Genotoxicity Associated with Nitric Oxide Production in Activated Murine Macrophages

by

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Abstract

It is estimated that about one-third of the world's total cancer cases are related to microbial infections. Mechanisms through which microbial infections contribute to the carcinogenic process are presently not understood. However, it is well established that inflammatory cells (neutrophils and macrophages) are recruited into infected areas, where they release large quantities of reactive oxygen species (ROS) and nitric oxide (NO•). ROS from inflammatory cells have been shown to induce DNA damage, gene mutation, and neoplastic transformation in target cells. Little is known about the genotoxicity of NO• produced by inflammatory cells, mainly due to the lack of suitable experimental systems.

The objective of this thesis work was to characterize potential genotoxicity of NO• under physiologically relevant conditions. An early observation was made that under some conditions, a subset of the mouse macrophage-like RAW264.7 cells survived stimulation for NO• production by interferon-γ (IFN-γ) and lipopolysaccharide (LPS). Subsequent characterizations revealed that cell toxicity and growth of RAW264.7 cells were markedly influenced by, among other factors, NO• production rate in the cells. At low levels (30 nmoles per 10^6 cells per day in cells stimulated with LPS alone), NO• production had no measurable effect on cell viability but appreciably slowed cell growth; at higher levels (70 nmoles per 10^6 cells per day in cells stimulated with IFN-γ alone), cells grew significantly slower than untreated ones with little cell death. At still higher levels (150 nmoles per 10^6 cells per day in cells stimulated with both LPS and IFN-γ), substantial cell death and growth arrest were observed. This information, combined with use of the endogenous *hprt* gene of the cells for measurement of genotoxic response, made it possible to examine genotoxicity associated with different levels and duration of NO• production in these cells. Results showed that prolonged, continuous production of NO• led to considerable genotoxicity in the cells. Higher levels of NO• production over as short as 36 hours led to significant genotoxicity accompanied by massive cell death.

To understand the molecular mechanisms through which NO• production leads to increased genotoxicity, the spectrum of mutations in NO•-associated hprt mutants was characterized using RT-PCR and DNA sequencing techniques. Results showed that the mutation pattern was not significantly different from the spontaneous mutation pattern of the cells, with the exception that limited numbers of small deletions and insertions were observed only in NO•-associated mutants. Analysis of *hprt* mutant populations collected *en masse* using Constant Denaturant Capillary Electrophoresis (CDCE) showed four to six putative "hot-spot" mutations in two untreated and two NO•-associated samples. Each of these mutations comprised from 0.1 to 1% of total mutants. On the basis of the elution time, two of these "hotspot" mutations seemed to be present in all four samples, indicating that the similarity between spontaneous and NO•-associated mutations may

extend to this level. These results suggest that NO• production leads to gene mutations in the cells through complex and mechanisms yet to be identified.

The RAW264.7 cell line provided a convenient experimental system for the study of NO•-associated cell toxicity and genotoxicity in mammalian cells because they served as target as well as producer cells. In order to relate findings from the use of this system to other cell types, experiments of two types were performed. First, effects of cell density on viability, growth, and *hprt* mutation in RAW264.7 cells stimulated to produce NO• were examined. If the cellular damage were induced by both intracellular and extracellular NO•, levels of NO•-associated damage would decrease as cell density is decreased and then remain unchanged below a certain density. It was discovered that cell death associated with NO• production was completely curtailed at low densities, indicating that damage in cells stimulated to produce NO• might have been mediated by NO• from neighboring cells. This finding illustrates the usefulness of RAW264.7 cells as a model system for the study of NO•-associated cytotoxicity and genotoxicity in mammalian cells.

Further experiments were conducted to compare cytotoxic and genotoxic responses to NO• production in macrophages and target cells co-cultured with them under same experimental conditions. This was achieved by genetically engineering macrophages and target cells to make them resistant to two different drugs, thereby allowing separation of one cell type from the other following co-culture treatment. Results of this endeavor showed that cell death and gene mutations occurred in target cells as well as in macrophages. Furthermore, cell line- and culture condition-specific responses were also observed.

Collectively, this work clearly demonstrates that NO• produced by macrophages can lead to genotoxicity in exposed cells and thus support a possible role for its involvement in the carcinogenic process. The results also provide evidence indicating that the contribution of NO• to the carcinogenic process may be influenced by other factors, including duration and rate of production, type of target cell, effector-to-target cell distance, and local environment.

Thesis Advisor: Gerald N. Wogan
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Abbreviations

NO•	nitric oxide
iNOS	inducible nitric oxide synthase
ROS	reactive oxygen species
IFN-γ	interferon-γ
LPS	lipopolysaccharide
NMA	N^G-methyl-L-Arginine monoacetate
MF	mutant fraction
6TG	6-thioguanine
hprt	hypoxanthine-guanine phosphoribosyltransferase
F_3TdR	triflurodeoxythymine
tk	thymine kinase
4-NQO	4-nitrosoquinoline-N-oxide
RT-PCR	reverse transcription-polymerase chain reaction.
TNF-α	tumor necrosis factor α
IL-1β	interleukin 1β
CDCE	constant denaturant capillary electrophoresis

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Chapter 1. Background and Literature Review

Introduction

Two decades of research have firmly established that genetic changes are at the root of cancer. It is now clear that cancer often initiates when critical genes in a particular cell are mutated either spontaneously or by the action of a carcinogen. The mutant cell acquires growth advantage causing it to multiply more quickly than normal cells. This process continues through multiple cell generations eventually giving rise to a cell which has accumulated genetic changes in a number of its growth-regulating genes [1-3]. These altered genes work in concert leading to fully malignant and aggressive growth of the cell.

The nature of genetic changes associated with the development of cancer has been the focus of research in a number of arenas. Chemical and retroviral carcinogenesis research in animals and cancer inheritance studies in humans, respectively, led to the discovery of two classes of cancer related genes: oncogenes and tumor suppressor genes. Oncogenes are mutated forms of normal cellular genes, protooncogenes. Both protooncogenes and tumor suppressor genes function in a normal cell to ensure tight controls of the proliferation process. However, they do so in different manners: protooncogenes encourage cell growth, whereas tumor suppressor genes inhibit it. Mutations in protooncogenes often result in the formation of protein products which are overly active or over-produced, driving excessive cell growth. The aggressive behavior of oncogenes is illustrated by their ability to transform normal cells. Mutations in or loss of tumor suppressor genes, in contrast, lead to the loss of functional growth inhibitory proteins, which consequently result in increased cell proliferation by removing the growth constraints imposed on normal cells [4].

The molecular mechanisms through which oncogenes and tumor suppressor genes contribute to cancer development have been studied in unprecedented detail since their discovery [4]. It is now clear that the protein products of these genes encompass virtually every critical protein involved in cell division, ranging from extracellular growth factors and their transmembrane receptors [5, 6] to relay proteins of the signal transduction cascades found in the cytoplasm [6], and from transcription factors to components of the cell cycle machinery located in the nucleus [7-9]. Still others act as cell cycle checkpoints, instructing growth arrest for repair or cell suicide following cellular insult [9-11]. By doing so, the genomic integrity of the cell is maintained.

When the functions of genes involved in the maintenance of genomic stability are lost, the corresponding cells would acquire genetic changes in other cancer genes more rapidly, thereby

accelerating the multistep carcinogenesis process. If enhanced genomic instability has been inherited, the affected individual will be destined to have early onset of the disease. Human cancer-prone syndromes such as Li-Fraumeni syndrome and ataxia-telangiectasia provide excellent examples that inherited genomic instability is associated with a predisposition to cancer [11]. It is conceivable that as more cancer susceptibility genes are identified and their normal functions characterized, many of them will prove to be directly involved in the safeguard of the genomic integrity.

Since inheritance of mutated tumor suppressor genes accounts for only a small proportion of cancers seen at the clinic, it is likely that most tumors collect all the mutations required for their malignant growth somatically. These mutations can be induced endogenously by chemicals produced in our own cells, or exogenously by carcinogens taken into our bodies from the environment through the everyday processes of eating, drinking, and breathing. Major exogenous risk factors include tobacco smoking, chronic inflammation, and an unbalanced diet. To date, the most extensively studied endogenous carcinogens are reactive oxygen species and hormones [12].

The distinction of carcinogens as exogenous and endogenous is less meaningful now, because it is becoming clear that aside from a few DNA alkylating agents and UV light, most of exogenous risk factors undergo various forms of biotransformation inside a human body to become carcinogenic [13]. For example, polyaromatic hydrocarbons and aflatoxins are not mutagenic until activated by the cytochrome P-450 enzyme system. Other exogenous agents are believed to increase cancer risk through their ability to induce the production of endogenous carcinogens, although the mechanisms of action of these agents have not been established. Belonging to this category are chronic microbial infections and inflammation.

Although the mechanisms through which chronic infection/inflammation contribute to carcinogenesis are largely unknown, it has been established that inflammatory cells such as neutrophils and macrophages are often recruited into the sites of infection/inflammation and secrete a large array of cytotoxic chemicals and enzymes as means of host defense [14]. Among the chemicals secreted by these cells are reactive oxygen species (ROS), which have been shown to be capable of inducing mutagenic DNA damage as well as cell death. More recently, nitric oxide (NO•) has been identified as another cytotoxic product secreted by macrophages. On the basis of its chemical properties, it has been proposed that NO•-derived intermediates may also induce cytotoxic and mutagenic DNA damage in target cells. It is this hypothesis which forms the scientific foundation for this thesis. A search for answers to this question is of increasing importance in the light that as many as one-third of the world's total cancer cases might be associated with chronic microbial infections [12]. An understanding of the mechanisms by which

infection/inflammation increases cancer risks would provide useful information which may lead to early diagnosis and more effective prevention.

In this literature review, evidence in support of microbial infections and chronic inflammation as cancer risk factors is first introduced, followed by a brief review of aspects of neutrophil and macrophage biology pertinent to this thesis work. A discussion of oxygen metabolism in phagocytes is also included because it may provide insights into the mechanism of action of NO• under physiological conditions. A majority of the discussion is devoted to the biology of NO•, including its discovery, biosynthesis, metabolism, its roles in mammalian physiology and in host defense in particular, and its roles in diseases. Our current knowledge of the genotoxicity of ROS and NO• is another focus of this review. The last section of the chapter discusses experimental approaches and rationale for the use of the mammalian *hprt* gene as a tool in mutation research.

Chronic infection/inflammation and cancer

Evidence from epidemiological studies

Epidemiological studies have documented an association between chronic infection/inflammation and occurrence of cancer. As summarized in Table 1, the association has been observed in many different tissues, and in most cases the causative agents for inflammation are well defined (viral or bacterial infections; parasitic infestations; and chemical or physical irritants). Some of the risk factors for which strong supportive evidence exists are discussed below.

Viral infections and liver cancer

One of the strongest associations between infection and cancer is chronic hepatitis B virus (HBV) infection with hepatocellular carcinoma (HCC) [15, 16]. A wide range of case control and prospective cohort studies conducted in different geographic areas around the globe showed significantly elevated HCC incidence in regions where chronic HBV infections are prevalent. Of note is a prospective study involving more than twenty thousand Taiwanese men, which found that the relative risk for HCC in hepatitis surface antigen (HBsAg)-positive individuals was 223-fold higher than in HBsAg-negative men [16]. Concurrent HBV infection and aflatoxin ingestion dramatically increases this cancer risk.

Association of chronic viral infection with tumorigenesis in the liver has also been observed in a number of animal models, including woodchuck, domestic duck, ground squirrel, goose, and grey heron [15, 16]. In fact, woodchuck hepatitis virus (WHV) has been isolated from feral woodchucks with chronic hepatitis and HCC. When chronic hepatitis was experimentally established in woodchucks by infection with WHV, all of the animals developed HCC within 17-36 months of infection [16].

Table 1. Chronic inflammation as risk factors in human cancers
(Adapted from refs. 17 and 151)

Cause of Inflammation	Cancer
Viruses	
Hepatitis viruses	Liver (hepatocellular carcinoma)
Human papilloma virus, herpes simplex virus type 2, cytomegalovirus	Cervix
Epstein-Barr virus	Burkitt's lymphoma, nasopharyngeal carcinoma
Human T-cell leukemia virus	Adult T-cell leukemia
Human immunodeficiency virus	Kaposi's sarcoma, non-Hodgkin lymphoma
Parasites	
Schistosoma haematobium	Bladder
Schistosoma mansoni	Liver, spleen
Schistosoma japonicum	Colon, liver
Opisthorchis viverrini, Clonorchis sinensis	Liver/biliary tract (cholangiocarcinoma)
Malania	Burkitt's lymphoma
Bacteria	
Helicobacter pylori	Stomach
Urinary infection	Bladder
Tuberculosis	Lung
Physical or chemical irritants	
Particles (Asbestos, silica)	Lung
Hot beverages, barrett's esophagus, reflux esophagus	Esophagus
Undefined	
Ulcerative colitis, Crohn's disease	Colon
Atrophic gastritis	Stomach
Chronic skin ulcers	Skin
Catherized patients with chronic cystitis, recurrent cystitis	Bladder
Hepatic cirrhosis	Liver

Parasitic infestations and cancers

In developing countries, parasitic infestations are widespread. Schistosomiasis, a common parasitic disease, occurs in 74 tropical and subtropical countries and affects about 10% of the world's population. Humans are infected by three major *Schistosoma* species, each of which is associated with increased cancer risk in the infested organ. In Egypt, the eggs of *Schistosoma haematobium* are deposited in the bladder, causing inflammation and bladder cancer. *Schistosoma japonicum* and *Schistosoma mansoni* infections have been associated with occurrence of colon and spleen cancers, respectively, although the supporting evidence is somewhat limited [17].

Opisthorchis viverrini, a liver fluke, infects millions of people in Thailand and Malaysia [12, 18]. The flukes reside in bile ducts and increase the risk of cholangiocarcinoma. The association is strong, with an odds ratio of 5.0 [18]. In China, millions of people are infected with another species of fluke, *Chlonorchis sinensis*, which also increases the risk of cholangiocarcinoma [12].

Bacterial infections and gastric cancer

More than one-third of the world's population is infected with *Helicobacter pylori* bacteria [12]. Infection by this microbe has been identified as one major risk factor for stomach cancer, ulcers, and gastritis [12, 19, 20]. Characterization of the carcinogenesis process revealed that infection by *Helicobacter pylori* and excessive salt intake contribute to the initial stages of gastritis and atrophy which, under the influence of additional factors including insufficient ascorbic acid and the formation of N-nitroso compounds in the stomach, can progress into gastric cancer [19].

Particle exposure and lung cancer

Non-microbial irritants have also been associated with human cancers. Inhalation of particles such as silica [21], carbon black [22], and asbestos [23] can lead to pulmonary diseases including lung cancer. In most cases, exposures to these materials occur under occupational settings, though the general public may be exposed as a result of air pollution. In the case of asbestos, people may also be exposed to this material by living or working in deteriorating buildings in which asbestos was used as construction material. Unlike microbial pathogens, however, the association of asbestos exposure and lung cancer is weak, and is complicated by other factors. In fact, lung tumors are rare among asbestos workers who do not smoke [23]. Thus, it appears that asbestos may synergize with smoking in causing lung cancer.

Chronic infection/inflammation and genetic instability in host cells

One hallmark of cancer cells is their genomic instability in contrast to their normal counterparts. It is well accepted that agents capable of inducing genomic instability or damage in normal cells are virtually carcinogens, whether or not they act directly on genomic DNA. Although the aforementioned epidemiological data do not prove a cause-effect relationship between chronic infection/inflammation and cancer, other studies do suggest a causative role in certain cancers. These studies clearly demonstrate the ability of infections or inflammation to increase genetic changes in the cells of affected humans or treated animals. For example, chronic inhalation of carbon black by rats increased the *hprt* mutation frequency in alveolar epithelial cells of the animals, and the eventual development of lung tumors [22]. Significant induction of SCEs was also observed in lymphocytes from pigs infected with *Taenia solium* metacestode [24]. Furthermore, rats treated with N-methyl-N-nitrosourea and with killed *Escherichia coli* or its membrane component lipopolysaccharide (LPS) showed a significant increase in the incidence and number of tumors of the urinary bladder compared to those receiving only the chemical [25, 26]. In humans, infections with *Mycobacterium leprae*, tuberculosis bacillus or mycoplasma have been associated with increases in chromosome aberrations and sister-chromatin exchanges (SCEs) in lymphocytes of infected individuals [27]. Elevated levels of genetic damage measured as micronucleus frequency are observed in the bladders of patients infected with the parasite *Schistosoma haematobium* [17, 28]. The link between infection and induction of genetic damage is strengthened by observations that drug treatments that kill the infectious agents also reduce the extent of genetic changes [17, 27].

Although animal studies have been instrumental in defining the role chronic inflammation plays in cancer development, attempts to identify a cause-effect relationship in human populations are often complicated by many factors. For example, infections by different microbial strains may have different pathogenic consequences; individuals infected at a young age may respond differently from those infected later in life; the genetic makeup may determine the vulnerability of the infected individual to tumor development; and finally, dietary factors and tobacco smoking may substantially influence the odds of tumor formation in infected individuals.

Despite pathogen and host variability, several common features exist among inflammation-associated cancers in humans. In most cases, tumors develop at sites of inflammation, inflammation conditions persist for long periods of time, and most notably, inflammatory cells are recruited into and activated at the inflamed areas. Therefore,

infection/inflammation-associated carcinogenesis is by and large a localized event. Although it is conceivable that cancer may develop as a result of direct pathogen-host cell interactions, more attention has been paid to the possible role inflammatory cells play in the development of inflammation-associated cancer.

Phagocytic cells in host defense

Inflammation is a complex process initiated by tissue damage caused either by endogenous factors such as tissue necrosis and bone fracture or by exogenous factors such as microbial infections and chemical irritation. Within minutes after injury, the inflammatory process begins with synthesis and activation of proinflammatory substances which alter blood flow and vascular permeability, attract circulating leukocytes into infected tissues, and stimulate leukocytes to destroy the inciting agent [14].

The inflammatory response draws on both innate and acquired immunity. Before the specialized and specific form of immunity, acquired immunity, comes into play during an inflammatory response, various non-specific components of innate immunity operate promptly against invading substances. One important non-specific component of innate immunity is phagocytosis, the ingestion and destruction by individual cells of invading foreign particles, such as bacteria, by individual cells. The phagocytic cells consist of neutrophils/polymorphonuclear leukocytes (PMNs), phagocytic monocytes or macrophages, eosinophils, and fixed macrophages of the reticuloendothelial system. Cells responding early upon the inception of inflammation are mainly neutrophils, which accumulate within 30-60 minutes. These cells phagocytize the invaders or damaged tissue and release lysosomal enzymes in an attempt to destroy the intruder. If the inflammation persists beyond this point, the inflamed area is infiltrated by mononuclear cells, which include macrophages and lymphocytes. The macrophage cells supplement the polymorphonuclear leukocytes in the elimination of foreign materials. Moreover, the macrophages participate in the processing and presentation of antigen to lymphocytes, and in so doing inducing acquired immunity. The specificity of acquired immunity is mediated by immunoglobins/antibodies produced by B lymphocytes or by receptors on T lymphocytes that bind to specific determinants/epitopes.

Inability of the immune system to remove the cause of inflammation results in chronic inflammation. Due to the persistent presence of the invading agent or stimulus, the inflammatory response outlined above continues. Under these circumstances, the non-specific activities of the phygocytic cells may become destructive.

Neutrophils

Neutrophils are the most well studied phygocytic cells, not only because they play a central role in host defense, but also because these cells are more readily available than other cell types. The availability of neutrophils has undoubtedly facilitated the discovery and subsequent characterization of the respiratory burst phenomenon. As was later discovered, much of the antibacterial activity in neutrophils is accomplished by respiratory burst-derived reactive oxygen species (ROS), which will be discussed Section 1.4 (see below).

Neutrophils reside in three body compartments, each of which contains cells at different stages of development. Bone marrow is the site of proliferation and terminal maturation of neutrophilic granulocytes. In fact, it is estimated that approximately 60% of the human bone marrow is dedicated to the production of neutrophils [29]. Proliferation, approximately five cell divisions, takes place only during the first three stages of neutrophil maturation (blast, promyelocyte, and myelocyte stages). After the myelocyte stage, the cells become fully differentiated (no longer capable of mitosis) and enter a large storage pool in bone marrow. About 5 days later, they are released into the second compartment, the blood, where they circulate for about 10 hours. It is estimated that more than 100 billion neutrophils enter and leave the circulation daily in normal adults, and this number may increase several folds in the setting of serious infection [29]. Once the cells migrate from blood into tissues, the final compartment, they are capable of surviving several days. The relative short life span of neutrophils indicates that at sites of chronic infection or inflammation, the neutrophil population will be constantly replenished. The final tissue destination of most neutrophils in the normal host has not been resolved.

Neutrophils synthesize proteins at regular intervals early in their maturation in bone marrow and store them for days as large cytoplasmic granules. There are two distinct types of granules: the azurophil or primary granule, which is formed during the promyelocyte stage and contains peroxidase, and the specific or secondary granule, which is formed later during the myelocyte stage and is peroxidase-negative. When appropriately stimulated, these cells may release the contents of their granules within seconds into an endocytic vacuole or, by fusion with the plasma membrane, to the exterior of the cell. Table 2 lists the components of human neutrophil granules [30]. Most of these granule-associated proteins exhibit catalytic activity. For example, the typical lysosomal hydrolases are capable of degrading microbial macromolecules, including nucleic acids, proteins, polysaccharides, peptidoglycans, and lipids. Because the activities of these components

are independent of oxygen, they are more often referred to as oxygen-independent antimicrobial systems [31].

Another important protein is the enzyme (myelo)peroxidase, which has no antimicrobial effect by itself, but can exert an antimicrobial effect indirectly by catalyzing the conversion of a substance with little antimicrobial activity to one that is strongly toxic. Since this peroxidase/H_2O_2/halide system requires hydrogen peroxide, it is also classified as an oxygen-dependent antimicrobial system (see Section 1.4).

Table 2. Constituents of azurophil and specific granules from human neutrophils

Azurophil grandules	Specific granules
Microbicidal enzymes	
Myeloperoxidase	Lysozyme
Lysozyme	
Neutral proteinase	
Elastase	
Cathepsin G	
Proteinase 3	
Acid hydrolases	
β-Glycerophosphatase	Collagenase
β-Glucuronidase	
N-Acetyl-b-glucosaminidase	
α-mannosidase	
Cathepsin B	
Cathepsin D	
Other	
Cationic proteins	Lactoferrin
Defensins	Vitamin B12-binding proteins
Bactericidal permeability increasing protein (BPI)	Plasminogen activator
	Histaminase
Azurophil-derived bactericidal factors (ADBF)	Receptors
	fmet-leu-phe
	CR3 (C3bi)
	Laminin
	Cytochrome b

Mononuclear phagocytes (monocytes and macrophages)

The importance of mononuclear phagocytes in host defense and in the development of immune diseases has been increasingly appreciated. A collection of comprehensive reviews has recently been published in a monograph [32], which is a valuable source of information for the summary presented below.

Distribution and development

Like neutrophils, all mononuclear phagocytes also originate from the bone marrow. Newly formed monocytes remain in the bone marrow for only a short period of time (<24 hr) and then migrate to the peripheral blood. Once in the blood, approximately 40% of the total monocyte pool stay in circulation, with the rest as marginating monocytes, presumably about to migrate to the tissues and body cavities. This distribution seems to be consistent with a reported half-time of 17 hr for the blood monocytes [14, 33, 34].

After leaving the circulation, monocytes differentiate into macrophages in tissues and organ cavities, where they remain for days before being replaced by influx monocytes and to a less extent by locally dividing macrophages. The ultimate fate of macrophages is not understood. One possibility is that macrophages die in lymph nodes. It is also conceivable that their death occurs in tissues and body cavities. The number of macrophages that die under normal conditions must be considerable given the fact that there is constant formation, circulation, and differentiation of monocytes. It is estimated that in a normal mouse, the turnover rate of macrophages is 1.5×10^6 cells per day [14].

During inflammation, the number of monocytes in circulation increases; the extent of this increase depends on the kind of inflammatory stimulus. In general, there is a two to three fold increase in number over the normal state. The increased monocyte production results from the temporal shortening of the cell cycle time of promonocytes in the bone marrow, cells one step removed from those in the inflamed areas. There is also an increase in the local production of macrophages at the site of inflammation [14].

Secretory products

The macrophage expresses more than 100 specific receptors on its surface, which can be classified into several broad categories (Table 3) [33]. These include receptors important for

endocytosis such as those for immunoglobulins and complement components, and for regulation of activation such as receptors for interferons.

Macrophages are powerful secretory cells as well; they are currently known to produce over 100 defined molecular products (Table 4) [33]. Some products, such as lysozyme, are apparently secreted constitutively, but most are released as a result of occupancy of a specific receptor or receptors. Many of these ligand-receptor interactions trigger a respiratory burst similar to that observed in neutrophils. One immediate response may be the activation of the membrane bound oxidase complex to produce reactive oxygen species through mechanisms similar to those used by neutrophils. A delayed response can be expression of genes for production of other toxic agents such as nitric oxide. Another consequence can be the phospholipase A_2-mediated release of arachidonic acid from cellular stores of phospholipids and its subsequent conversion via either lipooxygenase or cyclooxygenases to a series of leukotrienes or prostanglandins, respectively.

Macrophages employ these large arrays of surface receptors and secreted products to destroy a wide range of prokaryotic organisms, including viruses, bacteria, fungi, and protozoa. In general, the initial requirement for antimicrobial activity involves recognition. Macrophages recognize foreign invaders primarily by the action of opsonins, molecules which bind to specific sites on both the invader and the macrophage. Opsonins may be of several categories, the most well documented being immunoglobulin G and fragments of the third component of complement. Increasing importance is now being placed on the role of glycoproteins and glycolipids and the family of integrins and selectins in microbial as well as tumor cell recognition. Microorganisms may be destroyed in the surrounding environment by the secretion of toxic materials, but opsonic phagocytosis is the principal route to macrophage-mediated destruction of microbes. As mentioned earlier, the production and intracellular release of ROS represents a major antimicrobial mechanism of macrophages. Destruction of numerous pathogens such as leishmania, toxoplasma, trypanosomes, mycobacteria, and condida can be correlated with the ability of appropriately stimulated macrophages to secrete H_2O_2. In addition to ROS, NO• has recently been recognized as another major antimicrobial mechanism of macrophages (see Section 1.5).

Whereas some secretory products from macrophages are effective cytotoxic agents against a wide spectrum of microbes, other products are secreted only in response to certain types of microorganisms. For example, the production of cytokines in macrophages, appears to be a specific response to viral infection as well as tumor growth. These cytokines, which include platelet-derived growth factor, tumor necrosis factors (TNFs), colony-stimulating factors, the interleukins, and a group of interferons (IFNs), can act in concert with other virus-clearance mechanisms to abort or restrict viral infections. Of all the cytokines studied, IFNs are pre-eminent

in their ability to induce host cell defensive mechanisms during viral infections. Macrophages are quite intimately related to the IFN system. Contrary to most of other cells in the body, macrophages can be induced by a variety of microbial pathogens as well as viruses to make IFN-α, and -β, and can cooperate with T-cells in the production of IFN-γ. Further, many of the functional properties of the macrophage are under the influence of IFNs, especially IFN-γ.

Macrophages also produce mediators capable of inhibiting the division (cytostasis) or killing of tumor cells. Possible mediators of cytostasis include prostanglandins, secreted nucleotides such as thymidine, enzymes such as arginase that metabolize essential amino acids , or cytokines such as IFNs, TNF-α, or IL-1α/β. Also, NO• has now been shown to be a major mediator of cytostasis (see below). Cytostasis affects a very broad spectrum of target cells, including normal and transformed cells. It is also effective across allogenic or xenogenic barriers. In general, it requires a substantially large number of macrophages (60% or more of the total cell population) for cytostasis to occur.

In addition to cytostasis, macrophages secret toxic substances, which result in the eventual lysis of target cells. A number of toxic or lytic products secreted by macrophages have been identified. This include cytolytic protease, TNF-α, ROS, and NO•. Cytolytic protease is a neutral serine protease that is secreted only by fully activated macrophages. TNF-α has also received considerable attention as a cytolytic mediator. However, its relative significance must be assessed in light of the limited range of targets which are apparently sensitive to its destructive effect in vivo and *in vitro*. ROS or NO• alone may have limited cytolytic activity, they may interact to form more toxic agents such as peroxynitrite. Alternatively, these reactive species may act in concert with protein mediators to maximize cell killing.

Activation

In general, the activation of macrophages requires a cascade of separable events, and the acquisition of the competence to destroy neoplatic cells selectively in the absence of antibody provides an excellent example (Fig. 1) [35]. It was previously discovered that neither resident macrophages nor responsive macrophages (immature mononuclear phagocytes isolated from the sites of inflammation) could bind tumor cells [35]. Primed macrophages, i.e., those treated with IFN-γ, bound tumor cells but did not secrete lytic mediators. Secretion of lytic materials requires a triggering signal, such as lipopolysaccharide (LPS). Besides the change in the competence of macrophage-mediated tumor cytotoxicity, macrophages at different stages of the activation cascade also exhibit quantifiable differences in other capacities and functions. In many cases, these change correlate precisely with changes in ability to execute a complex function.

On a molecular level, activation is initiated when the inductive signal binds to the appropriate receptor(s) on the surface of a macrophage. This ligand-receptor interaction immediately triggers the activities of second messengers which, through a series of relay molecules, lead to the expression of certain "early" genes. The products of these genes in turn regulate the expression of the genes whose products are required for execution of macrophage functions.

As the understanding of the fundamental biology and significance of macrophage activation has grown, so has our appreciation of the complexity and breadth of activation. It is now apparent that the natural physiology of these cells is to lie relatively dormant in the tissues until they contact appropriate inductive signals. These signals then induce activation. Of particular note is the potential of macrophages for being activated in numerous different ways; each way represents the enhancement of one or more functions and the suppression of others. Macrophages should thus be viewed as pleuripotent cells which can be modulated in the tissues in a large number of ways. Indeed, the number of discrete states of activation may range between 100 and 1000 [35]. Of equal importance is the fact that activation is not permanent. Cessation of inductive signals plus application of suppressive signals returns the cells to the basal state. Such tight regulation is efficient for the cells because it focuses the cell's limited protein and metabolic repertoire.

Obviously, the complexity of activation in macrophages is advantageous to the host, because it provides a great opportunity for diversity in activation. This is consonant with the fact that these cells exhibit a very wide array of defensive and homeostatic functions. Diversity also provides great redundancy, and therefore provides backup and security in the defense of the host.

Table 3. Defined receptors on and molecules binding to macrophages from various species. (from ref. 33)

Regulatory proteins and cytokines
- IFN-α/β
- IFN-γ
- CSF-1
- GM-CSF
- TNF-α
- MIF
- IL-1
- IL-2
- IL-3
- IL-4
- IL-6

Immunoglobulins
- IgG_{2a}
- IgG_{2b}/IgG_1
- IgG_3
- IgE
- IgA

Complement components
- $C1_q$
- C3b
- C3bi
- C3d
- C5a

Hormones and additional proteins
- α_1-Antiprotease–protease complexes
- α_1-Antithrombin
- α_2-Macroglobulin–protease complexes
- Calcitonin
- Ceruloplasmin
- Coagulation factor VII
- Coagulation factor VIIa
- Estrogen
- Fibrin
- Fibrinogen products
- Fibronectin
- FSH
- Hemopexin
- Insulin
- Lactoferrin
- Laminin
- Maleylated proteins (multiple receptors)
- Parathormone
- Progesterone
- Thymosin-α_1
- Thymosin-β_4
- Thymotropin
- Transferrin

Glycoproteins and carbohydrates
- Mannose/fucose/G1NAC terminal glycoproteins
- Mannose-6-phosphate terminal glycoproteins
- Galactose terminal glycoproteins
- Heparin
- Glucose-modified proteins (advanced glycosylative endproducts)

Peptides and small molecules
- Adenosine
- Arg-vasopressin
- β-Endorphin
- Bomobesin
- Bradykinin
- Calcitonin
- Dexamethasone
- Epinephrine
- Gastrin-releasing peptide
- Glucagon
- Glucocorticosteroids
- Histamine (H_1 and H_2 receptors)
- Met-enkephalin
- Neurotensin
- *N*-Formylated peptides
- NK1 tachykinin
- NK2 tachykinin
- Platelet-activating factor
- Serotonin
- Somatomedin
- Somatotropin
- Substance P
- Tuftsin
- Vasoactive intestinal peptide
- 1,2,5-Dihydroxyvitamin D_3

Lipids and lipoproteins
- LDL
- β-VLDL
- Modified LDL (e.g., acetylated LDL)
- Leukotriene C
- Leukotriene D_4
- Leukotriene B_4
- Prostaglandin E_2

Pharmacologic agents
- Muscarinic and nicotinic Cholinergic agonists
- α_1/α_2-Adrenergic agonists
- β_1/β_2-Adrenergic agonists
- Benzodiazepine

Adhesion molecules
- LFA-1 (integrin $\alpha L\beta_2$)
- MAC-1 (integrin $\alpha M\beta_2$)
- p150/95 (integrin $\alpha X\beta_2$)
- ICAM-1 (counterreceptor for LFA-1)
- GPIV (binds to thrombospondin)

Table 4. Secretory products of macrophages. (from ref. 33)

Coagulation factors
- Factor X
- Factor IX
- Factor V
- Thromboplastin
- Prothrombin
- Thrombospondin
- Fibrinolysis inhibitor
- Tissue factor
- Factor VII/VIIA
- Factor X activator
- Prothrombinase

Components of complement cascade and regulators
- C_1
- C_4
- C_2
- C_3
- C_5
- Factor B
- Factor D
- Properdin
- C3b inactivator
- βIH

Other plasma proteins
- Haptoglobin
- Serum amyloid A
- Serum amyloid P
- Apolipoprotein E
- Acidic isoferritins
- Lipid transfer protein
- Transcolobamin II

Matrix proteins
- Fibronectin
- Gelatin-binding protein
- Thrombospondin
- Chondroitin sulfate proteoglycans

Enzymes
- Plasminogen activator
- Elastase
- Collagenases (types I, II, III, and IV)
- Angiotensin convertase
- Cytolytic proteinase
- Lysozyme
- Amyloid proteinase
- Lipoprotein lipase
- Phospholipase A_2
- Amylase
- Hyaluronidase
- Acid hydrolases
- β-Galactosidase
- β-Glucuronidase
- Nucleases
- Ribonucleases
- Acid phosphatases
- Sulfatases
- Cathepsins (B, L, H, L, N)

Inhibitors of enzymes
- α_2-Macroglobulin
- α_1-Antiprotease
- Lipomodulin
- α_1-Antichymotrypsin
- Inhibitors of plasminogen
- Inhibitors of plasminogen activator

Cytokines, growth factors, and hormones
- INF-α/β
- IFN-γ
- IL-1 (IL-1α and IL-1β)
- IL-6
- IL-8
- TNF-α
- *gro*
- MCP-1
- IP-10
- Inhibitors of IL-1
- TGF-β
- PDGF
- FGF
- Angiogenesis factor
- GM-CSF
- G-CSF
- Erythroid colony potentiating factor
- FIM
- Erythropoitin
- Lactoferrin
- 1α-Dihydroxyvitamin D_3
- Thymosin B_4
- Insulin-like activity

Reactive oxygen intermediates
- O_2^-
- H_2O_2
- OH·
- Hypohalous acids

Reactive nitrogen intermediates
- NO·
- NO_2
- NO_3

Lipids
- PGE_2
- $PGF_{2\alpha}$
- Prostacylin
- Thromboxane A_2
- Leukotrienes B, C, D, and E
- Mono-HETES
- Di-HETES
- PAF
- Lysophospholipids

Small molecules
- Purines
- Pyrimidines
- Glutathione
- Thymidine
- Uracil
- Uric acid
- Deoxycytidine
- Neopterin
- cAMP

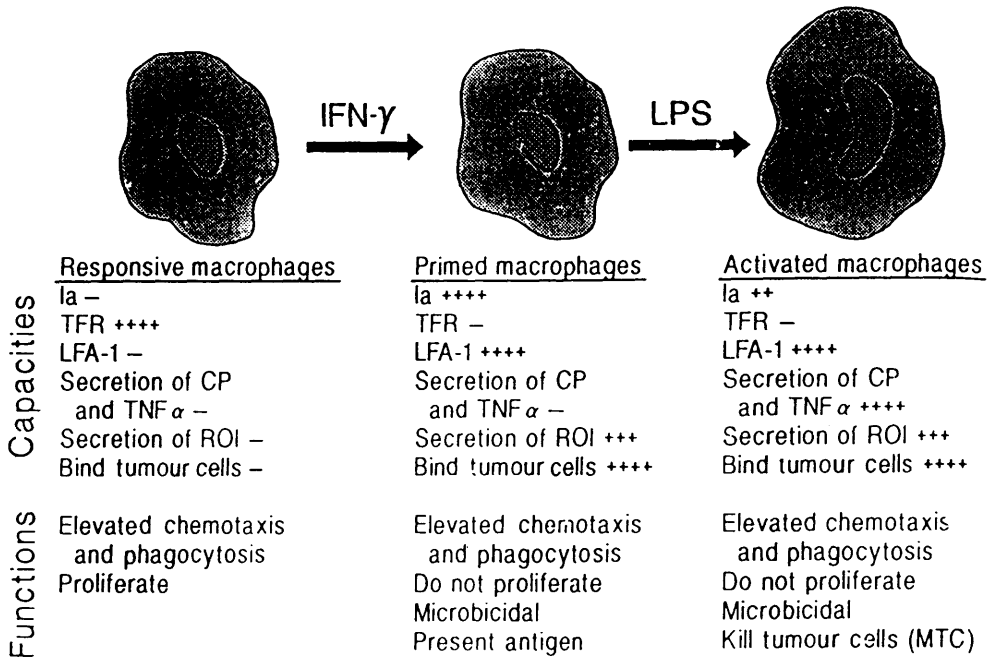

Fig. 1. A basic model of macrophage activation for macrophage-mediated tumor cytotoxicity. Shown are responsive, primed, and activated macrophages and two major signals, IFN-γ and LPS, which respectively prime and trigger cytolysis. Also shown are a few selected, objective markers of each stage plus certain functions which are turned on or turned off in each of the three stages. (from ref. 35)

Comparison and contrast of neutrophils and macrophages in host defense

Neutrophils and mononuclear phagocytes constitute the major innate defense against microbial invasion. The neutrophil, in general, is a more efficient phagocyte, except when the particle is large in relation to the cell or when the particle load is great. Under these circumstances mononuclear phagocytes are more effective than neutrophils. Since production of ROS through the respiratory burst in neutrophils occurs within minutes after stimulation, these cells probably constitute the most important player in acute response to bacterial infections. The fact that neutrophils generate ROS rapidly and are loaded with myeloperoxidase-rich granules suggests that ROS are their major anti-bacterial mechanisms.

Monocytes/macrophages also use ROS, but the magnitude of the respiratory burst decreases markedly when monocytes mature into macrophages. Resident macrophages, for example, have only a weak respiratory burst, though this can be increased several folds when they are activated *in vivo*. Furthermore, macrophages, as opposed to neutrophils and peripheral blood monocytes, have very low levels of myeloperoxidase (MPO) and therefore may rely upon the MPO-independent mechanism predominantly.

The mononuclear phagocytes also actively synthesize proteins, including granule-associated proteins. Its sustained biosynthetic capabilities provide the cells with means of replenishing antibacterial proteins and of assembling new granules, permitting repeated phagocytic events [31]. This is in contrast to neutrophils, which exhibit little biosynthetic activity beyond the promyelocyte/myelocyte stages of differentiation.

The lifespan of macrophages is also substantially longer than neutrophils. For example, bone marrow transplant studies have suggested that alveolar macrophages have a lifespan of three months. The relatively long lifespan of the macrophage and its sustained biosynthetic capacity equip the cell with continued microbicidal activity. Macrophages thus represent a major defense against invasion of the host by a wider variety of micro-organisms over a broader time horizon.

Finally, the macrophage presents antigen to helper T-cells and B-cells for induction of the host specific immune responses. Collectively, the macrophage serves as a bridge between the immediate, non-specific innate immunity (mainly mediated by neutrophils) and the delayed, specific acquired immunity (mainly mediated by B- and T-cells) of the host defense system.

Products of oxygen metabolism in phagocytes [36]

The conversion of oxygen molecules to water is a thermodynamically favorable reaction. However, this reaction requires a catalyst to overcome a high kinetic barrier. The basis of the kinetic inertness of oxygen is its electronic configuration, in which two of its valence electrons are unpaired and have parallel spins. For oxygen to react with a nonradical, the spin state of one of its unpaired electrons needs to be inverted. This can be accomplished by excitation, such that the newly paired electrons occupy the same orbital (delta singlet oxygen, $^1\Delta_g O_2$) or different orbitals (sigma singlet oxygen, $^1\Sigma^+_g O_2$).

Another way to increase the reactivity of oxygen is by partial reduction, resulting in the formation of highly reactive oxygen intermediates. More specifically, reduction of oxygen by the acceptance of one, two, or three electrons leads to the formation of superoxide ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO\bullet$), respectively (Fig. 2). As will be discussed below, these reactive oxygen species represent a major microbicidal mechanism in neutrophils and possibly to a less extent in macrophages.

ROS in neutrophils

Respiratory Burst and Superoxide

A striking feature of neutrophils is their response to certain stimulatory signals with a marked increase in oxygen consumption. It became clear later that the respiratory burst results from the use of oxygen molecules by the cells in the synthesis of a microbicidal agent, superoxide ($O_2^{-\bullet}$). The enzyme system responsible for the respiratory burst is NADPH oxidase, a multi-component transmembrane electron transport system. Upon activation, this enzyme system transfer one electron from a reduced pyridine nucleotide (predominantly nicotinamide adenine dinucleotide, or NADPH) on the cytoplasmic side of the membrane to an oxygen molecule in the extracellular fluid or in the phagosome, through a series of reactions involving the oxidation and reduction of a flavin, a β-cytochrome, and a quinone.

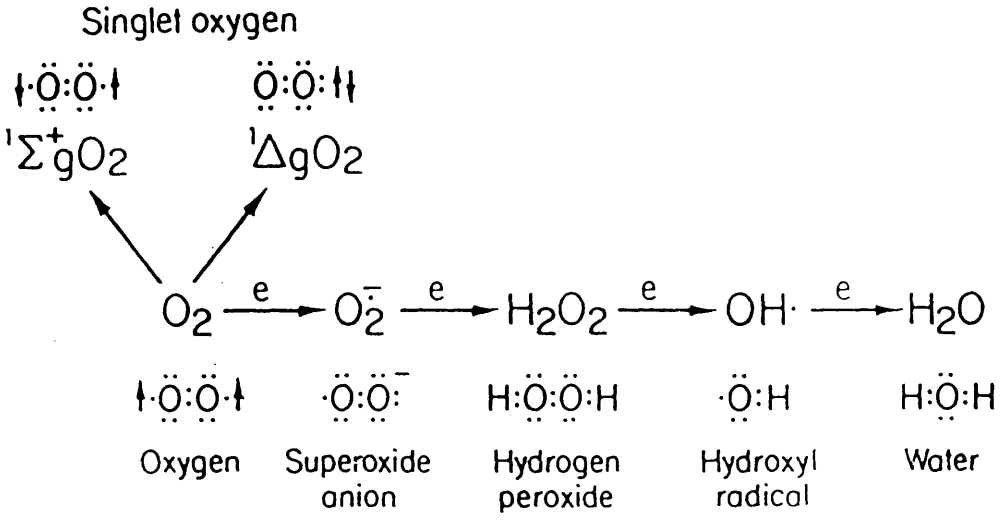

Fig. 2. Reduction and excitation of oxygen. (from ref. 36)

The oxidase is synthesized and stored in an inactive dormant form in the granules of a neutrophil. Within minutes after the cell is exposed to a stimulus, the components of the enzyme system assemble at the cell surface or on the phagosome membrane by degranulation, leading to the formation of a fully activated oxidase. The long-recognized stimuli are phorbol esters such as phorbol myristate acetate (PMA), 12-o-tetradecanoyl-phorbol-13-acetate (TPA), which are believed to activate the oxidase through protein kinase C.

Superoxide, the initial product of the respiratory burst, is in equilibrium with its protonated form with the pKa of the dissociation

$$HO_2\bullet \ <=> \ O_2^{-}\bullet \ + H+$$

being 4.88. Thus, the radical exists almost entirely as $O_2^{-}\bullet$ at neutral pH. $O_2^{-}\bullet$ is predominantly a reductant, although it can also act as an oxidant. Two molecules of $O_2^{-}\bullet$ can interact in a dismutaion reaction:

$$2\ O_2^{-}\bullet + 2H^+ \ => \ O_2 + H_2O_2$$

This reaction can occur spontaneously or be catalyzed by the enzyme superoxide dismutase (SOD). Three distinct SODs exist that vary in their metal component (copper-zinc SOD, manganese SOD, iron SOD) and in their distribution in cells. Spontaneous dismutation occurs at pH 4.8; at this pH the rate constant approaches that of SOD-catalyzed dismutaion ($1.9 \times 10^9\ M^{-1}s^{-1}$). The rate constant for SOD-catalyzed reaction is not affected by pH over the range of 5.0 to 10.0. Thus, at neutral or alkaline pH, SOD-catalyzed dismutaion predominates, whereas at acidic pH, both spontaneous and catalyzed dismutations can compete with each other.

$O_2^{-}\bullet$ may exert its toxicity through direct or indirect pathways. A direct toxic effect of $O_2^{-}\bullet$ is implicated when the toxicity of an $O_2^{-}\bullet$-generating system is inhibited by SOD but not by catalase or by HO• scavengers. A large body of evidence suggests that either or both pathways might be operative depending on the experimental systems tested. One indirect pathway involves the formation of hydrogen peroxide from superoxide through the dismutation reaction.

Hydrogen peroxide

H_2O_2 is a well known germicidal agent and is formed in large amounts by stimulated neutrophils and other phagocytes. The reactivity of H_2O_2 is relatively low compared to other reactive oxygen products of the respiratory burst such as HO• (see below). This low reactivity allows H_2O_2 to pass intact through cell membranes and through complex biological fluids and acts on a distal target which is beyond the reach of more reactive oxygen species.

The toxicity of H_2O_2 can be decreased by the action of the enzyme catalase, which breaks down H_2O_2 to oxygen and water. Alternatively, H_2O_2 can be detoxified by the glutathione cycle. Neutrophils protect themselves from the toxic effects of exogenous or endogenous H_2O_2 by their content of catalase and the components of the glutathione cycle. Similar protection mechanisms may exist in certain target cells.

The toxicity of H_2O_2 can be increased considerably by a number of mechanisms. One major mechanism is through the formation of the peroxidase/ H_2O_2/halide system. As mentioned earlier, myeloperoxidase (MPO) is synthesized and packaged into the azurophil (primary) granules of neutrophils during the promyelocyte stage of development. MPO is present in human neutrophils in exceptionally high concentrations, with estimates varying from 1 to 5% of the dry weight of the cell. The enzyme has an intense green color due to the use of two iron chlorins as its prosthetic groups. MPO forms three distinct complexes on reaction with H_2O_2: compounds I, II, and III. Of these, Compound I is the primary catalytic peroxide compound of MPO and is highly unstable. This compound oxidizes halides to form toxic agents, including hypohalous acids, halogens, long-lived oxidants such as chloramines or aldehydes, and possibly hydroxyl radicals and singlet oxygen. Among them, HOCl is probably the most abundant product because its formation accounted for 30 to 50% of the oxygen consumed by stimulated neutrophils.

The products of halide oxidation are powerful oxidants that can attack the target at a variety of chemical sites by direct halogeneration or oxidation. The reaction can be completed in less than a second, suggesting that the reaction may occur at the surface of the target. The nature of the cytotoxic lesion is not known, although it is high conceivable to involve more than one form.

Hydroxyl Radical

Another mechanism by which the toxicity of H_2O_2 can be increased is by reaction with ferrous iron to form OH• through Fenton chemistry:

$$H_2O_2 + Fe^{2+} \Rightarrow Fe^{3+} + OH^- + OH\bullet$$

When the iron concentration is limiting, the ferric iron needs to be reduced back to ferrous iron for the complete conversion of H_2O_2 to OH•. This can be accomplished by O2^{-}•:

$$O_2^{-\bullet} + Fe^{3+} \Rightarrow Fe^{2+} + O_2$$

The overall reaction

$$O_2^{-\bullet} + H_2O_2 \Rightarrow OH^- + OH\bullet + O_2$$

is also known as Haber-Weiss reaction. In the absence of trace metal catalysis, the direct reaction rate between $O_2^{-\bullet}$ and H_2O_2 is very low compared to the dismutation reaction of $O_2^{-\bullet}$. Other trace metals known to catalyze Haber-Weiss reaction include Cu^{2+} and Co^{2+}. Ascorbic acid, GSH or cysteine, and NADPH can replace $O_2^{-\bullet}$ as the reductant required for the formation of OH•. In iron-catalyzed Haber-Weiss reaction, addition of the chelator EDTA significantly increases the reaction rate.

Because both $O_2^{-\bullet}$ and H_2O_2 are produced by stimulated neutrophils, it is logical to assume that hydroxyl radicals are generated by the cells through the Haber-Weiss reaction. However, direct evidence is lacking in demonstration of its formation in vivo. This is mainly due to the fact that OH• is an extremely powerful oxidant, reacting with essentially the first molecule it encounters. The nondiscriminating reactivity, on the other hand, allows OH• to be readily scavenged by compounds in the body fluid or by nonessential components of the target.

Singlet oxygen

In aqueous solution the lifetime of sigma singlet oxygen does not exceed 10-11 sec, whereas that of delta form is approximately 2 msec. Thus, delta singlet oxygen seems to be more biologically relevant. It is a strong electrophile, reacting with compounds in areas of high electron density to form characteristic oxygenated products. It has been observed that certain dyes in the presence of light and oxygen are toxic to cells, leading to the proposition that the light sensitive dye reacts with oxygen to form $^1O_2$, which consequently attacks the cells or other targets. This fact, together with the chemiluminescence observed when neutrophils are stimulated, raised the possibility of the formation of $^1O_2$ by phagocytes and its involvement in microbicidal activity.

Several reactions have been proposed to account for the possible generation of $^1O_2$ by phagocytes. The well-established mechanism is by the reaction of hypochloride and H_2O_2:

$$OCl^- + H_2O_2 \Rightarrow Cl^- + H_2O + {}^1O_2$$

The reaction emits a weak red chemiluminescence, and spectroscopic studies have established that the metastable product formed is $^1\Delta_g O_2$. Since HOCl is one major reactive oxygen species derived from the respiratory burst, it is possible that HOCl reacts with excess H_2O_2 to form $^1O_2$. However, efforts attempting to detect the formation of $^1O_2$ by intact leukocytes have not yielded conclusive results. Thus a role of $^1O_2$ in the microbicidal activity of phagocytes has not been established.

ROS in mononuclear phagocytes

Blood monocytes

The respiratory burst as a result of stimulation in mononuclear phagocytes, and in the blood monocytes in particular, is similar to that in neutrophils. However, the magnitude of the respiratory burst in monocytes is less than that of equivalent numbers of neutrophils comparably stimulated. It has been estimated that the oxygen consumption and H_2O_2 production in blood monocytes are 40% and 20%, respectively, of that in similarly treated neutrophils. Monocytes also contain MPO in their cytoplasmic granules, but three times less than neutrophils. Similar to neutrophils, the MPO of monocytes is released into the phagosome following particle ingestion, where it can react with H_2O_2 and a halide to form a microbicidal system.

Macrophages

The transformation of monocytes into macrophages is accompanied by multiple synthetic and secretory activities, and a concomitant decrease in microbicidal potency. The basis for the decreased potency is, in part, due to the loss of granule peroxidase and a decrease in the magnitude of the respiratory burst in macrophages, thereby causing a decrease in oxygen-dependent mechanisms of cytotoxicity. However, the respiratory burst in macrophages can be increased by several folds when the cells are activated. Furthermore, this increase can also be suppressed or deactivated. The reversibility of the respiratory burst in macrophages appears to result from the change in the affinity of the NADPH oxidase for its substrate. It is interesting to note that a change in the activity or level of MPO under activation and deactivation has not been reported, raising the question whether the oxygen-dependent system in macrophages is similar to the one in neutrophils and monocytes.

Nitric Oxide (NO•)

Brief historic accounts

High levels of nitrate in some environments have been linked to elevated incidence of human gastric cancer [37]. It was postulated that nitrate served as a precursor of nitrite, the latter capable of reacting with amines in the formation of potent animal carcinogens, nitrosamines. Long-term metabolic balance studies on healthy young men showed that the amount of nitrate excreted in urine was an average of 4-fold greater than the amount ingested [37, 38]. The results pointed to the existence of nitrate biosynthesis in man. The possibility that microorganisms in the intestinal tract were generating nitrate by means of nitrification was ruled out by studies using germ-free rats, which showed no difference in nitrate biosynthesis between these animals and their conventional counterparts. An important clue came form the findings that rats receiving intraperitoneal injection of *E. coli* lipopolysaccharide (LPS) excreted significantly higher levels of nitrate, suggesting a role of the immune system in nitrate production. Subsequent studies by another group of investigators showed that one type of immune cells (macrophages) synthesized nitrite and nitrate when treated with LPS [39]. This group later showed that lymphokines such as interferon-γ (IFN-γ) could also stimulate this synthesis [40]. Furthermore, a number of macrophage-like cell lines, when stimulated with LPS or/and IFN-γ, produced nitrite and nitrate at levels comparable to primary macrophages [41]. The precursor for nitrite/nitrate in macrophages was later found to be L-arginine [42]. Collectively, these studies strongly suggested nitrite/nitrate synthesis as a common property of activated macrophages. More importantly, the observation that the nitrite/nitrate was produced during BCG infection of mice in a time course that paralleled the acquisition of increased nonspecific bacterial resistance led to the suggestion that nitrite/nitrate synthesis may be involved in microbicidal process [40].

Another avenue of research concerning the tumoricidal activity of activated macrophages [43] have found that activated macrophages inhibit certain metabolic pathways (DNA replication, and mitochondrial respiration) but not others (glycolysis) in the target cell. Inhibition of these metabolic pathways can lead to two phenotypically distinctable responses: cytostasis or cell growth arrest which can be rescued by addition of glucose, and cytolysis to which glucose has no effect [44]. Further analysis of cells undergoing cytostasis after co-cultivation with activated macrophages showed that these target cells experienced iron loss that was concurrent to inhibition

of DNA replication. Since mitochondrial respiration but not glycolysis involves iron-containing enzymes, these results raised the possibility that factors released by activated macrophages injured target cells by the inhibition of certain iron-containing enzymes, especially those involved in mitochondrial respiration. Indeed, the same group demonstrated that aconitase, a citric acid cycle enzyme with a catalytically active iron-sulfur cluster, was inactivated in target cells co-cultured with activated macrophages [45]. Studies employing conditioned mediums identified the requirement of L-arginine for expression of the activated macrophage cytotoxic effector mechanism that causes inhibition of mitochondrial respiration, aconitase activity, and DNA synthesis in tumor target cells. The L-arginine dependent inhibition of mitochondrial iron-sulfur enzymes was observed in the macrophage effector cells as well [46]. Pretreatment of cytotoxic activated macrophages with L-arginine or post-treatment of the target cells after co-cultivation is not effective. D-arginine is not effective, and N-monomethyl-L-arginine is a reversible potent inhibitor [47]. Subsequent biochemical studies showed that L-arginine was converted to L-citrulline and more notably nitrite [48].

The identification of the intermediate in the L-arginine-dependent synthesis of nitrite/nitrate in activated macrophages was also facilitated by findings from studies concerning guanylate cyclase (GC). In 1977, Murad and his associates showed that NO• activates GC [49]. Ignarro and colleagues found that NO• leads to the relaxation of bovine coronary artery as well as the activation of GC [50]. Furchgott and Zawaski demonstrated the requirement of endothelium for vascular smooth muscle relaxation [51]. Later, work done by Moncada and colleagues put all of the pieces together by providing the first definitive proof that endothelial cells are capable of NO• synthesis and NO• accounts for most of the biological properties of endothelium derived relaxation factor (EDRF) [52].

The fact that endothelial cells are capable of synthesizing inorganic and unstable nitric oxide suggested that this molecule was likely to be the intermediate that led to nitrite/nitrate formation in activated macrophages as described earlier. Marletta and Hibbs groups then independently provided direct proof that this was indeed the case [53, 54]. Convincibly, authentic NO• gas causes the same pattern of cytotoxicity in the tumor cells as is induced by activated macrophages [54]. Moncada and coworkers subsequently showed that NO• produced by the endothelial cell also derived from L-arginine [55]. In the meantime, neuronal cells were also shown capable of synthesizing NO• via glutamate stimulation of N-methyl-D-asparate receptors [56]. It was clear at that point that a number of mammalian cells have the ability to produce NO• using the same L-arginine to NO• pathway.

Biosynthesis

Since its discovery, NO• production has been documented in an increasing number of mammalian cell types [57], including neuron [58], neutrophil (see below), hepatocyte [59], chondrocyte [60], airway epithelium [61], and renal mesangial cell [62]. Following the cloning of nNOS gene [63], several other NOS genes have also been cloned and characterized [57, 64, 65]. Despite the diversity of cells capable of synthesizing NO•, there appear to be three distinct forms of NO• synthase (NOS): inducible and calmodulin-independent NOS (iNOS), constitutive, calmodulin-dependent NOS in endothelium (eNOS) and in neuron (nNOS). The three forms of NOS display about 50% identity in amino acid sequence, with macrophage and endothelial NOS shorter at the N- and C-termini than neuronal NOS.

All three NOS isozymes display significant sequence homology to only one other mammalian enzyme, cytochrome P-450 reductase (CPR) [58]. The CPR-like sequence of NOS comprises only the carboxyl half, with the rest of the enzyme containing a P-450-like heme moiety [66]. Since the function of CPR is to donate electrons to P-450, NOS represents the first self-sufficient mammalian P-450 [66, 67]. Beyond the cysteine-ligated heme moiety, however, NOS and P-450 share little homology. This probably evolved to accommodate the specific binding of substrates with different chemical properties: L-arginine is hydrophilic, whereas most substrates for P-450 are hydrophobic [66].

The reaction catalyzed by all three NOS is the same (Fig. 3). Overall, NO• synthase catalyzes the five-electron oxidation of L-arginine to citrulline and NO• [57, 67]. Co-substrates for the reaction include NADPH and molecular oxygen. Other required components for full activities include co-enzymes FAD and FMN, tetrahydrobiopterin (BH_4) [57, 66-69]. The heme moiety is directly involved both steps of the reaction shown in Fig. 3 [66, 70].

Although the three NOS isoforms catalyze virtually the same reaction, they do so in different ways, possibly reflecting their differences in function. For instance, the iNOS isoform exists as homodimers under native conditions, and dimerization appears to be necessary for enzymatic activity [68, 69]. The formation of dimers requires BH_4, and the amino acid residues essential for dimerization and binding of BH_4 has been identified [71]. Studies concerning the subcellular localization of iNOS yielded equivocal results, with data supporting both cytosolic and membrane-bound arguments [66, 72, 73]. In contrast, the nNOS isoform are cytosolic localization of nNOS seems to be well established, so is its existence as a dimer under native conditions, although it is not clear whether dimerization is required for activity or not [74]. The third isoform,

eNOS, has been shown to be membrane bound through myristoylation and palmitosylation [75]. It is not known whether the active form of eNOS is a monomer or a dimer [74].

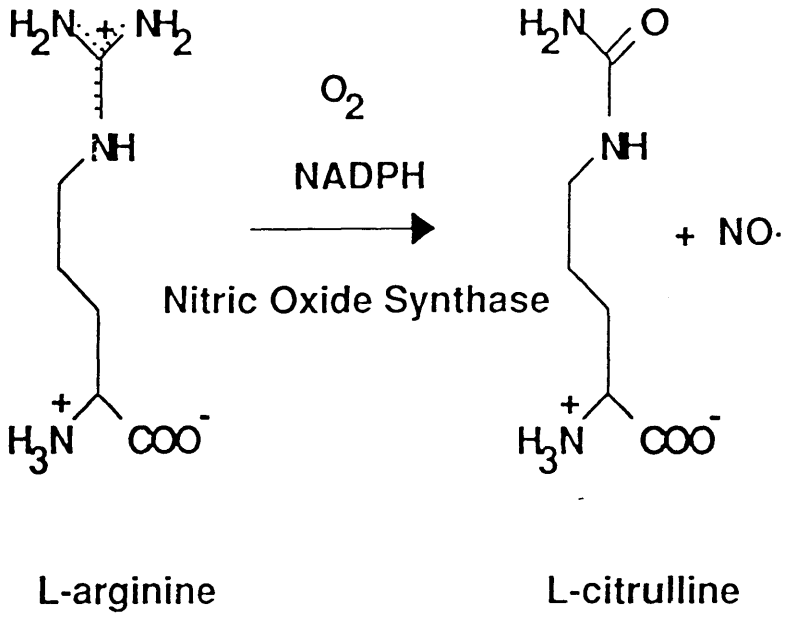

Fig. 3. Biosynthesis of nitric oxide by NO• synthases.

Regulation of enzymatic activity

The most important feature among these isoforms is probably their differential dependence on Ca^{2+}/calmodulin for activity. Both nNOS and eNOS are constitutively expressed, and is activated by calcium. It is clear now that calcium binds to calmodulin and in so doing initiates the association of calmodulin with NOS, leading to the activation of the enzyme [57, 76]. Due to this property, the activation of both nNOS and eNOS is rapid and transient. In contrast, expression of iNOS activity requires gene transcription and de novo protein synthesis [66, 74]. Once formed, the enzyme is tightly associated with calmodulin under physiological levels of intracellular calcium [66]. As a result, the activity of this inducible form is Ca^{2+} independent.

Other mechanisms for the regulation of the enzymatic activity of NO• synthases include protein stability, phosphorylation, availability of substrates and cofactors, and subcellular localization. It is interesting to note that in the absence of L-arginine NO• synthase produces superoxide or hydrogen peroxide instead of NO• [57, 76].

The activity of iNOS can also be regulated at the transcriptional level. The list of agents documented to induce iNOS expression in one or another cell type, often in synergy with IFN-γ or LPS, has been growing, so has the list of those capable of inhibiting iNOS expression induced by stimuli such as LPS and/or IFN-γ. Much of our understanding on the transcriptional induction of iNOS has derived from studies in murine macrophages. The inducibility of iNOS by agents of diverse properties imdicates that its promoter would be complex. This has been proven to be the case: numerous potential binding sites for transcription factors, including those involved in the inducibility of other genes by cytokines and bacterial productshave been located to a 1749-bp fragment from the 5'-flanking region of the mouse iNOS gene [57, 77]. Of particular interest is the presence of two discrete regulatory regions upstream of the putative TATA box. Region I contains LPS-related responsive elements, including a binding site for nuclear factor interleukin 6 (NF-IL6) and the kB binding site for the transcription factor NF-kB. Region II contains motifs for binding IFN-related transcription factors [77]. In the presence of IFN-γ and LPS, Region I alone increase the expression of a reporter gene in the mouse RAW264.7 cell line by 75-fold over the minimal promoter construct. Region II alone does not increase the expressed activity, but together with Region I it causes an additional 10-fold increase in expression. Delineation of these two cooperative regions explains at the level of transcription how these two stimuli act in concert to induce maximal expression of the mouse macrophage iNOS gene [77].

As expected, agents capable of stimulating macrophage antimicrobial or antitumor activity are in general good inducers of iNOS expression as well. These include exotoxins [78], relatively small molecules such as Taxol [79], and cytokines such as TNF-α, interleukins, and other isoforms of interferon [74, 80, 81]. Of note, inhibition of NO• synthesis by the use of enzyme inhibitors also enhances iNOS expression [82].

While macrophage activators are good iNOS inducers, anti-inflammatory agents are often turned out to be effective iNOS suppressors. For example, anti-inflammatory cytokines such as IL-4, IL-10, and TGF-β have been shown to suppress iNOS expression [74]. Curcumin, an anti-tumor promoter and anti-inflammatory agent, also inhibits induction of nitric oxide synthase in activated macrophages [83]. Aspirin-like drugs can also exert inhibitory effects on iNOS activity, although at the level of translational and post-translational modification [84]. Endogenous glucocorticoids attenuate the induction of iNOS by LPS *in vivo* [85] Finally, certain antioxidants can act as NO• scavengers [86, 87].

Metabolism

Once formed, NO• in biological systems can have three possible fates depending on the local environment of production: auto-oxidation, free diffusion, and reaction with superoxide.

Auto-oxidation

In an aqueous environment, NO• reacts with molecular oxygen:

$$2\ NO\bullet + O_2 \Rightarrow 2\ NO_2$$

$$NO\bullet + NO_2 \Rightarrow N_2O_3$$

$$N_2O_3 + H_2O \Rightarrow 2\ H^+ + 2\ NO_2$$

The rate of reaction of NO• with O_2 for nitrite formation is second-order in NO• and first-order in O_2 [88-90]. The formation of an intermediate N_2O_3 has been the subject of debate [89, 90]. A more recent study examining the N-nitrosation of morphine in oxygenated NO• solution seems to support its existence [91], although the absolute amount formed appears to be significantly lower in cell culture system than in simple NO• solution [92]. The consequence of N_2O_3 formation under physiological conditions is significant. As a powerful electrophilic nitrosating agent, N_2O_3

leads to formation of carcinogenic N-nitroso compounds and deamination and crosslinking of DNA [93]. However, N_2O_3 also reacts with thiols such as cysteine and glutathione to form S-nitrosated compounds at a rate comparable to that of auto-oxidation [94, 95]. Thus, high intracellular levels of glutathione may represent a physiological scavenger of N_2O_3 [94, 96].

Free diffusion

The second-order dependency of auto-oxidation dictates that the half-life of NO• be inversely proportional to its concentration. Maximal concentrations of NO• in the cellular micro-environment are estimated to be in the range of 0.45–10 mM, which correspond to half-lives of 1-500 sec for NO• in air-saturated aqueous solution [89]. Further, cell membrane permeability of NO• is similar to that of oxygen [93]. Therefore, it is possible for low amount of NO• to diffuse relatively far removed from the point of generation, as predicted by simulation models [97, 98]. (Interestingly, one of the models also predicts that NO• from a collection of NO•-producing cells will act in a mostly paracrine fashion [98].) Constitutive NOS in neuronal and endothelial cells generate low levels of NO•, which may have sufficient time to reach target sites even in the presence of O_2. Under certain activation conditions, macrophages synthesize more NO• than O_2^{-}• [92, 99], both of which may react with each other (see below). The excess amount of NO• from activated macrophages may undergo auto-oxidation; a small portion of this pool of NO• may diffuse freely into adjacent cells. Once inside a target cell, free NO• may react with a number of intracellular targets, including Fe-S center of aconitase, tyrosyl radical of ribonucleotide reductase, and protein thiols, or with superoxide generated within the target cell [74, 100, 101]. Free NO• may react with myoglobin and hemoglobin in extracellular spaces as well [74].

Reaction with superoxide

As mentioned earlier, activated macrophages can simultaneously produce NO• and O_2^{-}• [102]. At reduced L-arginine concentrations, purified neuronal NO• synthase can produce both O_2^{-}• and NO• [103]. NO• and O_2^{-}• are known to react to form peroxynitrite anion ($ONOO^{-}$), which is relatively stable under alkaline conditions. Once protonated, $ONOO^{-}$ decomposes rapidly with a half-life of 1.9 sec at pH 7.4 [104]. Decomposition of peroxynitrite generates hydroxyl radical, considered the strongest oxidant in biological systems [104]:

$$O_2^{-}\bullet + NO\bullet \Rightarrow ONOO^{-} + H^{+} \Rightarrow ONOOH \Rightarrow HO\bullet + NO_2\bullet \Rightarrow NO_3^{-} + H^{+}$$

In the presence of superoxide dismutase (SOD) or ferric ion, peroxynitrite undergoes heterolytic cleavage to form a nitronium-like species, leading to nitration of a wide range of phenolics

including tyrosine in proteins [105, 106]. Peroxynitrite can also directly oxidize protein and non-protein sulfhydryls [107, 108].

Significantly different reaction rates of NO• with O_2^{-}• have been reported. The rate constant was determined to be 3.7 x 10^7 $M^{-1}s^{-1}$ in one study [109], but 6.7 x 10^9 $M^{-1}s^{-1}$ in another [110]. The latter rate constant is close to the rate constant for diffusion, and is ~3.5 times faster than the SOD-catalyzed decomposition of superoxide [110]. Since both studies used well-defined inorganic systems, it is not clear which rate constant is a better description ofin vivo situations, although the higher rate constant has been cited frequently [88]. However, more than one study have reported that superoxide detection in macrophages is not affected by the inhibition of NO• production [99]. This observation seems to suggest that NO• may not react with O_2^{-}• as fast as people have thought, thus favoring a significantly lower rate constant under these conditions.

When O_2^{-}• and NO• react, the mechanisms and extents of $ONOO^-$ formation are strongly influenced by the relative fluxes of O_2^{-}• and NO• [103, 111], and by the presence of CO_2 [101]. Paradoxingly, equimolar fluxes of O_2^{-}• and NO• in an *in vitro* system or in macrophages have lower cytotoxicity in target cells and in macrophages themselves, respectively [112]. In addition to O_2^{-}•, NO• can react with H_2O_2 to form $^1O_2$ [113]. Collectively, these observations underscore the complexity of reactive species involved in host defense and in pathological events.

Physiology

Although the three "classics" of NO•-mediated functions, i.e. endothelium dependent relaxation, neurotrnsmission, and cell-mediated immune response, are well received, an expanding body of evidence suggests that virtually every mammalian cell is under the influence of NO• [114]. Further, it has been demonstrated in many systems that same physiological functions can be mediated by NO• derived from two or more different cellular sources. For example, in the regulation of smooth muscle relaxation, vascular NO• has been found to derived from adventitial nerves and epithelial cells as well as endothelial cells [114].

Regardless its sources, the physiological role of NO• appears to depend on the output of its corresponding NOS [65]. Because the activity of nNOS and eNOS is triggered by Ca^{2+} elevating agents, its output is transient (minutes) and low. NO• at this level of output has a relatively long half-life and are mainly involved in homeostatic processes such as neurotransmission, peristalsis,

and moment-to-moment blood pressure regulation. The neurotransmission and vasodilatation actions of NO• are largely mediated by the activation of soluble containing guanylate cyclase (GC) after the binding of NO• to the sixth coordination position of the heme iron of this enzyme. This leads to the synthesis of a secondary mediator, cGMP, and subsequent activation of cGMP kinases in responder cells [101]. An alternative mechanism in vascular control has recently been reported, which involves the use of S-nitrosohemoglobin as a vehicle in the transduction of NO•–related activities in the blood [115].

In contrast, NO• produced by iNOS is independent of Ca^{2+} elevating agents, and its output is high, often lasting days or longer. The sustained activity of iNOS is often reserved for host defense against infection and inflammation. As discussed earlier, high levels of output may confine the action of NO• within a limited tempo, thereby minimizing its potential toxic effects. The complex interaction between reactive oxygen and nitrogen species appears to equip mammalian cells with a primitive, broad-spectrum antimicrobial and antitumor defense, which will be discussed in greater details below.

Roles in host defense

Anti-microbial. NO• as an antimicrobial agent has been strongly suggested in co-cultures of activated murine macrophages with a variety of pathogens, including *Cryptococcus neoformans* growing extracellularly, extracellular schistosomula of *Schistosoma mansoni*, intracellular amastigotes of *Leishmania major*, intracellular trophozoites of *Toxoplasma gondii*, intracellular *Mycobacterium tuberculosis*, and intracellular *Mycobacterium leprae* [116]. The antimicrobial activity of NO• is probably mediated by a multiplicity of reactive chemical species, including $ONOO^-$, N_2O_3, and free NO• [101, 116, 117]. T-cell derived cytokines (i.e. IFN-γ) are major activators of murine macrophages for NO• production and antimicrobial activities [116, 118-121], although release of NO• during the T-cell independent pathway of macrophage activation has been documented in the killing of intracellular *Listeria monocytogenes* [122]. Mice deficient in inducible NO• synthase failed to restrain the replication of *Listeria monocytogenes in vivo* [123]. Anti-inflammatory cytokines such as IL-10 and TGF-β inhibit NO• production in macrophages, with concomitant loss of anti-microbial activities in the cells [124, 125].

The involvement of NO• in microbial killing is further substantiated by several pieces of evidence suggesting that certain microbes have evolved self defense mechanimsms against the

insult of NO•. For example, *Salmonella typhimurium* appears to defend itself from NO•-related cytostasis through intracellular synthesis of homocysteine [126]. The oxidative-stress response in *Escherichia coli* is also operative in conferring bacterial resistance to activated murine macrophages [127, 128].

While the killing of these microbes is mediated by NO• production in the host, and in macrophages in particular, other microbes seem to be killed through mechanisms independent of NO• production in macrophages. For example, Francisella tularensis can can be killed by INF-γ stimulated murine macrophages, but in the absence of NO• production [129]. Mice with a disrupted NF-IL6 gene had a compromised ability in bactrerial killing, even though IFN-γ and NO• productions in the animals remain intact [130].

Anti-neoplastic. As mentioned earlier, NO• has been identified as an effector molecule in the induction of cytostasis in target tumor cells co-cultivated with activated macrophages [54, 131]. NO• induces cytostasis in tumor cells through formation of iron-nitrosyl complex in mitochondrial enzymes, thereby inhibits cellular respiration [45, 46, 132, 133]. NO• or its derivatives can also induce apoptosis in tumor cells [134] as well as in the generator cells [135]. The cytotoxicity in cells exposed to NO• or $ONOO^-$ may be mediated by DNA strand breakage and the subsequent activation of the DNA repair enzyme poly (ADP ribose) synthetase (PARS), leading to the depletion of cellular NAD^+ and ATP pool [136, 137]. These effects could be inhibited by the use of PARS inhibitors or by the inactivation of PARS gene [136, 137]. The antitumor activity of NO• *in vivo* has also been documented in a UV light-induced murine skin cancer model [138]. However, in another *in vivo* model, NO• appears to have no effect on the growth of transplanted tumor cells [139].

It is worthwhile to point out that certain tumors or tumor cell lines have been documented to synthesize NO• [140-143]. It is not clear how these tumor cells escape the cytotoxicity of NO• they produce. One possibility is that the levels of NO• production is such that it is low enough not to cause toxicity but sufficient to induce other activities in neighboring cells. This property may provide the tumors growth advantages by increasing tumor blood flow, edema, and vascular permeability. Alternatively, these cells may have evolved self protection mechanisms against relatively high levels of endogenous NO•.

Diseases associated with abnormal NO• metabolism

With a defect in NO• production

The biological functions of nitric oxide have been clarified considerably by the generation of mice with various forms of NOS inactivation through gene knockout technology [123, 144-149]. These mice are viable, grossly normal, and fertile. The phenotypes of mice deficient in NOS are summarized in Table 5 [150]. Overall, some of the phenotypes confirm previous studies using NOS inhibitors or other mechanisms involving inhibition of NO• production, but others do not. In the case of $iNOS^{-/-}$ mice, which have been created by three independent research groups, the role of NO• in endotoxin-induced septic shock appears to be influenced by experimental conditions [123, 147, 148].

With overproduction of NO•

NO• is a double-edge sword. NO• produced in a controlled manner plays an important role in many aspects of mammalian physiology. However, excessive production of this highly reactive small molecule is potentially toxic. Indeed, NO• has been implicated in a number of pathophysiological conditions in humans as well as in animal models. Of great interest is the documentation of increased NO• formation in a number of chronic infection/inflammation conditions in humans, including those which have been linked to higher cancer risk [16, 18, 151-157]. Furthermore, the causative role of NO• in chronic inflammation is established by the use of NOS inhibitors or antioxidants [151, 154-156].

Endotoxin-induced septic shock may also involve overproduction of NO•, because endotoxin tolerance can be achieved through inhibition of NO• production by various mechanisms, including glucocorticoids [85], tyrosine kinase inhibitors [158], interleukin-1 receptor antagonist, and disruption of interferon regulatory factor-1 gene [159]. It should be pointed out that the causal effect of NO• in sepsis can not be firmly established from these studies, because most of these mechanisms inhibit production of other effectors in addition to NO•. However, results from the use of $iNOS^{-/-}$ mice unequivocally demonstrate the critical role of NO• in septic shock [123, 147].

NO• has also been implicated as a pathological factor in other diseases. Adjuvant-induced arthritis, a model of chronic inflammation that exhibits several pathological changes similar to those occurring in rheumatoid arthritis in humans, can be suppressed by selective inhibition of inducible NO• synthase [154]. In pharmacologically induced models of insulin-dependent diabetes and

nonobese diabetic mice, progressive insulitis, dysfunction, and eventual killing of pancreatic β cells correlate with the induction of iNOS; in some cases, these pathological changes can be abrogated by NOS inhibitors [114].

NO•-mediated activation of PARP appears to be responsible for NO•–elicited neurotoxicity [160]. One source of NO• in causing neurotoxicity may be from microglial cells [161]. NO•–related neurotoxicity may be mediated by its derivative peroxynitrite [162], although in vitro studies employing NO• donor compounds showed that NO• protects against neuronal cytotoxicity from reactive oxygen species [112]. In the absence of reactive oxygen speceis, NO• may trigger a switch to growth arrest during differentiation of neuronal cells [163]. In addition to neurons, NO• is implicated in the induction of other tissue injury [164].

NO• production in neutrophils

Several papers have reported NO• synthesis in neutrophils isolated from rats [165, 166] and humans [167-169]. The amount and kinetics of production suggests that NO• may be synthesized by a cNOS-like enzyme and as a result does not appear to contribute to the antimicrobial activities of peripheral blood neutrophils [74, 167, 168]. In fact, NO• from neutrophils has been found in neutrophil chemotaxis, in the regulation of neutrophil adhesion to endothelium, and in the inhibition of platelet aggregation [170]. However, motile, anucleate fragments isolated from human neutrophils showed NO•–dependent residual bacterial killing activity, which suggests that the presence of granules rather than of activable respiratory burst oxidase activity masks the expression of NO•–dependent antimicrobial functions in neutrophils [170]. Thus, it appears that the generation of NO• in granule–poor neutrophils may serve as an additional microbial killing mechanism.

Table 5. Phenotypes of mice deficient in nitric oxide synthase (NOS)
(Reproduced from ref. Snyder, 1995 #506)

NOS subtype	Phenotype
Neuronal (nNOS, type 1)	Pyloric stenosis Resistant to vascular stroke Inappropriate, excessive sexual and aggressive behaviour Normal hippocampal long-term potentiation and cerebellar long-term depression
Inducible (iNOS, type 2)	More susceptible to Listeria and Leishmania infection and lymphoma cell proliferation Resistant to endotoxin hypotension and carrageenan inflammation
Endothelial (eNOS, type 3)	Deficient acetylcholine vasodilation Elevated mean blood pressure L-nitroarginine-induced hypotension

Genotoxicity of ROS

Substantial evidence exist demonstrating the genotoxic and carcinogenic activities of ROS [171-174]. Much of the characterization has been conducted using ROS generating systems in aqueous solution. Mainly due to ease of preparing and stimulating human neutrophils for superoxide production, these cells have been frequently used as a cellular source of ROS. In some cases, murine macrophages have been used. The existence of a collection of well characterized antioxidants or scavengers have helped greatly in delineating the role of ROS under a variety of conditions. Finally, the identification of products resulting from oxidative damage to DNA (see Fig. 4) have made it possible to examine the role of ROS *in vivo*.

ROS from cell-free systems

Hydrogen peroxide

H_2O_2 solution has been found to be mutagenic to human lymphobalstoid TK6 cells [175]. Characterization of the H_2O_2 induced *hprt* mutations in the cells showed that 24% had large gene alterations as determined by Southern blot, compared to 39% and 43% in spontaneous and oxygen-induced mutants, respectively. Molecular analysis of the types and positions of induced mutations in exon 3 using denaturing gradient gel electrophoresis revealed several hot spots: AT to TA at position 259 (3% of total mutants), two GC to CG at 243 (0.6%) and 202 (0.4%), and a 5-bp deletion at 274-278 (0.3%). These are different from those observed in spontaneous mutant populations, where two hot spots were observed: a 1-bp deletion (-A) at 256 or 257 (1%), and a 2-bp deletion (-GG) at 237 and 238 (1%). On the basis of the data the authors concluded that ROS do not contribute to a significant extent, if any, to the spontaneous point mutations, although the formation of spontaneous large gene deletions may involve ROS.

H_2O_2 solution was also mutagenic to the SV40-based shuttle vector pZ189 transfected and replicated in simian cells (CV-1). H_2O_2-induced mutations in this system included large deletions, base changes and small deletions of 3-bp or less [176].

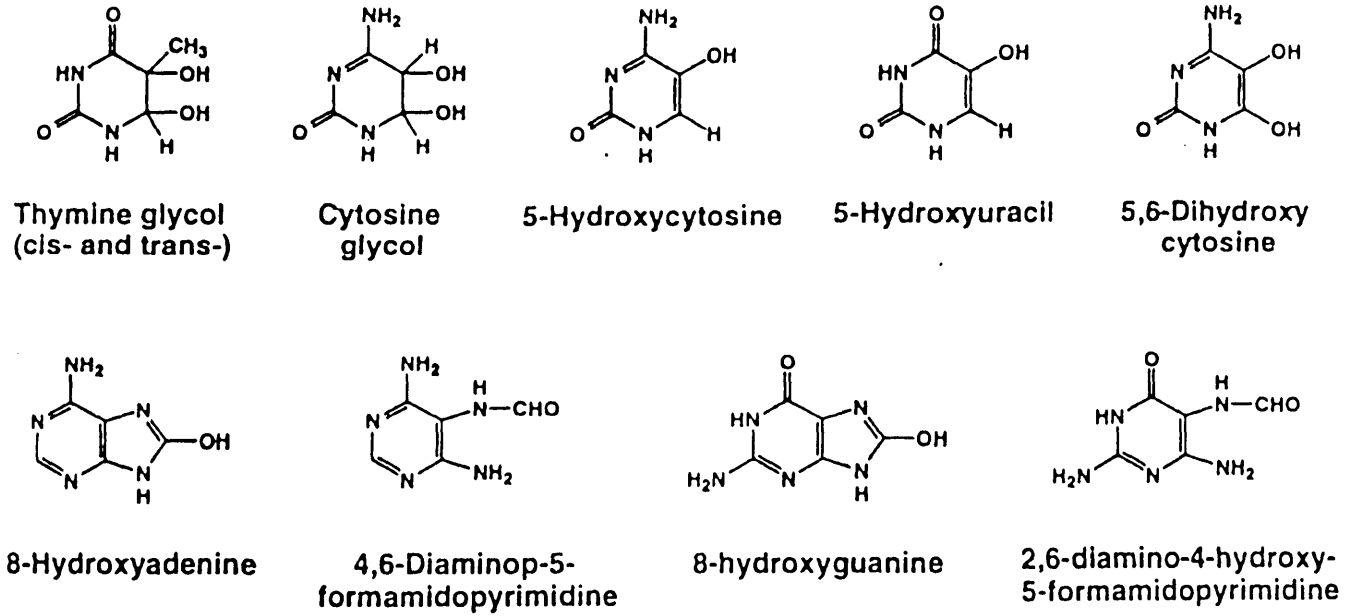

Fig. 4. Oxidized DNA bases. These modified bases can presumably be formed by the action of ROS on cells. Peroxynitrite, a reaction product of superoxide and nitric oxide, is also believed to induce oxidative damage to DNA.

Xanthine/xanthine oxidase

This system, with either xanthine, hypoxanthine, or acetaldehyde as substrate, forms $O_2^{-}\bullet$, H_2O_2 and OH•. For this reason it has been employed as a model of the oxygen-dependent microbicidal mechanisms in phagocytes. The use of SOD, catalase, and OH• scavengers has led to the identification of OH• as a major microbicidal product in this system. ROS generated by this system induced high levels of oxidized guanine and cytosine products [172, 177], sister-chromatin exchanges [178], and DNA breakage [179]. Reactive species generated from this system have also been found capable of promoting the transformation of cells initiated with g-irradiation or benzo[a]pyrene diol epoxide I [180], and of inducing neoplastic transformation of mouse fibroblasts [181].

Metal ions and oxygen

Single-stranded M13mp2 DNA incubated with 10 mM Fe^{2+} prepared as an aerobic solution showed a 20- to 80-fold increase in mutant fraction over untreated controls. The involvement of ROS under this condition was indicated by the ability of SOD, catalase, and mannitol to diminish the mutagenic response. Ninety-four percent of the detected mutations were single-base substitutions, the most frequent being G to C, followed by C to T and G to T [176]. Fe^{2+}, Cu^{2+}, or Ni^{2+}, together with g irradiation, could induce tandem double CC to TT mutations, which has been suggested to be a marker of ROS-induced mutation in cells that are not exposed to UV irradiation [182].

Hyperoxia

Exposure of Chinese hamster cells [182] or human lymphoblastoid TK6 cells, to a high concentration of oxygen to 10-40% survival failed to induce mutation in the *xprt* and *hprt* genes in CHO and TK6 cells, respectively [183]. Hyperoxia also failed to induce normal growing *tk* mutants in TK6 cells, but efficiently induced slow growing *tk* mutants [184]. The authors interpreted this observation as the inefficiency of endogenous activated oxygen species to induce point mutations or small deletions, but tend to generate gross rearrangements.

Singlet oxygen

When M13 mp19 RF DNA was exposed to $^1O_2$ produced by a separated-surface-sensitizer, followed by replication in bacteria, GC to TA was the major type of induced base substitution [185]. Another study using singlet oxygen released by

thermo-dissociation of the endoperoxide 3,3'-(1,4-naphthalidene) dipropionate also came to a similar conclusion, although the number of mutants characterized was limited [186].

Radiation

Since radiation poses as a significant risk factor in humans, extensive efforts have been spent in characterizing radiation-induced lesions in human cells. At genomic level, X radiation induces large deletions at both *hprt* and *tk* loci in TK6 cells. The size of deletion is typically in the order of hundreds of killo base pairs and the percentage of mutants having deletions of this size is significantly higher than that of spontaneous mutants [187-191]. At nucleotide level, radiation-induced mutations had an increased number of small deletions and decreased number of transitions as compared to spontaneous mutational spectrum [192]. However, when mouse fibroblasts containing in their genome multiple copies of a recoverable lamda phage shuttle vector were treated with X rays, a high proportion of TA to GC transversions (57%) was found [193].

Gamma rays also induced large gene deletion in the *hprt* gene in primary human skin fibroblasts [194]. DNA plasmids treated with γ-radiation in the presence of oxygen mostly contained GC to CG transversions [186].

Ionizing radiation also caused large deletions (77% of total mutants) in human lymphoblastoid cells. However, pre-exposure of the cells to a low dose of ionizing radiation reduced the mutation frequency and the proportion of deletion-type mutations in the endogenous *hprt* gene by 70% and 35%, respectively [195]. Possible radioadaptation mechanisms include induction of a repair mechanism responsible for strand breaks, increased cell ability to remove toxic radicals through expression of detoxifying enzymes or small molecules.

ROS from neutrophils

Human neutrophils and a human leukemia cell line HL-60 can be activated by a number of agents to produce physiological levels of ROS, and have been widely used in studying the role of ROS in carcinogenesis.

DNA oxidative damage and gross genetic alterations

Stimulated human phagocytes induced sister-chromatin exchanges in co-cultured Chinese hamster ovary cells [178], which can be significantly reduced by combinations of oxygen radical scavengers and antioxidants [196]. Activated neutrophils also induced DNA single-strand breaks (SSBs) in a co-cultured mouse plasmacytoma cell line[197]. Damage induction in target cells coincided with the oxidative burst in neutrophils in that it was induced within 5 min and remained high for more than 90 min. Induction of SSBs was completely inhibited by catalase and partially inhibited by SOD, mannitol, and reduced glutathione, but not by sodium azide [197]. When human embryonic kidney Ad293 cells were exposed to human neutrophils activated with phorbol-12-acetate-13-myristate (PMA), a significant increase in the level of base modifications in chromatin-associated DNA was detected. Among the highly induced damaged products was 8-hydroxyguanine (8OHdG) [198].

Increased DNA or chromosomal damage has been found in ROS producer cells as well. Increased levels of sister-chromatin exchanges and 8-hydroxyguanine have been respectively detected in human lymphocytes [199] and in HL-60 cells [200] activated with PMA for superoxide and hydrogen peroxide production .

Mutations

By the use of Ames microbial assay, Weitman and Stossel provided the first evidence that human leukocytes have the ability to induce mutation [201]. The mutational spectrum induced in naked single-stranded M13mp2 DNA exposed to activated neutrophils followed by replication in bacteria has been constructed [202]. The type and distribution of neutrophils-associated mutations, including the characteristic double CC to TT mutations, was quite similar to that induced by Fe(II)/O_2 [176], suggesting that these two systems may generate similar compositions of mutagenic oxygen species. Recently, the mutational specificity of neutrophils-derived ROS in the supF gene of the shuttle vector pSP189 plasmid has been determined [203]. This study differed from the previous one in that the target DNA was exposed to neutrophils after transfection into the

host Ad293 cells and is considered to mimic in vivo exposure. Overall, there is no obvious difference in the mutational pattern between untreated and treated targets. No small (1-3 bp) deletions were observed, nor were double CC to TT mutations.

Neoplastic transformation

Besides gene mutation, neutrophils stimulated with TPA were able to induce neoplastic transformation in co-cultured mouse $10T_{1/2}$ fibroblasts [181].

ROS from monocytes/macrophages

Activated macrophages have been shown to induce DNA strand breaks and 6-thioguanine-resistant variants in a co-cultured tumor cell line [204, 205]. The involvement of ROS in the studies was implicated from the ability of oxygen radical scavengers to inhibit the induction of DNA damage and drug-resistance in the target cells.

The role of oxidative damage in carcinogenesis

The discovery that oxidative stress can lead to strand breaks and oxidized base formation in DNA [173] and that these lesions are mutagenic if not properly repaired [206, 207] strongly suggests oxidative stress as an important risk factor in carcinogenesis. The identification of 8-hydroxyguanine (8OHdG) as an abundant base modification in mammalian cells under oxidative stress conditions [206], and the availability of means of quantitating this abnormal base with high sensitivity [173, 208], has made 8OHdG an important biomarker in studying the involvement of oxidative stress in cancer development. For example, significant and sustained accumulation of 8OHdG was found in hepatocytes of transgenic mice with chronic active hepatitis destined to develop hepatocellular carcinoma [209]. In individuals with Faconi's anemia (FA), a congenital disorder with higher cancer incidence, the 8OHdG level in FA homozygotes was significant higher than age-matched controls, indicative of genetic defect(s) underlying the abnormal oxidative metabolism [210]. Increased 8OHdG content was also detected in *Helicobacter pylori*-infected human gastric mucosa, thereby provides a mechanistic link between *H. pylori* infection and gastric

carcinoma [211]. Finally, urine obtained from smokers has elevated levels of altered nucleotides that are known to be produced by oxygen-free radicals, which reinforces the notion that cigarette smoking contributes to lung cancer through induction of oxidative stress in smokers in addition to injuring the normal epithelium in the lungs with carcinogenic agents [182].

Genotoxicity of NO•

DNA damage

As mentioned earlier, NO• produced at high levels by activated macrophages can be metabolized in three possible processes: auto-oxidation to form a deaminating intermediate (N_2O_3), diffussion as free NO•, and reaction with superoxide to form peroxynitrite. These products are potentially toxic to DNA as well as proteins and lipids. Peroxynitrite may dissociate to form hydroxyl radical, leading to DNA oxidation or/and strand breaks. Nitrosation by N_2O_3 of primary heterocyclic amines such as purines and pyrimidines can lead to deamination. NO•-induced modulation of enzymes involved in the maintenance of genomic integrity may indirectly contribute to increased genotoxicity in target cells. These hypotheses have been supported to varied extents by studies to be discussed below.

Earlier studies showed that high doses of NO• gas or donor drugs cause deamination to deoxynucleosides, deoxynucleotides, and naked RNA or DNA [212, 213]. Xanthine and hypoxanthine, from deamination of guanine and adenine, respectively, were formed in intact human cells exposed to NO• gas [213]. Oxidative damage in addition to deamination was detected in the DNA of murine macrophages activated for NO• production [99]. Oxidized DNA bases which can be formed in cells exposed to oxidants such as peroxynitrite are listed in Figure 4.

DNA strand breaks were also observed in intact DNA treated with NO• gas [213], and in rodent cells treated with NO• gas or peroxynitrite [214]. Single strand breaks may arise from the formation of abasic sites, or from other indirect mechanisms [214]. Finally, NO• can induce DNA cross-links, although the role these lesions play in NO•-associated cytotoxicity and mutagenesis has yet to be established [214].

The formation of various DNA lesions from NO• exposure is also supported by indirect evidence. For example, bacterial cells deficient in nucleotide excision repairs, and mammalian cells defective in repair of intra-strand cross-links or single-strand breaks, are invariantly more sensitive to cell killing by NO• [214]. Exposure to activated macrophages of a *lacZ* coding CMV plasmid in solution, in macrophages, or in a host cell line resulted in a decrease in the b-galactosidase activity of the *lacZ* gene. Inhibition of NO• production in macrophages by an NO• synthase inhibitor

recovered parts of the enzyme activity, suggesting that other factors in addition to NO• are also operative.

Endogenous formation of N-nitroso compounds

N-nitroso compounds (NOCs) form a group of carcinogens occurring in tobacco products, in nitrite-cured meat and other foods, in drugs, and in certain industrial settings [215]. Exposure to NOCs has been implicated as an etiological factor for cancer of the stomach, esophagus, nasopharynx, urinary bladder, and colon [215]. NOCs can potentially formed endogenously as well. As mentioned earlier, auto-oxidation of NO• leads to the formation of an effective nitrosating agent N_2O_3. Reaction of N_2O_3 with primary, secondary, and tertiary amines can form NOCs. NO•-associated formation of NOCs has been demonstrated in aqueous solution [91], in macrophages activated for NO• production [92], and in infected patients [16, 151].

DNA mutations

DNA lesions, if not properly repaired, will lead to mutations. Deaminated purines or pyrimidines, for example, are mutagenic (Figure 5). In fact, some of these mutations have been observed in various target genes treated with different forms of NO•. C to T transitions were observed in bacteria exposed to NO•-releasing compounds [212]. This type of mutation can presumably result from deamination of cytosine or 5-methylcytosine. However, more recent studies showed that a defect in uracil glycosylase has minimal impact on killing or mutagenesis by NO• [214, 216], suggesting that alternative mechanisms are operative in the formation of this mutation.

Extensive studies regarding the mutagenic specificity of NO• have been carried out using plasmic DNA containing the supF gene as the target [217-219]. When the plasmid DNA was treated with NO• gas prepared as saturated aqueous solution, followed by replication in bacteria and in human cells, three quarters of induced mutants had AT to GC transitions, with the other quarter being GC to AT transitions [217]. When NO• donor drugs were used under otherwise identical conditions, GC to AT transitions dominate the types of observed mutations, with

transversions at GC base pairs the next most prevalent. These data suggest that NO• in the form of gas or donor drugs has distinctly different chemical properties. It is not clear which form of NO• is a better mimic of NO• produced in mammalian cells.

A variation of this system has been used to investigate the mutagenicity of peroxynitrite [219]. In plasmids treated with ONOO and replicated in bacteria, the majority of mutations occurred at GC base pairs, predominantly involving GC to TA transversions (65%), with the rest being GC to CG transversions. A more complex mutational spectrum was observed in plasmids replicated in human cells. While GC to TA also accounted for the majority of base substitutions (63%), GC to CG and GC to AT each occurred at 11% frequency. A significant number of large deletions, insertions, tandem and multiple mutations were also observed. Thus, the mutation spectra induced by $ONOO^-$ are very different from those by NO•, but similar in many respects to those by singlet oxygen [219].

Less is known about the mutagenesis in intact mammalian cells exposed to NO• or its derivatives. In one study, NO• gas induced dose-dependent increase in mutant fraction in the endogenous *hprt* and tk genes of a human cell line [213]. A low level of NO• production by an IFN-γ/LPS responsive NOS in mouse fibroblasts appears to promote neoplastic transformation of the producer cells [220]. However, when human bronchial epithelial cells were engineered to stably express NO• synthase gene or exposed to an NO• donor drug, no increase in mutation in the endogenous *hprt* gene or in codon 248 of p53 gene was detected [221]. More recently, *in vivo* production of NO• has been found to be mutagenic in transgeic mice [222].

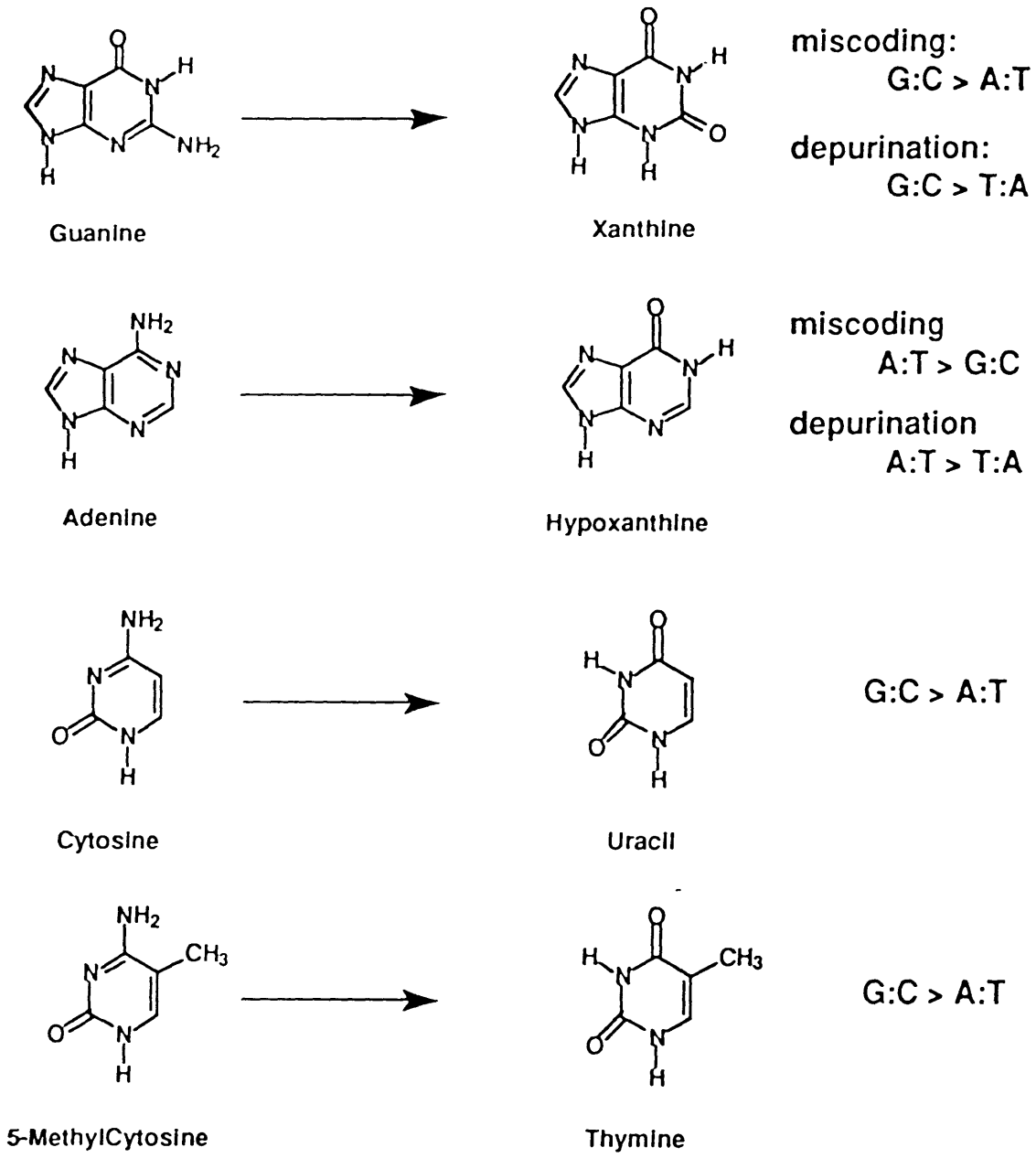

Fig. 5. Deamination products of DNA bases and their potential mutagenicity.

Mutation studies using the endogenous X-linked *hprt* gene in mammalian cells

Features of the *hprt* gene

The average mutation rate of a mammalian gene is in the order of 1 in 1-10 million. For autosomal genes, the odds that both alleles are mutated is at best 1 in 10^{12}. For practical purposes, this means that any endogenous gene suitable for mutation assay must be either heterozygous, in which one copy of the gene is inactivated, or hemizygous, in which the gene exists as a single copy as in the case of X-linked genes in cells from a male species. Further, there must be available a selection system which can distinguish a mutant cell from a wild type one. Probably the most convenient selection system is the one capable of killing all wild type cells, leaving only mutant cells alive for subsequent enumeration.

One of the genes having these features is the mammalian hypoxanthine-guanine phosphoribosyl transferase (*hprt*) gene. This X-linked gene encodes the enzyme *HPRT* which catalyzes one of the first steps in the salvage pathway for the purine bases hypoxanthine and guanine in mammalian cells . Although *HPRT* deficiency in man results in the clinical disorders of Lesch-Nyhan syndrome and gouty arthritis [223, 224], mammalian cells lacking this gene remain viable in culture. Remarkably, a purine analog, 6-thioguanine, is able to kill cells having a wild type *hprt* gene. The underlying mechanism for the action of 6-thioguanine is simple: a functional *HPRT* enzyme can metabolize this otherwise non-toxic compound into a highly toxic intermediate inside a cell, leading to cell death. In the presence of 6-thioguanine, therefore, the only surviving cells are those with a non-functional *HPRT* enzyme, which can arise from certain mutations in the *hprt* gene. Conversely, in the presence of cytidine/hypoxanthine/aminopterin/thymindine, or CHAT medium, only wild type cells survive. This reverse selection system is useful in establishing a cell population with a low background mutant fraction in the *hprt* gene.

Molecular analysis of mutations

The *hprt* gene also has other features that have made it a useful target for molecular analysis of mutations induced by mutagen treatment. Its coding sequence is 657 bp long [223-225], and the complete sequence of the 57-kb human *hprt* locus has been determined [226, 227]. This sequence information, in coupled with polymerase chain reaction amplification technology, has permitted detailed sequence analysis of the *hprt* cDNA and individual exons in genomic DNA (see below). Consequently, a large data base has accumulated describing mutations occurring in human cells exposed to mutagens in vitro as well as in mutant T-cells cloned from peripheral blood, reflecting mutations that have arisen in vivo in humans. This collection of data has been summarized in the form of a computerized database for ease of data entry, retrieval and analysis [228-230].

Characterization by RT-PCR/sequencing

For many mechanistic studies, it is important to know what types of mutations in mammalian cells are induced by a particular treatment. In most circumstances, molecular analysis of mutations in an endogenous gene such as *hprt* can provide useful information. Specifically, independent *hprt* mutants are first isolated from independently treated cells. Total RNA or mRNA is then isolated from each mutant and used for first strand cDNA synthesis by the action of a reverse transcriptase. The coding region of the *hprt* gene in the cDNA sample will be amplified by the polymerase chain reaction (PCR) technique using a pair of primers flanking the *hprt* coding region. If a PCR product is obtained, its sequence is determined for mutation identification. In a typical experiment, characterization of a few dozen *hprt* mutants is sufficient to draw informed conclusion. Because of its straightforwardness, this approach has been commonly used in mutation studies. As a result, most of the information regarding the types and positions of small changes detected in spontaneous and induced *hprt* mutants are obtained this way.

One limitation in using the RT-PCR/sequencing approach is the high occurrence of mutants with whole exon deletions, which by itself are non-informative with regard to the nature of genetic changes in these mutants. In most cases, loss of whole exon(s) is the consequence of a point mutation that affects one of the corresponding splicing sites [231-233]. PCR amplification and sequencing of the flanking intron regions are used to characterize this subset of mutants.

Characterization by Southern blot/multiplex PCR

Certain mutagens, e.g. radiations, predominantly induce large gene alterations. In many cell lines, a significant portion of spontaneous *hprt* mutants has large gene deletions. Southern blot in combined with multiplex PCR assay are often used in mapping and defining deletion endpoints in these mutants [191, 195, 234].

Molecular analysis of spontaneous *hprt* mutants in human cells using this approach has revealed that the proportion of deletion mutations is cell type dependent. In human B lymphoblastoid TK6 cells, 39-57% have large deletions affecting multiple exons [234, 235]. In human T lymphocytes, 10-20% of the *hprt* mutants isolated from normal young adults had large deletions, and the proportion increased to 85% in mutants from placental cord blood samples [236]. In the latter case, most of the alterations involved deletion of exons 2 and 3. In primary human fibroblasts, however, the proportion of deletion-type mutants is significantly smaller (2 out of 23) [194]. It appears that large gene deletions occur more frequently in B and T lymphocytes, as compared to fibroblasts. It is possible that a high frequency of large gene deletions is due to residual V(D)J recombinase activity in Band T cells.

Characterization by denaturing gradient gel electrophoresis (DGGE) or constant denaturing capillary electrophoresis (CDCE)

The previous approaches have provided much useful information for most researchers who have been interested in knowing the types of mutations occurring spontaneously or from a treatment in their systems. However, if one wants to know whether two mutagens which induce same types of mutations cause significantly different mutational spectra, or distributions of mutations within a defined DNA sequence with regard to position and type, hundreds of clones need to be characterized by these clone-by-clone approaches [237].

A less arduous way is the bulk approach, in which mutants in a selectable gene are collected en masse from large cell cultures and the mutant sequences are separated from the wild type sequence as well as from each other using various gel electrophoresis techniques. These separation methods include denaturing gradient gel electrophoresis (DGGE), constant denaturant gel electrophoresis (CDGE), and a capillary-based variant of the latter, constant denaturant capillary electrophoresis (CDCE). All of these techniques base on the observation that the melting of DNA is a cooperative or discontinuous process, which is influenced by the presence of loop, or a mismatched base pair. Therefore, electrophoresis of DNA under partial denaturing conditions (elevated temperature and/or media containing urea and/or formamide) will make it possible to

separate mutants differing by only a single nucleotide as individual bands or peaks, which are collected, further amplified, and sequenced [237].

This approach has been successfully used in defining mutational spectra for a number of environmentally important chemicals [238]. Information from this approach may prove to be useful in human risk assessment [239]. The improved sensitivity of CDCE system, which at present has a limit of detection of approximately 10^{-5}, will be applicable to the identification of low frequency mutations, to mutation spectrometry, and to the detection of rare variants. It may also hold promises for detection of mutations in a non-selectable gene.

Comparison and contrast of mutations detected in the *hprt* gene to those in other systems

For a number of mutagens, same types of point mutation are often obtained, regardless of the target genes or systems used. This is well exemplified by UV-induced mutations. The predominant type of base substitution has been found to be C to T transition at dipyrimidine sites in virtually all systems reported, including treated naked DNA replicated in different hosts, different target DNAs integrated into different hosts, intact bacteria, rodent and human cells [240, 241]. In UV-treated mutations, results from DGGE and RT-PCR approaches are remarkably consistent to the extent that a hot spot mutation detected by DGGE, a GC to AT transition at position 208 representing 2.7% of total induced mutants, is also found in one of the mutants analyzed by RT-PCR [240, 241]. In addition to UV, several DNA alkylating agents also induce similar types of mutations in different experimental systems. The findings are not surprising because these mutagens are unanimously ultimate mutagens, i.e., they act on DNA target without biotransformation.

For other mutagens, mutational spectra differ among different experimental systems. The previously discussed mutations induced by ROS are good examples. It is probably true that the extent of similarity in mutational spectrum among different experimental systems is a good indicator of the extent of biotransofrmation needed for the agent to become mutagenic. In this regard, it is of great interest to note that spontaneous mutational spectra appear to be different among cell lines, species, and position of integration of an exogenous DNA target [242-244]. Furthermore, data presently available indicate that there may not be any reproducible, spontaneously arising, hot spot mutation. For instance, two studies have detected different

spontaneous hot spot mutations in exon 3 of *hprt* gene in TK6 cells using the DGGE technology. One study reported two hot spots consisting of one base deletion (-A) at 256 or 257, and two-base deletion (-GG) at 237 and 238, each representing about 1% of total mutants [175]. The other study reported three hot spots in the same cells: two 12-bp deletions at positions 365-376 and 277/278-288/289, and an A:T to T:A transversion at position 290, which respectively represent 0.5%, 2.5%, and 2% of the total mutants [240]. In contrast to hot spots induced by UV light, none of these hot spot mutations has been seen in the spontaneous mutations reported by Lichtenauer-Kaligis et al. using clone-by-clone approach [243]. On the basis of these observations, it appears that spontaneous mutations might be caused by a multiplicity of factors, which may or may not be DNA damaging agents.

Summary

There is a strong body of evidence from human and animal studies in support of a causative role of chronic infection/inflammation in carcinogenesis. Mechanisms through which persisten infection/inflammation increase cancer risks are largely unknown. However, it has been established that phagocytic neutrophils and macrophages infiltrate the inflamed areas, where they produce large quantities of reactive oxygen species, which have been shown to induce gene mutations and cell transformation. The more recent identification of nitric oxide (NO•) as another reactive species produced by macrophages has spurred great interest in understanding its potential genotoxic properties. High levels of NO• have been found to be mutagenic to cells as well as to DNA targets *in vitro*. However, little is known about the genotoxicity of NO• produced by activated macrophages. The work described in this thesis addresses this issue at both cellular and molecular levels.

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Chapter 2. Genotoxicity associated with nitric oxide production by macrophages continuously stimulated with interferon-γ

Abstract

Mechanisms through which persistent infections/inflammation increase cancer risks are largely unknown. However, it has been established that phagocytic macrophages and neutrophils infiltrate the inflamed areas, where they produce large quantities of nitric oxide (NO•) and reactive oxygen species, which have been shown to induce lethal and mutagenic DNA damage. Less is known about the genotoxic potential of NO• produced chronically at lower levels. Here we report that the mouse macrophage-like RAW264.7 line can be activated with interferon-γ for long-term production of NO•, and that the endogenous X-linked *hprt* gene of the cells is mutated under these conditions. Activation of the cells with 10 units/ml interferon-γ continuously for 14 and 23 days produced a total of 9.8 and 14 μmoles NO• per 10^7 cells, respectively. Mutant fractions in the *hprt* gene of the cells were 16.6 and 31.3 (x 10^{-5}), respectively, as compared to 2.2 and 2.5 (x 10^{-5}) in the untreated cells. Addition of an NO• synthase inhibitor, N^G-methyl-L-Arginine, decreased NO• production and mutant fraction by 90% and 70%, respectively. The results demonstrate that long-term production of NO• is associated with increased genotoxicity in mammalian cells. This cell model system will prove to be an useful tool in understanding the molecular mechanisms through which NO• production leads to genomic instability in mammalian cells.

Introduction

Persistent infections or chronic inflammation resulting from noninfectious sources have been identified as important risk factors for several cancers, notably by hepatitis B and C viruses in liver cancer, *Helicobacter pylori* in stomach cancer, and asbestos in lung cancer [1]. Mechanisms through which infections or the inflammatory state increase cancer risk are presently not known. However, the prominent role of inflammatory cells such as macrophages and neutrophils in those processes has long been recognized, and as a consequence, these cells have been proposed to contribute to tumor development through their ability to process xenobiotics, increase cell proliferation, and deregulate cellular metabolism [2].

Much attention has been focused on the ability of reactive oxygen species (ROS) generated by these cells to cause DNA damage and mutations [3-5]. Neutrophils stimulated to produce ROS induced genetic changes in co-cultured target cells, including oxidative damage and gross genetic alterations [6-9], gene mutation in bacteria [10] and in mammalian cells [11], and cell transformation[12]. Likewise, ROS from macrophages were capable of inducing DNA damage and mutation in co-cultured target cells [13, 14]. Increased oxidative damage quantitated as 8-oxoguanine content was also detected in *Helicobacter pylori*-infected human gastric mucosa [15] and in hepatocytes of transgenic mice with chronic active hepatitis [16], thus providing evidence for the involvement of ROS in causing genetic alterations *in vivo*.

In addition to generating ROS, phagocytic monocytes share with many other cell types the capability to produce nitric oxide (NO•) [17-20]. High levels of NO• produced by macrophages have antimicrobial and antitumor activities [17, 21]. However, overproduction of NO• may exceed cellular defense mechanisms, leading to DNA damage and mutation. There are at least two major pathways by which NO• may cause DNA damage: deamination, which involves formation of potent nitrosating species such as N_2O_3, and oxidation mediated by peroxynitrite, a reaction product of NO• and superoxide [22-24]. Indeed, large quantities of NO• delivered over short periods of time cause damage and mutations in exposed target DNA or cells [25-28]. However, it is presently not known to what extent chronic exposure to physiological levels of NO• might contribute to the induction of mutations in human cells and tissues *in vivo*, as compared to the levels of mutations that arise from reactive oxygen radicals.

A number of macrophage cell lines have been found to produce NO• at levels comparable to primary cells when appropriately stimulated [29]. Commonly used stimuli for NO• production by

macrophages include the cytokine interferon-γ and bacterial lipopolysaccharide [20]. Thus, these macrophage cell lines can deliver NO• at physiologically relevant levels. One of the cell lines, the mouse RAW264.7 line [30], seems to produce higher levels of NO• than other cells [29]. Here we describe the development of RAW264.7 cells as the generator and target cells for studying the genotoxicity of NO• in mammalian cells. Continuous incubation with interferon-γ stimulated the cells to produce stable levels of NO• and caused a dose-dependent increase of the mutant fraction in the endogenous *hprt* gene.

Materials and methods

Cell culture and stimulation of RAW264.7 cells. Cells of the mouse macrophage-like RAW264.7 line were obtained from ATCC [29], and cultured in DMEM supplemented with 10% heat-inactivated calf serum, 100 units (U)/ml penicillin, 100 μg/ml streptomycin, and 1 mM L-glutamine (all reagents were purchased from BioWhittaker, Walkersville, MD). To compare cell growth under different stimulation conditions, 6 x 10^6 cells were cultured in each 100-mm tissue culture plate in 10 ml growth medium containing: no stimulus; 20 ng/ml *E. coli* lipopolysaccharide (LPS) (serotype 0127:B8, Sigma, St. Louis, MO); 20 U/ml recombinant mouse interferon-γ (IFN-γ) (Genzyme, Cambridge, MA) plus 10 U/ml Polymyxin B sulfate (Sigma); or 20 ng/ml LPS plus 20 U/ml IFN-γ. In another set of plates, the NO• synthase inhibitor N^G-monomethyl-L-Arginine monoacetate (NMA) (Chem-Biochem Research, Inc., Salt Lake City, UT) was added to the medium to a concentration of 2 mM. Every 24 hours, total NO• production (nitrite plus nitrate content in the medium of each plate) was determined by a previously described automatic procedure [31]. Cells were trypsinized and counted in a hemocytometer, and 6 x 10^6 cells from each plate were replated in fresh medium with the corresponding agent(s). Cultures costimulated with LPS plus IFN-γ contained less than 6 x 10^6 viable cells after the first day; in these instances, all surviving cells were replated. At the end of three days of continuous stimulation, cells were transferred to 10 ml fresh medium containing only 2 mM NMA, and incubated until the cells reached an exponential growth rate. During this recovery period, cell number was monitored and periodically adjusted to a density of 6 x 10^6 cells per plate in order to permit optimal growth. Some experiments involved repeated stimulation, in which case 6 x 10^6 cells were cultured with LPS plus IFN-γ for 3 days and allowed to recover and resume exponential growth after removal of the agents, as described above. This process was repeated twice. During each round of stimulation, total nitrite/nitrate levels in the medium were measured.

For continuous stimulation, 2 x 10^7 cells in a 150-mm plate were incubated for 21 to 23 days in 30 ml medium containing either 100 ng/ml LPS, or 20 U/ml IFN-γ plus 10 U/ml Polymyxin B, and in the presence or absence of 2 mM NMA under both conditions. Nitrite concentration in the medium was estimated daily using the Griess reagent [31], and medium was replaced when the concentration exceeded 60 μM. Cell numbers were also determined daily, and cell density was maintained at 1-2 x 10^7 cells per plate.

Spontaneous mutation frequency of stable hygromycin B-resistant RAW264.7 cells. One of our objectives was to study the genotoxic response in target cells co-cultivated with NO•-producing macrophages. Results of those studies will be reported in Chapter 5 of this thesis. For this purpose, we developed a co-cultured system that involved the use of hygromycin B-resistant RAW264.7 cells. To establish a stable hygromycin B-resistant phenotype, cells were transfected with the P7Hygro-9 vector (a gift of Deb Moshinsky) containing the hygromycin resistance gene in a BTX electroporator using parameters recommended by the manufacturer. Two days after electroporation, cells were grown in fresh medium containing 500 μg/ml hygromycin B (Boehringer Mannheim, Indianapolis, IN). After two weeks, growing cells were expanded in medium containing 100 μg/ml hygromycin B and frozen for later use. Integration of the hygromycin B-resistant gene did not affect generation time or NO• production in response to stimulation by LPS and/or IFN-γ (data not shown).

Hygromycin-resistant RAW264.7 macrophages were used in the following mutation studies. To establish a population of cells with a low background mutant fraction (MF), a multiplicity of 200 cells were inoculated in each well of a 6-well plate and allowed to expand for two weeks. Cells from each well were further expanded to approximately 10^8 cells, and 1.65 x 10^7 cells were plated in five 96-well plates in the presence of 2 μg/ml 6-thioguanine (Sigma) to select for cells with mutations in the endogenous hypoxanthine-guanine phosphoribosyltransferase (*hprt*) gene. The remaining cells were frozen for later uses. Two weeks later, mutant clones in the plates were scored, and the corresponding MFs calculated using a published method [32]. The population of cells with the lowest MF was used in subsequent mutation studies.

To measure spontaneous mutation rate, a total of 1 x 10^8 cells in four 150-mm plates were cultured with appropriate dilution for 3 weeks. At different time points, 3.3 x 10^7 cells were plated in ten 96-well plates in the presence of 2 μg/ml 6-thioguanine. To measure plating efficiency, cells were plated at 2 cells/well in five 96-well plates without the selecting agent. Two weeks later, clones in the plates were counted and the corresponding MF calculated, as described above. Spontaneous mutation rate was calculated from the slope of the linearly fitted curve on the plot of MF vs. time.

Treatment with the mutagen 4-nitroquinoline-N-oxide (4-NQO). 4 x 10^7 cells in one 150-mm plate were treated with 140 ng/ml 4-NQO (Sigma) at 37°C for one hour, then washed twice with 25 ml fresh medium, and subsequently incubated in fresh medium. MFs in the *hprt* gene at different time points were measured as described above over a period of 3 weeks, except that 5,000-20,000 cells/well were plated. As negative controls, parallel manipulations were

performed with untreated cells. The survival rate of 4-NQO-treated cells was estimated by extrapolating the growth curve to the zero time point.

Genotoxicity in RAW264.7 cells continuously stimulated with IFN-γ. A total of 1.5 x 10^8 cells in six 150-mm plates were cultured in 10 U/ml IFN-γ plus 10 U/ml Polymyxin B, in the presence or absence of 2 mM NMA, as described above. Untreated cells were grown in parallel as negative controls. At the end of 23 days of continuous stimulation, cells were transferred to fresh medium containing only 2 mM NMA, and allowed to resume growth. Two weeks following continuous stimulation, MF was measured, as previously described. To confirm the results, a repeated experiment was conducted under similar conditions, except the period of continuous stimulation was 14 days instead of 23 days.

Results

Growth patterns of RAW264.7 cells during and after stimulation with LPS and/or IFN-γ. It has been a common understanding that macrophages terminally differentiate and do not proliferate following activation [33]. However, our early observations suggested that under some conditions, some RAW264.7 cells survived stimulation by both IFN-γ and LPS and eventually attained growth rates equal to those of untreated cells. To characterize this response in further detail, RAW264.7 cells were stimulated with either 20 ng/ml LPS or 20 U/ml IFN-γ plus 10 U/ml Polymyxin B or both LPS and IFN-γ. After three days of stimulation, cells were cultured in medium lacking stimulating agents, but containing 2 mM NMA, added to block residual NO• production. Cells numbers were monitored from the beginning of the experiment until the growth rate of unstimulated cells was attained. Results are summarized in Fig. 1. Cells exposed to LPS or IFN-γ alone continued to divide during the 3-day stimulation period, although more slowly than cells cultured in the absence of either agent. Upon removal of the agents, cells attained exponential growth within 24 hours. In contrast, when cells were stimulated with both agents simultaneously cell number decreased not only during the three days of exposure, but also from two days following their removal. An additional four days were required for cells to resume exponential growth. During the 3-day stimulation period, NO• production by cells exposed under each condition was in good agreement with published results [29].

To assess contributions of NO• to this pattern of cell growth, cells were cultured in the presence of an NO• synthase inhibitor in addition to the stimulating agent(s). As shown in Fig. 1, the inhibitor had no effect on growth of unstimulated cells, demonstrating that it was not toxic to the cells under this condition. At the concentration used, NMA inhibited NO• production by stimulated cells by at least 90% (data not shown). In cells stimulated with either LPS or IFN-γ, NMA brought the growth rates closer to that of unstimulated cells. The effect of NMA was more significant on cells cultured in the presence of both LPS and IFN-γ, where the cell number increased by 40-60% at any time point during the three days of stimulation, and the cells resumed growth within 48 hours after removal of the agents. Taking together, the results demonstrated that RAW264.7 cells stimulated with LPS and IFN-γ continued to divide, and suggested that other factors are more cytostatic/cytotoxic than NO• in cells co-stimulated with both LPS and IFN-γ.

Macrophages have been long known to be highly heterogeneous with respect to their responses to different stimuli [33]. The possibility therefore existed that surviving cells in the

above experiments may have represented subpopulations unresponsive to stimulation, and thus incapable of NO• production, under the conditions used. We therefore carried out repeated stimulation experiments in which responsiveness was assessed by measurement of NO• production. As shown in Fig. 2, when cells surviving stimulation by LPS and IFN-γ were restimulated, they produced NO• in amounts comparable to cells stimulated for the first time. Indeed, greater amounts of NO• were produced in each succeeding round of repeated stimulation. Mechanisms underlying this phenomenon have yet to be elucidated. Nonetheless, the data demonstrate clearly that cells surviving stimulation by LPS and IFN-γ were fully capable of NO• production and not representatives of a nonresponsive subpopulation. In subsequent experiments, RAW264.7 cells subjected to 7 rounds of repeated stimulation showed undiminished capability for NO• production (data not shown).

Protracted, continuous stimulation of RAW264.7 cells by LPS or IFN-γ. On the basis of the above observations, we initiated a long-term stimulation experiment, with the results summarized in Fig. 3. Cells stimulated with 100 ng/ml LPS for 21 days produced NO• at a stable rate, 30 nmoles per 10^6 cells per day, with an estimated doubling time of 35 hours. The doubling time of untreated cells was 18-22 hours. Inclusion of 2 mM NMA in the medium blocked NO• production by 90%, but only slightly shortened the doubling time to 30 hours. When cells were stimulated with IFN-γ plus Polymyxin B for 23 days, the NO• production rate was 70 nmoles per 10^6 cells per day, which was also effectively blocked by NMA. In this instance, NMA had a more pronounced effect on cell growth; in its presence cells grew at a doubling time of approximately 35 hours in contrast to 70 hours in its absence. Collectively, these results demonstrate that RAW264.7 cells stimulated continuously with LPS or IFN-γ continue to grow and produce NO• over many cell generations. It is important to note that the rate of NO• production by cells stimulated with LPS decreased gradually after three weeks of continuous stimulation, whereas cells cultured in the presence of IFN-γ continued to divide and produce NO• at the same rate after six weeks of stimulation (data not shown).

Characterization of the spontaneous mutation rate and phenotypic expression time in RAW264.7 cells. The RAW264.7 line was originally established from a male mouse [30], suggesting that the cell has only one copy of the X-linked hypoxanthine-guanine phosphoribosyltransferase (*hprt*) gene. To assess the possibility of using this line for mutation study, we performed experiments to characterize the *hprt* gene. As the first step, we determined the concentration of 6-thioguanine required for selection of *hprt* mutants. By plating cells in 96-well plates at different concentrations of the selecting agent, it was found that there was no significant difference in the number of mutants when the concentration of the agent was 1 to 10 μg/ml (data not shown). Thus, 2 μg/ml 6-thioguanine was used in all *hprt* mutant selection

experiments. Next, we measured the spontaneous mutation rate and the mutagenic response of cells to treatment with 4-nitroquinoline-N-oxide (4-NQO), a well-characterized mutagen. Cells were grown without treatment or treated with 140 ng/ml 4-NQO for 1 hour. The treatment with 4-NQO reduced the cell survival by 50% . As summarized in Fig. 4, in untreated cells, the MF increased over time, with the spontaneous mutation rate estimated to be 1×10^{-6} per cell per day. In cells treated with 4-NQO, exponential increase of MF was observed 8 days following the treatment, and reached a plateau by 12 days. On the basis of these results, we considered 12 days as the phenotypic expression time of RAW264.7 cells in the *hprt* gene, and in subsequent studies, cells were typically cultured for two weeks following a treatment. Treatment with 4-NQO increased the MF from 2.3×10^{-5} of untreated control to 51×10^{-5}, a 22-fold increase. Thus, this line is suitable for mutation study in the endogenous *hprt* gene.

Genotoxicity in RAW264.7 cells continuously stimulated with IFN-γ for NO• production. On the basis of the above observation, we performed experiments to investigate whether long-term production of NO• was genotoxic to the generator cells, with results summarized in Fig. 5. Cells stimulated with INF-γ plus Polymyxin B for 14 days produced a total of 9.8 μmoles NO• per 10^7 cells, or 70 nmoles per 10^6 cells per day. In independent experiments, cells were stimulated under similar conditions for 23 days, producing a total of 14 μmoles per 10^7 cells, or 61 nmoles per 10^6 cells per day. In both cases, addition of NMA decreased NO• production to baseline level (data not shown). During stimulation, the growth patterns of cells were similar to the one described in Fig 3. The results demonstrate that establishment of the hygromycin-resistant phenotype did not affect the response of cells to stimulation by IFN-γ with respect to cell growth and NO• production.

As shown in Fig. 5, cells continuously stimulated for 14 days had a MF of 16.6×10^{-5}, compared to 2.2×10^{-5} in untreated cells and 4.6×10^{-5} in cells stimulated in the presence of NMA. A similar increase in MF was also observed in cells continuously stimulated for 23 days; in this case, NO•-producing cells had a MF of 31.3×10^{-5} as compared to 2.5×10^{-5} in untreated cells and 6.8×10^{-5} in the presence of NMA. Collectively, the data clearly demonstrate that there is a positive correlation between MF and the duration of stimulation in cells stimulated to produce NO•. Most notably, 70% of the increase in MF in stimulated cells are associated with NO• production.

Discussion

In this study we reported the development of the mouse macrophage-like RAW264.7 line as a model cell system for the study of nitric oxide-mediated genotoxicity in mammalian cells under long-term exposure conditions. Cells stimulated with IFN-γ alone continued to divide and produce NO• over many cell generations. Under this condition, a substantial increase in the mutant fraction in the endogenous *hprt* gene of cells was observed. Addition of the NO• synthase inhibitor N^G-methyl-L-Arginine blocked NO• production by 90% and reduced mutant fraction values by 70%. These results clearly demonstrate that NO• produced by stimulated macrophages under long-term conditions is genotoxic to mammalian cells, thereby providing evidence in support of a causative role of prolonged production of NO• in the process of cancer development.

It is presently not clear how NO• production led to increased genotoxicity in this system. This could be not caused by increased cell proliferation because cells producing NO• grew slower than those stimulated in the presence of NMA (Fig. 3). It has been shown that RAW264.7 cells stimulated with IFN-γ and LPS to produce NO• had increased DNA lesions [34]. These lesions can lead to mutations if not properly repaired [23, 35]. Another nonexclusive possibility is that NO• may inhibit the activity of certain enzymes, leading to increased genomic instability. Indeed, NO• has been documented to inhibit enzymes involved in DNA biosynthesis and repair (reviewed in [24]). Characterization of these NO•-associated mutations will provide important insights regarding NO• mutagenesis.

Since macrophages are both NO• producer and target cells in this study, it is not clear to what extent the genotoxicity in macrophages was due to NO• generated intracellularly. A simulation model predicted that for a collection of NO•-producing cells, NO• acts mainly in a paracrine fashion [36]. Further investigation is warranted because evidence in support of this prediction will validate the use of RAW264.7 cells for the study of NO• mutagenesis in exposed target cells.

Our observation that some stimulated macrophages did not terminally differentiate and die was in contrast to what has long been the dogma [33]. Mechanisms responsible for the discrepancy are not well understood. Perhaps macrophage cell lines behave differently from primary cells. However, it was noted that differences in cell culturing affected results

considerably. More specifically, we found that changing medium frequently during continuous stimulation was necessary in order to avoid cell death as a result of decreased medium pH (data not shown). Results shown in Figures 1 and 3 also suggest that NO• production rate affected cell viability and growth. It will be of great interest to know whether changing medium frequently has an effect on viability of primary macrophages in culture.

It has been reported that IFN-γ alone was not sufficient to induce expression of the inducible NO• synthase (iNOS) gene in RAW264.7 cells [37]. Consistent with findings by other investigators [29], we found that IFN-γ alone was able to induce NO• production. We believe this was not due to contamination by LPS, because experiments involving stimulation with IFN-γ alone were always performed with the presence of Polymyxin B, a LPS inhibitor, in the medium. In fact, the concentration of Polymyxin B used in this study has been found to be effective in blocking the activity of 100 ng/ml LPS as measured by NO• production in RAW264.7 cells, even in the presence of IFN-γ (data not shown). However, IFN-γ can synergize with a wide variety of agents in the stimulation of NO• by macrophages. These agents include exotoxins [38], relatively small molecules such as taxol [39], and several other cytokines [18, 40, 41]. Whether any of these or other unidentified agents were present in reagents used in this study has not been formally tested. Nevertheless, the ability of IFN-γ to stimulate NO• production over many cell generations in this system renders it a useful tool for the study of physiological and pathophysiological consequences associated with long-term production of NO•.

It is noteworthy that a number of non-macrophage cell types also express the iNOS gene, notably hepatocytes [42, 43] and lung epithelium cells [44]. The fact that substantial genotoxicity occurred in NO•-producing cells suggests a novel mechanism for cells like hepatocytes and lung epithelial cells to undergo transformation. Indeed, endogenous production of NO• has been implicated as a tumor promotion agent in neoplastic transformation of mouse fibroblasts [45]. It will be of great importance to investigate whether therapeutic agents such as taxol can also induce NO• production in these non-macrophage cells. Information from that study could lead to a more comprehensive assessment of potential toxicity associated with the use of these pharmacological agents.

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Figure legends

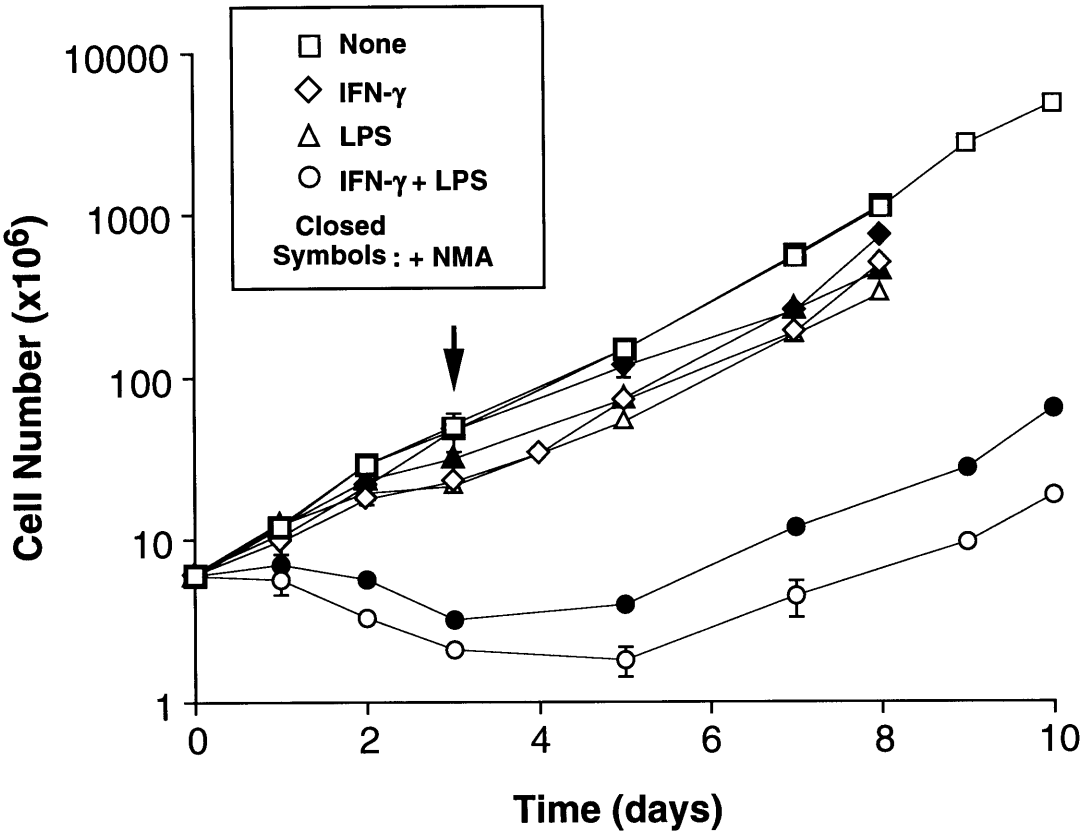
Fig. 1. Growth of RAW264.7 cells during and after three days of stimulation under different conditions. Cells were incubated with no stimulus; 20 ng/ml LPS; 20 U/ml IFN-γ plus 10 U/ml Polymyxin B; or both LPS and IFN-γ, in the absence or presence of 2 mM NMA. At the end of three days of stimulation (arrow), cells were incubated in fresh medium containing NMA until they reached exponential growth. Results are mean ± s.d. of triplicate experiments.

Fig. 2. Comparison of NO• production by RAW264.7 cells during different rounds of stimulation. Cells were stimulated with 20 U/ml IFN-γ plus 20 ng/ml LPS, as described in Fig. 1. The medium was replaced daily and NO• production was quantitated as total nitrite/nitrate content in the medium at the time points indicated. Total stimulation time was three days for each round. Each point represents the mean ± s.d. of triplicate experiments (no s.d. exceeded 0.1 μmoles/sample).

Fig. 3. Prolonged, continuous production of NO• in RAW264.7 cells stimulated with LPS or IFN-γ. Cells were incubated with 100 ng/ml LPS or 20 U/ml IFN-γ plus 10 U/ml Polymyxin B, in the absence or presence of 2 mM NMA. Total cell numbers and total nitrite/nitrate content in the medium were measured at time points indicated. Each point represents the mean ± s.d. of triplicate experiments.

Fig. 4. Time course of mutant fraction in the *hprt* gene of RAW264.7 cells with or without 4-NQO treatment. Hygromycin B-resistant RAW264.7 cells were treated with or without 140 ng/ml 4-NQO at 37°C for one hour. Mutant fraction was measured at the time points indicated. Each point represents the mean ± s.d. of duplicate experiments.

Fig. 5. Mutant fraction in the *hprt* gene of RAW264.7 cells stimulated with IFN-γ for long-term production of NO•. Hygromycin B-resistant cells were stimulated with 20 U/ml IFN-γ plus 10 U/ml Polymyxin B for the indicated periods of time. In the presence of 2 mM NMA, NO• production decreased to baseline level (data not shown). Results are mean ± s.d. of duplicate experiments.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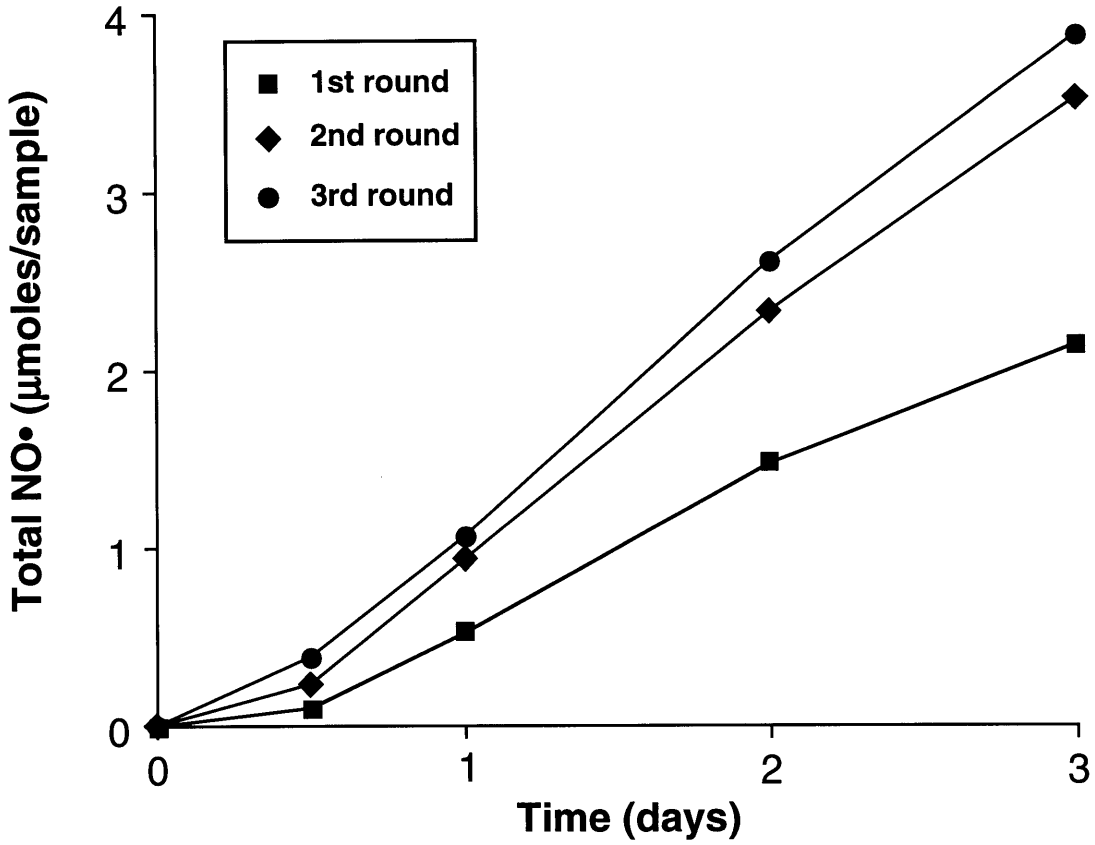
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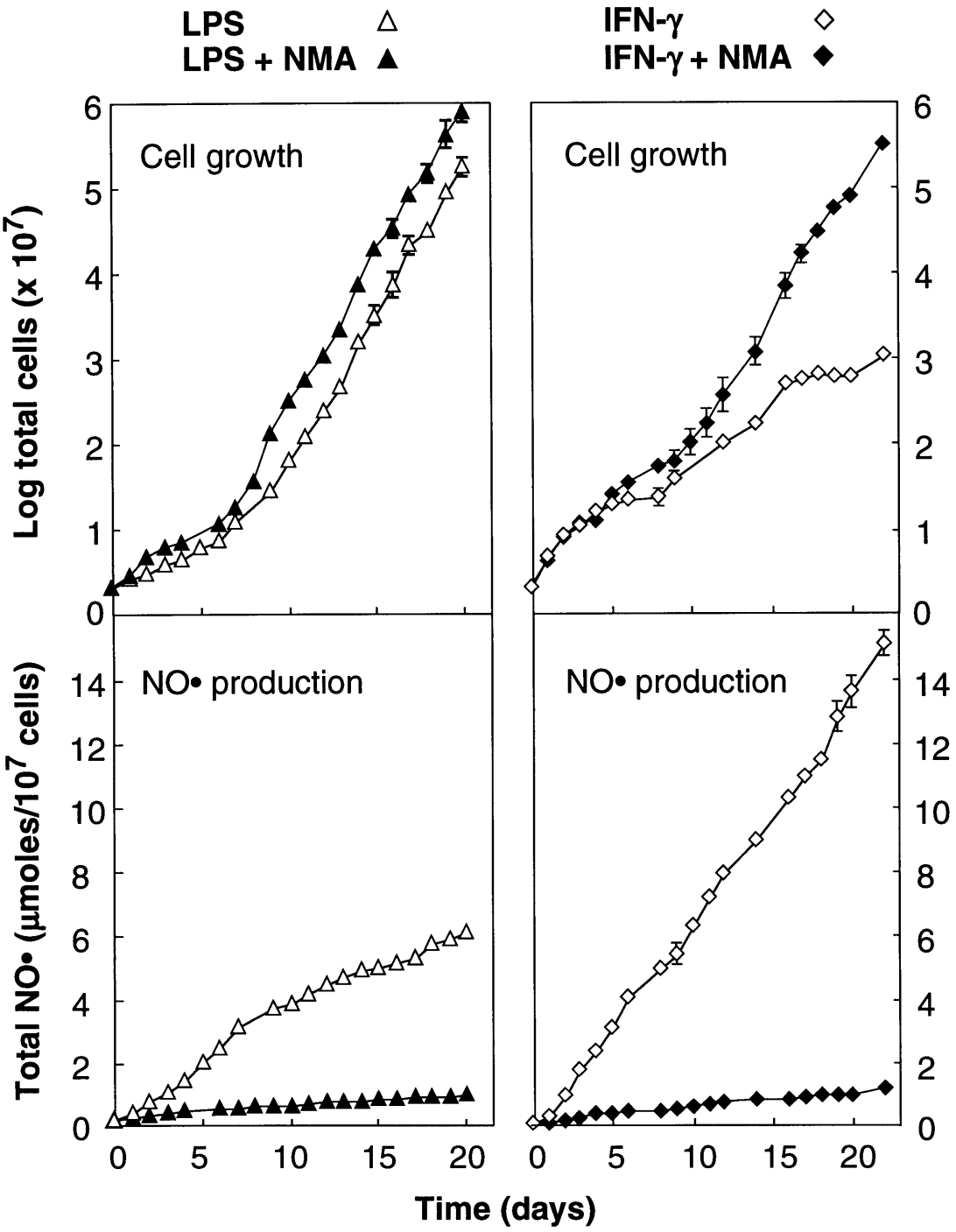
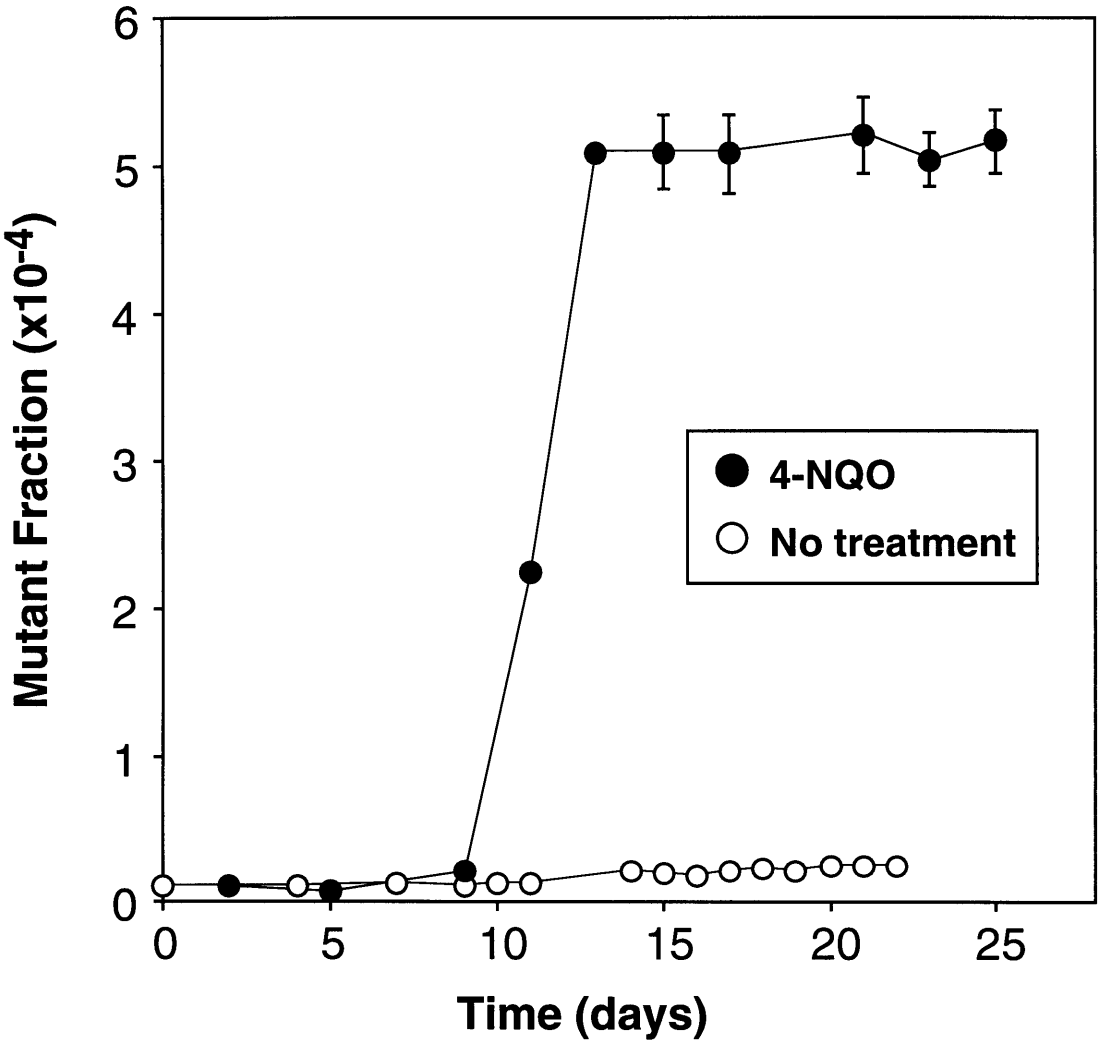
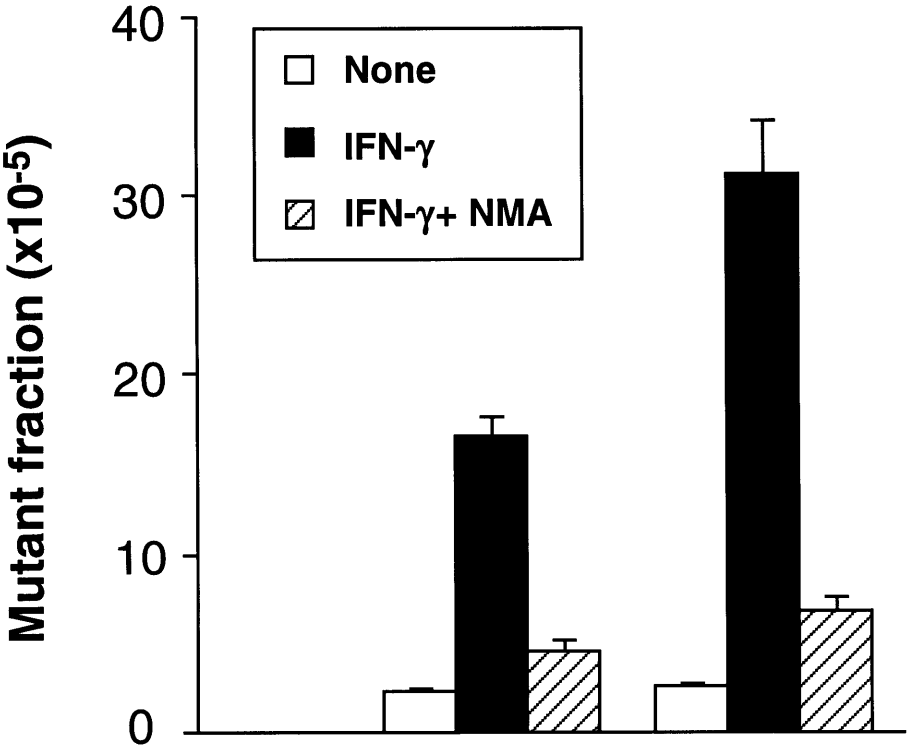
3
2
1
0
0
1
2
3
Time (days)
1st round
2nd round
3rd round


LPS
LPS + NMA
IFN-γ
IFN-γ + NMA
Cell growth
Cell growth
NO• production
NO• production
Log total cells (x 10^7)
Total NO• (μmoles/10^7 cells)
Time (days)
0
1
2
3
4
5
6
0
2
4
6
8
10
12
14
0
5
10
15
20


Mutant Fraction (x10-4)
6
5
4
3
2
1
0
0
5
10
15
20
25
Time (days)
4-NQO
No treatment



Total Stimulation Time (days)	14	23
Total NO• (μmoles/10^7 cells)	9.8	14.0

Chapter 3. Similar mutational patterns in nitric oxide associated and spontaneously arising *hprt* mutants in macrophages

Abstract

We have recently reported development of the mouse macrophage-like RAW264.7 cell line as a model cell system for studying the genotoxicity of nitric oxide (NO•) under physiologically relevant conditions. We further demonstrated that there was a 12-fold increase in mutant fraction in the endogenous *hprt* gene of cells activated by interferon-g (IFN-γ) for 23 days of NO• production. Here we report the use of reverse transcription-PCR and DNA sequencing techniques to characterize the nature of mutations in the coding region of the *hprt* gene in these mutant cells. The results revealed that the mutational pattern was not significantly different from the spontaneous mutational pattern of this cell line, with the exception that limited numbers of small deletions/insertions and multiple exon deletions were observed only in NO• producing cells. We also characterized a collection of independent *hprt* mutants following co-activation with IFN-γ and lipopolysaccharide (LPS) for NO• production over a period of 4 days, and found this mutational pattern to be remarkably similar to the one described above. Furthermore, addition of N-methyl arginine, an NO• synthase inhibitor, to the culture medium during the 4 days of co-activation reduced the increase in mutant fraction, but did not significantly affect the mutational pattern. These results are in contrast to NO• mutagenesis in other systems, and thus suggest that NO• induces gene mutations in macrophages through mechanisms yet to be identified.

Introduction

Epidemiological studies have estimated that one-third of the world's total cancer cases can be attributed to chronic microbial infections [1]. Mechanisms through which persistent infections/inflammation increase cancer risks are largely unknown. However, it has been established that phagocytic macrophages and neutrophils infiltrate the inflamed areas, where they produce large quantities of reactive oxygen species (ROS) and NO•. ROS from neutrophils and macrophages have been shown to induce gene mutations and cell transformation (reviewed in [2]). Studies in several *in vitro* experimental systems have demonstrated that NO• is capable of inducing cellular damage and mutations (reviewed in [3-5].

We recently reported development of the mouse macrophage-like RAW264.7 cells as a model cell culture system for the study of NO• genotoxicity under physiologically relevant conditions [6]. These cells can be stimulated by IFN-γ alone to produce NO• over many cell generations. Under these conditions, as high as a twelve-fold increase in mutant fraction occurred in the endogenous *hprt* gene of NO•-producing cells compared to controls. Recent work from this lab also showed a co-localization of increased genotoxicity with NO• production in transgenic SJL mice [7].

Although NO• has been shown to induce point mutations *in vitro*, little is known about the nature of mutations NO• induces in an endogenous gene in mammalian cells. The collection of *hprt* mutants isolated from our previous study [6] provides an opportunity to answer this question. As the first step, we examined the nature of mutations in these *hprt* mutants by focusing on the coding region of the gene using RT-PCR and DNA sequencing techniques. Results demonstrated that NO•-associated and spontaneous mutational patterns were not significantly different and suggest the involvement of unidentified mechanisms by which NO• mediates mutagenesis in these cells.

Materials and Methods

Isolation of independent spontaneous *hprt* mutants. The hygromycin-resistant mouse macrophage RAW264.7 cells were cultured as previously described [6]. To isolate a collection of independent spontaneous *hprt* mutants, approximately 200 cells were plated in each well of five 24-well plates and allowed to expand. At confluence (~$3x10^6$ cells), cells from each well were transferred to another well in 6-well plate. The cells were grown with appropriate dilution for an additional 20 divisions. A total of 1-3 $x10^6$ cells from each well were then plated in one 96-well plate with normal growth medium supplemented with 2 μg/ml 6-thioguanine (Sigma) for selection of *hprt* mutants. Two weeks later, one clone was picked from each 96-well plate and the cells were expanded in one well of a 24-well plates for total RNA isolation.

Isolation of individual *hprt* mutants from macrophages activated for long-term production of NO•. RAW264.7 cells which had been activated by interferon-γ (IFN-γ) for 23 days [6] were grown with appropriate dilution for 18 cell divisions for phenotypic expression. Cells were then plated in 96-well plates at a density of 7 $x10^7$ cells/well in the presence of 2 μg/ml 6-thioguanine. Two weeks later, individual clones of different sizes from the 96-well plates were picked and expanded in 24-well plates for total RNA isolation.

Isolation of independent *hprt* mutants from macrophages co-activated with LPS and IFN-γ for four days in the absence or presence of NMA. One clone of RAW264.7 cells which gave rise to no 6-thioguanine resistant clones, as assayed in the isolation of spontaneous *hprt* mutants experiment, was used to assure a low background mutant fraction. For isolation of independent *hprt* mutants, a total of 1.2 x 10^6 cells were plated in each well of twenty 6-well plates. Cells were stimulated in 2 ml of growth medium containing 20 ng/ml E. coli lipopolysaccharide (LPS) (Sigma) and 20 units/ml IFN-γ (Genzyme, Cambridge, MA). N-methyl-L-arginine (NMA) (Chem-Biochem Research, Inc., Salt Lake City, UT), an NO• synthase inhibitor, was added to half of the 120 wells to a final concentration of 1 mM. All cells were stimulated for 4 days with daily change of medium containing the corresponding reagents, except at the end of the first day of stimulation. Prior to each medium replacement, aliquots of medium from 4 stimulation wells and 4 stimulation plus NMA wells were saved for nitrite/nitrate analysis [8]. At the end of 4-day stimulation, cell numbers in 4 stimulation wells and 4 stimulation plus NMA wells were counted. The medium in all wells was replaced with regular growth medium supplemented with 1 mM NMA, and the cells were allowed to recover and resume normal growth. Each individual cell population was then transferred to a 10-cm plate and allowed to divide for an

additional 18 times. Approximately 10^6 cells from each population were then plated in one 96-well plate in the presence of 2 μg/ml 6-thioguanine for selection of *hprt* mutants. Two weeks later, one clone was picked from each 96-well plate and expanded in 24-well plates for total RNA isolation. The 6-thioguanine resistant colonies in the 96-well plates of each condition were pooled for calculation of an average mutant fraction.

Total RNA isolation. Total RNA from each clone was isolated by guanidium thiocyanate method [9] using a kit from Promega (Madison, WI). After cells reached confluence in the 24-well plates, the medium was removed and the cells were resuspended in 1 ml ice-cold phosphate-buffered saline (PBS) solution. The cells were pelleted and after brief vortexing were lysed with 300 ml prechilled Denaturing Solution. The cell lysate was then acidified by addition of 40 ml 2 M sodium acetate (pH 4.0), and 0.4 ml prechilled phenol:chloroform:isoamyl alcohol solution was added. After vortexing vigorously for 10-15 sec, the mixture was chilled on ice for 15 min and centrifuged at 12,000x g (4°C) for 20 min. The aqueous phase was removed and total RNA in the solution was precipitated by addition of 0.5 ml isopropanol and storage at -20°C overnight. After centrifugation at 4°C, the RNA pellet was washed with 1 ml cold 70% ethanol prepared with RNase-free water. The washed pellet was then air dried and dissolved in 42 ml RNase-free water with shaking for at least two hours at 37°C. RNA concentration and purity were determined using a UV spectrophotometer. The RNA solution was then adjusted to a working concentration of 500 ng/ml and stored at -20 °C.

Reverse transcription-PCR. A GeneAmp RNA PCR kit from Perkin Elmer (Foster City, CA) was used by following manufacturer's recommendations. Briefly, first strand cDNA was synthesized using 1 mg of total RNA in a volume of 20 μl containing 5 mM $MgCl_2$, 1x PCR Buffer II (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1 mM each of dGTP, dATP, dCTP, and dTTP, 2.5 mM oligo $d(T)_{16}$ primer, 20 units of RNase Inhibitor, and 50 units of M-MLV Reverse Transcriptase. The mixture was incubated at 42°C for 15 min and the reaction stopped by heating at 99°C for 5 min. Half of the cDNA solution was mixed with 40 μl PCR solution containing 2 mM $MgCl_2$, 1x PCR Buffer II, 400 nM each of primers UP1 and DN2 (see below), and 2 units of AmpliTaq DNA Polymerase. The 50 μl mixture was overlayed with 50 μl mineral oil, and PCR was carried out using the following conditions: 95°C for 5 min, 35 cycles of 1 min each at 95°C, 50°C, and 72°C, and 72°C for 5 min.

DNA sequencing of PCR products. An aliquot of each PCR sample was run on a 5% polyacrylamide or 2% agarose gel and stained with ethidium bromide. If a PCR product was detected, 5 μl of the corresponding PCR solution was treated with 10 units of exonuclease I and 2 units of shrimp alkaline phosphatase (Amersham, Arlington Heights, IL) at 37°C for 15 min to

digest single strand DNA and deoxynucleotides, respectively. The solution was then heated at 80°C for 15 min to deactivate the enzymes. The treated PCR product was mixed with 5 pmoles of sequencing primer UP5 or DN4 and submitted for automated cycle sequencing (Whitehead Institute Sequencing Facility, Cambridge, MA). Mutations were identified with the aid of Sequencher 3.0 DNA sequence analysis software (Gene Codes, Ann Arbor, MI).

The primers used in this study were designed using the published mouse *hprt* cDNA sequence [10] and are shown below, with the adenine residue of the methionine initiation codon numbered as 1:

UP1: 5' $_{-87}$ TTACCTCACTGCTTTCCGGA $_{-68}$ 3'

DN2: 5' $_{711}$ TACTGGCAACATCAACAGGA $_{692}$ 3'

UP5: 5' $_{-47}$ GGCTTCCTCCTCAGACCGCT $_{-28}$ 3'

DN4: 5' $_{691}$ CTCCTCGTATTTGCAGATTC $_{672}$ 3'

β-Actin expression. For those samples that failed to show a PCR product using the *hprt* primers UP1 and DN2, the other half of the cDNA solution was used for PCR with a pair of mouse β-actin primers (Strategene, La Jolla, CA) under the same reaction conditions to ensure the quality of RNA. Samples which showed no β-actin PCR product were excluded from this study.

CDCE analysis. Figure 1 shows the procedure used in the analysis of *hprt* mutant populations by CDCE technology [38, 39].

Results

Spontaneous *hprt* mutational pattern of RAW264.7 cells. We first characterized the spontaneous mutational spectrum in this cell line. Eighty four out of the 120 independent cultures gave rise to at least one 6-thioguanine-resistant clone. One clone from each culture was picked and expanded for total RNA isolation. Each individual total RNA sample was reverse transcribed into first strand cDNA and *hprt* was then amplified by PCR using two primers flanking the coding region of the gene. PCR reactions with a product were then identified by gel electrophoresis and treated with enzymes to remove the PCR primers and deoxynucleotides prior to DNA sequencing. As summarized in Table 1, 32% (27/84) of the mutants gave rise to a RT-PCR product. DNA sequencing on these products revealed that the majority of them (19/27) were base substitutions, with the remaining (8/27) being single exon deletions. The majority of base substitutions were transversions, although all six types of base substitutions were observed (Tables 2 and 3). Eight single exon deletions occured in 5 of the 9 exons in the gene. No small deletions/insertions or multiple exon deletions were observed in these 84 mutants.

***Hprt* mutational pattern in RAW264.7 cells activated for long-term, low-dose production of NO•.** To study the nature of mutations associated with NO• production, individual *hprt* mutants were isolated from two independent cultures of RAW264.7 cells which have a 12-fold increase in mutant fraction at the *hprt* locus over untreated controls (31.3 vs. 2.5 x 10^{-5}) as a result of NO• production in the cells [6]. Characterization of these mutants (23-day with NO•) by RT-PCR and sequencing revealed that 29 out of 70 (41%) and 24 out of 58 (41%) individual *hprt* mutants from these 2 cultures gave rise to a RT-PCR product. The comparable percentage of samples with a RT-PCR product between the duplicate cultures suggests that there was no apparent growth advantage or disadvantage of certain subsets of clones. The absence of significant clonal seletion in these *hprt* mutant populations was verified by the sequencing data (Table 4), which showed that all but one mutation event occured at no more than two individual clones from the same population of cells. Based on these observations, the mutation data from the two independent cultures were pooled for subsequent analysis.

As shown in Table 1, the distribution of *hprt* mutations in this collection of mutants is similar to that of spontaneous mutations by virtue of the fact that base substitutions (23/53) and single exon deletions (17/53) are the major types of change. As in the case of spontaneous mutations, the majority of base substitutions are transversions (Tables 2 and 4). Of note, single exon deletions in these mutants occurred at the same five exons as in the spontaneous mutants.

The high incidence of exon 4 or exon 5 deletions in these NO•-associated mutants was probably not due to clonal selection of these two types of mutants, because both were observed in the two independent cultures (data not shown).

Small differences between the NO•-associated and spontaneous mutations were also observed. Specifically, limited numbers of small deletions, insertions, and multiple exon deletions were observed in NO•-associated mutants. Among the seven deletions, three were a 1-bp deletion. Two identical deletions, the removal of the first 17 bp of exon 9, were also observed and were independent events because they came from different cultures. Table 4 summarizes the positions and the corresponding amino acid changes of these base substitutions, deletions, and insertions. Four individual mutants from one culture had the same A:T to C:G transversion at position 84, and therefore may be siblings. Three of them were excluded from the analysis in Table 2. Additionally, double mutations were also observed in two of these mutants.

***Hprt* mutations in RAW264.7 cells activated for short-term, high-dose production of NO•.** To further examine the nature of mutations associated with NO• production, we activated a multiplicity of independent RAW264.7 cells with both LPS and IFN-γ, so that the cells would produce NO• at a higher rate within a relatively shorter period of time. As summarized in Table 5, cells activated in this manner produced an average of 300 nmoles NO•/10^6 cells over the 4 days of stimulation. At the end of the stimulation, 44% of the starting cells survived, and these cells had an average mutant fraction of 14.0×10^{-5} in the *hprt* gene, as compared to 2.8×10^{-6} in untreated controls.

Comparison of the mutations in these independent *hprt* mutants with those from the long-term, low-dose experiment showed a striking similarity with respect to the types and proportions of mutations (Tables 1 and 2). Furthermore, one mutation, the deletion of a C at 503 or 504, was also observed in the long-term, low-dose experiment (Tables 4 and 6). These results indicate that the mutational pattern associated with NO• production in this system is dose rate independent.

***Hprt* mutations in RAW264.7 cells activated by LPS and IFN-γ in the presence of NMA.** We also isolated and characterized independent *hprt* mutants from cells activated with LPS and IFN-γ for 4 days, but in the presence of NMA, an NO• synthase inhibitor. Addition of NMA blocked 85% of NO• production in the cells. This was accompanied by an increase of cell survival and an decrease of mutant fraction (Table 5). As shown in Table 1, the percentage of samples with a RT-PCR product is comparable to that of spontaneous mutants, but somewhat lower than that of NO•-associated mutants. Again, base substitutions and single exon

deletions represent the two major types of mutations (11 out of 14), with the remaining being multiple exon deletions. No small deletions or insertions were observed.

CDCE analysis of *hprt* mutant populations. Analysis of *hprt* mutant populations collected *en masse* using Constant Denaturant Capillary Electrophoresis (CDCE) showed four to six putative "hot-spot" mutations in two untreated and two NO•-associated samples (Figure 2). Each of these mutations comprised from 0.1 to 1% of total mutants. On the basis of the elution time, two of these "hotspot" mutations seemed to be present in all four samples, indicating that the similarity between spontaneous and NO•-associated mutations may extend to this level.

Discussion

In this study, we characterized the nature of mutations in the coding region of the *hprt* gene in macrophage RAW264.7 cells activated to produce NO•. For comparison, we first characterized spontaneous *hprt* mutations in this cell line. As summarized in Table 1, spontaneous *hprt* mutations of RAW264.7 cells are comprised of only three types: no RT-PCR product (68%), base substitution (22%), and single exon deletion (10%). The genetic cause for mutants with no RT-PCR is presently not clear, but in other cell systems, this type of change has been found to result from whole gene deletion or partial deletions affecting either of the PCR primer binding sites [11]. Relatively high numbers of large deletions have also been detected in *hprt* mutants isolated from other cell systems. For example, in human B lymphoblastoid TK6 cells, approximately half of the spontaneous *hprt* mutants had large deletions spanning multiple exons [11, 12, 13]. In T-cell *hprt* mutants isolated from normal young adults, 15% (125/739) had gross structural alterations visible on Southern blots [14]. However, this fraction increased to 85% in mutants isolated from placental cord blood samples of newborns, most of which consisted of deletions of exons 2 and 3 [15]. It is now clear that the characteristic exons 2 and 3 deletions are derived from the age/development-dependent illegitimate action of V(D)J recombinase [16]. It is of great interest to know whether a similar mechanism is also operative in RAW264.7 cells in causing a high number of large deletion mutations, although it is noteworthy that both macrophages and T cells are of hematological origin.

For spontaneous mutants with a RT-PCR product, the mutational pattern in this cell line is closely comparable to that in the mouse T-lymphoma GRSL13 line in two respects [17]. First, the proportion of samples with whole exon deletions is similar (36 and 30% in GRSL13 and RAW264.7, respectively). Most (7/9 in GRSL13) or all (8/8 in RAW264.7) of these whole exon deletions are a single exon deletion. Single exon deletions have been found to result frequently from point mutations affecting the splicing site(s) of the corresponding exon (reviewed in [18]). Second, base substitutions account for most (14/16 in GRSL13) or all (19/19 in RAW264.7) of the remaining mutations. As far as base substitution mutations are concerned, a comparison with several other cell systems (summarized in [19]) reveals that the distribution in RAW264.7 cells is similar to that in hamster V79 cells. A comparison between two mouse cell lines (GRSL13 and RAW264.7) is not possible due to low numbers of base substitution events in both cases (see Table 2 and [17]). Collectively, the spontaneous mutational pattern in the macrophage-like RAW264.7 line seems to reflect both its tissue and species origins. The absence of mutants with

small deletions/insertions or multiple exon deletion in RAW264.7 cells renders its spontaneous mutational pattern the "simplest" one among all cell lines characterized thus far (reviewed in [19]).

As summarized in Tables 1 and 2, the NO•-associated mutational patterns are very similar to the spontaneous one. Remarkably, the mutational patterns in cells activated for long-term, low dose (23 days), and relatively short-term, high dose (4 days) of NO• production are also very similar to each other. This suggests that the NO•-associated mutational pattern is dose rate independent in this system, and for practical purposes, a short-term activation of cells for NO• production with both IFN-γ and LPS is effective for the study of NO• mutagenesis in this system.

Two small differences between the NO•-associated and spontaneous mutational patterns are noteworthy. One is the presence of six 1-bp frameshift mutations in NO•-associated mutants (Tables 4 and 6). Small deletions of 3 bases or less have been to be mutations characteristic of H_2O_2 exposure [20], although another study failed to detect a mutation hotspot of this type using another experimental system [21]. The formation of the 1-bp deletion in this system is unlikely to be mediated by reactive oxygen species, because this type of mutation was not detected in cells activated in the presence of NMA, which blocks NO• synthesis but presumably not superoxide production [22, 23] (see Tables 1 and 2). Thus, the mechanistic link between NO• production and the formation of 1-bp frameshift mutations is presently not understood. However, it is noted that 5 out of the 6 frameshifts occurred at positions with two or more identical nucleotides (Tables 2 and 4), where small insertion-deletion mismatches can form as a result of strand slippage during DNA replication [19] and references therein). These small loops are repaired by the mismatched repair (MMR) machinery in the cell [24], and small numbers of frameshifts are commonly parts of spontaneous mutational spectra [19]. On the basis of our findings, it is conceivable that RAW264.7 cells are highly proficient in MMR, which can be compromised in the presence of NO• production. In support of this proposition, NO• has been shown to inhibit enzymes involved in DNA synthesis or repair (reviewed in [3]).

In addition to 1-bp frameshifts, a small number of insertions/deletions are only seen in NO• producing cells. The mutation with deletion of the 5' 17-bp of exon 9 (I1-74 and I2-35 in Table 4) has also been observed in human cells several times. In a few cases, the cause of the mutation has been identified to be single base substitution or small deletion in intron 8, leading to a splicing error [18, 25]. Finally, small numbers of mutants with multiple exon deletions are present only in activated macrophages regardless of NO• production (Table 2). This type of mutation is often the consequence of genomic deletion occurring at the corresponding exons [25].

Collectively, there is no apparent difference in mutational pattern among untreated cells, cells activated for NO• production, and activated cells without NO• production. However, if the two NO•-associated sets of data are combined and compared to one consisting of data from untreated cells and cells activated in the presence of NMA, there is a significant increase of samples with a RT-PCR product ($P<0.05$, Chi square test). Likewise, the comparison reveals that the increase in NO•-associated small deletions/insertions is statistically significant ($P<0.01$). It is worth noting that the presence of low percentages of small deletions/inserions and multiple exon deletions makes the NO•-associated mutational pattern more comparable to spontaneous mutational spectra in other cell systems (Table V in [19]). Furthermore, when the 4 sets of base substitution data are pooled together and analyzed, the distribution resembles closely to what is expected of a random distribution in the mammalian *hprt* gene [19].

The findings from this study are in considerable contrast to what has been reported about the mutagenesis of NO• using in vitro experimental systems, in which NO• in the form of a gas, donor drug, or its metabolite peroxynitrite, has been shown to induce predominantly base substitutions of various types (reviewed in [5]). A number of reasons can account for this discrepancy. First, the supF target gene used in other stduies is small in size (<100 bp), thereby preventing the detection of large deletions. This is in contrast to the endogenous *hprt* gene, which is >33 kb long [10]. Secondly, the doses and dosing rates of NO• used in other studies are several magnititudes higher than what was used in this study. Additionally, different forms of NO• have been found to induce distinct base substitutions under otherwise identical conditions [26, 27]. We have also found that dose rate alone can affect the cytotoxic and mutagenic responses in human TK6 cells (unpublished results). Finally, differences in the way target genes are treated has been found to affect the mutational spectrum induced by reactive oxygen species [28, 29].

It is presently not clear how NO• induces mutations in our system. The fact that the mutagenesis of NO• is highly sensitive to differences in experimental systems suggests a complex mechanism of action of this free radical. The ability of NO• to induce DNA damage has been demonstrated in a number of studies using different experimental systems (reviewed in [3, 4]). Related to these studies is the finding that increased deamination and oxidation in the DNA of RAW264.7 cells were associated with NO• production [22]. The absence of any NO•-specific base substitution in this study suggests that these DNA lesions may be efficiently repaired by RAW264.7 cells.

Recently, it was reported that when the supF reporter gene was transfected into human cells and exposed to neutrophils stimulated to produce superoxide, there was up to a 10-fold increase in mutant fraction, but the mutational spectrum was indistinguishable from that of spontaneous [29].

The finding that NO• production also increased the mutant fraction without significantly changing the mutational spectrum in macrophages in this study raises the possibility that the underlying mechanisms in these two systems may be similar. In neutrophils, the conversion of superoxide to more reactive species such as hydroxyl radical is largely mediated by high concentrations of myeloperoxidase stored in the azurophil granules of the cells [30]. Much of the granule peroxidase is lost in macrophages, but the cells acquire multiple synthetic and secretory activities [31], including the capacity to produce NO•. In principle, NO• in macrophages can serve a function similar to peroxidase in neutrophils by virtue of its ability to facilitate the transformation of superoxide/hydrogen peroxide into more reactive oxygen species. NO• can react directly with superoxide to form peroxynitrite, leading to the formation of hydroxyl radical [32]. NO• can also react with hydrogen peroxide to form singlet oxygen [33]. Furthermore, NO• can cause the release of iron from mitochondrial enzymes [34], thereby augmenting Fenton chemistry in macrophages [35]. These highly reactive oxygen species are likely to induce oxidative stress by causing damage to components of outer membrane and the cytoplasm in target cells. Validation of this facilitator model awaits the examination of NO• mutagenesis in the absence of superoxide production in macrophages. Alternatively, NO• may directly induce oxidative stress response in macrophages through mechanisms analogous to those employed by bacteria [36, 37].

This study also demonstrates the feasibility of isolating individual clones from pools of *hprt* mutants without an adverse sibling problem. Successful factors include induction of large numbers of induced mutants (in this study, more than 40,000 mutants were induced during the 23 days of NO• production), use of duplicate cultures, and picking of clones of various sizes. In addition, the methodology we have developed for this study makes it possible to examine a large number of *hprt* mutants with reasonable amounts of time and effort. The accuracy of the whole experimental approach is highlighted by the fact that double mutations were only observed in two mutants from the 23-day NO• production samples, where there is an increased chance a single cell is mutated twice.

We have provided evidence suggesting that NO• mutagenesis in macrophages may be largely mediated by mechanisms other than DNA damage. An understanding of these undefined mechanisms will not only provide insights about the interations of NO• with other cellular components, but also help in deciphering the cause of spontaneous mutations in mammalian cells.

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Figure legends

Figure 1. Detection of mutation hotspots in *hprt* mutant populations using CDCE technology.

Figure 2. CDCE analysis of *hprt* mutant populations from untreated and NO•-producing macrophages.

1.5 x 10^8 untreated macrophages or cells stimulated to produce NO• for 23 days

↓ add 6-thioguanine

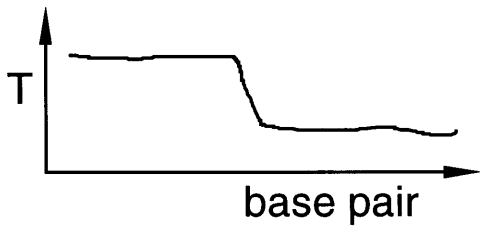
***hprt* mutants**

↓ DNA isolation

genomic DNA

↓ hi-fi PCR with primers flanking exon 3

exon 3, 184 bp



↓ 2nd PCR with a fluorescein primer

CDCE analysis

CDCE Analysis of Mutations in HPRT (Exon 3)

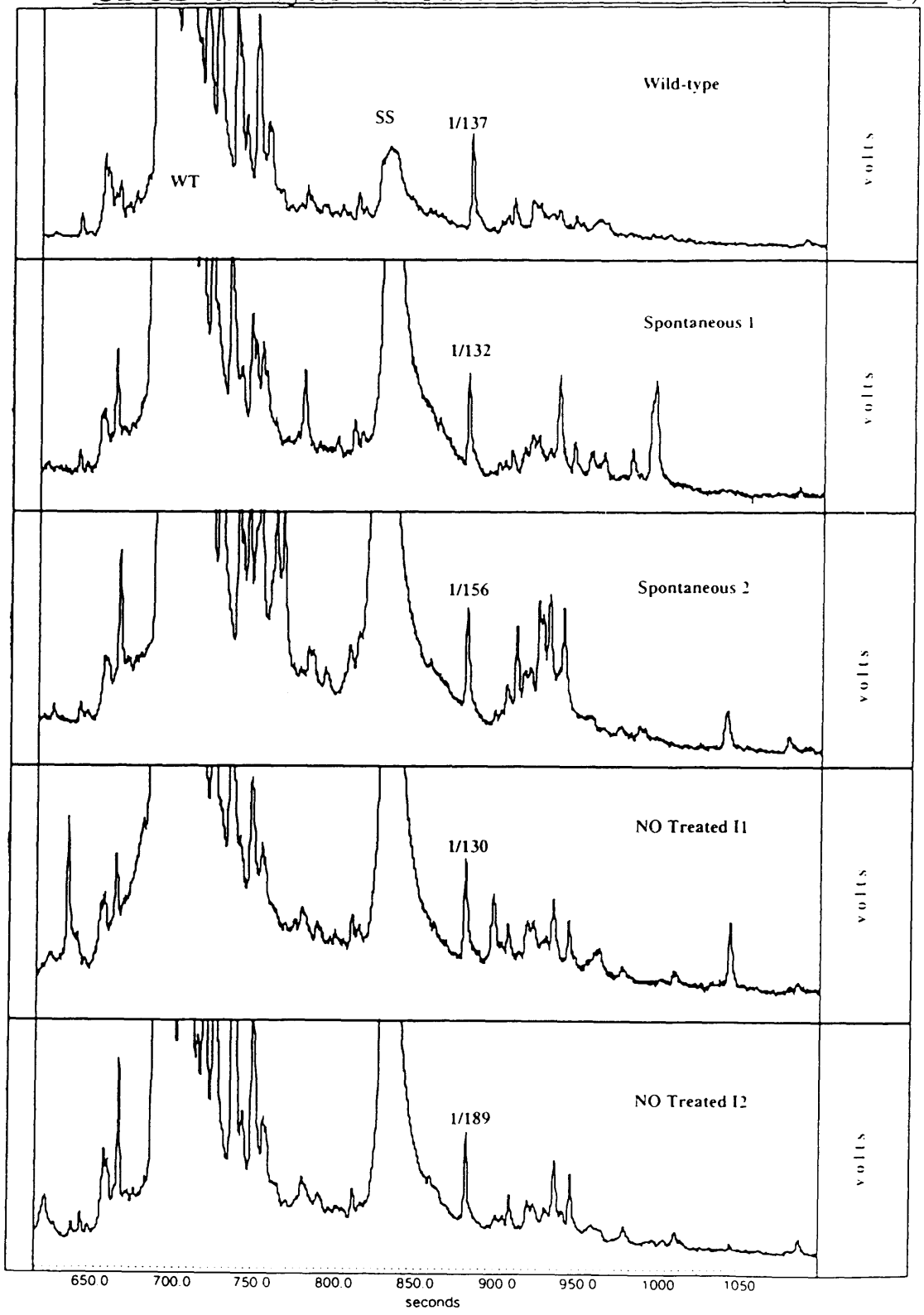


Table 1. Summary of RT-PCR results and classification of mutations in the coding region of the *hprt* gene in RAW264.7 cells

Classification	Spontaneous	w/NO• (23-day)	w/NO• (4-day)	w/ NMA (4-day)
Total mutants	**84**	**128**	**58**	**54**
w/o RT-PCR product	**57 (68%)**	**75 (59%)**	**33 (57%)**	**40 (74%)**
w/ RT-PCR product	**27 (32%)**	**53 (41%)**	**25 (43%)**	**14 (26%)**
Base substitution	19 (22%)	23 (18%)	9 (15%)	6 (11%)
Small deletion	0 (0%)	7 (6%)	3 (5%)	0 (0%)
Insertion or truncation	0 (0%)	3 (2%)	1 (2%)	0 (0%)
Single exon deletion	8 (10%)	17 (13%)	7 (12%)	5 (9%)
Multiple exon deletion	0 (0%)	3 (2%)	5 (9%)	3 (6%)

Table 2. Types of mutation in the coding region of the *hprt* gene in RAW264.7 cells

Type of mutation	Spontaneous	w/NO• (23-day)	w/NO• (4-day)	w/ NMA (4-day)
Base Substitution	**19**	**19**	**9**	**6**
Transversion				
A:T to C:G	5	6	3	3
A:T to T:A	1	5	1	1
G:C to T:A	3	4	2	0
G:C to C:G	3	0	3	0
Transition				
G:C to A:T	3	1	0	1
A:T to G:C	4	3	0	1
Deletion	**0**	**7**	**3**	**0**
1 bp deletion	0	3	2	0
other deletion (<72 bp)	0	4	1	0
Insertion	**0**	**3**	**1**	**0**
small insertion (<8 bp)	0	1	1	0
large insertion (+E4-6)	0	1	0	0
insertion/deletion	0	1	0	0
Single exon deletion	**8**	**17**	**7**	**5**
–Exon 2	2	2	3	2
–Exon 4	2	5	1	1
–Exon 5	2	7	1	1
–Exon 6	1	1	2	0
–Exon 7	1	2	0	0
–Exon 8	0	0	0	1
Multiple exon deletion	**0**	**3**	**5**	**3**
–Exon 2-3	0	0	3	1
–Exon 2-4	0	1	0	0
–Exon 3-4	0	1	0	0
–Exon 4-5	0	0	2	0
–Exon 5-6	0	0	0	1
–Exon 4-6	0	0	0	1
–Exon 2-7	0	1	0	0

Table 3. Base substitutions in spontaneous *hprt* mutants of RAW264.7 cells

Mutant	Position	Type of mutation	Surrounding sequence	Amino acid change
S25	1	A:T to G:C	CGTCATGCC	Met to Val
S43	1	A:T to G:C	CGTCATGCC	Met to Val
S54	1	A:T to T:A	CGTCATGCC	Met to Leu
S62	74	G:C to C:G	ATACCTAAT	Pro to Arg
S60	88	G:C to T:A	TGCCGAGGA	Glu to Stop
S24	104	A:T to C:G	AAAGTGTTT	Val to Gly
S16	119	G:C to T:A	CATGGACTG	Gly to Val
D-2	136	A:T to C:G	CAGGACTGA	Thr to Pro
S34	229	G:C to T:A	TGCTGACCT	Asp to Tyr
S29	260	G:C to C:G	AATAGAAAT	Arg to Thr
S15	370	A:T to C:G	CTCAACTTT	Thr to Pro
S57	407	A:T to C:G	GATATAATT	Ile to Arg
G-1	440	A:T to G:C	TTGCTTTCC	Leu to Pro
S37	464	G:C to C:G	AGCCCCAAA	Pro to Arg
S12	569	G:C to A:T	GTTGGATAT	Gly to Glu
S33	580	G:C to A:T	CCTTGACTA	Asp to Asn
S55	635	G:C to A:T	ACTGGAAAA	Gly to Glu
S47	647	A:T to G:C	AAATACAAA	Tyr to Cys
S72	647	A:T to C:G	AAATACAAA	Tyr to Ser

Table 4. Base sustitutions, deletions, and insertions in *hprt* mutants isolated from cells stimulated for long-term production of NO•

Mutant	Position	Type of mutation	Surrounding sequence	Amino acid change
I2-12	1	A:T to T:A	CGTCATGCC	Met to Leu
I1-2 I1-17	82	A:T to C:G	TCATTATGC	Tyr to Asp
I1-48 I1-49 I1-51 I1-86	84	A:T to C:G	ATTATGCCG	Thr to Stop
I2-73	133	A:T to T:A	GGACAGGAC	Arg to Trp
I1-1	145	G:C to A:T	AAGACTTGC	Leu to Phe
I2-4	160	A:T to T:A	TGTCATGAA	Met to Leu
I1-69	220	A:T to C:G	TAAGTTCTT	Phe to Val
	269	A:T to G:C	AGTGATAGA	Asp to Gly
I1-6 I1-45	380	G:C to T:A	ACTGGAAAG	Gly to Val
I2-50 I2-61	397	G:C to T:A	GATTGTTGA	Val to Phe
I1-65	407	A:T to C:G	GATATAATT	Ile to Arg
I1-15	533	A:T to G:C	GACTTTGTT	Phe to Ser
I2-42	542	A:T to G:C	GGATTTGAA	Phe to Ser
I1-19	547	A:T to T:A	TGAAATTCC	Ile to Phe
I1-23	563	A:T to T:A	TTTGTTGTT	Val to Asp
I1-42	594	G:C to T:A	AGTACTTCA	Tyr to Stop
I1-4 I1-13	614	A:T to C:G	CACGTTTGT	Val to Gly
I1-46	132	–C	GAA(C)AGG	Frameshift
I1-4	503 or 504	–C	GGA(C)(C)TCT	Frameshift
I1-74	526 or 527	–C	AGG(C)(C)AGA	Frameshift
I1-63	643-646	–4 bp		Frameshift
I1-74	610-626	–17 bp		Frameshift, deletion
I2-35	610-626	–17 bp		Frameshift, deletion
I1-71	160-230	–71 bp		Frameshift, deletion
I2-20	207-212	+G	GGGGGG	Frameshift
I1-47	exons 4-6	duplication		
I1-40	exon 2	replaced by a noval 116-bp fragment		

Table 5. Production of NO, cell viability and mutant fraction in the *hprt* gene of RAW264.7 cells stimulated by IFN-γ plus LPS for 4 Days

	without NMA	with NMA
Total NO_2^-/NO_3^- (nmoles/10^6 cells)	300	47
Survival (% of starting cells)	44	62
MF ($x10^{-6}$)*	14.0	7.3

* Background mutant fraction was 2.8×10^{-6}.

Table 6. Base substitutions and small deletion/insertions in *hprt* Mutants associated with 4 days of stimulation by LPS plus IFN-γ

Mutant	Position	Type of mutation	Surrounding sequence	Amino acid change
1. Mutations associated with NO• production				
A59	1	A:T to T:A	CGTCATGCC	Met to Leu
A25	23	A:T to C:G	AGCGTCGTG	Val to Gly
A31	65	A:T to C:G	TTGTTTTGT	Phe to Cys
A4	97	G:C to T:A	TTTGGAAAA	Glu to Val
A61	119	G:C to C:G	CATGGACTG	Gly to Ala
A15	299	A:T to C:G	TTTATCAGA	Ile to Ser
A45	419	G:C to T:A	ACTGGTAAA	Gly to Val
A56	569	G:C to C:G	GTTGGATAT	Gly to Ala
A9	606	G:C to C:G	ATTTGAATC	Leu to Phe
A8	-22 to 2	24 bp deletion		Loss of Met initiation codon
A24	503 or 504	–C	GGA(C)CTC	Frameshift
A52	532, 533, or 534	–T	AGAC(T)TTG	Frameshift
A27	606 and 607	7 bp insertion		Frameshift
2. Mutations associated with the presence of NMA				
AN30	1	A:T to T:A	CGTCATGCC	Met to Leu
AN47	194	A:T to C:G	GCCCTCTGT	Leu to Arg
AN24	473	A:T to C:G	ATGGTTAAG	Val to Gly
AN25	473	A:T to C:G	ATGGTTAAG	Val to Gly
AN50	530	A:T to G:C	CCAGACTTT	Asp to Gly
AN58	539	G:C to A:T	GTTGGATTT	Gly to Glu

Chapter 4 Evidence for macrophage self protection against cellular damage induced by intracellularly generated nitric oxide: validation of the use of macrophages for mutation study

Abstract

Macrophages play a prominent role in host defense by inducing cellular damage in infectious agents and tumors. One mechanism macrophages use to exert their cytotoxic and cytostatic effects on target cells is to release nitric oxide (NO•). NO•-mediated cellular damage also occurs in macrophages themselves. It is presently not known to what extent cellular injury in any given macrophage results from self-produced NO• (intracellular) or from NO• that diffuses from neighboring cells (extracellular). To investigate this, macrophages were stimulated for NO• production at different cell densities. When the mouse macrophage RAW264.7 cells were stimulated with interferon-γ and lipopolysaccharide at a subconfluent density of $2x10^7$ cells per 150-mm plate, NO• production in the cells reduced the number of viable cells to 46% of those in which NO• production was blocked by N-methyl-L-arginine (NMA), an NO• synthase inhibitor. At this density, NO• production also increased the time that was required for the cells to resume exponential growth from two days to seven days, and increased the mutant fraction in the *hprt* gene of these cells to 4.6×10^{-5}, compared to 2.7×10^{-5} in cells stimulated in the presence of NMA. At a lower density of $4x10^6$/plate, there was no significant increase in the number of viable cells, but the cells resumed exponential growth within two days following stimulation in both the presence and absence of NMA. Significant decreases in mutant fraction were observed in cells stimulated in both the presence and absence of NMA, although the value in the absence of NMA was still significantly higher than the one in the presence of NMA. At the two lowest densities of $8x10^5$ and $2x10^5$/plate, the numbers of viable cells were the same regardless of the presence of NMA. Thus, NO•-induced lethality, growth arrest, and gene mutation are cell density dependent in stimulated macrophages, suggesting that macrophages may have protection mechanisms against insult by intracellular NO•. Cellular damage observed in NO•-producing macrophages may have been induced by NO• from neighboring cells.

Introduction

Macrophages play a prominent role in host defense by inducing cellular damage in infectious agents and tumors [1, 2]. One mechanism macrophages use to exert their cytotoxic and cytostatic effects on target cells is by releasing nitric oxide (NO•) (reviewed in [3-5]). NO•-mediated toxicity in target cells includes growth arrest through the formation of an iron-nitrosyl complex in mitochondrial enzymes, thereby inhibiting cellular respiration [6-8], energy depletion through deregulation of ATP metabolism [9], programmed cell death [10-12], and gene mutations [13].

Collateral to toxicity in target cells, NO• causes cellular damage in the producer macrophages as well. Indeed, virtually all types of cell toxicity seen in target cells also occur in macrophages [6, 7, 13-19]. In some instances, NO•-mediated toxicity in macrophages has been interpreted as a self regulatory mechanism [14-17].

We have previously demonstrated that the rate of NO• production, as achieved by stimulation of macrophages with different combinations of IFN-γ and LPS, affected the cell viability, growth and genotoxic response in the producer cells [19, 20]. More recently, we found concurrent cytotoxic and genotoxic responses in macrophages and in co-cultured target cells [13]. It is noteworthy that results showing NO•-mediated toxicity in macrophages have derived from experiments involving subconfluent macrophage cultures. Collectively, these observations raise the possibility that at least some of the NO•-mediated cell toxicity in macrophages in culture could have been caused by NO• from neighboring macrophages.

To examine the relative contribution of intracellular NO• vs. extracellular NO• to the cytotoxicity and genotoxicity in the macrophages in culture, macrophages were stimulated for NO• production under different cell densities. it was reasoned that if the macrophage possesses mechanisms to synthesize and deliver NO• in a way as to minimize self intoxication, NO•-mediated damage in the cell will decrease and eventually be curtailed as the cell density decreases. On the other hand, if the cellular damage is induced by both intracellular and extracellular NO•, cellular damage associated with NO• production will first decrease as cell density decreases and then remain unchanged at a low cell density where an individual macrophage cell is exposed to the highest local concentration of self-generated NO•. Results from this study show cell density dependence of viability, growth, and gene mutation in macrophage stimulated to produce NO•, and thus suggest the existence of self protection mechanisms in macrophages against cellular damage induced by intracellularly generated NO•.

Materials and Methods

Cell culture and reagents. The mouse macrophage-like RAW264.7 cells with an integrated hygromycin B resistance gene were used in this study and cultured as previously described [20]. Other reagents used in the studies included recombinant mouse interferon-γ (IFN-γ) (Genzyme, Cambridge, MA), *E. coli* lipopolysaccharide (LPS) (serotype 0127:B8; Sigma, St. Louis, MO), N-methyl-L-Arginine monoacetate (NMA) (Chem-Biochem Research, Inc., Salt Lake City, UT), and 6-thioguanine (Sigma).

Stimulation of NO• production at different cell densities: effects on viability. A total of 2 $x10^7$, 4 x 10^6, 8 $x10^5$, or 2 x 10^5 cells in 25 ml fresh culture medium were inoculated into 150-mm tissue culture plates. The cells were then stimulated with 20 U/ml IFN-γ and 20 ng/ml LPS, in the absence or presence of 2 mM NMA. Unstimulated cells at each density were used as negative controls. Twenty-four hours after stimulation, cells were resuspended to separate siblings, and 24 hours later, cells were trypsinized, pelleted, and resuspended in fresh medium. Viable cells were then counted after staining with trypan blue. An aliquot of medium from each plate was saved for analysis of nitrite and nitrate content using an automated procedure [21].

Stimulation of NO• production at different cell densities: effects on gene expression. A total of $2x10^7$ cells or $2x10^6$ cells were inoculated into one or ten 150-mm plates, producing a cell density of $2x10^7$ or $2x10^5$ cells/plate, respectively. IFN-γ plus LPS were added to each plate to a final concentration of 20 U/ml IFN-γ and 20 ng/ml LPS in a total of 25 ml fresh culture medium. Untreated cells were cultured in parallel as negative controls. Twelve hours later, cells at each density were removed from the plates using trypsin and were collected by centrifuging at 1000x g for 10 min. Total cellular RNA was isolated by the guanidium thiocyanate method [22] using a kit purchased from Promega (Madison, WI); RNA solutions were adjusted to a concentration of 500 ng/μl.

Reverse transcription-PCR of the RNA samples was carried out using a GeneAmp RNA PCR kit purchased from Perkin Elmer (Foster City, CA) with the following modifications to the recommended protocol. First strand cDNA was synthesized using oligo $d(T)_{16}$ primer; the PCR reaction was carried out in a volume of 50 μl with a profile of 95°C for 5 min, 40 cycles of 1 min each at 95°C, 50°C, and 72°C, and 72°C for 5 min. Primers for macrophage inducible NO• synthase (iNOS) gene were designed according to the published mouse iNOS cDNA sequence [23]

and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). NOS1 (sense): 5' $_{613}$ CTCGGAACTGTAGCACAGCA $_{632}$ 3', NOS3 (antisense): 5' $_{1256}$ CTATGGAGCACAGCCACATT $_{1237}$ 3'. This primer pair gives rise to a 644-bp product. Primers for mouse IL-1β, TNF-α, and β-actin were purchased from Strategene (La Jolla, CA) and used as recommended. PCR products were resolved in a 6% polyacrylamide gel and visualized by ethidium bromide staining.

Stimulation of NO• production at different cell densities: effects on growth and mutation. A total of 4 x 10^7 cells were placed in two or ten 150-mm tissue culture plates, producing a cell density of 2 x 10^7/plate or 4 x 10^6/plate, respectively. These cells were then stimulated with 20 U/ml IFN-γ plus 20 ng/ml LPS, in the absence or presence of 2 mM NMA, as described above. Unstimulated cells were cultured in parallel as negative controls. At the end of 48 hours of stimulation, the medium was replaced with medium containing only 2 mM NMA, and the cells were allowed to recover and resume growth, with cell numbers monitored at various time points. Two weeks after termination of stimulation, the cells were plated in the presence of 2 μg/ml 6-thioguanine to select for *hprt* mutants, as previously described [19].

Results

To examine the relative contribution of intracellular NO• vs. extracellular NO• to the cytotoxicity and genotoxicity in cultured macrophages, we stimulated macrophages for NO• production under different cell densities. We reasoned that if the cell possesses mechanisms to synthesize and deliver NO• in a way as to minimize self intoxication, NO•-induced damage in the cell will decrease and eventually be curtailed as the cell density decreases. On the other hand, if the cellular damage is induced by both intracellular and extracellular NO•, the cellular damage associated with NO• production will first decrease as cell density decreases and then remain unchanged at a low cell density where an individual macrophage cell is exposed to the highest local concentration of self-generated NO•.

Cell density effects on viability in macrophages stimulated to produce NO•. Figure 1 shows the effect of cell density on the viability of stimulated macrophages. At the highest cell density examined, NO• production in the cells reduced the number of viable cells to 46% of those in which NO• production was blocked by NMA. As cell density was decreased, the proportion of viable cells increased, and at the two lowest densities, the number of viable cells were the same regardless of NO• production. Total NO• production, as reflected in nitrite/nitrate content of the medium was 120 and 146 nmoles/10^6 cells, at densities of 2 x10^7 and 4 x 10^6 cells/plate, respectively. Addition of 2 mM NMA blocked NO• production by 90% in cells at both densities (data not shown). NO• production in cells at densities of 8 x10^5 and 2 x 10^5 per plate was less than could be measured accurately by the analytical procedure used.

To rule out the possibility that the lack of cell death at the two lowest densities examined was due to the failure of cells to respond to treatment of IFN-γ plus LPS under these conditions, we investigated expression of genes thought to be induced only in responsive macrophages. As shown in Figure 2, iNOS and IL-1β mRNA were detected in cells stimulated with IFN-γ plus LPS for 12 hours at densities of 2x10^7 and 2x10^5 cells per plate, but not in unstimulated cells. mRNA of TNF-α and β-actin was present in both stimulated and unstimulated cells. Thus, cell density did not affect the response of cells to treatment with IFN-γ plus LPS; in addition, the expression of iNOS strongly suggests that cells stimulated at the two lowest densities may be producing NO• at levels comparable to those at higher densities. Collectively, these results demonstrate that NO•-associated lethality in stimulated macrophages is cell density dependent.

Cell density effect on growth in macrophages stimulated to produce NO•. Cell density had a more pronounced effect on the time required for the stimulated cells to resume normal growth after removal of IFN-γ and LPS from the medium. As shown in Fig. 3, approximately seven days were necessary for cells stimulated at a density of 2 x 10^7 cells/plate to resume exponential growth. When NO• production was inhibited by NMA, cells cultured at the same density resumed exponential growth after only two days following stimulation, suggesting that NO• was cytostatic for the cells at this density. However, when cells were stimulated at a lower density, 4 x 10^6 cells/plate, cells resumed exponential growth within two days in both presence or absence of NMA, indicating that NO• was not cytostatic at this cell density. Thus, the cytostatic effect of NO• was also cell density dependent.

Cell density effect on genotoxicity in macrophages stimulated to produce NO•. Cell density also had a more profound effect on the mutant fraction in the endogenous *hprt* gene of stimulated macrophages. As shown in Fig. 4, cells stimulated at a density of 2 x10^7 cells/plate had significantly higher mutant fraction values ($P<0.05$) than those at the lower density of 4 x 10^6 cells/plate, in both the presence and absence of NMA. At the density of 4 x 10^6 cells/plate, there was still significant difference in mutant fraction values ($P<0.05$) in cells stimulated in the absence or presence of NMA. Collectively, NO•-associated genotoxicity in stimulated macrophages was also cell density dependent.

Discussion

In this study, we examined the effects of cell density on viability, growth, and genotoxic response in macrophages stimulated to produce NO•. At a subconfluent density, 2 x 10^7 cells/plate, substantial cell death, cytostasis, and gene mutations in macrophages could be attributed to NO• production in the cells because much of the cellular damage could be blocked by the addition of an NO• synthase inhibitor. As cell density was decreased to 4 x10^6 cells/plate, NO• production had the same effect on viability, had a partial effect on gene mutations, but no longer had any effect on growth of cells following stimulation. As density was further decreased to 8 x 10^5 cells/plate or lower, NO• production no longer had an effect on viability. Collectively, these results demonstrate that the cellular damage observed in macrophages as a result of NO• production is cell density dependent, and thus suggest that much, if not all, of the cellular damage in any individual macrophage could have been caused by NO• from neighboring macrophage cells, i.e., extracellular NO•.

NO• production had differential effects on viability, growth arrest, and gene mutations when density was decreased from 2 x 10^7 cells/plate to 4 x 10^6 cells/plate, suggesting that these damages are caused by distinct mechanisms. Cell death can be caused by damage incurred at any subcellular locations of target cells, including the outer membrane. Since we observed an absence of growth arrest at the lower cell density, the mechanism responsible growth arrest must be different from the one for cell death. NO• induces growth arrest in tumor cells through the formation of an iron-nitrosyl complex in mitochondrial enzymes, thereby inhibiting cellular respiration [6-8]. It is possible that similar mechanism is operating in macrophages. In contrast, damage incurred even at the outer membrane can lead to cytotoxicity. It is, therefore, conceivable that the dose of NO• required to induce growth arrest in an individual macrophage may be higher. The partial reduction in gene mutation in cells stimulated at 4 x 10^6 cells/plate may be due to the formation of lipid peroxidation products (e.g., arachidonate) which subsequently cause DNA damage [24]. It will be of interest to examine the effect of cell density on levels of lipid oxidation, iron-nitrosyl complexes, and DNA damage in stimulated cells. This information may provide insights regarding the nature of NO•-derived reactive species responsible for the observed cell toxicity.

The observation that NO• production did not result in cell death at the two lowest densities suggests the presence of a self protection mechanism(s) in macrophages against intracellularly generated NO•. NO• may be synthesized in a macrophage and delivered to the extracellular space

in a vesicle form, although efforts trying to identify membrane-bound NO• synthase activity have yielded inconclusive results [25, 26]. Alternatively, macrophage may have high contents of detoxifying agents such as glutathione [27] or bcl-2 [28]. Another possibility is that NO• diffuses outside of the macrophage faster than it can react with intracellular targets, as predicted by a simulation model [29]. One potential extracellular target for NO• is superoxide generated by the same macrophage cell [30, 31]; both can react to form the highly potent damaging agent peroxynitrite [32, 33]. It follows, therefore, that a peroxynitrite-like agent formed by a single cell will be diluted in the extracellular space, thereby sparing the producer cell from damage. As cell density increases, the concentration of damaging agents in the extracellular space also increases and eventually reaches a threshold, leading to cell toxicity.

Results from this study are consistent with observations in an *in vivo* study, which showed that cells undergoing apoptosis were concentrated around cells expressing iNOS [12]. In addition to the formation of peroxynitrite around a macrophage cell, free NO• can react with intracellular superoxide in a target cell. Thus, responses to NO• or its derivatives are likely to be dependent on cell type. The apparent resistance of macrophages to NO• toxicity compared to other cell type ensures that the effector cells can kill their targets without killing themselves.

The lack of NO•-mediated toxicity in an individual producer cell also has several divserse implications. NO• production has been documented in an increasing number of tumor cells [34-36]. This has been interpreted as the ability of NO• to increase blood flow around tumors. Our results offer alternative or supplementary explanations. The ability of a tumor cell to produce NO• will allow it to kill neighboring cells, thus leaving more available rooms and nutrients for its own growth. It also equips the cell to fight off immune cells, such as T-cells and perhaps macrophages. During the process of tumor growth, tumor cells may have evolved other mechanisms to protect themselves from NO• produced by neighboring tumor cells [37]. Alternatively, some tumor cells may simply dissociate themselves from the rest of the tumor mass and travel to other locations, leading to metastasis. The notion that an NO•-derived toxic agent is formed outside of macrophages may form the basis for macrophages to become good hosts for intracellular parasites [2]. If this is the case, one approach to kill intracellular pathogens would be to localize infected macrophages followed by stimulating the cells to produce NO•.

To establish the role of NO• in self regulation of macrophages, it is necessary to conduct experiments which can distinguish the effects of intracellular NO• from extracellular NO•. This study offers an easy and informative approach for this purpose. Much of the reported NO•-mediated cell toxicity and gene mutations in cultured macrophages could have been cell culture artifacts. However, our findings suggest that NO•-mediated toxicity in macrophages can occur,

provided that macrophages are localized, such as in lymph nodes or spleen. Indeed, it may be possible to alleviate inflammatory conditions involving macrophages by localizing the cells and consequently destroying them with the NO• they produce. Finally, results reported here validate the use of RAW264.7 macrophages as an useful model system for the study of NO•-mediated cellular damage including genotoxicity, because they are probably exposed to NO• or its derivatives in a manner similar to target cells.

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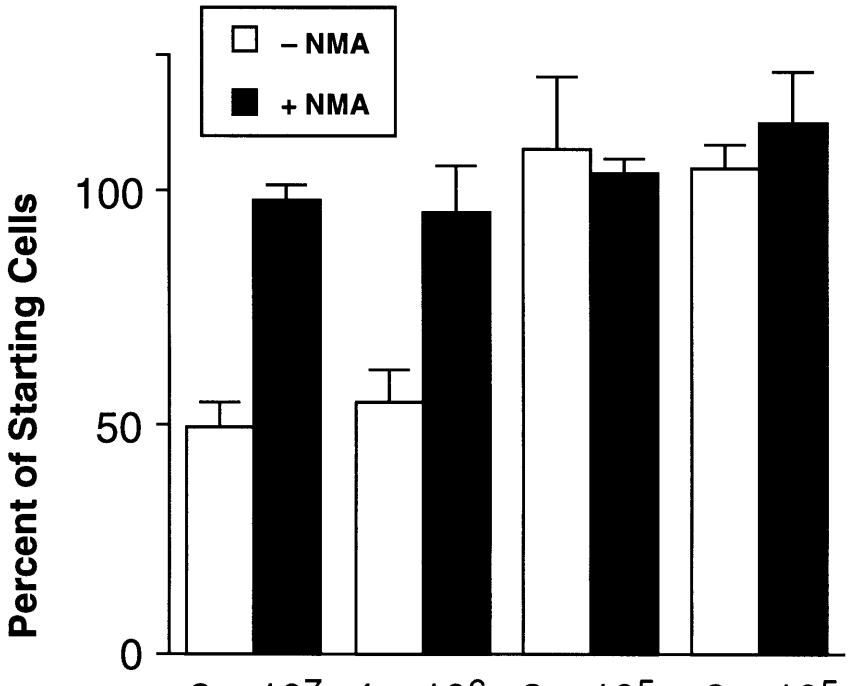
Figure legends

Fig. 1. Cell density effects on viability in macrophages stimulated to produce NO•. Cells were stimulated with IFN-γ plus LPS at the indicated densities, in the absence or presence of NMA. Two days later, viable cells were counted after staining with trypan blue. Results are mean ± s.d. of triplicate experiments. n.d., not determined.

Fig. 2. Cell density effects on gene expression in macrophages stimulated to produce NO•. RAW264.7 cells were incubated with IFN-γ plus LPS for 12 hours at different cell densities; untreated cells were cultured in parallel as negative controls. Expression of iNOS, IL-1β, TNF-α, and β-actin in these cells was examined using RT-PCR technique. H, high density ($2x10^7$ cells/plate), L, low density ($2x10^5$ cells/plate), U, untreated cells.

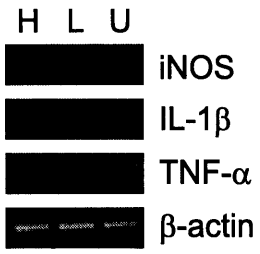
Fig. 3. Cell density effects on growth in macrophages stimulated to produce NO•. A total of 4×10^7 cells were stimulated with IFN-γ and LPS at each indicated density, in the absence or presence of NMA. Unstimulated cells were used as negative controls. Two days later, cells were grown in fresh medium containing only NMA, and allowed to recover and resume exponential growth, with the cell numbers monitored at the time points indicated. Results are mean ± s.d. of duplicate experiments.

Fig. 4. Cell density effects on genotoxicity in macrophages stimulated to produce NO•. Cells were stimulated and grown as in Fig. 2. Two weeks following stimulation, cells were plated in the presence of 6-thioguanine to select for *hprt* mutants. Calculation of mutant fraction was performed as previously described [19]. Results are mean ± s.d. of duplicate experiments.



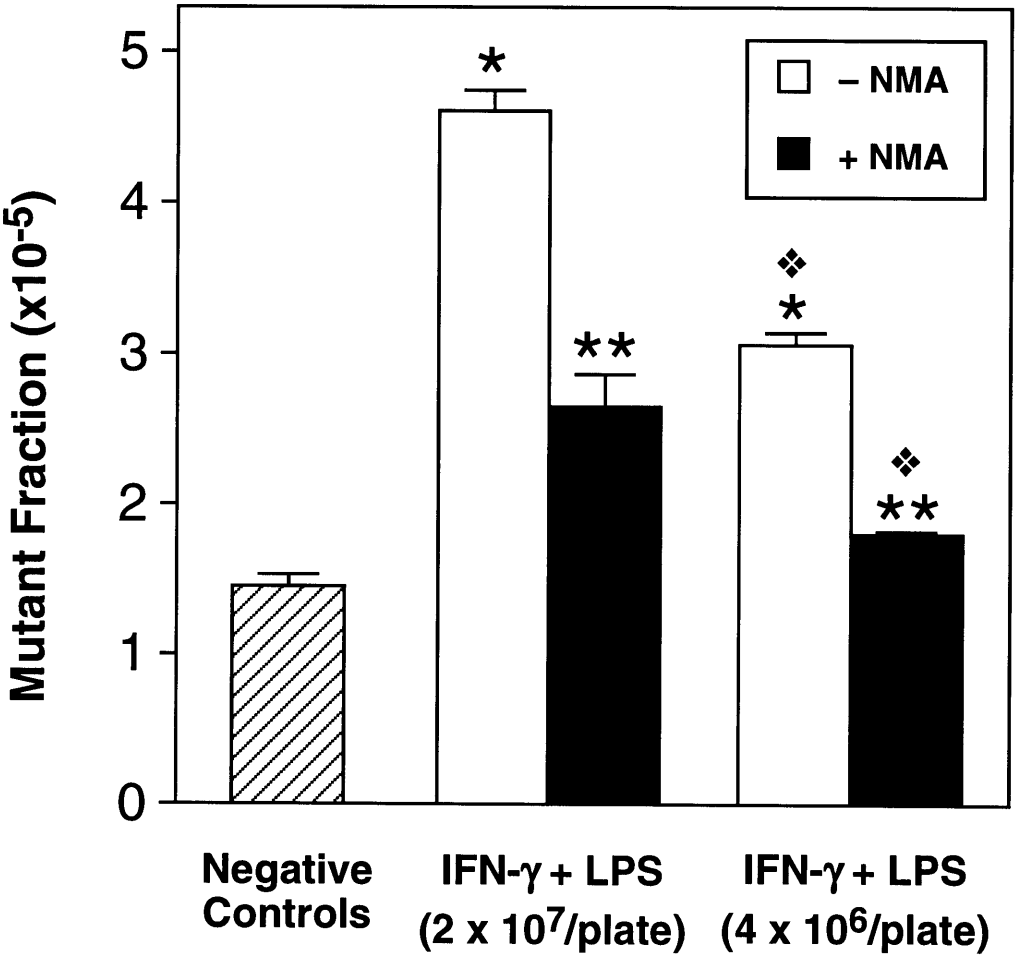
Cell Density (cells/plate)	2×10^7	4×10^6	8×10^5	2×10^5
Confluency (%)	20	4	0.8	0.2
NO• production (nmoles/10^6 cells)	120	146	n.d.	n.d.
iNOS mRNA	Yes	n.a.	n.a.	Yes

n.d. = not detected
n.a. = not analyzed

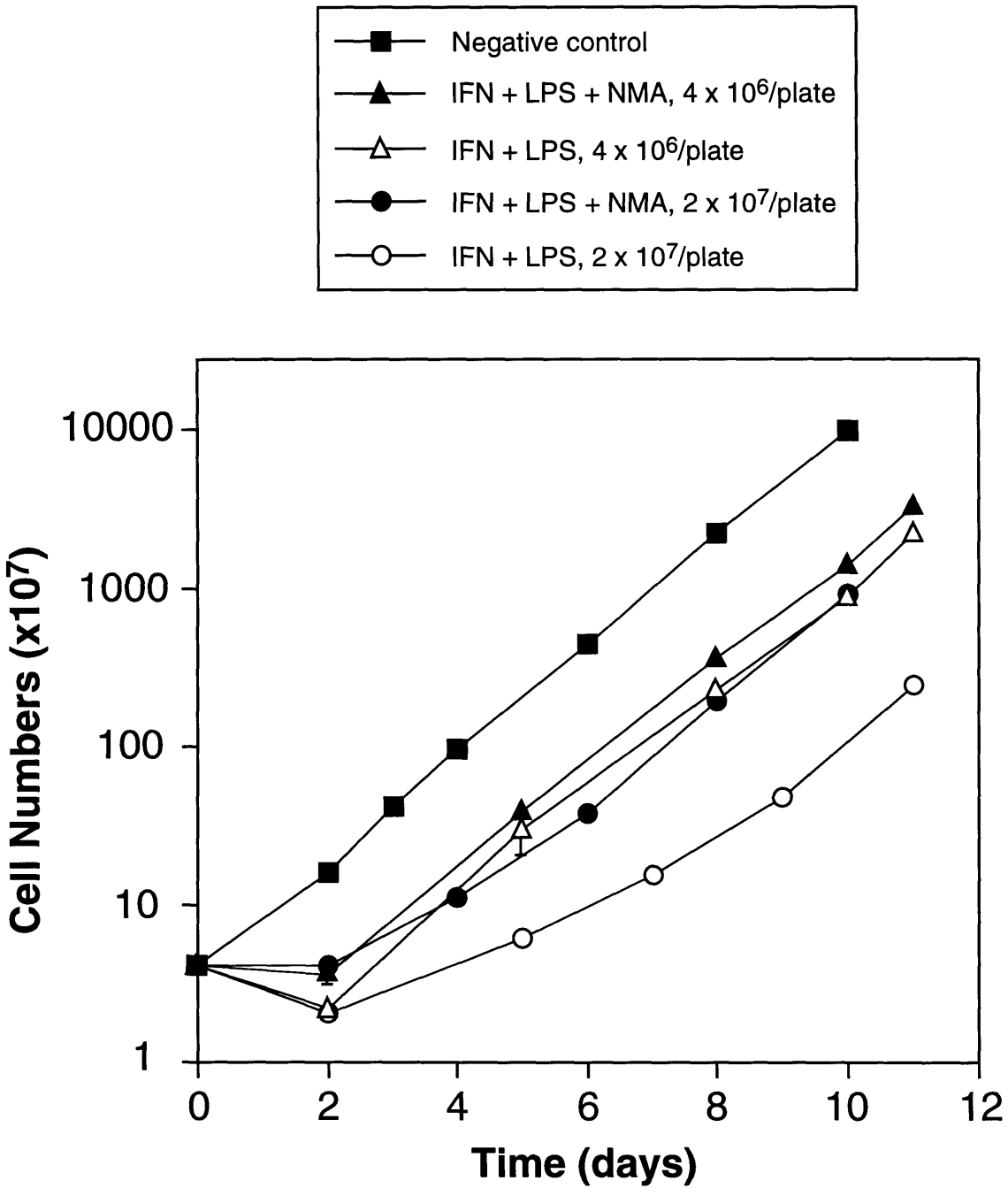


H = high density (2 x 10^7 cells/plate)
L = low density (2 x 10^5 cells/plate)
U = untreated cells

Stimulation time: 12 hr



* Significantly different ($P < 0.01$)
**, ❖ Significantly different ($P < 0.05$)


Negative control
IFN + LPS + NMA, 4 x 10^6/plate
IFN + LPS, 4 x 10^6/plate
IFN + LPS + NMA, 2 x 10^7/plate
IFN + LPS, 2 x 10^7/plate
10000
1000
100
10
1
Cell Numbers (x10^7)
0
2
4
6
8
10
12
Time (days)

Chapter 5 Genotoxicity associated with nitric oxide production in macrophages and co-cultured target cells

Abstract

We have recently reported development of the mouse macrophage-like RAW264.7 cell line as a model system for the study of nitric oxide (NO•)-associated genotoxicity under physiologically relevant conditions. Results from those studies clearly demonstrate that considerable genotoxicity is associated with NO• production in macrophages. Here we report development of a co-culture system which allows us to study in parallel the cytotoxic and genotoxic responses in co-cultured target cells and macrophages stimulated to produce NO•. Using this system we found that co-cultivation with macrophages stimulated to produce NO• for approximately 38-42 hours resulted in significant increases in mutant fraction in the endogenous genes of target human TK6 and hamster AA8 cells and in macrophages themselves, accompanied by a substantial decrease in cell viability. In most cases, addition of N-methyl-arginine, an NO• synthase inhibitor, abrogated much of the cytotoxicity and genotoxicity in target and macrophages cells, thereby establishing the role of NO• formation in the induction of the genotoxic and cytotoxic responses under these conditions. We also showed that NO•-associated genotoxic response in macrophages could be influenced by culture medium. Collectively, results from this study support a causative role of NO• production by stimulated macrophages in the development of inflammation-associated cancer.

Introduction

Although there is strong evidence from human and animal studies suggesting a causative role of chronic infection/inflammation in carcinogenesis, the molecular mechanisms through which infection/inflammation increase cancer risks are largely unknown [1-3]. However, it has been established that phagocytic neutrophils and macrophages infiltrate inflamed areas, where they produce a large array of cytotoxic chemicals and enzymes as a means of host defense [4]. Among the chemicals secreted by these cells are reactive oxygen species (ROS) [5], which have been shown to induce DNA damage, gene mutations, and cell transformation in target cells [6, 7]. The more recent identification of nitric oxide (NO•) as another reactive species produced by inflammatory macrophages has spurred great interest in understanding its potential genotoxic properties [8-11]. High levels of NO• have been found to be mutagenic to cells as well as to DNA targets in vitro (reviewed in [10, 11]). However, little is known about the genotoxicity of NO• produced by macrophages.

We recently reported development of the mouse macrophage-like RAW264.7 cell line as a model system for the study of NO•-associated genotoxicity under physiologically relevant conditions [12, 13]. We showed that considerable genotoxicity occurred in macrophages stimulated to produce NO• at a steady level over many cell generations or at higher levels over a relatively shorter period of time [13]. Furthermore, we provided evidence suggesting the existence of self protection mechanisms in macrophages against intracellularly generated NO• [12, 13]; thus, cell toxicity and genotoxicity in any individual macrophage was most likely caused by NO• from neighboring macrophages. To demonstrate that NO• produced by macrophages is indeed capable of inducing genotoxicity in neighboring target cells, we developed a co-culture system which allows the study of cytotoxic and genotoxic responses in target cells co-cultured with macrophages stimulated for NO• production. Results from this study clearly demonstrate that high levels of NO• production by stimulated macrophages over a relatively short period of time results in substantial cell toxicity and a significant increase of genotoxicity in macrophages and in co-cultured target cells, thereby supporting a causative role for NO• production by stimulated macrophages in the development of inflammation-associated cancer.

Materials and Methods

Cell culture and reagents. The hygromycin resistant mouse macrophage-like RAW264.7 cell line was cultured in DMEM containing 10% heat inactivated calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml hygromycin B, as previously described [12]. The Chinese hamster ovary AA8 cell line, obtained from the American Type Culture Collections (Rockville, MD), was cultured in αMEM medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. The human lymphoblastoid TK6 cell line (a gift of Dr. W. Thilly) was grown in RPMI-1640 medium supplemented with 10% horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. All of these reagents were purchased from BioWhittaker (Walkersville, MD). Other reagents used in this study included recombinant mouse interferon-g (IFN–γ) (Genzyme, Cambridge, MA) and *E. coli* lipopolysaccharide (LPS) (serotype 0127:B8; Sigma, St. Louis, MO), prepared as a 100x stock solution (2000 U/ml IFN–γ and 2000 ng/ml LPS) in DMEM medium containing no hygromycin; N-methyl-L-Arginine monoacetate (NMA) (Chem-Biochem Research, Inc., Salt Lake City, UT), prepared as a 400 mM stock solution in sterile water; hygromycin B solution (500 mg/ml) (Boehringer Mannheim, Indianapolis, IN); G418 sulfate (GIBCO-BRL, Gaithersburg, MD), 1mg/ml in αMEM medium (the solution was adjusted to pH7.5 using sterile 4N NaOH solution); triflurodeoxythymine (F_3TdR) (Sigma), 1mg/ml in sterile water; and 6-thioguanine (6TG) (Sigma), 10 mg/ml in DMSO.

Establishment of stable G418-resistant cells. To establish stable G418-resistant AA8 and TK6 cells, cells of each line were transfected with the pSV2neo plasmid (a gift of Z. Zhu) containing a neomycin resistance gene. A BTX electroporator was used following recommended conditions. Transfected cells were allowed to recover in their corresponding culture medium for two days, at which time, they were transferred to fresh medium containing 1 mg/ml G418. One week later, the medium was replaced and cells were incubated for another week. Viable cells were subsequently expanded in medium supplemented with 200 μg/ml G418, and this concentration of G418 was included in the culture medium for TK6 or AA8 except otherwise specified. To obtain cell populations with a low background mutant fraction by excluding preexisting mutants, 200 cells of each cell type were inoculated into 2 ml of appropriate culture medium, expanded by addition of fresh medium, and frozen for later use.

Co-culture of TK6 and RAW264.7 cells. A total of 40 co-culture plates were prepared, each of which contained 1 x 10^7 TK6 and 2 x 10^7 RAW264.7 cells in 30 ml hygromycin

B-free DMEM medium in a 150-mm tissue culture plate. To stimulate NO• production by macrophages, IFN-γ and LPS were added at a final concentration of 20 U/ml and 20 ng/ml, respectively, to the co-cultured plates. NMA solution was added to half of the plates at a final concentration of 2 mM. Stimulation was allowed to proceed for 30 hours in one set of plates (ten plates with and ten without NMA) and 42 hours in the other set. At the end of the stimulation at both time points, plates were shaken gently, and the suspension cells were separated from attached cells by removing the co-culture medium from the plates. Attached cells, the majority of which were macrophages, were allowed to recover and resume growth in the original plates in the presence of 2 mM NMA (to block residual NO• production) and 500 mg/ml hygromycin (to kill non-macrophage cells). Four days later, cells from every 5 plates of each condition were combined and a total of 4 x 10^7 cells were grown in 2 new plates for an additional ten days in the presence of 100 mg/ml hygromycin for phenotypic expression. Cells were then plated in 96-well plates in the presence of 6-thioguanine (6TG) to select for *hprt* mutants, as previously described [13].

Medium from five co-culture plates of each condition were combined and centrifuged to collect suspension cells, most of which were TK6. Each cell pellet was resuspended in 400 ml fresh RPMI-1640 medium plus 1 mg/ml G418 (to kill macrophages) in a sterile 500-ml glass bottle. Cells were incubated at 37°C overnight without stirring. Starting the following day, the cells were grown with stirring and appropriate dilution to maintain a cell density of 3-9 x10^5/ml. Six days after the termination of co-culture, 4.4 x 10^7 cells from each bottle were collected and resuspended with 220 ml RPMI-1640 containing 8% heat-inactivated horse serum instead of 10% horse serum. Triflurodeoxythymine (F_3TdR) was added to the cell solution to a final concentration of 1mg/ml and the cells were plated in ten 96-well plates at 40,000 cells/well to select for thymine kinase (*tk*) mutants. The remaining cells in each bottle were allowed to grow for another three days, and were plated in ten 96-well plates at 40,000 cells/ml in the presence of 2 μg/ml 6TG for selection of *hprt* mutants. Ten to fourteen days later, *tk* or *hprt* mutants were enumerated and mutant fraction calculated as described [14].

Co-culture of AA8 and RAW264.7 cells. Two independent experiments were conducted. In the first experiment, 40 co-culture plates were prepared, each containing 1 x 10^7 AA8 and 2 x 10^7 RAW264.7 cells in 15 ml G418-free αMEM and 15 ml hygromycin-free DMEM medium. Cells were stimulated with IFN-γ and LPS in the presence (20 plates) or absence (20 plates) of NMA as described in co-culture of TK6 and RAW264.7 cells. The plates were divided into two groups: one was incubated for 26 hours, the other 40 hours. At each time point, the medium in six plates of each condition (i.e., with or without NMA) was replaced with fresh αMEM medium plus 2 mM NMA and 1 mg/ml G418 (to kill macrophages). The six plates were

divided into two groups, maintained as duplicate cultures, and handled independently. Medium in the remaining four co-culture plates of each condition was replaced with DMEM plus 2 mM NMA and 500 μg/ml hygromycin (to kill AA8 cells). Likewise, the four plates were divided into two groups and handled as duplicate cultures. G418 resistant (AA8) cells and hygromycin-resistant (RAW264.7) cells were cultured for 10 and 14 days, respectively. Cells were then plated in the presence of 6-TG for determination of mutant fraction, as described above.

In the replica experiment, a total of 12 co-culture plates were prepared and stimulated with IFN-γ and LPS in the presence or absence of NMA for the two different periods of time, as described above. At the end of co-culture, medium in the plates was replaced with 30 ml αMEM containing 2 mM NMA and 1 mg/ml G418. Three days later, the medium, which contained dead macrophages, was replaced with fresh αMEM supplemented with 1mg/ml G418. Cells in each plate were cultured independently for another 7 days and plated for determination of mutant fraction, as described above.

During the co-culture experiments described above, untreated RAW264.7, TK6, or AA8 cells were handled in parallel as negative controls. An aliquot of medium at each condition was saved for analysis of total nitrite/nitrate using an automatic procedure [15].

Effect of AA8 cells or αMEM on mutant fraction in NO•-producing RAW264.7 cells. A total of 20 plates were prepared, each containing $2x10^7$ RAW264.7 cells in 15 ml hygromycin-free DMEM medium. These plates were divided into 5 groups of 4 plates each. To one group, an additional 15 ml DMEM medium was added to each plate, and no further treatment was performed (negative controls). A second group received IFN-γ and LPS in 15 ml DMEM medium per plate. A third group received IFN-γ, LPS, and NMA in 15 ml DMEM medium. The fourth group received IFN-γ and LPS in 15 ml αMEM medium, and the last group received $1x10^7$ AA8 cells, IFN-γ, and LPS in 15 ml αMEM medium. All plates were incubated for 36 hours; at that time, medium in all plates was replaced with fresh DMEM medium containing 500 μg/ml hygromycin. Cells were cultured for two weeks and the mutant fraction in the *hprt* gene of macrophages were determined as described above.

Statistical analysis. In the analysis of mutant fraction values from all co-culture experiments, Bartlett's test showed that there was no significant difference in variance of all mutant fraction values from experiments involving co-culture. ANOVA was then used to determine whether there was a significant difference among the means. Supplementary *t* testing was used to further determine whether any particular treatment resulted in a significant difference in mutant

fraction value from controls. In analysis of the differences in mutant fraction values as a result of change in cell density, F test and two-tailed *t* test were used [16].

Results

Development of a co-culture system for parallel study of genotoxic responses in target and effector cells. To investigate whether NO• and other reactive species produced by stimulated macrophages can increase the frequency of gene mutation in target cells under physiologically relevant conditions, we developed a co-culture system which allows the separation of target cells from macrophages following a co-culture treatment. As summarized in Scheme 1, macrophages and target cells were established as stable hygromycin and neomycin resistant cells, respectively. In co-culture experiments, subconfluent target and generator cells were mixed together in plates in the absence of either selecting drug. At the end of co-culture, plates were divided into two groups, one of which received hygromycin treatment for selection of macrophages, and the other G418 to select for target cells. In cases where target cells are grown in suspension, the majority of them separated from attached macrophages by removal of the co-culture medium. Target cells obtained in this manner were nonetheless grown in the presence of G418 to kill any remaining macrophage cells. Likewise, macrophage cells were always selected for in the presence of hygromycin to kill any target cells. Growth curves of cells under each selection condition were constructed to determine cell survival by extrapolating the growth curves to the zero time point. In general, one week after addition of selecting drugs, pure target or macrophage cells were obtained, and these were used for the mutation study.

Cytotoxic and genotoxic responses in human TK6 cells co-cultured with macrophages. We first examined the cytotoxic and genotoxic responses in human TK6 cells, which grow as a suspension culture, after co-culturing with macrophages stimulated to produce NO•. As summarized in Table 1, there were time- and dose-dependent decreases in cell survival with increasing amounts of NO• exposure. However, the genotoxic responses in two endogenous genes in TK6 cells differed. While there were highly significant differences ($P<0.01$) in mutant fraction values in the *tk* gene of cells, the differences in *hprt* gene were not significant ($P>0.05$). Neither co-culture with unstimulated macrophages nor incubation with IFN-γ and LPS had a significant effect on mutant fraction in the endogenous genes of TK6 cells (data not shown). Thus, genotoxic response in TK6 cells co-cultured with macrophages stimulated to produce NO• was locus-specific.

Addition of N-methyl-L-arginine (NMA), an NO• synthase inhibitor, to the culture medium blocked over 90% of the NO• production and cytotoxicity. Supplementary *t* test revealed that cells co-cultured with NO•-producing macrophages for 30 or 42 hours both had significant increases in

mutant fraction over untreated controls ($P<0.05$). In the presence of NMA, however, the differences were no longer significant. Furthermore, the mutant fraction value in cells co-cultured for 42 hours in the absence of NMA was significantly higher than in the presence of NMA. These observations suggested that much of the cytotoxic and genotoxic response in TK6 cells were closely associated with NO• production by stimulated macrophages.

Cytotoxic and genotoxic responses in AA8 cells co-cultured with macrophages. We also examined the cytotoxic and genotoxic responses in co-cultured AA8 cells, which grow as a monolayer, as macrophages do. Similar to TK6 cells co-cultured with macrophages, there were time- and dose-dependent decreases in cell survival with increasing NO• exposure (Table 2). Although there was an increase in mutant fraction values in cells co-cultured with stimulated macrophages compared to untreated cells, the differences were not statistically significant ($P>0.05$). In the replica experiment (Experiment 2 in Table 2), however, the differences were highly significant ($P<0.01$). When the mutant fraction values from both experiments were pooled and reanalyzed, the differences were still highly significant ($P<0.01$). As in the case of TK6 cells, neither co-culture with unstimulated macrophages nor incubation with IFN-γ and LPS had an effect on mutant fraction in the endogenous gene of AA8 cells (data not shown). Collectively, there were significant increases in mutant fraction in the *hprt* gene of AA8 cells co-cultured with macrophages stimulated to produce NO•.

As in the TK6 co-culture experiment, NMA blocked approximately 90% of the NO• production in cells (Table 2). NMA also blocked 90% of the cytotoxicity in cells co-cultured for 26 hours, but only 40% in those co-cultured for 36 hours. Analysis of the pooled data using the *t* test supplementary to ANOVA revealed that only cells co-cultured for 36 hours with NO•-producing macrophages had significant increases in mutant fraction over untreated controls but not over cells co-cultured in the presence of NMA. Interestingly, cells co-cultured for 36 hours in the presence of NMA also showed a significant increase in mutant fraction over untreated controls (Table 2). Together, these results suggest that factor(s) in addition to NO• produced by stimulated macrophages are also cytotoxic and genotoxic to AA8 cells co-cultured for 36 hours.

Cytotoxic and genotoxic responses in macrophages co-cultured with AA8 or TK6 cells. As mentioned earlier, this co-culture model system allowed us to examine the cytotoxic and genotoxic responses in macrophages in addition to those in target cells. As summarized in Tables 1 and 2, macrophages produced NO• in proportion to the length of stimulation by IFN-γ and LPS, regardless of the target cells with which they were co-cultured. Likewise, the viability of cells decreased as the length of stimulation increased, regardless of the co-cultured target cells (Table 3). However, the genotoxic response differed in cells co-cultured

with different target cells. Specifically, macrophages co-cultured with TK6 cells had significantly ($P<0.01$) higher mutant fraction means than untreated controls, whereas in cells co-cultured with AA8 cells, the differences in the means were not significantly different ($P>0.05$). Furthermore, the difference in genotoxic responses under these co-cultured conditions seemed to be abrogated in the presence of NMA (Table 3). Collectively, the data indicate concurrent cytotoxic and genotoxic responses in macrophages co-cultured with different target cells and suggest that co-culture conditions could influence the genotoxic response in macrophages.

Effect of αMEM medium on the mutagenic responses in RAW264.7 cells. A number of factors can presumably affect responses in macrophages co-cultured with different target cells. One is macrophage-target cell interactions, which may lead to differential responses in macrophages and/or target cells. It is noteworthy that both macrophages and AA8 cells grow attached, whereas TK6 cells grow in suspension. Therefore, it is conceivable that AA8 cells might have acted as physical blocks between macrophages, thereby reducing the amount of NO• or its derivative(s) produced by an individual macrophage to attack its neighboring macrophages. Also, difference in culture media used in these co-culture studies may affect experimental results. To distinguish between the latter two possibilities, we compared the genotoxic responses in macrophages stimulated in DMEM (100%), in αMEM and DMEM (50%:50%), and with AA8 cells in αMEM and DMEM (50%:50%). As shown in Figure 1, neither the composition of culture medium or AA8 affected NO• production by stimulated macrophages. However, there was significant ($P<0.05$) decrease in mutant fraction means in cells stimulated for NO• production in the presence of 50% αMEM compared to those in 100% DMEM, regardless of the presence of AA8 cells. This finding indicated that the presence of component(s) in the culture media are capable of modulating the NO•-associated genotoxic response in stimulated macrophages.

Discussion

We previously demonstrated that high levels of NO• production by stimulated macrophages over a relatively short period of time resulted in both cell toxicity and genotoxicity in the generator cells [12, 13]. To study whether NO• produced under these conditions also causes gene mutations in target cells, we developed a co-culture system which allowed the study of cytotoxic and genotoxic responses in cells co-cultured with macrophages stimulated for NO• production. Using this system we found that co-cultivation with stimulated macrophages for approximately 38-42 hours resulted in significant increases in mutant fraction in endogenous genes of target human and hamster cells, accompanied by a substantial decrease in cell viability (Tables 1 and 2). Except for the AA8 cells co-cultured for 38 hr, inhibition of NO• production by addition of NMA to the culture medium abrogated much of the cytotoxicity and genotoxicity in target and macrophages cells, thereby establishing the role of NO• formation in the induction of the genotoxic and cytotoxic responses in these target cells.

The molecular mechanisms through which NO• formation led to genotoxicity in these target cells are presently not known. NO• alone or in concert with ROS and lipid oxidation products such as metabolites of arachidonic acid could cause DNA damage, including deamination, oxidation, strand breaks, and cross-links [10, 17, 18]. Any or a combination of these NO•-related reactive species could also induce oxidative stress in target cells through mechanisms analogous to those in *E. coli* [19, 20], which can conceivably lead to increased gene mutations. We previously showed that there was no significant difference in mutational pattern in the *hprt* gene of macrophages stimulated to produce NO• or unstimulated controls [13]. Given the evidence that NO• produced by stimulated macrophages acts in a paracrine fashion, it is likely that similar yet to be identified mechanisms are operative in the formation of mutations in the target cells. More specifically, the mutational patterns in TK6 and AA8 cells co-cultured with NO•-producing macrophages may be similar to their corresponding spontaneous mutational patterns. Confirmation of this proposition awaits the molecular characterization of mutated target cells.

The background mutant fraction at the *tk* locus of TK6 cells in this study was 5 fold higher than the historic mean [21]. Nonetheless, the time- and dose-dependent increases in mutant fraction in cells co-cultured with stimulated macrophages were statistically significant (Table 1). However, the mutant fraction values in the *hprt* gene of the same treated TK6 cells showed no difference from those of untreated cells. A 3-fold higher increase of mutant fraction in the *tk* gene than the hprt gene was also previously observed in TK6 cells exposed to aqueous NO• solution

[21]. Locus-specific differences in response to mutagen treatment has also been reported by other studies. For example, WTK1, a human lymphoblast cell line derived from the same donor as TK6, was 20 fold more sensitive to the mutagenic effects of x rays at the *tk* locus, but only 4 folds more sensitive at the *hprt* locus for similar doses of X rays [22]. It is well established that X-rays induce large deletions encompassing multiple loci [22, 23]. Thus, it is conceivable that radiation-induced large deletions on the X chromosome are lethal and therefore would not be recovered. Indeed, there was evidence for the presence of an essential gene neighboring the *hprt* gene on the hemizygous X chromosome [24]. In another study, TK6 cells treated with X irradiation or radon showed a higher mutant fraction at the *tk* locus than at the *hprt* locus, although the lesion sizes in radon-induced mutants at both loci are smaller than those by X rays [25]. Locus-specific differences occur in spontaneous mutation rates as well; in WTK1 cells, the background mutant fraction in *tk* was an order of magnitude higher than that in TK6 cells, but no difference at the *hprt* locus was observed [22]; the historic background mutant fraction in the *tk* gene of TK6 cells is higher than in the *hprt* gene [21]. It appears that a higher mutant fraction at the *tk* locus than at the *hprt* locus is a specific response of TK6 cells to treatment by mutagens capable of inducing large gene deletions. By virtue of this observation, it is predicted that a significant portion of TK6 cells co-cultured with NO•-producing macrophages have undergone large gene deletions, which would be consistent with our previous finding that a high proportion of *hprt* mutations in NO•-producing macrophages was large gene deletions [13]. On the other hand, a significantly lower proportion of *hprt* mutations in AA8 cells is predicted to have large deletions, as is the case with spontaneous mutants in this cell type [26, 27]. This could also explain why a significant increase in mutant fraction value in the *hprt* gene could be detected in AA8 but not TK6 cells.

Although RAW264.7 cells stimulated with LPS plus IFN-γ also produced considerable amounts of superoxide [28-30], the fact that virtually all of the genotoxic responses in target cells could be abrogated by NMA suggested that superoxide or its metabolite(s) was not as genotoxic as NO• or its metabolite(s) under our experimental conditions. It has been reported that a minimum of 50:1 of macrophage:target cell ratio was necessary for induction of *hprt* mutants in target tumor cells co-cultured for 9 days with macrophages stimulated to produce reactive oxygen species [31]. In this study, the failure to detect significant genotoxicity in target cells in the presence of NMA may be attributed to a substantially lower macrophage:target cell ratio (2:1). It is likely that by increasing either or both co-cultivation time and macrophage:target cell ratio in our system, which in fact increases the amounts of superoxide delivered from stimulated macrophages, significant genotoxicity in target cells will be detected. It is noteworthy to point out that superoxide produced by neutrophils is more genotoxic, because co-culture of target cells with stimulated neutrophils for as short as one hour resulted in considerable DNA damage and mutations [32, 33]. The difference

in responses to ROS from neutrophils or macrophages may be attributable to higher levels of respiratory burst in neutrophils. Alternatively, ROS from neutrophils may have properties which are different from those of macrophages.

In contrast to genotoxic responses, co-culturing with macrophages stimulated to produce NO• clearly results in high levels of cell toxicity in both target cells. Similar to genotoxicity, much of the cytotoxicity in target cells could be diminished by NMA. NO• has been found to mediate cell death through a number of mechanisms, including induction of apoptosis [34-36], energy depletion as a result of acotinase inhibition [37], lipid oxidation [17], DNA strand breaks [18], and possibly protein modification through nitrotyrosine formation [38, 39]. Our results showed that TK6 cells were more sensitive than AA8 cells and macrophages to killing mediated by NO• (Tables 1-3). A similar observation has been reported in the same cell lines exposed to aqueous NO• solution, and a difference in glutathione metabolism between the two cell lines provided one explanation for the differential sensitivity of the cells to NO• mediated killing [40].

In the presence of NMA, however, TK6 appeared to be more resistant to killing by co-cultured macrophages than AA8 cells (Tables 1 and 2). This indicates that AA8 cells are more sensitive to superoxide-mediated killing than TK6 cells. Although the underlying mechanism is not known, it is noteworthy that TK6 cells grow in suspension whereas AA8 grow attached. Compared to NO•, superoxide has a much shorter half life (<50 msec) and a free diffusion path of only 2 um [41]. Thus, it is conceivable that superoxide produced by macrophages may not be able to reach TK6 cells that loosely float above the attached macrophage cell layer to an extent that is capable of conferring toxicity. On the other hand, many of attached AA8 cells are either in direct contact or in very close proximity to attached macrophages, thereby exposing them to higher levels of superoxide released from macrophages. Collectively, TK6 appears to be more sensitive than AA8 cells to NO• produced by macrophages, even though they were further away in distance from macrophages than AA8 cells. This is in agreement with our previous findings from animal studies, which showed that cells stained positive for apoptosis or nitrotyrosine were not often not those adjacent to cells that stained positive for iNOS protein [39]. Thus, cell properties as well as effector-to-target cell distance affect the fate of cells exposed to NO•.

The system described in this study allows comparison of cytotoxic and genotoxic responses in macrophages co-cultured with different target cells. As shown in Tables 1, 2, and 3, there were time-dependent increases in NO• production and decreases in cell viability in stimulated macrophages, regardless of co-cultured target cells. However, the genotoxic responses in macrophages differed. Further investigation of this phenomenon revealed that this difference was caused by the use of different medium compositions in the two co-culture studies (Figure 1).

Specifically, factor(s) present in αMEM could modulate genotoxic response to NO• formation in macrophages. Interestingly, the use of different medium compositions did not affect NO• production, cell viability, or growth of macrophages (Table 3, Figure 1, and unpublished results). This observation indicated that the cytotoxic and genotoxic effects associated with NO• production in macrophages are mediated by different mechanisms. The responsible factor(s) in αMEM medium used in this study is presently not identified, nor is it clear whether the factor(s) alone was sufficient for the effects or required interaction with factor(s) present only in DMEM medium. Identification of and an understanding of the mechanisms of action of the factor(s) will provide important insights regarding the molecular basis of NO• genotoxicity in this system.

The most unique feature of the co-culture model system described here is the ability to separate attached target cells from macrophages following co-cultavation of the two cell types under physiologically relevant conditions. The system can readily be adapted to study the effects of effector-target interactions on the cytotoxic and genotoxic responses in any two cell types; or can be used to co-culture macrophages with more than one type of target cells, thereby allowing the comparison of responses in two target cells under same treatment. What interests us is the use of the system to examine the effects of NO• production rate, interaction of NO• with oxygen species, and generator-to-target distance on the genotoxic response in target cells.

In this study we showed that target cells co-cultured with macrophages stimulated to produce NO• underwent a significant increase in gene mutations as well as cell toxicity. This supports the role of NO• production in the development of cancer through its ability to deregulate cellular metabolism, to induce massive cell turnover, and to induce genotoxicity in target cells. More importantly, these consequences can be brought about in target cells exposed to high levels of NO• produced by macrophages for as short as 40 hr, thereby raising the possibility that the role of NO• plays in carcinogenesis may be broader than previously thought.

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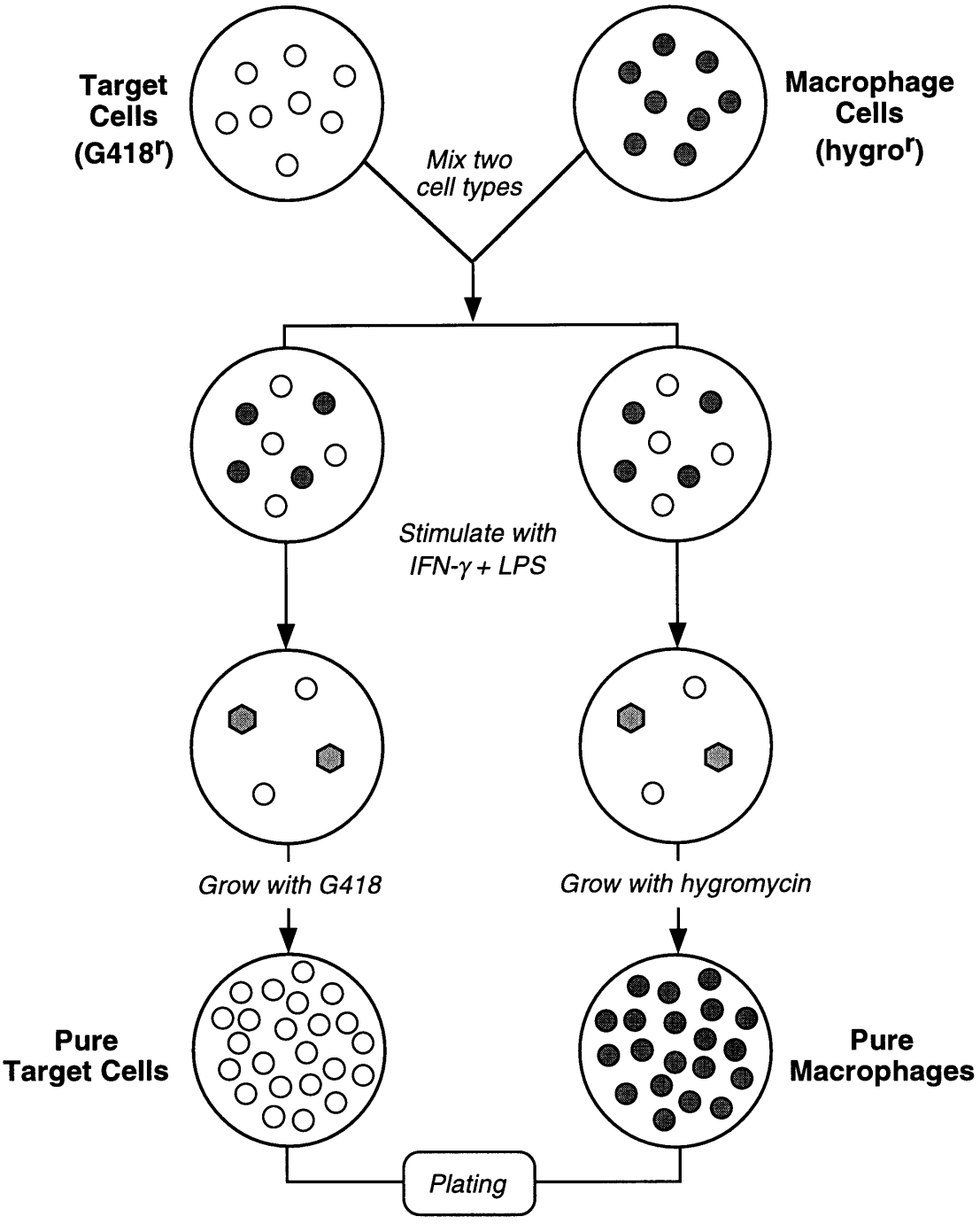
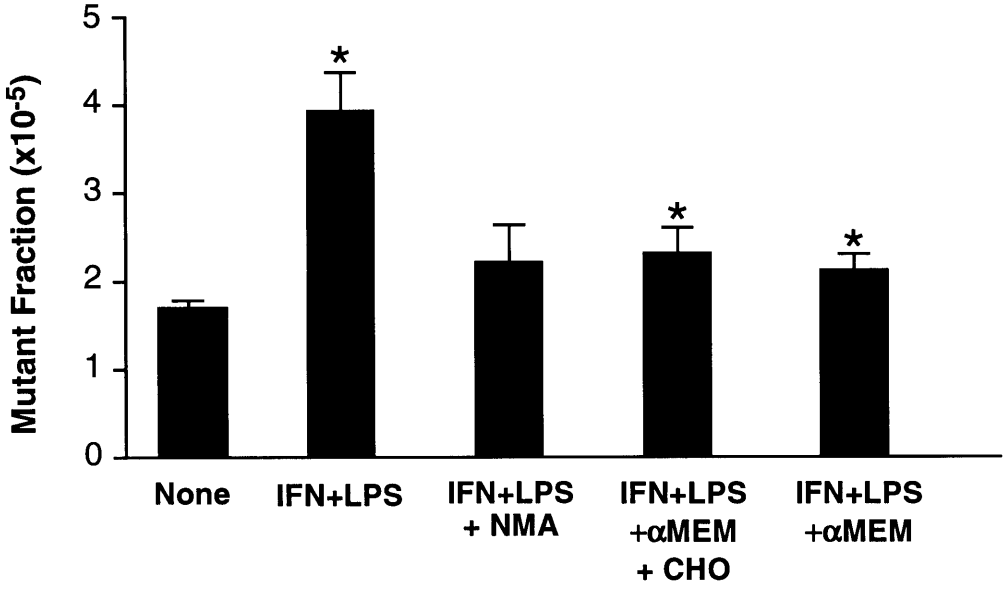
Figure legends

Scheme 1. Co-culture system for parallel mutation studies in macrophages and in target cells.

Fig. 1. Effect of αMEM and/or AA8 cells on the mutant fraction in the endogenous hprt gene of macrophages.


Target Cells (G418r)
Macrophage Cells (hygror)
Mix two cell types
Stimulate with IFN-γ + LPS
Grow with G418
Grow with hygromycin
Pure Target Cells
Pure Macrophages
Plating



Total NO• (μm)	**0**	**97**	**13**	**95**	**103**

* Significantly different ($P < 0.05$)

Table 1. Cytotoxicity and Genotoxicity in human TK6 target cells co-cultured with macrophages.

Condition	Nitrite & nitrate (μmoles/10^7cells)	Survival (%)	Mutant Fraction ($x10^{-6}$) *tk* gene		Mutant Fraction ($x10^{-6}$) *hprt* gene	
			Replicate	Mean[a] ± SEM	Replicate	Mean[b] ± SEM
None	0	100 ± 8	7.7	7.4 ± 0.4	3.6	3.7 ± 0.1
			7.2		3.8	
IFN-γ + LPS, 30 hr	1.0	16 ± 2	9.4	9.7[c] ± 0.4	4.4	3.9 ± 0.8
			10.0		3.3	
IFN-γ + LPS, 42 hr	1.7	2 ± 1	12.0	11.5[c,d] ± 0.7	2.8	3.6 ± 1.1
			11.0		4.3	
IFN-γ + LPS + NMA, 30 hr	0.13	96 ± 7	8.1	7.7 ± 0.6	4.1	3.9 ± 0.3
			7.3		3.7	
IFN-γ + LPS + NMA, 42 hr	0.26	94 ± 7	8.1	8.3[d] ± 0.2	4.1	4.1 ± 0.1
			8.4		4.0	

a. The differences in means are highly significant ($P<0.01$).

b. The differences in means are not significant ($P>0.05$).

c. Significantly higher than negative controls ($P<0.05$).

d. The difference in means is significant ($P<0.05$).

Table 2. Cytotoxicity and Genotoxicity in hamster cells co-cultured with macrophages.

Condition	Nitrite & nitrate (μmoles/10^7cells)	Survival (%)	Mutant Fraction ($x10^{-6}$) Experiment 1[a] Replicate	Mean[b] ± SEM	Experiment 2[a] Replicate	Mean[c] ± SEM
None	0	100 ± 4	1.0	1.1 ± 0.2	0.8	0.7 ± 0.2
			1.2		0.5	
					0.8	
IFN-γ + LPS, 26 hr	0.83	66 ± 9	2.7	2.1 ± 0.9	1.3	1.2 ± 0.1
			1.4		1.3	
					1.2	
IFN-γ + LPS, 38 hr	1.4	15 ± 5	3.1	3.0[d] ± 0.1	2.5	2.0[d] ± 0.5
			2.9		2.1	
					1.5	
IFN-γ + LPS + NMA, 26 hr	0.08	93 ± 7	1.7	1.6 ± 0.1	1.0	1.1 ± 0.1
			1.5		1.2	
					1.2	
IFN-γ + LPS + NMA, 38 hr	0.15	55 ± 5	2.8	2.3 ± 0.8	2.2	1.7[e] ± 0.5
			1.8		1.8	
					1.2	

a. If results from both experiments are pooled, the differences in means are highly significant ($P<0.01$).

b. The differences in means are not significant ($P>0.05$).

c. The differences in means are highly significant ($P<0.01$).

d. Significantly higher than negative controls ($P<0.01$).

e. Significantly higher than negative controls ($P<0.05$).

Table 3. Cytotoxicity and Genotoxicity in macrophages co-cultured with TK6 or CHO cells.

	with TK6 cells			with CHO cells		
	Survival (%)	Mutant Fraction ($x10^{-5}$)		Survival (%)	Mutant Fraction ($x10^{-5}$)	
Condition		Replicate	Mean[a] ± SEM		Replicate	Mean[b] ± SEM
None	100 ± 17	1.0	1.1 ± 0.1	100 ± 8	1.7	1.6 ± 0.1
		1.2			1.5	
IFN-γ + LPS, 26-30 hr	34 ± 6	4.4	4.4[c] ± 0.1	36 ± 6	1.8	2.3 ± 0.6
		4.3			2.7	
IFN-γ + LPS, 38-42 hr	10 ± 1	6.2	5.8[c] ± 0.6	14 ± 3	2.7	3.1 ± 0.5
		5.3			3.4	
IFN-γ + LPS + NMA, 26-30 hr	56 ± 6	1.2	1.3 ± 0.1	58 ± 6	1.6	1.7 ± 0.1
		1.3			1.8	
IFN-γ + LPS + NMA, 38-42 hr	54 ± 7	2.6	2.4 ± 0.3	47 ± 5	2.4	2.2 ± 0.3
		2.2			2.0	

a. The differences in means are highly significant ($P<0.01$).

b. The differences in means are not significant ($P>0.05$).

c. Significantly higher than negative controls ($P<0.01$).

Chapter 6 Molecular analysis of *hprt* mutants in human TK6 cells treated with aqueous nitric oxide solution

Abstract

Nitric oxide (NO•), an active free radical, has been found to be produced by many types of cell, notably by activated macrophages in large quantities. It has been proposed that high levels of NO• by activated macrophages could be hazardous to neighboring normal cells with respects to cell death and mutation. Aqueous NO• solution has been found to cause DNA base damage and strand breaks, and to induce DNA mutations in bacteria and human cells. The mutagenic specificity of aqueous NO• solution has been characterized using a shuttle vector treated in vitro. However, the mutagenicity of aqueous NO• solution in an endogenous gene in human cells has not been reported. Here we describe the cytotoxic and mutagenic responses of human lymphoblastoid TK6 cells after treatment with NO• gas using the Silastic® membrane delivery system. Independent *hprt* mutants were isolated and the mutations characterized using reverse-transcription-polymerase chain reaction (RT-PCR) and DNA sequencing.

Comparison of the mutations detected in this study to those occurring spontaneously in the cells revealed a striking similarity in mutation pattern, with the exception that a small increase of A:T to G:C transitions was observed in NO• treated cells. Results from this and previous studies suggest that NO•-induced cytotoxicity and genotoxicity in same target cells can be influenced by difference in NO• dosing rate. Furthermore, the choice of experimental system dramatically affect the conclusions regarding NO• mutagenesis.

Introduction

Numerous studies have demonstrated that NO• plays an important role in many biological functions. For example, it is formed as a second messenger in the brain, it serves as a vascular dilator, it is involved in the process of learning and memory, and finally it mediates the cytotoxic functions of activated macrophages. In neurons and endothelial cells, NO• is produced at a low yet constitutive rate. In contrast, production of NO• by macrophages is inducible and a burst of NO• is frequently observed upon activation of macrophages, leading to the killing of invading microorganisms and some tumor cells.

High production rate of NO• by activated macrophages is beneficial to animals including human being with respect to its role in immune defense against infection and tumor growth. It is logical, however, for one to ask: can this active molecule also be harmful to normal cells within a species? This question becomes more prominent when epidemiological studies correlated tumor formations with inflammatory responses in such organs as liver, stomach, and colon. As one part of an inflammatory response, macrophages migrate to and filtrate the inflamed tissue so as to chew up those infected or damaged cells. Since nitric oxide is produced by macrophages during this process, a high local concentration of nitric oxide within the inflamed tissue may spell out as a disaster to those normal cells within the same area. It is not clear at this point regarding the nature and extent of damage NO• imposes on normal cells within the vicinity of inflammation. Results from this and other related studies ongoing in this department will shed light on answers to this question.

Advances in cancer research have enabled elucidations of tumorigenesis at molecular levels. Particularly, the concept of oncogene has revolutionized the way scientists think about cancer formation and the approach they choose to address this problem. Many normal counterparts of oncogenes, called protooncogenes, are critical genes involved in the control and regulation of numerous cellular functions such as cell division, differentiation, and self defense. Once such genes are mutated, or more strictly speaking, their normal functions are aberrated, cells housing one or more of such mutated genes will grow in an abnormal manner and in many cases lead to formation of tumor. To convert a protooncogene into an oncogene, all it takes may be just a single point mutation in the DNA sequence of the gene.

Many environmental insults could result in such genetic changes. In particular, numerous epidemiological studies have found that certain types of cancer are correlated with exposure of

certain chemicals. For example, cigarette smoking is related to lung cancer, aflatoxin B1 with liver cancer, benzene with leukemia, to name just a few. It is the goal of genetic toxicologists to pinpoint the etiology of cancer at molecular level. One commonly employed approach leading to this goal is to treat certain cells, bacterial or mammalian, with a carcinogen in concern. Mutated cells may be selected by one mechanism or another, and the gene of interest studied by molecular biological techniques. Once the types and positions of mutation in this gene have been obtained from sequencing data, a mutational spectrum of the carcinogen in this gene may be constructed, which serves in a way as a fingerprint. Armed with this information, it will be easier to identify the cause of cancer in patients, ultimately by constructing the mutational spectrum of the same gene from a patient and compared to the existing mutational spectra of a variety of carcinogens.

To address whether production of NO• by activated macrophages cause mutations to normal cells which subsequently lead to tumor formation, it is necessary to study the type and position of mutation caused by NO• in a gene context. It is desirable that such a gene is an oncogene found from other studies to be critical in tumor formation. For example, p53, a tumor suppressor gene, has been found to be mutated in many types of tumors, including those of stomach and colon. If the type and position of mutation NO• causes in p53 gene can be constructed by treating the cells with NO•, it is possible to pinpoint the etiology of certain types of tumors, which in turn may suggest ways of intervention. Unfortunately, no method thus far allows selection of cells with mutation in an oncogene. Nevertheless, there exists a selection method for cells with mutant *hprt* gene. *Hprt* gene encodes a protein which participates in nucleotide metabolism. In addition to its normal function, this enzyme can convert a nucleotide analog, 6-thioguanine, into a toxic metabolite, thus committing suicide. When the gene is mutated, in this case by NO• treatment, the corresponding enzyme is nonfunctional, and therefore unable to metabolize 6-thioguanine. In other words, this cell will survive. Thus, by treating cells and allowing them to grow in the presence of 6-thioguanine, only those cells with mutated *hprt* gene will survive and grow into clones. Each individual mutant clone derived from NO• treatment may be studied independently, and the mutation data summarized afterward. This approach is commonly referred as clone-by-clone method. Alternatively, the pool of mutants from a single treatment may be studied as a mixture. Either way, a mutational spectrum may be constructed.

Once the mutational spectrum is obtained for nitric oxide, I would like to look into certain types of cancer to see if similar pattern of mutation in the *hprt* gene can be revealed. A positive result will lead to novel treatment of these types of cancer. For example, antibiotics will be able to prevent tumor formation in certain organs. In fact, a recent study showed that administration of antibiotics caused regression of precancerous lesions in a stomach, though the underlining molecular mechanism is yet to be elucidated.

In this report, I will describe the experimental procedures I used to study the mutational spectrum of NO• in the *hprt* gene of a human lymphoblastoid cell line, TK6 cells. Results will be discussed in relation to findings from other studies.

Materials and Methods

Treatment with aqueous NO• solution and isolation of independent mutant clones. A total of 100-200 cells from an exponentially growing wild-type TK6 stock were inoculated into 5 ml of growth medium in a 6-well plate. When a white cloud of cells could be seen from the bottom (this typically takes a week to 10 days), the cells were then transferred into 100 ml medium and grown with stirring. The cell population was allowed to expand until the total cell number reached $4x10^8$. The cells were then spun down at 500x g for 5 minutes. The pellet was resuspended into 200 ml of exposure medium. A total of 3×10^8 cells were exposed to NO• through 20 cm of Silastic® tube until the total NO_2^-/NO_3^- reached the desired concentration. In this experiment, the duration of exposure was one hour, and the final concentration was 0.7 mM. The experiment was done in duplicate. For positive control, 5×10^7 cells was exposed to 140 ng/ml of 4-nitrosoquinoline oxide (4-NQO) for one hour. For negative control, 5×10^7 cells were treated with argon gas via a Silastic® tube for the same amount of time.

At the end of treatment, the cells were spun down and resuspended to $5x10^5$ cells/ml with regular growth medium. Aliquots of $5x10^6$ cells each from the NO•-treated stock were transferred into twenty 30-ml vials and allowed to grow for 6 days. The cell numbers of the remaining NO•–treated cells and the positive and negative control cells were monitored over time. When all of the three cell populations reached exponential growth, the surviving fraction of NO•-treated cells was calculated as the total number of the treated cells divided by the total number of negative control cells. The surviving fraction of 4–NQO treated cells was calculated similarly. For mutation frequency measurement, cells from these three populations were respectively plated in three 96-well plates at $5x10^4$ cells/well in the presence of 1 μM 6-thioguanine. Colonies from the plates were scored at the end of 12–day incubation, and mutation frequency calculated according to published method (1).

Similarly, 6-thioguanine was added to each of the vials to a final concentration of 1 μM at the end of 6-day phenotypic expression. Cells from each vial were plated into a 96-well plate at $2x10^4$ cells per well, or 0.1 mutant per well based on a mutation frequency of 5×10^{-6}. The plates were incubated at 37°C. By 12 days, mutant clones appeared in 96-well plates, each of them was expanded in one well of a 24-well plate. When the cell density reached approximately 10^6 per ml (this could be measured by cell counter or judged from the yellowish medium color), the medium in each well was sucked off and 200 μl of freezing solution added. The plates were immediately

stored in -100°C freezer. Since each well in a 24-well plate holds 2 ml of medium, the total number of cells in each well was about 2 x 10^6.

Total RNA isolation. Total RNA from each mutant clone was isolated by the method of Chomczynski and Sacchi (2). Briefly, the frozen cells in each well of a 24-well plate were thawed and transferred to an 1.5-ml Eppendorf tube and washed with 1 ml cold phosphate-buffered saline. Cells were collected by centrifugation at 500x g for 5 minutes at 4°C. A total of 300 µl prechilled denaturing solution containing 4M gaunidinium thiocyanate, 25 mM sodium citrate, pH7.0, 0.5% sarcosyl, and 0.1M 2-mercaptoethanol, was added to the cell pellet and shaken to dissolve. The cell lysate was acidified by adding 40 µl 2M sodium acetate (pH4.0), followed by adding 400 µl phenol (pH 4.3) and 80 µl chloroform. The content was mixed by vortexing until milky and then placed on ice for 15 minutes. After spinning at 10,000x g for 20 minutes at 4°C, the clear aqueous phase was removed carefully and transferred to a new tube. RNA was precipitated by addition of equal volume of isopropanol. The pellet was washed with 1 ml cold 80% ethanol and dissolved with 300 µl DEPC-treated sterile water. A total of 30 µl 2M sodium acetate (pH 4.0) was added, vortexed, and the total RNA was reprecipitated by 700 µl of cold 100% ethanol, followed by 80% ethanol washing and air drying. Finally, the pellet was dissolved with 20 µl of DEPC-treated water. The RNA content was measured on a photospectrometer and its purity monitored by the ratios of A_{260}/A_{280} and A_{260}/A_{230}.

Reverse transcription-polymerase chain reaction. A total of 2 µg of total RNA isolated above was used to synthesize first strand cDNA in a volume of 20 µl containing 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH8.3), 1 mM each of the four deoxynucleotides, 1 unit RNase inhibitor, 2.5 units M-MLV Reverse Transcriptase, and 2.5 µM $d(T)_{16}$ primer. The mixture was incubated at 42°C for 30 minutes, and the reaction stopped by heating at 99°C for 5 minutes. One quarter of the reaction mixture was used for polymerase chain reaction (PCR). The reaction conditions were as follows: 2.5 units AmpliTag DNA polymerase, 50 nM each of the primers P3 and P4, 0.1mM each dNTP, 3mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl (pH 8.3). The 50 µl reaction mixture was overlaid with 20 µl of mineral oil. DNA amplification was achieved using a Thermal cycler with 30 cycles of 1 minute 95°C, 1.5 minutes 55°C. The sequences of the primers, adopted from Yang et al. (3), are shown below.

Primer P3: $_{-36}$ CCT GAG CAG TCA GCC CGC GC $_{-17}$

Primer P4: $_{701}$ CAA TAG GAC TCC AGA TGT TT $_{682}$

DNA sequencing. The PCR mixture was run on a 5% polyacrylamide gel. After ethidium bromide staining, the desired DNA band was cut, minced, and the DNA purified. one

half of the band was used as template for sequencing with dsDNA cycle sequencing system (GIBCO BRL). The sequencing reaction mixtures were prepared according to manufacture's instruction. Amplification-sequencing profile included 20-30 cycles of 30 seconds at 95°C, 30 seconds at 45°C, and 60 seconds at 70°C. The reaction was stopped by adding 5 µl stop solution containing 95% (v/v) formamide, 10 mM EDTA (pH 8.0), 0.1% (w/v) bromophenol blue, and 0.1% (w/v) xylene cyanol. After heating at 95°C for 5 minutes, the mixture was chilled on ice water and subjected to electrophoresis on 6% polyacrylamide/7M urea gel. The gel was dried and exposed to X-ray film at -80°C with intensifying screen until the desired intensity was achieved.

Sequencing primers used (3):

S1: $_{264}$ ATT TCT ATT CAG T $_{252}$

S2: $_{169}$ ATG GGA GGC CAT C $_{181}$

S3: $_{405}$ TAT AAT TGA CAC T $_{417}$

Results and Discussion

NO• cytotoxicity and genotoxicity. Three independent experiments were performed. Table 1 summarizes the survival and mutation frequency (MF) of TK6 cells after NO• treatment. It was noted that the spontaneous MFs in these cultures differed significantly. This could be just the variation from one experiment to another (W. Thilly, personal communication). When the three sets of data are averaged, there arrives an NO•–induced MF 3-fold higher than that of background.

Nueyun *et al.* reported that 12-20 % survival corresponded to about 10-fold increase in MF over background, using the gas-bubbling method (4). When this is compared to the results shown above, it is suggestive that TK6 may respond to NO• in a delivery system-specific manner. It is conceivable that the two different delivery systems impose different NO• chemistry on TK6 cells. More detailed studies are desirable to prove or disprove this.

Other experiments suggested that the cytotoxicity and MF of NO• toward TK6 cells were dose-rate dependent (S. Tannenbaum and T. deRojas-Walker, unpublished results). In other words, conditions may exist at which low cytotoxicity and significantly high MF over background can be attained. Identification of such a condition (i.e., the dose rate) is important for long-term-low-dose experiment.

NO mutagenesis. A total of 68 independent *hprt* mutants have been sequenced and summarized in Table 2. Among them, 41 produced a RT-PCR product. DNA sequencing on these RT-PCR products revealed that 38% (26/41) had mutations in the coding region of the *hprt* gene. The majority of them (18/26) were base substitutions, with the remaining being deletions (6/26) of 1-27 bp and a 13 bp insertion (1/26). The majority of base substitutions occur at A:T base pairs (12/16). The remaining samples (15/41) which had a RT-PCR product had whole exon deletions, all but one of which being single exon deletions. It has been found that single exon deletion mutants often result from point mutations that affect the corresponding splicing sites in *hprt* gene. Sequencing of genomic DNA from exon-4 deletion mutants confirmed this, in which 3 out of 4 mutants revealed a G to A mutation at the last base of Intron 3, a splicing site of Exon 4 in the *hprt* gene (data not shown). It is not clear, however, if these whole-exon deletion mutants are spontaneous or NO•–induced. Results from numerous other investigators, who used the same methodology, showed that this type of mutation was observed frequently. It will be of interest to see which of these mutants exist in the mixture of spontaneous mutants from a large culture

experiment. Information obtained thereof could become a foundation for interpreting experimental data derived from clone-by-clone methodology.

A comparison of these NO•-induced mutations with recently published spontaneous *hprt* mutations in TK6 cells revealed a striking similarity. Previous study in which *supF* gene was treated with aqueous NO• solution in vitro showed that A:T to G:C was the major type of mutations induced by NO•. In this study, a small increase in the number of A:T to G:C transitions in NO-induced mutants was also observed, although the difference was not statistically significant (the expected value for either of them was smaller than 5). More recently, we found that the mutation patterns in the *hprt* gene of NO•-producing macrophages and unstimulated macrophages were very similar as well. Collectively, these results from this and other studies suggest that: 1) NO• dosing rate affects the cytotoxic and genotoxic responses in same target cells; 2) for mutation study, the NO• delivery system used in this study is a good simulation of NO• produced by macrophages; and finally, experimental systems dramatically affect the conclusions regarding NO• mutagenesis.

The new system was more efficient in delivering NO•, and a reproducible toxicity and mutational frequency was observed. Under the conditions used in these experiments, however, the MF was not as high as the one using the old system. We recently reported that TK6 cells co-cultured with macrophages stimulated to produce NO• showed differential genotoxic responses in the two endogenous genes. Specifically, there was a significant increase in mutant fraction in the *tk* gene of cells, whereas no difference was found in the *hprt* gene of same cells. As previously discussed, NO• can increase large gene deletion mutations in an endogenous gene of mammalian cells, and it was observed that mutagens capable of inducing large gene deletions often had a differential effect on the mutant fraction values measure in *tk* and *hprt* genes of TK6 cells. Thus, it will of great interest to examine the mutagenic responses in the *tk* gene of cells in this study. On the basis of previous findings, it is conceivable that the mutant fraction measured at the *tk* locus of TK6 cells will be significantly higher than the 3-fold increase observed at the *hprt* locus reported in this study. Results from this study will provide important insights into the molecular mechanisms through which NO• production leads to gene mutations in mammalian cells. It may also prove possible to extract information regarding the mutagenic specificity of a treatment by comparing the relative responses to the treatment in *tk* and *hprt* genes of TK6 cells.

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Table 1. Summary of cell survival and mutant fraction in TK6 cells treated with NO through 20 cm of Silastic tube for 60-70 min.

NO dose	Survival(%)	Mutant fraction (x10-6)
Experiment 1		
0	100	8.7
0.8 mM	12	29
Experiment 2		
0	100	0.55
0.7 mM	12	6.0
Experiment 3		
0	100	3.4
0.7 mM	16	6.0

Table 2. *Hprt* mutants in TK6 cells treated with aqueous NO• solution.

Category	NO•-treated	Spontaneous[a]
No RT-PCR product	27 (.40)	31 (.31)
Missing exon(s)	15 (.22)	40 (.40)
Exon insertions	0 (00)	2 (.02)
Coding region	26 (.38)	27 (.27)
Total Mutant	**68**	**100**

(a) From Lichtenauer-Kaligis et al. (1995) *Mutagenesis* **10**, 137-143.

Table 3. Mutations in the coding region of *hprt* gene in TK6 cells treated with aqueous NO• solution.

Category	NO•-treated	Spontaneous[a]
A. Total Mutant	**26**	**42**
Deletion	7 (.27)	9 (.21)
Insertion	1 (.04)	2 (.05)
Multiple mutations	0 (00)	1 (0.2)
Point mutations	18 (.69)	30 (.71)
B. Total point mutations	**18**	**30**
A:T → G:C	5 (.28)	1 (.03)
A:T → T:A	6 (.33)	7 (.23)
A:T → C:G	1 (.06)	5 (.17)
G:C → A:T	4 (.22)	10 (.33)
G:C → T:A	2 (.11)	4 (.13)
G:C → C:G	0 (00)	3 (.10)

(a) From Lichtenauer-Kaligis et al. (1995) *Mutagenesis* **10**, 137-143.

Conclusions and Suggestions for Future Research

This thesis work has accomplished its objectives, in that it clearly demonstrates the genotoxic potential of NO• produced by macrophages and thus support a possible role for its involvement in the carcinogenic process. The results also provide evidence that NO• production leads to cytotoxicity through mechanisms more complex than previously thought. At this stage, little is known about the nature of these processes. A search for answers to these questions is certain to be an intellectually challenging and exciting undertaking. The experimental systems and approaches developed in this work will provide a set of useful tools to achieve this goal. Furthermore, what has been learned from this work may serve as a framework upon which future experiments can be devised.

An interesting finding was that macrophages, as NO• producers, are not more or less affected by NO•-mediated toxicity than are other cells; much of the damaging activity of NO• occurs outside of the producing cells. NO• is a relatively lipophilic, freely diffusible small molecule, which is readily released into the extracellular space once produced by a macrophage cell. Its biological function is by and large governed by the type of target molecule it first encounters. In the setting of infection or inflammation, one candidate target for NO• is superoxide, generated concurrently by the same individual macrophage, or produced by other inflammatory cells. A careful examination of oxygen metabolism in neutrophils suggests that like NO•, superoxide itself probably has little toxicity. In neutrophils, large quantities of superoxide are generated by the respiratory burst. The majority of superoxide is probably converted into other reactive oxygen species (ROS) through the peroxidase/H_2O_2/halide system, because myeloperoxidase (MPO) in neutrophils accounts for 1-5 % of the dry cell mass, and the formation of HOCl accounts for 30-50 % of the oxygen consumed by stimulated neutrophils. Evidently, neutrophils are committed to this function, because the biosynthetic capability is lost before they leave bone marrow.

The magnitude of the respiratory burst decreases markedly when monocytes mature into macrophages, but maturation is accompanied by increased biosynthetic capability. Both the respiratory burst and biosynthesis can be increased many folds when macrophages are activated. Macrophages, as opposed to neutrophils and peripheral blood monocytes, have very low levels of MPO and therefore must rely on MPO-independent mechanisms predominantly. MPO-independent mechanisms in macrophages are presently poorly understood. By virtue of the ability

of NO• to react rapidly with superoxide to form peroxynitrite leading to more reactive species like hydroxyl radicals and singlet oxygen, it is tempting to propose that NO• may represent such an MPO-independent mechanism in macrophages. In other words, NO• potentiates the action of superoxide. Indeed, when NO• production by stimulated macrophages is blocked by NMA, much of the cytotoxicity associated with NO• production is also lost, even though superoxide production remains intact.

Macrophages stimulated with IFN-γ and LPS generate twice as much NO• as superoxide. Excessive amounts of NO• produced by macrophages can also react with molecular oxygen, leading to the formation of N_2O_3. As a nitrosating agent, N_2O_3 can induce cell damage by reacting with cytosolic proteins and also with DNA. As the concentration of free NO• decreases through these reactions, the remaining small amount of NO• may become quite stable, capable of travelling several cell diameters away from its generator macrophage. Some of the free NO• may penetrate target cell membranes and eventually react with the iron-sulfur moiety in mitochondrial enzymes, leading to growth arrest in the target cell. Alternatively, the free NO• may react with superoxide or hydrogen peroxide formed inside target cells, thereby causing cell toxicity. Thus, high levels of NO• produced by macrophages enables them to use ROS as a major microbicidal mechanism without losing biosynthetic activities, and at the same time to kill microorganisms or tumor cells that may be resistant to killing by superoxide-derived species.

Several pieces of evidence from this work suggest that at least in macrophages, the several types of cellular damage (cell death, growth arrest, and genotoxicity) associated with NO• production are mediated by different reactive species. An important remaining question will be: how will a change in the extracellular level of superoxide affect the profile of NO•-associated cellular damage in macrophages? There are several ways to modulate the extracellular level of superoxide of macrophages. On an enzymatic level, superoxide dismutase and/or catalase can be added to the cell culture. It is not clear whether this would be effective in inactivating superoxide due to its potentially rapid reaction with NO•. On a biochemical level, a reversible NADPH oxidase inhibitor can be added to stimulated macrophage cultures. In this instance, experiments need to be done to determine the effectiveness and toxicity of the compound. On a genetic level, variant RAW264.7 cells defective in superoxide production may be selected. If successful, the variant cells will also prove to be a valuable resource for future research. At the other end of the spectrum, superoxide levels can be increased by addition of a generating system such as xanthine/xanthine oxidase, or simply by adding stimulated neutrophils. Alternatively, a RAW264.7 variant with increased superoxide production can be sought.

Another question to ask will be the effects of oxygen tension on the NO•-associated cellular damage in macrophages. Experiments of this sort may be relatively more straightforward. More specifically, during cell culture and in particular during stimulation of macrophages, the level of oxygen can be increased or decreased to produce a hyperoxia or hypoxia condition, respectively. This can be done alone or in combination with modulation of superoxide levels, as described above.

Examination of effects of each condition on a multiplicity of responses in stimulated macrophages, including cell viability, growth arrest, and genotoxicity will be of importance. As a matter of fact, assays addressing other biological endpoints need to be developed and incorporated into the current cellular damage profile. Among others, the expression of stress proteins and levels of lipid oxidation products in stimulated macrophages under different conditions will be of particular pertinence to the overall objective. It is likely that some of the conditions mentioned above may lead to decreased cell death but simultaneously to increased growth arrest or genotoxicity. Disproportionate responses to a particular condition will be most informative.

The co-culture model system developed in this thesis will add another dimension to the experiments described above. Specifically, responses in target cells to changes in superoxide level or/and oxygen tension can be compared with those in macrophages. Furthermore, it is even feasible to co-culture two types of target cells with macrophages and examine their relative responses. In a co-culture experiment, the ratio of target:macrophage cells and the cell density are additional variables that could be manipulated. In a longer term, it will be of great interest to co-culture two RAW264.7 variants, one defective in superoxide production and the other in NO• production and determine whether cytotoxicity differs in the two cell types. Results from this experiment will provide useful information regarding the kinetics of NO• and superoxide under physiologically relevant conditions.

The effect of αMEM medium on genotoxicity of NO•-producing macrophages warrants further investigation. First of all, the active component(s) responsible for the effect should be identified. This could turn out to a daunting task. Without additional information, it is still interesting to examine the effect of αMEM on genotoxicity in macrophages stimulated to produce NO• over many generations. Molecular analysis of *hprt* mutants isolated under this or a similar condition may shed light on the mechanisms of action of components in αMEM medium.

A long-term co-culture experiment should be performed to examine whether continuous production of NO• at lower levels can also result in genotoxicity in target cells, and if so, to what

extent compared with macrophages. Finally, the neoplastic transformation potential of macrophage-derived NO• should be explored.

On the basis of the findings that co-culture with neutrophils for 60 min or with macrophages for 36 hr result in substantial cell death and significant genotoxicity in target cells, it is possible that acute infections may also pose a significant risk in the carcinogenic process. Perhaps chronic infection serves more as a "smoking gun" than really required for cancer initiation. Alternatively, production of ROS and NO• by inflammatory cells during acute infections may provide "one hit" for the carcinogenic process, whereas chronic infection provides multiple hits. If that were true, a possibly effective cancer prevention strategy may emerge, namely providing our children with a high quality living environment to avoid major infections.

Biographical note

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