close association with glycoproteins termed MHC-I proteins, which consist of an α-chain (ca. 45 kDa) and β2-microglobulin (12 kDa). Thus the purification and characterization of these peptides require their isolation from either whole cells or purified MHC-I molecules. Buus et al. and Rötzschke et al. have shown that peptides complexed with MHC proteins can be dissociated by strong acid, i.e., acetic acid (1) or trifluoroacetic acid (TFA) (4). They separated peptides from MHC proteins by low-pressure gel filtration chromatography prior to RP-HPLC analysis. Harvesting the total peptide fraction by ultrafiltration with a Centricon-10 or Centriprep®-10 unit offers several advantages such as greater speed and economy, high recovery, smaller volume for HPLC injection and concomitant removal of particulates before HPLC injection.

MATERIALS AND METHODS

The human MHC-I proteins HLA-A2 and HLA-B7 were purified from JY cells (B lymphoblastoid cell line) by established procedures (3,6) with minor changes (7). Human β2-microglobulin was purchased from Calbiochem (San Diego, CA). Purified HLA-A2 and HLA-B7 were concentrated and freed of unbound peptides by ultrafiltration at 5000×g for 40 min using a Centricon-10 concentrator (Amicon, Beverly, MA). All centrifugations were performed in a Beckman JA-20 rotor (Beckman Instruments, Fullerton, CA). The proteins were then denatured by treatment with 0.5% TFA (4) for 60 min at 37°C and the dissociated peptides collected by Centricon ultrafiltration. The retentate, containing denatured protein, was subjected to TFA treatment and ultrafiltration a second time, and the two filtrates were combined for RP-HPLC.

Peptide purification was achieved on a Beckman HPLC system (Model 334) by means of C18 reverse-phase
chromatography with an acetonitrile gradient in 0.1% TFA (Vydac 218TP104; The Separations Group, Hesperia, CA) and/or C8 reverse-phase chromatography at pH 8.5 with an acetonitrile gradient in triethylamine acetate (Vydac 228TP104). Gradients were 51% acetonitrile per min. Peak detection was provided by means of UV absorbance at 220 nm and/or 280 nm as well as radioactivity when using radioiodinated peptides. Fractions were dried in a SpeedVac® (Savant Instruments, Farmingdale, NY) before bioassay and re-chromatography of the fractions exhibiting biological activity. Synthetic peptides were obtained by conventional solid-phase techniques at the M.I.T. Biopolymers Laboratory (Cambridge, MA).

RESULTS AND DISCUSSION

The extraction of bound peptides from purified HLA-A2 and HLA-B7 was achieved with TFA as first described by Ritzschke et al. (4). In several cases, a radioiodinated peptide thought to bind HLA-A2 was added to JY cells before HLA purification. The radioactive peptide was subsequently separated from purified HLA-A2 by TFA, providing a measure of the efficiency of peptide extraction by TFA (7). In all cases, the peptides obtained from HLA were a highly heterogeneous mixture. Multiple rounds of HPLC fractionation were required in order to isolate individual peptides.

Prior to RP-HPLC, the peptides dissociated from HLA by TFA treatment were separated from the protein by Centricon-10 ultrafiltration (Figure 1). This step 1) ensured that only compounds soluble in TFA would be loaded onto the HPLC system; 2) removed dust and particulates as required before HPLC injection; 3) separated a peptide fraction (<10 kDa) from the HLA sample (ca. 45 kDa and 12 kDa; see below); and 4) concentrated the HLA in the retentate for further analysis (calculation of yield, sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE], etc.). Moreover, the advantages over conventional gel filtration include greater speed and reproducibility, lower overall cost and avoidance of the dilution effect inherent in gel filtration. Centricon ultrafiltration has also been used by others in similar recent studies (5,9). For the most efficient extraction of HLA-bound peptides, TFA treatment and ultrafiltration were repeated and the two ultrafiltrates were combined for RP-HPLC. When radioiodinated peptides were used, over 95% of the radioactivity was measured in the ultrafiltrate by this method (7). Typical results are shown in Figure 2.

As an alternative to HLA purification followed by TFA treatment to remove bound peptides, whole cell lysates were treated directly with TFA (4,8). Centricon-10 ultrafiltration

![Figure 1. Peptide purification scheme based on acid denaturation, ultrafiltration and RP-HPLC (see text).](image)

![Figure 2. Representative results from RP-HPLC fractionation of a peptide mixture recovered from purified HLA-A2 by TFA extraction. Several peptide peaks are evident in the region from 30–50 min (25%–45% acetonitrile in the linear gradient used). The single radioactive peak represents an HLA-A2-binding peptide (ILKEPVHGV) stoichiometrically labeled on its histidine and added to JY cells before HLA-A2 purification (7). Based on the known specific radioactivity of this peptide (ca. 10¹² cpm/µg) and our yield of HLA-A2 (ca. 35 µg per 10⁹ JY cells), this peptide constitutes 0.1%–0.5% of the total peptide bound to cell surface HLA-A2.](image)
followed by RP-HPLC led to the characterization of several biologically active peptides that were dried and re-chromatographed using different sets of HPLC conditions (gradient, column and pH) until the desired peptides were sufficiently pure for sequence determination (8). Identification of the biologically important peptides after each round of chromatography depended on the use of a cytotoxic T cell assay to screen HPLC fractions. In each case the replacement of gel filtration with Centricon ultrafiltration saved considerable time and provided high yields of peptides ready for HPLC injection.

Because HLA consists of two noncovalently associated chains, α and β₂-microglobulin, we were concerned about the efficiency of separation of β₂-microglobulin (12 kDa) from the peptide fraction. As shown in Figure 3, virtually all (>98%) of the β₂-microglobulin is retained by the Centricon-10 membrane (nominal molecular mass cutoff 10 kDa).

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**Figure 3.** Retention of β₂-microglobulin (12 kDa) by the Centricon-10 membrane. 15 μg purified human β₂-microglobulin were denatured in 400 μl 0.5% TFA followed by Centricon-10 ultrafiltration to completion (no visible volume in the retentate). The membrane was washed with 400 μl 0.1% TFA to collect any retained material, and both the retentate (i.e., membrane wash) and filtrate were injected onto RP-HPLC. Upper chromatogram represents retentate and lower chromatogram is filtrate. Both chromatograms are at same scale.

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CONCLUSION

Although gel filtration is often used for the fractionation of peptides and proteins, non-ideal size exclusion effects due to interactions with column matrices are well known (2). We have found that Centricon ultrafiltration offers several important advantages for the separation of a peptide mixture from larger proteins, including speed, convenience and economy. Moreover, when utilized as a sample preparation step prior to RP-HPLC fractionation of peptides, ultrafiltration also filters out dust and other particulates and ensures that large hydrophobic proteins will not make it to the HPLC column, where they can be irreversibly retained. Human 32-microglobulin, a protein of mass 12 kDa, was completely (>98%) retained by the Centricon-10 membrane. The method is recommended for the purification of peptides (or other biomolecules) from starting mixtures as complex as whole cell lysates or cell supernatants. When large volumes are involved, the larger Centriprep units may be used and/or an additional drying step (lyophilization, SpeedVac) may be inserted.

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Effects of Peptide Length and Composition on Binding to an Empty Class I MHC Heterodimer


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ABSTRACT: Class I major histocompatibility complex (MHC) proteins present peptide antigens to T cells during the immune response against viruses. Peptides are loaded into newly synthesized class I heterodimers in the endoplasmic reticulum such that most or all cell surface class I molecules contain peptides derived from endogenous or foreign proteins. We previously reported the assembly of empty heterodimers of the murine class I MHC molecule H-2K\(\text{d}\), from denatured heavy and light chains from which endogenous peptides had been removed [Fahnestock et al. (1992) Science 258, 1658-1662]. Here we measure thermal stability profiles of empty versus peptide-filled molecules and compare the effects of human versus murine light chains on the overall stability of the K\(\text{d}\) heterodimer. The majority of empty heterodimers are stable at 37 °C regardless of the species of light chain, indicating that our previous report of the unexpectedly high thermal stability was an intrinsic property of the K\(\text{d}\) molecule and not due to use of a murine/human chimeric protein. Binding constants are derived for a series of peptides interacting with empty K\(\text{d}\) heterodimers. The dissociation constants of four known K\(\text{d}\)-restricted peptides range from 2.3 \(\times\) 10\(^{-7}\) to 3.4 \(\times\) 10\(^{-8}\) M. Using a series of 24 analog peptides, the effects of length and peptide composition on binding affinity of one K\(\text{d}\)-restricted peptide are explored, and the results are interpreted with reference to the known three-dimensional structures of class I MHC protein/peptide complexes.

Virally infected cells present antigenic peptides embedded in class I major histocompatibility complex (MHC)\(^1\) molecules to cytotoxic T lymphocytes (Townsend & Bodmer, 1989). Recognition by a T cell receptor on a cytotoxic T cell is specific for the particular combination of class I molecule and peptide on the surface of the infected cell. Crystallographic analyses of class I molecules have shown that peptides bind in a groove located between two \(\alpha\)-helices on the top surface of the molecule (reviewed in Bjorkman & Parham, 1990). Pockets at each end of the peptide-binding site contact main-chain atoms of the N- and C-termini of octamer and nonamer peptides (Garrett et al., 1989; Saper et al., 1991; Fremont et al., 1992; Madden et al., 1992; Matsumura et al., 1992a; Zhang et al., 1992). These pockets (A and F) are lined with highly conserved residues, while the intermediate pockets (B–E) contain residues that vary in class I sequences. Elution and sequencing of endogenous peptides bound to class I molecules revealed a preference for peptides that are eight or nine amino acids in length and elucidated allele-specific sequence motifs and the presence of "anchor" residues important for allele-specific binding (Van Bleek & Nathenson, 1990; Falk & Rammensee, 1990; Rötzschke et al., 1990a,b; Falk et al., 1991; Jardetzky et al., 1991). For example, peptides that bind to the murine class I molecule H-2K\(\text{d}\) are nonamers that most commonly have a tyrosine at position 2 and leucine or isoleucine at position 9 (the anchor residues) (Falk et al., 1991; Romero et al., 1991; Rammensee et al., 1993).

Class I molecules bind peptides in the endoplasmic reticulum during the assembly of the heavy chain with \(\beta\)-2 microglobulin (\(\beta\text{2m}\), the class I light chain (Townsend & Bodmer, 1989). Under normal circumstances, the majority of all class I molecules that reach the cell surface are occupied with a peptide, either derived from a self protein to which the immune system is tolerant, or derived from a foreign pathogen, to which the immune system will react. Class I molecules purified from wild type antigen presenting cells or transfected eukaryotic cells contain a mixture of endogenous peptides, and typically less than 1% of the purified protein will bind exogenous peptide (Chen & Parham, 1989). Assembly of class I heavy and light chains in the absence of peptide to make empty class I heterodimers was once thought to be a structural impossibility (Townsend et al., 1989). However, recent experiments using mutant cell lines have established that empty class I molecules can assemble and reach the surface of cells grown at 26 °C (Ljunggren et al., 1990; Schumacher et al., 1990; Townsend et al., 1990). The resulting empty class I heterodimers are less stable than their peptide-filled counterparts and rapidly become undetectable by conformationally sensitive antibodies at physiological temperature unless stabilized by addition of exogenous peptide (Ljunggren et al.,)

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\(^1\) Abbreviations: \(\beta\text{2m}, \beta\text{-2 microglobulin}; \text{CHO}, \text{Chinese hamster ovary}; \text{CD}, \text{circular dichroism}; K_d, \text{dissociation constant}; K_I/\text{ha2b}, K_I/\text{hamster 2b}; K_I/\text{hu2b}, K_I/\text{human 2b}; K_I/\text{human 2m}, K_I/\text{murine 2m}; \text{KHC}, \text{major histocompatibility complex}; T_m, \text{transition midpoint}.

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An empty form of a class I molecule would be the ideal reagent to use for peptide affinity measurements without the complication of displacing endogenous peptide. We previously reported the efficient reassembly of depleted endogenous peptide. We previously reported the efficient reassembly of separated and denatured class I heavy and light chains in the absence of peptide to make an empty form of H-2K^{\alpha}, which was shown to bind equimolar amounts of offered peptide (Fahnstock et al., 1992). Thermal stability profiles of empty K^{\alpha} molecules were compared to profiles of their peptide-filled counterparts to quantitate the increase in thermal stability provided by occupation of a peptide. The transition midpoint (T_m) of the melting curve of peptide-filled K^{\alpha} was substantially higher than the T_m of empty K^{\alpha} (57 °C compared to 45 °C), and a thermodynamic analysis indicated that the free energy of stabilization due to peptide binding was 4.4 kcal/mol (Fahnestock et al., 1992). Further analysis of the data indicated that 75% of empty K^{\alpha} molecules were in a native configuration at 37 °C, which was unexpected due to the reported instability of empty class I molecules at physiological temperature. However, because the reassembled class I heterodimers consisted of the murine K^{\alpha} heavy chain complexed to human \beta2m (K^{\alpha}/hu\beta2m), it was possible that the higher than expected stability of the empty molecules could reflect the greater stability reported for murine class I heavy chains that are complexed with human rather than with murine \beta2m (Hochman et al., 1988).

In this report, we extend our original studies in several ways: first, we report a method for purifying completely murine heterodimers (K^{\alpha}heavy chain complexed with murine \beta2m: K^{\alpha}/mu\beta2m) from transfected cells grown in serum free medium and record thermal stability profiles of K^{\alpha}/mu\beta2m for comparison to profiles of K^{\alpha}/hu\beta2m. Second, we explore the effects of varying peptide length and composition on the ability of a K^{\alpha}-restricted peptide to bind empty K^{\alpha} heterodimers. During preparation of the K^{\alpha}/mu\beta2m protein, we noted that the majority or all of the K^{\alpha}/mu\beta2m heterodimers secreted from transfected CHO cells were occupied with endogenous peptides, whereas 70% of secreted K^{\alpha}/hu\beta2m appeared to be empty (Fahnstock et al., 1992). However, the thermal stability profiles of the empty and peptide-filled forms of K^{\alpha}/mu\beta2m differ little from the profiles of their K^{\alpha}/hu\beta2m counterparts (the T_m of empty K^{\alpha}/mu\beta2m is 42 °C, the T_m of peptide-filled K^{\alpha}/mu\beta2m is 56 °C), suggesting that the higher than expected thermal stability originally reported for K^{\alpha} (Fahnstock et al., 1992) was an intrinsic property of this class I molecule and not due to the use of a nonphysiological chimeric protein. Finally, we use equilibrium dialysis to derive an affinity constant for the interaction between a radiolaabeled K^{\alpha}-restricted peptide and empty K^{\alpha} and measure the affinities of other K^{\alpha}-restricted peptides and peptides of altered length and amino acid sequence to evaluate the effects of altering the anchor residues and extending or deleting residues at both ends of the peptide. Many comparative studies on sets of related peptides use T cell assays to access effects of alterations in the peptide sequence. Such assays cannot distinguish between the effects of peptide alteration on binding to the MHC molecule and the effects on T cell recognition of the resulting peptide/MHC complex. In order to separate these two binding events, a direct measurement of peptide/MHC binding affinities is required, as is reported here. The peptide binding studies are interpreted and rationalized using the three-dimensional structure of the class I peptide binding site.

**MATERIALS AND METHODS**

**Cell Lines.** Stable CHO cell lines expressing secreted K^{\alpha}/hu\beta2m or K^{\alpha}/mu\beta2m were generated using a glutamine synthetase-based amplification system (Bebbbingon & Hentschel, 1987) as described (Fahnstock et al., 1992, 1994). In this system, amplification of transfected genes depends upon increasing concentrations of the drug methionine sulfoximine. In order to produce a secreted K^{\alpha} heterodimer, a stop codon was introduced into the K^{\alpha} heavy chain gene after the codon for amino acid 284 (Fahnstock et al., 1992). An expression plasmid containing the truncated heavy chain gene was cotransfected with an expression plasmid containing the complete cDNA sequence of murine (\alpha allele; Daniel et al., 1983) or chimpanzee \beta2m. The protein sequence of mature chimpanzee \beta2m is identical to human \beta2m (Lawlor et al., 1990); thus the protein product from the chimpanzee gene is referred to throughout this paper as human \beta2m. Transfected CHO cells secreting K^{\alpha}/hu\beta2m were maintained in glutamine-free aMEM (Irvine Scientific) supplemented with 10% dialyzed fetal bovine serum ( Gibco/BRL) and 100 \muM methionine sulfoximine (Sigma). Transfected CHO cells secreting K^{\alpha}/mu\beta2m were maintained in serum-free conditions as modified from Hamilton and Hamm (1977) (glutamine-free aMEM supplemented with bovine serum albumin, insulin, vitamins, trace metals, and 100 \muM methionine sulfoximine). Penicillin (100 units/mL) and streptomycin (100 \muG/mL) were included in all media used.

**Protein Purification.** Protein was purified from cells secreting K^{\alpha} heterodimers by immunoaffinity chromatography as described (Fahnstock et al., 1992). A column constructed with the monoclonal antibody 34-1-2 (Ozato et al., 1982) was used for purification of K^{\alpha}/hu\beta2m from supernatants of confluent cells grown in 10-cm plates. Typical yields were 9–10 mg of K^{\alpha}/hu\beta2m/L of supernatant, with no detectable exchange of human \beta2m for endogenous hamster \beta2m or bovine \beta2m in the medium (Fahnstock et al., 1992). For purification of K^{\alpha}/mu\beta2m from serum free medium, an immunoaffinity column constructed with the monoclonal antibody M1/42 (Stallcup et al., 1981) was used. This antibody recognizes murine class I MHC heavy chains only when associated with murine \beta2m (M.L.F., unpublished observations), allowing separation of K^{\alpha}/mu\beta2m from K^{\alpha} heavy chains associated with hamster \beta2m (K^{\alpha}/ha\beta2m). A mobility difference on SDS–PAGE gels between hamster and murine \beta2m (Fahnstock et al., 1994) allowed K^{\alpha}/mu\beta2m and K^{\alpha}/ha\beta2m to be easily distinguished. Protein eluted from the M1/42 column was verified to be free of hamster \beta2m by N-terminal sequence analysis (M.L.F. and P.J.B., unpublished data) and gel migration behavior. The flow-through material was saved and purified by passage over the 34-1-2 immunoaffinity column as a source of K^{\alpha}/ha\beta2m. To obtain sufficient quantities of K^{\alpha}/mu\beta2m, cells expressing secreted K^{\alpha}/mu\beta2m were introduced into a hollow fiber bioreactor device (Cell Pharm I; Unisyn Fibertec, San Diego, CA) in serum free medium, and supernatants were collected daily. The final yield of purified K^{\alpha}/mu\beta2m protein was 2.5–3.0 mg/L of supernatant. An additional 1–2 mg/L was purified as K^{\alpha}/ha\beta2m.

**Acid Elutions of K^{\alpha} Heterodimers.** Purified K^{\alpha}/hu\beta2m, K^{\alpha}/mu\beta2m, or K^{\alpha}/ha\beta2m was analyzed for the presence of bound peptides using established methods (Van Bleek & Nathenson, 1990; Jardetzky et al., 1991). Briefly, 0.25 mg of protein (quantitated by a BCA assay, Pierce Chemical Co.) was concentrated to 100 \muL in a Centricon 10 (molecular weight cutoff of 10 000) ultrafiltration device (Amicon;
Peptide Binding to Empty Class I MHC Molecules

Beverly, MA). After dilution with 1.0 mL of 50 mM ammonium acetate, pH 7.5, the proteins were again concentrated to 100 μL, and this procedure was repeated. The washed protein was then treated with 1.0 mL of 12% acetic acid and concentrated again to 100 μL in the ultrafiltration unit, and this elution step was repeated. Using this procedure, the acetic acid eluates were lyophilized and analyzed by automated Edman degradation using an Applied Biosystems Model 477A protein sequencer.

Reassembly of Empty Heterodimers from Separated Heavy and Light Chains. Empty K²/huB²m or K²/m82m heterodimers were prepared from denatured protein as described (Fahnestock et al., 1992). Briefly, heavy and light chains were first denatured in 6.0 M guanidine hydrochloride and separated from endogenous peptides by gel filtration chromatography on a Superose 12 FPLC column (Pharmacia). Heavy and light chain peaks were pooled and renatured by dialysis against 20 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride containing 8 M urea and then twice against the same buffer without urea. Renatured material was concentrated 8–10-fold in a Centricon-10 (molecular weight cutoff 10 000) ultrafiltration device (Amicon; Beverly, MA) and passed over a Superdex FPLC size exclusion column (Pharmacia). Fractions corresponding to heterodimer were pooled and concentrated.

Circular Dichroism Spectra and Thermal Stability Analyses. A Jasco J-720 spectropolarimeter equipped with a Peltier thermal control unit and a rectangular 1-mm pathlength cuvette was used for CD measurements. Spectra were recorded from protein samples (0.25–0.4 mg/mL) in 5 mM phosphate buffer, pH 7.0. For measuring melting curves, the CD signal was monitored at 223 nm while the sample temperature was raised from 25 to 75 °C at a rate of 20 °C/h. Tm's were calculated by estimating the half-point of the ellipticity change between the pure native and pure denatured states.

Peptide Synthesis and Labeling. All peptides were synthesized by automated solid phase methodology on an Applied Biosystems Model 432A synthesizer using the manufacturer's standard Fmoc protocol. Preloaded resins were purchased from Bachem Bioscience Inc. (Philadelphia, PA), and Fmoc-protected amino acids and additional reagents were from Applied Biosystems (Foster City, CA). Cleavage from the resin and simultaneous side chain deprotection was accomplished by treatment with 90% trifluoroacetic acid, 2.5% thioanisole, 2.5% mercaptoethanol and 5% phenol for 2 h at room temperature. The precipitated, crude peptides were purified to homogeneity using a preparative C-4 Vydac column (Hesperia, CA) and a 1% aqueous trifluoroacetic acid/acetoniitrile gradient. Peptide composition was confirmed by amino acid analysis. Lyophilized peptide samples were resuspended in water. The concentrations of peptides were estimated spectrophotometrically using the extinction coefficient of tyrosine at 274 nm (1420 M⁻¹ cm⁻¹) (Wetlaufer, 1962) or by a BCA assay (Pierce Chemical Co.). A K²-restricted peptide from influenza virus nucleoprotein (amino acids 147–155; sequence TYQRTRLV; Röttinger et al., 1990a) was titrated by catalytic exchange (Amersham TR7 Tritium Labeling Service) to a specific activity of 1.6 × 10⁶ counts per minute/μmol. Aliquots were purified by reverse phase chromatography on an FPLC Peptide C-4 R/PC column (Pharmacia) prior to use. A portion of the K²-restricted peptide was iodinated with 127I on the anchor tyrosine residue, using a technique that makes a stoichiometrically iodinated and thus chemically homogeneous peptide (Tsomides & Eisen, 1993). Mono- and di-iodinated species were separated using a C4 reverse phase HPLC column (Vydac) and analyzed by Edman degradation.

Equilibrium Dialysis. Equilibrium dialysis was performed in phosphate-buffered saline containing 0.3% gelatin and 0.02% NaN₃ using a Hoefer Microdialyzer EMD 101 (Hoefer; San Francisco, CA). For Scatchard analyses, eight inside compartments [each filled with 100 μL of K²/huB²m (2 μM)] were separated from eight outside compartments [each filled with 100 μL of varying concentrations of 3H-labeled peptide (0.25–5.0 μM)] by a membrane with a molecular weight cutoff of 14 000. Dialysis was established at room temperature for 24 h. Equilibrium was established during this time period, as no change of binding values was observed with longer incubations. Samples (50 μL) from each inside and outside compartment were then collected and transferred to a vial containing 10.0 mL of Safety-Solve (Research Products International Corp.) for scintillation counting on a Beckman LS 5000TD scintillation counter. The concentration of free peptide after reaching equilibrium was calculated using a specific activity of 1.61 × 10⁹ counts per minute/μmol. The concentration of peptide bound to protein was estimated after subtracting the counts per minute measured in the chamber without protein from the counts per minute measured in the chamber that included protein. The data were plotted as [bound]/[free] versus [bound] and the Kd calculated as −1/slope. For inhibition studies using unlabeled peptide, the inside compartments were filled with 100 μL of K²/huB²m (2 μM), and the outside compartments were filled with 100 μL of a solution containing 3H-labeled peptide (2 μM) and varying concentrations of unlabeled inhibitor peptide (0.0–2 mM). For each concentration of inhibitor, the percent inhibition of peptide binding was calculated by comparing results to a pair of chambers that did not include inhibitor, and the percent inhibition was plotted versus the concentration of inhibitor on a logarithmic scale. The 50% inhibition value was determined from this graph and used to calculate the Kd of the inhibitor using the following equation (Müller, 1983):

$$K_d = (I_50 - [H-P]) × (1 - 1.5b + 0.5b^2)$$

where b = percent peptide binding in the absence of inhibitor (75.2% ± 10.7%), I_50 is the concentration of inhibitor required for 50% inhibition, and [H-P] is the total concentration of 3H-labeled peptide (1.0 μM after equilibrium). For each inhibition curve, seven concentrations of inhibitor were tested in duplicate or triplicate (although unpredictable leakage of the samples in the chambers sometimes prevented all samples from being analyzed), with the eighth chambers of the dialysis blocks tested under identical conditions in the absence of inhibitor. For the two peptides that bound with a higher affinity than the 3H-labeled peptide, the Kd was estimated using the following relationship (Cheng & Prusoff, 1973):

$$K_d = I_50/(1 + [H-P]/K_{d(labeled peptide)})$$

where the K_{d(labeled peptide)} refers to the dissociation constant determined for the labeled peptide by Scatchard analysis.

RESULTS

The Majority of K²/m82m, but Not K²/huB²m, Heterodimers Secreted from Transfected CHO Cells Are Occupied with Endogenous Peptides. K² heterodimers purified from CHO cells transfected with the K² and the murine β²m genes contain a mixture of murine and bovine β²m (Fahnestock et al., 1992), which suggests that a large portion of murine β²m was replaced in an exchange reaction by serum-derived bovine β²m present in the medium (Bernabeu et al., 1984). When K² protein was purified from transfected CHO cells
Table 1: Picomoles of Amino Acids Recovered from Acid Elutions of...
Peptide Binding to Empty Class I MHC Molecules

**FIGURE 2:** Representative Scatchard analysis of 3H-labeled peptide binding to empty K\textsubscript{d}/hu#2m heterodimers. The dissociation constant, \( K_D \), is calculated as the negative of the inverse of the slope of the line. For this experiment, \( K_D = 6.0 \times 10^{-8} \) M. Data from six separate experiments were analyzed in a similar way, obtaining a value for the \( K_D \) of \((6.9 \pm 2.3) \times 10^{-8} \) M.

The dissociation constant, \( K_D \), was determined by plotting the percent inhibition of binding versus inhibitor concentration (Figure 2). The concentrations resulting in 50% inhibition were then used to calculate dissociation constants for the inhibitor peptides as described in Materials and Methods. As a test of the validity of the \( K_D \) values derived from the inhibition studies, an unlabeled version of the \( ^3 \)H-labeled peptide was used to compete with labeled peptide for binding. The \( K_D \) derived from the inhibition analysis was \( 1.0 \times 10^{-7} \) M as compared to the value of \((6.9 \pm 2.3) \times 10^{-8} \) M derived from the Scatchard analysis (Figure 2), demonstrating that the two methods of affinity constant derivation give comparable values.

PepTides were synthesized that correspond to extensions of the K\textsuperscript{a}-restricted influenza nucleoprotein derived peptide, with additions at either the N- or C-termi

Determine if the peptide NP15 and the large nonpolar residue tryptophan (NP16) reduced the affinity by ~400 fold, whereas replacement by a positively charged (lysine; NP17) or negatively charged (aspartic acid; NP18) residue reduced the affinity by 5 orders of magnitude or to the point that no inhibition was observed with 1 mM peptide. By contrast to the effects of substitutions at positions 2 or 9, single substitutions of residues at positions 3–7 with cysteines (peptides NP19 through NP23) had very little effect on binding. However, simultaneous replacement of the same five internal residues with prolines or glycines was detrimental to binding, resulting in a 1000-fold reduction in the binding affinity (five prolines; NP24) or a 2000-fold reduction in binding affinity (five glycines; NP25).

None of the altered peptides in the influenza nucleoprotein analog series were found to have an increased binding affinity. However, two other K\textsuperscript{a}-restricted peptides of unrelated sequence (KD1, Faust et al., 1991; KD2, Romeo et al., 1991) were found to bind with slightly higher affinities than the influenza nucleoprotein peptide to empty K\textsuperscript{a} heterodimers.

**DISCUSSION**

Class I MHC molecules bind peptides during their assembly in the endoplasmic reticulum (Townsend & Bodmer, 1989); thus purified preparations of class I molecules contain mixtures of endogenous peptides. For this experiment, the presence of endogenous peptides complicates binding measurements of defined peptides, largely preventing binding, so that the percent of class I molecules that can accept exogenous peptide is low (Chen & Parham, 1989). The ideal reagent for the comparison of binding affinities of different peptides is a class I protein devoid of endogenous peptides, i.e. an empty molecule. One strategy to produce empty molecules has been to express the protein in Drosophila cells (Jackson et al., 1992; Matsumura et al., 1992b), with the assumption that invertebrate cells do not have the proper mechanism for intracellular peptide loading and that the resulting empty molecules will be stable at the reduced temperature of insect cell culture (27 °C). Indeed, class I molecules produced in Drosophila cells are capable of quantitative binding of exogenous peptide (Matsumura et al., 1992b), allowing the solution of several defined peptide/MHC crystal structures (Fremont et al., 1992; Matsumura et al., 1992a).

We previously reported an alternative method for producing an empty form of the murine class I K\textsuperscript{a} heavy chain complexed with human B2m, referred to as K\textsuperscript{a}/huB2m (Fahnestock et al., 1992). The method relies upon a biochemical separation of endogenous peptides from denatured class I heavy and light chains, followed by reassembly in the absence of added peptide. Initial attempts to produce the completely murine heterodimer (K\textsuperscript{a}/mB2m) were complicated by exchange of murine B2m for exogenous hamster B2m and bovine B2m in the medium (Fahnestock et al., 1992). For this study, we developed a method to purify K\textsuperscript{a}/mB2m from CHO cells transfected with the K\textsuperscript{a} and murine B2m genes and compared the percent of purified K\textsuperscript{a}/mB2m molecules that are occupied with peptide to the percent of K\textsuperscript{a}/huB2m and K\textsuperscript{a}/haB2m that contain peptide. While only ~30% of purified K\textsuperscript{a}/huB2m molecules contain peptides (Fahnestock et al., 1992), the majority or all of the K\textsuperscript{a}/mB2m and K\textsuperscript{a}/haB2m molecules were occupied (Table 1). A possible explanation for this difference is related to our observation that CHO cells transfected with the K\textsuperscript{a} and
FIGURE 3: Determination of the 50% inhibition values for unlabeled peptides competing for binding of 3H-labeled peptide NP1 to empty Kd/hu2m heterodimers. Only the data points corresponding to between 10% and 90% inhibition are plotted, since this portion of the inhibition curve can be approximated by a straight line (Miller, 1983). See Table 2 for peptide names and sequences. (A) Comparison of binding of unaltered Kd-restricted peptides. (B) Comparison of binding of NP1 analog peptides with additions to the N- or C-terminus. (C) Comparison of binding of NP1 analog peptides with insertions or deletions. (D) Comparison of binding of NP1 analog peptides with altered residues at the position 2 anchor residue. (E) Comparison of binding of NP1 analog peptides with altered residues at position 9 (the C-terminus). (F) Comparison of binding of NP1 analog peptides with substitutions at positions 3-7. Data for peptides that did not compete for binding of 3H-labeled NP1 are not shown on the graphs.

human β2m genes consistently produce a much higher yield of Kd than CHO cells transfected with the Kd and murine β2m genes: ~100 mg of Kd/hu2m are recovered/L of supernatant harvested from cells grown in a hollow fiber bioreactor device (Fahnestock et al., 1992) compared to ~2-3 mg of Kd/mβ2m or 1-2 mg of Kd/haβ2m. The high proportion of empty Kd/huβ2m molecules produced under these conditions may reflect a limiting peptide supply inside the transfected CHO cells under conditions of overproduction of a class I molecule.

In a previous study, we found a surprisingly high thermal stability for empty Kd/hu2m (Tm of the heavy chain denaturation = 45 °C), which suggested that ~75% of these empty heterodimers are folded at 37 °C (Fahnestock et al., 1992). These results contrast with studies of the behavior of empty murine class I molecules at the cell surface, which become undetectable at 37 °C by immunoprecipitation with conformationally dependent antibodies unless stabilized by appropriate peptides (Ljunggren et al., 1990; Schumacher et al., 1990; Townsend et al., 1990). In order to ascertain if the unexpected stability observed at temperatures above 37 °C was due to increased stabilization caused by pairing of the murine heavy chain with the human β2m light chain (Hochman et al., 1988), we compared the thermal stability of empty Kd/mβ2m to empty Kd/hu2m. The stability profile of empty Kd/mβ2m (Tm of the heavy chain denaturation = 42 °C) revealed only a slight decrease in thermal stability compared to empty Kd/hu2m (Figure 1A,B). The equilibrium constant derived at 37 °C (as described in Fahnestock et al., 1992) suggests that ~70% of the empty Kd/mβ2m heterodimers are folded at the physiological temperature, as compared to ~75% of empty Kd/hu2m heterodimers (Fahnestock et al., 1992). These results are compatible with studies of a soluble form of the murine class I molecule Kb produced in Drosophila cells (Matsumura et al., 1992b). The Kb protein (which behaves as if it is empty) was shown to be immunoreactive with a conformationally sensitive antibody at temperatures up to 47 °C, as long as the immunoprecipitation was carried out in the absence of detergent. Upon addition of 1% Triton X-100, the immunoreactivity was lost at 37 °C. If the presence of the detergent interferes with the association of the heavy and light chains, the empty heterodimers would dissociate at temperatures lower than 37 °C, reconciling our stability data and the Kb data (Matsumura et al., 1992b) with observations of the instability of empty cell surface class I molecules in detergent lysates (Ljunggren et al., 1990; Schumacher et al., 1990).
Table 2: Analysis of Dissociation Constants of K\textsuperscript{d}-Binding Peptides\textsuperscript{a}

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Length</th>
<th>Sequence</th>
<th>Category</th>
<th>(K_D) (M)</th>
<th>(IC_{50}) (M)</th>
<th>Ratio (relative to NPI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPI</td>
<td>9</td>
<td>TYQRTRALV</td>
<td>viral</td>
<td>(6.9 \times 10^{-4})</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>NP2</td>
<td>10</td>
<td>ATYQRTRALV</td>
<td>NH(_2) addition</td>
<td>338</td>
<td>5.2 \times 10^{-5}</td>
<td>754</td>
</tr>
<tr>
<td>NP3</td>
<td>11</td>
<td>DATYQRTRALV</td>
<td>NH(_2) addition</td>
<td>754</td>
<td>1.2 \times 10^{-4}</td>
<td>1739</td>
</tr>
<tr>
<td>NP4</td>
<td>10</td>
<td>TYQRTRALV</td>
<td>COOH addition</td>
<td>1091</td>
<td>1.7 \times 10^{-4}</td>
<td>2464</td>
</tr>
<tr>
<td>NP5</td>
<td>13</td>
<td>DATYQRTRALV</td>
<td>both additions</td>
<td>no binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP6</td>
<td>8</td>
<td>YQRTRALV</td>
<td>NH(_2) deletion</td>
<td>1441</td>
<td>2.2 \times 10^{-4}</td>
<td>3188</td>
</tr>
<tr>
<td>NP7</td>
<td>8</td>
<td>YQRTRAL</td>
<td>COOH deletion</td>
<td>414</td>
<td>6.4 \times 10^{-5}</td>
<td>928</td>
</tr>
<tr>
<td>NP8</td>
<td>7</td>
<td>YQRTRAL</td>
<td>both deleted</td>
<td>no binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP9</td>
<td>10</td>
<td>TYQRTRALV</td>
<td>insertion</td>
<td>63</td>
<td>9.6 \times 10^{-4}</td>
<td>139</td>
</tr>
<tr>
<td>NP10</td>
<td>9</td>
<td>TYQRTRALV</td>
<td>Y \rightarrow F</td>
<td>37</td>
<td>5.6 \times 10^{-4}</td>
<td>81</td>
</tr>
<tr>
<td>NP11</td>
<td>9</td>
<td>TYQRTRALV</td>
<td>Y \rightarrow T</td>
<td>no binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP12</td>
<td>9</td>
<td>TYQRTRALV</td>
<td>Y \rightarrow A</td>
<td>no binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP13</td>
<td>9</td>
<td>TYQRTRALV</td>
<td>moniodinated</td>
<td>304</td>
<td>4.7 \times 10^{-5}</td>
<td>681</td>
</tr>
<tr>
<td>NP14</td>
<td>9</td>
<td>TYQRTRALV</td>
<td>diiodinated</td>
<td>646</td>
<td>1.0 \times 10^{-4}</td>
<td>1449</td>
</tr>
<tr>
<td>NP15</td>
<td>9</td>
<td>TYQRTRALV</td>
<td>V \rightarrow S</td>
<td>197</td>
<td>3.0 \times 10^{-5}</td>
<td>435</td>
</tr>
<tr>
<td>NP16</td>
<td>9</td>
<td>TYQRTRALV</td>
<td>V \rightarrow W</td>
<td>172</td>
<td>2.7 \times 10^{-5}</td>
<td>391</td>
</tr>
<tr>
<td>NP17</td>
<td>9</td>
<td>TYQRTRALV</td>
<td>V \rightarrow K</td>
<td>1763</td>
<td>2.7 \times 10^{-4}</td>
<td>3913</td>
</tr>
<tr>
<td>NP18</td>
<td>9</td>
<td>TYQRTRALV</td>
<td>V \rightarrow D</td>
<td>no binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP19</td>
<td>9</td>
<td>TYQRTRALV</td>
<td>Q \rightarrow C</td>
<td>2.2</td>
<td>1.9 \times 10^{-7}</td>
<td>2.8</td>
</tr>
<tr>
<td>NP20</td>
<td>9</td>
<td>TYQRTRALV</td>
<td>R \rightarrow C</td>
<td>2.6</td>
<td>2.5 \times 10^{-7}</td>
<td>3.6</td>
</tr>
<tr>
<td>NP21</td>
<td>9</td>
<td>TYQRTRALV</td>
<td>T \rightarrow C</td>
<td>1.4</td>
<td>6.2 \times 10^{-4}</td>
<td>0.90</td>
</tr>
<tr>
<td>NP22</td>
<td>9</td>
<td>TYQRTRALV</td>
<td>R \rightarrow C</td>
<td>7.1</td>
<td>9.5 \times 10^{-7}</td>
<td>14</td>
</tr>
<tr>
<td>NP23</td>
<td>9</td>
<td>TYQRTRALV</td>
<td>A \rightarrow C</td>
<td>2.5</td>
<td>2.3 \times 10^{-7}</td>
<td>3.3</td>
</tr>
<tr>
<td>NP24</td>
<td>9</td>
<td>TYPPEndLV</td>
<td>five changes</td>
<td>438</td>
<td>6.8 \times 10^{-5}</td>
<td>986</td>
</tr>
<tr>
<td>NP25</td>
<td>9</td>
<td>TYGGGGGLV</td>
<td>five changes</td>
<td>956</td>
<td>1.5 \times 10^{-4}</td>
<td>2174</td>
</tr>
<tr>
<td>KD1</td>
<td>9</td>
<td>SYFEITHI</td>
<td>self</td>
<td>0.72</td>
<td>4.7 \times 10^{-4}</td>
<td>0.68</td>
</tr>
<tr>
<td>KD2</td>
<td>9</td>
<td>SYIPSJEKI</td>
<td>parasitic</td>
<td>0.49</td>
<td>3.2 \times 10^{-4}</td>
<td>0.46</td>
</tr>
<tr>
<td>KD3</td>
<td>8</td>
<td>YIPSJEKI</td>
<td>parasitic</td>
<td>2.5</td>
<td>2.3 \times 10^{-7}</td>
<td>3.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Peptide name, length, sequence, and category are summarized with the concentration at which peptide inhibited 50% of the binding of a \(^3\)H version of NPI (\(IC_{50}\)) (see Figure 3 for determination of \(K_D\) values). \(K_D\) values are calculated as described in the Materials and Methods using the \(IC_{50}\) values and an average percent bound in the absence of inhibitor of 75.2% (average of 24 experiments with an error range of ±10.7%). Peptide NPI is a known \(K^d\)-restricted epitope from influenza nucleoprotein (Rötzer et al., 1990); peptides NP2 through NP25 are analogs of NPI, peptides NP13 and NP14 are stoichiometrically iodinated (Tsomides & Eisen, 1993) forms of NPI, peptide KD1 represents the sequence of a prominent self peptide eluted from \(K^d\) molecules on P815 cells (Falk et al., 1991), and peptides KD2 and KD3 are nonamer and octamer versions of a \(K^d\)-restricted peptide from \(P. berghei\) circumsporozoite protein (Romero et al., 1991). "No binding" indicates that a peptide did not inhibit binding of the \(^3\)H-labeled NPI peptide at concentrations up to 1 mM.

Our data predict that at least the extracellular portion of an empty class I heterodimer can exist as a native folded structure under physiological conditions. However, in vivo studies show that surface class I expression is greatly reduced in mutant mice in which the gene encoding the peptide transporter associated with antigen processing was disrupted (Ashton-Rickardt et al., 1993). Thus, there may be other mechanisms in addition to the decreased thermal stability of empty class I molecules that keep large numbers of them from remaining at the surface of cells.
Most studies to detect peptide binding to MHC molecules have used peptides labeled by iodination of tyrosine residues (Benjamin et al., 1991; Cerundolo et al., 1991; Christinck et al., 1991), either within the antigenic peptide sequence or as an N- or C-terminal addition. The iodine atom is large, similar in size to a benzene ring (Bolton & Hunter, 1973). Thus iodination would be expected to introduce steric hindrance as well as ionic effects that may interfere with the hydrogen bonding potential of the tyrosine hydroxyl. When a tyrosine for labeling is added to the beginning or end of the peptide, the peptide is no longer the optimal length for binding to class I molecules (Rötzschke & Falk, 1991), and the interactions of one of the termini with conserved pockets in the class I peptide binding site (Fremont et al., 1992; Guo et al., 1992; Madden et al., 1992; Matsumura et al., 1992a) are disrupted. In addition, traditional iodination procedures label only ~1% of the peptide molecules, leaving the remainder unlabeled, complicating the interpretation of inhibition or other experiments performed using a mixture. Other studies use peptides modified by biotinylation at their N-termini (Levy et al., 1991), which would also disrupt interactions with the conserved pocket for the N-terminal residue. To avoid these problems, measurements of binding affinity were done with a peptide labeled with 3H to high specific activity by catalytic exchange.

The binding affinity of a 3H-labeled Kβ-restricted peptide (peptide NP1, Table 2) from influenza virus nucleoprotein (Rötzschke et al., 1990a) for empty Kβ MHC heterodimers was measured using equilibrium dialysis. The KD was determined from multiple experiments as (6.9 ± 2.3) × 10^-8 M, in close agreement with the binding affinity of this peptide reported by others (Ojcius et al., 1993) and with the value (KD = 5.0 × 10^-8 M) derived from measuring the binding of the same 3H-labeled peptide to lipid-linked Kβ molecules expressed on the surface of CHO cells (Fahnestock et al., 1994).

Binding affinities of a series of unlabeled analog peptides were determined by quantitation of inhibition of binding of the peptide labeled. Using the knowledge of the three-dimensional structures of class I molecules, we can attempt to interpret differences in peptide binding affinities caused by altering the Kβ-restricted peptide on a structural basis. Although the three-dimensional structure of Kβ has not yet been determined, the crystal structures of other class I molecules have revealed a common mode of peptide binding (Garrett et al., 1988, Saper et al., 1991; Fremont et al., 1992; Madden et al., 1992; Matsumura et al., 1992a; Zhang et al., 1992), so that the structure of Kβ and peptides bound to it can be modeled upon that of known structures of Kβ/peptide complexes (Fremont et al., 1992; Matsumura et al., 1992a; Zhang et al., 1992). To facilitate the following discussion, a view of the peptide binding site of Kβ, including the location of the pockets A-F, is shown in Figure 4.

Peptides with one or two extensions to the N- or C-terminus of the Kβ-restricted peptide were tested for binding affinity. The particular residues introduced in the extended peptides were charged amino acids on either side of the nonamer peptide, based upon the sequence of influenza nucleoprotein (Winter & Fields, 1981). Extending the sequence beyond nine residues significantly lowered the binding affinity, with additions at the C-terminus being more detrimental to binding. The specific residues added to the C-terminus of the peptide may have had a particularly deleterious effect upon binding. Peptides eluted from Kβ proteins exhibit a preference for an aliphatic amino acid at their C-terminus (Falk et al., 1991; Romero et al., 1991). The side chain of the C-terminal peptide residue fits into pocket F, consisting in Kβ of residues Ser 77, Ala 81, Phe 95, and Phe 116, assuming a three-dimensional structure similar to that of Kβ (Fremont et al., 1992; Matsumura et al., 1992a; Zhang et al., 1992). The residue immediately following the end of the Kβ-restricted nonamer peptide in influenza nucleoprotein is an arginine (Winter & Fields, 1981). If the additional length of the dodecamer peptide is accommodated by bulging in the middle (Guo et al., 1992), the charged C-terminal peptide residue would have to fit into the F pocket, which is fairly hydrophobic in the Kβ molecule and therefore not likely to tolerate charged hydrophilic groups. Indeed, in nonamer peptides, the substitution of the final amino acid for a charged amino acid reduces the KD to millimolar values (see peptides NP17 and NP18, Table 2), demonstrating the poor complementarity in peptides of optimal length between a charged C-terminal residue in the peptide and pocket F in the Kβ molecule. At the peptide N-terminus, the side chain is exposed to solvent, with contacts to pocket A made with main-chain peptide atoms (Fremont et al., 1992; Matsumura et al., 1992a). If the Kβ-restricted peptide with a single extension at the N-terminus (peptide NP2) binds such that its N-terminal residue is positioned in pocket A and the extra length is accommodated with a bulge in the middle, there would be a threonine at position 2 instead of the required tyrosine. However, this mode of binding seems unlikely since analysis of a nonamer peptide with a threonine at position 2 (peptide NP11) reveals that the threonine substitution abrogates binding, even though both threonine and tyrosine are polar amino acids containing a hydroxy group. These observations imply that peptides NP2 and NP3 retain the anchor tyrosine in pocket B and that their extra length is accommodated by extending the peptide beyond pocket A, resulting in significantly lower affinities.

For peptide binding to Kβ, the opposite phenomenon was observed: peptides with extensions at the N-terminus bound more poorly than peptides extended at the C-terminus (Matsumura et al., 1992b). Thus it appears that individual class I molecules will differ with regard to their tolerance for extensions of peptides at the N- or C-terminus, with the particular residues added being important determinants of the binding affinity. Our data suggest that versions of a
Peptide Binding to Empty Class I MHC Molecules

restricted peptide with extensions at either end bind with significantly lower affinity and imply that adding extra residues for the purpose of labeling a peptide should be avoided.

Although other class I molecules bind either octamer or nonamer peptides (e.g. K\(^\alpha\); Fremont et al., 1992; Matsumura et al., 1992a,b), deletion of either the N-terminal, C-terminal, or both residues of the viral nucleoprotein nonamer peptide greatly reduced the binding affinity. The N-terminal deletion had the greater effect, presumably because the resulting tyrosine at position 1 cannot simultaneously act as the first residue by fitting into pocket A and as the anchor residue by fitting into pocket B. Deletion of the C-terminal residue leaves a leucine as the final residue, and this side chain fulfills the greatly reduced the binding affinity. The N-terminal deletion or both residues of the viral nucleoprotein nonamer peptide et al., nonamer peptides (e.g. Kb; Fremont et al., 1993) do not show a large discrepancy in binding affinities, with the octamer binding with only 10-fold lower affinity than the nonamer. Thus for K\(^\alpha\)-restricted peptides, the preference for a nonamer or octamer appears to be dependent on the particular peptide sequence.

As might be predicted, changing the tyrosine residue at position 2 affects binding affinity. A peptide with phenylalanine at this position (peptide NP10) still binds with fairly high affinity, as would be predicted by the finding that some of the peptides eluted from K\(^\alpha\) molecules contained phenylalanine at position 2 (Falk et al., 1991). However, no detectable binding is seen with an alanine-substituted peptide (peptide NP12) or a threonine-substituted peptide (peptide NP11). The poor binding of stoichiometric iodinated derivatives (Tsomides & Eisen, 1993) of the K\(^\alpha\)-restricted peptide (peptides NP13 and NP14) demonstrates that the addition of the large iodine atom, as is commonly done for labeling purposes, has dramatic effects on the binding properties of the iodinated peptide compared to the peptide in its native state. These results suggest that great care must be used in labeling peptides for binding studies to make sure that the labeled peptide is not chemically altered in such a way as to change its binding properties.

The effects of single substitutions at the interior nonanchor positions were next explored using five peptides in which the natural residues at positions 3–7 were progressively altered to cysteine. On the basis of the structure of K\(^\alpha\), positions 3, 6, and 7 are predicted to fit into pockets D, C, and E respectively, while positions 4 and 5 are predicted to be solvent exposed (Matsumura et al., 1992a). As expected, altering peptide residues 4 and 5 to cysteine has very little effect upon binding (peptides NP20 and NP21, Table 2). Changing residues 3 and 7 (peptides NP19 and NP23) also has only small effect upon binding, suggesting that pockets D and E in K\(^\alpha\) can accommodate a number of different residues, in accordance with sequencing data of peptides eluted from purified K\(^\alpha\) (Falk et al., 1991). At position 6, a 14-fold reduction in affinity is seen upon substitution with cysteine (NP22), suggesting a slight preference in pocket C for residues other than cysteine. Indeed, in peptides eluted from K\(^\alpha\) molecules, lysine and phenylalanine were frequently found at this position (Rammensee et al., 1993), implying that pocket C prefers side chains larger than cysteine. Knowledge of the binding affinities of these cysteine-substituted peptides will allow greater ease of interpretation of future work involving covalent coupling of cysteine-substituted peptides to a solid support for kinetic and equilibrium analyses of peptide binding using a surface plasmon resonance assay similar to that reported by Khliko et al. (1993).

A previous report describes competitor analogs of K\(^\alpha\)-restricted peptides synthesized using polyproline or polyglycine spacers (Maryanski et al., 1990). Dodecamer pentaprolines and pentaglycine analogs were found to compete for binding of a 13-mer K\(^\alpha\)-restricted peptide, with the pentaproline analog being the more active. We synthesized comparable nonamer pentaproline and pentaglycine versions of the K\(^\alpha\)-restricted influenza nucleoprotein peptide (peptide NP1) in order to measure their binding affinities for empty K\(^\alpha\). The pentaproline (peptide NP24) and pentaglycine (peptide NP25) analogs both showed reduced binding compared to the original peptide, possibly confirming the importance of secondary anchor residues in peptide binding, as was noted in the binding of peptides to the human class I molecule HLA-A2 (Ruppert et al., 1993). By contrast, the dodecamer pentaproline analog of the 13-mer K\(^\alpha\)-restricted peptide was as active as the original peptide in a functional competition assay (Maryanski et al., 1990), perhaps because this pentaproline analog was closer to the optimal nonamer length than the original peptide. We and the previous workers (Maryanski et al., 1990) both observed that the polyglycine analog was a less effective inhibitor, which may be due to the absence of a proline at position 4 (a frequently observed residue at this position in sequences of peptides eluted from purified K\(^\alpha\) molecules; Rammensee et al., 1993), or the greater flexibility allowed by a series of glycines that could adversely affect binding.

The four unaltered K\(^\alpha\)-restricted peptides (NP1, KD1, KD2, and KD3) represent three different sequences with only the anchor positions in common, but all bind with fairly comparable affinities (K\(_D\) = 10⁻⁷⁻¹⁰⁻⁸ M). These values represent slightly lower affinities than those have been reported for restricted peptides binding to K\(^\alpha\) (Matsumura et al., 1992b) but comparable or higher than affinities of HLA-A2 restricted peptides (Ruppert et al., 1993). These results suggest that optimal peptides binding to K\(^\alpha\) and other class I molecules have K\(_D\) values in the nanomolar range and that alteration of anchor residues or the length of the peptide drastically affect the binding affinity.

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REFERENCES

MAXADILAN BINDS TO MEMBRANE FRACTIONS OF BRAIN TISSUE

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Summary: Maxadilan is a potent vasodilator peptide isolated from salivary glands extracts of the hematophagous sand fly. Besides effects on the cutaneous vasculature, it has also been shown to relax rabbit aortic rings while elevating levels of cAMP. As a result of the effects on the skin and aorta, it was elected to undertake an examination of the tissue distribution of binding sites for maxadilan. In addition to specific binding in rabbit aorta and spleen, binding was detected in brain from various species including human, bovine, rabbit, rat and mouse with a KD of between 85 and 201 pM. Competitive displacement of [125I] maxadilan by a number of known vasoconstrictor peptides, vasodilator peptides and small molecule receptor ligands did not occur in the rabbit brain preparation. These results suggest the presence of specific binding sites in mammalian tissue for maxadilan whose endogenous ligand remains unknown.

When an insect probes for a blood meal in skin, blood vessels are injured and the basic hemostatic processes of vasoconstriction, coagulation, and platelet aggregation are initiated (1). To overcome these processes and ensure success at obtaining blood, insects have developed a number of potent anti-hemostatic compounds in their saliva. The ultimate goal of blood-feeding by arthropods is to allow completion of the life cycle in which the female needs the stimulus of a blood meal in order to lay eggs. Males typically do not blood feed. The small size of many arthropods and rapidity with which they can feed suggests that these compounds are potent substances that can be exploited in the development of pharmacological agents (1).

During the last several years a number of antihemostatic compounds have been found in salivary glands of arthropods and it would appear that these substances aid in bloodfeeding. Some of these substances inhibit platelet aggregation and others effect the coagulation pathway (2). Two vasodilator peptides have been described - a nitrovasodilator from Rhodnius prolixus (3) and maxadilan, a peptide from the sand fly Lutzomyia longipalpis. which acts directly on smooth muscle cells (4, 5).

Maxadilan is a vasodilator peptide isolated from salivary gland extracts of the sand fly, the vector of the protozoan disease leishmaniasis. Leishmaniasis occurs in many parts of the

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world including Central and South America, the Middle East and Indian subcontinent. The disease is typically manifest as ulcers on the skin or infection of the liver and spleen. It has been shown that sand fly salivary gland extracts exacerbate the infectious process by decreasing or turning off the local immune response of the host. Besides its vascular effects, maxadilan has immunomodulatory properties and appears to be the substance responsible for the exacerbative effect (submitted). The ability of maxadilan to dilate blood vessels and modulate immune responses is similar to the effects of CGRP, which has the additional property of being a neuropeptide (6, 7).

Maxadilan is produced as a 63 amino acid peptide which undergoes C-terminal cleavage and amidation to a 61 amino acid peptide. It contains four cysteine residues which participate in the formation of disulfide bonds between positions 1-5 and 14-51. The peptide acts to raise level of cAMP in rabbit aorta suggesting that it interacts with a cell-surface receptor to mediate its effects (5).

In previous studies, it was demonstrated that maxadilan was functionally active on rabbit vessels and human skin but not on rat aorta, dog mesentery or pig or cow coronary vessels (5). To obtain a picture of the tissue distribution of binding sites for this peptide, maxadilan was stoichiometrically labeled with $^{125}$I to high specific radioactivities (2000-4000 Ci/mmol). The iodinated peptide was incubated with membrane fractions prepared from a variety of rabbit tissues. These results, which revealed binding to membrane fractions obtained from rabbit brain, prompted an investigation of binding of maxadilan in the brain of various species.

Materials and Methods

Preparation of peptide - Recombinant maxadilan produced in E. coli contained the four additional amino acid residues glycine, serine, isoleucine and leucine at the N-terminus as a result of construction in the pGEX vector designed for cleavage with thrombin (8). Termed "GSIL-maxadilan", it was purified to homogeneity using reverse phase HPLC.

Maxadilan was labeled with $^{125}$I using Iodo-Beads (Pierce, Rockford, IL). Free iodine was removed by passing the peptide over a Sep-Pak C18 cartridge (Waters, Marlborough, MA) and unlabeled peptide was separated from radiolabeled products by reverse phase HPLC (Figure 1) (9). Specific activity was 2000-4000 Ci/mmol in different preparations. As maxadilan does not contain tyrosine residues, the reaction was carried out at pH 8.2 to promote iodination on either or both of the histidine residues in the peptide (10). Biological activity was assessed by stoichiometrically labeling maxadilan with cold $^{127}$I followed by injection into rabbit skin. This material maintained complete functional activity relative to the unlabeled peptide. It was inferred from this result that the $^{125}$I labeled material was also biologically active.

Preparation of membranes - Rat, rabbit, mouse and bovine tissue was obtained from Pel-Freez (Rogers, AR). Rat brain material was also obtained from animals purchased from Charles River Laboratories. Human brain material was kindly provided by Tessa Hedley-Whyte of the neuropathology service at MGH. Membrane fractions from these tissues were prepared as described previously (11). Briefly, tissue was placed in 10 vol of ice-cold 50 mM Tris-HCl buffer (pH 7.6) containing 0.32M sucrose, 5 mM EDTA, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 2 mg/ml actin and 10 mg/ml phenylmethylsulfonyl fluoride. Tissue was homogenized with a polytron PT 3000 (Brinkmann Instruments, Westbury, NY) for 30 s at power level 8 at 4°C. The homogenate was centrifuged for 10 min at 1000 x g at 4°C. The supernatant was removed, and the pellet resuspended in 15 ml of homogenizing buffer, homogenized again using the Polytron at the same setting as the first homogenization, and the homogenate was centrifuged at 1000 x g for 10 min at 4°C. The combined supernatant was centrifuged at 30,000 x g for 20 min at 4°C.

The pellet was washed two times by successive suspension in 50 mM Tris-Cl buffer containing 1 mM MgCl₂, 0.3 % BSA, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 2 mg/ml bacitracin and 10 mg/ml phenylmethylsulfonyl fluoride.
Maxadilan (100μg) was iodinated with 20mCi carrier-free Na125I and two Iodo-Beads in 0.1M sodium phosphate, pH 8.2. After removal of unreacted iodide, the peptide was loaded on a reverse phase C4 column to separate labeled and unlabeled maxadilan. Since unlabeled peptide is essentially removed by this method, specific radioactivity of the labeled peptide may be calculated for that of carrier-free 125I.

**Binding of [125I] maxadilan** - Crude membranes (250-400 mg) were incubated for 2 hr at 4°C in a final volume of 0.5 ml consisting of 50 mM Tris-HCl buffer (pH 7.6) containing 0.3 % BSA, 1mM MgCl2, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 2 mg/ml bacitracin, 10 mg/ml phenylmethylsulfonyl fluoride and 70 pM maxadilan in the absence or presence of 1 μM maxadilan. At the end of incubation, samples were assayed for protein-bound radioactivity by vacuum filtration through GF/C Whatman glass microfiber filters pretreated with 0.5% polyethylenimine. Filters were then washed with 3 x 3 ml of incubation buffer at 4°C. The radioactivity trapped on the filters was measured using a gamma counter. Non-specific [125I] maxadilan binding was determined by the addition of 1μM unlabeled maxadilan and represented between 10-20% of total binding depending upon the tissue preparation. In the Results, specific binding (total cpm minus non-specific cpm) is shown. Proteins were estimated by the method of Bradford using bovine serum albumin as standard.

**Chemicals** - All peptides were obtained from Peninsula Laboratories (Belmont, CA). All other reagents were of analytical grade from Sigma (St. Louis, MO).

**Results**

**Binding of maxadilan to rabbit tissues**

Crude membrane homogenates were prepared from various rabbit tissues and assayed for specific maxadilan binding (Figure 2). Specific binding was noted in aorta, spleen and brain. Although binding to brain and spleen were seen consistently, binding to aorta was reproducible for some but not all batches of iodinated maxadilan.

**Binding of maxadilan to brain and spleen tissues**

Because of the specific binding of maxadilan to rabbit brain and spleen, such tissues from mouse, rat, bovine and human sources were examined. In all cases, specific binding was noted in the brain (Figure 3). In the case of spleen, specific binding was only seen in rabbit. The equilibrium binding and Scatchard analysis of rabbit brain membrane revealed a Kd of 136 pM (Figure 4). The distribution of [125I] maxadilan binding sites was examined in rat brain, where binding appeared to be essentially equal across the areas examined including cortex, hippocampus,
Figure 2. Specific binding of maxadilan to membrane fractions of rabbit tissues. Crude membrane fractions were prepared from the indicated tissues and incubated with $[^{125}I]maxadilan$ and excess unlabeled peptide in a competition assay. Specific binding was noted in brain and spleen.

brain stem, striatum and cerebellum (Figure 5). Specific binding was noted to all areas of human brain examined including substantia nigra, pons, vermis (anterior portion), medulla/olive, thalamus, parietal, hippocampus and cerebellar gray and white matters (data not shown). The least binding

Figure 3. Specific binding of maxadilan to membrane fractions of brain and spleen of various species. Maxadilan binds to all brains examined, including mouse, rat, rabbit and bovine. Binding to human brain occurred (not shown). Binding to spleen was only noted in rabbit tissue.
Figure 4. Equilibrium binding and Scatchard analysis of binding of maxadilan to rabbit brain. Saturable binding occurs and it appears that a single class of high affinity receptors is present.

...was noted in cerebellar white matter. This finding correlates well with preliminary autoradiographic data in which preferential binding occurs in gray matter of rat brain sections (not shown). The dissociation constants and Bmax values for binding to brain membranes from the...
various species and rabbit spleen are noted in Table 1. In all cases, it appears that maxadilan binds with high affinity to a single class of receptors.

**Competition between maxadilan and selected receptor ligands**

Because maxadilan receptors were present both in the vasculature and the brain, a number of known vasoconstrictor or vasodilator peptides, also found in brain, and small molecules were examined for their ability to compete with binding of [125I] maxadilan to rabbit brain. In a preliminary experiment, a competitive binding assay between a fixed quantity of [125I] maxadilan and increasing concentrations of unlabeled maxadilan was performed (Figure 6) yielding an IC50 of maxadilan of 2.0+/-.3nM. None of the peptides tested, including, CGRP, amylin, endothelins and VIP competed with maxadilan (Figure 7). Results of an analogous experiment performed with small molecule agonists, including receptor ligands (carbachol, dopamine, histamine, isoproterenol, norepinephrine, serotonin, glycine, GABA and glutamate) and ion channel blockers (TEA, verapamil, and nifedipine) revealed that none of these small molecules had the ability to compete with labeled maxadilan binding to brain extracts (not shown).

**Discussion**

Maxadilan is a vasodilator peptide present in sand fly salivary glands and the fly probably uses this peptide as an aid in obtaining a blood meal. When sand flies transmit leishmaniasis, their salivary gland extracts exacerbate this process and recent data indicates that maxadilan participates in this process (submitted). To examine these cutaneous effects in more detail, an iodinated probe would be useful. The data presented here reveal that the peptide has been labeled to very high specific radioactivities (equimolar or greater ratio of 125I to peptide) with maintenance of biological activity despite a lack of tyrosines residues.

Because vasoactive molecules might be expected to interact with receptors in a variety of tissues, a survey was undertaken to examine the binding of labeled maxadilan to membrane fractions obtained from various rabbit tissues, instead of using this probe to assess binding sites in skin by autoradiography. Maxadilan was found to bind with high affinity to a single class of receptors in rabbit aorta and spleen and in brain from a variety of species. The binding in spleen is consistent with the interaction of maxadilan with cells of the immune system. The studies in rat and human brain reveal wide distribution of binding and the preferential binding to white

<table>
<thead>
<tr>
<th>species/tissue</th>
<th>Kd (pM)</th>
<th>Bmax (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse brain</td>
<td>90</td>
<td>80</td>
</tr>
<tr>
<td>rat brain</td>
<td>201</td>
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<td>14</td>
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<tr>
<td>bovine brain</td>
<td>103</td>
<td>27</td>
</tr>
</tbody>
</table>

Table 1

Kd and Bmax of maxadilan binding to membrane homogenates
Figure 6. Competitive binding assay between labeled and unlabeled maxadilan in rabbit brain. Crude rabbit brain membranes were incubated with [125I] maxadilan (70pM) and the indicated concentrations of cold maxadilan. The IC50 for maxadilan was approximately 2nM.

matter suggests that maxadilan is interacting with a neuronal component in the central nervous system. The result that a high degree of specific binding occurred in brain may, in retrospect, not be surprising as a number of neuropeptides have vascular effects (12). However, the

Figure 7. Competition between maxadilan and endogenous brain-derived peptides. The ability of a number of peptides to compete with labeled maxadilan was determined by competition. 1: no peptide, 2: maxadilan, 3: VIP, 4: CGRP, 5: amylin, 6: neurotensin, 7: bradykinin, 8: bombesin, 9: oxytocin, 10: somatostatin, 11: angiotensin II, 12: parathyroid hormone, 13: substance P, 14: endothelin I, 15: endothelin II, 16: endothelin III.
observations with maxadilan come from the opposite direction - it is an exogenous peptide whose primary effect is exerted on the vasculature to aid in blood-feeding, and it incidentally binds to brain membranes. A possible interpretation of the observation that some but not all batches of the labeled peptide bind to rabbit aorta while all preparations bind to rabbit brain is that subclasses of a "maxadilan receptor" exist and the iodination process alters the structure of the peptide enough to "distinguish" between such subclasses.

That maxadilan raises levels of cAMP and appears to bind to a membrane receptor suggests that it interacts with a member of the G-protein coupled receptor family (13). Coupled with earlier observations of slight primary sequence homology to CGRP, it is possible that maxadilan interacts with a subclass of CGRP or related receptors (4). It should be possible to identify a cell line, rather than whole tissue, which binds and signals upon incubation with maxadilan. Such a cell line would be useful for functional studies and for expression cloning of a maxadilan receptor. It is also possible that a maxadilan-like peptide is present in mammals, a scenario which could be examined in a number of ways including probing western blots of mammalian brain using antibody to maxadilan or Southern blots of mammalian genomic libraries using the gene encoding maxadilan as a probe. A positive result could lead to the identification of a previously unknown neuropeptide.

Identification of a maxadilan receptor or endogenous maxadilan-like peptide will advance our understanding of how this arthropod-derived molecule works. This result will shed light on how maxadilan exerts its long-lasting erythema effect and elucidate further the potential therapeutic role of this potent vasodilator.

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References
In Vitro Radiolabeling of Peptides and Proteins

Radiolabeling of peptides or proteins is often performed to enhance the sensitivity of detection, to quantitate the binding of peptides to other molecules, or for radioimmunoassays. The choice of radioisotope depends in part on the amino acid residue(s) to be modified (Table 3.3.1). The most common and straightforward labeling strategy is radioiodination with $^{125}$I because of its relative ease of use, the high resulting specific radioactivities (curies per millimole), and the convenience of detecting $\gamma$ emissions. Radioiodination may also be performed with $^{131}$I, which has a shorter half-life (8 days versus 60 days for $^{125}$I), giving higher specific radioactivities but limiting the useful lifetimes of the labeled products; in addition, $^{131}$I has higher energy $\gamma$ emissions, necessitating more shielding to protect workers.

Iodination at tyrosine or histidine residues using Iodo-Beads (Basic Protocol 1) is the most convenient method, but chloramine T or Iodogen (Alternate Protocol 1) may give higher recoveries when working with small quantities of peptide, and lactoperoxidase (Alternate Protocol 2) results in less oxidative damage and lower specific radioactivities, and is commonly used when labeling whole cells. Modifications for achieving equimolar or higher (stoichiometric) rather than trace iodination are presented in Support Protocol 1, and the optional separation of unlabeled peptide from iodinated products by HPLC is described in Support Protocol 2. For peptides that do not contain tyrosine or histidine, or in which labeling of these amino acid residues interferes with biological activity, $^{125}$I or $^{131}$I may be added onto primary amino groups (lysine residues and the peptide N-terminus) using Bolton-Hunter reagent (Basic Protocol 2).

As an alternative to iodination, peptides or proteins can be labeled with $^{14}$C or $^3$H by acetylation of primary amino groups using anhydride (Basic Protocol 3), by reductive alkylation of primary amino groups using aldehyde and borohydride (Alternate Protocol 3), or by alkylation of cysteine sulfhydryl groups (Alternate Protocol 4). Figure 3.3.1 summarizes these radiolabeling strategies as well as the basic iodination reactions. Two methods are available to label a peptide without altering its structure: $^3$H labeling by catalytic reduction of (nonradioactive) $^{127}$I-labeled peptide (Basic Protocol 4) and de novo peptide synthesis using a radiolabeled form of any amino acid (Basic Protocol 5). However, these methods tend to be more expensive and laborious. Finally, selective

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>Label</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>$^{125}$I, $^{131}$I</td>
<td>Chloramine T, Iodo-Beads</td>
<td>Greenwood et al. (1963)</td>
</tr>
<tr>
<td>Histidine, tyrosine</td>
<td>$^{125}$I, $^{131}$I</td>
<td>Chloramine T, Iodo-Beads</td>
<td>Wolff and Covelli (1969)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>$^{125}$I, $^{131}$I</td>
<td>Lactoperoxidase</td>
<td>Marchalonis (1969)</td>
</tr>
<tr>
<td>Lysine, N-terminus</td>
<td>$^{125}$I, $^{131}$I</td>
<td>Bolton-Hunter</td>
<td>Bolton and Hunter (1973)</td>
</tr>
<tr>
<td>Lysine, N-terminus</td>
<td>$^{14}$C, $^3$H</td>
<td>Anhydride</td>
<td>Fraenkel-Conrat and Colloms (1967)</td>
</tr>
<tr>
<td>Lysine, N-terminus</td>
<td>$^{14}$C, $^3$H</td>
<td>Aldehyde/borohydride</td>
<td>Tack and Wilder (1981)</td>
</tr>
<tr>
<td>Cysteine</td>
<td>$^{14}$C, $^3$H</td>
<td>Iodoacetic acid</td>
<td>Chersi et al. (1988)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>$^3$H</td>
<td>Reduction of $^{127}$I</td>
<td>Amersham or Du Pont NEN literature</td>
</tr>
<tr>
<td>Any residue</td>
<td>$^{14}$C, $^3$H</td>
<td>Peptide synthesis</td>
<td>Stewart and Young (1984)</td>
</tr>
<tr>
<td>Carbohydrate groups</td>
<td>$^3$H</td>
<td>Periodate/borohydride</td>
<td>Gahmberg and Andersson (1977)</td>
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Figure 3.3.1 Common peptide radiolabeling reactions. (A) Iodination on tyrosine. (B) Iodination on histidine. (C) Bolton-Hunter iodination on lysine and N-terminus. (D) Acetylation on lysine and N-terminus. (E) Reductive alkylation on lysine and N-terminus. (F) Alkylation on cysteine.
labeling at the N-terminus of a peptide can be performed during peptide synthesis (Alternate Protocol 5).

All peptide labeling procedures described in this unit are also applicable to larger proteins; however, with larger proteins there is increased potential for idiosyncratic results because of tertiary structure and for undesired side reactions because of sensitive amino acid residues (e.g., tryptophan, methionine, and cysteine in the case of oxidative iodinations). To some extent these problems may be minimized through the use of milder labeling procedures (e.g., lactoperoxidase-catalyzed iodination).

**NOTE:** Various methods for separating radiolabeling agents (e.g., free iodide) from labeled peptide products are described in Basic Protocol 1 and in **UNIT 8.2 & 8.3**, and any of these methods may be used in conjunction with any of the radiolabeling protocols in this unit. The choice of separation method should depend primarily on the properties of the peptide or protein (see Critical Parameters, discussion of iodination work-up).

**CAUTION:** When working with $^{125}$I, $^{131}$I, $^{14}$C, $^{3}$H, or other radiochemicals, always observe appropriate safety precautions, including the use of gloves (consider use of a glove box with a charcoal filter, particularly for >5 mCi iodide), shielding, dosimetry monitoring, and proper disposal of radioactive waste. Consult a radiation safety specialist to ensure that all procedures are safe and in accordance with Nuclear Regulatory Commission guidelines. Oxidation converts iodide to a volatile and hazardous form; peptide-bound iodine is not volatile, but emits $\gamma$ radiation and remains a potential hazard. Be sure to perform all steps that may give rise to volatile iodine in an externally vented hood. See **APPENDIX 2B** for additional guidelines.

### IODINATION AT TYROSINE OR HISTIDINE RESIDUES USING IODO-BEADS

Iodo-Beads are nonporous polystyrene beads with an immobilized oxidizing agent (N-chlorobenzenesulfonamide) capable of converting Na$^{125}$I (or Na$^{131}$I) to its reactive form (Markwell, 1982). Iodination occurs by electrophilic addition to tyrosine at the two positions ortho to the hydroxyl group, and on histidine at two imidazole carbon positions (Means and Feeney, 1971; Fig. 3.3.1). After reaction of a peptide with oxidized iodide, the mixture is transferred to a solid-phase extraction device (e.g., a Sep-Pak cartridge) to remove free iodide from the labeled peptide.

#### Materials

- **Iodo-Beads** (Pierce)
- 0.1 M sodium phosphate, pH 6.0 or pH 8.5
- Carrier-free sodium iodide-125 (Na$^{125}$I; Amersham, Du Pont NEN, or ICN Biomedicals) or sodium iodide-131 (Na$^{131}$I)
- 0.1 M sodium hydroxide
- Peptide, ideally dissolved in ≤0.4 ml of water or 0.1 M sodium phosphate
- Methanol
- HPLC solvent A: 0.1% (v/v) trifluoroacetic acid (TFA) in water
- HPLC solvent B: 0.1% (v/v) TFA in acetonitrile
- Filter paper
- 100-μl syringe with beveled tip, or hot pipettor (i.e., dedicated to radioactive use)
- Luer-tipped syringes (1-, 5-, and 20-ml) and one 23-G needle
- Solid-phase extraction device (e.g., Sep-Pak or Sep-Pak Plus C18 cartridge, Waters)
- Polypropylene or other plastic tubes (12 × 75 mm)
1. Wash Iodo-Beads with 1 ml of 0.1 M sodium phosphate, pH 6.0 (for labeling primarily on tyrosine) or pH 8.5 (for labeling on histidine and tyrosine). Dry on filter paper and add to 0.5 ml of same buffer in capped microcentrifuge tube or small reaction vial.

   For trace labeling use two Iodo-Beads; for stoichiometric labeling more may be needed (see Support Protocol 1).

   Iodo-Beads are compatible with most salts (including azide), chaotropic agents, and detergents, but not with reducing agents or organic solvents that dissolve polystyrene such as dimethyl sulfoxide (DMSO) and dimethylformamide (DMF). Iodo-Beads must be stored in the presence of desiccant, and have a manufacturer-suggested shelf life of 1 year.

2. Add Na\(^{125}\)I (or Na\(^{131}\)I) to Iodo-Beads in the reaction tube using a 100-\(\mu\)l syringe with beveled tip or a hot pipettor. Cap tube and wait 5 min at room temperature, agitating the mixture occasionally. Rinse syringe with 0.1 M sodium hydroxide (\(^{125}\)I is less volatile at high pH) and then with water before storing.

   The amount of radioactive iodide depends largely on the desired specific radioactivity, subject to safety and cost limitations; 1 to 5 mCi is typical. Use a fresh batch to minimize loss of radioisotope as volatile molecular iodine. Na\(^{125}\)I is supplied in various concentrations of NaOH; the more dilute NaOH solutions are preferable since they are less likely to alter the pH of the reaction.

3. Add peptide to the reaction. Cap tube and wait 30 min, agitating occasionally.

   The amount of peptide is inversely related to the final specific radioactivity, unless iodinated products are separated from unlabeled peptide (see Support Protocol 2); 0.05 to 2.0 mg is typical. Reaction time can be 45 min or longer if desired, or just 5 to 10 min if oxidation-sensitive residues in the peptide (tryptophan, methionine, cysteine) are of concern, and should be optimized empirically if needed.

4. During the iodination reaction (or before if more convenient), prepare a Sep-Pak C18 cartridge by attaching a syringe and passing 5 ml methanol through the cartridge, followed by 5 ml water and 10 to 20 ml HPLC solvent A. Maintain a flow rate of ~2 ml/min, do not reverse the orientation of the cartridge when changing solvents, and avoid introducing air bubbles or allowing the cartridge to dry out.

   Although this Sep-Pak method offers the most efficient separation of unbound radioactive iodide from the peptide, anion-exchange (UNIT 8.2) or gel-filtration (UNIT 8.3) chromatography may be used as alternatives, especially for larger peptides at risk of denaturation under the conditions used in steps 4 to 8 (see Alternate Protocol 1, steps 1 and 4). Dialysis is another alternative for large peptides, but takes much longer.

5. Remove the mixture from the reaction tube using a 1-ml syringe fitted with a 23-G needle. Carefully remove the needle and slowly load mixture onto the preconditioned Sep-Pak cartridge, collecting the eluate into a radioactive liquid waste container.

6. Wash Sep-Pak cartridge with 20 ml HPLC solvent A, collecting the eluate as radioactive waste. Neutralize waste with 5 ml of 0.1 M sodium hydroxide.

   Unbound radioactive iodide will pass through the Sep-Pak cartridge under these conditions, while peptide will remain in the cartridge.

7. Elute two fractions from the Sep-Pak cartridge into 12 × 75-mm plastic tubes, the first using 4 ml of a 1:1 mixture of HPLC solvent A and HPLC solvent B, and the second using 4 ml HPLC solvent B.

   Usually the first tube contains most of the labeled peptide, but a two-step elution improves overall recovery. Because unbound iodide is now removed, the tubes may be transferred out of the glove box or other iodination facility, as long as appropriate shielding is maintained.
8. Dry the tubes in a Speedvac evaporator. Redissolve in water or desired buffer and pool the two fractions.

9. To determine the specific radioactivity of the labeled peptide, estimate peptide recovery or analyze an aliquot of the sample for peptide concentration (e.g., by a colorimetric assay) and simultaneously determine cpm (in a γ-radiation counter, possibly after serial dilutions in the presence of a carrier protein to reach a level of radioactivity within the linear counting range of the γ counter).

Specific radioactivity (in cpm/μg) = measured radioactivity (in cpm/μl) + peptide concentration (in μg/μl). To convert to millicuries, use 1 mCi = 2.22 × 10⁹ dpm and cpm = dpm × counting efficiency of γ counter (often 50% to 75%, depending on the instrument).

Alternatively, if iodinated products are to be separated from unlabeled peptide by HPLC and the molar ratio of incorporated iodide to peptide is known (or estimated), the specific radioactivity of the peptide may be calculated from that of carrier-free ¹²⁵¹ (or ¹³¹¹; see Support Protocol 2).

IODINATION AT TYROSINE OR HISTIDINE RESIDUES USING CHLORAMINE T OR IODOGEN

Instead of using Iodo-Beads, Na¹²⁵¹ can be oxidized to its reactive form using soluble chloramine T (N-chlorobenzensulfonamide sodium salt; Greenwood et al., 1963), the solid-phase reagent Iodogen (1,3,4,5-tetrachloro-3α,6α-diphenylglycoluril; Fraker and Speck, 1978), or the enzyme lactoperoxidase (see Alternate Protocol 2). Although slightly less convenient than Iodo-Beads, these methods may give higher recoveries when working with small quantities of peptide (<0.05 mg), which can adsorb to Iodo-Beads. The reagents are inexpensive and stable, and with Iodogen or lactoperoxidase, expose the peptide to relatively mild reaction conditions.

Additional Materials (also see Basic Protocol 1)
Anion-exchange resin, e.g., Dowex 1-X8, chloride form, 100 to 200 mesh (Bio-Rad)
BSA solution: 1.0 mg/ml BSA in PBS, with 0.02% sodium azide (NaN₃)
1.0 mg/ml chloramine T in 0.1 M sodium phosphate, prepared immediately before use
Iodogen (Pierce), dichloromethane, and glass tubes or vials (optional alternate to chloramine T)
Saturated solution of tyrosine in water (~0.4 mg/ml at 25°C)
2.5 mg/ml sodium metabisulfite (Na₂S₂O₅) in 0.1 M sodium phosphate, prepared immediately before use (optional alternate to tyrosine solution)
Pasteur pipet or 1-ml syringe with glass wool plug inserted at bottom
Gel-filtration column, e.g., Sephadex G-10 or G-25 (Pharmacia Biotech), or Excellulose Desalting Column (Pierce; optional alternate to anion-exchange column)

1. Suspend Dowex resin in water and pour into plugged Pasteur pipet. Wash with ≥20 ml BSA solution. Alternatively, equilibrate a gel-filtration column with BSA solution. Avoid introducing air bubbles into the column and do not allow column to dry out.

Because short peptides (<20 residues) are difficult to separate completely from unbound radioactive iodide by gel-filtration columns, an anion-exchange column may be preferred. Highly negatively charged (e.g., phosphorylated) peptides and very short peptides (<10 residues) may be retained on an anion-exchange column and should therefore be desalted by solid-phase extraction (see Basic Protocol 1). The desalting method should be established in advance.
2a. Chloramine T procedure: Place peptide in a small tube and buffer with 0.1 M sodium phosphate, pH 6.0 or 8.5 (see Basic Protocol 1, step 1); bring the total volume to 100 to 300 μl. Add Na^{121} (or Na^{32}I), followed by 10 μl chloramine T (freshly prepared), and mix. Wait 1 min for reaction to occur.

2b. Iodogen procedure: Add sodium iodide to the peptide (as described in step 2a) in a glass tube previously coated with the solid-phase reagent Iodogen by dissolving the solid in dichloromethane and blowing off the solvent under a stream of dry nitrogen. Wait 10 min at room temperature.

Iodogen-coated tubes may be stored for many months at room temperature in a desiccator, protected from light. Because the oxidizing agent is water insoluble, peptide damage is kept to a minimum.

3. Add 50 μl saturated tyrosine solution to quench the reaction. Alternatively, the reaction may be terminated by adding 10 μl sodium metabisulfite, although this reducing agent is potentially more damaging to peptides.

4. Load mixture onto a Dowex or gel-filtration column and collect 0.5-ml fractions in 12 x 75-mm plastic tubes, using BSA solution for elution. Monitor fractions with a hand-held γ counter, or count 1-μl aliquots of each fraction in a γ counter to locate the peptide-containing fractions.

Radiolabeled peptide should pass through the column; unreacted iodide is retained longer and need not be eluted. Dispose of the entire column as radioactive sharps waste.

IODINATION AT TYROSINE OR HISTIDINE RESIDUES USING LACTOPEROXIDASE

Although the degree of oxidative damage to peptides undergoing iodination is difficult to predict, the lactoperoxidase method results in less oxidative damage than chemical oxidative procedures (Marchalonis, 1969). Iodination is confined to tyrosine. Specific radioactivities are likely to be lower by this method. Because lactoperoxidase (mass 77,500 Da) will be iodinated during the reaction, it should be used only if the labeled peptide or protein of interest can be separated from labeled lactoperoxidase by gel filtration (UNIT 8.3), ion-exchange chromatography (UNIT 8.2), or some other means. Alternatively, it may be possible to use lactoperoxidase coupled to beads, or biotinylated lactoperoxidase and avidin-coated beads to remove the labeled enzyme (Sigma). Because the enzyme does not gain access to the cytoplasm, lactoperoxidase-catalyzed iodination is useful for selectively radiolabeling membrane proteins on whole cells, provided iodide concentrations are kept low and the proportion of dead cells is very small (Marchalonis et al., 1971).

Additional Materials (also see Basic Protocol 1)

- 0.2 mg/ml lactoperoxidase in PBS
- 0.01% hydrogen peroxide (H$_2$O$_2$) in PBS (diluted from 30% stock immediately before use)
- 2 U/ml glucose oxidase in PBS and 0.1 M D-glucose in PBS (optional alternate to H$_2$O$_2$)

1. Place peptide in a tube with PBS, ideally at ≥1.0 mg/ml in a small volume.

2. Add 10 μl of 0.2 mg/ml lactoperoxidase.

Azide should not be present in the reaction as it inhibits lactoperoxidase.
3. Add Na\textsuperscript{125}I (or Na\textsuperscript{131}I), cap tube, and mix.

4. Add 10 \mu l of H\textsubscript{2}O\textsubscript{2}. Wait 10 min at room temperature.

   The H\textsubscript{2}O\textsubscript{2} concentration is critical and may need fine-tuning for best results. Alternatively, use a coupled glucose/glucose oxidase system to generate H\textsubscript{2}O\textsubscript{2} in situ, i.e., treat with 10 \mu l of glucose oxidase and 10 \mu l of glucose for 10 min at 4\degree C (Hubbard and Cohn, 1975).

5. Terminate the reaction and remove unbound radioactive iodide (see Alternate Protocol 1, steps 1, 3, and 4).

**EQUIMOLAR IODINATION TO GIVE HIGH YIELDS OF PRODUCT**

Iodination of microgram to milligram amounts of a peptide using low millicurie amounts of carrier-free Na\textsuperscript{125}I (or Na\textsuperscript{131}I), such as those suggested in Basic Protocol 1 and Alternate Protocols 1 and 2, results in trace labeling (iodination of only a small fraction of the peptide molecules). For example, in a reaction between 1 mCi of \textsuperscript{125}I (\textsim 600 pmol) and 500 \mu g of a peptide of mass 1000 Da (500 nmol) or a protein of mass 100,000 Da (5 nmol, or \textsim 200 nmol of tyrosine residues on average), there is at most only enough \textsuperscript{125}I to label well under 1\% of the tyrosines. For proteins, trace labeling is often advantageous as it minimizes the chance that the protein's activity will be lost (Eisen and Keston, 1949; Hunter and Greenwood, 1962); moreover, a sizable fraction of protein molecules will have one iodine atom on at least one of its tyrosines. However, for short peptides over 99\% of the molecules remain unmodified, greatly diluting the specific radioactivities that are possible and complicating the interpretation of experiments in which labeled and unlabeled peptides differ significantly in their activities (see Support Protocol 2).

To label a higher proportion of a peptide, nonradioactive Na\textsuperscript{127}I ("carrier") can be added to \textsuperscript{125}I, giving a higher yield of iodinated product and resulting in a mixture of chemically equivalent \textsuperscript{125}I- and \textsuperscript{127}I-labeled peptides. Perform all steps for iodination as described using Iodo-Beads (see Basic Protocol 1) or chloramine T or Iodogen (see Alternate Protocol 1), with two modifications. First, add 0.1 M Na\textsuperscript{127}I in water to the Na\textsuperscript{125}I prior to using Na\textsuperscript{125}I in the indicated step. The amount of \textsuperscript{127}I should be in moderate molar excess over the peptide (2- to 5-fold) to achieve high yields of iodinated products (70\% to 95\%). Further increasing the amount of \textsuperscript{127}I unnecessarily diminishes the products' specific radioactivities: e.g., at a 4-fold molar excess of \textsuperscript{127}I, only up to 25\% of the \textsuperscript{125}I added can be incorporated, at a 10-fold molar excess only 10\% can be incorporated, etc. Second, a sufficient number of Iodo-Beads (or other oxidizing reagent) must be used to oxidize the total iodide (essentially \textsuperscript{127}I). Use the manufacturer-determined oxidizing capacity of 0.55 \pm 0.05 \mu mol per Iodo-Bead. Note that Iodo-Beads rapidly develop a yellow-brown color when stoichiometric amounts of iodide are added; failure to observe this color indicates deterioration of the beads.

For maximum specific radioactivities, label a small amount of peptide (e.g., 30 \mu g) with up to an equimolar amount of \textsuperscript{125}I (e.g., 40 mCi) and separate the unlabeled peptide from iodinated products by HPLC (see Support Protocol 2).

It is also possible to iodinate with \textsuperscript{127}I only, in which case the products will be nonradioactive but chemically equivalent to the corresponding radiolabeled products. This approach is useful for practicing the iodination procedure and for assessing the effects of iodination on a peptide's activity (Tsomides and Eisen, 1993), as well as for preparing an \textsuperscript{127}I-labeled peptide to be reductively tritiated (see Basic Protocol 4).
SEPARATION OF UNLABELED PEPTIDE FROM IODINATED PRODUCTS BY HPLC

Whether trace iodinated with $^{125}$I only or stoichiometrically iodinated with a mixture of $^{125}$I and $^{131}$I, some peptide will remain unlabeled, and it may be desirable to separate this unlabeled component from the iodinated products. To obtain chemically homogeneous radiolabeled peptides, iodinated products can be resolved from each other and from unlabeled peptide by reversed-phase HPLC. (This optional procedure is distinct from the obligatory separation of peptide from unbound radioactive iodide by solid-phase extraction, anion-exchange chromatography, gel filtration, exhaustive dialysis, or other means as detailed in steps 4 to 8 of Basic Protocol 1.) Because iodine is a bulky, hydrophobic atom relative to hydrogen, the retention times of iodinated peptides will be greater than those of their unlabeled counterparts on reversed-phase HPLC columns (e.g., C18 for short peptides, C4 for longer or more hydrophobic peptides).

In most iodinations, more than one product will be generated, even if the peptide has only a single tyrosine (see Fig. 3.3.1). Because the $pK_a$ of monooiodotyrosine is lower than that of tyrosine (Edelhoch, 1962), electrophilic addition of the second iodine atom may be accelerated relative to the first; however, this reaction will not go to completion even when iodide is present in molar excess over the peptide. When more than two or three iodinatable residues (tyrosine and histidine) are present (as in a protein), the labeled products may be too complex to allow for individual resolution by HPLC, but unlabeled peptide can still be removed from the radiolabeled mixture. Even peptides as long as 50 to 100 residues can be separated from their iodinated derivatives by reversed-phase HPLC.

For peptides with a single iodinatable residue, the two products are usually easily separated by HPLC and can readily be identified by their specific radioactivities, which are in the ratio 1:2 for monooiodinated and diiodinated peptide, respectively. Figure 3.3.2 shows the result of stoichiometrically labeling 2.0 mg of an eight-residue peptide with 20

![Figure 3.3.2](image-url)
mCi Na$^{125}$I mixed with 5 μmol Na$^{127}$I and separating the unreacted, monoiodinated, and diiodinated products by HPLC. If Edman degradation is available, labeled products may be characterized directly by detecting the monoiodinated and diiodinated derivatives of tyrosine and/or histidine (Tsomides and Eisen, 1993). Note that peptides containing tryptophan, methionine, or cysteine residues may undergo oxidation during iodination. The resulting oxidized products (both radiolabeled and unlabeled) may appear as separate peaks on HPLC, complicating the identification of products.

When carrying out stoichiometric iodination with a mixture of $^{125}$I and $^{127}$I, specific radioactivities of the product mixture or of individual HPLC-purified products can be measured as described for the Iodo-Beads procedure (see Basic Protocol 1, step 9). However, when trace labeling with $^{125}$I only and removing unlabeled peptide by HPLC, specific radioactivities may simply be calculated as the specific radioactivity of carrier-free $^{125}$I (~2000 Ci/mmol) multiplied by the molar ratio of $^{125}$I to peptide (e.g., 1 for a monoiodinated derivative, 2 for a diiodinated derivative, etc.). When a mixture of $^{125}$I-labeled products is separated from unlabeled peptide by HPLC but not resolved into its individual components, the same calculation may be applied by taking the average molar ratio of $^{125}$I to peptide to be the number of iodinatable residues. For example, the specific radioactivity of a peptide of mass 1000 Da with two tyrosine residues that is labeled with $^{125}$I and separated from unlabeled peptide by HPLC is ~5 x 10$^8$ cpm/μg, assuming an average of two $^{125}$I per labeled peptide.

NOTE: Although HPLC fractions can be analyzed for radioactivity by transferring them to a γ counter, it is preferable to use an on-line radioisotope detector connected in series with an ultraviolet absorbance detector (see Fig. 3.3.2).

**IODINATION AT LYSINE RESIDUES OR N-TERMINUS USING BOLTON-HUNTER REAGENT**

Many peptides do not contain tyrosine or histidine. Other peptides’ function may be altered if crucial tyrosine or histidine residues are iodinated or if damage to oxidative-sensitive residues occurs during iodination. An alternate strategy is to label primary amino groups on lysine residues and/or at the N-terminus of the peptide using Bolton-Hunter reagent 3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester (Bolton and Hunter, 1973). This reagent introduces a side chain similar to that of tyrosine (see Fig. 3.3.1). Of course, this method carries the potential drawback of altering peptide function if lysine residues (or the N-terminus) are critical for activity; unlike the methods above, peptide charge will be decreased by one unit for each site modified (at pH values below the amino group’s $pK_a$ value).

Because lysine’s ε-amino group and the N-terminal α-amino group have different $pK_a$ values (~10 vs. 8 to 9), it is possible, in principle, to achieve selective labeling by adjusting the pH (lower pH should favor the N-terminus). However, this is difficult in practice, and a mixture of products is to be expected from lysine-containing peptides unless the N-terminal residue is proline (secondary amine) or a protecting group is present on either lysine or the N-terminus.

$^{125}$I-labeled Bolton-Hunter reagent is commercially available in monoiodinated (~2000 Ci/mmol) or diiodinated (~4000 Ci/mmol) form. A cost-saving modification is to prepare $^{125}$I-labeled Bolton-Hunter reagent from the less expensive unlabeled reagent by iodination using Iodo-Beads (see Basic Protocol 1) or chloramine T (see Alternate Protocol 1) followed by extraction of the product into anhydrous benzene. (In this case it is crucial to work quickly to minimize hydrolysis, and specific radioactivities will be lower unless unreacted Bolton-Hunter reagent is removed from the labeled product.) Alternatively, the
peptide can first be modified with unlabeled Bolton-Hunter reagent and then radiiodinated using any of the methods previously described. Because 125I-labeled Bolton-Hunter reagent suffers some degradation by competing hydrolysis during its reaction with peptide, this alternate approach gives a higher incorporation of radioactivity; however, it also results in the radiolabeling of any tyrosine or histidine residues present in the peptide, as well as exposure of the peptide to oxidative conditions.

**Materials**

- 0.1 M sodium borate, pH 8.5
- 0.1% (w/v) gelatin in 0.1 M sodium borate, pH 8.5
- 125I-labeled Bolton-Hunter reagent (Amersham, Du Pont NEN, or ICN Biomedicals); or substitute unlabeled Bolton-Hunter reagent and iodinate later
- Peptide, ideally ≥1 mg/ml in ≤100 μl of 0.1 M sodium borate, pH 8.5
- 1 M glycine in 0.1 M sodium borate, pH 8.5
- Gel-filtration column, e.g., Sephadex G-10
- Dry nitrogen tank and outlet tubing fitted with needle
- Polypropylene or other plastic tubes (12 x 75 mm)

1. Equilibrate a gel filtration column with 0.1% gelatin/0.1 M sodium borate, pH 8.5, to prevent loss of radiolabeled peptide by nonspecific adsorption.

   *BSA is not recommended as a carrier protein in place of gelatin because albumin binds to Bolton-Hunter reagent. If gelatin is undesired in the final preparation (e.g., iodination is to be performed later), condition the column and then reequilibrate in buffer of choice. Alternatively, use a Sep-Pak cartridge instead of gel filtration to separate by-products and salts from the labeled peptide (see Basic Protocol 1, steps 4 to 8).*

2. Immediately prior to use, evaporate 125I-labeled Bolton-Hunter reagent to dryness by inserting a needle through the rubber septum and admitting a gentle stream of dry nitrogen, using the manufacturer-supplied charcoal trap as a vent to contain any released volatile radioiodine. Do not splash solution onto upper parts of the vial.

   *If using unlabeled Bolton-Hunter reagent, prepare a stock solution in anhydrous organic solvent and dilute into borate buffer at the time of reaction to achieve a 3- to 5-fold molar excess over peptide. Unlabeled Bolton-Hunter reagent is also available as a water-soluble (sulfo) derivative (at higher cost), obviating the need for organic solvent and offering an advantage for labeling cell surface proteins because the sulfo derivative is membrane impermeable.*

3. Chill peptide in borate buffer to 0°C on ice. Add peptide to vial containing dried 125I-labeled Bolton-Hunter reagent (or freshly dissolved unlabeled Bolton-Hunter reagent) and wait 15 to 30 min at 0°C, agitating the reaction occasionally.

   *Incorporation is greatest when using high peptide concentrations. Phosphate or bicarbonate buffers, pH 8.5, may be used instead of borate, but avoid amine-containing buffers (ammonium, glycine, Tris) as well as azides and thiols. Being an N-hydroxysuccinimide ester, Bolton-Hunter reagent is sensitive to hydrolysis, with a half-life of a few hours at pH 7.0 and only ~10 min at pH 8.5; however, its reaction with amino groups is greatly accelerated at the higher pH since more amine is in the deprotonated (active) form.*

4. Add 100 μl of 1 M glycine in 0.1 M sodium borate, pH 8.5, to quench the reaction and wait 5 min, or use a corresponding amount of Tris or any other amine for quenching.

   *Longer incubations at 0°C (>4 hr) may improve yields and obviate the need for quenching by consuming all of the reagent.*
5. Load mixture onto gel filtration column and collect 0.5-ml fractions in 12 × 75-mm plastic tubes, using borate (or other) buffer for elution. Monitor fractions with a hand-held γ counter, or count aliquots of each fraction in a γ counter to locate the peptide-containing fractions.

6. Resolve products by HPLC if desired and determine specific radioactivities (see Support Protocol 2).

**14C OR 3H LABELING AT LYSINE RESIDUES OR N-TERMINUS BY ACETYLATION USING ANHYDRIDE**

There are two main advantages of labeling with 14C or 3H instead of 125I. First, for peptides that are unacceptably altered as a result of iodination, different sites may be targeted for modification (i.e., lysine or cysteine), or the peptide may even be labeled without changing its structure by replacing carbon or hydrogen atoms with the corresponding radioisotope (see Basic Protocol 4 and Basic Protocol 5). Second, the half-lives of 14C and 3H are much longer than that of 125I (5760 and 12.26 years, respectively, vs. 60 days), so labeling need not be done often. However, the specific radioactivities of peptides labeled with 14C or 3H are generally correspondingly lower, and the labeling techniques are not as widely applied as radiiodination. Also, counting β emissions involves the use of a liquid scintillation cocktail and is therefore slightly more cumbersome than counting γ emissions.

Acetylation with [14C]- or [3H]anhydride is a simple reaction that will modify amino groups (lysine and/or the N-terminus of a peptide; Fig. 3.3.1; Fraenkel-Conrat and Colloms, 1967). The most common side reaction is O-acetylation of tyrosine, which is reversible under mild alkali conditions (Means and Feeney, 1971).

**CAUTION:** All procedures should be carried out in an externally vented hood.

**Materials**

- Saturated sodium acetate solution
- Peptide, ideally ≥1 mg/ml in ≤100 μl H2O or sodium acetate
- [14C]- or [3H]acetic anhydride (Amersham, Du Pont NEN, ICN Biomedicals, or Sigma)
- Acetic anhydride, unlabeled (optional)

1. Add 1 vol of saturated sodium acetate solution to the peptide (unless already in this buffer) and cool to 0°C on ice.

2. Add [14C]- or [3H]acetic anhydride, with or without 2 molar equivalents of additional unlabeled acetic anhydride, in four equal installments at 10- to 15-min intervals.

   *Because the anhydride is rapidly hydrolyzed, addition over several installments is the preferred method. The use of excess unlabeled anhydride increases the extent of reaction but dilutes the specific radioactivity of the products (analogous to stoichiometric vs. trace iodination; see Support Protocol 1).*

3. Remove by-products by gel filtration (UNIT 8.3), dialysis (APPENDIX 3B), or solid-phase extraction (see Basic Protocol 1, steps 4 to 8), and count fractions in an appropriate liquid scintillation cocktail.

   *Resolution of individual products may be possible by HPLC (see Support Protocol 2).*
**ALTERNATE PROTOCOL 3**

**14C OR 3H LABELING AT LYSINE RESIDUES OR N-TERMINUS BY REDUCTIVE ALKYLATION**

An alternative two-step procedure used to label peptides is treatment with an aldehyde followed by reduction of the resulting Schiff base with sodium cyanoborohydride (Fig. 3.3.1; Tack and Wilder, 1981; Jentoft and Dearborn, 1983). Most aldehydes give the monosubstituted alkylamine product, but formaldehyde goes on to give the dimethyl product. 14C can be incorporated via the aldehyde, or 3H can be added via labeled sodium cyanoborohydride. The latter gives higher specific radioactivities, reportedly ≥50 Ci/mmol. One advantage of this approach over acetylation is that the net charge on amino groups is not substantially altered (there is only a small change in pKₐ value).

**CAUTION:** All procedures should be carried out in an externally vented hood.

**Materials**

- 0.2 M sodium borate, pH 9.0
- Peptide, ideally ≥1 mg/ml in 0.1 to 1.0 ml of 0.2 M sodium borate, pH 9.0
- [3H]sodium borohydride (Amersham, Du Pont NEN, or ICN Biomedicals) or [3H]sodium cyanoborohydride (Amersham)
- 0.01 M NaOH
- 37% (12.4 M) formaldehyde stock
- [14C]formaldehyde and unlabeled sodium cyanoborohydride (optional alternative for labeling with 14C instead of 3H)

1. Add an equal volume of 0.2 M sodium borate, pH 9.0, to the peptide (unless already in this buffer) and cool to 0°C on ice.

2. Dilute formaldehyde stock (12.4 M) to 0.1 to 0.2 M and add to peptide on ice to give a 2- to 6-fold molar excess over amino groups (e.g., 10 to 20 μl).

   More concentrated formaldehyde in this step may lead to peptide cross-linking.

   For 14C labeling, use [14C]formaldehyde in this step.

3. Immediately dissolve [3H]sodium borohydride (or [3H]sodium cyanoborohydride) in 0.01 M NaOH to a final concentration of 1.0 Ci/ml and add to peptide on ice for 10 min. Use a molar ratio of sodium borohydride to formaldehyde of between 0.25 and 0.40 (e.g., 30 to 50 μl).

   Sodium cyanoborohydride is a superior reagent for this purpose because it reduces the Schiff base formed initially between the peptide and formaldehyde without reducing disulfides or aldehydes. [14C]sodium borohydride or [3H]sodium cyanoborohydride should be stored frozen in small aliquots rather than frozen and thawed repeatedly.

   For 14C labeling, use unlabeled sodium cyanoborohydride in this step.

4. Remove by-products by gel filtration (UNIT 8.3), dialysis (APPENDIX 3B), or solid-phase extraction (see Basic Protocol 1, steps 4 to 8), and count fractions in an appropriate liquid scintillation cocktail.

   Resolution of individual products may be possible by HPLC (see Support Protocol 2).
14C OR 3H LABELING AT CYSTEINE RESIDUES USING IODOACETIC ACID OR IODOACETAMIDE

To label cysteine residues, a peptide is reduced and allowed to react with either iodoacetic acid (to give the carboxymethyl derivative) or iodoacetamide (to give the carboxamidomethyl derivative, which is uncharged; see Fig. 3.3.1). For large peptides containing disulfide bonds, this procedure is often used to denature the peptide irreversibly. Carboxymethylcysteine content is readily determined by amino acid analysis following acid hydrolysis. [14C]- or [3H]iodoacetic acid and [14C]iodoacetamide are commercially available for radiolabeling. Possible side reactions are alkylation of histidine, methionine, or lysine, each of which can usually be controlled by pH and other reaction conditions (Crestfield et al., 1963; Means and Feeney, 1971).

CAUTION: All procedures should be carried out in an externally vented hood.

Materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M Tris-Cl/6 M guanidine-HCl/0.002 M disodium EDTA, pH 8.6</td>
<td>Peptide, ideally 1 mg/ml in 0.1 to 1.0 ml of 0.5 M Tris-Cl/6 M guanidine-HCl/0.002 M disodium EDTA, pH 8.6</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>1.0 M NaOH</td>
</tr>
<tr>
<td>[14C]- or [3H]iodoacetic acid or [14C]iodoacetamide (Amersham, Du Pont NEN, or ICN Biomedicals)</td>
<td>Iodoacetic acid or iodoacetamide, unlabeled (optional)</td>
</tr>
</tbody>
</table>

1. Peptides containing disulfide bonds or significant tertiary structure require reduction and/or denaturation prior to alkylation of cysteine residues. For this, prepare a solution of peptide in 0.5 M Tris-Cl/6 M guanidine-HCl/0.002 M disodium EDTA (pH 8.6), incubate for 1 hr at 37°C, add dithiothreitol (50-fold molar excess over disulfide), and incubate overnight at 37°C.

2. Cool to room temperature and add desired amount of radiolabeled iodoacetic acid (or iodoacetamide) dissolved in 1/10 vol of 1.0 M NaOH for 30 min in the dark. Optionally, include additional unlabeled iodoacetic acid (or iodoacetamide) at a 2-fold molar excess over the dithiothreitol added to increase the extent of the reaction (analogous to stoichiometric vs. trace iodination; see Support Protocol 1).

3. Remove by-products by gel filtration (UNIT 3), dialysis (APPENDIX 3B), or solid-phase extraction (see Basic Protocol 1, steps 4 to 8), and count fractions in an appropriate liquid scintillation cocktail.

Resolution of individual products may be possible by HPLC (see Support Protocol 2).

3H LABELING AT TYROSINE RESIDUES BY REDUCTION OF 127I-LABELED PEPTIDE

It is possible to label one or more tyrosine residues of a peptide stoichiometrically with 3H to a relatively high specific radioactivity (e.g., 50 Ci/mmol). Since 3H substitutes for hydrogen, the peptide’s structure remains unaltered. However, this approach is more laborious and expensive than the previous methods, requiring the preparation of a 127I-labeled peptide that is then catalytically reduced in the presence of tritium gas, leading to replacement of each 127I (or other halogen atom) with 3H.

The 127I-labeled peptide precursor can be produced in two ways: by stoichiometric iodination with 127I (see Basic Protocol 1, Support Protocol 1, and Support Protocol 2) or by incorporating a suitably protected 127I-labeled tyrosine derivative (either mono-
iodinated or diiodinated) during de novo peptide synthesis (e.g., N-Boc-O-3-bromo-benzyl-3,5-diiodo-L-tyrosine, available from Peninsula). The $^{127}$I-labeled peptide is then submitted to a specialized facility for reductive tritiation (Amersham and Du Pont NEN offer this service).

INTRODUCTION OF LABELED AMINO ACID RESIDUES DURING PEPTIDE SYNTHESIS

The most specific and least disruptive labeling procedure is incorporation of a suitably protected radioactive amino acid into any desired position of a peptide during its solid-phase synthesis. However, because this requires an entire synthesis rather than use of just an aliquot of peptide, and because the protected derivatives of radioactive amino acids are not generally available and therefore must be prepared, it is not commonly done. Furthermore, introduction of a radioactive amino acid into a peptide synthesizer will be possible only if the operator of the instrument is prepared to work with radioactive samples. However, amino acids containing $^{14}$C, $^3$H, or $^{35}$S (for methionine) are available, procedures for attaching various protecting groups to these amino acids are quite well established, and the result may be an abundant supply of a radiolabeled peptide unperturbed in structure and not obtainable by any other means. Specific radioactivities are typically two to three orders of magnitude lower than for radioiodinated peptides.

The simplest approach is to select a radioactive amino acid that requires protection during solid-phase peptide synthesis only on its α-amino group and not on its side chain (e.g., $^{14}$C- or $^3$H-labeled alanine, isoleucine, leucine, valine, or phenylalanine, all available from Amersham, Du Pont NEN, ICN Biomedicals, or Sigma). If classical t-butyloxycarbonyl (t-BOC) chemistry is to be used during peptide synthesis, a variety of procedures are available for attaching t-BOC to α-amino groups (Stewart and Young, 1984); one such procedure is outlined below for leucine.

**Materials**

- L-Leucine, unlabeled
- L-$^{[14]}$C- or L-$^{[3]}$H]leucine, 1 to 5 mCi
- Triethylamine
- 2-(t-butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC-ON, Aldrich)
- Dioxane
- Diethyl ether, anhydrous
- 1.0 M HCl, 4°C
- Separatory funnel

1. Add 131 mg L-leucine (1 mmol) and the desired number of millicuries L-$^{[14]}$C- or L-$^{[3]}$H]leucine (supplied in water) to a 25-ml round-bottom flask.

   *Other amino acids or their side chain protected derivatives can be substituted for leucine. Unlabeled amino acid is used because the amount of $^{14}$C- or $^3$H-labeled material is very small in molar terms (e.g., 2 mCi $^[3]H$leucine is <1 μmol); safety and cost considerations generally preclude the use of stoichiometric amounts of radiolabeled amino acid.*

2. Add 210 μl triethylamine (1.5 mmol).

3. Add 271 mg BOC-ON (1.1 mmol).

4. Add a mixture of 600 μl dioxane and enough water to bring the total volume of water to 600 μl; e.g., if the radiolabeled amino acid is supplied in 400 μl water, add 600 μl dioxane and 200 μl water.
5. Stir with a magnetic stir bar 3 hr at room temperature. The yellow, heterogeneous reaction will become homogeneous.

6. Add 2 ml water and 2 ml diethyl ether and transfer the reaction mixture to a separatory funnel.

   The aqueous (bottom) layer contains the triethylammonium salt of the t-BOC-amino acid product; the ether (upper) layer contains an oxime by-product from the reaction (bright yellow).

7. Drain the aqueous layer and wash it three times with an equal volume of diethyl ether in the separatory funnel to remove unwanted oxime.

8. Add a small volume of 4°C 1.0 M HCl to bring the aqueous layer to pH 2 and precipitate the t-BOC-amino acid product.

9. Add an equal volume of diethyl ether to the aqueous layer in the separatory funnel, shake, and drain off the aqueous layer. Save the ether layer and repeat ether extraction of the aqueous layer two more times. Pool the combined ether layers into an Erlenmeyer flask.

   The t-BOC-amino acid product now partitions into the ether layer after being converted by HCl from a salt to the free acid. Some t-BOC-amino acids (serine, threonine) remain soluble in water and are best extracted if the aqueous layer is first saturated with NaCl before adding ether.

10. Allow the product to crystallize for several hours on ice or overnight at 4°C. First blowing off some ether with a gentle stream of dry nitrogen gas may be helpful. Wash the white, crystalline product carefully with cold diethyl ether.

   The t-BOC-amino acid should be checked for nonreactivity of its amino group by a ninhydrin (Sarin et al., 1981) or other analytical test prior to using it in peptide synthesis.

### SELECTIVE LABELING ON N-TERMINUS DURING PEPTIDE SYNTHESIS

The N-terminus of a peptide that contains one or more lysine amino groups can be selectively labeled after the peptide has been synthesized by solid-phase methods but before amino acid side chains (including that of lysine) have been deprotected. For example, N-terminal acetylation of a newly synthesized protected peptide can be used to label the peptide with ¹⁴C or ³H without affecting lysine residues that might be important for activity (see Basic Protocol 3). Alternatively, addition of a Bolton-Hunter group allows subsequent iodination at this position (see Basic Protocol 2); tyrosine, if present, will also be iodinated unless radiolabeled Bolton-Hunter reagent is used.

Various other reagents can be used to modify a peptide at its N-terminus. For example, a dinitrophenyl (DNP) group can be attached specifically to the N-terminus of a protected resin-bound peptide using dinitrofluorobenzene (100 mol equivalents), N,N-diisopropylethylamine (20 mol equivalents), and dichloromethane as the solvent for the coupling reaction (2 hr at room temperature with shaking in the dark; T. Tsomides, unpub. observ.).

Specific details of the protocols to be used for N-terminal labeling reactions depend on the particular chemistries involved in the peptide’s synthesis and cleavage. In particular, any label attached selectively to the N-terminus must be stable during the peptide side chain deprotection step (e.g., hydrogen fluoride or trifluoroacetic acid). In the example above, DNP is stable during deprotection with trifluoromethane sulfonic acid.
COMMENTARY

Background Information

Detection of minute quantities of a peptide or protein is often made possible by radiolabeling procedures. The most common labeling strategy, radiiodination with $^{125}\text{I}$, is relatively straightforward to perform and low in cost. Thus, a peptide with two tyrosine residues can be radiolabeled with $^{125}\text{I}$ to a specific radioactivity of $5 \times 10^9 \text{cpm/\mu g}$ (see Support Protocol 2), allowing its detection at subfemtomole levels, or several orders of magnitude lower than absorbance- or fluorescence-based detection methods (UNIT 3.1).

The main drawback of radiiodination (other than the need to work safely with a potentially hazardous radioisotope) is that some peptides may be unreactive or may be damaged by the introduction of iodine atoms (each as large as a phenyl ring) or by the oxidative conditions typically employed during the labeling reaction. These problems may be more acute for a large protein with native tertiary structure and more oxidation-sensitive residues (tryptophan, methionine, and cysteine) than for a short peptide; nevertheless, even small peptides can be significantly altered in their biological activities by iodination. In most cases these problems can be obviated by modifying the radiolabeling strategy (see Table 3.3.1).

Standard radiiodination methods rely on the oxidation of $^{125}\text{I}$ (in the form of Na$^{125}\text{I}$) to its reactive state (which can be considered "$I^-$") for electrophilic addition to the phenolic and imidazole side chains of the amino acids tyrosine and histidine, respectively (see Fig. 3.3.1). At a pH between 6 and 7, tyrosine labeling predominates, whereas at a higher pH there is also labeling on histidine (Wolf and Covelli, 1969; Tsumi and Eisen, 1993). Different methods of oxidizing the $^{125}\text{I}$ include IodoBeads (a convenient immobilized form of chloramine T; Basic Protocol 1), soluble chloramine T, and Iodogen (a water-insoluble reagent plated onto tubes prior to use; Alternate Protocol 1). A gentler oxidative procedure uses the enzyme lactoperoxidase along with hydrogen peroxide (Alternate Protocol 2). All four methods can be expected to give reasonable specific radioactivities (lowest for lactoperoxidase), and their relative merits have been assessed for some peptides (Thean, 1990; Rizzino and Kazakoff, 1991).

For peptides that lack tyrosine and histidine or that are unacceptably altered by standard iodination procedures, an alternate strategy is to label free amino groups (lysine side chains and the peptide N-terminus, unless it is proline or blocked) using an iodinated acylating agent (e.g., Bolton-Hunter reagent; see Basic Protocol 2 and Fig. 3.3.1; Bolton and Hunter, 1973). This mild coupling reaction avoids direct contact of the peptide or protein with harsh oxidizing conditions, but the resulting labeled product is likely to have a lower specific radioactivity (because Bolton-Hunter reagent suffers competing hydrolysis during the reaction); a second drawback is the relatively high cost of this reagent. A similar though less commonly tried modification using an iodinated amidinating reagent (Wood et al., 1975) has the potential advantage that the positive charge on lysine ($pK_a=10$) is retained in the final labeled product (an amide with $pK_a=11.5$ to 12.5).

Besides labeling with $^{125}\text{I}$, it is straightforward to radiolabel peptides or proteins with $^{14}\text{C}$ or $^3\text{H}$ using various methods (see Table 3.3.1). This may be desirable if all iodination options fail, or if it is critical to obtain a radioactive product devoid of structural alterations. Usually the products of $^{14}\text{C}$ or $^3\text{H}$ labeling will have much lower specific radioactivities than the products of $^{125}\text{I}$ labeling, reflecting the different disintegration rates for these radioisotopes (~35,000 and 75 times lower for $^{14}\text{C}$ and $^3\text{H}$, respectively, than for $^{125}\text{I}$). Also, counting $\beta$ emissions requires liquid scintillants and is therefore less convenient than counting $\gamma$ emissions, which requires no additives to the sample. However, the longer half-lives of $^{14}\text{C}$ and $^3\text{H}$ (5760 and 12.26 years, respectively) render the resulting products useful for much longer than $^{125}\text{I}$-labeled products (~60 days).

A selection of protocols for labeling a peptide with $^{14}\text{C}$ or $^3\text{H}$ is given in this unit: acetylation on amino groups with acetic anhydride (see Basic Protocol 3), reductive alkylolation on amino groups with formaldehyde and sodium cyanoborohydride (see Alternate Protocol 3), and alkylolation on cysteine with iodoacetic acid or iodoacetamide (see Alternate Protocol 4). Each of these procedures results in modification of the peptide structure, albeit on different residues than does standard radiiodination (Fig. 3.3.1). To label a peptide without altering its structure, two alternatives are available: $^3\text{H}$ labeling by catalytic reduction of (nonradioactive) $^{125}\text{I}$-labeled tyrosine (see Basic Protocol 4), and de novo peptide synthesis using a radiolabeled (and suitably protected) amino acid. 

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Critical Parameters and Troubleshooting

Amount of radioisotope

The amount of radioisotope to be used is often a compromise among the desire for high specific radioactivities (curies per millimole or cpm per microgram), a practical limit to the amount of radioactivity that can be afforded and safely handled, and (particularly for a large protein) any risk of altering tertiary structure and safely handled, and (particularly for a large protein) any risk of altering tertiary structure by excessive substitution or radiation damage. Commonly, 1 to 5 mCi of $^{125}$I (or $^{131}$I) is used; however, it is possible to scale up the $^{125}$I and increase specific radioactivities proportionately, because iodide is almost always limiting relative to iodinatable sites (e.g., 2 mCi $^{125}$I is only ~1.2 nmol). Conversely, lowering the amount of peptide or protein will also improve specific radioactivities, but lowering the amount of substrate too far will cause unacceptable losses of material by nonspecific adsorption to tubes; a practical lower limit is 5 to 10 μg of peptide.

Sensitive amino acid residues

During oxidative iodinations, the amino acid residues at greatest risk for damage are tryptophan, methionine, and cysteine. The problem is greatest for tryptophan, as an oxidized indole ring cannot be restored by subsequent treatment with a reducing agent such as dithiothreitol. When these residues are critical for peptide function, the lactoperoxidase or Bolton-Hunter methods may be preferred for $^{125}$I labeling.

Iodination work-up

Several options are available for the removal of unbound radioactive iodide from a peptide once the reaction is complete, including solid-phase extraction (e.g., using Sep-Pak cartridges; Basic Protocol 1), anion-exchange chromatography (UNIT 8.2), gel filtration (UNIT 8.3), and dialysis (APPENDIX 3A). The choice of desalting procedure will depend on the peptide, particularly its size and charge. For small peptides the Sep-Pak method works well, because the peptide binds to the Sep-Pak cartridge, allowing extensive washing to remove all traces of free iodide (Tsomides and Eisen, 1993). For larger peptides and proteins, anion-exchange chromatography (e.g., Dowex column) and gel filtration are popular, as the peptide is eluted immediately while iodide is retained on the column. To ensure that the removal of unbound iodide is complete, many investigators precipitate a radiolabeled protein with trichloroacetic acid (TCA) to determine the proportion of TCA-precipitable counts. Dialysis is a time-consuming but effective method for removing traces of iodide, provided the peptide is large enough to be retained by the dialysis membrane used. Note that for highly negatively charged peptides, there may be some retention on anion-exchange columns. For peptides of 10 to 20 amino acids, separation of labeled peptide from free iodide may be poor on an anion-exchange or gel-filtration column because the peptide’s molecular mass is so close to the exclusion limit of the column. For such peptides the Sep-Pak method may be advantageous.

Once unbound radioactive iodide has been removed, the labeled peptide mixture can be used as is, or the individual products can be separated from each other as well as from the unreacted peptide that is almost always present (see Support Protocol 2). If unlabeled peptide is removed by reversed-phase HPLC, the specific radioactivity of the labeled peptide will be maximized and will equal the specific radioactivity of carrier-free $^{125}$I (~2000 Ci/mmol) multiplied by the number of $^{125}$I atoms incorporated per peptide molecule.

Poor iodination results

Table 3.3.2 gives potential causes and possible remedies for poor iodination results.

Anticipated Results

In radioiodinating a purified protein, it is customary to aim for incorporation of up to one $^{125}$I atom per protein molecule, i.e., ~20 μCi/μg for a protein of molecular mass 100,000 Da. For short peptides it is rare to label all the molecules, but specific radioactivities are typically on the same order (e.g., 20 μCi/μg for a peptide of mass 1000 Da, representing only 1% of the peptide labeled and 99% unlabeled). More labeled peptide is obtained when nonradioactive $^{125}$I is added during the iodination reaction (see Support Protocol 1). For example, Figure 3.3.2 shows the results of iodinating an eight-residue peptide with 20 mCi $^{125}$I and 5 μmol indolyl-2-carboxylic acid, followed by separating the products from unlabeled peptide by HPLC.
Table 3.3.2 Troubleshooting the Iodination Reaction

<table>
<thead>
<tr>
<th>Problem</th>
<th>Potential cause</th>
<th>Possible remedy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor iodine incorporation</td>
<td>Peptide contains reducing agent, e.g., from synthesis/cleavage</td>
<td>HPLC-purify or desalt peptide prior to use</td>
</tr>
<tr>
<td></td>
<td>Peptide contains detergent or other component that becomes iodinated</td>
<td>HPLC-purify peptide</td>
</tr>
<tr>
<td></td>
<td>Tyrosine/histidine residues are unreactive because of tertiary structure</td>
<td>Treat peptide with denaturing agent</td>
</tr>
<tr>
<td></td>
<td>Iodo-Beads are unreactive</td>
<td>Test for color (see Support Protocol 1); use fresh lot</td>
</tr>
<tr>
<td>Poor peptide recovery</td>
<td>Solubility problem</td>
<td>Change solvent system or adjust pH</td>
</tr>
<tr>
<td></td>
<td>Failed elution from Sep-Pak</td>
<td>Replace with ion exchange, gel filtration, or dialysis</td>
</tr>
<tr>
<td></td>
<td>Nonspecific losses</td>
<td>Increase amount of peptide or add carrier protein (after iodination reaction)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Use chloramine T instead of Iodo-Beads</td>
</tr>
<tr>
<td>Labeled peptide unreactive</td>
<td>Tyrosine is crucial for activity</td>
<td>Iodinate for shorter time and/or use less label</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Use alternate strategy, e.g., Bolton-Hunter reagent</td>
</tr>
<tr>
<td>Other peptide damage</td>
<td></td>
<td>Use alternate strategy</td>
</tr>
</tbody>
</table>

Time Considerations

Each of the listed protocols is easily completed within a few hours (not including HPLC purification if chosen), and with practice can be done in even less time. The resulting radioactive peptides will, of course, decay according to the half-life of the radioisotope used (or in some cases faster, if there is radiation damage). To calculate specific radioactivity after $t$ days, simply multiply the initial specific radioactivity by $2^{(-t/t_\text{half})}$, where $t_\text{half}$ is the half-life of the radioisotope (60 days for $^{125}$I).

Literature Cited


**Key References**


Excellent overview of side chain chemistries, including but not limited to radiolabeling procedures.

Tsomides and Eisen, 1993. See above.

*Describes use of Sep-Pak to remove free iodine, HPLC to separate iodinated products, Edman degradation to identify products, and pH dependence of tyrosine vs. histidine iodination using Iodo-Beads.*

Contributed by Ton N.M. Schumacher and Theodore J. Tsomides
Massachusetts Institute of Technology
Cambridge, Massachusetts
CHAPTER 12

Role of Ligand Density in T Cell Reactions

Theodore J. Tsomides

BACKGROUND

Studies in the early 1970s revealed a requirement that T lymphocytes responding to antigens on other cells (B lymphocytes or macrophages) must share MHC specificities with the antigen-bearing cells they recognize in order to become functional helper T cells.3 Zinkernagel and Doherty unequivocally showed that virus-specific cytotoxic T lymphocyte (CTL) responses also require shared MHC specificities between the T cell and the virus-infected target cell ("MHC restriction").4 Subsequently, molecular genetic studies led to characterization of the highly polymorphic genes of the MHC and to sequencing of the genes encoding subunits of the antigen-specific T cell receptor (TCR). Details of the tripartite interaction between antigen, MHC protein, and TCR did not begin to emerge until the mid-1980s, when landmark studies of Townsend et al showed that the antigens recognized by T cells are short peptides (generally 8-25 amino acids in length),5 and x-ray crystallographic structure determinations provided striking images of MHC molecules complexed with mixtures of cellular peptides6-7 or individual synthetic peptides.8,9 The 1990s have seen an explosion of information regarding peptide-MHC interactions and an accompanying heightening of interest in applying this information to clinical areas such as autoimmunity, vaccine design, and immunotherapy (e.g., refs. 12-15).

The modern view of MHC function is conveyed by the term "antigen presentation." Class I MHC (MHC-I) molecules sample the contents of a cell by binding to short peptides that result from partial degradation of cytosolic proteins. These peptides are translocated into the endoplasmic reticulum (see chapter 3), where they come into contact with newly formed MHC-I molecules that are then transported to the cell surface.10 Hence the MHC-I molecules of a cell undisturbed by any pathologic process are loaded with peptides derived from normal cellular proteins. These noncovalent peptide-MHC-I complexes will not ordinarily be recognized by the host's own T cells because of immune tolerance to self, but they will be recognized by T cells from an MHC-different (allogenetic) individual. Some proportion of MHC-I molecules at the surface of a tumor cell or a cell invaded by a virus or other pathogen will carry peptides that are recognized by host T cells.

either because the peptide sequences are foreign to the responding organism or because tolerance to native sequences is somehow breached. The minimum number of peptide-MHC-I complexes per target cell needed to elicit T cell function varies in different systems (see below), but for the sake of argument suppose that an average minimum number is \( \approx 100 \). Since nucleated cells of vertebrate organisms express about half a dozen different MHC-I proteins at their surface, each typically at a level of \( 10^2 \) to \( 10^3 \) molecules per cell, the universe of antigens potentially recognized by MHC-I-restricted T cells (mostly CD8\(^+\) CTL) will then consist of up to a few thousand different peptides representing the internal contents of each cell of an organism.

Class II MHC (MHC-II) molecules also bind peptides and present them to T cells (mostly CD4\(^+\) helper T cells), but in this case the peptides are derived from extracellular or membrane-associated proteins. MHC-II function will not be treated here (see chapters 6 and 13), although similar principles probably apply to T cell recognition of peptide-MHC-I and peptide-MHC-II complexes. This chapter will consider the peptides recognized (as peptide-MHC-I complexes) by CD8\(^+\) CTL and the broad question of what factors may limit the efficacy of CTL-mediated lysis of target cells. A viewpoint that the density of naturally processed peptide-MHC-I complexes is often a critical determinant of CTL function will be presented.

**THE STUDY OF PEPTIDES RECOGNIZED BY CD8\(^+\) CTL**

The peptides recognized by CD8\(^+\) CTL were first studied by adding synthetic peptides to appropriate target cells (generally transformed cell lines expressing the MHC-I protein restricting a particular CTL clone), thereby sensitizing the target cells for lysis by CTL.\(^{1,7}\) By screening sets of synthetic peptides (e.g., derived from viral protein sequences), optimally active peptides could be identified that sensitized target cells at extracellular concentrations < 1 \( \mu M \). Often these peptides were nine amino acids in length. However, it remained unclear whether the same peptides arose physiologically (e.g., during viral infection) or merely mimicked the naturally processed peptides, which might have different N- or C-termini (reviewed in ref. 18).

Why is this distinction between naturally processed and active synthetic peptides of more than passing interest? One of the factors likely to influence the degree of lysis by CTL is the density of peptide-MHC-I complexes on target cells, termed ligand density or epitope density (Fig. 12.1). Ligand density on natural CTL targets such as tumors, virally infected cells, or self tissues in autoimmunity cannot be determined until the precise identity of a relevant naturally processed peptide is known. Therefore studies using only synthetic peptides, while of enormous value in mapping out T cell epitopes and defining the conditions required for CTL lysis, fail to permit analysis of the role of ligand density in host T cell reactions in situ.

Information regarding naturally processed peptides was obtained following the realization that purified or cell surface MHC molecules (both MHC-I and MHC-II) harbor tightly-bound endogenous peptides that can be removed by acid treatment.\(^{19,20}\) The first natural peptides were isolated from virally infected cells and identified using virus-specific CTL.\(^{21,22}\) Other natural peptides were isolated and sequenced based on their relative abundance in purified MHC preparations rather than on their recognition by specific CTL.\(^{23,24}\) Falk et al showed that acid treatment of MHC-I molecules followed by Edman sequencing of the resulting peptide mixtures yielded patterns which they called motifs: e.g., for the human MHC-I molecule HLA-A2.1, bound peptides
Role of Ligand Density in T Cell Reactions

Target cell
- density of peptide-MHC complexes on target cells or antigen presenting cells
- suitable peptides not generated or transported into endoplasmic reticulum
- peptides present at cell surface but quantity insufficient for effective T cell response
- antigen escape mutations thwart effective T cell response
- poor MHC-I expression (e.g., neuronal cells)
- physiological lifetime of peptide-MHC complexes (peptide dissociation rate)

T cell-target cell interaction
- affinity of the TcR for peptide-MHC complexes
- kinetics (on-rate and/or off-rate) of the TcR-peptide-MHC reaction
- coreceptor and adhesion molecule interactions between T cell and target cell

T cell
- density of TcR on the T cell
- density of adhesion molecules on the T cell
- functional status of T cell (e.g., lymphokine requirements, lytic capacity of a CTL)
- T cell tolerance/anergy

tended to be nonamers with Leu or Met at position two and Val or Leu at position nine.24 These motifs accorded well with structural information from x-ray crystallography25-31 and encouraged the prediction of T cell epitopes from parent protein sequences.28,32 More refined techniques for predicting which peptides can bind strongly to a given MHC protein and be recognized by T cells have since been developed (see chapter 10). However, the number of proved naturally processed peptides recognized by known CTL has remained surprisingly limited (Table 12.1).

In order to characterize a naturally processed T cell epitope, several steps are required. First, the peptide must be identified. This requires a sufficient number of cells from which to isolate the peptide and a means for its detection. For example, in order to identify a naturally processed tumor peptide recognized by anti-tumor CTL, a relatively large number of tumor cells is needed, depending upon the peptide’s abundance, the sensitivity of the detection method, and the overall efficiency (yield) of the isolation procedure. Assuming that an unknown peptide is present at a level of 100 molecules per cell (cf. Table 12.1) and that a 50% yield is possible, more than 10^11 cells are needed to obtain just 10 pmol of peptide. Peptide sequencing by Edman degradation generally requires at least this much peptide;34-36 mass spectrometry, while not as routinely available, offers the chance to sequence far lesser amounts of peptide.34,36,37 Once a suitable starting number of cells is available, the purification scheme must be chosen. This involves several choices, including whether to isolate MHC molecules by immunoaffinity methods or simply lyse whole cells without MHC purification; each approach has its advantages and disadvantages.34-36 A third strategy has been described in which intact cells are treated with a citrate/phosphate buffer to release MHC-bound peptides without rupturing the cells, which then regenerate peptide-MHC complexes.36,37

Peptide detection during the purification must rely on a suitable assay, and by far the most sensitive is bioassay with a specific clone of T cells: sensitivities of ≤10^-12 M have been obtained for several CTL, and a theoretical limit of 10^-15-10^-14 M has been postulated.40 Thus, crude mixtures of peptides obtained from whole cells or from isolated MHC molecules are fractionated by HPLC, each fraction is tested for
Table 12.1. Measured ligand densities for naturally processed peptides recognized by CD8* CTL

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Source</th>
<th>MHC-I</th>
<th>Peptide recovery per cell</th>
<th>Estimated yield</th>
<th>Calculated ligand density</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TYQRTRLV</td>
<td>influenza NP 147-155</td>
<td>H-2K&lt;sup&gt;d&lt;/sup&gt;</td>
<td>220-540</td>
<td>&gt;100%</td>
<td>220-540</td>
<td>22, 51</td>
</tr>
<tr>
<td>ASNENMETM</td>
<td>influenza NP 366-374</td>
<td>H-2D&lt;sup&gt;p&lt;/sup&gt;</td>
<td>55</td>
<td>25%</td>
<td>220</td>
<td>22, 51</td>
</tr>
<tr>
<td>ROVYVQGL</td>
<td>vesicular stomatitis virus NP 52-59</td>
<td>H-2K&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5-10% of total peptide</td>
<td></td>
<td></td>
<td>21</td>
</tr>
<tr>
<td>SIINFEKL</td>
<td>ovalbumin 257-264</td>
<td>H-2K&lt;sup&gt;e&lt;/sup&gt;</td>
<td>88</td>
<td></td>
<td>&gt;88</td>
<td>70</td>
</tr>
<tr>
<td>GYKDGNHEYI</td>
<td>L. monocytogenes listeriolysin 91/99</td>
<td>H-2K&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td>15-20%</td>
<td>800</td>
<td>32, 87</td>
</tr>
<tr>
<td>KYGVSVQDI</td>
<td>L. monocytogenes p60 217-225</td>
<td>H-2K&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
<td>50%</td>
<td>3500</td>
<td>87</td>
</tr>
<tr>
<td>LSFPFPFDL</td>
<td>mouse α-ketoglutarate dehydrogenase</td>
<td>H-2L&lt;sup&gt;d&lt;/sup&gt;</td>
<td>400</td>
<td>10%</td>
<td>4000</td>
<td>33</td>
</tr>
<tr>
<td>VAITRIEQLSPFPFDL</td>
<td>mouse α-ketoglutarate dehydrogenase</td>
<td>H-2L&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7000</td>
<td>5%</td>
<td>140,000&lt;sup&gt;f&lt;/sup&gt;</td>
<td>34</td>
</tr>
<tr>
<td>KYQAVTTL</td>
<td>P198 gene from mutagenized P815 cells</td>
<td>H-2K&lt;sup&gt;e&lt;/sup&gt;</td>
<td>100</td>
<td>&gt;100%</td>
<td>&gt;100</td>
<td>49</td>
</tr>
<tr>
<td>ALWCGFPVL</td>
<td>lymphoblastoid (IY) cells</td>
<td>HLA-A2.1</td>
<td>1500</td>
<td>90% (assumed)</td>
<td>1600&lt;sup&gt;f&lt;/sup&gt;</td>
<td>36</td>
</tr>
<tr>
<td>YLEPGPVTI</td>
<td>gp100 from melanoma</td>
<td>HLA-A2.1</td>
<td>0.23</td>
<td>≤12%</td>
<td>2</td>
<td>37</td>
</tr>
<tr>
<td>ILKDPVFHG</td>
<td>HIV-1 reverse transcriptase 476-484</td>
<td>HLA-A2.1</td>
<td>3</td>
<td>25%</td>
<td>12</td>
<td>41</td>
</tr>
<tr>
<td>SLYNTVL</td>
<td>HIV-1 gag 77-85</td>
<td>HLA-A2.1</td>
<td>100</td>
<td>25%</td>
<td>400</td>
<td>41</td>
</tr>
</tbody>
</table>

<sup>*</sup> naturally processed peptides for which ligand densities have not been determined are not listed.

<sup>f</sup> includes peptide not bound to L<sup>d</sup>.

activity in a T cell assay, and the active fraction(s) are subjected to further rounds of purification and finally to direct sequencing. If there is insufficient purified peptide for sequence determination by chemical methods (Edman degradation, mass spectrometry), it may be possible to identify the peptide indirectly by comparing the HPLC retention times of the naturally occurring sample (determined by T cell assay) with those of several synthetic peptides that are good candidates for the naturally processed peptide.<sup>22, 25, 26</sup> At least two or three different HPLC columns or conditions should be used in order to assign a peptide sequence by this indirect method. The various strategies discussed above are outlined in Figure 12.2.

In addition to these biochemical approaches, Boon and colleagues pioneered a molecular genetic approach to identifying natural T cell epitopes.<sup>22, 25, 26</sup> This involves transfecting cDNA from a target cell of interest (e.g., a tumor cell) into a cell that lacks the epitope and screening large numbers of transfectants for their ability to stimulate the relevant T cells. Ultimately the gene encoding an antigen recognized by these T cells can be sequenced. This technique has been used to identify the first precursors of T cell antigens on tumor target cells. Candidate peptides based on the gene sequences are then synthesized and tested for activity.<sup>22, 25, 26</sup> An important advantage is that the method is not limited by the abundance of naturally processed peptides, which is
sometimes very low (Table 12.1). However, in order to determine whether an active synthetic peptide corresponds to a naturally processed one, it is necessary to analyze the chromatographic pattern of the synthetic peptide relative to a naturally occurring sample. This has been done for a mouse tumor-specific peptide, but not for any of the human tumor antigens so far identified by the genetic approach.

Once a naturally processed peptide has been identified, its abundance at the cell surface (ligand density, expressed as an average number of peptide-MHC complexes per cell) can be measured. This is usually accomplished by synthesizing the same peptide and titrating the synthetic peptide in a T cell assay to establish a standard curve. A synthetic peptide can be characterized in terms of its concentration required to elicit 50% of maximal activity in a T cell assay (SD₅₀ value). The amount of a naturally occurring sample that contains the peptide at or near its SD₅₀ value is then determined, allowing a quantitation of ligand density.

Alternatively, if Edman degradation or mass spectrometry is used to sequence a naturally processed peptide, peptide abundance may be estimated directly.

Because losses are unavoidable during peptide isolation and analysis, the overall yield of recovered peptide must be estimated in order to calculate an absolute abundance. One practical method for estimating yield is to add a precisely known amount of synthetic peptide to a cell homogenate and extract the synthetic peptide exactly as is done for the naturally processed peptide. This method does not give a yield for the efficiency of peptide separation from MHC protein (usually by extraction with trifluoroacetic acid, TFA), but serial TFA extraction has been shown to result in the release of 100% of tightly-bound radioactive peptide from HLA-A2.1, so the yield obtained in such a "spiking" experiment can be considered an overall yield of peptide recovery. Naturally processed peptides for which ligand densities have been determined are listed in Table 12.1.

WHAT DETERMINES THE EFFICACY OF CTL-MEDIATED TARGET CELL LYSIS?

MHC-I-restricted CD⁸⁺ CTL play a central role in immune responses against many viruses; influenza virus for example, by destroying virus-infected cells. CTL specific for diverse human immunodeficiency virus (HIV) products (e.g., gag, pol, nef, env) also appear within 1-2 weeks of infection, and the importance of these CTL is suggested by their unusually high frequencies: they can often be detected...
in freshly isolated peripheral blood without the in vitro antigenic stimulation required to demonstrate CTL activity in other viral infections. Yet the presence of these CTL is clearly insufficient to eradiclate HIV in most cases. Similarly, despite the relative ease of isolating CTL from certain tumors such as human melanoma, there is a failure of T cells to contain these tumors. Why are CTL responses sometimes highly effective, sometimes not? The factors that might limit T cell responses can be grouped into three broad categories (Fig. 12.1): (1) factors intrinsic to the target cell, such as the number of specific peptide-MHC complexes and their physiological lifetime at the cell surface; (2) the quality of the T cell-target cell interaction, including the intrinsic affinity and kinetics of the reaction between TCR and peptide-MHC complexes; (3) factors related to individual T cells (e.g., TCR density, adhesion molecule density) or to the T cell compartment as a whole (e.g., tolerance/anergy).

Measurements of the affinities of different TCR for their peptide-MHC ligands pose a significant technical challenge, both because TCR and peptide-MHC are large membrane proteins and because special techniques are required to generate sufficient quantities of homogeneous peptide-MHC complexes. By several approaches, the intrinsic equilibrium constants for some TCR-peptide-MHC reactions have now been determined, and they span a large range from —10^{-5} to 10^{-7} M^{-1} (expressed as association constants) (reviewed in ref. 57). While not apparently as high as the intrinsic equilibrium constants of some antibodies for antigens (up to 10^{10} M^{-1}), this wide variation nevertheless contrasts with earlier expectations that TCR reactions would generally be of low affinity. Furthermore, the kinetic measurements made for TCR-peptide-MHC reactions indicate that these reactions approach equilibrium rapidly (usually within ~1 minute), so that the extent to which TCR molecules become engaged by peptide-MHC complexes in a T cell-target cell encounter is largely dependent upon intrinsic equilibrium constants. Thus it is important to evaluate the functional importance of differences in TCR affinity among T cells.

It is often assumed, implicitly or explicitly, that "high-affinity T cells" (i.e., high affinity of the TCR for its peptide-MHC ligand) are better effector cells than "low-affinity T cells." To date, very little data are available to evaluate this idea. For example, it seems intuitively reasonable that there may be an inverse correlation between the density of peptide-MHC complexes on target cells and the required affinity of a CTL's TCR to effect lysis of the cells. Figure 12.3 illustrates an empirical relationship between TCR affinity and ligand density for several peptides, and shows that peptide-MHC complexes present at lower densities are indeed recognized by TCR with higher intrinsic affinities. It is important to note that correlations between TCR affinity and peptide activity (e.g., SD_{50} value) are worse than those between TCR affinity and ligand density because SD_{50} is only a crude indicator of ligand density (due to variations in peptide-MHC binding affinities, see below). In particular, a synthetic peptide with relatively low affinity for the restricting MHC-I molecule must be added to target cells at a significantly higher concentration (SD_{50} value) in order to match the ligand density of a different peptide, regardless of the affinity of a TCR for each peptide-MHC complex. Similar considerations apply to efforts aimed at demonstrating a correlation between dissociation rate of the TCR-peptide-MHC reaction and peptide activity. The correlation between TCR affinity and ligand density is probably too imperfect to permit the prediction of either from the other: for example,
Fig. 12.3. Relationship between TCR affinity (equilibrium association constant of the TCR-peptide-MHC-I reaction) and ligand density at half-maximal T cell lysis (as calculated in Table 12.2). CTL is clone 2C except for ligand SIINFEKL*Kb, which is recognized by ovalbumin-specific CTL clone 4G3. Original references cited in Eisen HN, Skulev Y, Tsomides TI. The antigen-specific T-cell receptor and its reactions with peptide MHC complexes. In: Haber E, ed. Adv Protein Chem Academic Press 1996; in press.

in Figure 12.3 the TCR of CTL clones 2C and 4G3 differ 10-fold in affinity for the peptide-MHC ligands QLSPFPFDL*Ld and SIINFEKL*Kb, respectively, but the densities of these two ligands at half-maximal lysis are essentially identical. However, for a single CTL recognizing different peptides in association with the same MHC protein (e.g., clone 2C in Fig. 12.3), a rough inverse correlation between TCR affinity and ligand density is apparent.

ROLE OF LIGAND DENSITY

CTL function depends critically upon ligand density. This is most apparent in cytotoxicity assays in which appropriate synthetic peptides are added at increasing concentrations to target cells, thereby increasing the degree of target cell lysis. One way to quantitate the dependence of T cells on ligand density is to determine the minimum number of peptide-MHC complexes required to elicit a T cell response. The first estimates of minimum ligand densities involved incubating target cells (or antigen presenting cells) with 125I-labeled peptides at minimally active concentrations. The restricting MHC protein, together with its associated peptides, was then immunoprecipitated and the number of 125I-labeled peptide-MHC complexes per target cell determined. These results indicated that around 100-400 complexes were required for cytokine production by CD4+ T cell hybridomas or for lysis of target cells by CD8+ T cell lines.

A more recent approach to determining minimum ligand densities invokes the law of mass action as applied by Karush:

\[ r = \frac{K_a \cdot c \cdot n}{(1 + K_a \cdot c)} \]

where \( r \) is the number of peptide-MHC complexes per target cell at a free peptide concentration \( c \) that sensitizes target cells for lysis by a given T cell, \( K_a \) is the equilibrium association constant for the peptide-MHC reaction, and \( n \) is the number of MHC binding sites per target cell accessible to extracellular peptide at saturating concentrations. Because the peptide-MHC reaction probably never reaches true equilibrium, and because the peptide is subject to proteolytic degradation during whole cell binding assays, the minimum value of \( r \) that is calculated
MHC Molecules: Expression, Assembly and Function

using this equation (using for c the peptide concentration that leads to half-maximal lysis, i.e., the SD0 value) represents an upper limit to the number of peptide-MHC complexes required for T cell activation.

To calculate r values by means of this equation, we developed a quantitative whole cell binding assay to measure equilibrium constants for different peptide-MHC-I combinations as well as the number of specific peptide-binding sites per cell. Stoichiometrically radiolabeled indicator peptides were found to bind specifically to cells bearing the correct MHC-I molecules, and by Scatchard analysis values for K, and n were determined. By inhibition analysis, K, values for unlabeled peptides could also be measured, and the use of nonradioactive 125I-labeled peptides confirmed the equivalence between K, values calculated by means of inhibition and those measured directly with 125I-labeled peptides. The K, values for peptide-MHC reactions ranged from 10^4-10^9 M⁻¹. Notably, the best MHC-binding peptides were not always those that elicited T cell activity at the lowest concentrations. For example, peptide 125I-[ILKEPVHG] (two iodine atoms incorporated on histidine at position seven) bound approximately 10 times better than ILKEPVHG to HLA-A2.1, yet had an SD0 that was 200-fold higher. Table 12.2 shows that calculated ligand densities for half-maximal T cell lysis vary over several orders of magnitude depending on the particular T cell, peptide, and MHC protein and can be as low as ten or fewer complexes per cell in optimal cases.

Because of unavoidable assumptions in the calculation of r above, and in view of the intriguing result that such low numbers of peptide-MHC-I complexes can trigger T cell function, a direct verification of ligand density was performed. A peptide recognized efficiently by CTL clone 2C (QLSPYPFDL) was stoichiometrically labeled with carrier-free 125I to a specific radioactivity of ≈2000 Ci/mmol, or 2.5 x 10⁹ cpm/nmol. This unusually high specific radioactivity permits direct counting of the peptide molecules bound to target cells when

| Table 12.2. Ligand densities leading to half-maximal lysis of target cells* |
|------------------|--------|------|----------------|-----------------|-----------------|
| Synthetic peptide | MHC-I | CTL | K₅₀ for pep-MHC-I reaction (M⁻¹) | Peptide conc. for half-maximal lysis (SD₅₀) | Number of MHC-I peptide-binding sites per cell (n) | Ligand density (r) |
| ILKEPVHG | A2.1 | 68A62 | 5 x 10⁻⁹ | 5 x 10⁻¹² | 1.5 x 10⁴ | 1 |
| DNP | A2.1 | 68A62 | 5 x 10⁻⁷ | 1 x 10⁻⁹ | 1.5 x 10⁴ | 750 |
| ILKEPVHG | A2.1 | 68A62 | 2 x 10⁻⁷ | 1 x 10⁻⁷ | 1.5 x 10⁴ | 10,000 |
| SIINFEKL | Kb | 4G3 | 1.5 x 10⁸ | 3 x 10⁻¹² | 2 x 10⁴ | 9 |
| LSPFFPFDL | Kb | 2C | 1 x 10⁵ | 1 x 10⁻⁶ | 2 x 10⁴ | 1800 |
| LSPFFPFDL | Ld | 2C | 7 x 10³ | 1 x 10⁻⁹ | 3 x 10⁴ | 21 |
| LSPYPFDL | Ld | 2C | 5 x 10³ | 1 x 10⁻⁹ | 3 x 10⁴ | 15 |
| SPPFDLLL | Ld | 2C | 4 x 10⁵ | 1 x 10⁻⁸ | 3 x 10⁴ | 8600 |
| QLSPPFDL | Ld | 2C | 1 x 10⁸ | 5 x 10⁻¹² | 3 x 10⁴ | 15 |

* Data from ref. 40 and references cited therein and unpublished work.
peptide is added at its SDso concentration. Minimum ligand densities thus determined were found to agree closely with those calculated from \( K_a, SDso, \) and \( n \) (manuscript in preparation).

T cell recognition of target cells expressing fewer than 10 peptide-MHC-I complexes has several interesting implications: (1) It creates some uncertainty regarding the importance of TCR crosslinking in signal transduction. At the single cell level, it seems almost incredible that a CTL can consistently ferret out such a small number of complexes, and then undergo signal transduction and activation as a result of ligation of a correspondingly small number of TCR. (2) It suggests that the surveillance function of CTL can be performed when target cells express very little specific ligand, at least by certain CTL. (Whether cloned T cells mirror the activities of CTL in vivo remains an important question.) (3) The universe of T cell antigens presented at the cell surface is larger than previously thought, since peptides present at \( < 100 \) copies per cell (at least half of the total) can be recognized. (4) The biochemical isolation of naturally processed peptides can be excruciatingly demanding, since at 1 copy per cell (to take the extreme case), 10 pmol of peptide (a minimal amount for Edman sequencing) would require starting with \( > 6 \times 10^{12} \) cells.

It is worth noting in this context that screening for T cell epitopes with a panel of synthetic peptides at high concentrations may be misleading, because at typical screening concentrations (10-100 \( \mu M \)), many peptides will form artificially high ligand densities. Peptides with even modest affinities for the restricting MHC molecule (\( K_a \) values of \( 10^5-10^8 \ M^{-1} \)) will occupy over 90% of available MHC binding sites at equilibrium, resulting in orders of magnitude more specific peptide-MHC complexes than the few hundred per cell that have been measured for several naturally processed peptides (Table 12.1). For example, at high concentrations many synthetic peptides of different lengths from HIV-1 reverse transcriptase will sensitize target cells for lysis by HIV-specific CTL clone 68A62, but only one of those peptides is detectable in HIV-infected target cells and therefore relevant to the immune response. Similar observations of nonphysiologic crossreactions have been made in influenza and other systems. Thus, epitope identification based on synthetic peptides, while useful as a preliminary guide, may not accurately represent CTL-target cell interactions as they occur in vivo.

The preceding discussion of ligand density pertains to synthetic peptides added to target cells. Whether the densities of endogenous peptide-MHC ligands are limiting in vivo is much less clear, and is difficult to investigate. As mentioned earlier, the first requirement is precise knowledge of the naturally processed peptide(s) that constitute a T cell ligand. This may be achieved by indirect methods (compare the chromatographic profile of the naturally processed peptide with those of candidate synthetic peptides), or by direct biochemical isolation of an active naturally processed peptide, or by a genetic approach (clone the gene, test synthetic peptides, and verify active synthetic peptides by chromatographic comparison to a sample containing the naturally processed peptide). Measured ligand densities for proved naturally processed peptides recognized by CD8+ CTL vary from \( \geq 2 \) to several thousand complexes per target cell, as listed in Table 12.1.

An example of the role of endogenous ligand density is provided by two human HIV-specific CTL clones. 68A62 (anti-reverse transcriptase) and 115p (anti-gag). Whereas CTL 115p efficiently lysed a panel of HIV-infected target cells, CTL 68A62 exhibited lower levels of activity against infected target cells and barely lysed one of the
infected cell lines (JA2), even though the same CTL clone could lyse uninfected target cells pulsed with as little as 1 pM of synthetic peptide ILKEPVHG from reverse transcriptase (termed IV9). The level of expression of endogenous peptide IV9 was found to be quite low in HIV-infected JA2 cells: ~12 molecules per cell, versus ~400 molecules of the gag peptide, suggesting that low peptide abundance is likely to be responsible for poor killing of infected JA2 cells by what would have to be considered a highly efficient clone of CTL. A different HIV-infected target cell expressing higher levels of endogenous IV9 was lysed to a far greater extent by CTL 68A62. Thus, in spite of the extreme sensitivity of CTL 68A62 to synthetic peptide IV9 and the demonstration of naturally processed IV9 in HIV-infected cell extracts, these CTL lyse certain infected cells quite inefficiently, raising the possibility that low ligand densities of naturally processed HIV peptides may limit the effectiveness of some HIV-specific CTL in vivo. The generality of this observation and its implications for efforts to enhance CTL immunity against HIV remain unknown.

In a second example, a peptide recognized by an alloreactive (H-2b anti-H-2d) mouse T cell clone (2C) was found: (1) to be indigenous in H-2b mice ("self"),(33) (2) to bind a self MHC-I product (Kb) almost as well as the allogenic MHC-I product (Ld),40 and (3) to elicit cytolytic activity from the same alloreactive T cell clone in the context of either Ld or Kb, but with a marked difference in the peptide concentrations required.42 Since H-2b mice (as well as H-2b mice transgenic for the TCR of alloreactive clone 2C) show no signs of autoreactivity, these studies indicate that lack of recognition by specific T cells of a self peptide-MHC-I complex in vivo is due to its low cell surface density, not to its absence or to the absence of potentially autoreactive T cells; as a corollary, increased expression of the complex might well trigger an autoimmune response.41

Recently, several groups have identified peptides recognized by human anti-melanoma T cells.42-45 A striking feature of these results is that the peptides are all apparently nonmutated sequences of normal self proteins. Two obvious questions are why these nonmutated sequences are immunogenic, and what prevents autoimmune reactions from occurring against other tissues expressing the same antigens. There are several potential explanations, although none is yet proved: (1) The nonmutated sequences might be overexpressed on tumor cells, resulting in recognition by T cells that do not react against tissues expressing lower levels (ligand densities) of the same peptides. (2) The local inflammatory reaction to tumors may result in paracrine effects on lymphocytes and thus in activities not otherwise observed. (3) The peptides may have low binding affinities for MHC-I molecules, and this property may be related to their failure to induce a more sturdy self-tolerance. (4) CTL against the nonmutated sequences may have low-affinity TCR, because cells with high-affinity TCR would have been deleted or anergized.

Apart from ligand density, an equilibrium parameter, the kinetics of peptide-MHC reactions (particularly the dissociation rates) may be important factors in limiting T cell responses. The first kinetic data, obtained for MHC-II, indicated unusually slow on- and off-rates for the peptide-MHC reaction (half-life = 5-10 hours at 37°C).46 Later studies found evidence for rapid formation of a quickly dissociating peptide-MHC-II complex that slowly converts to a stable complex (half-life >30 hours).47 Others have found that the half-lives of peptide-MHC-II complexes vary widely, with selective persistence of the most stable complexes helping to account for the striking immunodominance of
Role of Ligand Density in T Cell Reactions

certain peptides. Thus, a rapidly-dissociating peptide-MHC-II complex might be ineffective at eliciting T cell responses, including those that result in T cell tolerance. For example, it has been suggested that rapid dissociation of a self peptide derived from myelin basic protein might fail to induce tolerance toward this peptide, leaving potentially autoreactive T cells that could cause autoimmune encephalomyelitis if activated by crossreacting viral peptides. It remains to be seen whether such a mechanism is correct and whether it may also apply to peptides interacting with MHC-I proteins. The time constant for peptide-MHC-I association (the time needed to reach 63% of equilibrium binding) and the half-life of cell surface complexes were both found to be several hours using an 125I-labeled peptide. In general, the long half-lives of peptide-MHC reactions make it seem as though kinetics do not usually limit T cell responses to endogenous ligands.

CONCLUDING REMARKS

The promise of T cells, especially CD8+ CTL, to destroy virus-infected cells and tumor cells is motivating intensive efforts to develop peptide-based vaccines that can stimulate the production of CTL capable of preventing infection or treating chronic infection (e.g., HIV) or established tumors. For this reason it is extremely important to understand the reasons why CTL are not always completely effective. Among the possible reasons listed in Figure 12.1, low densities of endogenous peptide-MHC ligands are especially likely to be an important factor in limiting T cell responses in vivo. Since it cannot be taken for granted that peptides derived from a viral protein or a tumor antigen will be displayed on the affected cell’s surface at a level that is sufficient to induce cell lysis, it is important to establish not only the identities but also the abundances of naturally processed viral and tumor peptides recognized by CTL. High-affinity T cells may be able to compensate for low ligand densities to a certain extent (Fig. 12.3), so that measurements of TCR affinities will continue to be important. Ideally, for peptide-based vaccines to be effective, they should be able to elicit the production of many cytolically active T cells that satisfy the following criteria: (1) their antigen-specific receptors have high affinities for specific peptide-MHC ligands; (2) the optimal epitopes for these CTL are present as naturally processed ligands on target cells in vivo; and (3) the peptide-MHC ligands exist at levels of abundance that are sufficient to trigger potent cytotoxic responses.

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Role of Ligand Density in T Cell Reactions


Antigen-Specific T Cell Receptors and Their Reactions with Natural Ligands: Complexes Formed by Peptides with Major Histocompatibility Complex (MHC) Proteins

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Ig, immunoglobulin; mAb, monoclonal antibody; CDR, complementarity determining region(s); MHC, major histocompatibility complex (genes or proteins); MHC-I, class I MHC (gene or protein); MHC-II, class II MHC (gene or protein); CTL, cytotoxic T lymphocyte(s); TcR, antigen-specific T cell receptor(s); DTH, delayed-type hypersensitivity; APC, antigen presenting cell; ER, endoplasmic reticulum; pep-MHC, noncovalent complex between peptide and MHC protein; peptide–MHC, reaction between peptide and MHC protein; TcR•pep-MHC, noncovalent complex between TcR and pep-MHC; TcR–pep-MHC, reaction between TcR and pep-MHC.
I. Overview

The most distinctive feature of the vertebrate immune system is its ability to recognize an enormous number of organic molecules and molecular complexes, termed antigens, distinguishing broadly between those that are foreign to the responding animal (nonself) and those that are indigenous (self). This property is due to antigen-specific receptors on lymphocytes, small cells that comprise ~5 percent of all cells in the body (i.e., estimated at $10^{11}$ to $10^{12}$ out of ca. $10^{13}$ cells in an adult human). The receptors on the two major classes of lymphocytes, B and T cells, are similar structurally but profoundly different functionally. On B cells the receptors are immunoglobulins (Ig) embedded in the cell surface as integral membrane proteins; in response to recognition of antigens, B cells produce large amounts of the receptors and secrete them as soluble antibody molecules. The antigen-specific receptors on T cells (T cell receptors or TcR) are also Ig-like cell surface integral membrane proteins; their recognition of antigens triggers T cells to exercise a great variety of functions, but not to secrete the receptors.

Both Ig and TcR molecules are heterodimers, each subunit consisting of two or more domains, with each domain having a characteristic three-dimensional shape called the “Ig fold.” The N-terminal domains (termed variable or V domains) differ in amino acid sequence from one lymphocyte clone to another, the variable domains of each heterodimer pairing to form a single antigen binding site that determines the unique ability of each clone to recognize and respond to one or a few similar antigens out of the millions of different ones to which an individual animal can respond. The enormous diversity of B and T cell receptors arises from the many germline gene segments that encode them; as each lymphocyte matures, different combinations of these segments are joined (combinatorial diversity) and additional variations in sequence are introduced at the junctures (junctional diversity), leading to an immense number of variable domain sequences.

Despite extensive similarities in amino acid sequence between Ig and TcR and in the organization and recombination of the gene segments that encode them, these receptors differ remarkably in the universe of antigens they recognize. The antigens recognized by antibodies (or their membrane-bound form on B cells) vary enormously: physically they may be soluble, colloidal, particulate, or parts of virions or microbial or eukaryotic cells, and chemically they may be proteins, peptides, carbohydrates, lipids, nucleic acids, or any of a limitless number of diverse small organic molecules. In contrast, the TcR reviewed here normally recognize and respond only to complexes formed between small peptides and a specialized set of proteins encoded by the major histocompatibility complex (MHC). Because MHC molecules (sometimes called histocompatibility antigens) are integral membrane proteins, these complexes (termed pep-MHC) are confined to cell surfaces, and a T cell’s TcR is therefore normally able to recognize antigenic complexes only on the surfaces of other cells, called antigen presenting cells or target cells. Inasmuch as both TcR and their natural pep-MHC ligands are embedded in cell surface membranes, analysis of their interaction at the molecular level poses a major challenge. Later in this review we focus on how this challenge is being met and emphasize recent results that illuminate the way in which TcR react with (i.e., "recognize") their natural pep-MHC ligands, particularly the TcR on those T cells (called cytotoxic T lymphocytes, or CTL) that destroy other cells (termed target cells).
II. History and Background

The immune system has been under study for around a hundred years, but it is only in the past thirty years that T and B cells have been distinguished and only in the past ten that TcR molecules and the genes encoding them have been identified. Nevertheless, for decades before T cells and TcR emerged as recognized entities, their existence was foreshadowed by certain antigen-specific inflammatory responses produced by injecting antigens into the skin of individuals with previous exposure to that antigen, either through natural infection or deliberate inoculation (“immunization”). Around 1890, Koch first showed that the injection of tubercle bacilli (or a mixture of proteins called “tuberculin” from supernatants of tubercle bacilli cultures) elicited an intense inflammatory response in guinea pigs if they had been previously infected with these microorganisms. The responses appeared 12-48 hr following antigen injection, and similar “delayed-type hypersensitivity” (DTH) responses, always specific for the original inciting antigen, were subsequently demonstrated with crude protein mixtures from many other microbes (bacteria, fungi, and later viruses) and sometimes following deliberate immunization with purified proteins (e.g., ovalbumin) or even with small organic molecules applied to the skin, providing they reacted \textit{in situ} to form covalent derivatives of skin proteins (as with 2,4-dinitrochlorobenzene, or a catechol in the case of poison ivy).

In contrast to the late appearance of DTH responses, many other antigen-specific skin responses appear almost immediately, e.g., within 1 min or sometimes up to 2-3 hr. Because the transfer of serum antibodies from an immunized to a nonimmunized (naïve) individual confers on the recipient the same prompt antigen-specific responses, it was clear that these rapid (“immediate-type”) responses were mediated by antibodies. But serum failed to transfer the delayed-type responses (e.g., to tuberculin). Although efforts were made to reconcile these failures with a role for special antibodies, it came to be widely believed that antigen-specific cells rather than soluble antibody molecules were the direct mediators of DTH responses. This belief was supported by the finding that DTH responses could be transferred to naïve recipients with inflammatory cells from immunized donors, although the transferred cells were complex mixtures of leukocytes that probably included some antibody-forming cells. The resolution of all doubt ultimately came after T cells were distinguished from B cells, largely through studies involving extirpation of the thymus from newborn mice. These studies led to the establishment of a clear dichotomy between those lymphocytes that develop from immature precursors in the thymus (T cells) and those that develop to maturity in the bone marrow (B cells). B cells were shown to be the source of Ig and antibodies, and T cells were shown to be required for the optimal production of antibodies. In addition, highly purified populations of T cells could transfer DTH responses, suggesting that T cells bear receptors that recognize antigens.

Early studies on the nature of the antigen-recognizing receptors on T cells were marked by intense disagreements arising largely, it appears in retrospect, because the T cell populations studied were often contaminated by small numbers of B cells and because reliance was placed almost exclusively on serological analyses using anti-Ig antisera. Antigen-specific molecules on T cells were variously suggested to be Ig molecules firmly attached to T cells, or to be some other (non-Ig) type of cell associated protein, or not even to be protein but rather some other type of informational macromolecule. The debate was ultimately resolved by the development of monoclonal
antibody (mAb) technology and the ability to grow T cell clones in culture. Using mAb raised against T cell clones, one a malignant lymphoma and the others normal T cells, three independent studies succeeded in immunoprecipitating a T cell surface protein that was unique for cells of the immunizing clone (Allison et al., 1982; Meuer et al., 1983; Haskins et al., 1983). The clonally specific (or clonotypic) protein was in each case a disulfide-linked heterodimer (=90 kDa), consisting of a relatively acidic membrane-bound α chain and a more basic membrane-bound β chain.

The idea that these clone-specific heterodimers were antigen-specific receptors was strengthened by two findings. First, antibodies against them could block antigen-driven responses of the corresponding T cell clones (Lancki et al., 1983). Second, amino acid sequences of proteolytic fragments from isolated α and β subunits suggested that, like Ig heavy and light chains, they had some regions where amino acid sequences varied from clone to clone and others where these sequences were invariant (Kappler et al., 1983; Meuer et al., 1984; McIntyre and Allison, 1983). The isolation and sequencing of cDNA clones for the β subunit (Yanagi et al., 1984; Hedrick et al., 1984; Saito et al., 1984a) and then for the α subunit (Saito et al., 1984b; Chien et al., 1984) finally demonstrated unambiguously that the clonally diverse heterodimers greatly resembled Ig and had all the characteristics expected of cell surface integral membrane proteins serving as antigen-specific receptors. Moreover, introduction of genes for both the α and β subunits into a T cell clone having unrelated specificity transferred the donor cell's antigen-specific responsiveness to the recipient cell (Dembic et al., 1986; Saito et al., 1987).

In the course of searching a T cell cDNA library for clones for the α and β subunits, cDNA for a third related subunit was found (Saito et al., 1984a). Termed γ, the third gene turned out to encode an Ig-like chain that paired with the product of a fourth gene, δ, to form a γδ heterodimer (Chien et al., 1984). Closely similar to the αβ TcR, γδ heterodimers are another type of antigen-specific receptors, found on a subset of T cells located primarily in epithelia. γδ+ cells constitute about 1-5% of peripheral T cells in mice and humans; these TcR will not be considered further here, as their natural ligands are not as well defined as those recognized by αβ TcR and the function of γδ T cells is still not entirely clear.

Ten years before the molecular identity of the αβ TcR began to take shape, several observations pointed to the special character of its natural ligands. By transferring T cells to athymic ("nude") mice, Kindred and Shreffler (1972) saw that these cells reacted only when the T cells and the recipients had the same MHC type. The ineffectiveness of MHC-disparate T cells could not be attributed to their immune elimination in the recipient, because athymic mice do not reject allografts. It was therefore concluded that the MHC "must play an active role in ensuring cooperation between B and T cells."

Shortly thereafter, in vitro studies of T cell enhancement of the production of antibodies by B cells (Katz et al., 1973) and of T cell responses to antigens presented by macrophages (Rosenthal and Shevach, 1973) also indicated that successful T cell responses required the responding T and B cells, or T cells and macrophages, to have the same MHC type. Then, in a seminal publication, Zinkernagel and Doherty (1974) reported that virus-infected target cells were lysed by T cells from a mouse infected by that virus only if the target cells expressed the same MHC products as the infected
animal. Work with different antigens revealed similarly that antigen-specific lysis of antigen-bearing target cells by cytotoxic T cells depended upon target cell expression of a proper MHC protein (Shearer, 1974; Bevan, 1975; Gordon et al., 1975). This dual requirement of antigen recognition, referred to as “MHC restriction” (Zinkernagel and Doherty, 1979), has since been found to be characteristic of all αβ TcR mediated reactions.

To explain MHC restriction, two models were proposed. In the two-receptor model, T cells have one receptor for an MHC product and another one for antigen, and both have to be occupied for a successful T cell response (Cohn and Epstein, 1978). According to the alternative one-receptor model, each T cell has a single type of receptor that recognizes an antigen-MHC complex. The one-receptor model was ultimately shown to be correct by various approaches. In one, a hybridoma resulting from the fusion of two cells recognizing antigen “A” and MHC “X” or antigen “B” and MHC “Y” responded specifically to cells expressing either A + X or B + Y but not to those expressing A + Y or B + X, indicating that the T cell did not see the antigen and the MHC product separately (Kappler et al., 1981). The antigen-MHC complex was sometimes referred to as “altered self” because in this context the restricting MHC is indigenous (self) with respect to the responding T cells and is therefore nonimmunogenic, while the antigen is foreign (nonself) and somehow alters the character of self MHC so as to elicit a reaction.

Mature T cells bearing αβ TcR fall into two groups, each marked by one of two cell surface glycoproteins. Termed CD4 and CD8 (formerly called Lyt-1 or Lyt-2,3 in the mouse), both are present on immature (“double positive”) T cells in the thymus, and one of these is lost by what appears to be a stochastic process (Corbella et al., 1994) to yield “single positive” (CD4+ or CD8+) mature T cells. Some mature CD4+ T cells, termed helper (Th2) cells, are required for optimal B cell responses, while other helper cells (Th1) produce cytokines that cause inflammation, as in DTH responses. In contrast, CD8+ T cells, which also produce some cytokines, behave primarily as cytotoxic T lymphocytes (CTL), lysing target cells that bear appropriate antigen-MHC complexes.

The CD4/CD8 T cell dichotomy also extends to the types of MHC protein that restrict antigen recognition. Class I MHC proteins (MHC-I) restrict antigen recognition by CD8+ T cells, and class II MHC proteins (MHC-II) restrict antigen recognition by CD4+ T cells (Table I). Virtually all cells express MHC-I and can present peptides to CD8+ cells, whereas only specialized cells (primarily macrophages, dendritic cells and B cells) express MHC-II for interactions with CD4+ cells. By common usage, cells that present pep-MHC-II complexes to CD4+ cells are called “antigen presenting cells” (APC), whereas those that present pep-MHC-I complexes to CD8+ CTL are termed “target cells.”

Once it became established that a single heterodimeric receptor on T cells reacts with an antigen displayed on the surface of another cell, and that the antigen includes both a self (restricting) MHC protein and a bona fide foreign element (such as a virus-encoded product), it was reasonable to assume that the MHC protein and the foreign protein combine to form an antigenic complex in the plasma membrane of the target cell (e.g., Cohen and Eisen, 1977). However, the subsequent finding that T cells responding to viral infections are paradoxically specific for intracellular proteins that are not found...
at the cell surface (e.g., influenza virus nucleoprotein: Townsend and McMichael, 1985; Yewdell et al., 1985) led Townsend and colleagues to the discovery that short peptides (ca. 8-25 amino acids in length) derived from internal viral proteins, can sensitize target cells bearing appropriate MHC molecules for lysis by CD8+ T cells (Townsend et al., 1986).

It has since become clear that: (1) the natural ligands for TcR are cell surface complexes each consisting of a short peptide and an MHC protein (pep-MHC); (2) under normal conditions the peptides are produced intracellularly by limited proteolysis (“processing”) and are transported into the endoplasmic reticulum (ER) where they can bind to newly formed MHC proteins; and (3) this physiological pathway can be circumvented by adding to whole cells synthetic peptides that bind directly to a subset of cell surface MHC molecules that are free of naturally processed peptides or bind them weakly. The idea that fragmented protein antigens, i.e., peptides, can be recognized by T cells had actually emerged from earlier studies of Unanue and Allen (1987) on antigen presentation to CD4+ T cells by MHC-II+ APC. Indeed the first unequivocal and quantitative demonstration of binding between a defined peptide and an MHC molecule was obtained for MHC-II (Babbitt et al., 1985).

In mice and humans, MHC-I proteins are encoded by genes at three linked loci (termed K, D, and L in mice and A, B, and C in humans), and MHC-II proteins by several genes at two or three linked loci (termed IA and IE in mice and DP, DQ, and DR in humans). Most of these genes are extremely polymorphic, some having as many as 50 (or more) allelic variants. Any particular individual inherits alleles for only a small number of MHC proteins, at most six MHC-I and six MHC-II in humans, due to heterozygosity at each gene locus. Some inbred mouse strains have only two MHC-I and a single MHC-II protein. Remarkably, however, these few proteins can effectively present an enormous number of different peptides to a vast number of TcR, each MHC protein having the capacity to bind to thousands of different peptides with equimolar (1:1) stoichiometry. Although some peptides can be bound by more than one MHC protein, each MHC protein binds to distinctly different sets of peptides. How each of these protein molecule is able to bind so many different peptides and yet retain a significant degree of selectivity has become clear as a result of striking advances over the last five years, particularly in the solution of several three-dimensional structures of crystallized pep-MHC complexes (see Section V, below).

III. TcR Genes

The genes that encode TcR α and β chains are assembled in developing T cells in the thymus by the juxtaposition of variable (V), diversity (D), and joining (J) gene segments to form VJ or VDJ or VDDJ exons, separated by a short intron from an exon for the constant (C) domain. The great similarity in organization of TcR and Ig gene segments is striking (Davis, 1990) and include the canonical heptamer and nonamer signal sequences adjacent to TcR V, D and J segments; these signal sequences are almost the same for TcR and Ig genes and, indeed, are interchangeable (Yancopoulos et al., 1986).

TcR β gene segments are spaced over 700-900 kb of human (or mouse) DNA, with an array of multiple VB segments separated from two tandemly duplicated sequences each including one DB, six JB, and one CB segment. All of these segments
have the same transcriptional orientation except for one downstream Vβ segment (Fig. 1). TcR α gene segments are also distributed in a long linear array, but surprisingly these were found to be interrupted by J and C sequences of the TcR δ chain (Chien et al., 1984). The pairing of a δ with a γ chain forms the γδ heterodimeric receptor on the small subset of T cells (γδ+7) referred to above. Because a single locus contains gene segments for both α and γ chains, the rearrangements and expression of α and γ genes are regulated by an intricate process (Winoto and Baltimore, 1989; Diaz et al 1994). Evidently, a V gene from the same upstream array in this locus can join either to Jδ (or Dδ and Jδ segments), to form a complete VδCδ (or VδDδCδ) sequence for a δ chain, or to one of many Jα segments to form a complete VJαCα sequence for an α chain. In T cells that express an αβ TcR, the joining of a V gene to a Jα eliminates δ gene sequences and thus precludes expression of a δ chain. In T cells that express γδ receptors, the joining of a V segment to Dδ and/or Jδ takes place first and evidently suppresses subsequent rearrangements involving α gene segments.

Despite many similarities in the organization of Ig and TcR genes and their extensive amino acid sequence homology, they differ in some important respects as noted below.

**Localization of Hypervariable Sequences in TcR V Domains.** In Ig there are three especially variable ("hypervariable") regions in V domains of light and heavy chains, and x-ray crystallographic studies show that these form the boundaries of antigen binding sites of antibodies (Davies et al., 1990). Because they determine the complementary fit of antibody to antigen, they are referred to as "complementarity determining regions," or CDR (i.e., CDR1, CDR2, and CDR3).

Comparison of the amino acid sequences of α and β variable (V) domains from many TcR have shown that, in contrast to Ig, the CDR1 sequences of αβ TcR vary very little, the CDR2 sequences vary only slightly more, but that the CDR3 sequences vary greatly in length and sequence. The great variation of CDR3 reflects the very large number of TcR J gene segments as compared with those for Ig genes (e.g., ca. 50 Jα and 12 Jβ segments in contrast to 4 JH and 5 JK segments for murine Ig). As discussed below, the extensive variation in CDR3 and J sequences suggests that the most diverse element of the pep-MHC ligand that binds to a T cell receptor, the peptide moiety, makes contact with the receptor's CDR3 region, whereas the less variable flanking α-helical regions of the MHC protein make contact with CDR1 and CDR2.

**Alternative RNA Splicing.** Alternative splicing of RNA transcripts of Ig genes plays a critical role in modifying the 3' ends of messenger RNA in order to generate proteins that are secreted as antibodies or retained as cell surface antigen-specific B cell receptors. In contrast, RNA splicing is not involved in the expression of TcR genes, and TcR molecules are not secreted; they invariably remain as integral membrane proteins on T cell surfaces.

**Isotype Switching.** Gene sequences for the V domains that determine the specificity of antibodies for antigens can be spliced to one of several different immunoglobulin C regions to yield various forms of heavy chains called isotypes. Different Ig isotypes have different functional roles: for example, certain ones bind to receptors on B cells and macrophages, certain others can be transported across the placenta, and others bind to receptors on mast cells to mediate anaphylactic reactions.
During the course of normal B cell differentiation, "switching" of isotypes can occur (with no change in antigen specificity). For TcR, there is no comparable switching because there are no forms homologous to Ig isotypes.

**Somatic Mutation.** A distinctive feature of the antibodies produced in response to antigenic stimulation is the progressive increase over time in intrinsic affinities of the antibodies made against an antigenic epitope. These changes are the result of extensive somatic mutation that takes place in the V, D, and J regions of rearranged Ig genes during prolonged or repeated antigenic stimulation, together with selection by the antigen of B cells producing high-affinity antibodies (for a minireview see Foote and Eisen, 1995).

Comparisons among the sequences of several hundred V regions from TcR α and β mRNA or cDNA failed to reveal systematic evidence for somatic mutation, as the sequences nearly always matched one or another of the germline sequences (Davis, 1990). The important implication is that progressive increases or "maturation" in the intrinsic affinities of TcR does not occur, and thus TcR affinities for their ligands will generally reflect the distribution of affinities arising from germline sequences and variations introduced by joining V, D, and J gene segments. Recently, however, Zheng et al. (1994), in a careful analysis of lymphocytes in lymph node germinal centers (where B cells undergo somatic mutation), found evidence for somatic mutation of TcR V regions. Whether antigen selection of the mutated T cells takes place, as it does for B cells, is not yet clear.

**Allelic Exclusion.** In a newly developing, immature thymocyte, productive (in-frame) rearrangement of a Vβ gene segment in one chromosome prevents rearrangement of additional Vβ gene segments on the other chromosome (Van Meerwijk et al., 1991; Malissen et al., 1992). However, Vα segments on both chromosomes rearrange at the same time and can continue to rearrange until a complete αβ TcR is formed and the cell is stimulated (by "positive selection" in the thymus) to mature into a single positive T cell (CD4+ or CD8+). As a result, almost one-third of mature T cells in the periphery carry two productively rearranged α genes and have on their surface two TcR species, differing in α chain but having the same β chain (Padovan et al., 1993; Heath and Miller, 1993; Mason, 1994).

Because antibodies can bind via their constant (Fc) region to certain receptors (called FcR) on target cells, some antibodies to a TcR can mimic the TcR's natural ligand and trigger "redirected lysis" of an FcR+ target cell by CTL expressing that TcR (Kranz et al., 1984b), even though the target cell lacks a pep-MHC complex that is recognized by the TcR. Thus, a target cell expressing FcR was shown to be lysed by a CTL having two TcR, call them α1β1 and α2β1, in the presence of either antibody to α1 or antibody to α2, indicating that each TcR of such a two-receptor T cell can, when ligated via its Vα domain, trigger T cell activation (Padovan et al., 1993). This circumstance raises interesting possibilities, not yet critically tested, that a mature two-receptor T cell that is stimulated to proliferate via one TcR will result in an increased number of cells expressing both TcR molecules, perhaps increasing the possibility of reactions with different pep-MHC. Because the two receptors on such a T cell would be expected to have different specificities, they might increase opportunities for reactions against autoantigens or MHC-disparate cells (see Section IX, below). Experiments with mice transgenic for a TcR β subunit suggest, however, that in antigenically stimulated T
cells there may be unknown mechanisms that hinder expression of a nonutilized TcR
(Hardardottir et al., 1995), as with Ig receptors on B cells transfected with multiple κ
chain genes (Lozano et al., 1993).

IV. TcR Protein

Once the primary structures of a large number of T cell receptors became known
through the sequences of their V, D, J and C genes, it became possible to look for clues
regarding their tertiary structures. It was obvious on the basis of sequence homologies
that TcR belong to the so-called immunoglobulin superfamily. That is, the domains
of a heterodimeric TcR (VαVβ and CαCβ) were expected to have the same chain topology
and secondary structure elements as the canonical Ig fold (reviewed elsewhere in this
volume). However, this observation did not lead to an understanding of how TcR
recognize an antigenic universe consisting of relatively few MHC proteins combined
with a great many different peptides (thousands for each MHC protein).

Further analyses of sequence homologies between Ig and TcR (roughly 25-30%
identity between the two groups) led to more detailed structural predictions. Following
the first complete cDNA sequence of the TcR, Novotny et al. (1986) aligned the
sequences of TcR α, β, and γ chains with those of Ig of known three-dimensional
structure and concluded that a TcR molecule was likely to possess a single antigen
binding site that is essentially no different from that of an Ig. Later, Chothia et al.
(1988) identified 40 amino acid residues critical for the conserved structure of Ig
variable domains, either because they serve as framework residues or because they are
crucial for interdomain contacts (V-L-V-H or V-H-C-H1), and compared these with the
corresponding residues in ≈200 TcR Vα and Vβ sequences. They found a high
percentage of the same or very similar residues at these positions, often 90+% identity,
and they also found important similarities in the distribution of hypervariable regions
between Ig (three such regions per V-L or V-H chain) and TcR (Vα and Vβ chains). As
mentioned above, these noncontiguous hypervariable regions combine in the three-
dimensional folded structure of an Ig to form the antigen binding site, and are termed
complementarity determining regions, or CDR. Unlike Ig, however, the first and
second predicted CDR of a TcR are much less variable than the third, called CDR3
(approximately residues 95-105 from the N-terminus).

Building upon the freshly made discovery that peptides are an integral part of
the antigenic structures recognized by T cell receptors, Davis and Bjorkman (1988)
proposed a model for T cell recognition in which two CDR3 regions (from Vα and Vβ)
interact primarily with peptide, while CDR1 and CDR2 (each from both Vα and Vβ)
interact with the two α-helices flanking the peptide binding site. These authors also
emphasized the greater diversity in a TcR's CDR3 regions than in CDR1 and CDR2;
the latter are fully encoded by germline V genes, of which there are relatively few,
whereas CDR3 is formed by the joining of V and J genes (in TcR α and γ) or V, D and J
genes (in TcR β and δ). Because of prevalent N-region additions at these junctions (due
presumably to terminal deoxynucleotidyl transferase), as well as other mechanisms for
diversification, TcR variability is highly concentrated in the CDR3 region (Davis and
Bjorkman, 1988).

Since the MHC sites in contact with a TcR are generally less variable than the
peptide residues recognized by a TcR, the above model provided a rationale for the
greater sequence diversity of CDR3 than of CDR1 or CDR2; it also accommodated functional data from studies in which variations only in the CDR3 residues within a set of T cell clones affected peptide recognition but not MHC specificity (Fink et al., 1986; Winoto et al., 1986). Furthermore, this model, independently suggested by Chothia et al. (1988) and by Claverie et al. (1989), was consistent with the dimensions observed in the first crystal structure of an MHC protein (Bjorkman et al., 1987a): the peptide binding groove is \( \approx 10 \) Å wide and \( 25 \) Å long (Fig. 2), while the spacing between the predicted CDR3 segments in the TcR is \( \approx 10-15 \) Å. The two CDR3 regions were therefore projected to interact with \( \approx 5 \) residues of a bound peptide (Claverie et al., 1989; see also Section IX, below).

In a revealing study, Jorgensen et al. (1992) immunized mice transgenic for either the \( \alpha \) or the \( \beta \) chain of a particular TcR with a series of variant peptides. Analysis of the T cell responses in these mice indicated that some peptide variants elicited T cells having complementary changes in the CDR3 regions of their TcR; furthermore, the contact sites for two peptide side chains could be assigned to either the TcR V\( \alpha \) or V\( \beta \) chain.

To date no three-dimensional structure of a complete TcR molecule has been reported. Such a structure is eagerly awaited, not only to illuminate the structure of the TcR itself, but hopefully to explain in molecular terms how the TcR’s universe of antigens is limited to pep-MHC complexes, while that of antibodies is virtually limitless. The recently reported structure of a TcR \( \beta \) chain revealed Ig-like domains and three CDR loops plus an additional hypervariable loop whose significance is not yet clear (Bentley et al., 1995).

V. TcR Ligand: Peptide-MHC Complexes (Pep-MHC)

Beginning with Townsend and colleagues (Townsend and Bodmer, 1989), a common method for studying the ligands recognized by T cells on various target cells (e.g., virus-infected, tumor, or allogeneic cells) has been to add synthetic peptides to different target cells that express an appropriate MHC protein, forming pep-MHC complexes that can elicit a measurable T cell response. This approach has been successful in part because the added synthetic peptide need not match precisely the natural ligand (e.g., a processed viral peptide); it can be substantially longer, for example, and then undergo limited trimming by proteases in the assay medium or on cell surfaces to reach the optimal length for binding to an MHC protein. However, in some instances it is important to know the exact identity of a naturally processed peptide, in order, for instance, to determine the number, or density, of specific pep-MHC complexes on the surfaces of target cells (reviewed in Tsomides and Eisen, 1993a).

An understanding of the relationship between the synthetic peptides that can be used to elicit T cell responses and the naturally processed peptides that are generated within cells has rested on two principal lines of investigation: (1) x-ray crystallographic determination of MHC structures; and (2) direct biochemical isolation and sequencing of endogenous peptides associated with MHC molecules. The nature of the MHC and its ligand was greatly illuminated when the first crystal structure of an MHC protein, the human MHC-I protein HLA-A2, was solved by Bjorkman et al. (1987a). The molecule consisted of four domains of \( \approx 90 \) amino acids each, three derived from an \( \alpha \) chain (\( \alpha_1 \),
α2, and α3) and one comprising β2-microglobulin (β2m). While the membrane proximal α3 and β2m domains resembled Ig domains, α1 and α2 paired to form a novel structure: an 8-stranded antiparallel β-sheet underlying two long α-helices (Figs. 2 and 3). Between the two α-helices was a continuous region of unassigned electron density later shown to represent a complex mixture of peptides that copurified with HLA-A2; since A2 purification takes several days at least, this observation proved to be the first clue that some naturally processed peptides can form extremely stable complexes with MHC-I proteins, often having dissociation half-lives in the tens of hours (Cerundolo et al., 1991; Tsomides et al., 1991; Olsen et al., 1994). The crystal structure of HLA-A2 furthermore suggested that the unknown peptides harbored between the two α-helices might be between 8 and 20 amino acids in length, based on the approximate dimensions of the binding site (25 x 10 x 11 Å) (Bjorkman et al., 1987b).

More precise definition of some natural ligands was achieved through an acid elution technique used to separate peptides from MHC molecules (Buus et al., 1988; Rötzschke et al., 1990a), followed by characterization of the resulting peptides. The first naturally processed peptides to be identified were viral products obtained from infected cells (van Bleek and Nathenson, 1990; Rötzschke et al., 1990b), and these were 8 or 9 residues long. Other MHC-I associated peptides present at relatively high levels were resolved by HPLC and sequenced by Edman degradation (Jardetzky et al., 1991; Corr et al., 1992) or by a mass spectrometric approach pioneered by Hunt and colleagues (1992a); these proved to arise from a variety of intracellular components and were often 8 or 9 residues in length, though sometimes longer (Henderson et al., 1992; Wei and Cresswell, 1992). Rammensee and colleagues then performed a critical experiment in which they subjected the total pool of peptides eluted from MHC-I molecules to sequencing by Edman degradation (Falk et al., 1991a). While they found each amino acid at virtually every cycle during the Edman procedure, they discovered several key facts about naturally processed peptides: (1) for a given MHC-I protein (such as HLA-A2), the eluted peptides contained a predominance of one or two amino acids at certain key "anchor" positions (e.g., leucine or methionine at position two, and valine or leucine at position nine); (2) amino acid yields dropped precipitously after 9 cycles, suggesting that most bound peptides are nonamers (octamers for certain MHC-I molecules); and (3) the "motifs" characterizing peptide length and anchor positions were distinctive from one MHC-I protein to another.

These motifs have been highly useful for the rapid identification of candidate peptides recognized by T cells when the parent protein sequence is known (Rötzschke et al., 1991a; Pamer et al., 1991). However, this approach is not infallible for several reasons: (1) some MHC-binding peptides do not conform to the expected motif and therefore will be missed (e.g., Udaka et al., 1992); (2) synthetic peptides that are found to be active need not always correspond to naturally processed peptides, even when optimized for activity, since heteroclitic reactions can occur for T cell receptors (Bodmer et al., 1988) as well as for antibodies; and (3) nonphysiological cross-reactions can be observed when target cells are sensitized with high doses of synthetic peptides, due to the resulting artificially high pep-MHC densities (Milligan et al., 1990; Schild et al., 1990; Dutz et al., 1994; Tsomides et al., 1994). Despite these caveats, several naturally processed peptides recognized by T cells have turned out to match precisely the peptides predicted from motifs (Rötzschke et al., 1990b; van Bleek and Nathenson, 1990; Rötzschke et al., 1991a; Pamer et al., 1991; Tsomides et al., 1994) (Table II), leading to the reasonable assumption that this will often be the case.
All the biochemical information about naturally processed peptides bound to MHC-I molecules fit neatly with the emerging crystallographic data. Monopeptidic pep-MHC complexes were obtained and crystallized, revealing important structural similarities and differences among peptides binding to the same MHC-I molecule (Fremont et al., 1992; Zhang et al., 1992; Madden et al., 1993). Pockets within the MHC binding site were shown to accommodate peptide side chains at the positions described as anchors (Garrett et al., 1989; Matsumura et al., 1992a; Young et al., 1994). Peptides longer than 9 residues could fit by bulging out in the middle, with both peptide termini substantially buried in the binding site (Guo et al., 1992). Within a short time, a coherent picture of how a single MHC molecule can bind to a highly degenerate but restricted set of peptides was assembled.

Comparable findings (peptide motifs, MHC pockets) were later made for MHC-II molecules, although in this case the situation proved more complex. Naturally processed peptides binding to MHC-II molecules turned out to include sets of nested peptides sharing a core sequence but having different N- and C-termini (Demotz et al., 1989; Nelson et al., 1992; Chicz et al., 1992). These peptides tended to be longer than those eluted from MHC-I proteins, ranging from about 12-25 residues (Rudensky et al., 1991; Hunt et al., 1992b). Again, this information correlated well with the results of crystallography. The structure of an MHC-II molecule complexed with endogenous peptides (Brown et al., 1993) or with a single viral peptide (Stern et al., 1994) revealed two major differences from MHC-I: (1) the MHC-II binding groove allows bound peptides to extend out at both ends, rather than having their termini tucked into the binding site, thereby explaining the length heterogeneity among naturally occurring MHC-II-associated peptides (see Fig. 3B); and (2) many of the peptide-MHC-II contacts involve the peptide backbone rather than specific peptide side chains, implying different mechanisms for degenerate peptide binding by MHC-I and MHC-II proteins (Table I).

Once it was appreciated that purified MHC-I molecules are normally occupied by stably bound endogenous peptides, the failure of early attempts to demonstrate significant peptide binding to purified MHC-I molecules (Chen and Parham, 1989; Tsomides and Eisen, 1990) became understandable. Soon, techniques based on whole cell binding assays (Christinck et al., 1991), MHC-I immunoprecipitation from cell lysates (Cerundolo et al., 1991), or in vitro binding to empty MHC-I molecules purified from transfected Drosophila (Matsumura et al., 1992b; Saito et al., 1993) or other (Boyd et al., 1992; Ojcius et al., 1993; Fahnestock et al., 1994) cells enabled the measurement of equilibrium binding constants for a wide variety of peptide–MHC-I reactions. Parker et al. (1992; 1994) used β2m dissociation as a surrogate indicator for the stability of pep-MHC-I complexes, and Olsen et al. (1994) found that peptides bind well to purified MHC-I molecules at reduced temperatures. Each of these experimental systems suffers from certain limitations, a shared one being that the binding of synthetic peptides to fully formed MHC-I molecules may not accurately mirror events in the ER, where this reaction ordinarily takes place. Nevertheless, these equilibrium values seem to reflect the specificities otherwise observed for peptide–MHC-I reactions, and similar measurements have proved useful as indicators of potential peptide immunogenicity (Feltkamp et al., 1993; Celis et al., 1994; Sette et al., 1994).

Given the ability to measure equilibrium constants for the peptide–MHC reaction, the number of pep-MHC complexes per target cell required to trigger the
activity of a given T cell clone can be estimated in assays where T cell activity depends upon the concentration of synthetic peptides (added to extracellular medium). The estimates are based on the Karush (1970) form of the law of mass action (Day, 1990):

\[ r = \frac{K \cdot c \cdot n}{1 + K \cdot c}, \]  

Eq. 1

In Eq. 1, \( r \) is the number of pep-MHC complexes per target cells, \( K \) is the equilibrium association constant for a peptide–MHC reaction, \( c \) is the free concentration of peptide that sensitizes target cells for a particular level of lysis (e.g., half-maximal) by a given CTL, and \( n \) is the total number of MHC binding sites per target cell accessible to extracellular peptide. For several reasons, the calculated values of \( r \) should be regarded as only reasonable approximations, e.g., because the peptide may be subject to proteolytic degradation during the assays. More importantly, the number of accessible sites \( n \) changes over time as newly synthesized MHC molecules migrate to the target cell surface and some (unknown) proportion have accessible peptide-binding sites, either because the sites are empty or occupied by rapidly dissociating natural peptides that exchange readily with peptides in the extracellular medium. Nevertheless, Eq. 1 yields reasonable approximations for those peptides whose reactions with MHC (on intact cells) reaches steady state within a short time (e.g., 2-3 hours).

As discussed below, and in contrast to earlier studies reporting values of a few hundred (Harding and Unanue, 1990; Demotz et al., 1990; Vitiello et al., 1990; Christinck et al., 1991), the minimum number of activating complexes per target cell was recently found to vary over several orders of magnitude depending on the particular T cell, MHC-I molecule, and peptide used, from several thousand per target cell to fewer than ten in optimal combinations (Kageyama et al., 1995) (see Section VI.D, below).

How do the pep-MHC complexes recognized by T cells arise? CD8+ T cells react with pep-MHC-I complexes that form within a cell's ER as newly synthesized MHC-I molecules assemble (reviewed by Monaco, 1992; Yewdell and Bennink, 1992; Germain and Margulies, 1993; Heemels and Ploegh, 1995). The peptides are generated by limited proteolysis in the cytosol and translocated into the ER by MHC-encoded peptide transporters, TAP ("transporter associated with antigen processing") (Spies et al., 1991), or in some cases via TAP-independent pathways (Anderson et al., 1991; Henderson et al., 1992; Zweerink et al., 1993; Hammond et al., 1993; Zhou et al., 1993). Once inside the ER, peptides may or may not be subject to further proteolysis (Falk et al., 1990; Yewdell et al., 1994) before binding to nascent MHC-I molecules, which are then exported to the cell surface as mature pep-MHC complexes.

Given several thousand intracellular proteins potentially available for degradation in any given nucleated cell, and many-fold higher numbers of peptides theoretically available for transport into the ER and subsequent binding to MHC-I molecules, it is apparent that competition among peptides must be a significant feature of antigen presentation. It may be that only tightly-binding peptides compete effectively for MHC-I binding sites, explaining the slow dissociation rates that have been measured. Certainly peptide selectivity exists at the levels of MHC binding (Falk et al., 1991a; Schumacher et al., 1991) and TAP-mediated peptide translocation into the ER (Shepherd et al., 1993; Neefjes et al., 1993; Schumacher et al., 1994), and perhaps
also at the level of proteolysis (Goldberg and Rock, 1992), but much remains to be clarified about the generation of MHC-I binding peptides in vivo.

What is clear is that the pep-MHC-I complexes ultimately arriving at a cell's surface represent a sampling of that cell's contents, with some peptides present at relatively high copy numbers, e.g., several hundred to perhaps several thousand identical pep-MHC-I complexes per cell, and a greater number of peptides relatively scarce, e.g., between 1 and 100 complexes per cell (van Bleek and Nathenson, 1990; Falk et al., 1991b; Hunt et al., 1992a; Udaka et al., 1992; Tsomides et al., 1994). Most natural MHC-I-binding peptides arise from an individual's normal self proteins and are not recognized efficiently by that individual's mature T cells, which are purged of most self-reactive cells as they develop in the thymus ("negative selection") or rendered unresponsive (anergic) in the periphery (Schwartz, 1989). However, peptides from a foreign protein, e.g., originating from a virus or other intracellular microbe (Townsend and Bodmer, 1989), or an anomalous self protein, such as a tumor cell's mutated protein (Lurquin et al., 1989; Mandelboim et al., 1994), can (in association with a self MHC-I protein) elicit and react with CD8+ T cells. Peptides from certain normal (nonmutated) self proteins in some tumors can also be recognized, in association with MHC-I, by T cells that react against the tumor (Boon, 1994); for example, CD8+ T cells that can be isolated from excised human melanomas (Kawakami et al., 1994a, b; Coulie et al., 1994; Castelli et al., 1995; Bakker et al., 1994; Tsomides et al., 1996) or lymph nodes (Cox et al., 1994) have recently been shown to recognize peptides from nonmutated melanocyte-specific proteins in association with HLA-A2. The underlying requirement for all of these responses to be productive is that the abundance of the naturally processed pep-MHC complex is sufficient and the TcR affinity and kinetics are favorable (see Section VI, below).

CD4+ T cells, in contrast to the CD8+ cell, react with pep-MHC-II complexes on specialized APC (e.g., macrophages, dendritic cells, B cells). Peptides that bind to MHC-II proteins generally arise from integral membrane proteins or from endocytosed proteins, either soluble or membrane-associated (Tables I and II). These peptides are produced in special endosomal organelles by a set of cellular proteases that differ from those that generate MHC-I-binding peptides. Newly synthesized MHC-II molecules, complexed with a nonpolymorphic invariant chain, traffic into this endocytic pathway (Neefjes et al., 1990) and come into contact with the available peptides. There, the invariant chain is partially degraded, allowing some of the peptides to bind to MHC-II molecules under acidic conditions (pH optimum =5.0) and leading to egress of pep-MHC-II complexes to the cell surface. Studies aimed at quantitating the binding between synthetic peptides and MHC-II molecules succeeded before those involving peptide–MHC-I reactions (Babbitt et al., 1985; Buus et al., 1986; Jardetzky et al., 1990; Roche and Cresswell, 1990; Roof et al., 1990; Rothbard and Gefter, 1991). As with MHC-I binding peptides, immunodominance describes the phenomenon whereby only one or a few peptides from a given protein is bound efficiently by a given MHC-II molecule and presented to T cells, accounting for the bulk of a polyclonal T cell response to that protein.

Of considerable biological interest are the kinetics of the reactions between peptides and MHC proteins. The first kinetic data, obtained for MHC-II, indicated unusually slow on- and off-rates for the peptide–MHC reaction (e.g., $t_{1/2}$ off = 5-10 hr at 37°C, Buus et al., 1986). Sadegh-Nasseri and McConnell (1989) subsequently found
evidence for rapid formation of a quickly dissociating complex that slowly converted to a stable form ($t_{1/2}$ off >30 hr); the long half-life for peptide dissociation was shown to limit the association rate for an added peptide (Tampé and McConnell, 1991). These kinetically distinguishable pep-MHC-II complexes correlated with different behaviors in SDS-polyacrylamide gel electrophoresis: the short-lived complex dissociated into MHC-II $\alpha$ and $\beta$ subunits, whereas the more stable complex migrated as an intact heterotrimer ($\alpha, \beta, \text{peptide}$) (Sadegh-Nasseri and Germain, 1991, 1994; Stern and Wiley, 1992).

More recently, Nelson et al. (1994) found that the dissociation half-times of pep-MHC-II complexes vary widely, with selective persistence of the most stable complexes on APC helping to account for the striking immunodominance of certain peptides. Conversely, a rapidly-dissociating pep-MHC-II complex might be ineffective at eliciting T cell responses, including those that result in T cell tolerance. Thus, Fairchild et al. (1993) and Mason and McConnell (1994) have suggested that because of rapid dissociation a self peptide from myelin basic protein might fail to induce T cell tolerance toward this peptide. The failure would leave potentially autoreactive T cells that could eventually cause autoimmune encephalomyelitis if activated by cross-reacting viral peptides (see Oldstone, 1987; Wucherpfennig and Strominger, 1995; Section VI.F). It remains to be seen whether such a mechanism may also apply to peptides interacting with MHC-I proteins. A related possible mechanism for evading tolerance could be extrathymic post-translational modification of a self peptide, leading to inadequate negative selection of T cells specific for the modified peptide (Wu et al., 1995).

VI. T Cell Responses to Pep-MHC

The intensity of mature T cell responses to pep-MHC complexes on other cells is greatly affected by the cell surface abundance of these complexes and by small changes in peptide sequence, as illustrated in Fig. 4. When the same T cells and target cells (or APC) are used in the representative assays shown, synthetic peptides with closely related sequence can differ as much as a million-fold in the concentrations required for half-maximal intensity of the responses they elicit (SD$_{50}$ values, Tsomides et al., 1991). In attempts to account for these enormous differences, some T cell–target cell systems have been analyzed extensively in terms of: (1) the affinity of a T cell's TcR for particular pep-MHC complexes; and (2) the abundance of these complexes on target cells ("epitope density").

As discussed below (Section VI.C), TcR reactions with pep-MHC complexes approach equilibrium rapidly, indicating that intrinsic equilibrium constants for these reactions ("TcR affinities") can be relevant to understanding the responses illustrated in Fig. 4.

A. Affinity: Intrinsic Equilibrium Constants of TcR–Pep-MHC Reactions

Several approaches have been used to determine the equilibrium constants of TcR–pep-MHC reactions. They include: (1) competition assays in which soluble pep-MHC complexes compete with $^{125}$I-labeled antibodies (or Fab' fragments) for binding to TcR on intact T cells; and (2) direct binding of soluble, $^{125}$I-labeled pep-MHC complexes to TcR on intact cells, or unlabeled complexes to TcR molecules.
that are produced in soluble form by genetic engineering and immobilized on a solid support. All of these approaches have so far yielded reasonably consistent results (Table III), establishing confidence in: (1) the accuracy of the measured TcR equilibrium constants; and (2) the functional similarity of the genetically engineered, soluble analogs of TcR and MHC proteins to their native, membrane-associated forms.

The first measurement of an intrinsic TcR-pep-MHC equilibrium constant involved competition for the TcR on intact CD4+ T cells between a soluble pep-MHC-II complex and 125I-labeled Fab fragments of an antibody to the TcR's Vβ domain (Matsui et al., 1991). The free pep-MHC concentration that inhibited binding of the 125I-Fab by 50% was 5x10⁻⁵ M. This value (Kd) and the other equilibrium constants discussed here are all expressed below as association constants (Ka = 1/Kd) (Table III). A slightly higher Ka was found in another study that used a soluble TcR to inhibit the response of an intact CD4+ T cell (measured by the T cell's cytokine production) to an intact APC; by comparing the inhibitory effect of soluble TcR with the inhibitory effect of an anti-MHC Fab' fragment, it was estimated that the TcR's intrinsic affinity for the pep-MHC-II complex was ≈10⁵ M⁻¹ (Weber et al., 1992), a result that should be viewed with the understanding that it was indirect and based not on a measured physical interaction between TcR and its ligand but on production of IL2.

The equilibrium constants determined by Matsui et al. (1991) and Weber et al. (1992) (2x10⁴ M⁻¹ and ≈10⁵ M⁻¹, respectively) were consistent with the often expressed expectation that TcR affinities would generally fall in the low range exhibited by antibodies having germline V domains (i.e., not somatically mutated) sequences (e.g., Eisen, 1986; Davis, 1990). However, TcR with much higher intrinsic affinities were subsequently found by Sykulev et al. (1994a): in a binding assay involving the 125I-labeled Fab' fragment of an anti-clonotypic antibody to the TcR on a CD8+ T cell clone (2C) in competition with a soluble pep-MHC complex (formed by the MHC-I protein Ld and an octapeptide from α-ketoglutarate dehydrogenase, LSPFPFDL, termed p2Ca (Udaka et al., 1992, 1993)), the TcR affinity was found to be 1.5x10⁶ M⁻¹ (Table III).

Later, the same TcR was found to have a 10-fold higher affinity (1.5x10⁷ M⁻¹) for a related pep-Ld complex in which the peptide differed from p2Ca only by an additional glutamine residue at the N-terminus (QLSPFPFDL, QL9) (Sykulev et al., 1994b). Given this high intrinsic affinity, it proved possible to measure the direct binding of QL9-Ld complexes (trace-labeled with 125I) to TcR on intact 2C cells, obviating the need for indirect measurement by competition with an anti-receptor antibody. The values obtained by direct and indirect approaches were in close agreement, indicating the feasibility of measuring TcR affinities on diverse T cell clones without requiring rare (e.g., clonotypic) anti-TcR antibodies. Thus, in a direct binding assay with an 125I-labeled soluble pep-MHC complex formed by the MHC-I protein Kb and an octapeptide from ovalbumin (SIINFEKL, pOV8), the affinity of the TcR of an ovalbumin-specific CD8+ CTL clone (4G3) was found to be 1.5x10⁶ M⁻¹, another high-affinity reaction (Table III).

Whether the low affinities of the reactions between TcR on CD4+ T cells and pep-MHC-II complexes and the much higher affinities of the reactions between TcR on CD8+ T cells and pep-MHC-I complexes represent consistent differences between CD4+ and CD8+ clones is, perhaps, possible. The differences found so far more likely reflect a wider range of intrinsic affinities for TcR–pep-MHC reactions than had been
expected. Among the relatively small number of intrinsic affinities determined for TcR reactions with pep-MHC ligands, the highest values are similar to those often encountered for mAb against protein antigens (ca. $10^7$ M$^{-1}$). However, in view of the absence of extensive somatic mutation in TcR genes, it is doubtful that TcR affinities will match the highest intrinsic affinity values found for antibody reactions with protein antigens, which are often in the range of $10^8$ to $10^9$ M$^{-1}$ and can be as high as $10^{10}$ M$^{-1}$ (see Foote and Eisen, 1995, and Section VI.E.1, below).

B. Kinetics

Given the structural similarities between TcR and Ig molecules, the question arises as to whether TcR reactions with their natural ligands are similar kinetically to those of antibodies with antigens. To study TcR kinetics, the main approaches taken so far are much like those used to measure TcR affinities, e.g., they involve TcR molecules on intact T cells or genetically engineered TcR that are produced as soluble molecules and subsequently immobilized on a solid support for analysis by an optical method (surface plasmon resonance). Both approaches were used to study the reaction between the TcR of the CD8$^+$ T cell clone 2C (2C TcR) and the octapeptide p2Ca that it recognizes in association with L$d$. Based on competition between soluble p2Ca-L$d$ complexes and an $^{125}$I-labeled Fab' fragment of a clonotypic antibody for binding to the 2C TcR, the association and dissociation rate constants were found to be $1.1 \times 10^4$ M$^{-1}$ sec$^{-1}$ and $5.5 \times 10^{-3}$ sec$^{-1}$, respectively, at 25°C. For the higher affinity reaction of the same TcR with the closely related pep-MHC complex QL9.L$d$, the on-rate constant was slightly higher and the off-rate constant considerably lower than for p2Ca-L$d$ (Sykulev et al., 1994a, b) (Table IV).

Using soluble p2Ca-L$d$ complexes and soluble 2C TcR (Slanetz and Bothwell, 1991), Corr et al. (1994) measured their interaction by surface plasmon resonance. In this procedure, pep-MHC complexes flow over TcR produced in soluble form and bound covalently to a dextran surface by carbodiimide chemistry. The binding of pep-MHC complexes to TcR results in a local increase in refractive index, which is measured by a shift in resonance angle. Experimental data were fit to a single exponential equation, as for a conventional bimolecular reaction, as well as to a double exponential equation to improve the fit. Both analyses yielded essentially the same dissociation rate constant ($2.0-2.6 \times 10^{-2}$ sec$^{-1}$), which was 4-5 times larger than had been measured using the TcR on intact 2C cells. This disparity could be due to different pep-L$d$ complexes used in the two studies; the surface plasmon resonance study made use of a diverse population of L$d$ molecules that had been isolated from mammalian cells and probably contained a variety of endogenous peptides in addition to the cognate peptide p2Ca, i.e., pep-L$d$ complexes that might have bound weakly and dissociated more rapidly from the TcR than homogeneous p2Ca-L$d$ complexes used to study the TcR on intact cells. Of the two association rate constants found by surface plasmon resonance (Corr et al., 1994), the lower value agreed with the on-rate constant measured with intact 2C cells (Sykulev et al., 1994a).

The rate constants measured for the TcR of another high-affinity CD8$^+$ CTL clone, 4G3, and its natural ligand, the ovalbumin peptide SIINFEKL plus K$b$ (pOV8-K$b$), were determined by direct binding of pOV8-K$b$ to the TcR on intact 4G3 cells and were similar to those found for the 2C TcR reaction with one of its known natural ligands, p2Ca-L$d$ (Table IV).
Matsui et al. (1994) also used surface plasmon resonance to analyze the reactions between soluble TcR from a CD4+ T cell clone (2B4) and each of three closely related cytochrome c peptides complexed with IEk, an MHC-II protein. In keeping with the very low affinity of this clone's TcR for these pep-MHC complexes (Table III), the on-rate constants were extremely low (0.9-1.7x10³ M⁻¹ sec⁻¹) and the dissociation rates were rapid (t₁/₂ off values of 2-12 sec, Table IV) (see also Section VI.E.2).

Temperature Effects. All of the kinetic measurements recorded in Table IV were made at 25°C. The few made at both this temperature and 37°C showed: (1) a small increase in association rate constant at the higher temperature (presumably due to higher diffusion coefficients at 37°C than at 25°C); and (2) only an approximately 5-fold increase in dissociation rate constant. The equilibrium constant in this case was thus only slightly lower at 37°C than at 25°C (Table V).

Summary of On- and Off-Rates for TcR-Pep-MHC Reactions. The kinetic measurements made to date indicate that the association rate constants for TcR-pep-MHC reactions range from ≈10³ to ≈10⁵ M⁻¹ sec⁻¹. In contrast, the intrinsic on-rate constants for antibody reactions with protein antigens are generally about 10⁵ to 10⁶ M⁻¹ sec⁻¹, the latter probably representing an upper limit arising in part from diffusion rates of proteins (Mason and Williams, 1980; Foote and Milstein, 1991; Foote and Eisen, 1995).

TcR tend to dissociate faster from pep-MHC complexes than antibodies from protein antigens, with TcR t₁/₂ off values varying from a few seconds to 1-2 min, and t₁/₂ off values for antibody–protein antigen complexes generally ranging from a few min to an hr or more. These differences reflect the generally higher intrinsic affinities of antibodies than TcR for their respective ligands, probably due to antigenic selection of B cells having somatically mutated Ig genes and the absence of a comparable process for T cell selection in vivo. High intrinsic affinities can greatly enhance the efficacy of antibody molecules in solution, but they are less likely to have a correspondingly large impact on cell surface TcR, which is enormously multivalent (ca. 10⁵ TcR molecules per mature T cell). It has been suggested that, overall, an affinity ceiling for TcR (=10⁷ M⁻¹) is about 1000-fold lower than the affinity ceiling for antibodies (=10¹⁰ M⁻¹) (see Foote and Eisen, 1995, and Section VI.E.1, below).

In general, the on- and off-rates of TcR reactions with pep-MHC complexes are much faster than the rates involved in the reaction of peptides with MHC proteins. For some peptide–MHC reactions, association rate constants are slow (10⁰-10⁴ M⁻¹ sec⁻¹, Corr et al., 1994), and several hours (at room temperature) are often required to approach equilibrium (Kageyama et al., 1995). These reactions also tend to have long dissociation half-times, ranging from =1 hr to >100 hr (Buus et al., 1986; Cerundolo et al., 1991; Tsomides et al., 1991; Olsen et al., 1994), although in some instances they may be as short as 20 min (Fairchild et al., 1993; Mason and McConnell, 1994), or even less (Vturina et al., in preparation).

C. Time Required to Approach Equilibrium

T cells move continuously over other cells (Chang et al., 1979), but within 2-6 min of establishing contact with a suitable target cell, a T cell's response to antigen can...
be detected by an increase in the T cell's intracellular Ca\(^{2+}\) concentration (Poenie et al., 1987; Su et al., 1993). Can the reaction between TcR on the T cell and pep-MHC on the target cell reach a steady state within this brief interval?

Equilibrium is approached asymptotically at a rate that is generally defined by the time constant \(\tau\), the time required to form 63\% (i.e., 1-1/e) of the equilibrium number of receptor-ligand complexes. By measuring the net rate of accumulation of specific TcR•pep-MHC complexes on intact CD8\(^{+}\) 2C cells, Sykulev et al. (1994a, b) found that \(\tau\) was \(=3\) min for p2Ca-L\(^{d}\) at 25°C and \(=1\) min for QL9-L\(^{d}\) at 37°C. \(\tau\) varies with the pep-MHC concentration, becoming longer as the ligand concentration decreases, but it cannot be longer than approximately the half-time for dissociation (the \(t_{1/2}\) off value). This limit follows because \(1/\tau = (k_{+1})(L)+(k_{-1})\), where \((L)\) is the free pep-MHC concentration and \((k_{+1})\) and \((k_{-1})\) are on- and off-rate constants, respectively, for the TcR•pep-MHC reaction. Thus, for the most stable TcR•pep-MHC binding reaction measured so far (2C TcR + QL9-L\(^{d}\)), the \(t_{1/2}\) off was 0.7 min at 37°C (Table V), indicating that equilibrium for this reaction is approached in less than a minute. For reactions where \(t_{1/2}\) off values are shorter, equilibrium is approached even faster.

D. Epitope (Pep•MHC) Density

Many observations imply that the intensity of a T cell response depends upon the number of pep-MHC complexes ("epitopes") the T cell recognizes on another cell. For example, in the assays shown in Fig. 4 the extent of the CTL cytotoxic response varies with peptide concentration, which affects the total number of the corresponding pep-MHC complexes on target cells ("epitope density"). In another example, HIV-infected cells expressing low levels of processed viral peptides were lysed more poorly by HIV-specific CTL than infected target cells expressing higher levels of the same peptides (Tsomides et al., 1994). Other studies pointing to a critical role for epitope density include the effects of peptide concentration on the fate of developing thymocytes (positive and negative selection, Ashton-Rickardt et al., 1994) and the demonstration of nonphysiological T cell cross-reactions when high concentrations of synthetic peptides were added to target cells (Milligan et al., 1990; Schild et al., 1990; Dutz et al., 1994).

The first attempts to measure epitope densities involved incubating target cells (or APC) with \(^{125}\)I-labeled peptides at minimally active concentrations. The restricting MHC protein, together with its associated peptides, was then immunoprecipitated and the number of \(^{125}\)I-labeled pep-MHC complexes per target cell determined. The results indicated that a minimum of around 100-400 complexes were required to elicit cytokine production by CD4\(^{+}\) T cell hybridomas (Harding and Unanue, 1990; Demotz et al., 1990) or lysis of target cells by CD8\(^{+}\) T cell lines (Christinck et al., 1991).

Subsequent studies of antigen recognition by CD8\(^{+}\) CTL made use of mutant target cells (RMA-S, T2) with a defect in the transporter (termed TAP) that translocates peptides from the cytosol, where they are generated, into the ER (Townsend et al., 1989; Ljunggren et al., 1990; Schumacher et al., 1990; Cerundolo et al., 1990). In such cells newly synthesized MHC-I proteins arrive at the cell surface membrane deficient in bound peptides (e.g., peptide-free or with weakly bound peptides derived, perhaps, from protein signal sequences), and they then undergo rapid denaturation (at 37°C) unless stabilized by the binding of synthetic extracellular peptides. By raising the extracellular
concentration of peptide, the cell surface epitope density is increased, resulting in
greater target cell lysis by CTL. The free concentration of peptide required to sensitize
target cells for half-maximal lysis (called the $SD_{50}$ value, Tsomides et al., 1991) is a
useful indicator (though not in itself a measure) of the density of pep-MHC complexes
needed to elicit CTL-mediated lysis. Similarly, for stimulated CD4+ T cells that
respond by proliferating and producing cytokines, increasing the concentration of
extracellular peptide increases the responses.

Cytotoxicity assays are typically carried out for 4 hr (Fig. 4), and for many
peptides their reactions with MHC on the target cell reaches equilibrium (steady state)
during this time. Hence, for these peptides an estimate of the average epitope density
($r$) on target cells may be calculated from the free concentration of synthetic peptide ($c$)
in extracellular medium, the equilibrium association constant for the peptide–MHC
reaction ($K$), and the total number of pep-MHC sites available for binding peptide ($n$),
the later being determined with saturating concentrations of the peptide. (Eq. 1 above, $r$
$= K\cdot c\cdot n/(1+K\cdot c)$). Using this approach, Kageyama et al. (1995) determined the epitope
densities required to effect half-maximal lysis of target cells in a study involving sixteen
peptides and three MHC-I proteins on intact cells ($K_b$, $L^d$, and HLA-A2). With
different combinations of peptides, MHC proteins, and T cell clones, the epitope
densities required for half-maximal lysis were found to vary from several thousand
pep-MHC complexes per target cell to fewer than ten. The significance of this wide
range emerged from the subsequent development of a quantitative model for CTL–
target cell interactions (see Fig. 5 and below).

To check the accuracy of epitope densities determined according to Eq. (1), the
amount of an $^{125}$I-labeled peptide ($I_1$-QLSPFPFDL, termed $I_1$-QL9-Y5) that binds to $L^d$
(an MHC-I protein) on intact cells was measured directly, taking advantage of a method
for preparing radioiodinated peptides with extremely high specific radioactivities
($3.5\times10^{18}$ cpm per mole of monoiodinated peptide, the same as the specific activity of
carrier-free $^{125}$I (Tsomides and Eisen, 1993b)). The epitope densities found by this
direct approach and those calculated by Eq. (1) (Sykulev et al., submitted) agreed well
(to within a factor of two). When the same high specific activity peptide ($^{125}$I-$I_1$-QL9-
Y5) was added to target cells at a concentration that resulted in half-maximal
cytotoxicity ($SD_{50} = 5$ pM), only an average of 3 peptide molecules were bound to $L^d$
per target cell. Since these few peptide-$L^d$ complexes were probably distributed at
random over the target cell surface it appears that in CTL:target cell conjugates a single
pep-MHC on the target cell may be able to trigger a T cell's cytotoxic response (Sykulev
et al., submitted). A single complex can bind at any instant to only a single TcR
molecule (i.e., engage in univalent binding). It seems therefore that univalent ligation
of a TcR, without aggregation or cross-linking several TcR molecules, can elicit a
cytotoxic T cell response. Whether more complex responses, which require the
activation of previously silent genes (as in cytokine production), can also be triggered
by univalent TcR ligation is not clear.

To measure epitope densities of naturally processed (“endogenous”) peptides on
the cells that produce them requires a different approach. At low pH, peptides
dissociate rapidly from pep-MHC complexes (Buus et al., 1988; Röttschke et al.,
1990a), and in one effective procedure cells are treated briefly (ca. 1 min.) with isotonic
buffer at pH=3, stripping peptides from cell surface MHC (Storkus et al., 1993). The
peptides can then be fractionated by HPLC and particular peptides detected by bioassay.
with specific clones of CTL: sensitivities of \( \leq 10^{-12} \) M peptide have been obtained for several CTL-peptide-target cell systems (Bodmer et al., 1988; Reddehase et al., 1989; Tsomides et al., 1991; Rötzschke et al., 1990b; Falk et al., 1991b; Rötzschke et al., 1991a), and a theoretical limit for peptide at \( 10^{-13}-10^{-14} \) M has been postulated (Kageyama et al., 1995). In each case the efficiency of peptide recovery must be quantitated for an accurate assessment of natural peptide abundance (reviewed in Tsomides and Eisen, 1993a; for examples see Table II, above). While current efforts emphasize identification of the natural epitopes involved in T cell responses to tumor cells, allografts, cells infected by viruses or other pathogens, target cells of autoimmune reactions etc., a reasonable next step will be to measure the abundance of these epitopes in order to find out whether epitope densities commonly limit T cell responses (Tsomides, 1996).

E. TcR–Pep·MHC Engagement: An Affinity Model

The intensity of the T cell response has been widely assumed to be determined, in large measure, by TcR affinity for pep·MHC complexes and the number of these complexes on target cells (epitope density). The preceding section dealt with epitope density; here we focus on TcR affinity. Since the TcR–pep·MHC reaction approaches equilibrium rapidly (see Section VI.C, above), equilibrium constants for this reaction could govern the outcome of T cell–target cell encounters. However, TcR molecules and pep·MHC complexes are confined to the surface membrane of T cells and target cells, respectively. Hence the question arises as to whether their interaction can be described in terms of the law of mass action, which is generally concerned with interactions that involve freely diffusable reactants. Nevertheless, in developing a quantitative model for the antigen recognition step in the activation of T cells, Sykulev et al. (1995) assumed from the law of mass action that

\[
(TcR \cdot pep \cdot MHC) = (K) (TcR) (pep \cdot MHC),
\]

where \((TcR \cdot pep \cdot MHC)\) and \((pep \cdot MHC)\) refer, respectively, to the number per target cell of pep·MHC complexes that are or are not engaged by TcR molecules, and \((TcR)\) is the concentration of unengaged TcR molecules (assumed to be the same as the total TcR concentration because only a very small proportion of a mature T cell's TcR molecules engage pep·MHC complexes in any particular cell–cell interaction). \(K\), the "TcR affinity", is the equilibrium association constant for the TcR–pep·MHC reaction. The total epitope density, designated \((pep \cdot MHC)_0\), was assumed to equal the sum of TcR-bound and unbound pep·MHC; hence by substituting \((pep \cdot MHC)_0 - (TcR \cdot pep \cdot MHC)\) for \((pep \cdot MHC)\), they obtained:

\[
\log (pep \cdot MHC)_0 = \log (TcR \cdot pep \cdot MHC) - \log \{(K) (TcR)/[1+(K) (TcR)]\}
\]

On the assumption that \([TcR \cdot pep \cdot MHC]\) is fixed for a given level of T cell response (e.g., half-maximal cytotoxicity) and a particular T cell–target cell system, Eq. 2 describes the relationship between total epitope density and TcR affinity. A satisfactory fit was found between Eq.3 and experimentally determined values for total epitope density required for half-maximal T cell cytotoxic responses and TcR affinity for several TcR–pep·MHC reactions (Fig. 5). As shown in this Figure and predicted by Eq.3, over a wide range of affinity values (\(\leq 10^4\) to ca. \(10^6\) M\(^{-1}\)), the log (base 10) epitope density was a linear function of log (base 10) TcR affinity with a slope of minus
one. The agreement between this relationship and the law of mass action suggests that in a T cell–target cell conjugate TcR molecules on the T cell and pep-MHC complexes on the target cell come to occupy the same volume and react as though in solution. The validity of this model should become evident when its predictive ability is put to the test: since the product of epitope density and TcR affinity is constant (over the linear range shown in Fig. 5), with a peptide whose target cell epitope density at a half-maximal cytotoxic response is known, the model can predict the affinity of the responding T cell's TcR. Whether the predicted affinity values will prove to be correct remains to be seen.

1. **TcR Affinity Ceiling**

Eq. 3 predicts that at TcR affinities above some upper level ("affinity ceiling") the epitope density values plateau at a lower limit (Fig. 5), which corresponds for a specified level of response (half-maximal cytotoxicity) to the minimal number per target cell of ligated pep-MHC complexes. From the limited amount of data available at high affinity values the minimum epitope density value was seen to fall between 1 and 10 pep-MHC complexes per target cell (for TcR affinities above approximately 5x10^6 M^-1). The model's prediction was supported by the subsequent finding (made with the high specific activity radiolabeled peptide) that an average of 3 pep-MHC complexes per target cell were sufficient to elicit half-maximal target cell lysis by a CTL clone whose TcR's affinity for these complexes was sufficiently high (above 10^6 M^-1).

Although the precise value of the TcR affinity ceiling is not well defined by the available experimental data (Fig. 5), in principle it equals the reciprocal of the effective TcR concentration. This concentration remains to be defined. From the best fit between experimental data and Eq. 3, plotted as the solid line in Fig. 5, the concentration may be about 10^-7 M, a value that can be justified by the number of TcR molecules per mature T cell (=10^5, see Matsui et al., 1991; Sykulev et al., 1994a,b), and the fact that with extensive changes of T cell shape and surface area, the cell surface TcR molecules occupy over time a volume that approximates the T cell's volume (ca. 10^-6 μl per cell).

2. **Kinetic Control of T Cell Responses**

Equations 2 and 3 and the results shown in Fig. 5 assume that in T cell–target cell encounters the TcR–pep-MHC reaction essentially reaches a steady state, and that TcR affinity is a critical determinant of the response. Under some conditions, however, it is possible that rates of dissociation of pep-MHC from TcR, rather than equilibrium constants, might determine the response. In particular, if TcR–pep-MHC bond lifetimes were close to the bond residence time required to activate a signal transduction pathway, dissociation rate differences could be decisive. This possibility is evident from a study by Matsui et al. (1994). Markedly different concentrations of three similar peptides were needed to elicit equivalent responses by a CD4+ T cell hybridoma whose TcR had about the same low affinity (=10^4 M^-1) for all three peptides in association with the MHC-II protein IE^k. Based on the belief that these peptides interacted equally well with IE^k (and thus formed the same epitope densities at the same peptide concentrations), it was concluded that the different peptide concentrations required to elicit the T cell response might relate to the differences found in t_{1/2} off values of the TcR from the respective pep-MHC complexes (2-12 sec). Thus, while the affinity
model described above emphasizes the average number of TcR–pep-MHC bonds present at any instant under steady state conditions, an alternative view suggests that under some circumstances the TcR–pep-MHC bond lifetime might play a decisive role. Just how short the lifetime would have to be to serve as an independent controlling factor in a T cell response is not clear.

In Section VI.D it was noted that a single pep-MHC on a target cell can activate a T cell cytotoxic response. At any instant a single complex can bind to only one TcR molecule, but over time it can bind to one or a few TcR molecules repetitively or serially to many different TcR molecules, as proposed by Valitutti et al. (1995). Since it was suggested that many serial engagements enhance T cell responses, Valitutti et al. (1995) predicted that high TcR–pep-MHC dissociation rates, which are generally associated with low-affinity reactions, would promote T cell responses. Other studies, however, show that high-affinity TcR–pep-MHC reactions, which are generally associated with slow dissociation rates, enhance T cell cytotoxic responses (Sykulev et al., 1994a,b).

F. Specificity, Degeneracy, and Molecular Mimicry

It seems to be widely believed that high levels of specificity in immune reactions are manifestations of high-affinity interactions. However, measurements of TcR affinities have shown that exquisite specificity can be manifested by very low-affinity reactions. The lowest TcR intrinsic affinity measured so far, for the reaction of the 2C TcR with the naturally occurring peptide p2Ca (LSPFPFDL) in association with K\text{b}, is $3 \times 10^3$ M$^{-1}$ (Table III). Even with this low affinity it was possible for 2C CTL to lyse target cells bearing p2Ca–K\text{b} complexes (Sykulev et al., 1994a; Dutz et al., 1994), and, moreover, to exhibit a striking degree of specificity: as shown in Fig. 6, p2Ca and another naturally occurring octapeptide that differ only by a phe$\rightarrow$tyr substitution (LSPYPFDL, called p2Ca-Y4) are clearly distinguished by 2C CTL in cytotoxicity assays with K\text{b}$^+$ target cells (T2-K\text{b}) (Wu et al., 1995). Though both peptides bind equally well to K\text{b}$^+$, T2–K\text{b}$^+$ cells sensitized with p2Ca were lysed, whereas those sensitized with p2Ca-Y4 were not. This pronounced difference could be explained if the 2C TcR affinity for the p2Ca–K\text{b} complex ($3 \times 10^3$ M$^{-1}$) was just above the affinity threshold required for a cytotoxic response (we assume it to be $\approx 1 \times 10^3$ M$^{-1}$) and the 2C TcR affinity for the p2Ca-Y4–K\text{b} complex were just below it. It is also possible that it is not the lower affinity per se, but rather perhaps a faster off-rate that is responsible for failure of the ineffective complex to elicit a cytotoxic response. (Low-affinity reactions of antibodies are also capable of exhibiting high levels of specificity (Sykulev et al., 1992, 1993); such reactions underline the important practical use of antibodies against polysaccharides of blood group substances and bacterial cell walls as diagnostic reagents for typing blood cells or Salmonella.)

In distinguishing sharply between two pep-MHC complexes that differ only by a single oxygen atom in their respective peptide adducts, the TcR of CTL clone 2C matches the highest level of specificity exhibited by the most discriminating antibodies or enzymes. Comparable levels of discrimination are also suggested by the widely different concentrations of closely related synthetic peptides that are required to elicit responses by a given T cell clone (e.g., Fig. 4). However, unless the cell surface pep-MHC densities formed by the different peptides are known, large differences in T cell responses could result from differences in epitope densities of the corresponding
complexes rather than differences in TcR affinities for these complexes.

**Degeneracy.** In contrast to the high level of specificity described above (e.g., Fig. 6), the specificity of a given TcR can also appear quite low, as evidenced by reactions with a wide variety of different pep-MHC complexes. Thus, a T cell arising in the course of viral infection characteristically reacts with a peptide of viral origin plus one of the infected individual's own MHC proteins (a "foreign-self" complex). And such a T cell, like most others, can also react with a different MHC protein from another individual of the same species (allogeneic MHC, or "alloMHC") (Nahill and Welsh, 1993; see Paradox of Alloaggression, below), and each of these alloreactions may involve a particular peptide in association with the alloMHC molecule (e.g., Uda and others, 1992; see Section IX, below). Moreover, this T cell would have been stimulated to mature in the thymus (positive selection) by reaction of its TcR with thymic peptides in association with indigenous MHC proteins (Schwartz, 1989; von Boehmer, 1994; Ashton-Rickardt and Tonegawa, 1994; Hogquist et al., 1994).

The cross-reactions exhibited by T cells with sets of peptides having closely related or overlapping sequences, as in Fig. 4, are readily understandable. More interesting are the cross-reactions of viral or bacterial peptides with T cells that react with apparently unrelated self peptides, e.g., myelin basic protein found in the central nervous system (Wucherpfennig and Strominger, 1995; see also Selin et al., 1994). These cross-reactions can involve peptides having no amino acids in common except the few that serve as "anchors" to bind to MHC protein, a situation aptly described by the term "molecular mimicry" (Oldstone, 1987). Another example involves CTL clone 2C, which recognizes the naturally occurring α-KGDH peptide p2Ca (LSPFPFDL, see Table II) in association with Ld: this T cell also reacts with the Nε-2,4-dinitrophenylated form of an HIV peptide (ILKEPVHGV) in association with the same MHC protein (Ld) (Tsomides, unpublished data). Growing evidence suggests that cross-reactions due to molecular mimicry play an important role in stimulating autoreactive T cells (those having specific reactivity with a self peptide plus a self MHC protein), thereby triggering autoimmune disorders (Wucherpfennig and Strominger, 1995).

Since Vα and Vβ domains have so much amino acid sequence similarity to VL and VH domains, why should the range of cross-reactions appear to be so much greater for TcR than antibody molecules? Cross-reactions are certainly commonplace with antibodies, but they usually are readily understandable with even a superficial view of the cross-reacting structures. This is the case even for "strange" cross-reactions, e.g., as seen in the ability for antibodies raised against the 2,4-dinitrophenyl group to bind vitamin K3 (2-methyl-1,4-naphthaquinone) (Michaelides and Eisen, 1974). However, as noted above, the variety of pep-MHC that can be recognized by any particular TcR seems far greater than the variety of antigens that react with an antibody. One possible explanation is that the Vα-Vβ binding site is much more flexible than the VL-VH binding site and can adapt its conformation to fit many different ligands. Such flexibility could account for the difficulties currently being experienced in obtaining well-ordered TcR crystals for structure analysis.

A more likely explanation is that T cell reactions with pep-MHC can be detected with far greater sensitivity than antibody–antigen reactions: e.g., peptides at 10^-12 M can elicit half-maximal responses (see Fig. 4) and TcR–pep-MHC reactions having
affinity values as low as $10^{-3}$ M can result in target cell lysis (Fig. 6); an antibody reaction having such a low intrinsic affinity would ordinarily not be detectable. The great multivalency of T cells ($\approx 10^5$ TcR molecules per T cell), the abundance of some pep-MHC ligands on many target cells plus the ability of very few cognate pep-MHC complexes ($<10$ per target cell) to elicit T cell responses, and the amplifying effect of signal transduction in T cells must all contribute to the extraordinary sensitivity of T cell reactions and the correspondingly great range of their cross-reactions.

VII. TcR Accessory Proteins

A. CD3 and $\zeta$

The antigen-specific heterodimeric ($\alpha\beta$) TcR exists at the cell surface as part of a large multichain complex that includes the four invariant chains of CD3 ($\gamma$, $\delta$, and two $\epsilon$ chains) plus the structurally distinct $\zeta$ molecule, which is found in most T cells as a disulfide-linked homodimer. On some T cells an alternatively spliced form of $\zeta$, termed $\eta$, is present as $\zeta\eta$ heterodimers (reviewed by Ashwell and Klausner, 1990; see also Irving and Weiss, 1991). Immunoprecipitation (Blumberg et al., 1990) and quantitative immunofluorescence analysis (Punt et al., 1994) showed that there are two $\epsilon$ chains per $\alpha\beta$ TcR molecule on intact T cells; the exact stoichiometry of the $\eta$ chain homodimer or $\zeta\eta$ heterodimer with other chains of the TcR complex is not clear.

When cDNA for the $\alpha$, $\beta$, and $\gamma$ TcR subunits were first isolated and sequenced (Hedrick et al., 1984; Saito et al., 1984a, b), the deduced amino acid sequences revealed, curiously, a positively charged residue in the otherwise hydrophobic transmembrane domain. The CD3 $\gamma$, $\delta$, and $\epsilon$ subunits and $\zeta$ chain turned out to have in their hydrophobic transmembrane domains a negatively charged amino acid residue, suggesting that these charged sites are involved in assembling TcR heterodimers with the CD3 and $\zeta$ complex (see Davis, 1990).

CD3 chains immunoprecipitate together with $\alpha\beta$ TcR molecules. $\zeta$ molecules do not coprecipitate with $\alpha\beta$, but their essential involvement in the TcR complex is indicated by the fact that their expression, like that of CD3 $\gamma$, $\delta$, and $\epsilon$, is required for cell surface expression of the $\alpha\beta$ TcR. Minor truncations or alterations of $\zeta$ structure can abolish TcR function, as revealed by loss of responsiveness to antigens (reference).

The binding of antibodies to TcR or CD3 molecules on intact T cells activates two major signal transduction pathways, one resulting in hydrolysis of phosphatidylinositol-4,5-diphosphate and transient increases in intracellular Ca$^{2+}$, the other leading to tyrosine phosphorylation of cytoplasmic domains of CD3 subunits and the $\zeta$ dimer (reviewed by Weiss and Littman, 1994). None of these subunits exhibit intrinsic kinase activity; they appear to be substrates for Fyn, a member of the Src family of tyrosine kinases that coprecipitates with CD3 (Samelson et al., 1990). Similarly, the Src family member Lck interacts with cytoplasmic domains of CD4 or CD8 (Rudd et al., 1988).

A critical role for the cytoplasmic domains of CD3 and $\zeta$ chains in TcR-mediated signaling is evident from experiments involving T cell hybridomas transfected with genes for chimeric proteins that consist of various extracellular domains linked to cytoplasmic domains of CD3 or $\zeta$; antibodies to the extracellular domains of these
chimeric proteins elicited T cell responses (Wegener et al., 1992; Letourneur and Klausner, 1992). Mutational analyses of the cytoplasmic domains revealed an “antigen recognition activation motif” (ARAM) that includes tyrosine and leucine (i.e., D/E—Y—L/I(—)6,8Y—L/I) (reviewed by Weiss, 1993). The ARAM may serve as a phosphorylation site, allowing the recruitment of other intracellular molecules via SH2 domain–phosphotyrosine interactions. Thus, TcR ligation results in intracellular tyrosine phosphorylation of CD3 and ζ and recruitment of signaling proteins that bind specifically via their SH2 domains to phosphotyrosine. One of these signaling proteins, ZAP70, is associated with the phosphorylated form of ζ (Chan et al., 1992). There are three copies of ARAM per ζ chain but only one copy in each of the CD3 chains, structural features that may be related to different requirements for inducing diverse signaling pathways. For example, Sloan-Lancaster et al. (1994) found a different extent of ζ chain phosphorylation after challenging a clone of CD4+ T cells with different peptides of related sequence in association with a MHC-II protein (see Section VIII, below).

B. CD4 and CD8

As noted in Section II, the mutually exclusive expression of cell surface glycoproteins CD4 and CD8 on mature T cells distinguishes between the two major T cell lineages. During development in the thymus, immature T cells express both markers (CD4+CD8+ or double positive cells); these cells make up the majority of thymocytes and appear to undergo commitment stochastically to either the CD4+ or CD8+ single positive phenotype as they mature (Davis et al., 1993; Chan et al., 1993). CD4 is a single chain transmembrane glycoprotein, while CD8, which consists of two membrane-associated polypeptides, can be either an αα homodimer or an αβ heterodimer; functional distinctions between them are not yet clear (see Weiss and Littman, 1994). In humans and mice, ~60% of peripheral αβ TcR+ cells are CD4+ cells and the rest are CD8+. The preponderance of CD4+ over CD8+ cells is not universal, however, and in the African green monkey ~80% of peripheral T cells are CD8+ and ~10% CD4+ (Ennen et al., 1994).

CD4 and CD8 are both members of the Ig superfamily, and each has a positively charged CDR-like loop that protrudes from the N-terminal domain (CD4 or CD8α) and is thought to make contact with an invariant region of MHC-II or MHC-I molecules, respectively (Wang et al., 1990; Ryu et al., 1990; Leahy et al., 1992). The binding of a soluble, genetically engineered MHC-II protein (HLA-DR4) to a soluble, immobilized preparation of CD4 was reported to have an equilibrium constant of 1x10^5 M⁻¹ (Cammarota et al., 1992).

If a CD8 molecule on a T cell binds to precisely the same pep-MHC-I complex on the target cell that is bound by the T cell's TcR, the CD8–MHC-I interaction would be expected to enhance the overall stability of the TcR•pep-MHC-I complex, and the same would be expected for CD4 and pep-MHC-II complexes. However, no evidence for such stabilization was found when the binding of soluble pep-MHC complexes (either class II or class I) to TcR on intact CD4+ or CD8+ T cells was measured (Matsui et al., 1991; Sykulev et al., 1994a). These negative results are in accord with the finding of the same equilibrium constant for the TcR•pep-MHC-II reaction as measured with soluble pep-MHC and either intact CD4+ T cells or genetically engineered, immobilized TcR (Matsui et al., 1994). Recently, however, Luescher et al. (1995) have
succeeded in showing with intact T cells and a photoaffinity-labeled soluble pep-MHC-I complex that CD8 can modulate the TcR-pep-MHC interaction. Different monoclonal antibodies to CD8α and CD8β chains appeared to have different effects on the CD8–MHC-I interaction; while most anti-CD8 antibodies were inhibitory, some enhanced CD8–MHC binding, suggesting that small conformational changes in the CD8 protein might influence its interaction with MHC. It is possible that the extent of interaction of CD4 and CD8 with MHC proteins may depend upon the particular TcR and pep-MHC complexes involved.

VIII. Altered Peptide Ligands: Partial Agonists and Antagonists

Until recently, the TcR was generally viewed as an on-off switch, with its binding site vacant or occupied by ligand, and the liganded form, in sufficient amount and stability, causing the T cell to become activated. Surprisingly, however, it has turned out that ligand binding can result in various manifestations of T cell activation, depending upon the ligand (Evavold and Allen, 1991; De Magistris et al., 1992; Alexander et al., 1993; Jameson et al., 1993; Racioppi et al., 1993; Sloan-Lancaster et al., 1993; Vignali and Strominger, 1994). This phenomenon was strikingly evident with a peptide derived from hemoglobin, Hb64-76 (GKKVITAFVEGCK), in association with IEk (Sloan-Lancaster et al., 1993). Antigen presenting cells bearing this pep-MHC complex stimulated a characteristic activation response by a CD4+ T cell clone: T cell proliferation, cytokine secretion (IL2, IL3, δ-IFN), and hydrolysis of intracellular inositol phosphates. However, when the peptide was modified by a single ala→ser substitution at position 70 and presented with IEk, the same T cells responded only partially; they failed to proliferate, produce cytokines, or hydrolyze inositol phosphates, but they were able to express high levels of IL2 receptors and LFA-1 (an adhesion molecule), indicating that they could make a limited response to the altered peptide. Moreover, T cells exposed to the altered peptide were unable to proliferate in response to the unaltered, optimally active (agonist) peptide Hb64-76 for several days.

The persistent absence of proliferation in response to an immunogenic peptide appears to correspond to what was described earlier as T cell anergy, a state resulting from the stimulation of CD4+ T cells via TcR ligation in the absence of a second “costimulatory” signal (see Mueller et al., 1989). Costimulation for most T cells is provided by engagement of the T cell surface molecule CD28 by a protein ligand on APC called B7 for which various subtypes have been described (reviewed by Linsley and Ledbetter, 1993; June et al., 1994; Jenkins, 1994). Anergy has been described for various CD4+ T cells (Th0, Th1, and Th2) as well as for CD8+ T cells. Its hallmark is aberrant T cell responsiveness to TcR engagement, notably failure of ligand binding to drive T cell proliferation but preserved ability to trigger other manifestations of T cell activation, such as production of cytokines or lysis of target cells.

The anergic state induced by an altered peptide ligand (a partial agonist or an antagonist peptide) has been found to be characterized by aberrant signal transduction (Sloan-Lancaster et al., 1994; Madrenas et al., 1995). Binding of agonist pep-MHC complexes to a TcR results in a distinctive pattern of tyrosine phosphorylation of cytoplasmic domains of CD3 components and ζ chains. Since some altered peptides (partial agonists) induced a modified pattern of ζ chain phosphorylation, anergy is likely to result from activation of an anomalous signal transduction pathway.
The responses elicited by altered peptide ligands parallel to a large extent the activation of T cells by various monoclonal antibodies to the TcR or CD3. However, the signal transduction events evoked by these surrogate ligands do not completely mimic those resulting from physiological ligands (pep-MHC complexes). For example, pep-MHC complexes and antibodies to the ε subunit of CD3 can both trigger tyrosine kinase activity, but this response is inhibitable by cAMP only when the physiologic stimulus is used (Klausner et al., 1987). Moreover, various anti-TcR and anti-CD3 antibodies can differ considerably from one another in the concentrations required to elicit a given T cell response; although these differences seem not to correlate with variations in the antibodies' affinities for TcR, it should be borne in mind that Fab fragments were used to measure the affinities, but intact (bivalent) antibodies were used to study the effects on T cell activation (or inhibition) (Rojo and Janeway, 1988; Rojo et al., 1989; Yoon et al., 1994).

Given the complexity of the multisubunit structure that includes the αβ TcR, various CD3 subunits and ζ chains, and probably CD8 and CD4 as well, it is likely that there are many different opportunities for various ligands that bind to the TcR complex to activate different signaling pathways. Whether the different responses elicited by agonist and partial agonist peptides (in pep-MHC complexes) and by various anti-TcR antibodies result from differences in affinity or kinetics (e.g., bond lifetimes), or both, remains to be determined. Not surprisingly, an additional complexity is that the different effects elicited by various altered peptide ligands can differ among the various T cell clones that are raised against the same pep-MHC. The biologic significance of these complex effects is indicated by the finding that some naturally processed peptides of viral origin act as antagonists (or partial agonists), even at extremely low concentrations, for some anti-viral CTL (Klenerman et al., 1994; Bertoletti et al., 1994).

IX. MHC Restriction by Self and Nonself MHC: The Paradox of Alloaggression

All of the αβ TcR molecules on an individual's mature T cells are imprinted, as it were, with the capacity to recognize that individual's own MHC proteins (syngeneic MHC or "synMHC") in the form of pep-MHC complexes. The basis for this imprinting is emerging from studies of T cell maturation in the thymus (e.g., reviewed by von Boehmer, 1994). Immature double positive (CD4+CD8+) thymocytes are stimulated to mature into single positive (CD4+ or CD8+) T cells through “weak” interactions of their TcR with pep-MHC complexes on thymic epithelial cells (positive selection), whereas double positive cells that interact “strongly” with these complexes on thymic cells of hematopoietic origin undergo programmed cell death (negative selection). Although the peptides involved in these reactions have not been identified, and the kinetics, affinity, and specificity of the reactions in the thymus are all unknown, it is clear that the MHC proteins involved in both positive and negative selection are clearly indigenous, i.e., synMHC. As long as they can associate with a synMHC, an enormous number of different foreign peptides can be recognized by one or another of the clonally diverse TcR on the mature T cells that migrate out of the thymus; in other words, antigen recognition is normally restricted by synMHC.

Paradoxically, however, the same mature T cells can also react specifically with different MHC proteins from other individuals of the same species (alloMHC), and these reactions, like those restricted by synMHC, generally involve pep-MHC complexes (Rötzschke et al., 1991b; Udaka et al., 1992), if only in some cases because
the peptide's presence is required to stabilize the MHC molecules at 37°C. As described in Section II, MHC genes are the most polymorphic known; for example there are over 50 allelic variants of the murine gene K^b. The frequency of an individual's T cells that can react with any particular alloMHC is so high (e.g., =1:100, Fischer Lindahl and Wilson, 1977; Erard et al., 1985), that virtually every TcR must be able to recognize not only a peptide restricted by synMHC but also a peptide (probably a different one) restricted by an alloMHC molecule.

CTL clone 2C, referred to above (e.g., Tables III-V), is an example of such an alloreactive clone. It arose in an H-2^b mouse immunized with H-2^d cells and responds specifically to L^d (one of the three MHC-I proteins on H-2^d cells) in association with a peptide (termed p2Ca) that derives from the cellular protein α-ketoglutarate dehydrogenase (α-KGDH) (Kranz et al., 1984a; Udaka et al., 1992, 1993). Genes for the α and β subunits of the TcR on 2C cells have been expressed as transgenes in mice with different MHC haplotypes (Sha et al., 1988a, b). In mice with the H-2^d haplotype, double positive thymocytes that express transgenes for the 2C TcR undergo programmed cell death and fail to develop into mature T cells (negative selection), probably because of strong interactions in the thymus between this receptor and p2Ca-L^d complexes. However, in mice that lack L^d and have the H-2^b haplotype (the background haplotype in which the 2C cell arose), double positive thymocytes having the 2C TcR are positively selected by K^b (synMHC), presumably as a result of weak interactions with pep-K^b complexes (whose peptides are still unknown). Thus, most peripheral T cells in H-2^b 2C TcR transgenic mice express the 2C TcR, and despite having been positively selected by K^b they are able to react strongly with L^d in association with p2Ca (and related peptides from α-KGDH). To compound this paradox the 2C TcR's affinity for the pep-alloMHC ligand p2Ca-L^d is unusually high; indeed, it has been suggested that TcR affinity for pep-alloMHC complexes will generally tend to be higher than for pep-synMHC (Sykulev et al., 1994b, and see Fig. 7).

Why should a TcR restricted by synMHC also be restricted by an ostensibly unrelated alloMHC, and why might TcR affinity for pep-alloMHC tend to be higher than for pep-synMHC? The answers probably stem from the fact that TcR genes and MHC genes segregate independently. In an outbred population the TcR genes inherited from each parent must encode a TcR repertoire that is so highly diversified it can recognize and be restricted by virtually any MHC protein of the species. (If this were not the case, many of each individual's MHC proteins would be wasted, i.e., incapable of participating in the recognition of antigenic peptides.)

This broad repertoire is then narrowed down in each individual by positive and negative selection in the thymus to become restricted for antigen recognition by any one of the allelic MHC gene products in that individual. Through negative selection, immature T cells whose TcR happen to have high affinity for synMHC will be actively eliminated, while those that have low affinity for synMHC will escape this fate, whether or not they happen to have high affinity for alloMHC - because, by definition, alloMHC are not present in the normal animal. Hence a mature T cell, having survived negative selection and been positively selected, will have a very low but presumably significant affinity for synMHC (see below), but may have a high affinity for one or more alloMHC proteins. The resulting hypothetical distribution of TcR affinities for synMHC and alloMHC before and after thymic selection is illustrated in Fig. 7.
Differences between TcR affinities for pep-synMHC and pep-alloMHC complexes might be clarified if they could be resolved into two separate affinities, one for the peptide moiety and one for the restricting MHC protein. Some help in arriving at an estimate for these individual affinities may eventually be provided by the three-dimensional structures of TcR*pep-MHC complexes in which the intrinsic affinity of TcR for the pep-MHC is known. Meanwhile, a crude model can be put forward by considering the pronounced amino acid sequence similarities between TcR and antibodies (see Section IV, above) and the crystal structures of antigen-antibody complexes (Amit et al., 1986; Davies et al., 1990).

In the complex formed by lysozyme and the Fab fragment of an anti-lysozyme antibody, approximately 17 amino acid side chains of the antigen, presenting a solvent accessible area of 650 Å², make contact with the antibody's binding site. The intrinsic affinity for the interaction of lysozyme with this Fab fragment at 25°C is about 3x10^8 M⁻¹ (Bhat et al., 1994; Foote and Winter, 1992), or 11,450 calories per mole of antigen bound. Hence the antigenic epitope contributes, per mole bound, ≈20 cal per Å² of accessible ligand area, or close to the 25 cal per Å² estimated for various protein–protein interactions (Janin and Chothia, 1978). In applying this estimate to TcR–pep-MHC reactions, it is notable that x-ray crystallographic analyses of several pep-MHC complexes have shown that about 70 to 80% of the solvent accessible area of the peptide moiety is buried in the MHC binding groove (Fremont et al., 1992; Madden et al., 1993; Zhang et al., 1992). For example, the exposed area of octamer or nonamer peptides bound to Kb or Db and available to interact with a TcR was only about 175 Å², despite marked differences in peptide sequence (Young et al., 1994), suggesting that perhaps quite generally peptides may be expected to contribute ≈3500 cal per mol bound, with the rest of the binding energy of the TcR–pep-MHC reaction coming from the MHC component.

Consider, for example, the reaction of the alloreactive CTL 2C with QL9-Ld, a pep-alloMHC complex (Table III). With an affinity of 1.5x10⁷ M⁻¹ (Sykulev et al., 1994b), ΔG° = 9700 cal per mole ligand bound (at 25°C) or, deducting 3500 cal for the peptide’s contribution, ≈6000 cal per mole alloMHC (Ld) bound. It follows that the hypothetical equilibrium constant for the reaction of the 2C TcR with Ld, independent of associated peptide, is ≈3.4x10³ M⁻¹. Since this low affinity value can support a cytotoxic response (Sykulev et al. 1994a), providing the epitope density is high enough (Dutz et al., 1994; see also Fig. 6), these estimates can explain why some alloreactive CTL appear to react with alloMHC, regardless of associated peptide adducts (Rötzschke et al., 1991b; Elliott and Eisen, 1990; Vturina et al., in preparation). Although these notions are consistent with the view that thymic selection generates mature T cell populations having, on average, higher affinities for pep-alloMHC than for pep-synMHC, careful analyses of many synMHC- and alloMHC-restricted T cell clones and ultimately of polyclonal T cell populations will be required for an adequate test of the hypothesis.

X. Concluding Remarks

In the approximately ten years since the TcR was first identified, the naturally processed peptides that serve in association with MHC-I or MHC-II proteins as the receptor's natural ligands have been extensively characterized. A start has been made towards establishing the abundance of these ligands on target cells and APC.
determining the kinetics and affinities of their reactions with TcR, and correlating these values with the magnitude of TcR-mediated responses by intact T cells. More extensive analyses of this kind, as well as measurements of TcR*pep-MHC bond lifetimes, should provide a solid basis for understanding the great variations in magnitude of T cell responses and help explain why different pep-MHC having only slightly altered peptides can elicit very different responses.

Much of the progress made to date has been facilitated by the use of a small number of stable T cell clones or T cell hybridomas. Are these cells authentic representatives of normal T cells? Most T cells undergo senescence or apoptosis in vivo in response to repeated ligand binding to their TcR, and only a rare T cell can be successfully established as a cultured clone by current techniques, e.g., only one or two clones from an optimally immunized mouse. Given the idiosyncratic nature of each clone it would be useful to be able to generate and study large numbers of individual clones and even more useful to develop rigorous methods for analyzing polyclonal T cell populations. Beyond these challenges is the far greater one of applying the accumulating information about the TcR and its reactions to manipulate T cells and their responses in vivo, augmenting them against epitopes on pathological targets (e.g., cancer cells and HIV-infected cells) or suppressing them in autoimmune disorders.
Figure Legends

**Figure 1.** Arrangements of V (variable), D (diversity), J (junction), and C (constant) TcR gene segments in three loci in the mouse genome. Transcriptional orientation is indicated by arrow. (From Davis, 1990)

**Figure 2.** Ribbon diagram of MHC-I and MHC-II proteins. Shown for the MHC-I heterodimer are three domains of α or heavy chain (the polymorphic α1 and α2 domains, which together form the peptide-binding groove, and the conserved α3 domain) and the β2-microglobulin or light chain. Shown for the MHC-II heterodimer are the two domains for each subunit, i.e. the polymorphic α1 and β1 domains, which form the peptide-binding groove, and the conserved α2 and β2 domains. Although there is virtually no sequence identity between MHC-I and MHC-II proteins, their three-dimensional structures are remarkably similar (Brown et al., 1988). Courtesy of L.J. Stern and D.C. Wiley.

**Figure 3.** Ribbon diagram of a top view of the peptide-binding sites of MHC-I and MHC-II proteins (HLA-A2 and HLA-DR1, respectively). Note that the peptide adduct is generally larger (10-20 amino acids) in MHC-II than in MHC-I (8-10 amino acids) proteins. Courtesy of L.J. Stern.

**Figure 4.** Responses of T cells to target cells sensitized with various concentrations of cognate peptides or their structural analogs. (A) Cytotoxic responses by a CD8+ CTL clone (2C) to a naturally occurring octapeptide p2Ca from α-ketoglutarate dehydrogenase and three overlapping nonapeptides from this protein (QL9, SL9 and LL9) presented by the MHC-I protein Ld on Ld-transfected target cells (T2-Ld) (Sykulev et al., 1994b). (B) IL-2 production by a CD4+ T cell hybridoma (2B4) in response to peptides from cytochrome c of mouse (MCC) or pigeon (PCC) presented by the MHC-II protein IEk on IEk-transfected CHO cells (Matsui et al., 1994).

**Figure 5.** Relationship between target cell epitope density required for a half-maximal T cell response (target cell lysis) and the intrinsic affinity of TcR–pep-MHC reactions. The solid line corresponds to the best fit between Eq. 3 and experimentally determined values for epitope density and affinity (K), and to a TcR concentration of $10^{-7}$ M. Curves that fit the experimental data about as well, but are based on limiting epitope densities of 1 or 3 pep-MHC complexes per target cell are shown by the dashed lines. (From Sykulev et al., 1995)

**Figure 6.** TcR on CD8+ CTL clone 2C distinguishes sharply between two naturally processed peptides, p2Ca and p2Ca-Y4, in association with the same syngeneic MHC-I protein, Kb. (Upper) Specific lysis of T2-Kb target cells by CTL 2C in the presence of various concentrations of p2Ca (▲) and p2Ca-Y4 (△). (Bottom) Binding of p2Ca and p2Ca-Y4 to Kb as shown by peptide-stabilized expression of Kb on T2-Kb cells, measured by fluorescent staining with a monoclonal anti-Kb mAb and flow cytometric analysis. (From Wu et al., 1995)

**Figure 7.** Hypothetical distribution of intrinsic TcR affinities in populations of T cells recognizing pep-synMHC (solid line) and pep-alloMHC (dashed line) complexes before (A) and after (B) thymic selection.
Eisen et al. Figure 1
HLA-Aw68 (class I)

HLA-DR1 (class II)

Eisen et al. Figure 2
Eisen et al. Figure 3
Eisen et al. Figure 4
Eisen et al. Figure 5
Eisen et al. Figure 6
Eisen et al. Figure 7
### TABLE I. Comparison between peptide binding to MHC-I and MHC-II molecules.

<table>
<thead>
<tr>
<th></th>
<th>MHC-I</th>
<th>MHC-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distribution</td>
<td>All nucleated cells</td>
<td>Specialized APC</td>
</tr>
<tr>
<td>Domain structure</td>
<td>$\alpha_1$, $\alpha_2$, $\alpha_3 + \beta_2$</td>
<td>$\alpha_1$, $\alpha_2 + \beta_1, \beta_2$</td>
</tr>
<tr>
<td>Accessory molecule</td>
<td>CD8</td>
<td>CD4</td>
</tr>
<tr>
<td>Typical T cell response</td>
<td>Cytotoxic activity</td>
<td>B cell help, DTH</td>
</tr>
<tr>
<td>Origin of most bound peptides</td>
<td>Cytosolic (endogenous)</td>
<td>Extracellular/membr. proteins</td>
</tr>
<tr>
<td>Length of bound peptides</td>
<td>Usually 8-9 residues</td>
<td>Variable, $\approx$12-25 residues</td>
</tr>
<tr>
<td>Pockets in MHC groove</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Peptide N- and C-termini</td>
<td>Buried in groove</td>
<td>May extend outside groove</td>
</tr>
<tr>
<td>Many critical contacts</td>
<td>Peptide side chains</td>
<td>Peptide backbone</td>
</tr>
<tr>
<td>Equilibrium constants ($K_a$)</td>
<td>$\approx 10^4$-$10^9$ M$^{-1}$ measured</td>
<td>$\approx 10^6$-$10^8$ M$^{-1}$ measured</td>
</tr>
</tbody>
</table>

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### Table II. Examples of naturally processed peptides associated with MHC molecules.$^a$

<table>
<thead>
<tr>
<th>MHC protein</th>
<th>Peptide origin</th>
<th>Peptide sequence$^b$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-2K$^d$</td>
<td>Influenza NP 147-155</td>
<td>TYQRTRALV</td>
<td>Rötzschke et al., 1990b</td>
</tr>
<tr>
<td>H-2D$^b$</td>
<td>Influenza NP 366-374</td>
<td>ASNENMETM</td>
<td>Rötzschke et al., 1990b</td>
</tr>
<tr>
<td>H-2K$^b$</td>
<td>Vesicular stomatitis virus NP 52-59</td>
<td>RGYVYQGL</td>
<td>Nathenson, 1990</td>
</tr>
<tr>
<td>H-2K$^b$</td>
<td>Ovalbumin 257-264</td>
<td>SIINFEKL</td>
<td>Rötzschke et al., 1991a</td>
</tr>
<tr>
<td>H-2K$^d$</td>
<td>L. monocytogenes listeriolysin 91-99</td>
<td>GYKDGYEI</td>
<td>Pamer et al., 1991</td>
</tr>
<tr>
<td>H-2L$^d$</td>
<td>Mouse α-ketoglutarate dehydrogenase</td>
<td>LSPFPFDL</td>
<td>Udaka et al., 1992</td>
</tr>
<tr>
<td>H-2L$^d$</td>
<td>Mouse α-ketoglutarate dehydrogenase</td>
<td>VAITRIEQLSPFPFDL</td>
<td>Udaka et al., 1993</td>
</tr>
<tr>
<td>HLA-A2.1</td>
<td>HIV-1 reverse transcriptase 476-484</td>
<td>ILKEPVHGV</td>
<td>Tsomides et al., 1994</td>
</tr>
<tr>
<td>HLA-A2.1</td>
<td>HIV-1 gag 77-85</td>
<td>SLNTVATL</td>
<td>Tsomides et al., 1994</td>
</tr>
</tbody>
</table>

| Class II    | IA$^b$ | Murine leukemia virus envelope | 145-157 | HNEGFYVCPGPHR | Rudensky et al., 1991a |
|            |       | Murine leukemia virus envelope | 145-158 | HNEGFYVCPGPHRP |
|            |       | I-E α chain | 56-73 | ASFEAQGALANIADKA |
|            |       | Invariant chain | 39-53 | KPVSQMRLAMTLLMR |
|            | IE$^b$ | Murine leukemia virus envelope | 454-467 | SPSYVVHQFERRAK | Rudensky et al., 1991a |
|            |       | Murine leukemia virus envelope | 454-468 | SPSYVVHQFERRAKY |
|            |       | Murine leukemia virus envelope | 454-469 | SPSYVVHQFERRAKYK |
|            |       | BSA | 141-154 | GKYLYEIAARRHPYF |
|            | IA$^k$ | Hen egg lysozyme | 48-60 | DGSTDYGILQINS | Nelson et al., 1992 |
|            |       | Hen egg lysozyme | 48-61 | DGSTDYGILQINSR |
|            |       | Hen egg lysozyme | 48-62 | DGSTDYGILQINSRW |
|            |       | Hen egg lysozyme | 52-64 | DYGILQINSRWWC |
|            | HLA-DR1 | Invariant chain | 105-118 | KMRMATPLLMQALP | Chicz et al., 1992 |
|            |       | Invariant chain | 105-119 | KRMATPLLMQALPM |
|            |       | Invariant chain | 96-118 | LPKPPKPVSK...P |
|            |       | Invariant chain | 96-119 | LPKPPKPVSK...PM |
|            |       | Invariant chain | 96-120 | LPKPPKPVSK...PMG |
|            |       | Invariant chain | 97-118 | PKPPKPVSK...P |
|            |       | Invariant chain | 97-119 | PKPPKPVSK...PM |
|            |       | Invariant chain | 97-120 | PKPPKPVSK...PMG |
|            |       | Invariant chain | 98-118 | KPPKPVSK...P |
|            |       | Invariant chain | 98-119 | KPPKPVSK...PM |
|            |       | Invariant chain | 99-118 | PPKPVSK...P |

$^a$ adapted from Tsomides and Eisen (1993a).

$^b$ only MHC-I binding natural peptides that are recognized by established T-cell clones are shown; MHC-I binding sequences identified on the basis of their relatively high abundance are not included.

$^c$ for brevity, invariant chain residues 106-117 are represented by an ellipsis (...).
TABLE III. Equilibrium association constants for the binding of peptide-MHC complexes to TcR.

| T-cell clone (CD4 or CD8) | Peptide<sup>a</sup> | MHC | Equilibrium constant (K<sub>a</sub>)<sup>b</sup> Method<sup>b</sup> Reference<sup>c</sup> |
|--------------------------|----------------------|-----|----------------------------------|--------------------------|
| Name Sequence Class Allele |                      |     |                                 |                          |
| 5C.C7 (CD4) MCC ANERADLIAYLKQATK II IE<sup>k</sup> 2.0x10<sup>4</sup> competition (1) |
| 228.5 (CD4) MCC(99E) ANERADLIAYLEQATK II IE<sup>k</sup> 1.9x10<sup>4</sup> competition (1) |
| 14.3.d (CD4) HA<sub>110-120</sub> CFERFEIFPKKE II IE<sup>d</sup> 2.0x10<sup>5</sup> competition (2) |
| 2C (CD8) QL9 QLSPPFPFDL I L<sup>d</sup> 1.0x10<sup>7</sup> competition (3) |
| QL9 QLSPPFPFDL I L<sup>d</sup> 2.0x10<sup>7</sup> direct (3) |
| SL9 SPFPFDLLL I L<sup>d</sup> 1.4x10<sup>4</sup> competition (4) |
| p2Ca-A3 LSAFPFDL I L<sup>d</sup> 2.0x10<sup>6</sup> competition (4) |
| p2Ca-A5 LSPFAFDL I L<sup>d</sup> 1.6x10<sup>4</sup> competition (4) |
| p2Ca-A8 LSPFPFDL I L<sup>d</sup> 1.7x10<sup>6</sup> competition (4) |
| p2Ca LSPFPFDL I K<sup>b</sup> 3.0x10<sup>3</sup> competition (4) |
| pOV8 RGYVYQGL I K<sup>b</sup> <3.0x10<sup>3</sup> competition (4) |
| p2Ca LSPFPFDL I L<sup>d</sup> 1.0x10<sup>6</sup> s.p.r. (5)<sup>d</sup> |
| p2Ca LSPFPFDL I L<sup>d</sup> 8.0x10<sup>6</sup> s.p.r. (5)<sup>d</sup> |
| 4G3 (CD8) pOV8 SIINFEKL I K<sup>b</sup> 1.5x10<sup>6</sup> direct (3) |
| 2B4 (CD4) MCC ANERADLIAYLKQATK II IE<sup>k</sup> 1.5x10<sup>4</sup> s.p.r. (6) |
| MCC(102S) ANERADLIAYLKQASK II IE<sup>k</sup> 1.0x10<sup>4</sup> s.p.r. (6) |
| PCC ANERADLIAYLKQTAK II IE<sup>k</sup> 1.9x10<sup>4</sup> s.p.r. (6) |

<sup>a</sup> peptide MCC is from mouse cytochrome c, PCC from pigeon cytochrome c, HA from influenza virus hemagglutinin, p2Ca and related peptides from mouse α-ketoglutarate dehydrogenase (Udaka et al., 1992, 1993), pVSV from vesicular stomatitis virus, and pOV8 from ovalbumin.

<sup>b</sup> competition: soluble pep-MHC inhibits the binding of an 125I-labeled Fab' fragment of an anti-TcR Ab to TcR on intact T cells, or binding of soluble TcR to pep-MHC on intact APC inhibits the response of CD4<sup>+</sup> T cells to the APC; direct: binding of 125I-labeled soluble pep-MHC to TcR on intact T cells.

<sup>c</sup> s.p.r.: surface plasmon resonance of soluble pep-MHC binding to immobilized soluble TcR.

<sup>d</sup> K<sub>a</sub> values calculated from association and dissociation rate constants using two different models (see Table IV).
**TABLE IV.** Kinetic constants for the binding of peptide-MHC complexes to TcR.

<table>
<thead>
<tr>
<th>T-cell clone (CD4 or CD8)</th>
<th>Ligand (peptide-MHC)</th>
<th>(k_+) (M(^{-1})sec(^{-1}))</th>
<th>(t_{1/2}) (sec)</th>
<th>(\tau) (sec)</th>
<th>Method(^b)</th>
<th>Reference(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2C (CD8)</td>
<td>QL9-L(^d)</td>
<td>5.3x10(^4)</td>
<td>222</td>
<td>234</td>
<td>direct</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td>QL9-L(^d)</td>
<td>3.8x10(^4)</td>
<td>275</td>
<td>396</td>
<td>competition</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td>p2Ca-L(^d)</td>
<td>1.1x10(^4)</td>
<td>126</td>
<td>182</td>
<td>competition</td>
<td>(2)</td>
</tr>
<tr>
<td></td>
<td>p2Ca-L(^d)</td>
<td>2.1x10(^4)</td>
<td>27</td>
<td>38(^d)</td>
<td>s.p.r.</td>
<td>(3)(^e)</td>
</tr>
<tr>
<td></td>
<td>p2Ca-L(^d)</td>
<td>2.6x10(^5)</td>
<td>35</td>
<td>50(^d)</td>
<td>s.p.r.</td>
<td>(3)(^e)</td>
</tr>
<tr>
<td>4G3 (CD8)</td>
<td>pOV8-K(^b)</td>
<td>2.2x10(^4)</td>
<td>30</td>
<td>48</td>
<td>direct</td>
<td>(1)</td>
</tr>
<tr>
<td>2B4 (CD4)</td>
<td>MCC-IE(^k)</td>
<td>0.09x10(^4)</td>
<td>12</td>
<td>18(^d)</td>
<td>s.p.r.</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td>MCC(102S)-IE(^k)</td>
<td>0.2x10(^4)</td>
<td>2-5</td>
<td>3-10(^d)</td>
<td>s.p.r.</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td>PCC-IE(^k)</td>
<td>0.17x10(^4)</td>
<td>8</td>
<td>11(^d)</td>
<td>s.p.r.</td>
<td>(4)</td>
</tr>
</tbody>
</table>

\(^a\) peptide sequences given in Table III.

\(^b\) methods outlined in footnote to Table III; s.p.r. = surface plasmon resonance.

\(^c\) (1) Sykulev et al., 1994b; (2) Sykulev et al., 1994a; (3) Corr et al., 1994; (4) Matsui et al., 1994.

\(^d\) \(\tau\) values were not reported; they are calculated here using the relationship \(1/\tau = (k_+)(L)+(k_{-1})\), where \(L\) is the free pep-MHC concentration and \(k_+\) and \(k_{-1}\) are on- and off-rate constants, respectively, for the TcR-pep-MHC reaction; under physiological conditions \((k_+)(L)<<(k_{-1})\) and \(\tau\) is thus effectively determined by \(1/k_{-1}\).

\(^e\) \(k_+\) and \(t_{1/2}\) values were derived from nonlinear least squares fit analysis of experimental data, with the TcR-pep-MHC reaction modeled as either a classic bimolecular reaction (upper line) or a two-step reaction (fast and slow, lower line); in the latter case only the fast component of \(k_+\) and the slow component of \(t_{1/2}\) are given.
<table>
<thead>
<tr>
<th>Binding parameter</th>
<th>2C TcR + QL9-L\textsuperscript{d}</th>
<th>25\textdegree C\textsuperscript{b}</th>
<th>37\textdegree C</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_a$ (M\textsuperscript{-1})</td>
<td>1.5x10\textsuperscript{7}</td>
<td>6.0x10\textsuperscript{6}</td>
<td></td>
</tr>
<tr>
<td>$k_{+1}$ (M\textsuperscript{-1}sec\textsuperscript{-1})</td>
<td>5.3x10\textsuperscript{4}</td>
<td>9.0x10\textsuperscript{4}</td>
<td></td>
</tr>
<tr>
<td>$\tau$ (min)</td>
<td>3.9</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>$k_{-1}$ (sec\textsuperscript{-1})</td>
<td>3.1x10\textsuperscript{-3}</td>
<td>1.5x10\textsuperscript{-2}</td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}$ (min)</td>
<td>3.7</td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} data from Sykulev et al., 1994b. QL9 sequence given in Table III. 
\textsuperscript{b} room temperature (22\textdegree C-25\textdegree C).
XI. References


Today 15, 321-331.


44, 251-259.


APPENDIX 2

List of publications
abstracts/meeting reports:


research articles:


Sykulev, Y., Joo, M., Vturina, I., Tsomides, T.J. and Eisen, H.N. Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. Submitted.


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APPENDIX 3

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