EVALUATION OF HOLLOW FIBER BIOREACTORS AS AN ALTERNATIVE TO MURINE ASCITES PRODUCTION FOR SMALL SCALE MONOCLONAL ANTIBODY PRODUCTION

by

Lynn Rae Jackson, DVM

Submitted to the Division of Toxicology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Toxicology

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

May, 1994

Signature
of Author _____
Division of Toxicology
May 6, 1994

Certified by _____
Professor James G. Fox
Thesis Supervisor

Accepted by _____
Professor Steven R. Tannenbaum
Chairman, Department Committee on Graduate Students

ABSTRACT

Monoclonal antibody (MAb) production via the growth of ascitic tumors in mice has been the time-honored technique for producing small scale, research laboratory quantities of MAbs. There are many disadvantages associated with MAb production in murine ascites, including animal welfare concerns. Small scale, commercially available, hollow fiber bioreactor systems, which appear to have advantages over other in vitro cell culture techniques, have recently been introduced. To the author's knowledge, these bioreactor systems have not been independently evaluated as a potential alternative to the in vivo production of MAbs in murine ascites. The first objective of this study was to characterize the clinicopathologic changes in mice associated with the in vivo production of MAbs. Five different hybridoma cell lines were grown in groups of 20 mice. Mice were primed with 0.5 ml pristane intraperitoneally 14 days prior to inoculation of 1×10^6 hybridoma cells. Abdominal paracentesis was performed a maximum of 3 times for collection of ascites from each mouse; ascites volumes were recorded. Clinical observations, body weight measurements, and postmortem examinations were performed. Incidence and severity of clinical abnormalities increased with time. Disseminated intraabdominal seeding of tumor and/or solid tumor masses were observed at postmortem examination. The second objective of the study was to compare MAb production in mice versus hollow fiber bioreactor systems to determine the feasibility of these systems as potential alternatives to the use of mice. Three hybridoma cell lines were grown in each of three different commercially available hollow fiber bioreactor systems and in groups of 20 mice. Bioreactors were harvested 3 times weekly for 65 days and were monitored by cell counts, cell viability and media glucose consumption. Time and materials logs were maintained. Time spent in labor and materials costs were greater for the bioreactors than the mice. The mean antibody concentration, as determined by ELISAs, in mouse ascites versus the range of mean concentrations for the 3 bioreactor systems for each cell line were as follows: cell line 2B11, 4.22 mg/ml vs. 0.71 to 1.31 mg/ml; cell line 3C9, 4.07 mg/ml vs. 2.16 to 4.45 mg/ml; and cell line RMK, 8.37 mg/ml vs. 2.30 to 11.08 mg/ml. The total quantity of antibody produced in 20 mice versus the mean production for 3 bioreactors in 65 days was as follows: cell line 2B11, 454.50 mg vs. 168.35 mg; cell line 3C9, 445.57 mg vs. 564.52 mg; and cell line RMK, 996.64 mg vs. 1023.06 mg. Significant clinicopathologic changes in mice were associated with MAb production in ascites. Although time and materials costs were greater for the bioreactors, these results suggest that hollow fiber bioreactor systems merit further investigation as potentially viable alternatives to the use of mice for MAb production.

ACKNOWLEDGEMENTS

First I would like to thank my advisor, Professor James Fox for his continued support and encouragement throughout the time over which this research project was conducted.

I would like to thank Dr. Sally Seaver for her insights regarding the experimental design of this study, and Professor Gerald Wogan for making his hybridoma laboratory available for my use.

I wish to give special thanks to all the technical representatives from the hollow fiber bioreactor companies which generously provided instrumentation for use in this study along with many hours of technical advise.

I am especially grateful to all those at DCM, and in particular, Drs. Robert Marini, Karl Andrutis, and my fellow postdocs, for fostering my progress with their ever present words of encouragement and their willingness to take extra burdens upon themselves to compensate for my inflexible schedule and absences due to class time, thesis writing, and other degree candidate obligations.

My most special thanks go to the two people who must be deservingly credited with originating the conceptual framework for this work, Dr. Neil Lipman and Laura Trudel. Without Neil's support, guidance, and friendship, and without Laura's technical assistance, patience, and love, this project would never have been completed.

And finally, I would like to thank Marian Walke for generously providing her time and expertise to help design and execute graphics for this thesis as well as other presentations of these data.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (cont'd)

LIST OF TABLES

SECTION 1: INTRODUCTION

Mice which have been injected intraperitoneally with an ascitogenic priming agent, followed by inoculation with hybridoma cells, produce ascites which contains high concentration of monoclonal antibody secreted by the hybridoma cells. This fluid can subsequently be aspirated from the abdominal cavity and then processed and purified to obtain the antibody. This has been the time-honored technique for producing small scale, research laboratory quantities of monoclonal antibodies.

From personal observation of mice used for ascites production, and scientific reports, it was apparent that significant morbidity and mortality may be associated with this procedure. There is considerable information in the literature regarding antibody production parameters, but relatively little information which addresses the clinicopathologic changes in mice associated with this procedure.

In vitro methods are available for growth of hybridoma cells, but there are significant disadvantages associated with these techniques which have discouraged their widespread use.

As veterinarians and others with concerns for the welfare and humane care of animals used in research, we sought to characterize the clinicopathologic changes in mice associated with ascites production and identify a viable *in vitro* alternative to the use of mice for small scale production of monoclonal antibodies.

SECTION 2: BACKGROUND AND SIGNIFICANCE

History of Antibody Production in Murine Ascites

In 1957, Munoz first described a technique for obtaining antibodies from mouse ascitic fluid. He demonstrated that intraperitoneal injection of complete Freund's adjuvant-antigen mixtures in mice resulted in the production of large volumes of ascites which contained high concentrations of antigen-specific antibody (57). In 1961, Lieberman et al reported that ascites was produced following intraperitoneal injection of incomplete Freund's adjuvant and heat-killed Staphylococcus aureus in mice preimmunized subcutaneously with horse serum and incomplete Freund's adjuvant. Seventeen strains of mice were evaluated in that study. Strain variation and individual variation in ascites production was demonstrated, with CAF_1 mice producing relatively large volumes of ascites. BALB/cAnN mice which received incomplete Freund's adjuvant alone also produced ascites, and 10 of 19 animals that survived beyond 6 months developed plasma cell tumors (58).

Following observations of plasmacytoma induction by intraperitoneal injection of mineral oil into BALB/c mice, Anderson and Potter, in 1969, identified a component of mineral oil, pristane (2,6,10,14-tetramethylpentadecane), a saturated branched-chain hydrocarbon, as a potent inducer of plasmacytomas in BALB/c mice following 3 intraperitoneal

injections. Nineteen of 30 animals developed tumors within one year. One of these plasmacytomas was found to produce monoclonal antibody (55). Cells obtained from a plasmacytoma induced by intraperitoneal injection of mineral oil into a BALB/c mouse were referred to as MOPC for mineral oil plasmacytoma, and continuous plasmacytoma cell lines subsequently derived and established from this tumor are currently in use as fusion partners for production of hybridomas (59). In 1972, Potter et al demonstrated that plamacytomas could be propagated as ascites tumors in mice which had been pretreated with an intraperitoneal injection of mineral oil (60).

In 1976, Köhler and Milstein first described fusion of BALB/c mouse plasmacytoma cells and spleen cells from immunized mice, with subsequent selection to produce a hybridoma cell line secreting antibody of predefined specificity (1). Subcutaneous injection of these hybridoma cells into BALB/c mice lead to solid tumor formation and intraperitoneal injection resulted in the development of ascites tumors. The sera of these animals was found to contain specific antibody, and they reported that these cells could be grown in vitro to provide antibody (1,2).

Monoclonal Antibody Production in Murine Ascites

There have been many developments in the production and application of monoclonal antibodies since Köhler and Milstein first described the technique for producing

hybridoma cells which secreted monoclonal antibodies (1,2). Ascites production following intraperitoneal inoculation of hybridoma cells into pristane primed, histocompatible mice has been the time-honored technique for producing monoclonal antibodies (3,4,5). Ascitic tumors form in the abdominal cavity of the mouse, and the antibody-rich ascitic fluid is harvested by paracentesis.

Although an extremely useful technique, there are a number of disadvantages associated with production of monoclonal antibodies via the induction of ascitic tumors in mice which have been cited. Many of the criticisms which have been raised reflect humane concerns regarding the potential for different aspects of this procedure to induce pain or distress in the animals. These concerns are magnified by the large number of mice used in this process.

Pristane, a hydrocarbon derived from mineral oil, is commonly used to prime the peritoneal cavity prior to hybridoma cell inoculation. Pristane is thought to act by inducing granulomatous inflammation and interfering with peritoneal lymphatic drainage (6) thus increasing the volume of ascites produced. Injection of large volumes of pristane intraperitoneally in mice has been associated with weight loss, hunched appearance, and inactivity (7). The exact volume of pristane administered was not indicated, however. Peritonitis is known to cause abdominal pain in animals (8).

According to the material safety data sheet, pristane

can cause severe irritation if it comes into contact with the eyes, mucous membranes, skin, respiratory, or gastrointestinal tract, and it is classified as a tumorigenic agent, therefore requiring that appropriate precautions be taken when handling the agent.

Inoculation of large numbers of hybridoma cells has been shown to cause significant mortality in mice. In a study reported by Chandler, 3 groups of 20 pristane primed BALB/c mice were inoculated with 1 $\times 10^5$, 5 $\times$ 10^5, or 2 $\times$ 10^6 hybridoma cells, respectively. In the group which received 2 $\times$ 10^6 cells, 11 of 20 mice died before the second abdominal tap. Chandler also reported that for most cell lines, even when employing a standard inoculum of 5 $\times$ 10^5 cells/mouse, only 10-25% of the animals usually survive to the third abdominal tap (4).

In humans, mild ascites is often asymptomatic and generally not painful, but large amounts of ascites can cause severe abdominal discomfort, respiratory distress, anorexia, nausea, heartburn, and difficulty in ambulation (9,10). Ascites also causes abdominal pain in animals (8). Increased abdominal pressure created by ascites results in cranial displacement of the diaphragm which decreases ventilatory capacity and compromises gas exchange. Respiratory distress may be exhibited clinically as tachypnea or dyspnea. Additionally, increased abdominal pressure can produce venous stasis in the abdomen, decrease arterial blood pressure, and decrease renal blood flow (8).

The potential for abdominal distention and pain induced by the increasing abdominal tumor mass has also been addressed (5,11,12). Abdominal carcinomatosis in humans has been reported to be painful (9). While abdominal pressure may be at least temporarily reduced by removal of ascitic fluid, the tumor remains and continues to increase in mass over time. Abdominal carcinomatosis in humans and animals is generally associated with progressive weakness, debilitation, and cachexia (8,9).

In both human and veterinary medicine, it is not generally recommended that ascites fluid be removed by paracentesis. Potential complications associated with rapid or repeated removal of ascites include hypovolemia from compensatory fluid shifts and hypoalbuminemia (13,14). McGuill and Rowan have suggested that the possibility of physiologic stress due to hypovolemia as well as the possibility of stress induced by the collection procedure should be considered with regard to repeated abdominal paracentesis in mice (5). The number of abdominal taps permitted for collection of ascites from mice is now limited to 1 to 3 taps by most Institutional Animal Care and Use Committees.

As a result of the animal welfare concerns which have been raised, several European countries have established guidelines or regulations which restrict or prohibit ascites production in rodents. The United Kingdom Coordinating Committee on Cancer Research (UKCCCR)

"Guidelines for the Welfare of Animals in Experimental Neoplasia" states that predictable death is generally an unacceptable end point, that the volume of ascitic fluid should not become excessive or cause gross abdominal distention, and that solid tumor growth and cachexia should not become clinically significant. The guidelines state that ascitic volumes should not usually exceed 20% of normal body weight, that ascites should normally be drained only once, and that general anesthetics should be employed during paracentesis (12).

The Switzerland "Rules for the Judgement of Approval Requests for Animal Experiments for the Production of Monoclonal Antibodies" states that the ascites procedure is incompatible with the animal protection law, the Netherlands "Code of Practice for the Production of Monoclonal Antibodies" states that the in vitro method will be preferred to the in vivo method, and the German Federal Health Office in Berlin has accepted the UKCCCR guidelines (11).

Although murine monoclonal antibodies can be produced in high concentrations by growth of cells as ascitic tumors in histocompatible mice, similar attempts to grow heterohybridomas and xenogenic hybridomas have been much less successful (15,16,17,18,19,20). Methods such as X-ray irradiation of mice (16,17); treatment with immunosuppressive drugs such as antilymphocyte globulin, methotrexate, cyclophosphamide (16) or hydrocortisone (17);

prior subcutaneous adaptation of hybridoma cells (15); and the use of T-cell deficient (15,16,18) or T-cell and B-cell deficient SCID mice (19) have been employed in attempts to improve production. Even when utilizing one or more than one of these methods simultaneously, the efficiency of take for human hybridomas in mice may be as low as 50% (20). The additional expense of purchasing and maintaining aseptic housing and handling practices for immunodeficient mice impacts production costs.

Other concerns regarding antibody raised *in vivo* in murine ascites, particularly in relation to products intended for human clinical use, include the potential for contamination by murine adventitious agents, endogenous murine immunoglobulin and other murine proteins and nucleic acids, endotoxin, and residual pristane (21). The potential presence of adventitious agents and endogenous immunoglobulin impose additional difficulties in purification and quality control testing of the product (22). It should be noted, however that all of these concerns are not unique to antibody production in mouse ascites; for example, if the hybridoma itself is of animal cell origin there are concerns regarding adventitious agents regardless of the method of production (23). Cell culture media additives such as serum and antibiotics pose similar concerns for contamination of the product (22).

Using immunocytochemical techniques, Spicer *et al* recently identified contaminants in several murine ascites-

derived monoclonal antibodies which demonstrated binding to Golgi zones on epithelial cells that was unrelated to the antigenic specificity of the antibody. Hybridoma cell culture supernatants examined from two of the hybridomas showed no Golgi staining. Additional studies suggested that the ligand reacting with the Golgi complex could also be found in some mouse sera and was related to the presence of mouse anti-blood type A_1 antibody. Based on these findings, the authors warn against making the assumption that ascites-derived monoclonal antibodies will always demonstrate absolute antigen specificity in immunocytochemical staining (24). These findings point to possible sources of lot to lot variability in murine ascites-derived monoclonal antibodies.

Truitt et al reported gross morphological changes and changes in the DNA content of human/human hybridoma SA13 ascites passaged cells from nude mice by comparison to cells grown only in culture. Ascites passaged cells had greater aneuploid DNA level, and the authors hypothesized that the hybridoma cells may have taken up mouse DNA or over-replicated human chromosomal material. They suggested that this could be an indication of genetic instability resulting from the introduction of cultured cells into the foreign environment of the mouse peritoneal cavity (15).

To study the possible effects of pristane on the DNA of murine hybridoma cells, Garrett et al, using flow cytometric profiles of propidium iodide-stained hybridoma

cells obtained from ascites 1 month post pristane and 2 weeks post hybridoma cell inoculation, showed decreased fluorescent intensities compared to the parent hybridoma cell line. Diphenylamine analyses demonstrated that there was no change in the DNA content of the cells. There was a direct correlation between altered propidium iodide staining characteristics, which suggested a conformational change in the DNA, and the presence of cell-associated pristane. The authors proposed that pristane may have interacted directly with the DNA of hybridoma cells or via and indirect effect on DNA mediated by pristane-induced release of factors from inflammatory cells (25). These results also point to the potential for genetic instability of hybridoma cells grown in vivo.

Mammalian cells are used to produce complex proteins because they competently fold and post-translationally modify the proteins which are important factors in maintaining biological activity (26). Recent evidence suggests monoclonal antibody glycosylation patterns may differ dependent on the production method employed (27). In comparison of monoclonal antibodies derived from mouse ascites and cultured cells, differences in both the type and distribution of oligosaccharides present on the antibody have been documented (27,28,29). Additional findings included differences in solubility and RPLC peptide maps (28), and in binding activity, charge heterogeneity, conformational stability, and

pharmacokinetics (29).

All of the findings cited thus far substantiate the need for alternatives to the use of mice for monoclonal antibody production.

History and Applications of Monoclonal Antibodies

Monoclonal antibodies are valuable tools used in a variety of clinical and research applications. Historically, in the mid to late 1970's most monoclonal antibodies were used in research, and quantities of 100 mg were considered to be very large. Mouse ascites and conventional suspension culture were the production systems of choice. By the early 1980's, use of monoclonal antibodies in immunopurification of biologicals and in *in vitro* diagnostic kits dramatically increased the need for large quantities of antibodies. Beginning in the mid 1980's, monoclonal antibodies were evaluated for *in vivo* use for diagnostic imaging and therapeutic applications. These applications further increased the need for large scale production of monoclonal antibodies in the range of 100s of kilograms or more per product, and encouraged development of novel *in vitro* culture methodologies (30).

Current applications for monoclonal antibodies include *in vitro* affinity purification, and immunodiagnostics for tissue typing, therapeutic drug monitoring, and quantitation of hormones, growth factors, immunoglobulins, enzymes, and microorganisms. Monoclonal antibodies are

used *in vivo* as diagnostic imaging agents, and monoclonal antibody-toxin conjugates are being used for cancer therapy (30,31). Monoclonal antibodies are also being used as catalysts and templates for organic chemical reactions (31).

Monoclonal Antibody Production In Vitro

In vitro cell culture systems are now widely used for large scale production of monoclonal antibodies (26,30,32). In the research laboratory, small quantities, i.e. milligram quantities, of many different monoclonal antibodies are generally required. Disadvantages associated with many *in vitro* techniques have made them unsuitable for widespread use as alternatives to murine ascites for small scale monoclonal antibody production.

There are many advantages of monoclonal antibody production in mice against which *in vitro* technologies must compete. Production is rapid in mice with ascites often developing within 2-10 days following hybridoma inoculation (33). Mice can support the growth of most cell lines (4) and minimal materials and technical expertise are required. Antibody in mouse ascites is highly concentrated, ranging from approximately 0.5-20 mg/ml, and the concentrations in cell culture supernatants have been reported to be between one-hundreth and two-thousandths of the concentration achieved in mice (11). Because of low antibody concentrations in cell culture supernatants, large volumes

of media and extensive concentration of the antibody are required (34). Antibody production in mice has also been reported to be less costly than *in vitro* production (35).

A variety of *in vitro* cell culture techniques have been employed to produce monoclonal antibodies from hybridoma cells on a laboratory scale. The methods commonly used include stationary cultures in T-flasks, and suspension cultures in roller bottles and spinner flasks, all of which are generally handled as batch cultures (11,20,22). Other techniques include growth of cells in dialysis tubing (36), use of oscillating bubble dialysis chambers (34), microencapsulation of cells in gelled sodium alginate spheres coated with a biopolymer to form a semi-permeable membrane (30,37), and entrapment of cells in agarose beads (38). Laboratory scale stirred tank reactors (39,40), fermentors (41), and ceramic-matrix bioreactors (42) have also been used Advantages and disadvantages have been cited for all these production systems, but one disadvantage was consistent; the antibody product obtained from these *in vitro* systems was far less concentrated than that produced in mouse ascites.

Hollow Fiber Bioreactors

Recently, commercially available, laboratory scale hollow fiber bioreactor systems have been introduced. Initial investigations of these systems suggested that they had advantages over other *in vitro* systems and might

provide an economically feasible alternative to mice for small scale production of monoclonal antibodies.

The hollow fiber/artificial capillary cell culture system was first introduced by Dr. Richard Knazek et al in 1972 (43). These systems were modeled after and intended to simulate the in vivo capillary system in an attempt to maintain a more physiologic environment for cultured cells with regard to nutrient supply, metabolic waste removal, and pH, while providing a stable pericellular microenvironment (44).

Schematic diagrams of a hollow fiber bioreactor in longitudinal and cross-sectional views are presented in Figures 1 and 2, respectively. The bioreactors consist of bundles of hollow fibers through which culture media is continuously perfused in the intracapillary space. The cells are grown in the extracapillary space which surrounds the fibers. The walls of the hollow fibers serve as semi-permeable ultrafiltration membranes. The pore size of the membrane, depending on the molecular weight cut-off, is small enough to retain cells and secreted antibody highly concentrated in the relatively small volume of the extracapillary space, while permitting gas, nutrients, and metabolic waste products to freely diffuse across the membrane due to hydrostatic pressure differences and concentration gradients. Similarly, macromolecules in the perfusing media remain within the fiber lumina. Harvest ports provide access to the extracapillary space for

examination of cells and harvest of cell secreted products (22,45).

Figure 1.
Schematic Diagram of a Hollow Fiber Bioreactor

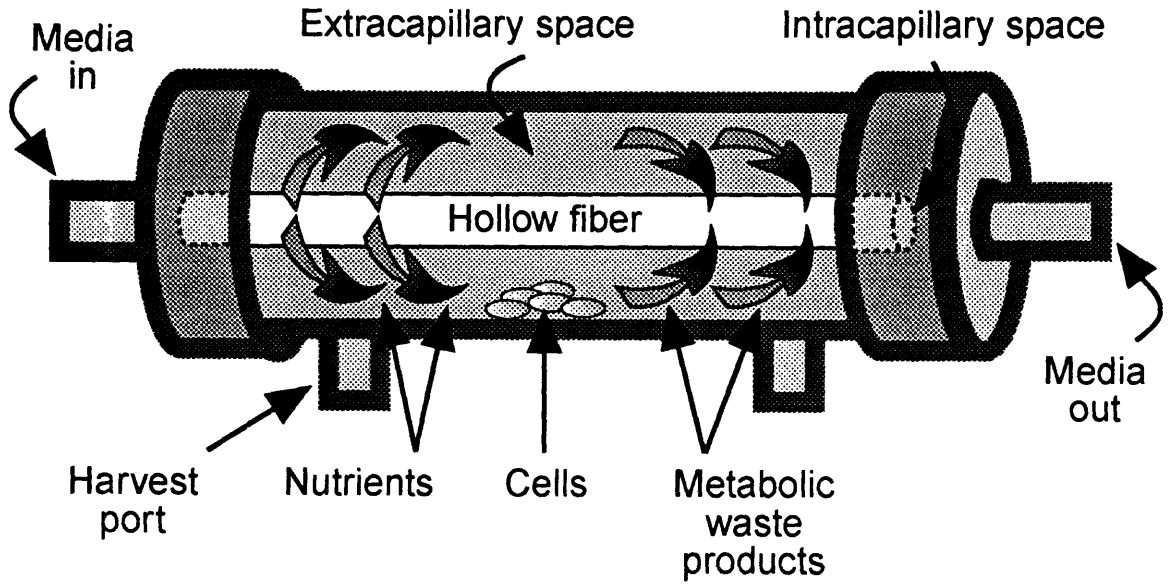
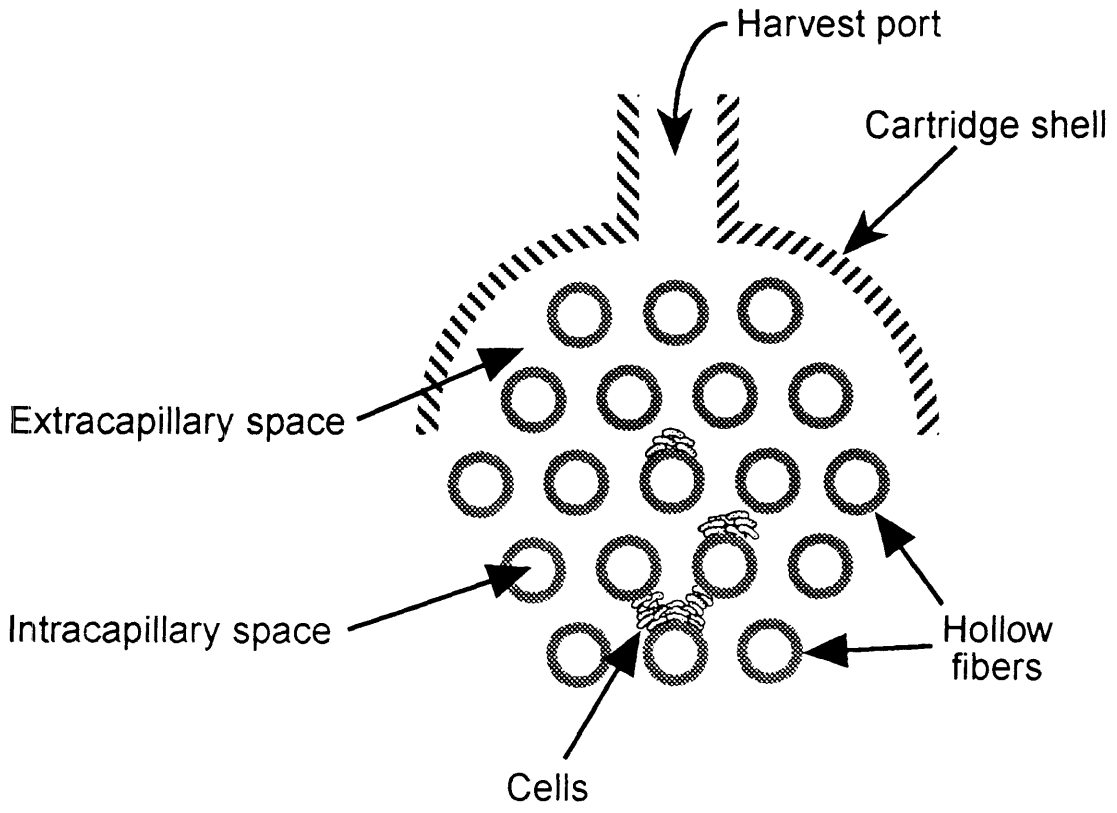


Figure 2.
Schematic Diagram of a Hollow Fiber Bioreactor in Cross Section



In many reports comparing antibody production using different cell culture methodologies, hollow fiber bioreactors have consistently produced the most concentrated product, ranging from 0.7 to 2.3 mg/ml (42,46,47). Antibody concentration up to 17 mg/ml has been reported (48). Antibody production rates for different cell lines in hollow fiber bioreactors are correlated with production rates in static culture (47).

In addition to the production of a highly concentrated antibody product in a small volume, precluding the necessity for extensive downstream concentrating techniques, there are many other reported advantages of hollow fiber bioreactor systems in comparison to other in vitro systems and in vivo systems. Hollow fiber bioreactors have been shown to successfully support the growth of many cell types from different species to include both anchorage-dependent and anchorage-independent, primary and established, normal and malignant, fibroblast and epithelial cells (45). The design and operation of hollow fiber bioreactors using continuous perfusion of media simulates the in vivo environment and provides an uninterrupted supply of essential nutrients and removal of metabolic waste products necessary for optimal production (32). The cells are protected from shear (40,46), which is important because many hybridomas have been found to be sensitive to shear, requiring low-shear mixing systems in agitated cultures (22). Resistance to shear appears to be

clone-specific (22).

Cells can be grown to very high densities ($\geq 10^7$-10^8 cells/ml) and cell viability and production can be maintained for long periods of time (22,32,45,49), from weeks to months. Continuous production for up to 6 months has been reported (47). The most frequent production kinetics found in mouse and human hybridoma cell lines is categorized as type II in which the highest monoclonal antibody-specific production rate is at the beginning of the exponential growth phase and at the stationary and decline phases (22). Growth of cells in continuous perfusion culture with maintenance of production for long time periods, as opposed to batch processes, takes maximum advantage of the production kinetics of hybridomas (22,26). Stability of the cell line must be ensured for continued antibody production (22,50).

Purity of the antibody product has been facilitated by gradual serum reduction in the culture medium which has been achieved in hollow fiber systems without significant inhibitory effect on production (42,45,46,50,51). Decreased serum utilization also decreases downstream processing and production costs (42).

The ability to frequently or continuously remove the product from the bioreactor during the run has a number of advantages which include decreased potential for degradation of the product resulting from prolonged exposure to the culture supernatant containing cellular

proteases, it minimizes the potential for feedback inhibition, and minimizes losses in the event of mechanical failure or microbial contamination (22,26).

Use of microporous hollow fiber bioreactors with large fiber pore sizes, between 0.2 and 0.5 μ, have been described (26,32,51,52). In comparison to ultrafiltration membranes, greater antibody production has been achieved with these microporous membranes (26,52), however the product is not retained within the small volume of the extracapillary space and must be concentrated from the large volume of perfusing media. Studies conducted by Kidwell demonstrated $TGF\beta_1$ production by 2 hybridoma cell lines inhibited cell growth and antibody production, and suggested that retention of inhibitory factors in the cell space of low MWCO bioreactors was probably accountable for production differences seen between microporous and ultrafiltration cartridges (52).

There are cited disadvantages associated with the use of hollow fiber bioreactors. As with all *in vitro* systems, there is the potential for microbial contamination (22,53), and technical expertise in cell culture is required for successful operation without contamination (53). The potential for mechanical failure also exists (22). Equipment and materials required have been considered expensive compared to those required for *in vivo* production (34).

Because of the nonhomogeneous nature of the hollow

fiber bioreactor, cell gradients with variable density, viability, and productivity exist within the reactor making it difficult to obtain a representative sample of cells from the reactor for monitoring (40,48). In studies conducted by Piret and Cooney, axial sectioning analyses of hollow fiber bioreactors demonstrated downstream concentration of viable cells, the monoclonal antibody product, and high molecular weight proteins. These findings were attributed to convective fluxes and gravity sedimentation phenomena. The authors speculated that downstream polarization of high molecular weight growth factors was responsible for the downstream concentration of viable cells. Reversing the direction of media flow through the reactor in 5 minute cycles improved protein and cell distribution within the reactor, and the number of cells recovered from the reactor and the amount of antibody produced in 6 days was 3 times greater than that produced by a corresponding reactor without alternation of media flow direction (48). Concentration gradients of nutrients and metabolites are also generated within hollow fiber bioreactors (40).

Hollow fiber bioreactor technology is relatively new and rapidly evolving and as a consequence there are frequent modifications in both system components and recommended operational protocols as more information becomes available regarding their use.

After reviewing the hollow fiber bioreactor literature,

it appeared that these systems had potential as a viable alternative to the use of mice for small scale monoclonal antibody production, and as such might serve as a reduction or replacement technology.

SECTION 3: OBJECTIVES AND EXPERIMENTAL DESIGN

The First Objective

One of the objectives of the current study was to characterize the clinicopathologic changes in mice associated with the *in vivo* production of monoclonal antibodies from ascitic tumors. There are numerous publications describing the use of mice for monoclonal antibody production, but the focus has generally been on production parameters. Although abdominal distention with ascites formation, the presence of tumor in the abdominal cavity, and death have been described in many publications, to the author's knowledge, detailed information describing the clinicopathologic changes in the animals is absent from the literature.

Abdominal lesions have been described in nude rats in which complete Freund's adjuvant was used as the priming agent in repeated doses ranging from 0.5 to 5.0 ml. Extensive adhesions were observed in the abdominal cavity at postmortem examination. With mineral oil or pristane priming, tumors grew in nodular patches on the mesentery and infiltrated the peritoneal cavity (16,18). Results of gross and microscopic examinations of mice with abdominal plasmacytomas induced by mineral oil or pristane have been reported (54,55,60).

A brief protocol review described a veterinarian's evaluation of 10 ascites mice from the time of pristane injection through ascites production to provide information for an Institutional Animal Care and Use Committee. Animals were observed twice daily for food intake and behavior. It was reported that for 12 hours after pristane injection and after the third collection of ascites fluid by peritoneal tap, the mice exhibited decreased food intake and decreased exploratory behavior (56).

As there is considerable variation in the biological behavior of different hybridoma cell lines in mice (4), it was decided that 5 different hybridoma cell lines would be evaluated in the current study. Clinical parameters to be monitored would include body weight measurements, and daily observation of general appearance, haircoat, posture, level of activity, and assessment of degree of abdominal distention. Gross postmortem examinations would be performed for all animals. The days of abdominal taps and volume of ascites collected would be recorded, and the quantity of antibody produced would be determined for each tap from each mouse. Based on results obtained among the different hybridoma cell lines to be evaluated, an attempt would be made to correlate specific experimental manipulations of animals with observed morbidity and mortality, with the goal of developing specific criteria for handling these animals in such a manner as to minimize morbidity and mortality.

Relevant scientific literature was reviewed to assist in developing an experimental protocol for the mice that would be applicable to current practices in common use for production of monoclonal antibodies by the induction of ascitic tumors in mice.

Parameters Affecting Monoclonal Antibody Production in Murine Ascites

Mouse Strain, Sex, and Age

As most hybridoma cells have been derived from the fusion of BALB/c plasmacytomas and BALB/c spleen cells, hybridoma recipient mice must be histocompatible. In 2 reports, ascites volumes and antibody production were greater in BALB/c-derived cross-bred F_1 hybrids by comparison to BALB/c parental mice (35,61). In a third report, no significant difference was observed in ascites volume or antibody production between the F_1 hybrids and the parental BALB/c mice (4).

In one study, ascites production and antibody production were shown to be greater in male mice (33), while another report suggested that there was no predictable difference between male and female mice with regard to these production parameters (4). It was suggested, however that some cell lines may grow slightly better in one sex of mouse than the other, and for some cell lines, solid tumors without ascites production tend to

form in one sex while ascites tumors tend to form in the opposite sex (4). BALB/c male mice are aggressive and tend to fight, often requiring single housing, which has been a deterrent to their use (4).

Retired female breeders have been used because of their availability, large size, and tendency not to fight (4). Use of retired female breeders has been recommended in the UKCCCR guidelines because their abdominal musculature is assumed to be more distensible, thereby allowing larger ascites volumes to be tolerated without discomfort (12).

The optimum age range, at the time of hybridoma cell inoculation, for maximizing antibody production was found to be 6-11 weeks. Older animals produced as much or more ascitic fluid but the antibody concentration was significantly lower (33). A single hybridoma cell line was used in this study.

Pristane Priming

Pristane is the ascitogenic agent most frequently used to "prime" the peritoneal cavity for successful growth of hybridomas as ascitic tumors. In mice which do not receive pristane prior to hybridoma cell inoculation, solid tumors may form but little or no ascites is produced (4,25,33,62). The reported biologic effects of pristane include the induction of granulomatous inflammation in the peritoneum primarily consisting of neutrophils and macrophages which have ingested oil (6,63), immunosuppression (64,65) which

has been shown to result from a decrease in the proliferative response of splenic T and B cells to mitogens and depression of natural killer cell activity (65), induction of potential growth factors (63,66), and lymphatic obstruction by oil-ladened neutrophils and macrophages (6).

Results of previous studies indicate a wide variation in recommended optimal time schedules and volumes of pristane to be used for injection prior to hybridoma cell inoculation. Times ranging from 3 days to 3-4 weeks prior to hybridoma cell inoculation have been reported to be successful for hybridoma growth, with optimum times of 10 days (62,67), 14 days (33), 3-4 weeks (4), and 30 days (63). It should be noted that in the study reported by Chandler (4), which suggested that 3-4 weeks was the optimal time period, the number of animals studied was considerably larger than in the other reports and priming periods ranging from 5-54 days were examined. One cell line was evaluated in 89 groups of animals each ranging in size from 200-1000 mice (4). The author suggested that the longer time period between pristane priming and hybridoma inoculation may be required for the peritoneal inflammatory cells to establish an ideal microenvironment for hybridoma growth.

In a study conducted by Brodeur et al pristane volumes ranging from 0.2 to 2.0 ml were investigated, and volumes of 0.5 ml were found to result in the largest volumes of

ascites and greatest antibody production (33). In a study reported by Hoogenraad and Wraight, pristane volumes ranging from 0 to 0.5 ml were evaluated, and no significant difference was found between 0.1 and 0.5 ml of pristane with regard to ascites volume produced, however antibody concentration data were not presented (62). In an unpublished study, with large numbers of animals, volumes of pristane less than 0.5 ml have been shown to result in suboptimal antibody production (personal communication).

Agents other than pristane have been evaluated for priming the peritoneal cavity prior to hybridoma inoculation. One study compared pristane to complete and incomplete Fruend's adjuvant, proteose-peptone, thioglycollate, corn oil, and mineral oil as possible alternatives to the use of pristane (68). Based on the number of taps/mouse, ascites volume, and antibody concentration, incomplete Freund's adjuvant was found to be as good or better than pristane as a priming agent in mice. In this study, 2 hybridoma cell lines were used. Priming agents were administered 7 days prior to hybridoma cell inoculation, and only a single dose of 0.25 ml was evaluated for each agent.

In another study, using a 0.5 ml dose of incomplete Freund's adjuvant and 2 hybridoma cell lines, it was found that the time interval between priming and hybridoma inoculation could be reduced to 1 day and that as few as 1×10^5 hybridoma cells could be inoculated without

significant negative impact on ascites production (69). Results from this study also demonstrated development of ascites tumors in control mice which received only incomplete Freund's adjuvant and no hybridoma cells. Jones et al reported that when 0.1 ml pristane or incomplete Freund's adjuvant was administered 7 days prior to hybridoma cell inoculation, ascites volumes were significantly greater in incomplete Freund's adjuvant-primed mice with no significant difference in antibody concentrations in comparison to pristane-primed mice, therefore resulting in greater antibody production. The time interval over which animals were tapped was also shorter for the incomplete Freund's adjuvant-primed mice (70). A single hybridoma cell line was used in this study.

Hybridoma Cell Inoculum

From results of experiments reported by Chandler, the optimum number of hybridoma cells for inoculation was found to be 5×10^5 cells. Cell inoculums of 2×10^6 were associated with significant mortality, and with doses of 1×10^5 cells, fewer mice developed ascites and the ascites volume yield was significantly lower. Differences in number of cells inoculated in these experiments did not significantly alter antibody concentration (4). Studies reported by Brodeur et al demonstrated that there was an inverse relationship between the number of hybridoma cells inoculated and the duration of time between injection of

cells and onset of ascites production as well as duration of survival of the mice (33). Ascites formed sooner with larger cell inoculums, but shortened survival times could limit the number of taps possible. Based on ascites volumes and antibody concentration, they reported optimal cell dosage to be between 0.6 and 3.2 x 10^6 cells. Both of the studies cited here (4,33) were conducted using a single hybridoma cell line.

Abdominal Paracentesis

It has been recommended that abdominal paracentesis be performed every 1-3 (71) or 2-3 (72) days with a maximum of 1 (12) to 3 or more (4) taps/mouse. The timing of taps has generally been determined based on subjective assessment of degree of abdominal swelling (33,62,67,69,70). Although generally performed by needle paracentesis with ascites dripping out due to gravity and pressure (33,68), a vacuum aspiration technique has also been described and has been considered advantageous based on more thorough removal of ascites in a shorter period of time, with potentially less stress on the animals (4).

Criteria for Establishing the Experimental Design

From information obtained in this literature review, there appeared to be a lack of consensus regarding optimization of many procedural parameters for producing monoclonal antibodies from growth of ascitic tumors in

mice. It was difficult to make meaningful comparisons between study results, because for the studies reviewed here there were differences in the sex, age, and number of animals used, the hybridoma cell lines used, the volumes and schedules for priming agents used, the number of hybridoma cells inoculated, and the production parameters evaluated.

The experimental regimen selected for use in this study, as presented in materials and methods in the following section, was determined in consideration of results reported in the literature, the requirement that the regimen be representative of current practices in common use at MIT and other academic and commercial institutions, and the obligation that the selected procedures be in compliance with guidelines established by the MIT Committee on Animal Care.

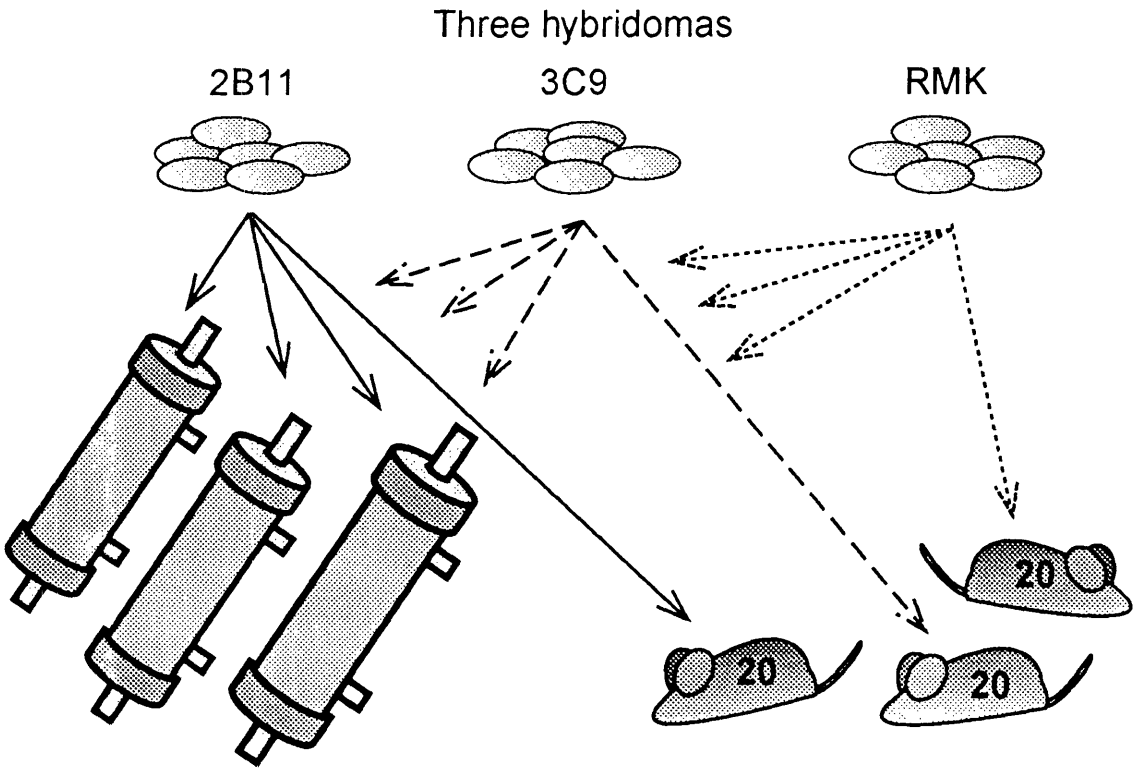
The Second Objective

Another objective of the current study was to compare monoclonal antibody production in mice versus hollow fiber bioreactor systems to determine the feasibility of these systems as potential alternatives to the use of mice. To the author's knowledge, small scale hollow fiber bioreactors have not been independently evaluated as a potential alternative to the *in vivo* production of monoclonal antibodies in murine ascites. Data obtained from 3 of the hybridoma cell lines to be evaluated in mice

according to the preceding section would be used in this comparative study. Each of these 3 hybridoma cell lines would be grown in mice and in each of 3 commercially available hollow fiber bioreactor systems. The overall experimental design for this component of the study is illustrated diagramatically in Figure 3.

Figure 3.
Experimental Design

Monoclonal Antibody Production in Mice vs. Hollow Fiber Bioreactors



The following parameters would be used to compare monoclonal antibody production in mice and bioreactors: amount and concentration of antibody produced, production time, equipment and materials required, technical expertise, humane aspects of the process, time/labor required, and associated costs.

The hollow fiber bioreactor literature has been reviewed in the previous section. It should be noted that of the hollow fiber bioreactor publications cited, only 2 described commercially available systems similar to the small scale systems that were used in the current study (45,46). Most of the hollow fiber bioreactor literature pertains to large scale production.

Criteria for Establishing the Experimental Design

While techniques for operating bioreactor systems were performed as recommended by each respective manufacturer, protocols for sample harvesting and system monitoring were formulated with the intention of maintaining ease of use, and minimizing financial and time expenditures associated with the performance of monitoring procedures. The author's belief was that minimizing procedural complexities would provide greater incentive for widespread use in the event that the production data from the bioreactors was favorable.

Although all of the systems evaluated differed in both components and operational procedures, whenever possible,

standardization of materials and operations was incorporated into the experimental design. Cell culture media used was the same for all systems, and was the media in which the cells had been previously adapted for in vitro growth. The media was supplemented with antibiotics. Although the use of antibiotics in cell culture medium is controversial (73,74), it has been recommended that antibiotics be used in continuous cultures because of the increased vulnerability of the systems to contamination over long periods of operation (22). Consideration was given to the possible use of HEPES in the medium which would provide sufficient pH control to operate systems in a warm room, thus precluding the necessity for space requirements in CO_2 incubators. HEPES has been found to negatively affect some cells types in culture (75), therefore it was decided that HEPES would not be used in this study.

Glucose is one of the most important energy and carbon sources for cultured cells (74), and glucose consumption is an indicator of cellular metabolic activity in mammalian cells (75). As previously described, because of the heterogeneous nature of hollow fiber bioreactors, cellular activity cannot be measured directly; it is generally measured indirectly by for example, measurement of glucose utilization in the media (42,47,48,49). In studies conducted by Evans, glucose measurements were found to be more valuable for evaluating bioreactor productivity than

measurement of other metabolites. Glucose utilization was maintained during monoclonal antibody production, while neither ammonia, lactic acid, or pH were found to correlate with monoclonal antibody production or inhibition of monoclonal antibody secretion (47). Based on this evidence, media glucose was selected to be monitored as a measure of cellular activity. Media glucose has traditionally been assayed using standard commercially available enzymatic test kits. In a recent report by Kurkela et al, media glucose was measured using a commercially available blood glucose meter and the results correlated closely with enzymatically measured glucose concentrations (42). Because of greater ease of use, it was decided that glucose measurements would be primarily monitored with a blood glucose meter. Maintenance of media glucose at ≥ 150 mg/dl has been described as the optimal condition for monoclonal antibody production in hollow fiber bioreactors (49,53) and was selected as the parameter to be used for determination for fresh media exchanges.

Sandwich ELISAs have been shown to be sensitive, reproducible, and economical tests for measurement of murine monoclonal antibodies in hybridoma supernatants and in ascites (77). It was therefore decided that ELISAs would be developed for measurement of antigen-specific antibody in ascites samples and in bioreactor harvests.

The Third Objective

If results of the data comparing monoclonal antibody production in mice and hollow fiber bioreactors suggested that these bioreactors might serve as a viable alternative to the use of mice, then funding would be sought to establish a hollow fiber bioreactor core laboratory at MIT to serve as a model for laboratories at other institutions with similar needs for a diverse number of monoclonal antibodies in small scale production lots. Experimental studies would be conducted in this laboratory to continue evaluations of hollow fiber bioreactors with larger numbers of cell lines to improve production through optimization of environmental conditions for the cells and operational protocols.

SECTION 4: MATERIALS AND METHODS

HYBRIDOMA CELL LINES

Five different hybridoma cell lines were selected for use in this study. These cell lines were selected in part because of a demonstrated need for particular monoclonal antibodies for experimental use at this institution, but also in an attempt to choose hybridomas representative of varied plasmacytoma fusion partners, varied isotypes of secreted antibody to include IgM and IgG, and different subclasses of IgG, and both mouse x mouse and a rat x mouse heterohybridoma. Cell line designation, antibody specificity, plasmacytoma fusion partner, and antibody isotype and subclass are presented in Table 1 for each of the five cell lines selected.

Hybridomas 2B11, 3C9, 3D6, and 2C6D9 are BALB/c mouse x mouse hybridomas which were obtained from Laura Trudel in the hybridoma laboratory of Dr. Gerald Wogan, MIT Division of Toxicology. Hybridomas 2B11, 3C9, and 3D6 secrete mouse antibodies directed against aflatoxin B_1, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, and 4-aminobiphenyl, respectively (88,89,90). Aflatoxin is a naturally occurring environmental toxin produced by some strains of Aspergillus flavus. The heterocyclic amine, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), is a food mutagen produced by pyrolysis, and 4-aminobiphenyl (4-ABP)

is an aromatic amine found in fossil fuel combustion products and cigarette smoke. These compounds are currently under study by investigators in the MIT Division of Toxicology. Hybridoma 2C6D9 secretes mouse antibody directed against bovine serum albumin (BSA).

Hybridoma RMK, which was generously provided by Ann Marshak-Rothstein from the hybridoma laboratory at Boston University, is a rat x mouse heterohybridoma which secretes rat antibody directed against the kappa light chain of mouse immunoglobulin. This antibody has particular utility for use in immunoassays to quantitate mouse immunoglobulins (78,79) as 95-98% of all mouse immunoglobulins contain kappa light chains (80).

Each of the five hybridoma cell lines was grown in a group of 20 mice to provide data on monoclonal antibody production in murine ascites. Additionally, three of these cells lines, 2B11, 3C9, and RMK, were also grown in each of three different hollow fiber bioreactor systems to provide comparative production data from these <u>in vitro</u> systems.

Legend

Table 1

Selection of Hybridoma Cell Lines

Cell line designation, antibody specificity, plasmacytoma fusion partner, and antibody isotype and subclass are presented for each of the five different cell lines used in this study.

Key to abbreviations:

MeIQx = 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline

K = kappa light chain of mouse immunoglobulin

BSA = bovine serum albumin

4-ABP = 4-aminobiphenyl

Table 1.
Selection of Hybridoma Cell Lines

	cell line	antibody	plasmacytoma	isotype
1.	2B11	anti-aflatoxin	Sp2/0-Ag14	IgM
2.	3C9	anti-MeIQx	P3-X63Ag8	IgG_{2b}
3.	RMK	rat anti-mouse K	Sp2/0-Ag14	IgG_{2a}
4.	2C6D9	anti-BSA	Sp2/0-Ag14	IgG_1
5.	3D6	anti-4-ABP	Sp2/0-Ag14	IgG_1

ASCITES PRODUCTION

Animals

Each mouse x mouse hybridoma, #1, 2, 4, and 5 as indicated in Table 1, were grown as ascites tumors in 20 CAF_1/J male pathogen free mice (Jackson Laboratories, Bar Harbor, ME). An additional 12 mice served as the CAF_1 control group.

The rat x mouse heterohybridoma, #3 as indicated in Table 1, was grown as ascitic tumors in 20 Fox Chase SCID™, C.B.-17/IcrTac-scidDF male pathogen free mice (Taconic Farms, Germantown, NY). An additional 12 mice served as the SCID control group.

Animals were 7 weeks of age at receipt. Animals were maintained in AAALAC accredited animal facilities at MIT. Animals were housed 4/cage in polycarbonate shoebox cages with wire bar lids and Micro-barrier™ filter tops (Allentown Caging, Allentown, NJ). Lab grade pine shavings (Northeastern Products Corporation, Warrensburg, NY) were used for bedding. Cages with bedding for SCID mice were autoclaved for 20 minutes at 121°C prior to use. CAF_1 mice were fed pelleted rodent chow (Prolab 3000, Agway, Syracuse, NY) and fresh distilled water in bottles ad libitum. SCID mice were fed autoclavable pelleted rodent chow (Prolab 3500, Agway, Syracuse, NY) and autoclaved distilled water in bottles ad libitum. A 12 hour light/dark cycle was maintained.

Animal husbandry was performed according to the standard operating procedures of the institution and as described in the Guide for the Care and Use of Laboratory Animals, NIH Publication No. 86-23, Revised 1985. Animal husbandry procedures and experimental manipulations with SCID mice were conducted in a horizontal laminar positive flow mass air displacement unit (EdgeGARD, Baker Company, Sanford, ME) using aseptic technique.

All mice were held in quarantine for a minimum of 6 days following arrival to allow for acclimation. Following release from quarantine, animals were randomized into groups by weight and identified by ear punch. The protocol for animal use for monoclonal antibody production in murine ascites, Protocol #91-003, was approved by the MIT Committee on Animal Care.

Pristane Priming

On study day -14, each test mouse received a single intraperitoneal injection of 0.5 ml pristane (2,6,10,14-tetramethylpentadecane, Sigma Chemical Company, St. Louis, MO). Animals in the control groups received a single 0.5 ml intraperitoneal injection of 0.9% NaCl, USP (Abbott Laboratories, North Chicago, IL). Injections of pristane or saline were administered at the caudal left quadrant of the ventral abdomen using sterile syringes and sterile 25 gauge 5/8 inch needles.

To determine whether sterility was maintained in

bottles of pristane under routine use and storage conditions, samples were obtained, using aseptic technique, from 4 different bottles of pristane (Sigma Chemical Company, St. Louis, MO) that were currently in use in the animal facilities at MIT. Aerobic and anaerobic bacterial cultures were performed. For aerobic cultures, 1 ml of pristane was inoculated into 5 ml tryptic soy broth (Remel, Lenexa, KS) and incubated 18-24 hours at 35°C. Broth samples were then plated on blood agar/MacConkey agar biplates (Remel, Lenexa, KS) and incubated 18-24 hours at 35°C. For anaerobic cultures, 1 ml of pristane was plated on blood agar/MacConkey agar biplates and incubated 46-48 hours at 35°C in an anaerobic jar with a BBL^{R} Gas Pak Plus^{TM} (Becton Dickinson, Cockeysville, MD). All samples were protected from light. Results were reported as bacterial growth or no bacterial growth.

Clinical Observations

All mice were observed at least once daily beginning on day -14 following administration of pristane. During the time interval when abdominal taps were performed, animals were generally observed twice daily. Clinical parameters evaluated included general appearance, character of the haircoat, posture, level of activity, and assessment of degree of abdominal distention. Clinical observations for test and control animals were recorded.

Body Weight Measurements

Body weight measurements were made and recorded for each mouse on study day -14 prior to pristane injection, on day -7, on day 0 prior to hybridoma cell inoculation, on days 2, 4, 7, 9, and daily thereafter until study completion. On days when test animals were tapped to collect ascitic fluid, before- and after-tap body weight measurements were recorded.

Hybridoma Cell Inoculation

Hybridoma cells had previously been maintained frozen in liquid nitrogen in a solution composed of 95% fetal bovine serum (Endlo™, JRH Biosciences, Lenexa, KS) and 5% DMSO (Sigma Chemical Company, St. Louis, MO). Cells were defrosted and grown in static suspension culture prior to inoculation into mice. Cell culture medium used was Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 grams glucose per liter (Gibco, Grand Island, NY). Medium was supplemented (10 ml/L) with 10 mM MEM non-essential amino acid solution and 200 mM L-glutamine (Gibco, Grand Island, NY) for a final concentration of 6 mM L-glutamine. Fetal bovine serum (Endlo™, JRH Biosciences, Lenexa, KS) was added to 20% v/v final concentration. Cells were expanded in culture to provide > 20 x 10^6 cells in log phase growth for each group of mice. Antibody secretion into the cell culture supernatant was verified by ELISA for each hybridoma cell line prior to preparation for inoculation

into mice. Materials and methods for ELISAs are presented in a following section under the heading "antibody quantitation."

On study day 0, cells were counted using a hemocytometer, and as determined by trypan blue dye exclusion, a hybridoma cell suspension containing 2×10^6 live cells/ml was prepared. Each test mouse received a single intraperitoneal injection of 1×10^6 cells in cell culture medium in a total volume of 0.5 ml. Mice in control groups received a single intraperitoneal injection of 0.5 ml cell culture medium without cells. All injections were administered in the left caudal quadrant of the ventral abdomen using sterile syringes and sterile 25 gauge 5/8 inch needles.

Abdominal Paracentesis

Abdominal paracentesis was performed when there was visible moderate to marked abdominal distention. Body weights of the mice were monitored such that animals were tapped before exceeding a 20% increase in body weight from day 0 in accordance with the MIT Committee on Animal Care Guidelines for the Utilization of Rodents in Experimental Neoplasia and Ascites Production and the United Kingdom Coordinating Committee on Cancer Research guidelines (12). Abdominal taps were performed every 1 to 3 days depending on the clinical appearance of the mouse and the rate of ascites production as assessed by the degree of abdominal

distention. Each mouse was tapped a maximum of three times.

Abdominal taps were performed using sterile 18 gauge 1 1/2 inch needles inserted into the peritoneal cavity through the left lateral abdominal wall. Ascites was then collected by permitting the fluid to drip from the hub of the needle and directly from the paracentesis site into a sterile centrifuge tube. The animals were restrained manually by grasping the loose skin over the shoulder blades. Gentle digital pressure was applied to the abdomen and the position of the mouse was altered as needed to facilitate removal of all the ascites fluid. When no more fluid could be obtained, animals were weighed, returned to their cages, and post-tap clinical observations were made.

Abdominal paracentesis was also performed for mice in the control groups. All control mice were tapped 3 times during a time interval approximating the abdominal taps for the test groups. Abdominal paracentesis was performed in the same manner as for the test animals using sterile 18 gauge 1 1/2 inch needles inserted into the peritoneal cavity through the left lateral abdominal wall. The mice were handled and restrained in a manner similar to and for a time period approximately equal to that necessary to collect ascitic fluid from test mice. Animals were then returned to their cages and post-tap clinical observations were made.

The total volume of ascites fluid collected and the day

of collection were recorded for each tap for each mouse. Samples were centrifuged at 550xg for 10 minutes and the ascites fluid supernatant was then aspirated with a Pasteur pipette and transferred to a sterile centrifuge tube, leaving the cell pellet and lipid layer, if present. The pellet remaining in the original tube was reamed with a pipette and the sample was centrifuged once or twice more as needed to completely separate the remaining ascitic fluid. Centrifugation has been reported as one method useful for clarification of ascites fluid (81). The resultant volume of ascitic fluid following centrifugation was recorded for each tap for each mouse. Samples were frozen at -20°C until analysis.

Euthanasia

Mice surviving to the third abdominal tap were euthanized just prior to performing paracentesis. During the study, any animal with clinical abnormalities which the author interpreted to be indicative of severe distress or suggestive that the animal might not survive to the next observation period was humanely euthanized. Six mice from each control group were euthanized at the time the last test animals were euthanized. Euthanasia was performed using CO_2 in accordance with accepted American Veterinary Medical Association guidelines (82).

Necropsy

A complete gross necropsy was performed on all animals in test groups and on six animals from each control group. Observations noted at necropsy were recorded for each animal. Organs and tissues were examined *in situ* then dissected free and fixed in 10% neutral buffered formalin. The remaining animals from each control group which were not euthanized for necropsy at study completion were released from the study for other experimental use.

Histology

Representative tissues from selected animals were processed for microscopic examination. Organs and tissues were trimmed and sectioned. Slides were stained with hematoxylin and eosin, and were examined microscopically by Dr. James Murphy. Microscopic lesions observed were recorded.

Antibody Quantitation

Enzyme linked immunosorbent assays (ELISAs) were used to screen the cell culture supernatant of hybridoma cells for secreted antibody prior to inoculation of cells into mice and bioreactors, and to quantitate in absolute units the antigen-specific antibody present in each ascites sample from each mouse, and each harvest from each bioreactor run. Laura Trudel developed quantitative indirect antibody ELISAs (83) and performed all of the

assays for the mouse and bioreactor samples generated in this study.

Antibody standards were prepared using the Monoclonal Antibody Purification System (Affi-Gel^{R} Protein A MAPS^{R} II Kit, Bio-Rad Laboratories, Richmond, CA) following the manufacturer's instructions for purification of monoclonal antibody from ascites for each of the IgG producing cell lines. Resulting antibody concentrations were determined by O.D. at 280 nm using the extinction coefficient for IgG; 1.4 O.D. units equal to 1.0 mg/ml. The antibody was diluted in Tris-2% BSA Buffer with 0.02% sodium azide and stored at 4°C. For the IgM cell line, 2B11, antibody was purified first by 40% ammonium sulfate precipitation of ascitic fluid dialyzed into PBS, followed by reprecipitation by dialysis against 2% boric acid, pH 6.0 (84). Antibody concentration was determined by O.D. at 280 nm using the extinction coefficient for IgM; 1.2 O.D. equal to 1.0 mg/ml. The antibody was diluted and stored as above.

Three of the antigens used to determine antigen-specific antibody concentrations of the ascites and bioreactor samples were hapten-protein conjugates; Aflatoxin B_1-BSA (88), MeIQx-BSA (89), and 4-ABP-bovine gamma globulin (90). The rat anti-mouse kappa antibodies were screened on affinity purified mouse anti-rat IgG (Boehringer Mannheim, Indianapolis, IN). Antibodies for 2C6D9 were screened on 0.1% BSA (Boehringer Mannheim,

Indianapolis, IN).

The assays were developed by first performing checkerboard titrations for each of the 5 antigens in PBS and were adsorbed overnight at 4°C onto 96 well polyvinyl chloride plates (Dynatech, Alexandria, VA). The plates were washed with deionized water 3 times and dried. Plates were then blocked with 0.2% BSA (Boehringer Mannheim, Indianapolis, IN) in PBS for one hour at room temperature to control nonspecific binding of antibodies to the plastic. The plates were then washed 3 times, dried, covered with parafilm and stored at -20°C.

Standard curves were constructed using concentrations of purified antibodies starting at 1 µg/ml with two-fold dilutions to 25 ng/ml in 10% horse serum/PBS (JRH Biosciences, Lenexa, KS). One hundred microliter samples were applied to the plate and incubated at 37°C for 2 hours. Plates were washed with 0.05% Tween 20 (Sigma Chemical Company, St. Louis, MO) in PBS 3 times and dried.

Appropriate concentrations of alkaline phosphatase conjugated goat anti-mouse IgG and IgM and mouse anti-rat IgG (H&L) (Boehringer Mannheim, Indianapolis, IN) were determined for each assay by testing varying concentrations on the standard curve dilutions and selecting the concentration that provided the highest signal with the lowest background noise. Specifically, 100 µl of the enzyme antibody conjugate dilution was added to each well and incubated for 1 hour at 37°C. The plate was washed and

dried as above and 100 µl of substrate, 1 mg/ml p-nitrophenyl phosphate (Sigma Chemical Company, St. Louis, MO) in 0.1 M Diethanolamine buffer, pH 9.8 (Sigma Chemical Company, St. Louis, MO) was added to the plate and incubated for 15 to 45 minutes at room temperature until the lowest dilution on the standard curve read 1-1.5 O.D. at 405 nm with a Dynatech MR 7000 automatic ELISA Reader (Dynatech, Alexandria, VA). The ELISA reader was programmed to calculate the curve fit for the standards and to determine the mean and standard deviation of the duplicates, and concentration of antibody. The assay sensitivity was high, therefore the samples required dilution ranging from 1:3000 to 1:250,000 in 10% horse serum/PBS. The dilution factor was manually recorded and final concentrations were calculated.

Materials and Time Logs

For the three cell lines which were selected for use in the segment of the study designed to compare monoclonal antibody production in mice and hollow fiber bioreactors, detailed logs were maintained to document materials used and time spent in labor in all phases of the ascites production process to include pristane priming, expansion and preparation of hybridoma cells for inoculation, inoculation of hybridoma cells into mice, performing clinical observations, abdominal taps, and euthanasia. Times recorded were inclusive of set up, experimental

manipulations, and clean up, but did not include the time taken to walk to and from the animal facility, as the animals were housed in a different building than the offices and laboratory.

Time spent in centrifugation and separation of ascites was not included in the time/labor figures because every ascites sample was handled individually as part of the protocol design. For most applications, murine ascites would be collected, clarified, and processed in batches, so the inclusion of these figures in the time/labor calculations was not deamed appropriate.

Statistical Analyses

Statistical analyses were performed by Dr. Ray Gleason.

Abdominal Tap Days, Ascites Volumes, and Antibody Quantitation

Differences among mean levels were tested for statistical significance using a one-way repeated measures analysis of variance (ANOVA) followed by pairwise comparisons among group means using the Newman-Keuls test.

Body Weights on Days -14, -7, and 0

Unpaired t-tests were used to compare mean body weights between pristane-treated and control mice on days -14, -7, and 0.

Body Weight Changes from Day 0 to Tap 1

Differences among mean body weight changes and mean percent changes in body weight between day 0 and the first abdominal tap for the five test groups were tested for significance using a one-way ANOVA followed by pairwise comparisons among group means using the Newman-Keuls test.

Post Abdominal Tap Body Weights vs. Control Body Weights

For comparison of mean post-tap body weights among test and control groups for each tap, the control mean weights used were those for the mean study day on which the tap was performed for the test group. Mean body weights were tested for significance at each tap using unpaired t-tests.

Mean and Percent Changes in Pre and Post Abdominal Tap Body Weights

Mean body weights among treated groups were tested for significance using a one-way ANOVA followed by pairwise comparisons among group means using the Newman-Keuls test.

Mean Body Weights and Percent Changes Among Taps Within Groups

Comparisons of mean and mean percent changes in body weight among taps within groups were made using a one-way repeated measures ANOVA followed by pairwise comparisons between taps using the Newman-Keuls test.

Statistical Significance

The alpha level for statistical significance was set at 0.05. Data are presented as means with the standard deviation as a measure of dispersion.

HOLLOW FIBER BIOREACTOR SYSTEMS

Three different hollow fiber bioreactor systems were selected for use in this study. System selection criteria included the following: instruments were to be commercially available, designed to produce monoclonal antibodies on a laboratory scale (≈ 100-600 mg antibody/ month/bioreactor), capital costs for equipment were to be ≤ $7,500, and there was to be a demonstrated willingness on the part of the manufacturer to provide equipment on loan for use in the study. Three different bioreactor systems were identified for which all of the above criteria were satisfied.

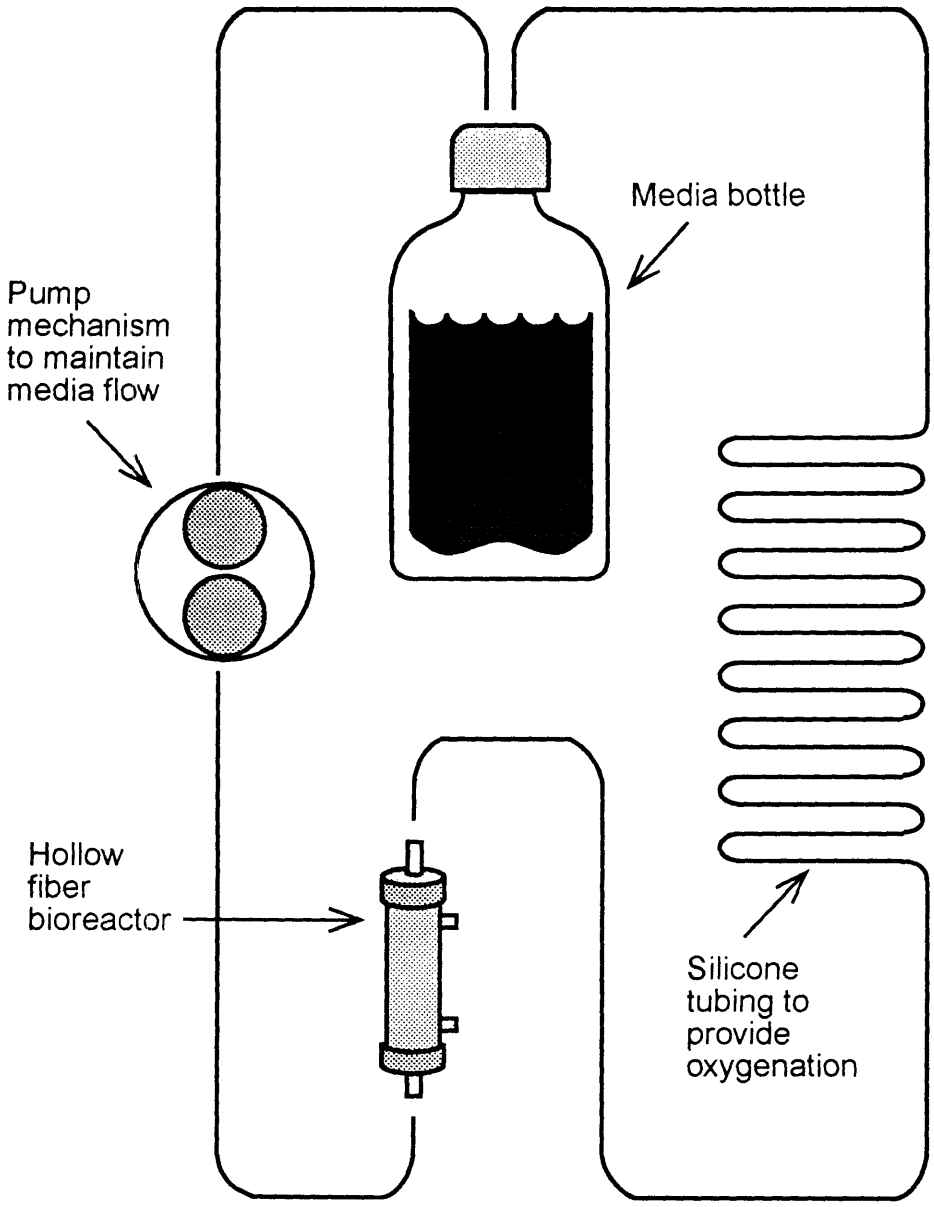
The manufacturers' names have been withheld from this document for 2 reasons. The objective of this study was to compare monoclonal antibody production in mice to monoclonal antibody production in hollow fiber bioreactors, and in so doing to evaluate hollow fiber bioreactors as a potential alternative to the use of mice. It was not the objective of this study to compare different hollow fiber bioreactor systems. Although attempts were made, to the extent possible, to reduce experimental variables between the different bioreactor systems, there are many significant differences in both system components and specifications, and in the operational protocols used to manage these systems, that make direct comparisons between systems inappropriate. In an effort to avoid comparisons

between manufacturers, the bioreactor systems, for purposes of this document, will be referred to as systems A, B, and C. Three hybridoma cell lines, 2B11, 3C9, and RMK, were grown in each of the three different bioreactor systems.

System Components

All 3 systems had the same basic functional components. The basic design of the bioreactor circuit is illustrated schematically in Figure 4. All systems were operated in a media recirculation mode, i.e., the media flow path was in a continuous unidirectional circuit. Included within the circuit was a bottle containing cell culture media, a variable speed pump to maintain media flow, a hollow fiber bioreactor cartridge, and a means of providing oxygen and CO_2 to the system to support cellular metabolism and maintain physiologic pH of the media. All systems had an instrument control module for regulation of media flow rate and other features unique to each system.

Figure 4.
Schematic Diagram of a
Hollow Fiber Bioreactor Circuit



System A

System A had a base unit with a central pump and stations designed to mount up to 4 hollow fiber cartridge modules, each with independent media flow path and media bottle. Three bioreactor cartridges were maintained simultaneously during this study. The pump motor rotated a cam on the motor shaft which forced pump pins to depress thick-walled pump tubing on each module, forcing media by positive displacement, to flow from the media bottle through 8 feet of gas permeable silicone tubing, through the hollow fiber bioreactor cartridge, and back to the media bottle. There were no media sampling ports in the system so media samples were obtained directly from the media bottle. The cap assembly for attachment of tubing to the media bottle was designed to fit the collar of commercially available 0.5 and 1 liter media bottles so no additional bottles were needed.

The base unit weighed 14 lbs. with dimensions of 15.5 x 15.5 x 7.5 inches and was designed to operate within a CO_2 incubator. An AC motor cable connected the base unit to an electronic control unit which was placed outside the incubator. A power cord connected the electronic control unit to a standard electrical outlet. A single pump speed setting could be selected at the control unit. Different media flow rates could be obtained for each bioreactor module, ranging from approximately 0 to 50 ml/min, by altering the length of the pump pins at any individual pump

station. The control unit also had a pause mode for temporarily stopping the pump motor. As a safety feature, the motor would automatically start again after 4 minutes.

The bioreactor cartridge modules with tubing were supplied sterile. The cap assemblies were not supplied sterile and were autoclaved at 121°C for 30 minutes prior to use.

System B

System B had a tray assembly which housed the entire system. The media pump, operated by a magnetic positive displacement mechanism, pumped media from a main media reservoir bottle to a hollow fiber bioreactor cartridge(s). The system was designed to maintain 1 to 3 bioreactors in series. The system was operated with a single bioreactor for this study to preclude any potential downstream effects of metabolic products from one cell line upon another. There was a media sampling port with a luer connector downstream from the bioreactor and a flowmeter followed by 5 meters of gas permeable silicone tubing from which the media was then directed back to the main media reservoir bottle. A media transfer bottle was incorporated into the system with a fill/drain valve to facilitate media exchanges. The main media reservoir bottle and media transfer bottle were 1 and 0.5 liter glass bottles, respectively, to which cap assemblies with 0.2 μ vent filters (Acrodisc CR, Gelman Sciences, Arbor, MI) were

attached.

The unit weighed 12 lbs. with dimensions of 14 x 11.5 x 11 inches and was designed to operate within a CO_2 incubator. A control panel which was attached to the tray assembly was also housed within the incubator and was connected via a power cord to a standard electrical outlet. Media flow rate could be regulated within a range of 0 to 250 ml/min and directional flow of media could be controlled to move media to and from the transfer bottle for exchanging media.

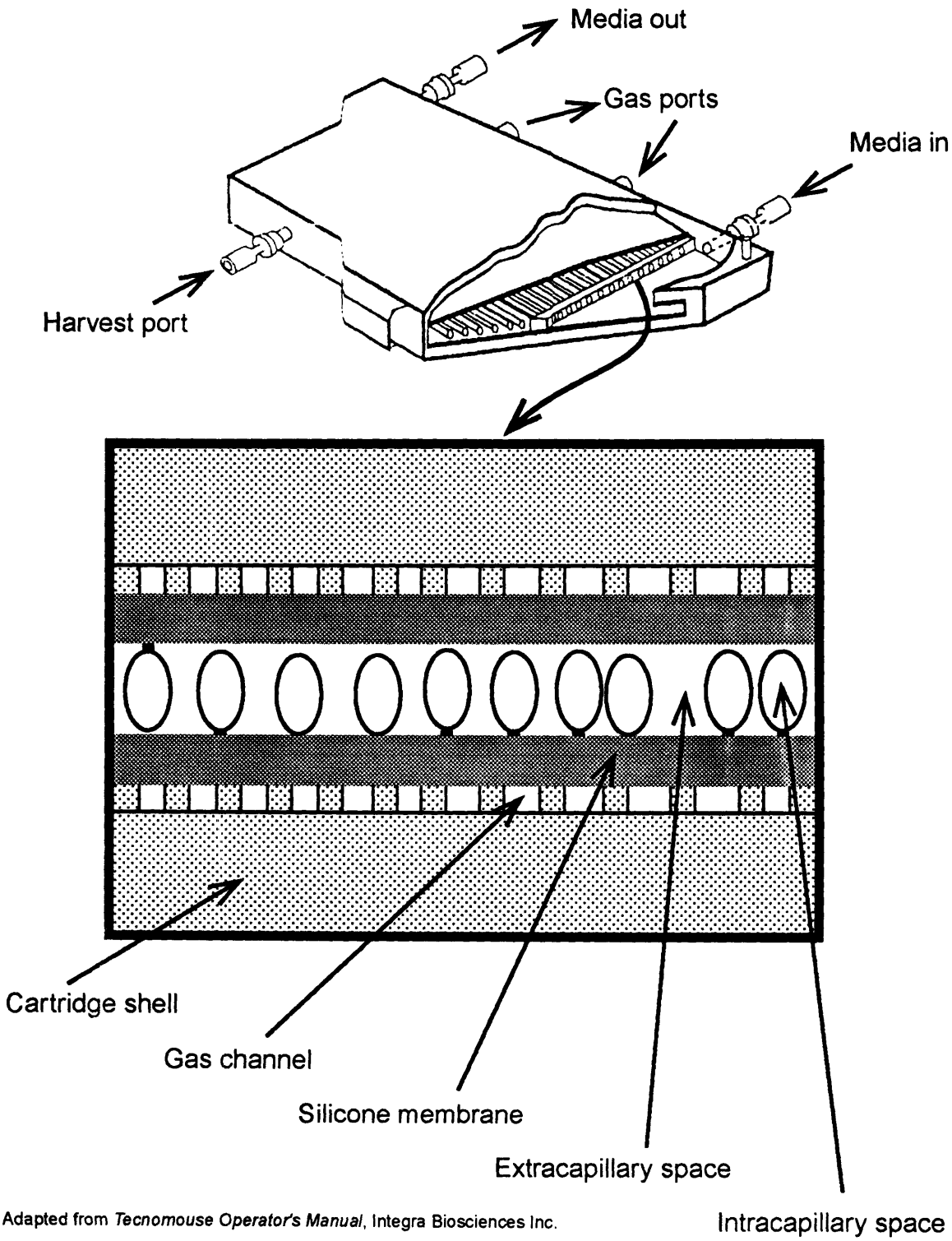
The bioreactor cartridges were supplied sterile. The entire system of tubing and media bottles, exclusive of the bioreactor, required set up and autoclave preparation, and was then autoclaved at 121°C for 45 minutes prior to use. Cleaning and reautoclaving of media bottles with cap assemblies were required for subsequent media exchanges. At the completion of each run, the system, exclusive of the bioreactor, was flushed and reautoclaved.

System C

System C consisted of an instrument module on which was mounted a removeable rack for placement of 1 to 5 bioreactors each with independent media flow path, and a pump head which contained individual pump cassettes for each bioreactor. Media tubing was extended through the cassette and rotating cylinders within the pump head depressed the tubing by a peristaltic mechanism, forcing

media to flow from the media bottle, through an in-line 0.2 μ filter, through the hollow fiber bioreactor cartridge, and back to the media bottle. The purpose of the filter was to trap any microorganisms potentially introduced into the system during media changes and thereby protect the bioreactor from contamination. Media sampling ports with luer connectors were located downstream from the bioreactors. Two liter media bottles were supplied with the system to which cap assemblies with 0.2 μ vent filters (Acrodisc CR, Gelman Sciences, Arbor, MI) were attached. Hollow fiber bioreactors used in this system were hybrid reactors. Unlike the conventional hollow fiber bioreactors containing a cylindrical bundle of fibers within a hard plastic shell (Figures 1 and 2), the fibers in the hybrid reactor were arranged in single parallel rows and encased in a silicone membrane, all within a plastic cartridge (Figure 5). A gas dome with individual gassing ports for each bioreactor was located at the back of the bioreactor cartridge housing, and incubator air was pumped via the dome and ports to channels outside the gas permeable silicone membrane, thus providing direct gassing to the cells in the extracapillary space media of the bioreactor.

Figure 5.
Schematic Diagram of a Hollow Fiber Hybrid Reactor



Adapted from *Tecnomouse Operator's Manual*, Integra Biosciences Inc.

The instrument module weighed 11 lbs. with dimensions of 15 x 12 x 9.5 inches and was designed for operation within a CO_2 incubator. Additional space within the incubator was required for placement of media bottles. An AC motor cable connected the instrument module to an electronic control module which was placed outside the incubator, and a power cord connected the control module to a standard electrical outlet. Media flow rate, which was the same for all bioreactors, could be selected ranging from 0 to 150 ml/hr. The module could be programmed to change the media flow rate in fixed or variable increments as a step function over a specified time interval. The gas supply setting was dependent on the number of bioreactors in use. An alarm was displayed in the event of a power failure. A printer port was available for printer interface, to print out media flow programs.

The bioreactor cartridges with tubing were supplied sterile. Two liter media bottles and cap assemblies were not supplied sterile and were autoclaved at 121°C for 30 minutes prior to use. Cleaning and reautoclaving of media bottles with cap assemblies were required for subsequent media exchanges.

Bioreactor Cartridge Specifications

The specifications for the 3 hollow fiber bioreactors used in this study are presented in Table 2.

Table 2.
Bioreactor Cartridge Specifications

	System A	System B	System C
Number of fibers	1300	3150	400
Fiber wall thickness (microns)	8	25	20
Fiber ID (microns)	200	210	200
Fiber OD (microns)	216	260	240
Fiber length (cm)	9.6	10.8	15.0
Fiber material	modified cellulose	cellulose	regener-ated cellulose acetate
Fiber type	isotropic	isotropic	isotropic
Extracapillary space surface area (m^2)	0.10	0.14	0.04
Extracapillary space volume (ml)	7	12	7
Molecular weight cut-off (kD)[1]	50	10	10
Capacity (cells)	$\geq 10^9$	$\geq 10^9$	$\geq 10^9$

[1] defined at 95% retention

All fibers were constructed from cellulose-based materials, and all fibers were isotropic, which defines the fiber wall as being homogeneous in architecture from the inner to the outer surface. There were significant differences in the number and length of fibers in the cartridges with the hybrid reactor in system C having the fewest in number and longest fibers. There were differences in fiber wall thickness, however the internal luminal diameter of the fibers was very similar for all 3 systems. There were also significant differences in ECS volume and surface area. The capacity for all cartridges was specified as $\geq 10^9$ cells.

The molecular weight cut-off (MWCO) for all cartridges was defined at 95% retention, and cut-offs were selected to retain the antibody product concentrated within the small volume of the ECS. The only bioreactor cartridge available for system C had a 10 kD MWCO, and a cartridge with the same MWCO was selected for system B. The smallest MWCO cartridge available for system A was 50 kD.

System Operations

All systems were housed in humidified CO_2 incubators maintained at 37°C with 5% CO_2 in air. During the study, incubator temperatures were monitored daily 5-7 days/week via thermometry and $\%CO_2$ was monitored using a fyrite CO_2 test kit (Bacharach Instrument Company, Pittsburgh, PA).

Techniques for bioreactor system set up, operation, and

maintenance were performed as recommended by each respective manufacturer according to instructions contained in the operator's manuals and recommendations made by technical service representatives. Each bioreactor run was 65 days. Pilot runs were conducted with each system to gain competence in technical procedures and to evaluate media conditions and operational protocols.

All manipulations of cells and bioreactor systems were performed in a Class II biological safety cabinet (SterilGARD, Baker Company, Sanford, ME) with the exception of system C for which system set up and cell inoculations were performed in a safety cabinet but subsequent manipulations of the bioreactor system were performed within the incubator in which the system was housed. The individual bioreactor cartridge modules for system A and the bioreactor tray assembly for system B were easily disconnected and moved to and from the incubator and safety cabinet. Because of tubing and gassing port connections to the instrument module and the larger 2 liter media bottles used in system C, movement of bioreactors was more difficult, and the manufacturer recommended that manipulations be performed within the incubator.

All systems were handled using strict aseptic technique. Latex gloves were worn and ports of entry which were accessed, such as the luer connections on ECS harvest ports and ICS media sampling ports, and media bottle cap connections, were thoroughly cleaned using individually

packaged alcohol pads (Baxter Inc., McGaw Park, IL) before disconnection and manipulation. All materials used were sterile. An aliquot was taken from each media bottle after addition of supplements and incubated at 37°C for 24 to 48 hours to check for sterility prior to use. As an additional precaution for system C, because manipulations were not performed within a safety cabinet, luer caps with injection septa provided with the system were placed at all ECS and ICS sample ports and were cleaned with 10% povidone-iodine (Betadine, Purdue Frederick Company, Norwalk, CT) prior to each use. To maintain the integrity of the septa, non-coring needles (Huber needles, Access Technologies, Skokie, IL) were used.

Media Composition

The same cell culture medium was used for all three systems and cell lines and was the medium to which the cells had been previously adapted in culture. Medium used was Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 grams glucose per liter supplemented (10 ml/L) with 10 mM MEM Eagle non-essential amino acid solution, 200 mM L-glutamine, and 10,000 unit/ml penicillin/10,000 µg/ml streptomycin mixture (BioWhittaker, Inc., Walkersville, MD) for a final concentration of 6 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Intracapillary space (ICS) perfusing media was supplemented initially with 1% fetal bovine serum (Endlo™, JRH Biosciences, Lenexa, KS)

and was subsequently reduced to 0% after bioreactor media consumption exceeded 1 liter/week. Use of ICS media with 1% serum was resumed if significant decreases in cell viability and/or glucose consumption were observed following removal of serum from the ICS; subsequent reduction in ICS serum concentration was from 1% to 0.5% before complete removal of serum from the media. Extracapillary space (ECS) media was initially supplemented with 10% fetal bovine serum and was subsequently reduced to 5% after demonstration of stability in cell viability and glucose consumption following removal of serum from the ICS media. Use of ECS media with 10% serum was resumed if significant decreases in cell viability and/or glucose consumption were observed following reduction of serum to 5%. Serum reductions in the ICS and/or ECS media were not performed if cell viability and glucose consumption remained low. The same lot of fetal bovine serum (lot# 2B2032) was used for all bioreactor media.

Cell Inoculation

Hybridoma cells from lines 2B11, 3C9, and RMK had been previously frozen in a freezing solution composed of 95% fetal bovine serum (Endlo™, JRH Biosciences, Lenexa, KS) and 5% DMSO (Sigma Chemical Company, St. Louis, MO) and had been maintained frozen in liquid nitrogen. Cells were defrosted and grown in static suspension culture prior to inoculation into bioreactors. Cells were placed in

supplemented DMEM as described above with 20% fetal bovine serum after defrosting. Approximately one week after defrosting, the serum was reduced to 10%. Cells were adapted to growth in media with 10% serum for 3-8 weeks. Cells were expanded in culture to provide the necessary number of cells in log phase growth for each bioreactor. Antibody secretion into the cell culture supernatant was verified by ELISA as previously described for each hybridoma cell line prior to preparation for inoculation into bioreactors. Bioreactor systems were set up and flushed with media on day -1, and visually inspected for sterility prior to inoculation of cells on day 0.

Concentration of bioreactor cell inoculums was standardized such that the number of cells inoculated per unit volume of extracapillary space was identical for all systems. On study day 0, cells were counted using a hemocytometer, and a cell suspension containing 8.6×10^6 live cells/ml as determined by trypan blue dye exclusion was prepared in 100% conditioned media for inoculation.

Limiting Dilution

All cell lines were tested for stability by limiting dilution (85) at the beginning of each run using cells retained from bioreactor inoculations, and at the end of each run using cells obtained from the last bioreactor ECS harvests. In brief, cells were diluted in BALB/c thymocyte-containing media and plated into 96 well plates.

The supernatant from wells which developed clones were tested by ELISA as previously described, and stability of each cell line was measured as the % of clones which tested positive for antibody secretion.

Media Monitoring

Media was recirculated in all 3 systems. Media flow rates were 50 ml/min, 100 ml/min and 100 ml/hr, for systems A, B, and C, respectively. The concentration of glucose in the perfusing ICS media was determined 3 times weekly at the time of product harvests using a commercially available glucometer (One Touch™, Lifescan, Inc., Milpitas, CA). Ten microliters of media at 37°C was placed on a test strip and measurements were recorded in mg/dl from the LED display on the instrument. Samples were tested in duplicate and the mean glucose value for each sample was used to calculate glucose consumption rate. Cleaning and maintenance of the glucometer, and calibration checks as determined by measurement of control samples, were performed according to the manufacturer's instructions.

Fresh ICS media was supplied as necessary to maintain media glucose concentrations at ≥150 mg/dl. At the minimum, media was exchanged once weekly because of concerns for spontaneous decomposition of glutamine at 37°C (86,87).

Glucose standard solutions and selected ICS media samples from pilot and study runs were retained (N=33), and

mean glucose measurements from the duplicate tests obtained using the glucometer were compared with glucose measurements obtained using a standard diagnostic test kit (Procedure #115, Sigma Diagnostics, St Louis, MO). Agreement between the two methods was assessed statistically using the method of Bland and Altman (91), Pearson's product moment correlation, and coefficient of variation.

Antibody Product Harvests

Antibody product was harvested from the bioreactor extracapillary space per manufacturer's instructions three days a week (Monday, Wednesday, and Friday) starting with the initial harvest on day 4 (systems A and B) or day 7 (System C). For system A, spent media with cells was aspirated from the ECS and then the ECS was subsequently refilled with fresh media. For systems B and C, fresh media was simultaneously flushed into the ECS at one harvest port as spent media with cells was aspirated from another harvest port. For system A, the volume of product harvests was dependent on the volume of media and cells in the ECS. For system B, 5.0 ml aspirates were obtained on harvest days 4 through 11, and 10.0 ml aspirates were obtained from days 14 through 65. For system C, 5.0 ml aspirates were obtained for all harvests.

Live and dead cell counts, as determined by trypan blue staining using a hemocytometer, were recorded for each

harvest. Cell viability (%) was calculated. Samples were centrifuged at 550xg for 10 minutes, the supernatant was aspirated from the cell pellet, and aliquots were stored at 4°C and -20°C. Sample volumes before and after centrifugation were recorded for each harvest.

Antibody Quantitation

Antibody from each harvest sample from each bioreactor was quantitated using the identical indirect antibody ELISAs that were used to measure antibody in mouse ascites samples. Materials and methods for ELISAs have been previously described. Samples were batched and analysed at the conclusion of the bioreactor runs.

Materials and Time Logs

Detailed logs were maintained to document materials used and time spent in labor for all aspects of bioreactor operation to include system set up and media flush procedures, cell expansion, preparation, and inoculation, sample collections from the ICS and ECS, media glucose testing, cell counts, media preparation, media exchanges, and cleaning and autoclaving of glassware. Times recorded were inclusive of set up, experimental manipulations, and clean up.

SECTION 5: RESULTS

PART 1: MONOCLONAL ANTIBODY PRODUCTION IN MURINE ASCITES

CLINICAL OBSERVATIONS

The incidence of clinical abnormalities observed in ascites mice during the study are presented in Table 3. All animals in the control groups remained clinically normal throughout the study. No abnormal clinical observations were made for any test animals during the two week period following injection of pristane. Clinical abnormalities observed in mice during the ascites production process were similar among groups, yet differed in the rapidity of progression and severity, and the incidence of specific abnormalities (see Table 3). These results are discussed more completely under the individual group results which follow.

The onset of clinical abnormalities in all groups was generally related to the development of ascites as determined by visible abdominal distention and increasing body weight. The range of days over which the first abdominal tap was performed was indicative of the time of initial ascites development for each group. This data is presented in Figure 6. Initial clinical abnormalities noted in all groups included roughened haircoats, hunched posture, and progressively increasing abdominal distention.

For animals in all groups, the severity of clinical abnormalities increased progressively over time, and as the number of abdominal taps increased. Decreases in activity, palpable abdominal masses, thin appearance, and evidence of dehydration as determined by decreased skin turgor and enophthalmos were most often noted later in the time course of study.

There were a number of clinical abnormalities seen during the observation period following abdominal paracentesis. These abnormalities were observed in some animals from all groups and included roughened haircoats, hunched posture, decreased activity often associated with the observation of mice huddling together in the cage, tachypnea and in some cases dyspnea, and pallor which presented as a blanching of normal pink color which was most evident on the muzzle and ears. These signs were most often transient, but in 19 animals, from 4 of the 5 test groups, these signs were severe and persistent leading to death or euthanasia following the first or second abdominal tap. Twelve of these 19 animals were from Group 2C6D9. All animals that died following paracentesis did so within approximately 30 minutes from the time of tap.

Persistent leakage of ascites fluid from the abdominal tap site was observed in one group of animals. This observation is discussed in more detail in the individual group results for Group RMK.

Small subcutaneous soft tissue nodules were grossly

visible and palpable in some animals from all groups, at the caudal left ventral abdomen, the site of intraperitoneal injection of pristane on day -14, and hybridoma cells on day 0. Nodules observed post-pristane were apparent in 8 animals from 4 groups, and were first noted on study day 0 prior to hybridoma cell injection. The nodules were raised and ranged in size from 2 to 6 mm in diameter. In 5 of the 8 animals which developed subcutaneous nodules post-pristane, the nodules decreased in size or became inapparent within 7-14 days from the initial observation.

Nodules observed post-hybridoma cell injection were first noted 13 to 20 days following hybridoma cell inoculation in 14 animals from 3 groups. The nodules were raised and ranged in size from 2 to 5 mm in diameter. The nodules increased in size over time in 9 animals. No change in nodule size over time was noted for the remaining 5 animals.

Individual group results follow Table 3.

Legend

Table 3

Incidence of Clinical Abnormalities in Ascites Mice

For each clinical observation listed, incidence is presented as the total number of animals in each group which were observed to have the indicated clinical sign.

Detailed descriptions of each clinical observation are provided in the text.

Table 3.
Incidence of Clinical Abnormalities in Ascites Mice

	CAF_1 Mice					SCID Mice	
	Control	2B11	3C9	2C6D9	3D6	Control	RMK
Number of animals	12	20	20	20	20	11	20
Daily observations							
Clinically normal	12	0	0	0	2	11	0
Subcutaneous mass at injection site post-pristane	0	4	1	2	0	0	1
Subcutaneous mass at injeciton site post-hybridoma	0	0	10	0	3	0	1
Rough hair coat	0	20	20	20	18	0	20
Hunched posture	0	20	20	20	18	0	20
Decreased activity	0	3	8	18	12	0	5
Palpable abdominal mass	0	1	6	0	6	0	0
Thin appearance	0	4	3	1	2	0	1
Dehydration	0	3	0	18	1	0	0
Animals euthanized prior to taps	0	1	0	0	0	0	0
Animals died prior to taps	0	0	0	1	0	0	0
Abdominal distension	0	19	20	19	18	0	20
Animals tapped	0	19	20	19	18	0	20
Animals euthanized post-tap 1	0	0	0	1	0	0	0
Animals died post-tap 1	0	0	0	1	0	0	0
Animals euthanized post-tap 2	0	4	1	7	1	0	0
Animals died post-tap 2	0	0	1	3	0	0	0
Animals euthanized at tap 3	0	15	18	7	17	0	20
Observations during post-tap period							
Rough hair coat	0	19	10	19	5	0	0
Hunched posture	0	19	17	19	6	0	10
Decreased activity	0	19	17	19	8	0	9
Increased respiratory rate	0	19	4	19	3	0	11
Pallor	0	19	7	19	10	0	3
Ascites leak from tap site	0	0	0	0	0	0	9

CLINICAL OBSERVATIONS (cont'd)

Group 2B11

One mouse in this group was euthanized following pristane and hybridoma cell injections, but before abdominal paracentesis. Seven days following hybridoma cell inoculation, this mouse had a palpable caudal abdominal mass. On day 9 the mouse appeared thin, had lost weight, was dehydrated, had a roughened haircoat and hunched posture, and was euthanized. Postmortem lesions for this mouse are described under Necropsy Observations.

All animals in this group exhibited clinical abnormalities following abdominal tap including hunched posture, roughened haircoat, decreased activity, increased respiratory rate, and pallor of the muzzle and ears. These signs were generally transient and of relatively mild severity with the exception of 4 animals with persistent and severe abnormalities necessitating euthanasia following the second abdominal tap.

Group 3C9

This group had the greatest incidence of subcutaneous soft tissue masses at the site of intraperitoneal injection. Masses developed in 10 of 20 animals between days 13 and 20 following hybridoma cell inoculation. Six animals in this group developed palpable abdominal masses during the study.

Clinical abnormalities following abdominal tap were generally transient and mild to moderate in severity with two exceptions. One animal died within 30 minutes following tap 2, and one animal was euthanized immediately following tap 2 because of marked pallor, dyspnea, and recumbency.

Group 2C6D9

Clinical abnormalities were the most rapidly progressive and severe in this group as is demonstrated by the greatest incidence of mortality and euthanasia prior to tap 3. Ten days post inoculation of hybridoma cells, and prior to any abdominal taps, one mouse in this group evidenced weight loss, appeared thin, had a roughened haircoat, hunched posture, and a palpable caudal abdominal mass. This animal was found dead in the cage the following day. Postmortem lesions for this animal are described under Necropsy Observations.

Of the 19 animals in this group which were tapped to collect ascites, all demonstrated transient or persistent clinical abnormalities post tap which included roughened haircoat, hunched posture, decreased activity, pallor of the muzzle and ears, increased respiratory rates and in some cases dyspnea. One animal died within 30 minutes of tap 1, and one animal was euthanized following tap 1. Three animals died within 30 minutes of tap 2, and an additional 7 animals were euthanized following tap 2

because of persistent and severe clinical abnormalities. The incidence of animals exhibiting decreased activity and signs of dehydration was also greatest in this group.

Group 3D6

Two animals in this group remained clinically normal throughout the study, produced no ascites, and so were not tapped. Postmortem lesions were, however, observed in both of these animals and are described under Necropsy Observations. Clinical abnormalities following abdominal tap in this group were generally transient and of mild to moderate severity. One animal was euthanized following tap 2 because of more severe and persistent clinical abnormalities.

Group RMK

There was 100% survival to tap 3 in this group. Overall, by comparison to other groups, clinical abnormalities were generally least severe in this group. Clinical abnormalities observed following abdominal taps were also generally transient and mild. It was the impression of the author that a number of these animals could have been tapped at least one additional time without unduly compromising their clinical condition.

Nine animals in this group had persistent leakage of ascites from the abdominal tap site. This observation was noted only in this group. The fur on the left lateral

abdomen around and ventral to the tap site was observed to be wet with clear fluid during observations made the day following abdominal tap. The leakage of ascites had usually resolved by 2 days following abdominal tap.

Abnormal clinical signs were observed in one control animal for this group during the quarantine period. This animal appeared thin, had hunched posture, roughened haircoat, dyspnea, decreased activity, and was euthanized. The prominent findings on post mortem examination included hemothorax and hemoperitoneum suggestive of trauma or a congenital cardiovascular anomaly. Bacterial cultures and mycoplasma cultures submitted at the time of necropsy were negative. As a result of this animal being euthanized, the control group for Group RMK was reduced to 11 animals.

TIME INTERVALS FOR ABDOMINAL TAPS

The time interval, represented by the range of days during which abdominal taps 1, 2, and 3 were performed for each group, and the mean ± 1 standard deviation for each tap interval are presented in Figure 6. Significant differences were observed between groups in the range of days during which abdominal taps were performed. For Group 2B11 and 2C6D9, the development of ascites was relatively synchronous among animals within each group, and consequently, the days on which abdominal taps 1, 2, and 3 were performed were nearly the same for all animals in each

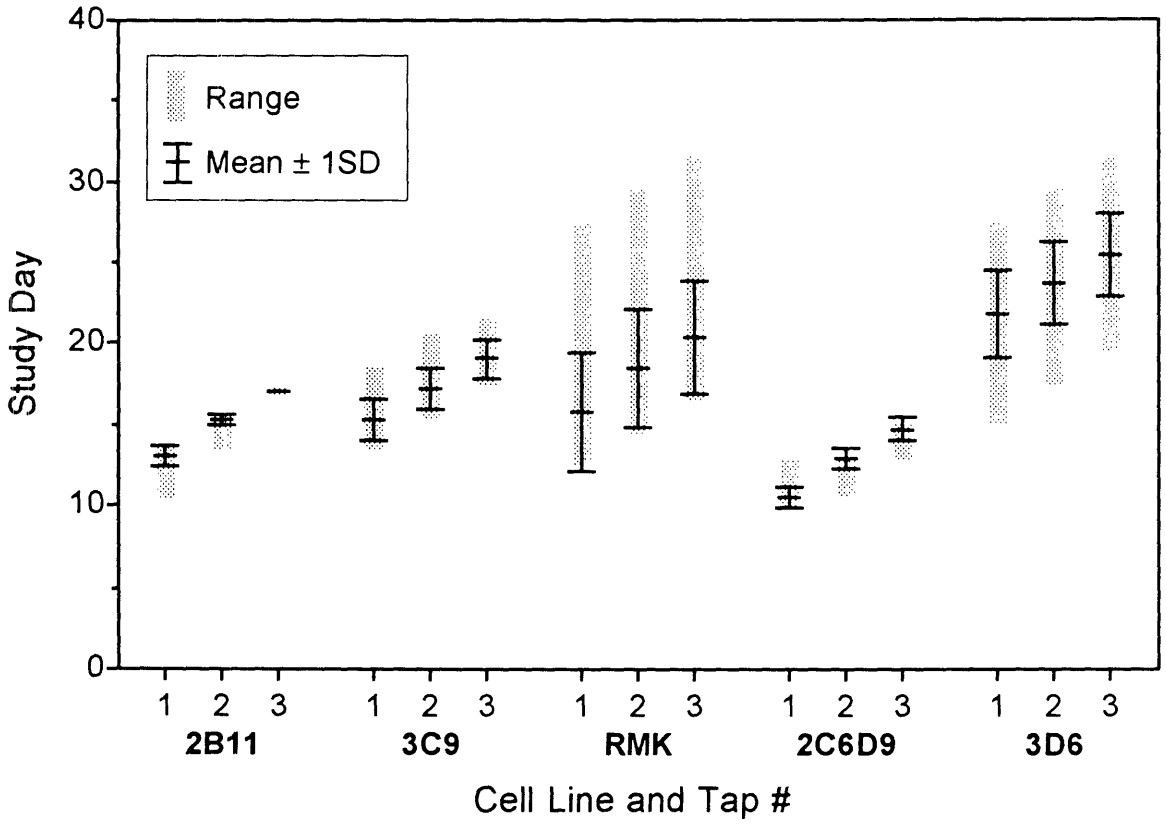
group. The range of days during which each tap was performed was progressively greater for Groups 3C9, 3D6, and RMK, respectively, indicating greater variability among animals in the time of ascites development, and consequently, the time of abdominal taps.

In comparisons of mean tap days between groups, the same trend was observed for taps 1, 2, and 3. For tap 1, the mean day of tap for Group 3D6 (day 22) was significantly greater than that for Groups RMK (day 16) and 3C9 (day 15), which were significantly greater than that for Group 2B11 (day 13) which was significantly greater than that for Group 2C6D9 (day 11).

For tap 2, the mean day of tap for Group 3D6 (day 24) was significantly greater than that for Groups RMK (day 18) and 3C9 (day 17), which were significantly greater than that for Group 2B11 (day 15) which was significantly greater than that for Group 2C6D9 (day 13).

For tap 3, the mean day of tap for Group 3D6 (day 26) was significantly greater than that for Groups RMK (day 20) and 3C9 (day 19), which were significantly greater than that for Group 2B11 (day 17) which was significantly greater than that for Group 2C6D9 (day 15).

Figure 6.
Time Intervals for Abdominal Taps



BODY WEIGHT MEASUREMENTS

A significant difference in mean body weights was observed for the CAF_1 mice versus the SCID mice at day 0; the mean body weight for the SCID mice was significantly lower than the mean body weight for the CAF_1 mice. As a result of this difference, no body weight comparisons were subsequently made between CAF_1 mice (Groups 2B11, 3C9, 2C6D9, and 3D6) and SCID mice (Group RMK).

Study Days -14 Through 0

No significant differences were observed in mean body weights for the CAF_1 control versus pristane treated test animals or the SCID control versus pristane treated test animals at days -14, -7, and 0.

No significant difference in body weights among CAF_1 control and test animals, and SCID control and test animals were noted at day 0.

Study Day 0 to the First Tap

Mean body weights in grams ± 1 standard deviation and mean percent increase in body weights between day 0 and the before tap weight at the first abdominal tap ± 1 standard deviation are presented for each group as follows:

Group	Day 0	Tap 1	% Increase
2B11	30.26 ± 1.73	34.79 ± 2.27	15.00 ± 3.46
3C9	29.71 ± 2.00	35.26 ± 2.01	19.24 ± 4.27
2C6D9	30.75 ± 2.35	33.86 ± 3.36	10.11 ± 7.66
3D6	29.65 ± 1.93	34.85 ± 2.78	17.59 ± 6.36
RMK	25.07 ± 2.25	30.41 ± 3.04	23.77 ± 6.27

The mean percent increase in body weight was significantly greater for Group RMK, and significantly less for Group 2C6D9, in comparison to other groups. The mean percent increase in body weight between day 0 and the before tap weight at the first abdominal tap for all groups combined was 17.14%.

Study Day 0 Through the End of Study

Because of considerable variability in the time of development of ascites and the range of days during which animals were tapped, both within and between groups, as presented in Figure 6, and because of the impact of this variability on body weight measurements, body weight comparisons within and between groups over time were not generally considered to be meaningful.

For comparisons of mean body weights between individual test and control groups for each specific tap (1, 2, or 3), test group body weights were compared to the control group body weight on the mean study day which the specific tap was performed for the test group. Control group weights

were compared to the post-tap weights (after removal of ascites), for the test groups as these weights were considered to more accurately reflect the actual body weight of the animals.

Mean post-tap body weights for the test groups ± 1 standard deviation at taps 1, 2, and 3 were as follows:

Group	Tap 1	Tap 2	Tap 3
2B11	30.79 ± 2.15	28.27 ± 2.56	28.89 ± 2.79
3C9	32.84 ± 2.11	30.98 ± 2.75	29.87 ± 3.02
2C6D9	30.36 ± 2.91	27.79 ± 2.38	27.61 ± 1.62
3D6	32.38 ± 2.39	31.21 ± 2.08	30.57 ± 2.14
RMK	27.80 ± 2.46	29.66 ± 3.21	30.36 ± 3.45

Group 2B11

Because the development of ascites, and consequently, days of abdominal taps were relatively synchronous in this group, mean body weights for this test group and the corresponding control group have been graphed over time, to include the mean percent drop in body weight for the test group at taps 1, 2, and 3 (Figure 7). Body weights for the test group began to increase on day 10 relative to the control weights, continued to increase, and then reached a plateau between day 11 and the first abdominal tap on day 13. The mean percent drop in body weight was significantly greater at the first tap (11.50%) in comparison to taps 2 and 3 (6.04 and 5.24%, respectively). There was no

significant difference between the post-tap weight for the test group and the control group weight following tap 1. The post-tap weights for the test group were significantly lower than the control weights following taps 2 and 3. Likewise, post-tap weights within Group 2B11 were significantly lower following taps 2 and 3 when compared to tap 1.

Group 3C9

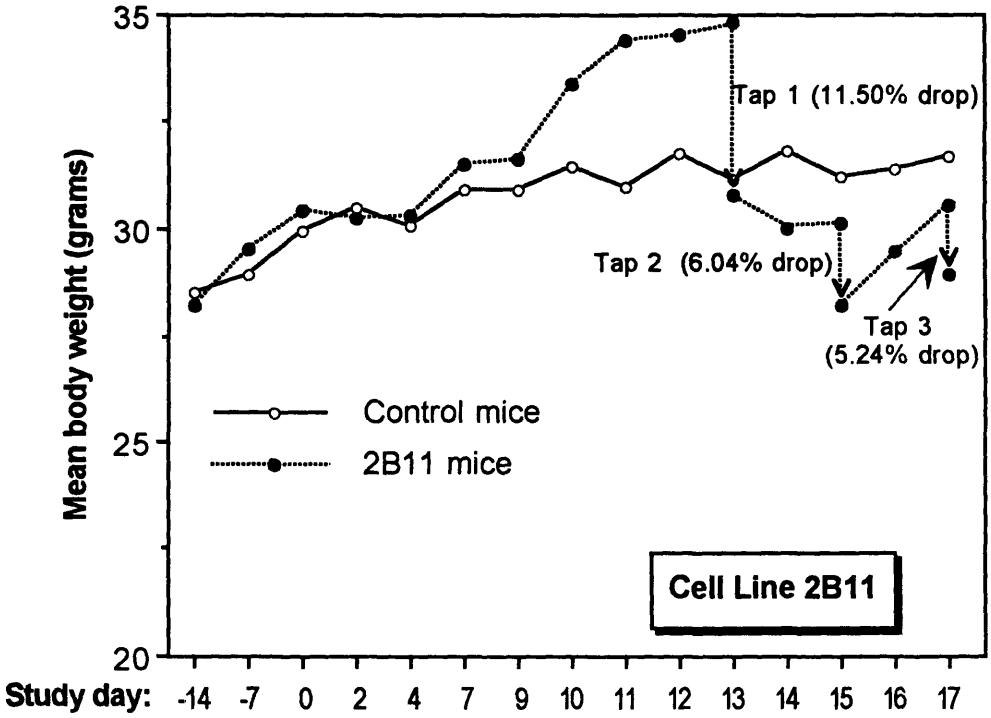
The mean percent drops in body weight were not significantly different for tap 1 (7.18%), tap 2 (7.11%), and tap 3 (5.75%) in this group. There were no significant differences between the post-tap weights for the test group following taps 1, 2, and 3 and the corresponding control weights, however, post-tap body weights did significantly and progressively decrease within Group 3C9 from taps 1 through 3.

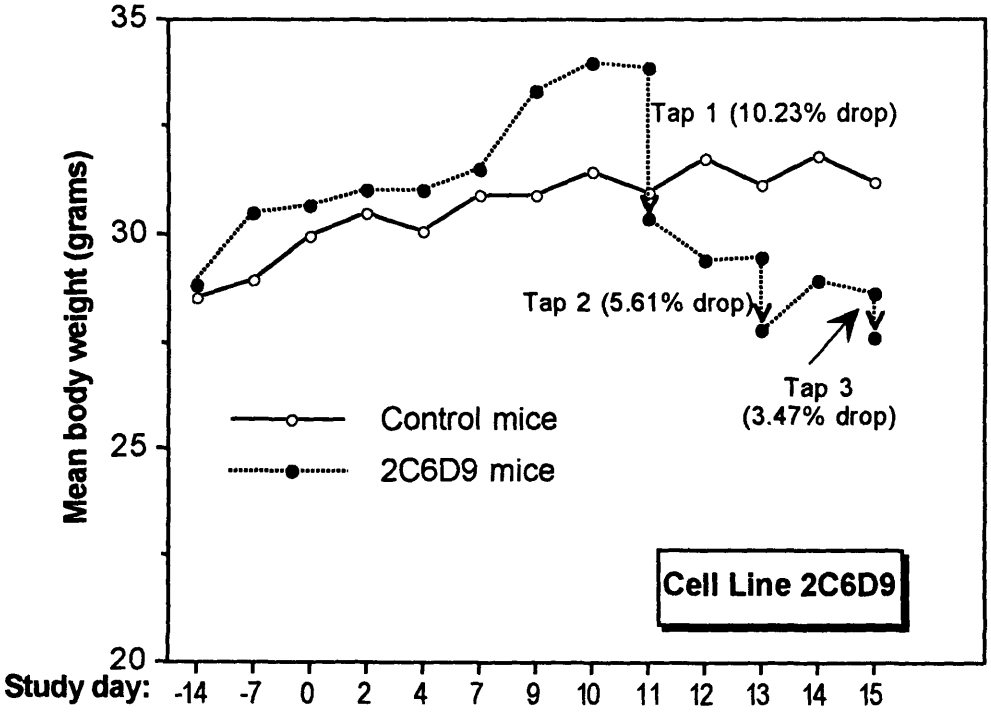
Group 2C6D9

Development of ascites, and consequently, days of abdominal taps were relatively synchronous in this group. Mean body weights for this test group and the corresponding control group have been graphed over time, to include the mean percent drop in body weight for the test group at taps 1, 2, and 3 (Figure 7). Body weights for the test group began to increase on day 9 relative to the control weights, continued to increase between days 9 and 10, and then

reached a plateau between day 10 and the first abdominal tap on day 11. The mean percent drop in body weight decreased significantly from the first through the third abdominal tap (10.23, 5.61, and 3.47%, respectively). There was no significant difference between the post-tap weight for the test group and the control group weight on day 11 following tap 1. The post-tap weights for the test group were significantly lower than the control weights following taps 2 and 3. Likewise, post-tap weights within Group 2C6D9 were significantly lower following taps 2 and 3 by comparison to weights following tap 1.

Figure 7.
Ascites Mice Body Weights





Numbers in parentheses indicate % change in body weight before tap/after tap

Group 3D6

The mean percent drops in body weight were not significantly different for tap 1 (7.00%), tap 2 (5.40%), and tap 3 (5.21%) in this group. There were no significant differences among the post-tap weights for the test group following taps 1, 2, and 3 and the corresponding control weights, however, post-tap body weights did significantly decrease within the test group from tap 1 to 2.

Group RMK

The mean percent drops in body weight were significantly greater for tap 1 (8.45%) and tap 3 (6.99%) in comparison to tap 2 (4.31%) in this group. There were no significant differences between the post-tap weights for the test group and the corresponding control weights following tap 1, however, post-tap body weights for the test group were significantly greater than the corresponding control weights following taps 2 and 3. Likewise, post-tap weights within Group RMK were significantly greater at taps 2 and 3 in comparison to weights at tap 1.

Correlation Between Body Weights and Clinical Observations

In examination of individual animal data, no definitive correlation could be made between the magnitude of the percent drop in body weight at the time of abdominal taps and the presence or absence of post-tap clinical

abnormalities such as pallor, tachypnea, hunched posture and decreased activity.

ASCITES MICE SURVIVAL

The survival of ascites mice over time is presented in Table 4. Survival is represented by the number of animals tapped in each group at taps 1, 2, and 3, respectively. For Group RMK, no animals died or were euthanized during the study, therefore survival was 100% at all timepoints. Two animals in Group 3D6 did not produce ascites and so were not tapped. Survival percentages for Group 3D6 corrected for these two animals not tapped would be 100, 100, and 95%, respectively, for taps 1, 2, and 3.

Number of animals tapped for all groups for taps 1, 2, and 3, respectively, ranged from 90-100%, 85-100%, and 35-100%, respectively. For all groups combined, overall survival to tap 1 was 98%; to tap 2 was 96%; and to tap 3 was 79%.

Table 4.
Ascites Mice Survival

Number of Animals Tapped

cell line	animals tap 1	animals tap 2	animals tap 3
2B11	19/20 (95%)	19/20 (95%)	15/20 (75%)
3C9	20/20 (100%)	20/20 (100%)	18/20 (90%)
RMK	20/20 (100%)	20/20 (100%)	20/20 (100%)
2C6D9	19/20 (95%)	17/20 (85%)	7/20 (35%)
3D6[1]	18/20 (90%)	18/20 (90%)	17/20 (85%)

[1] Two mice inoculated with 3D6 cells did not produce ascites.

BACTERIAL CULTURE OF PRISTANE

No bacterial growth was identified in any samples obtained from the 4 bottles of pristane which were submitted for aerobic and anaerobic bacterial cultures.

NECROPSY OBSERVATIONS

Incidence of gross lesions observed in mice at necropsy are presented in Table 5.

Legend

Table 5

Incidence of Gross Lesions at Necropsy in Ascites Mice

For each gross lesion listed, incidence is presented as the total number of animals in each group which were observed to have the indicated lesion.

Detailed descriptions of each gross lesion are provided in the text.

Table 5.
Incidence of Gross Lesions at Necropsy in Ascites Mice

	CAF_1 Mice					SCID Mice	
	Control	2B11	3C9	2C6D9	3D6	Control	RMK
Number of animals	6	20	20	20	20	6	20
Mean day of necropsy	31	16	19	13	26	32	20
Abdominal cavity							
Ascites	0	0	0	0	0	0	3
Hemoperitoneum	0	9	16	14	12	0	1
Disseminated tumor	0	19	20	18	17	0	19
Solid tumor mass	0	13	13	10	19	0	3
Peritesticular mass	0	3	11	0	5	0	1
Adhesions	0	12	2	9	12	0	1
Diaphragm – nodules	0	0	1	4	1	0	17
Diaphragm – plaques	0	0	10	0	12	0	0
Hepatomegaly	0	4	1	2	7	0	2
Splenomegaly	0	2	5	0	7	0	6
Renomegaly	0	1	0	0	3	0	1
Ureter dilated	0	1	1	0	0	0	1
Bladder distended	0	0	0	2	1	0	1
Bowel dilated	0	0	0	0	5	0	0
Ventral edema	0	0	0	0	0	0	7
Thoracic cavity							
Enlarged lymph nodes	0	12	9	0	11	0	9
Mediastinal mass	0	0	0	0	1	0	3
Injection site							
Subcutaneous mass	0	2	10	1	2	0	4
Abdominal wall mass	0	1	10	0	1	0	2
Peritoneal mass	0	3	1	0	0	0	2
Abdominal tap site							
Subcutaneous hemorrhage	0	18	20	16	17	0	18
Muscle hemorrhage	0	3	0	3	2	0	0
Subcutaneous mass	0	0	0	0	0	0	12
Edema	0	0	0	0	0	0	4

Lesions in the Abdominal Cavity

Hemoperitoneum was frequently observed in all groups of CAF_1 mice, with incidences ranging from 9-16/20 mice. Hemoperitoneum was only observed in 1/20 SCID mice from Group RMK, and an additional 3 mice from this group had clear ascitic fluid in the abdominal cavity at the time of necropsy.

With the exception of one animal from Group 2B11 and one animal from Group 2C6D9, all test animals examined from all groups had disseminated tumor and/or solid tumor masses in the abdominal cavity. The disseminated tumor was characterized by intraabdominal seeding with multi-lobulated, nodular to irregular, reddish to tan-white soft tissue, primarily involving the mesentery from stomach to rectum, and also involving dorsal lumbar, perirenal, peritesticular, and caudal abdominal/cranial pelvic areas with an apparent tropism for abdominal fat. Solid tumors were characterized as distinct and generally larger soft tissue masses, which differed from, but often occurred with, the diffusely seeded tumor. Solid tumor masses ranged in size from approximately 0.5 cm^3 to 3.5 x 2.2 x 1.5 cm. The most common location of solid tumors was the left caudal, ventrolateral abdomen, which was the site of hybridoma cell injection. Solid tumors were also observed in other abdominal and pelvic locations. Disseminated and solid tumors were seldom attached to the peritoneum.

Presence of solid tumor masses in the peritesticular

fat was also frequently observed with incidences ranging from 1 to 11/20 mice in 4 of the 5 test groups. The highest incidence of peritesticular tumor masses was 11/20 in Group 3C9. No peritesticular tumor masses were observed in Group 2C6D9.

Abdominal adhesions were observed in animals from all test groups with incidences ranging from 1-12/20 mice. Adhesions were observed in only 1 SCID mouse from Group RMK, and in 2 mice from Group 3C9, with incidences of 9, 12, and 12/20, respectively for Groups 2C6D9, 2B11, and 3D6. Abdominal adhesions were characterized by fibrous tissue attachments between abdominal structures which included the tumor, abdominal wall, diaphragm, liver, gastrointestinal tract, kidneys, ureters, bladder, seminal vesicles, and testicles.

Singular or multiple tumor nodules were observed attached to the hepatic surface of the diaphragm in animals from 4 of the 5 test groups. The incidence of diaphragmatic nodules was much greater in SCID mice from Group RMK (17/20), than the incidences observed in 3 of the 4 groups of CAF_1 mice, Groups 3C9, 3D6, and 2C6D9, which ranged from 1-4/20 mice, respectively. Nodules were frequently attached to the hepatic-diaphragmatic ligament, were usually tan in color and well-circumscribed, ranging in size from approximately 0.1 to 0.5 cm^3.

Plaques or linear lesions that appeared to be within the diaphragm were observed in 10/20 mice from Group 3C9,

and 12/20 mice from Group 3D6. Similar lesions were not observed in any other groups. Plaques were characterized as raised, white to tan foci, usually multiple, that appeared to be within the diaphragm, and were ≤ 0.3 cm^2 in diameter. Linear lesions were also white to tan and raised, and appeared to be within the diaphragm. Microscopic examination of selected tissues performed to define this gross lesion confirmed infiltration of tumor cells into the diaphragm.

Hepatomegaly, splenomegaly, and renomegaly were observed with variable incidence in the test groups. Ureteral dilation and bladder distention were infrequently observed among test groups. Five of 20 animals in Group 3D6 had intestinal dilation. Organ dilation or distention was generally related to distal constrictive lesions created by tumor growth or adhesions.

Edema of the ventral body wall was observed in 7/20 SCID mice from Group RMK. Edema was not observed in mice from any other groups. Edema in subcutaneous tissues and muscle was primarily observed in the ventral abdominal wall, but in some animals edema of the ventral neck, thoracic wall, and/or rear limbs was also observed.

Lesions in the Thoracic Cavity

Enlarged mediastinal lymph nodes were frequently observed in mice from 4 of the 5 test groups. Incidences of 9, 9, 11, and 12/20 mice were observed for Groups RMK,

3C9, 3D6, and 2B11, respectively. No mediastinal lymph node enlargement was observed in mice from Group 2C6D9. Enlarged lymph nodes were characterized as single to multiple, tan to white nodes located in the mediastinum at the base of the heart, near the tracheal bifurcation. Enlarged lymph nodes ranged in size from approximately 0.2 to 0.3 cm^3.

Mediastinal masses were observed in 3 SCID mice from Group RMK and 1 CAF_1 mouse from Group 3C9. Mediastinal masses were not observed in mice from other groups. Masses were red to tan-white in color, and were located in the cranial mediastinum or at the base of the heart. Heart base masses measured approximately 0.4 x 0.3 x 0.2 cm, and one of the mediastinal masses filled the entire cranial mediastinum. Microscopic examination of selected tissues performed to define the mediastinal lymph node enlargement and mediastinal masses demonstrated accumulations of tumor cells within mediastinal lymph nodes.

Lesions at the Injection Site

Gross lesions were observed at the site of pristane and hybridoma cell injection in mice from all test groups. The injection site, which was the same for all mice in all groups, was the left caudal, ventrolateral abdominal wall.

Subcutaneous and abdominal wall masses were observed most frequently in Group 3C9 with incidences of 10/20 and 10/20. Five mice in this group had both subcutaneous and

abdominal wall masses. Incidences of these lesions ranged from 0-4/20 in mice from other test groups. Subcutaneous and intramuscular masses within the abdominal wall were reddish to tan, soft tissue masses ranging in size from approximately 0.2 cm^3 to 0.6 x 0.6 x 0.3 cm.

Masses within the abdominal cavity and attached to the peritoneum at the injection site were observed in mice from 3 test groups. Incidences were 1, 2, and 3/20 for Groups 3C9, RMK, and 2B11, respectively. Peritoneal masses were reddish, soft tissue masses ranging in size from 0.3 x 0.2 x 0.1 cm to 0.6 x 0.6 x 0.1 cm.

Microscopic examination of selected tissues performed to define the injection site masses demonstrated that the masses were tumors.

Lesions at the Abdominal Tap Site

Gross lesions were observed at the site of paracentesis for collection of ascites in mice from all test groups. The abdominal tap site, which was identical for all mice in all groups, was the left midlateral abdominal wall.

Subcutaneous hemorrhage was very frequently observed at the tap site for mice in all test groups with incidences ranging from 16-20/20. Muscle hemorrhage was observed less frequently, with incidences ranging from 2-3/20 for mice in Groups 2B11, 2C6D9, and 3D6.

Subcutaneous soft tissue masses were observed at the abdominal tap site in 12/20 SCID mice from Group RMK.

Similar lesions were not observed in other groups. These subcutaneous lesions were characterized by raised, tan to white soft tissue observed between the skin and abdominal wall at the tap site, ranging in size from 0.2 to 1.0 cm^2 in diameter. Microscopic examination of these lesions demonstrated that the tissue was composed of hybridoma tumor cells.

Edema was observed in the subcutaneous tissues and muscle of the abdominal wall at the tap site in 4/20 SCID mice from Group RMK. Edema was not observed at the abdominal tap site in mice from any other groups.

No significant gross lesions were observed at necropsy in any CAF_1 or SCID control mice.

Necropsy Observations for Selected Animals

One mouse from Group 2B11 was euthanized on day 9 prior to abdominal paracentesis. At necropsy, there was hepatomegaly and bilateral renomegaly. Abdominal adhesions were observed between the liver, stomach, and jejunum, and there were many adhesions between pelvic structures. The intestinal tract was generally devoid of ingesta. There was right hydroureter, and a soft tissue mass was observed at the neck of the bladder. The bladder wall was thickened and reddened on cross-section, suggesting the presence of an intramural mass within the bladder wall. Microscopic examination of tissues from this mouse confirmed that the urinary bladder wall was diffusely invaded by tumor

resulting in destruction of the normal architecture of the bladder wall and occlusion of the lumen of the bladder. Tumor was also identified in accessory sex glands, abdominal lymph nodes, mesenteric fat, kidney, and diaphragm.

One mouse from Group 2C6D9 was found dead on day 11 prior to abdominal paracentesis. Hepatomegaly was noted and there were abdominal adhesions present between the small intestine and colon. The bladder was reddened and distended, and there was an irregular reddened soft tissue mass at the neck of the bladder. The bladder wall was thickened and reddened on cross-section, suggesting the presence of an intramural mass within the bladder wall. Autolysis precluded histologic evaluation of tissues.

Two mice in Group 3C9 remained clinically normal and produced no ascites during the study. Lesions observed at necropsy for one mouse included a 1.8 x 1.0 x 0.7 cm thin-walled, translucent cystic mass in the left caudolateral abdomen, attached to the left peritesticular fat. There was a 0.4 x 0.4 cm slightly raised, reddened mass within the peritesticular fat and attached to the cystic mass. There was also mild bilateral renomegaly.

The other mouse in Group 3C9 had a 1.8 x 0.8 x 0.7 cm whitish-tan soft tissue mass in the mesentery of the descending colon, and a segment of the proximal jejunum was adhered to the surface of the mass. There was a reddened soft tissue mass just caudal to the preceding mass, and

also within the mesentery of the descending colon, with dimensions of 0.5 x 0.5 x 0.3 cm. There was a small reddened focus within the right peritesticular fat which measured 0.2 x 0.1 cm in diameter. Hepatomegaly, renomegaly, and splenomegaly were observed. There were multiple white, raised plaques within the diaphragm 0.1 to 0.3 cm in diameter. In the thoracic cavity, there was a white soft tissue mass at the base of the heart which measured 0.4 x 0.4 x 0.2 cm.

ASCITES VOLUMES AND ANTIBODY QUANTITATION

Ascites volumes and antibody quantitation data are presented in Table 6. Data are presented for taps 1, 2, and 3, respectively, for each cell line. Ascites volumes are post-centrifugation volumes.

Ascites Volumes

Different trends were observed between groups when mean ascites volume/mouse obtained over time from taps 1 through 3 were compared. For Group 2B11, the mean volume/mouse obtained at tap 1 (3.2 ml) was significantly greater than mean volumes obtained at taps 2 and 3 (1.5 and 1.2 ml, respectively). For Group RMK, mean volumes were significantly greater at taps 1 and 3 (2.5 and 2.1 ml, respectively) by comparison to tap 2 (1.2 ml). For Group 2C6D9, significant and progressive decreases in mean

volumes were observed from tap 1 through 3 (2.6, 1.2, and 0.6 ml, respectively). No significant differences were observed in mean volumes over time for Group 3C9 (2.0, 2.1, and 1.6 ml, respectively) and Group 3D6 (2.1, 1.6, and 1.4 ml, respectively).

Antibody Concentration

Different trends were observed between groups in comparison of antibody concentrations in ascites over time from taps 1 through 3. For Groups 2B11 and RMK, there were no significant differences in comparison of antibody concentrations at taps 1 and 2, and the concentrations were significantly greater at tap 3. Mean concentrations were 3.61, 4.32, and 6.36 mg/ml for Group 2B11 and 4.39, 5.69, and 15.03 mg/ml for Group RMK for taps 1, 2 and 3, respectively. The large standard deviation (± 10.94 mg/ml) in the mean antibody concentration for Group RMK at tap 3 reflects the variability in concentrations among individual animals, inclusive of outliers with 0 and 44.45 mg/ml antibody concentrations in ascites. For Group 3C9, the mean concentration of antibody was significantly greater at tap 2 (6.12 mg/ml) by comparison to taps 1 and 3 (2.50 and 3.84 mg/ml respectively). For Group 3D6, there was a significant and progressive increase in mean antibody concentration from tap 1 through 3 (3.83, 6.25, and 7.56 mg/ml, respectively). For Group 2C6D9, mean concentrations at taps 2 and 3 (6.82 and 6.53 mg/ml, respectively) were

significantly greater that the mean concentration at tap 1 (5.17 mg/ml).

Total Antibody Production and Mean Production Per Mouse

Total antibody production and mean antibody production in mg/mouse for each tap reflect combined contributions of ascites volumes and antibody concentrations. For Groups 2B11 and 2C6D9, antibody production was greatest at tap 1. For Group 3C9, production was greatest at tap 2, and for Group RMK, production was greatest at tap 3. For Group 3D6, production at taps 2 and 3 were not significantly different, but production at tap 3 was significantly greater than production at tap 1.

Total antibody produced was 454.50 mg for Group 2B11, 445.57 mg for Group 3C9, 422.90 mg for Group 2C6D9, and 489.36 mg for Group 3D6. The greatest production was achieved in Group RMK where 996.64 mg of antibody was produced.

Table 6. MAb Production in Murine Ascites

Ascites Volumes and Antibody Quantitation

cell line	tap #	number of mice tapped	total vol (ml)	$\bar{x}$ volume (ml/mouse) +/- 1 SD	$\bar{x}$ Ab conc (mg/ml) +/- 1 SD	total Ab (mg)	$\bar{x}$ Ab (mg/mouse) +/- 1 SD
2B11	1	19	61.4	3.2 +/- .73	3.61 +/- 1.18	229.30	12.07 +/- 5.13
	2	19	27.7	1.5 .74	4.32 1.30	116.93	6.15 3.60
	3	15	18.6	1.2 .84	6.36 1.99	108.27	7.22 4.97
	Totals		107.7			454.50	
3C9	1	20	40.5	2.0 +/- .80	2.50 +/- 1.20	104.14	5.21 +/- 3.29
	2	20	41.0	2.1 .72	6.12 1.56	237.36	11.87 3.57
	3	18	28.0	1.6 .87	3.84 1.01	104.07	5.78 3.48
	Totals		109.5			445.57	
RMK	1	20	50.2	2.5 +/- .92	4.39 +/- 2.97	213.05	10.65 +/- 7.56
	2	20	24.0	1.2 .88	5.69 2.75	135.87	6.79 5.56
	3	20	41.5	2.1 1.10	15.03 10.94	617.72	30.89 20.83
	Totals		115.7			996.64	
2C6D9	1	19	49.5	2.6 +/- .75	5.17 +/- 1.85	264.27	13.91 +/- 5.95
	2	17	20.2	1.2 .45	6.82 1.96	132.67	7.80 3.31
	3	7	4.5	0.6 .27	6.53 2.74	25.96	3.71 1.14
	Totals		74.2			422.90	
3D6	1	18	37.5	2.1 +/- 1.28	3.83 +/- 1.58	121.56	6.75 +/- 4.07
	2	18	28.3	1.6 .89	6.25 1.69	163.17	9.06 4.86
	3	17	24.5	1.4 1.06	7.56 2.38	204.63	12.04 9.11
	Totals		90.3			489.36	

Ascites Volume Summary

Murine ascites volume summary data is presented in Table 7. The mean volume per tap for taps 1, 2, and 3 combined, were similar among groups, ranging from 1.7 to 2.0 ml/tap. These data suggest that a correlation exists between total ascites volumes and % of possible taps performed. Group RMK which had 100% survival and 100% of possible taps performed had the largest total volume of ascites. Group 2C6D9, which had the lowest survival and lowest % of possible taps performed (72%) also had the lowest total volume of ascites.

Table 7.
Murine Ascites

Volume Summary Data

cell line	no. of taps total	% of taps[1]	total vol (ml)	$\bar{x}$ vol/tap (ml)
2B11	53	88%	107.7	2.0
3C9	58	97%	109.5	1.9
RMK	60	100%	115.7	1.9
2C6D9	43	72%	74.2	1.7
3D6	53	88%	90.3	1.7

[1] % = percentage of taps performed/possible maximum of 60.

Another factor which impacted final ascites volumes was the volume of the cell pellet which was discarded after centrifugation of raw ascites samples. For taps 1, 2, and 3, the percentage of the total volume of ascites collected which was discarded as cell pellets ranged from 20-26% for Group 2C6D9, 13-16% for Group 2B11, 12-17% for Group 3C9, 12 to 14% for Group 3D6, and 7-8% for Group RMK. These data relate to gross observations regarding the general appearance of the ascites fluid. While there was variability between animals and taps within groups, the ascites obtained from mice in Group 2C6D9 was generally very hemorrhagic. Ascites collected from mice in Group RMK was generally clear. Ascites collected from other groups was generally mildly to moderately hemorrhagic.

PRODUCTION TIME

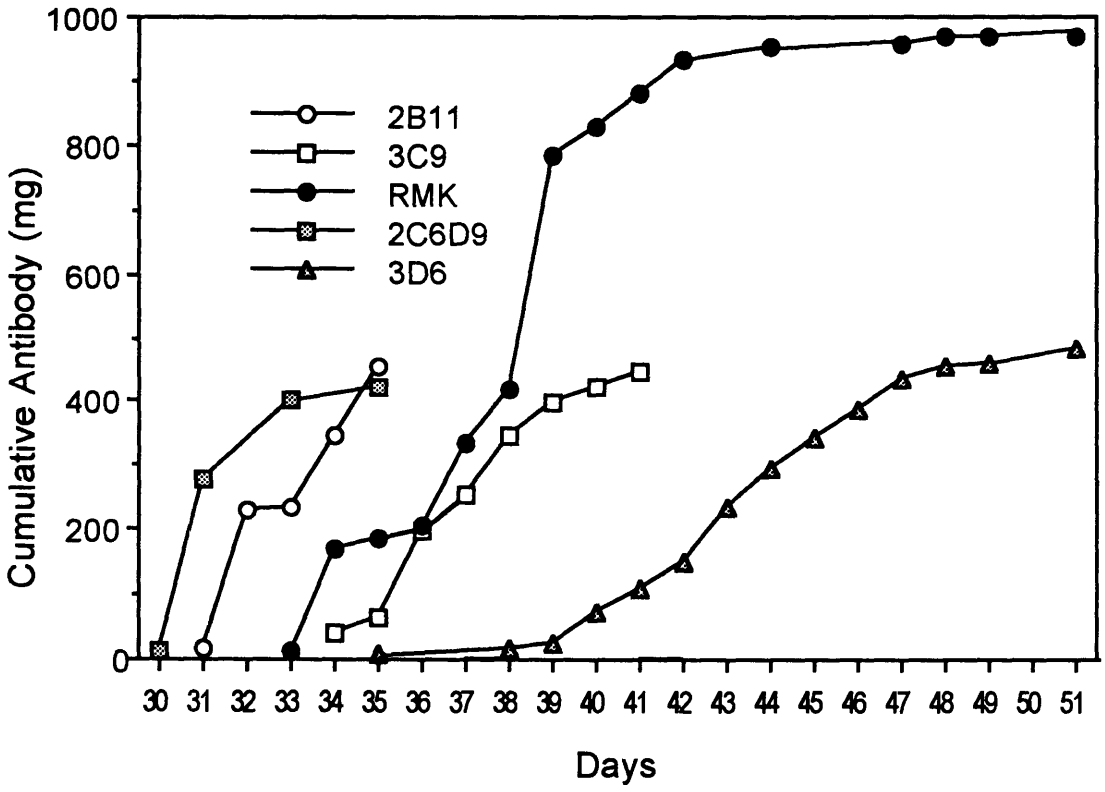
Cumulative antibody production over time for all groups of mice is presented in Figure 8. Day 0 was considered as the day that the mice arrived at the animal facility, therefore, production time is inclusive of the 6 day quarantine period, the two weeks following injection of pristane, and the time following hybridoma cell inoculation and prior to abdominal taps. Production began for each group at the time the first mouse was tapped.

Considerable variability between groups can be observed in production time. Production was complete for Groups

2B11 and 2C6D9 by day 35, while production for Group 3D6 was just beginning on day 35 and extended until day 51. Production began for Group 3C9 on day 34 and was complete on day 41. Production began on day 33 for Group RMK and production was not complete until day 51. The relatively flat slope of the production line for Group RMK from approximately day 42 through day 51 indicates that mice producing ascites late in time contributed relatively little to the total production.

Figure 8.
Production Time

Cumulative Antibody Production in Ascites



PART 2: MONOCLONAL ANTIBODY PRODUCTION IN HOLLOW FIBER BIOREACTORS

SYSTEM OPERATIONS

All systems were operated for 65 days without contamination. Cell growth within the bioreactors was monitored visually for systems A and B which had transparent bioreactor cartridge shells. Visualization was not possible for system C as the cartridge shell of the hybrid reactor was opaque. Cells grew to confluency around the bundle of hollow fibers and also settled in the gravity dependent areas of the bioreactors.

The media contained phenol red as a pH indicator, and by visual examination of the media color, pH gradients were observed for all systems between the ICS media and the ECS media. While the frequency of media changes in the ICS was generally sufficient to maintain relative pH neutrality of approximately 7.0 to 7.4, as determined by the red to orange color of the media, the ECS media at the time of product harvests was often visibly yellow indicating more acidic pH of approximately 6.5. ECS harvests from the hybrid reactors generally appeared less acidic than those from the conventional bioreactors.

It was necessary to check tubing luer connections frequently during the runs as they were observed to loosen over time, presumably related to vibrations induced by the media pumps.

Technical difficulties were encountered in the operation of all systems which required contact with technical service representatives. These problems are described below for the individual bioreactor runs.

System A

Cell Line 2B11

Cell viability dropped to 0% on day 46. Cells were subsequently expanded in static culture for reinoculation, and on day 53 the bioreactor was flushed and reinoculated with 60 x 10^6 cells according to instructions provided by the technical service representative. Operation of this bioreactor was continued through day 91.

Cell Line RMK

No problems were encountered during this run.

Cell Line 3C9

On study day 49, small pieces of black debris were observed in the media of the media bottle. Some debris was aspirated from the bottle and examined microscopically. The black debris ranged from approximately ≤ 1 to 3 mm in diameter and appeared amorphous with some linear strands. The morphology was not suggestive of bacterial or fungal contamination. Samples submitted for bacterial and fungal cultures were negative.

Black debris continued to be observed following media bottle changes and pinpoint black solid debris was subsequently observed on day 56 at the downstream potted ends of some hollow fibers in the bioreactor. On day 58, pinpoint black debris was also noted in some fibers at the upstream potted ends. The impact of the apparent lodging of debris in fibers on media flow rate, if any, is unknown. No debris was observed in ECS samples.

After completion of the run and disassembly of the system, a pipe cleaner was inserted into the metal tubing of the media bottle cap assembly and black material was removed from the inside of the metal tubing, suggesting that something was leaching from the metal components of the tubing. This possibility was subsequently confirmed in discussions with the technical representative.

System B

In all system B runs, cloudiness was observed in the ICS media which contained 1% serum. Media samples were examined microscopically and were submitted for aerobic bacterial and fungal cultures. No bacteria or fungi were observed microscopically, and culture results were negative. The technical representative stated that cloudiness of the media was common and resulted from denaturation of serum proteins caused by the media pump mechanism.

Cell Line 2B11

During the initial set up and media flush, the media pump failed before the ECS of the bioreactor had completely filled with media. The system was subsequently disassembled and returned to the manufacturer. The pump was repaired and the bioreactor cartridge was replaced by the manufacturer.

The 65 day run was completed after the second system set up. On day 25, media was observed leaking from a crack in the plastic luer connector of one of the cartridge ECS harvest ports. Parafilm was placed over the harvest port luer plug and around the cartridge. Subsequent ECS harvests were obtained from other harvest ports. The parafilm stayed in place for the remainder of the run, prohibited further leakage of media, and prevented contamination of the system.

Cell Line 3C9

Thermoregulation problems with the CO_2 incubator in which the system was housed were first noted on day 51. Alternative incubator space was not available for this system, and attempts to repair the temperature sensor were unsuccessful. Incubator temperature varied between 37.0 and 38.5°C during the remainder of the run. The mean temperature during this period of time was 37.6°C.

On day 63, the CO_2 tank step down regulator malfunctioned and resultant pressure changes apparently

caused disconnection of the gas tubing from the CO_2 tank to the incubator in which the system was housed. The CO_2 measured inside the incubator at the time the malfunction was discovered was 0%, and the color of the media was slightly purple indicating that the pH had become basic in the absence of CO_2. The author had been working in the laboratory that afternoon and became aware of the problem when frost was noticed on the regulator, therefore the problem was not present for an extended period of time. The gas tubing was reconnected and the regulator was replaced.

Cell Line RMK

No problems were encountered during this run.

System C

During system set up and media flush procedures, media flow could not be established in one of the three bioreactor circuits. All system components were thoroughly examined in attempt to identify the source of the problem. After ruling out malfunction of other components, and after discussions with the technical service representative, the bioreactor cartridge in the circuit was replaced. Media flow was then successfully established in all circuits and the media flush procedure was initiated.

Cell Line 2B11

No problems were encountered during this run.

Cell Line 3C9

Cell viability dropped to 0% on day 16. Cells were subsequently expanded in static culture for reinoculation, and on day 23 the bioreactor was flushed and reinoculated with 100 x 10^6 cells according to instructions provided by the technical service representative. Operation of this bioreactor was then continued through day 65.

Cell Line RMK

No problems were encountered during this run.

LIMITING DILUTIONS

The stability of each cell line was measured after limiting dilution as the percent of clones which tested positive for antibody secretion. Percent positive clones from limiting dilution of cells retained from bioreactor cell inoculums on day 0 were 93, 93, and 100% for cell lines 2B11, 3C9, and RMK, respectively. Percent positive clones from limiting dilution of cells retained from bioreactor ECS harvests on day 60 were 100% for all cell lines from all systems with the exception of cell line 3C9 from system C where no wells developed clones after limiting dilution.

HARVEST VOLUMES AND ANTIBODY QUANTITATION

Harvest volumes and antibody quantitation data are presented in Table 8. Product harvests began on day 4 for systems A and B so there were a total of 27 harvests over the 65 day run. Product harvests began on day 7 for system C so there were a total of 26 harvests over the 65 day run. Fewer harvests were made for cell line 3C9 in system C. No harvests were made between the time of bioreactor cell death and the 7th day post reinoculation of cells (harvest days 21, 23, 25, and 28).

Harvest Volumes

Harvest volumes presented in the table are post-centrifugation volumes. Total harvest volumes were similar for systems A and C which would be expected as the bioreactors for these two systems had the same extracapillary space volume (7 ml). The larger total harvest volumes for system B, approximately double the volume for systems A and C, reflects the larger extracapillary space volume of the bioreactor in this system (12 ml).

The similarities in mean volume per harvest for all cell lines within system B, and for all cell lines within system C, reflect the standardized harvest volumes for simultaneous ECS harvest and fresh media flush of the bioreactors. The greater variability in mean volumes per harvest for cell lines in system A reflects the variability

of the volume of media and suspended cells in the ECS of the bioreactors. Harvests for this system were obtained by first aspirating the media with cells from the ECS and then refilling the ECS with fresh media.

Antibody Concentration and Total Antibody Production

Mean antibody concentrations for cell line 2B11 in the three bioreactor systems ranged from 0.71 to 1.31 mg/ml, with total antibody production ranging from 91.11 to 249.49 mg in 65 days. For cell line 2B11 in system A, which was reinoculated with cells on day 53, an additional 42.79 mg of antibody was produced between days 67 and 91 (data not presented).

Mean antibody concentrations for cell line 3C9 in the three bioreactor systems were greater than those for cell line 2B11, ranging from 2.16 to 4.45 mg/ml. Total antibody production was 236.60 mg for system A and 380.71 mg for system C. System B had the greatest mean antibody concentration and the greatest total harvest volume resulting in significantly greater total production of 1076.26 mg of antibody in 65 days.

Mean antibody concentrations for cell line RMK varied significantly between bioreactor systems. Systems A and C had very high mean concentrations of 11.08 and 10.11 mg/ml, respectively. Total antibody production was also very high in these systems. System A produced 1228.44 mg and system C produced 1286.92 mg of antibody in 65 days. By

comparison, the mean concentration of antibody for system B was much lower at 2.30 mg/ml as was total production at 553.81 mg.

Antibody Concentration and Antibody per Harvest

Mean antibody per harvest ranged from 3.37 mg to 49.50 mg among all the different cell lines and systems tested, and followed the same trends as those for total antibody production. Total antibody production and mean antibody per harvest reflect combined contributions of harvest volumes and antibody concentrations.

Antibody concentration and total antibody in bioreactor harvests over time for the 65 day run for each cell line in each system are presented in Figures 9 and 10. For cell line 2B11, the trend in antibody concentrations over time were similar among systems. Concentrations gradually increased, plateaued, and then decreased over the 65 day runs.

Greater fluctuations in antibody concentration, both within and between systems were observed for cell line 3C9. Concentrations for system B peaked early in the run, decreased then stayed relatively constant in the middle of the run, then declined at the end of the run. Concentrations decreased early in the run for system C in association with bioreactor cell death. After cartridge reinoculation, concentrations increased and stayed relatively constant for the duration of the run. No clear

pattern could be discerned in antibody concentrations over time for system A.

For cell line RMK, concentrations peaked early in the run for systems A and C and stayed relatively constant until the latter part of the run where greater fluctuations were observed prior to decline in concentrations at the end of the run. Concentrations were relatively constant throughout the run for system B, and were consistently lower than concentrations for the other 2 systems.

Peaks observed in fluctuating antibody concentrations over time were frequently associated with Monday harvests after the longer 3 day weekend time interval as compared to the 2 day time interval between other weekday harvests. Trends in total antibody per harvest over time were similar to the trends described for antibody concentrations over time.

Table 8.

MAb Production in Hollow Fiber Bioreactors

Harvest Volumes and Antibody Quantitiation

cell line	bioreactor system	number of harvests	total vol (ml)	$\bar{x}$ volume/ harvest (ml) +/- 1 SD	$\bar{x}$ Ab conc (mg/ml) +/- 1 SD	total Ab (mg)	$\bar{x}$ Ab/ harvest (mg) +/- 1 SD
2B11	A	27	136.1	5.0 +/- 1.03	0.71 +/- 0.60	91.11	3.37 +/- 2.73
	B	27	243.0	9.0 1.71	0.99 0.53	249.49	9.24 5.51
	C	26	126.7	4.9 0.11	1.31 0.94	164.46	6.33 4.44
3C9	A	27	114.8	4.3 +/- 1.32	2.16 +/- 2.15	236.60	8.76 +/- 9.21
	B	27	243.0	9.0 1.74	4.45 4.00	1076.26	39.86 38.66
	C	22	107.2	4.9 0.11	3.57 1.65	380.71	17.30 7.86
RMK	A	27	122.6	4.5 +/- 1.38	11.08 +/- 5.73	1228.44	45.50 +/- 25.52
	B	27	245.2	9.1 1.80	2.30 0.81	553.81	20.51 7.79
	C	26	127.1	4.9 0.09	10.11 4.00	1286.92	49.50 19.80

Figure 9. Antibody Concentration in Bioreactor Harvests

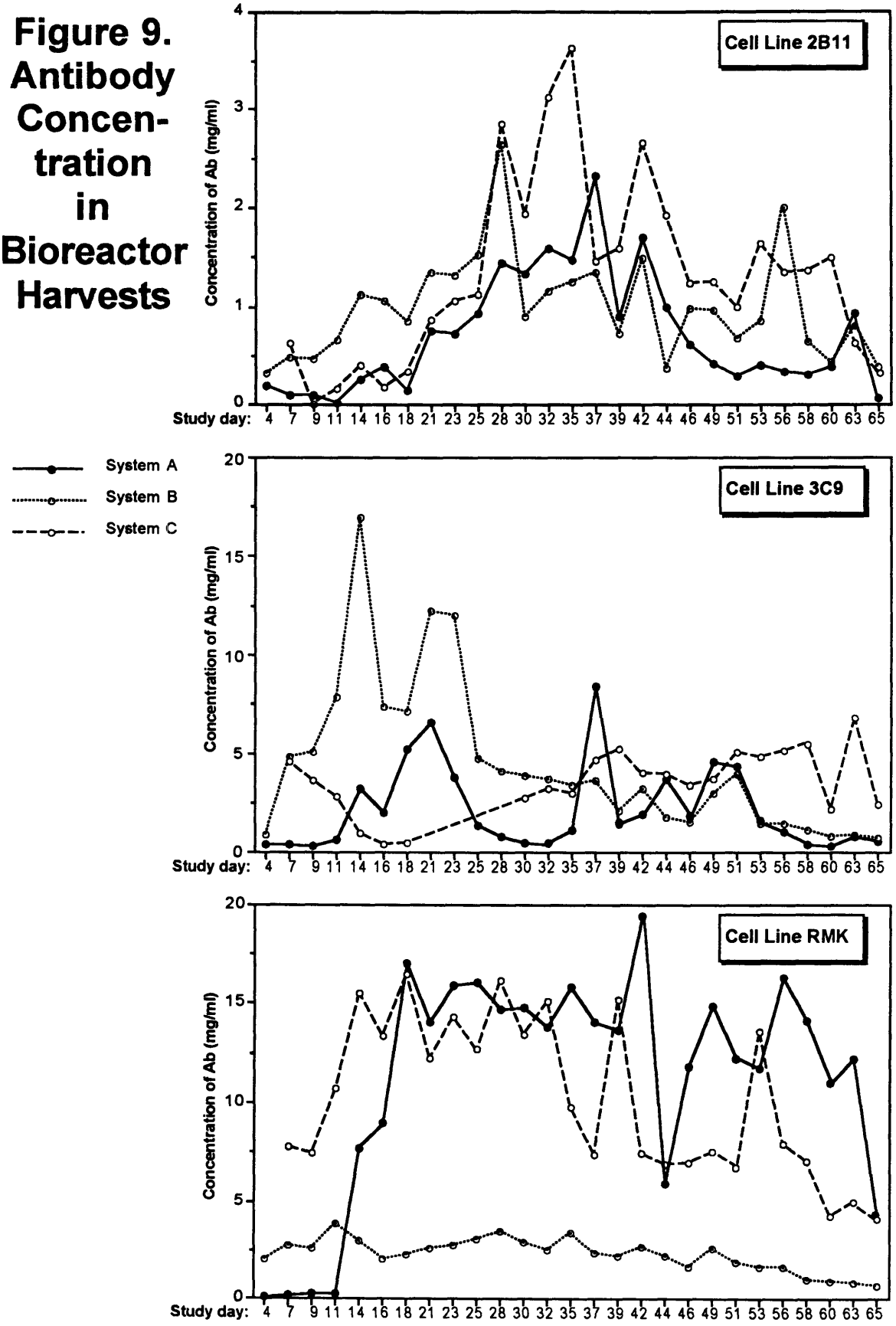
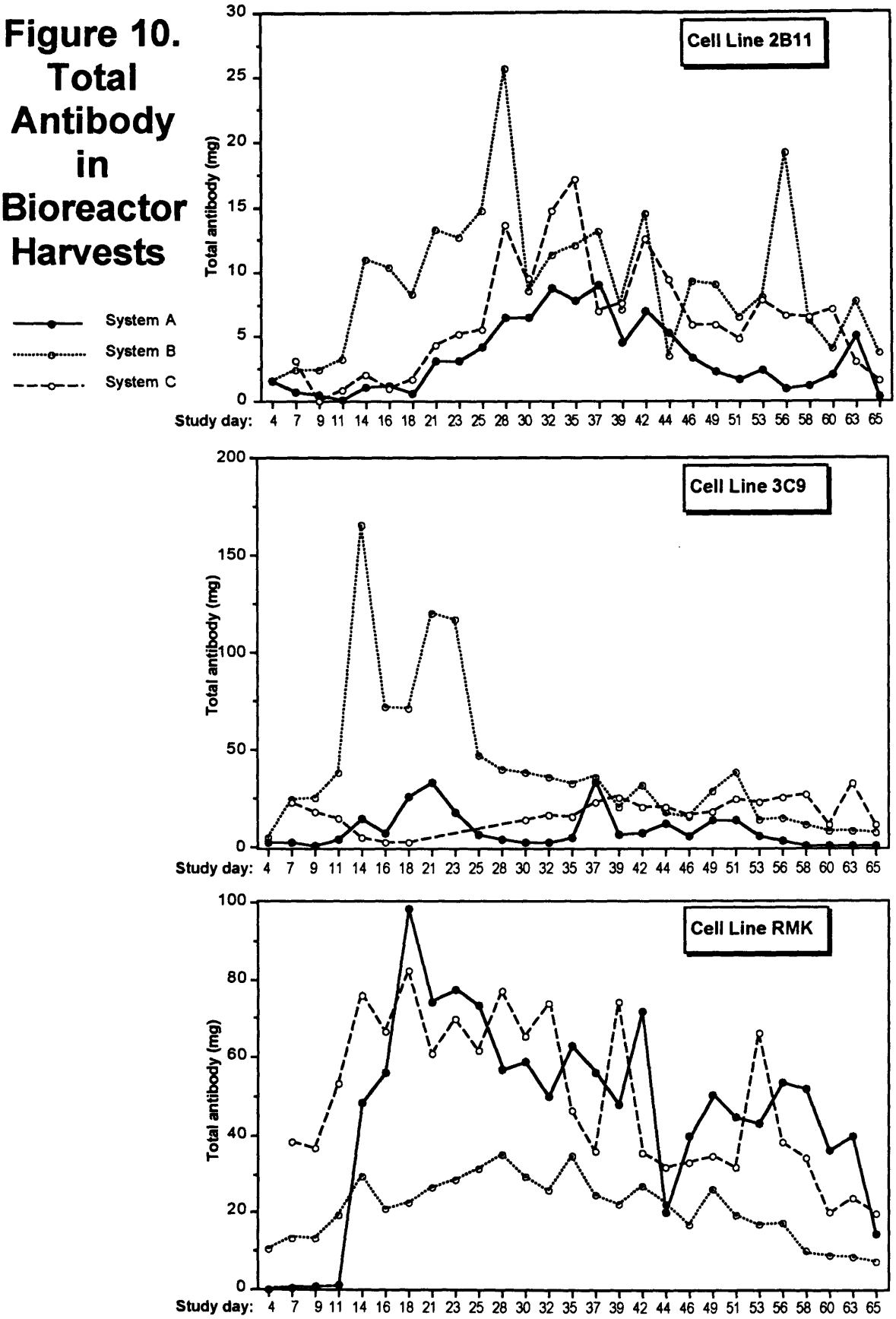


Figure 10. Total Antibody in Bioreactor Harvests



MEDIA CONSUMPTION

ICS Media Consumed

The total volume of ICS media used from initial system set up and media flush procedures through the end of the 65 day runs is presented below. The milligram amount of antibody produced per liter of media consumed is also indicated for each run.

Cell Line	System	ICS Media (L)	Mg Antibody/L Media
2B11	A	5.75	15.85
	B	10.00	24.95
	C	22.00	7.48
3C9	A	8.25	28.68
	B	14.00	76.88
	C	20.00	19.04
RMK	A	15.75	78.00
	B	17.50	31.65
	C	22.00	58.50

Mean media consumption for all cell lines in the 3 systems was 15 liters of media in 65 days. The mean quantity of antibody produced per volume of media consumed for all cell lines in the 3 systems was 37.89 mg/L.

ECS Media Consumed

The total volume of ECS media used from initial system set up and media flush procedures through the end of the 65 day runs was relatively insignificant by comparison to ICS media consumption. The total volume of ECS media consumed for system A ranged from 139.5 to 157.7 ml. The volumes of ECS media consumed for systems B and C were 270 and 180 ml, respectively, as these volumes were standardized. An additional 60 ml of ECS media was used for the bioreactor ECS flushes prior to reinoculation of cartridges for cell line 2B11 in system A and cell line 3C9 in system C.

MEDIA GLUCOSE CONSUMPTION, CELL COUNTS, AND CELL VIABILITY

It is the opinion of the author that total cell counts varied considerably dependent on how vigorously the ECS of the bioreactors was flushed during sample harvests; more vigorous flushes yielded more cells. ECS harvests were performed less vigorously early in the runs before the bioreactors reached cell confluency, and were more vigorous in mid to late parts of the runs in an attempt to prevent overgrowth of cells in the bioreactors.

Mean values for total cell counts (x 10^6/ml), % viable cells, and glucose consumption (mg/day) for the 65 day runs for each cell line in the 3 bioreactor systems were as follows:

Cell Line	System	Total Cells	% Viable	Glucose
2B11	A	9.06	31	133
	B	21.23	42	353
	C	18.41	53	479
3C9	A	20.76	40	269
	B	20.09	43	455
	C	9.79	20	353
RMK	A	16.40	45	552
	B	9.89	47	566
	C	14.40	49	564

For cell line 2B11 in system A and cell line 3C9 in system C, in which bioreactor cell death occurred during the runs, mean cell viability was lowest. Mean cell viability and glucose consumption were very similar in all systems for cell line RMK. Greater variability in these measurements was observed among systems for the other 2 cell lines.

Bioreactor antibody concentration in relation to media glucose consumption, cell counts, and cell viability over time are presented for each cell line in each system in Figures 11 through 19. Changes in media serum concentration over time are also indicated.

Cell Line 2B11

System A

The trends in glucose consumption and antibody concentration over time were similar. Glucose consumption decreased at the midpoint of the run prior to decreases in cell viability and antibody concentration. Bioreactor cell death as indicated by decrease in cell viability to 0%, was associated with decreased glucose consumption and decrease in antibody concentration. A significant increase in total cell counts was observed on day 49. Glucose consumption began to increase following reinoculation of cells on day 53. Because of relatively low glucose consumption and cell viability during this run, 1% serum was maintained in the ICS media and 10% serum was maintained in the ECS media throughout the run.

System B

Considerable fluctuation in glucose consumption and antibody concentration were observed over time. A decrease in glucose consumption and cell viability was observed between days 35 and 39. Following serum reduction in the ICS media on day 51, there was a gradual decrease in glucose consumption and total cell counts, and a transient increase followed by decrease in antibody concentration.

System C

Large fluctuations in glucose consumption were observed over time, although the overall trend followed that of antibody concentration. Reduction of serum in the ECS media on day 42 was associated with a subsequent decrease in antibody concentration. Antibody concentration continued to decrease following increase in serum in the ECS on day 56.

Cell Line 3C9

System A

The trends in glucose consumption and antibody concentration over time were similar. These measurements showed gradual fluctuations, increasing and decreasing over time. Glucose consumption decreased and total cell counts increased following serum reduction in the ICS media on day 44. Replacement of serum in the ICS media on day 53 was associated with a subsequent increase in glucose consumption. Antibody concentration decreased from day 51 through the end of the run.

System B

The trends in glucose consumption and antibody concentration over time were similar. Antibody concentration and glucose consumption peaked early in the run on days 14 and 16, respectively, and then decreased

gradually over the remainder of the run. Serum was removed from the ICS media on day 11 and reduced in the ECS media on day 49.

System C

Glucose consumption increased rapidly and then decreased rapidly at the beginning of the run. Decrease in glucose consumption and antibody concentration were associated with bioreactor cell death as indicated by cell viability decreasing to 0%. Following reinoculation of cells on day 23 glucose consumption gradually increased, and then decreased markedly following reduction of serum in the ICS on day 46. Antibody concentration remained relatively constant following reinoculation of cells.

Cell Line RMK

System A

The trends in glucose consumption and antibody concentration over time were similar. Glucose consumption and antibody concentration increased rapidly at the beginning of the run. Serum was removed from the ICS media on day 14. Following serum reduction in the ECS media on day 23, glucose consumption gradually decreased, but there was little change in antibody concentration. Following increase in serum in the ECS media on day 35, glucose consumption increased. Subsequent reduction of serum in

the ECS media on day 39 was again associated with a decrease in glucose consumption. Antibody concentration, with some fluctuations, remained high until the end of the 65 day run.

System B

Glucose consumption increased rapidly early in the run and then decreased following serum reduction in the ECS media on day 11. Glucose consumption decreased transiently then increased following increase of serum in the ECS media on day 18. A decrease in glucose consumption was again observed following decrease in serum in the ECS media on day 46. Antibody concentration remained relatively constant throughout the run, as did total cell counts.

System C

Large fluctuations in glucose consumption were observed over time, although the overall trend followed that of antibody concentration. Glucose consumption and antibody concentration increased rapidly early in the run. A marked decrease in glucose consumption and cell viability were observed following removal of serum from the ICS media on day 14, while antibody concentration remained relatively constant. Glucose consumption and cell viability increased following replacement of serum in the ICS media on day 21. Subsequent lesser reductions of serum in the ICS media were not associated with decreased glucose consumption.

Figure 11.
Bioreactor Antibody Concentration, Glucose Consumption, Cell Counts, and Cell Viability

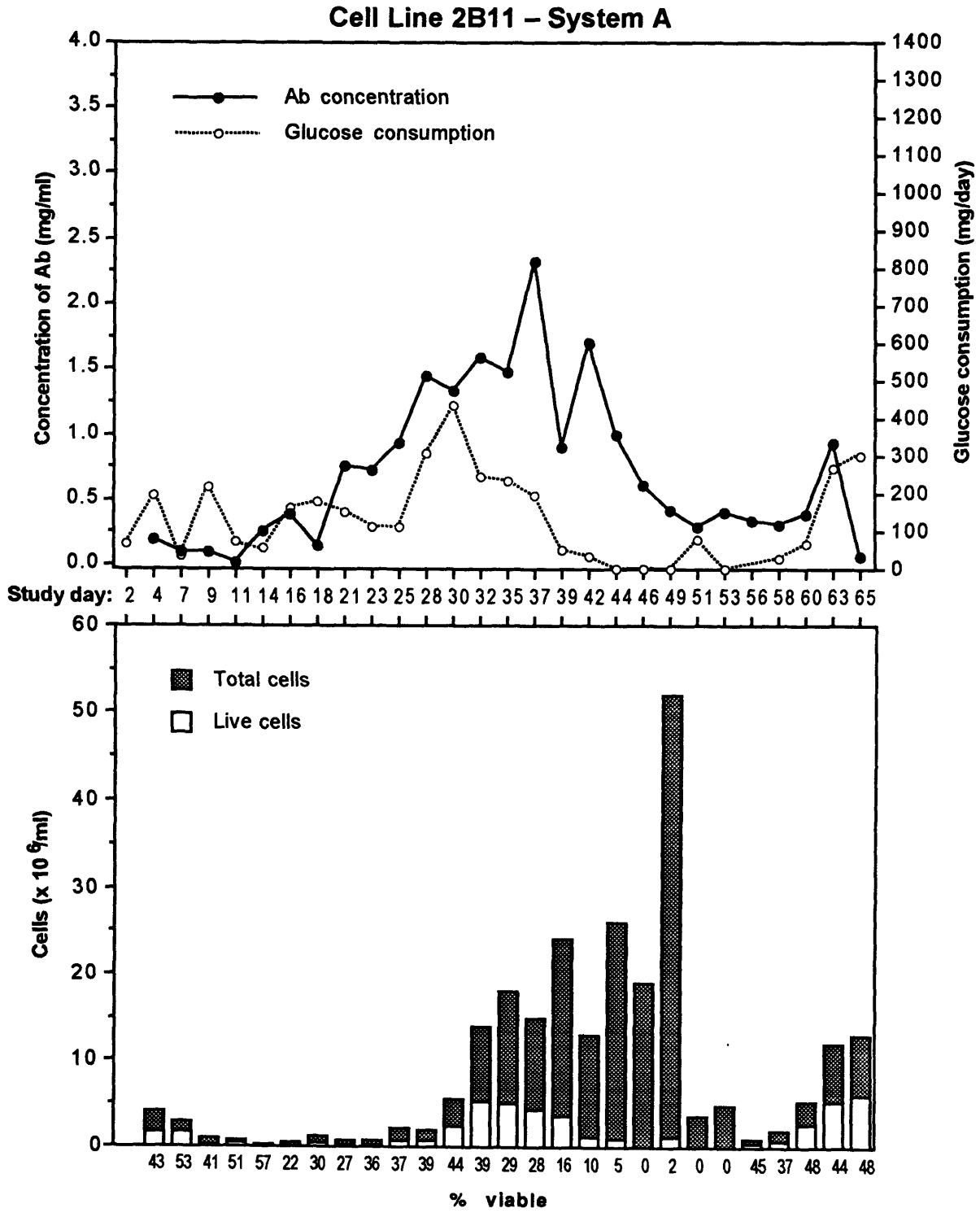


Figure 12.
Bioreactor Antibody Concentration, Glucose Consumption, Cell Counts, and Cell Viability

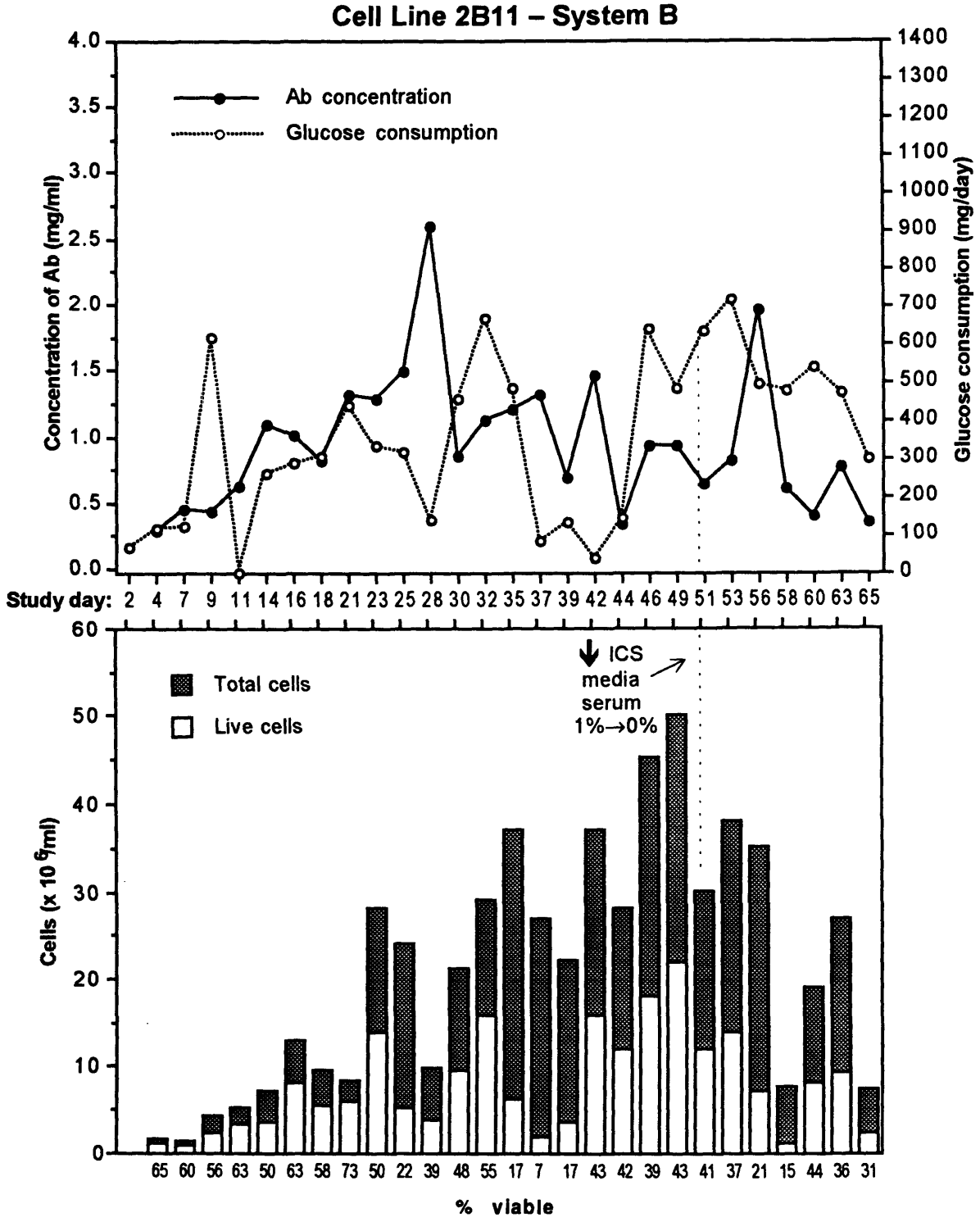


Figure 13.
Bioreactor Antibody Concentration, Glucose Consumption, Cell Counts , and Cell Viability

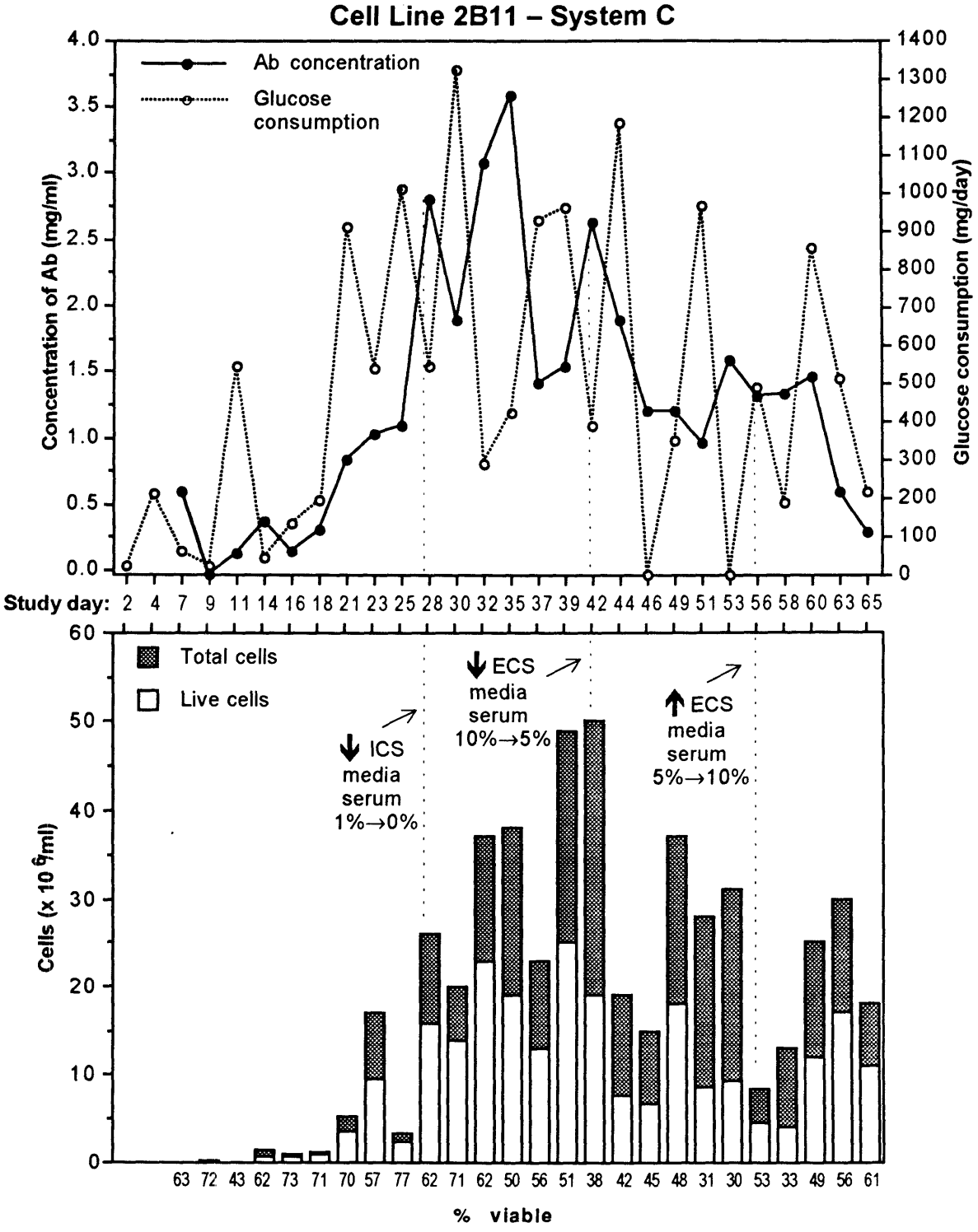


Figure 14.
Bioreactor Antibody Concentration, Glucose Consumption, Cell Counts, and Cell Viability

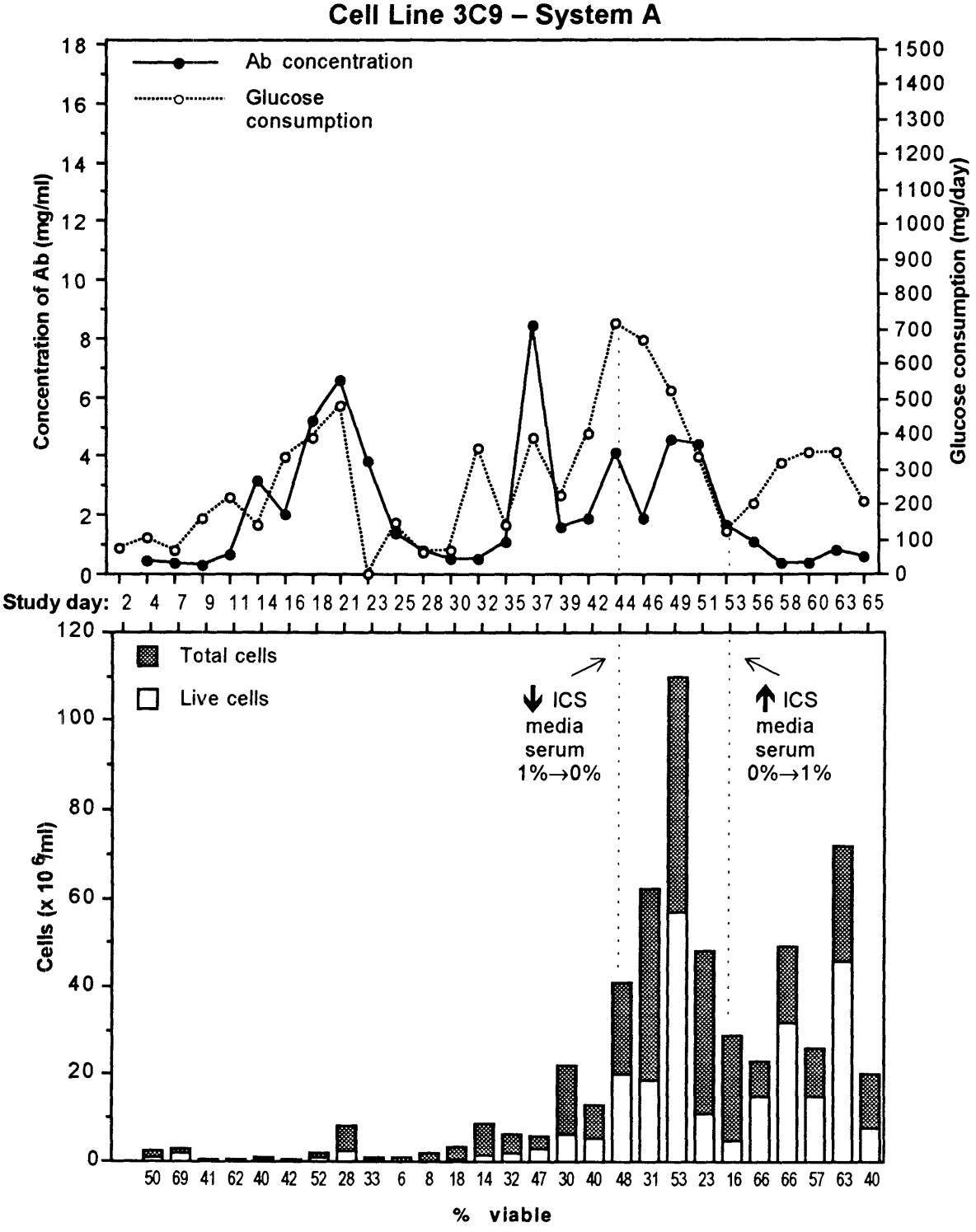


Figure 15.
Bioreactor Antibody Concentration, Glucose Consumption, Cell Counts, and Cell Viability

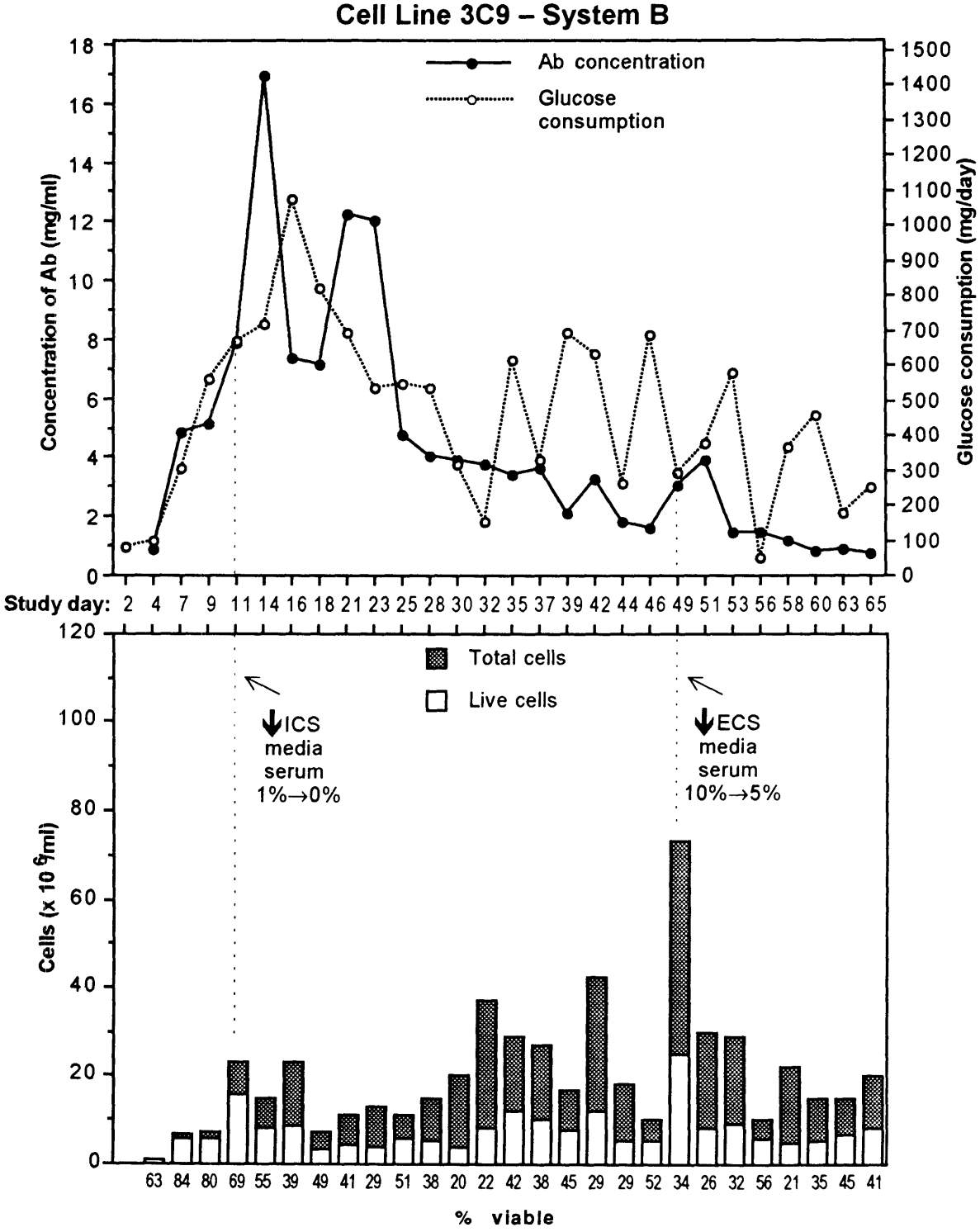


Figure 16.
Bioreactor Antibody Concentration, Glucose Consumption, Cell Counts, and Cell Viability

Cell Line 3C9 – System C

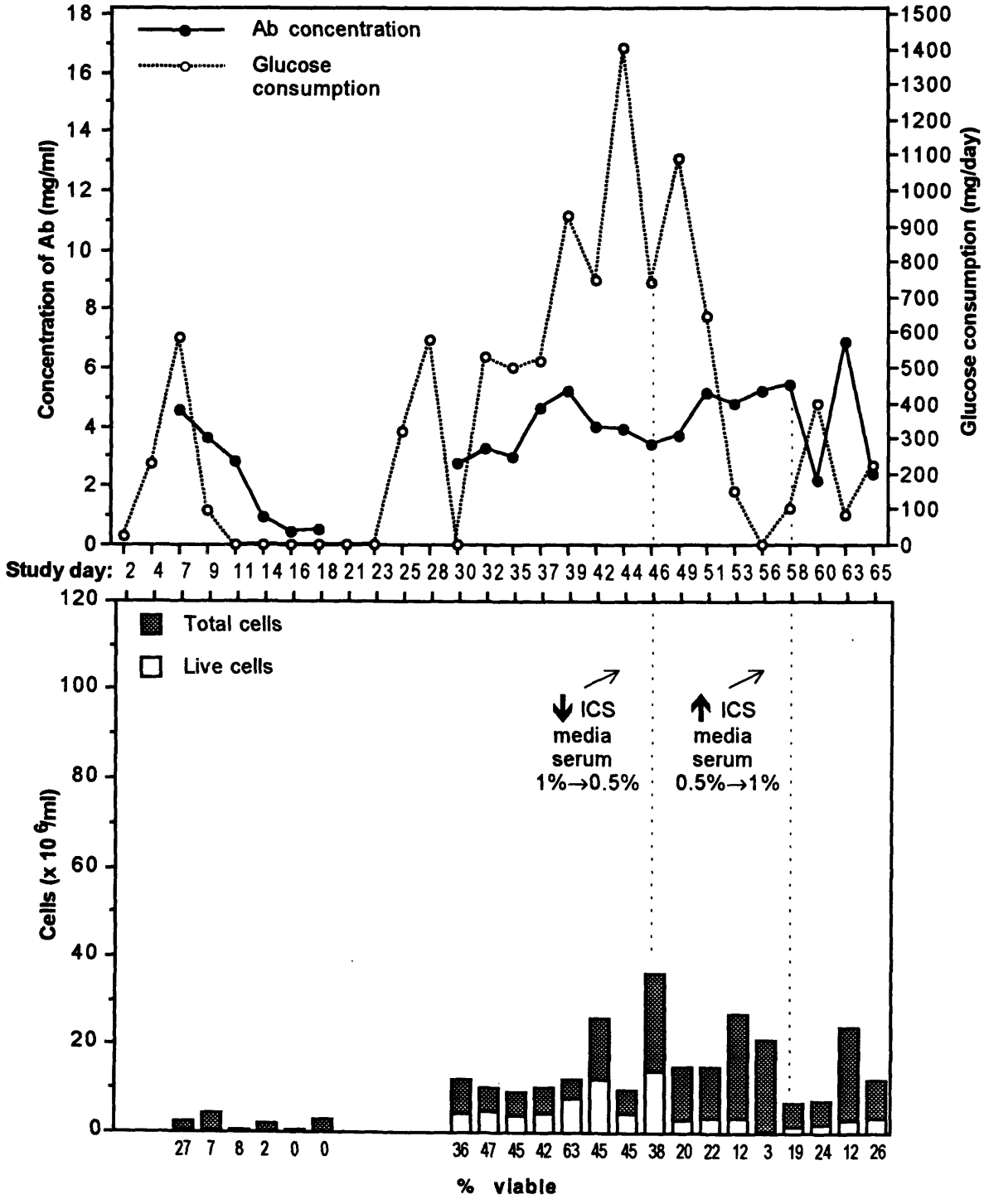


Figure 17.
Bioreactor Antibody Concentration, Glucose Consumption, Cell Counts, and Cell Viability

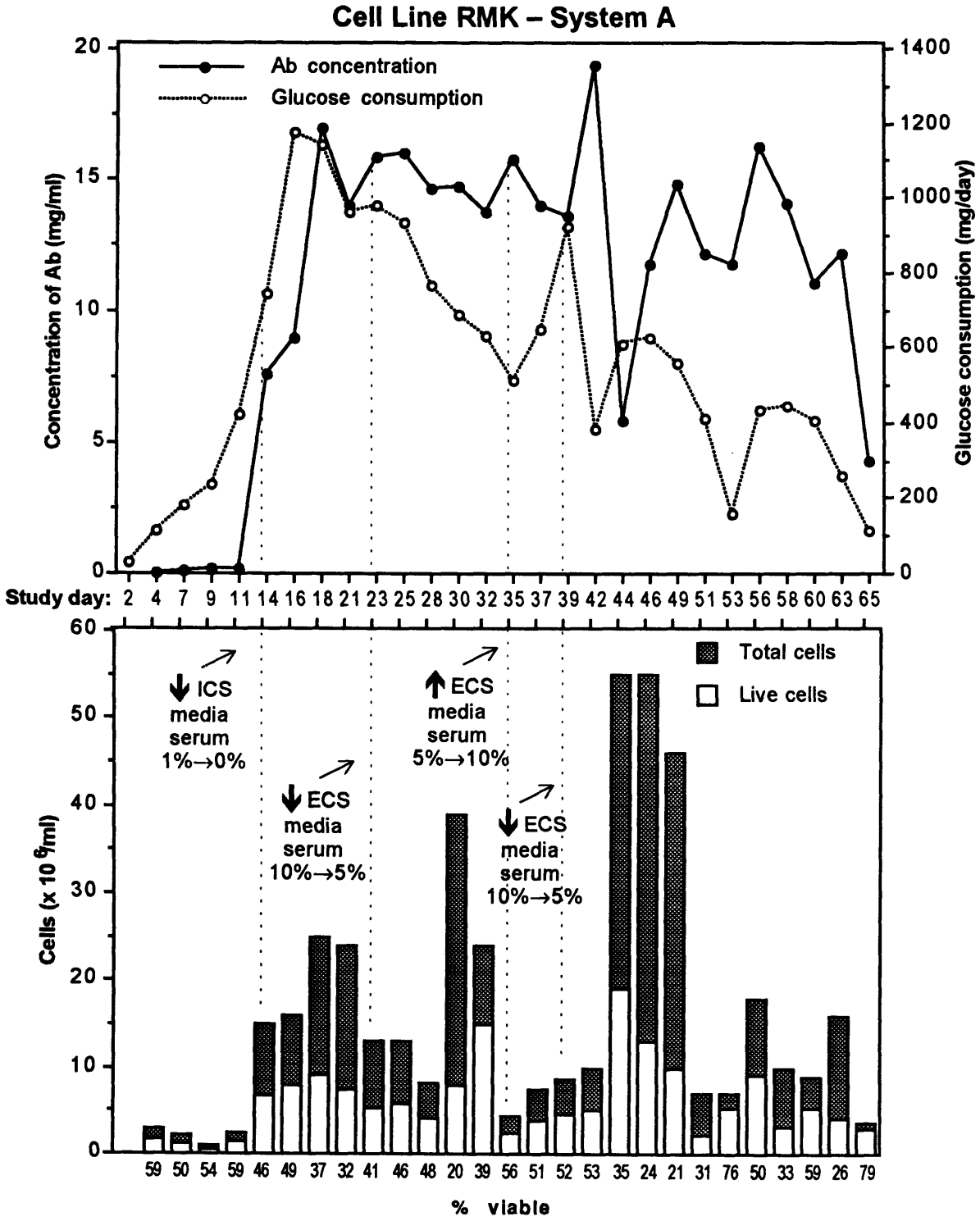


Figure 18.
Bioreactor Antibody Concentration, Glucose Consumption, Cell Counts, and Cell Viability

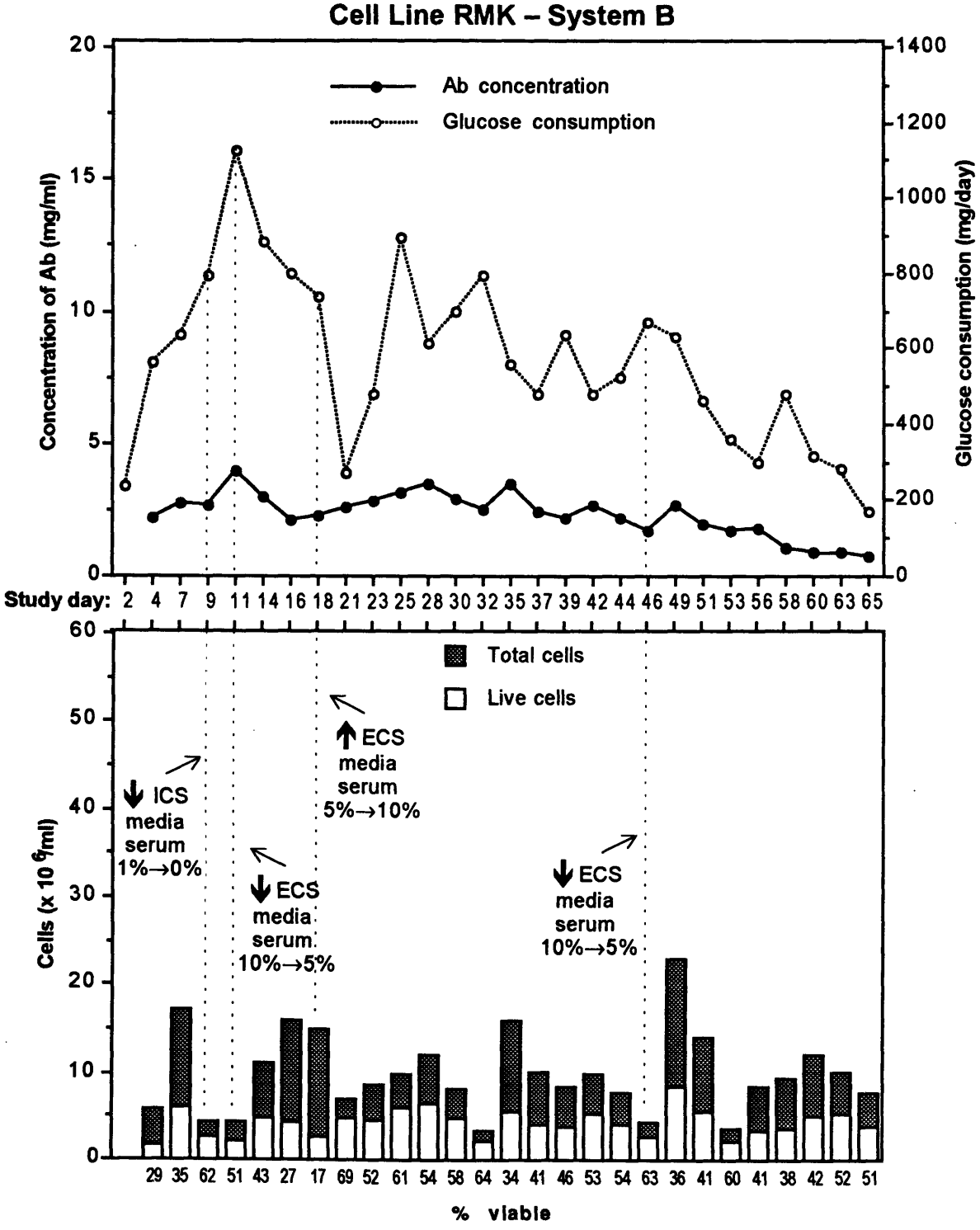
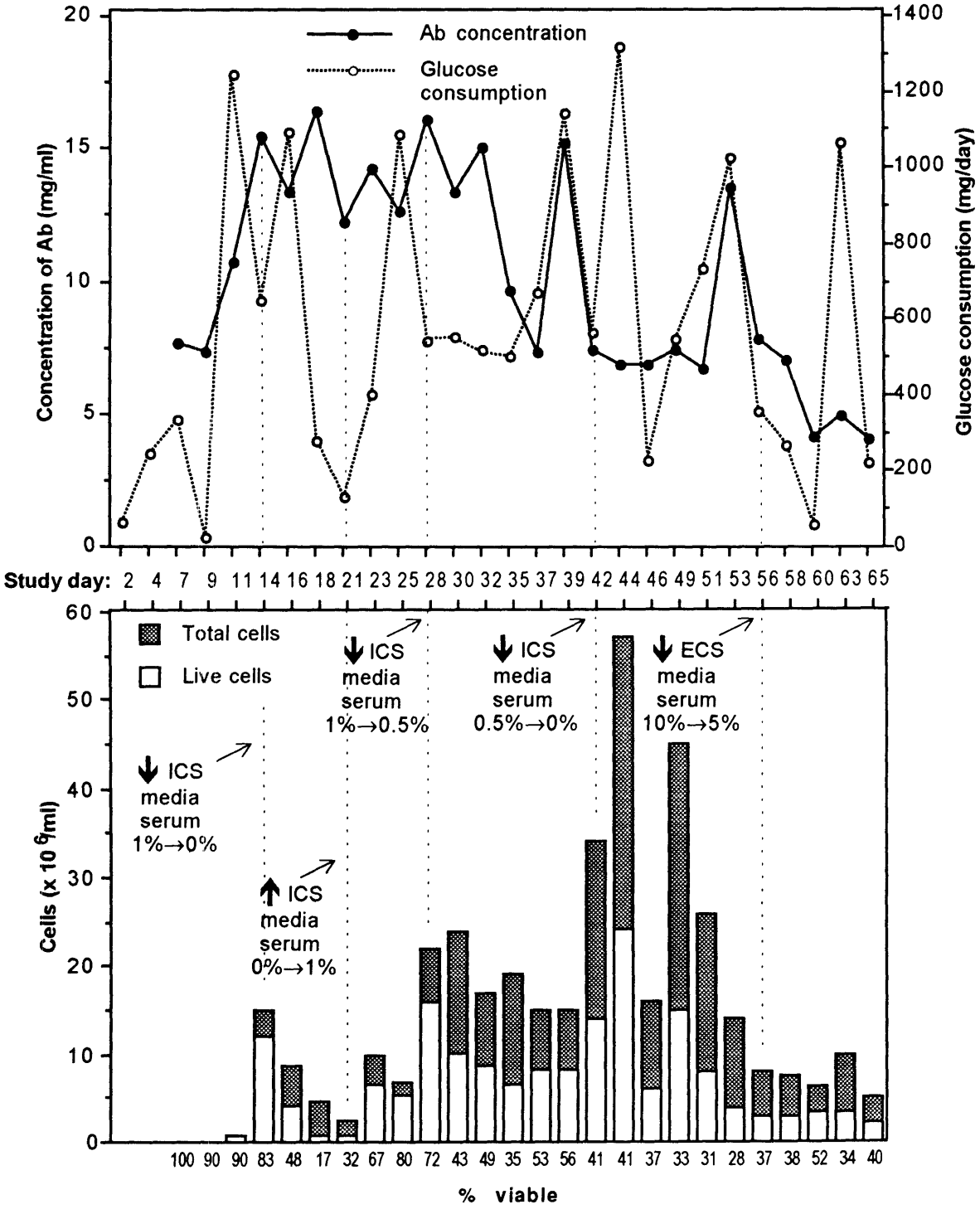


Figure 19.

Bioreactor Antibody Concentration, Glucose Consumption, Cell Counts , and Cell Viability

Cell Line RMK – System C



COMPARISON OF GLUCOMETER AND TEST KIT GLUCOSE MEASUREMENTS

Agreement between glucose measurements made using the glucometer and the Sigma glucose test kit, as determined by Pearson's correlation coefficient, was 0.982. The coefficient of variation was 6.2%. The difference in the means demonstrated a +18 mg/dl bias for measurements made using the glucometer. Results obtained using the method of Bland and Altman (91) suggested that the limits of agreement between the two methods could vary by as much as +61.8 to -25.8 mg/dl within a 95% confidence interval.

PART 3: COMPARISON OF MONOCLONAL ANTIBODY PRODUCTION IN MURINE ASCITES AND HOLLOW FIBER BIOREACTORS

EQUIPMENT AND MATERIALS REQUIRED

Much of the equipment and materials requirements for producing monoclonal antibodies do not differ for production in mice and bioreactors. Equipment and materials in common include a cell culture laboratory, CO_2 incubator, hybridomas, cell culture media, water bath, cell culture plasticware, pipets and centrifuge tubes, needles and syringes, biological safety cabinet, microscope, hemocytometer, centrifuge, and autoclave. There are significant differences in the time usage and quantity of some of the equipment and materials used. For example, cell culture equipment and materials are used for only a short time as necessary to expand hybridomas for injection into mice, whereas CO_2 incubator space requirements and cell culture material requirements are much greater for the bioreactors.

Equipment and materials specific to production in mice include mice, pristane, and an animal facility and staff. Equipment and materials specific to production in the bioreactors include the bioreactor systems and a glucometer with test strips for measurement of media glucose.

TECHNICAL EXPERTISE REQUIRED

Technical expertise required for monoclonal antibody production in mice includes growing hybridomas in culture, and performing intraperitoneal injections and abdominal paracentesis. The ability to make accurate clinical assessments of the condition of the animals is also necessary to minimize morbidity and mortality.

Technical expertise required for monoclonal antibody production in bioreactors includes growing hybridomas in culture, operation of bioreactor systems, and measurement of media glucose. Measurement of media glucose with the glucometer was a very simple technique. It is the opinion of the author that the degree of technical expertise required to successfully operate the bioreactor systems without contamination over a period of 65 days is significantly greater than the technical expertise required to perform procedures with the mice. It is also the opinion of the author that more time is required to develop technical expertise in the operation of bioreactor systems than that required to develop expertise in performance of procedures with mice. The background and technical experience of the individual would need to be taken into consideration with regard to development of technical expertise specific to manipulations with mice or bioreactors.

MATERIALS COSTS

Costs for materials used in monoclonal antibody production in mice and bioreactors are presented in Tables 9 and 10. Material costs are for antibody production in groups of 20 mice, and for bioreactor systems operation for 65 days.

Material costs for antibody production in immunodeficient SCID mice were significantly greater than for production in conventional CAF_1 mice. These costs reflect the higher purchase price for SCID mice and higher per diem for maintenance and husbandry for these mice under aseptic conditions. It should be noted that the per diem cost for SCID mice is inclusive of all disposable garments such as surgical masks and bonnets, lab coats, and shoe covers which were worn each time the animal room was entered.

Material costs for monoclonal antibody production in hollow fiber bioreactors are presented in Table 10. Total material costs for all 3 systems, exclusive of capital costs for bioreactor system instrumentation and the glucometer, were higher than total material costs for antibody production in CAF_1 mice, but lower than material costs for antibody production in SCID mice. Actual total material costs for production in the bioreactors would be dependent on the method used to amortize capital costs.

Table 9.
MAb Production in Murine Ascites
Material Costs

		Group		
		2B11	**3C9**	**RMK**
Purchase/Shipping				
Groups 2B11 and 3C9				
20 CAF_1/J 7-wk male mice @ 7.50	$ 150.00			
Shipping charge & processing fee	41.50	191.50	191.50	
Group RMK				
20 C.B.-17/Icr Tac-scid DF 7-wk male mice @ 27.00	540.00			
Shipping charge & processing fee	49.00			589.00
Per Diem Fees				
5 cages, 37 days @ 0.402 per diem/cage		74.37		
5 cages, 41 days @ 0.402 per diem/cage			82.41	
5 cages, 51 days @ 1.070 per diem/cage				272.85
Pristane Priming				
Cost includes pristane, sterile needles and syringes, alcohol prep pads, and latex gloves		4.31	4.31	4.31
Hybridoma Inoculation				
Cells: $1x10^6$ cells/mousex20 = $20x10^6$ cells.		nci	nci	nci
Cost includes media for cell expansion and animal inoculation, cell culture plasticware, pipettes, centrifuge tubes, sterile needles and syringes, alcohol prep pads and latex gloves		5.95	5.95	5.95
Abdominal Tap				
Cost includes sterile needles and syringes, alcohol prep pads, latex gloves, centrifuge tubes, and Pasteur pipettes				
53 taps @ 0.41 /tap		21.73		
58 taps @ 0.41 /tap			23.78	
60 taps @ 0.41 /tap (plus sterile gloves)				35.82
Total Material Costs	**$**	**297.86**	**307.95**	**907.93**

Costs reflect MIT DCM actual costs.

nci = no cost included

Suppliers Baxter Inc: Needles, syringes, alcohol prep pads
MIT Lab Supplies: Cell culture plasticware
Sigma Chemical Co: Pristane
Henry Schein Inc: Sterile latex gloves

Table 10.

MAb Production in Hollow Fiber Bioreactors

Material Costs

	System A	System B	System C
System setup: Cost for sterile latex gloves	0.66	0.66	0.66
ECS media flush: including pipettes, centrifuge tubes, needles, syringes, alcohol prep pads, and latex gloves	1.71	1.71	2.11
Hybridoma inoculation Cells: $60x10^6$ - $103x10^6$/bioreactor	nci	nci	nci
Cost includes media, cell culture plasticware, pipettes, centrifuge tubes, syringes, alcohol prep pads, and latex gloves for cell expansion, preparation and inoculation	9.62	13.00	9.62
ECS and ICS sample collections: including pipettes, centrifuge tubes, needles, syringes, alcohol prep pads and latex gloves	39.96	41.85	48.62
Cell counts and glucose tests: including eppendorf tubes, pipettes, and glucose test strips	41.16	41.16	41.16
ECS and ICS media and preparation: including media & pipettes for addition of supplements	115.98	115.40	248.60
CO_2 tank	4.67	7.00	4.67
Bioreactor Cartridge (1)	250.00	115.00	195.00
Subtotal for operations $	**463.76**	**375.78**	**550.44**
Additional costs for problems discussed in text	4.23	5.22	3.56
Total for operations $	**467.99**	**381.00**	**554.00**
Capital costs:			
System A (including 4 bioreactor cartridges)	4,950.00		
Systems B & C (no cartridges included)		4,000.00	7,235.00
Glucometer	62.99	62.99	62.99
Total for capital costs $	**5,012.99**	**4,062.99**	**7,297.99**

Costs reflect MIT DCM actual costs per bioreactor, averaged for the 3 runs in each system.
nci = no cost included

Suppliers Baxter Inc: Needles, syringes, alcohol prep pads
MIT Lab Supplies: Cell culture plasticware
BioWhittaker Inc: Cell culture media and supplements
Henry Schein Inc: Sterile latex gloves

TIME SPENT IN LABOR

Time spent in labor for monoclonal antibody production in mice and hollow fiber bioreactors is presented in Tables 11 and 12. Labor time is for antibody production in groups of 20 mice, and for bioreactor systems operation for 65 days.

Total labor time was similar for all groups of mice. The smallest labor time was for Group 2B11 where the development of ascites, and consequently abdominal tap times were relatively synchronous among animals in the group. This group also had the shortest duration of time for completion of all abdominal taps. The largest labor time was for Group RMK where there was greater variability in the development of ascites, and consequently abdominal tap times among animals in the group. This group also had the longest duration of time for completion of all abdominal taps. Additional time was also required for donning appropriate garb and performance of techniques to assure maintenance of asepsis when handling these animals.

Time spent in labor for monoclonal antibody production in hollow fiber bioreactors is presented in Table 12. Total labor time was relatively similar for all systems. Greater total labor time for system B reflects greater time for preparation of the system for autoclaving, system set up and media flush procedures, and media bottle changes. Operational methodology for performing media changes was more time consuming for this system as compared to the

others.

Overall, the time spent in labor for operation of bioreactor systems was approximately double the amount of time spent in labor for the mice.

Table 11.
MAb Production in Murine Ascites
Labor Time

	Group		
	2B11	**3C9**	**RMK**
Pristane Injection	20 min	23 min	27 min
Hybridoma Cell Expansion > 20 x 10^6 cells	30 min	30 min	30 min
Preparation of Hybridoma Inoculum	30 min	30 min	30 min
Hybridoma Inoculation	20 min	20 min	25 min
Daily clinical observations, abdominal taps, and euthanasia	6 hr 9 min	6hr 56 min	7hr 8 min
Total Time	**7 hr 49 min**	**8 hr 40 min**	**9 hr 0 min**

Times not included: preparation of the animal protocol for IACUC submission, ordering animals, uncrating animals at receipt, walking to and from the animal facility, centrifugation and processing of ascites, record keeping, disposal of animal carcasses.

Table 12.
MAb Production in Hollow Fiber Bioreactors
Labor Time

	System		
	A	B	C
Preparation of unit for autoclaving	0 min	30 min	0 min
Preparation of media bottles and cap assemblies for autoclaving	10 min	10 min	15 min
Preparation of extra luer plugs for autoclaving	10 min	10 min	0 min
Media preparation with supplements	45 min	1 hr 0 min	1 hr 5 min
System set up & media flush	20 min	*1hr 37 min	38 min
Check for sterility, check connections	5 min	5 min	5 min
Hybridoma cell expansion:			
Systems A, C >60 x 10^6 cells	40 min	—	40 min
System B >103 x 10^6 cells	—	50 min	—
Preparation of hybridoma inoculum	35 min	40 min	35 min
ECS media flush and cell inoculation	20 min	35 min	35 min
ECS and ICS sample collections	7 hr 12 min	7 hr 12 min	6 hr 56 min
Media bottle changes	1 hr 10 min	3 hr 7 min	1 hr 43 min
Cell counts and glucose tests	4 hr 30 min	4 hr 30 min	4 hr 20 min
Change injection septa on ports	—	—	20 min
Media bottle & cap assembly cleaning and preparation for autoclaving	0 min	1 hr 33 min	52 min
Subtotal	**15 hr 57 min**	**21 hr 59 min**	**18 hr 4 min**
Additional problems as discussed in text	45 min	1 hr 23 min	1 hr 17 min
Total Time	**16 hr 42 min**	**23 hr 22 min**	**19 hr 21 min**

* Takes ~40 min for cartridge ECS to fill.

Times indicated for each system are times per bioreactor, which were averaged for the 3 runs in each system.

Times not included: cleaning incubator, measuring incubator CO_2, changing CO_2 tanks, filling incubator water pan, conversations with technical representatives, returning instrumentation for repairs, time to warm media before use, tracking media and supplement stocks, ordering, walking to and from autoclave, centrifugation and processing of ECS samples, record keeping, making cell count and glucose consumption calculations.

SUMMARY: MONOCLONAL ANTIBODY PRODUCTION IN MICE AND BIOREACTORS

Ascites and bioreactor harvest volumes, mean antibody concentrations, mean antibody per harvest or tap, and total antibody produced are presented for mice and bioreactors in Table 13. The total volume of product for all cell lines was similar for the mice and systems A and C, which had the smaller bioreactor ECS volume. The total volume of product for all cell lines was greater for system B, which had the larger bioreactor ECS volume.

For cell line 2B11, the mean concentration of antibody in ascites was significantly greater than the mean concentration of antibody produced by any of the 3 bioreactor systems. Total antibody production in 20 mice was also significantly greater than the total antibody produced by any of the 3 bioreactor systems in 65 days.

For cell line 3C9, the mean concentration of antibody for system B exceeded the mean concentration in ascites. Total antibody produced by system B in 65 days was more than double the amount of antibody produced in 20 mice. Mean antibody concentrations and total antibody production for systems A and C were less than those for the mice, but both concentrations and total production were greater than those achieved for cell line 2B11.

For cell line RMK, the mean concentration of antibody for systems A and C exceeded the mean concentration in ascites. Total antibody production for both these systems

also exceeded the total antibody produced in 20 mice. The mean concentration of antibody for system B was significantly lower than that for the other bioreactor systems and ascites. Total antibody production for system B was less than half that produced by systems A and C. Overall, antibody production in mice and bioreactors was greater for cell line RMK as compared to other cell lines.

Table 13.

Summary Data

MAb Production in Mice and Bioreactors

cell line	system	number of harvests or taps	total vol (ml)	$\bar{x}$ Ab conc (mg/ml)	$\bar{x}$ Ab per harvest or tap (mg)	total Ab (mg)
2B11	A	27	136.1	0.71	3.37	91.11
	B	27	243.0	0.99	9.24	249.49
	C	26	126.7	1.31	6.33	164.46
	mice	53	107.7	4.22	8.58	454.50
3C9	A	27	114.8	2.16	8.76	236.60
	B	27	243.0	4.45	39.86	1076.26
	C	22	107.2	3.57	17.30	380.71
	mice	58	109.5	4.07	7.68	445.57
RMK	A	27	122.6	11.08	45.50	1228.44
	B	27	245.2	2.30	20.51	553.81
	C	26	127.1	10.11	49.50	1286.92
	mice	60	115.7	8.37	16.11	996.64

PRODUCTION TIME

Cumulative antibody production for each cell line over time is presented for mice and bioreactor systems in Figure 20. Day 0 for the mice was defined as the day that the animals arrived at the animal facility. Day 0 for the bioreactor systems was defined as the day of system set up.

For cell line 2B11, it is clear that a significantly greater amount of antibody was produced in murine ascites in a shorter period of time by comparison to any of the 3 bioreactor systems.

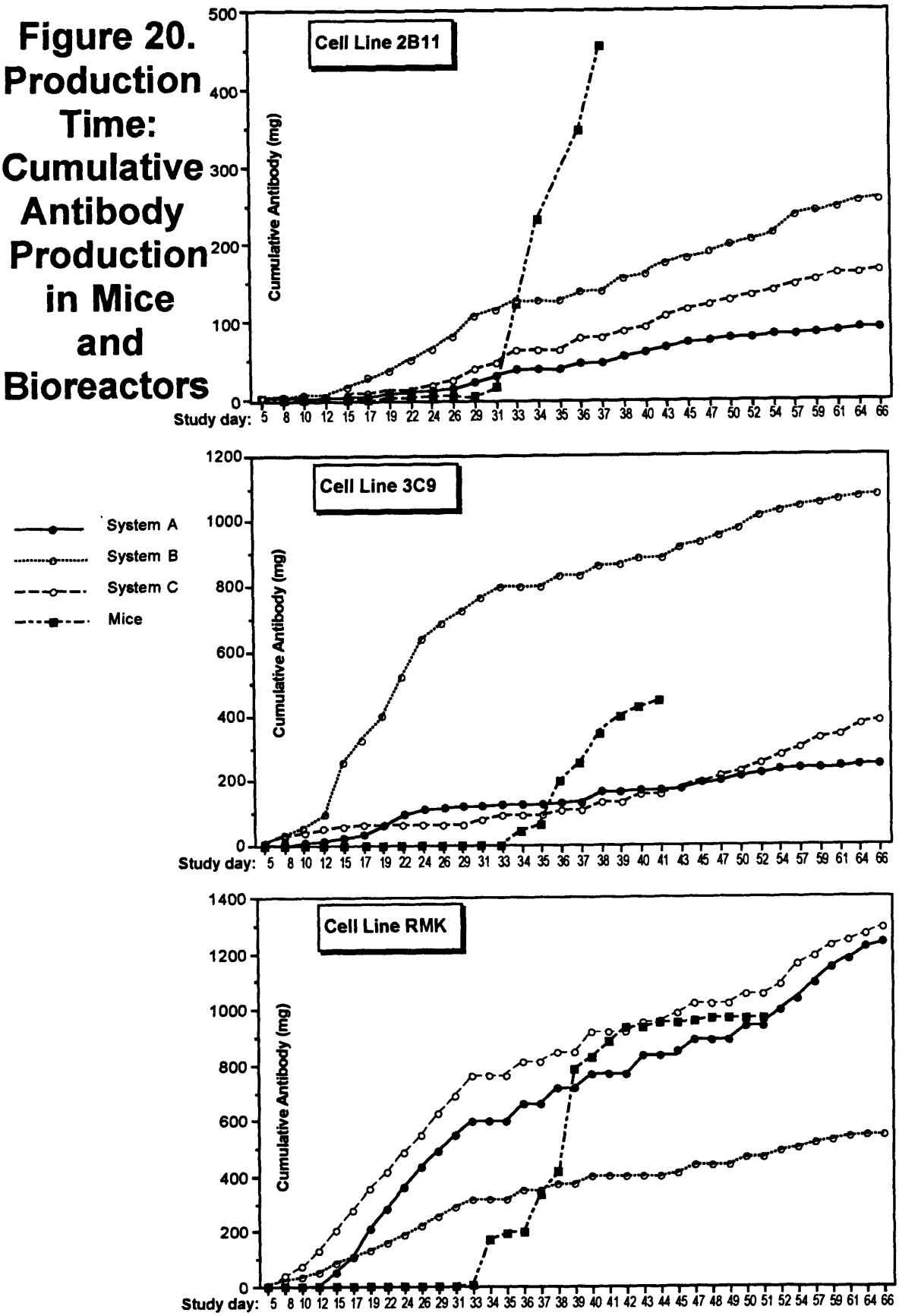
For cell line 3C9, system B produced a greater amount of antibody in a shorter period of time by comparison to production in the other bioreactor systems and mice. Greater antibody production was achieved in murine ascites in a shorter period of time by comparison to production for systems A and C.

For cell line RMK, production was achieved earlier for systems A and C. Production in these 2 systems and murine ascites reached near equivalence at approximately day 39; bioreactor production exceeded production in mice by day 66. Less antibody was produced over time for system B as compared to the other bioreactor systems and mice.

Because of the time associated with quarantine of the animals, the time between pristane and hybridoma injection, and the time between hybridoma injection and the first abdominal taps for the mice when the antibody product was first obtained, earlier initiation of antibody production

for all cell lines was achieved by the bioreactor systems in comparison to the mice.

Figure 20. Production Time: Cumulative Antibody Production in Mice and Bioreactors



BIOREACTOR ANTIBODY PRODUCTION IN MOUSE EQUIVALENTS

Bioreactor antibody production in mouse equivalents is presented in Table 14. For each cell line, antibody production in the mice in mg/mouse was determined by taking the total amount of antibody produced and dividing by 20, which was the number of mice used in each group. These figures were then used to calculate mouse equivalents based on bioreactor antibody production for each cell line in each system . There was great variability both within and between cell lines when comparing mouse equivalents for the bioreactor systems. For cell line 2B11, in which bioreactor antibody production was lowest, the antibody produced in each of the 3 bioreactor systems was equivalent to the use of from 4 to 11 mice. For cell line 3C9, antibody production in the bioreactor systems ranged from 10 to 48 mouse equivalents. For cell line RMK, antibody production in the bioreactor systems ranged from 11 to 27 mouse equivalents.

Table 14.

Summary Data

Bioreactor Antibody Production in Mouse Equivalents

cell line	bioreactor system	total Ab (mg)	mouse equivalents
2B11	System A	91.11	4
22.73 mg Ab/mouse	System B	249.49	11
	System C	164.46	7
3C9	System A	236.60	10
22.28 mg Ab/mouse	System B	1076.26	48
	System C	380.71	17
RMK	System A	1228.44	25
48.33 mg Ab/mouse	System B	553.81	11
	System C	1286.92	27

SECTION 6: DISCUSSION

Results of clinical observations, body weight measurements, and necropsy observations demonstrate significant clinicopathologic changes in mice associated with monoclonal antibody production via growth of ascitic tumors. A variety of pathologic processes were identified in association with different aspects of the production technique.

During the two weeks following injection of pristane and prior to inoculation of hybridoma cells, no clinical abnormalities were observed in any mice. Additionally, no significant differences in body weights were observed between test and control animals during this period. These findings suggest that the injection of 0.5 ml pristane intraperitoneally does not cause clinically detectable morbidity. However, as body weight measurements were recorded once weekly during this period, the possibility that there could have been a decrease in body weights and subsequent recovery within the first or second week following pristane injection cannot be definitively ruled out.

On day 0, prior to hybridoma cell inoculation, subcutaneous nodules were observed in some animals at the site of pristane injection. These nodules decreased in size or became clinically inapparent over time in greater

than half of the animals which demonstrated this lesion. Based on observations of the granulomatous inflammation in the peritoneum induced by pristane (6,63), it is suspected that these subcutaneous lesions were granulomas induced by a small amount of pristane which, particularly due to its high viscosity, may have been deposited at a subcutaneous site along the needle track during intraperitoneal injections. Subsequent resolution of the granulomatous inflammation over time may have accounted for the reduction in size of these lesions in some animals. The incidence of this lesion in this study was 8/100 mice or 8%. Histologic examination of these lesions would be required for confirmation.

Subcutaneous nodules were also observed in some animals at the site of intraperitoneal injection following the inoculation of hybridoma cells. The nodules increased in size over time in greater than half of the animals in which the lesion was observed. Histologic examination of these nodules demonstrated subcutaneous foci of tumor growth. Increasing size of these tumors over time would not be unexpected, and hybridomas have been previously reported to grow in subcutaneous sites (2,15,16,18). As the necropsy observations in the study suggest that the hybridoma cells have a tropism for fat, it does not seem surprising that subcutaneous tissues primarily composed of fat would provide a satisfactory microenvironment for the growth of these cells. Tumor growth within the abdominal wall at the

injection site was also observed. These subcutaneous and intramuscular sites of tumor growth likely resulted from hybridoma cells which were deposited along the needle track during intraperitoneal injections. At the time of necropsy it was not possible to distinguish subcutaneous masses at the injection site which arose before or after hybridoma cell inoculation. However, fifty percent of the animals in Group 3C9 had subcutaneous masses at necropsy which were first observed during clinical observations 13 to 20 days following hybridoma cell inoculation. Tumor growth was also observed within the abdominal wall at the injection site in fifty percent of the animals in this group. The greater incidence of tumor growth in the subcutaneous tissues and abdominal wall at the injection site in these animals suggests that hybridoma cell line 3C9 may have a greater propensity for growth in subcutaneous or intramuscular sites.

In addition to subcutaneous and intramuscular growth of tumor at the injection site, solid tumor masses were also observed with greatest frequency in the left caudal ventrolateral abdomen, and some tumors were attached to the peritoneum at the injection site. These findings suggest that initiation of solid tumor growth may begin locally, at the site of hybridoma cell injection. It might be interesting to investigate whether dividing the hybridoma inoculum for administration in more than one site would result in the growth of a larger number of smaller tumors

instead of the relatively large solitary tumors most frequently observed in this study. The potential benefit to the mouse, if any, would also need to be determined.

Necropsy observations and histologic findings for the two mice which died or were euthanized prior to development of ascites and abdominal paracentesis, demonstrated the presence of intramural tumor within the bladder wall. Inadvertent injection of hybridoma cells into the wall of the bladder could potentially account for the apparent rapid infiltrative growth of tumor at this site. Inaccurate injection technique, therefore, could be one possible source of subsequent morbidity and mortality. The incidence of this lesion was 2/100 mice or 2%.

The onset of clinical abnormalities such as roughened haircoat and hunched posture were associated with the initial development of ascites, and probably tumor growth, as determined by the progressive increase in abdominal distention and body weights. The mean percent increase in body weights between day 0 and the before tap weights at the first abdominal tap, for all groups of test mice combined, was 17.4%. This figure correlates relatively well with the recommendation in the UKCCCR guidelines (12) which states that ascites volumes should not usually exceed 20% of normal body weight. The plateau in mean body weights in the 1-2 days immediately preceding tap 1, as illustrated graphically for Groups 2B11 and 2C6D9, suggests that additional delay in the first abdominal paracentesis

would not have resulted in collection of greater ascites volumes. The plateau in body weights could indicate that there is little further distensibility in the abdominal wall, and consequently the pressure created by the large volume of ascites may prohibit further ascites production.

The smallest mean percent increase in body weight from day 0 to tap 1 (10.11%) was observed in Group 2C6D9. Clinical abnormalities in this group were the most severe and rapidly progressive, and mortality was greatest in this group. This group was also was the first group tapped following hybridoma inoculation (day 11). The largest mean percent increase in body weight from day 0 to tap 1 (23.77%) was observed in Group RMK. Clinical abnormalities in this group were the least severe and most slowly progressive, and 100% of the animals in this group survived to the end of the study. This group also had the largest range of days over which the first abdominal tap was performed (days 13 to 27). These factors are likely related to the observed percent increases in body weight for these groups between day 0 and the first tap.

Although there were significant differences between groups, the general progression of clinical symptomatology over time among groups was similar. The differences which were observed between groups in the incidence, severity, and rapidity of progression of clinical abnormalities during the study did have significant impact on survival, and ultimately, on the amount of antibody produced.

The clinical abnormalities which appeared to have the greatest impact on survival were those observed during the time immediately following abdominal paracentesis. These abnormalities included roughened haircoat, hunched posture, decreased activity, increased respiratory rate and in some cases dyspnea, and pallor. Control animals which received sham paracentesis demonstrated none of these clinical abnormalities. This observation would suggest that these signs were associated with the removal of ascitic fluid, rather than with stress resulting from restraint and the paracentesis procedures.

The abnormal clinical signs observed following paracentesis are compatible with signs of circulatory shock. Rapid removal of ascitic fluid in humans has been associated with hypotension (9), hypovolemia (9,10,13), and protein depletion (13). Similar complications have been observed in animals following removal of ascitic fluid (14). In this study, these signs were most often transient suggesting that the mice were able to compensate via physiologic mechanisms which possibly included tachycardia and peripheral vasoconstriction. The recommendation for use of general anesthetics during abdominal paracentesis in mice might be questioned in view of these findings, as many general anesthetic agents cause myocardial depression (92) and hypotension (93) and could potentially exacerbate any existing cardiovascular deficits. Additionally, in human and veterinary clinical practice, general anesthetics are

not routinely employed for paracentesis, although local anesthetic agents are sometimes used. Supportive therapy such as supplemental oxygen or warm fluids for volume expansion could be considered, but may be of questionable practicality. The additional stress resulting from administration of therapeutic agents would need to be weighed in view of the potential benefit.

In examination of individual animal data, no definitive correlation could be made between the magnitude of the percent drop in body weight at the time of abdominal taps and the presence or absence of clinical abnormalities following paracentesis. However, these data have not yet been subjected to rigorous statistical correlation analyses.

It is important to note that all of the animals in this study which died did so within approximately 30 minutes following abdominal tap, with the exception of one animal which died early in the study and was found to have a bladder tumor. With the exception of one animal euthanized early in the study with a bladder tumor, animals were euthanized during this study because the clinical abnormalities observed following abdominal paracentesis were indicative of severe distress, or were persistent rather than transient and of sufficient severity to suggest that the animals might not survive to the next observation period. These findings point to the need for careful observation of animals for at least 30 minutes following

abdominal paracentesis to permit identification of animals with severe or persistent post-tap clinical abnormalities which warrant humane euthanasia. Careful clinical monitoring of these mice following abdominal paracentesis and appropriate use of euthanasia were successful in preventing mortality at other timepoints during this study, and were essential for providing humane care for the animals. It should also be noted that abdominal paracentesis and clinical observations were performed as necessary 7 days a week without regard for weekends or holidays.

Overall survival to tap 3 for all groups combined was 79%, which is significantly higher than another report which states that generally only 10-25% of animals survive to the third tap (4). This information further supports the value of careful clinical monitoring of the animals to reduce mortality. Greater survival should also positively impact antibody production, as results from this study demonstrate a relationship between percentage of possible taps performed and total ascites volumes.

Six animals out of 100 died during this study for an overall mortality rate of 6%. In the individual groups, mortality ranged from 0 to 25%. These findings suggest that even with frequent and careful clinical observations, some mortality may occur, and that significant differences in mortality may be observed between different cell lines.

The most severe and rapidly progressive clinical

abnormalities were observed in mice from Group 2C6D9. Mortality was highest in this group and the largest number of animals euthanized were from this group. The greatest incidence of decreased activity and dehydration was exhibited by animals from this group. The presence of dehydration suggests that these animals may have stopped drinking water. Clinical abnormalities were observed earlier, abdominal taps were performed earlier, and the duration of survival was shorter for mice in this group as compared to mice in other groups. The ascites obtained from animals in this group was generally very hemorrhagic. Previous studies have demonstrated erosion of blood vessels by invasive abdominal plasmacytomas which was associated with hemoperitoneum (54,60). Gross necropsy observations for animals in this group did not demonstrate pathology significantly different from other groups. More complete histologic examination of tissues from these animals might provide more information regarding pathologic mechanisms. If tumor invasion through the intestinal wall could be demonstrated, for example, endotoxemia could be postulated as potentially having contributed to the clinical observations made in these animals. The available data suggest that this hybridoma cell line produced pathology which progressed rapidly enough to significantly affect survival of the animals. One consideration for future use of this cell line in mice would be to use a smaller cell inoculum with the intent of trying to slow the progression

of tumor pathology.

Clinical abnormalities in animals from Group RMK were the least severe and most slowly progressive, and 100% of the animals in this group survived to the end of the study. The ascites collected from these animals was generally clear, and hemoperitoneum was not observed at necropsy in any animals from this group. It was the impression of the author that a number of the animals in this group could have been tapped at least one additional time without unduly compromising their clinical condition. In addition to maintenance of good clinical condition over time, these animals had very high concentrations of antibody in ascites from the third abdominal tap, and if concentrations remained high for subsequent taps, considerably more antibody might have been produced. Another important observation related to the significantly higher concentration of antibody in ascites from the third tap from these animals, is that approximately 2/3 of the total antibody produced by this group was obtained from ascites from tap 3. These findings should be taken into consideration by Institutional Animal Care and Use Committees. If, for example, the number of abdominal taps for these animals had been restricted to a single tap, 93 mice would have been needed to produce the equivalent amount of antibody produced by 20 mice in 3 taps. Although lot to lot variation in murine ascites production parameters has been observed within cell lines (4), the

potential usefulness of quantitating production by tap is clearly apparent. Contrary to the example provided by Group RMK, there might be cell lines in which production is sufficiently greater at tap 1 or progression of pathology is sufficiently rapid or severe to warrant euthanasia following the first tap.

These results suggest that decisions on the limitation of the number of abdominal taps performed would best be made in view of the clinical condition of the mice and antibody production parameters to include ascites volumes and antibody concentration for taps over time. Based on the clinical appearance of most animals by tap 3, it is the author's opinion that as a general recommendation, three abdominal taps should be considered as a maximum, but that additional taps could be considered for cell lines with clinical effects and production parameters similar to those for Group RMK.

Results of clinical observations in this study also demonstrate variability among individual animals within groups, therefore, decisions regarding the number of abdominal taps performed and time of euthanasia need to be made for each individual animal based upon ongoing assessment of clinical condition.

Gross lesions observed at necropsy clearly demonstrate significant pathology in these mice associated with monoclonal antibody production from ascitic tumors. All mice had disseminated intraabdominal tumor and/or solid

tumor masses. Abdominal adhesions were also commonly observed.

Hemoperitoneum frequently observed in mice from all groups except Group RMK could have been the result of trauma associated with paracentesis or could reflect the presence of hemorrhagic ascites associated with tumor invasion of blood vessels. The presence of subcutaneous tumor at the tap site of mice in Group RMK suggests that tumor cells seeded in the subcutaneous tissues during collection of ascites from the abdominal cavity. Although this lesion was observed grossly only in mice from Group RMK, more complete histologic examination of tap site tissues from mice in all groups would be necessary to confirm the restriction of this lesion to Group RMK. The relationship, if any, between the presence of subcutaneous tumor at the tap site and persistent leakage of ascites from the tap site after paracentesis is unknown. Chronic leakage of ascites from paracentesis sites has been observed in people with cirrhosis following repeated paracentesis (13). Leakage of ascites from the tap site and dependent subcutaneous edema were only observed in SCID mice. These findings may be related to a greater tendency for the development of hypoproteinemia in these mice resulting from the combination of normally lower serum total protein related to agammaglobulinemia, and the loss of large quantities of albumin in ascites. Albumin in mouse ascites fluid has been reported to be between 1.1 and

1.8 g/dl (81).

Histologic evaluation of enlarged mediastinal lymph nodes revealed tumor cells within these nodes. Metastasis of tumor cells from the abdominal cavity to the thoracic lymph nodes, presumably via the lymphatics, has been previously reported for plasmacytomas (54,60).

Gross necropsy observations for the two animals from Group 3D6 which remained clinically normal throughout the study, demonstrated that despite significant pathologic changes in the abdomen and chest, animals can continue to appear clinically normal. Development of nonsecreting solid tumors rather than ascites tumors (62) and descriptions of less than 100% development of ascites in mice after injection of pristane and hybridoma cells (11) have been described. The overall incidence of mice which did not produce ascites was 2/100 or 2% for mice which survived to the time of ascites development.

Overall, these data suggest that with ongoing ascites production, repeated abdominal paracentesis, and progressive tumor growth, the clinical condition of ascites mice generally worsens over time. Despite progressive tumor growth and ascites production, body weights of the mice after the first abdominal tap generally decreased over time suggesting a significant loss of normal body mass in the face of increasing weight contribution by the tumor. Group RMK was an exception in that body weights continued to increase from taps 1 through 3, suggesting that the

clinical condition and hence normal body mass of these mice was maintained in the face of increasing weight contribution by the tumor. In view of all of these findings, it is clear that this is not a benign process, and the author would have to assume that there must be stress associated with this process.

Others have demonstrated significant differences in the behavior of different cell lines in mice (4), and differences between cell lines were observed in this study. Our findings must be viewed in reference to the fact that only 5 cell lines were evaluated in this study, and these results likely do not encompass the range of biologic behaviors possible among different hybridoma cell lines. Differences observed between groups of CAF_1 mice and the SCID mice cannot be differentiated based on the origin of the difference in the strain of mouse or origin of the difference in the hybridoma cell line.

Subsequent clinicopathologic studies of ascites mice should include assessments of hematologic and biochemical parameters to better evaluate blood loss and organ function over time. Measurement of blood pressure and hematologic parameters prior to and following abdominal paracentesis would be useful to define the pathophysiology of the observed post-tap clinical abnormalities, and to determine whether hypovolemia and hypotension develop following abdominal tap. Measurement of protein in serum and ascites over time would assist in identifying the potential

contribution of hypoproteinemia in relation to persistent leakage of ascites from the tap site and the development of edema in SCID mice.

The concentration of the antibody product from the hollow fiber bioreactor systems in this study compare favorably to results obtained by others (42,46,47,48), and overall, compare favorably to concentrations in mouse ascites, particularly in view of the low concentrations attained using alternative in vitro culture methodologies (42,46,47).

Variation observed in antibody production between systems and cell lines may be related to differences in system components such as the molecular weight cut-off of the bioreactor fibers, or operational parameters such as media flow rates and volumes of media exchanged, as well as differences in the requirements of different cell lines. To test these possible influences, one would need to perform multiple runs with a single cell line, changing one variable at a time, for example, using different bioreactor cartridges in sequential runs within the same system, or using the same bioreactor cartridge and changing media flow rate in sequential runs within the same system.

Antibody productivity has been demonstrated to be higher in bioreactors with 30 or 70 kD MWCO as compared to bioreactors with 10 kD MWCO (47). There is a trade-off to be considered, however, as leakage of IgG product from the ECS to the ICS was barely detectable for the 30 kD MWCO

bioreactor but was substantial for the 70 kD MWCO bioreactor (47). Improved production with larger MWCO bioreactors may result from reduced resistance to media flow (26) and/or enhanced diffusion of low molecular weight growth inhibitors such as $TGF\beta_1$ which is produced by some hybridoma cells (52), from the ECS to the ICS.

With the exception of cell line RMK, total antibody production was less in system A as compared to other systems. The bioreactor for system A had a larger MWCO of 50 kD by comparison to the other systems which had MWCO of 10 kD. Lower production could have resulted from some leakage of antibody from the ECS to the ICS for cell line 3C9 which was an IgG producing hybridoma, but leakage of the larger IgM antibody of cell line 2B11 would not be expected. Antibody was not measured in the ICS media of any systems in this study. Alternatively, growth factors such as insulin and transferrin, with molecular weights of approximately 6 and 78 kD, respectively, have been shown to increase hybridoma cell growth in hollow fiber bioreactors (94,95). Greater diffusion of these serum factors away from the cells in the system A bioreactor with the larger MWCO may also have contributed to the lower production observed. Lastly, decreased frequency of media exchanges during these runs based on glucose consumption may have resulted in limitation of other essential nutrients such as glutamine, which decomposes spontaneously over time at 37°C (86,87) and was not measured in ICS samples in this study.

Based on the larger ECS volume for growth of cells in the bioreactor for system B, one might expect greater antibody production in this system by comparison to the others. This was true for cell lines 2B11 and 3C9, but for cell line RMK, production was lowest in system B. Obviously, factors other than cell space affect production. The impact of the different method of gassing provided by the hybrid reactor for system C cannot be differentiated from other potential variables affecting production.

Antibody production for all systems was lowest for cell line 2B11, which was the only IgM product evaluated in this study. This data suggests that this cell line may not adapt well to growth in hollow fiber systems, or alternatively, it may indicate that media conditions, bioreactors, and/or operational protocols were not sufficiently optimized for successful cell growth and antibody production for this cell line. The latter explanation is more likely, as the author is aware that this cell line has been grown successfully in large scale hollow fiber bioreactor production systems. Anecdotal reports, however, suggest that some hybridoma cell lines will not grow in hollow fiber bioreactor systems.

To the extent possible, conditions were standardized in this study for bioreactor system operations for all cell lines. No attempts were made in this study to optimize conditions for individual cell lines. There are many operational variables which could be manipulated to include

media constituents, media flow rates and incremental changes in flow rate over time, volume of media exchanges, and harvest frequencies. Any or all of these factors have the potential to affect antibody production. Particularly in view of the observation of grossly visible acidification of the ECS media between harvests and the potential for increasing concentrations of cell-secreted inhibitory substances such as $TGF\beta_1$ over time within the ECS, more frequent ECS harvests could potentially have significant impact on total production. Operational changes such as this also would affect time spent in labor and material costs.

Decline in antibody production toward the end of the 65 day runs was observed for all cell lines in all systems. More novel operational strategies for maintaining production over long periods of time includes periodic agitation and flushing of the bioreactor with the intent of removing cells to prevent overgrowth of cells in the bioreactor (26). As long as sterility is maintained, and components are manufactured to provide sufficient longevity, reuse of bioreactors may also be possible with trypsinization to remove cells, followed by reinoculation of cells. The author would also like to investigate the effect of intermittent pulses of ECS media with higher concentrations of serum to evaluate the potential for a beneficial effect on maintenance of cell growth and production over longer periods of time.

Results from this study demonstrated excellent stability of these cell lines in terms of continued antibody secretion over time for the duration of study. Cell line stability is an important factor in consideration of longer continuous production times, but the problem of decreasing stability over time might be overcome by periodic flushes and reinoculation of the bioreactor.

Glucose consumption appeared to be a relatively good measure of cellular activity in the bioreactors, but changes in glucose consumption did not always correlate well with changes in antibody concentration. These findings suggest that antibody concentrations should be monitored frequently during bioreactor runs, rather than relying on indirect measurements. For systems B and C, ECS harvests were obtained by simultaneous aspiration of ECS media and infusion of fresh media. Consequently, the ECS samples assayed for antibody concentration were actually mixtures, which may have contributed to the variability in some measurements over time. Peaks in antibody concentration, often associated with Monday harvests after the longer 3 day weekend time interval, also contributed to fluctuations in antibody concentration over time.

Large fluctuations in glucose consumption over time were also observed, particularly in system C. This may have been related to the larger 2 liter media exchanges for this system, potentially resulting in greater dilution of beneficial cell secreted products. Although good

correlation was demonstrated by Pearson's correlation coefficient (0.982), results obtained using the method of Bland and Altman (91) suggested that the limits of agreement for glucose measurements between the glucometer and the Sigma test kit could vary considerably. This finding suggests another possible source for fluctuations in glucose consumption.

Changes in serum concentration in the ICS and ECS had variable impact on glucose consumption, antibody concentration, and cell viability within different cell lines and systems over time. Overall, decreases in glucose consumption and cell viability appeared more frequently following serum reductions than decreases in antibody concentration. These findings again suggest the importance of monitoring antibody concentration during the bioreactor runs.

In comparison of antibody production in bioreactors and mice, advantages and disadvantages are associated with each method. Advantages for the bioreactors include the potential for more rapid initiation of production, and sustained production over longer periods of time. However, larger total quantities of antibody were more often produced in a shorter period of time by the mice. The potential for contamination or mechanical failure of the systems themselves or supporting systems such as the CO_2 incubator, account for less absolute certainty in projected production times. Lot to lot variability in mice may also

create uncertainty in projected production times.

Bioreactors can be operated on a 3 day/week schedule. It is the impression of the author that mice producing ascites must be monitored at least once each and every day, including weekends, and holidays.

An animal facility is not required for production of antibody in hollow fiber bioreactors, however more extensive use of a cell culture laboratory is required for operating bioreactors.

It is the author's opinion that operation of bioreactors requires greater technical expertise than that necessary to perform procedures in mice. The author also recognizes that this factor is dependent on the background and experience of the technician. The author's extensive background and experience with animals and previously limited experience in cell culture may be a source of bias. It might also be noted that in view of the limited previous cell culture experience of the author, the author was able to successfully operate these bioreactor systems.

Time spent in labor and equipment and material costs were greater for operation of bioreactors. True equipment costs however, would be dependent on the method of amortization of capital costs for instrumentation. As these small scale systems have only recently been commercially introduced, the longevity of the instrumentation has yet to be determined. It should also be noted that time spent in labor and material costs were

determined for antibody production in 20 mice and bioreactor operation for 65 days, both of which are somewhat arbitrary.

Some of the problems encountered in the operation of these small scale bioreactor systems may be related to the recent development of this technology. These problems did impact labor time, and to a lesser extent material costs for the bioreactors. Presumably as the technology advances there will be improvements in both system components and operational techniques as more experience is gained in the use of these systems.

Results of this study suggest that the behavior of different hybridoma cell lines will likely vary in both mice and bioreactors necessitating flexibility and modifications in procedural parameters for individual cell lines in an effort to decrease animal distress and increase antibody production. In view of the demonstrated differences between cell lines, it is important to acknowledge that the results of this comparative study are based on evaluation of only 3 hybridoma cell lines.

In consideration of humane aspects, the author considers production of monoclonal antibodies in hollow fiber bioreactors to be more humane than production in mice. It should be noted, however, that the production of hybridoma cells still requires the use of mice, and serum used in the cell culture media is also an animal product, so the use of animals has not been completely eliminated by

this technology. A greater number of hybridoma cell lines need to be critically evaluated in hollow fiber bioreactors to more fully assess the potential of these systems to serve as alternatives for the reduction or replacement of the use of mice for monoclonal antibody production.

Results of this study demonstrate significant clinicopathologic changes in mice associated with monoclonal antibody production in ascites. Every attempt should be made to improve procedural protocols to maximize production so that the smallest number of animals can be used without compromising humane care. Further investigations should be conducted to determine the optimal time interval between pristane priming and subsequent inoculation of hybridoma cells, as the recommendations in the current literature are widely disparate.

As hybridomas obviously grow well and secrete large amounts of antibody in the microenvironment of the pristane-primed mouse peritoneal cavity, future studies should be designed to identify the specific factors involved in the enhancement of cell growth and secretion at this site. These factors could be added to the cell space of bioreactors, or alternatively, one could consider co-cultivation of hybridoma cells with other cells, such as fibroblasts, which secrete growth factors. It is the author's belief that hollow fiber bioreactor technology holds great potential promise for future applications.

SECTION 7: CONCLUSIONS

Significant clinicopathologic changes in mice were demonstrated in this study in association with monoclonal antibody production via the induction of ascitic tumors. Monoclonal antibodies from 3 cell lines were produced in 3 different, commercially available, laboratory scale hollow fiber bioreactor systems. Results of this study suggest that hollow fiber bioreactor systems merit further investigation as viable alternatives to monoclonal antibody production in murine ascites.

BIBLIOGRAPHY

1. Köhler G and Milstein C. (1975) Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity. Nature 256:495-497.

2. Köhler G and Milstein C. (1976) Derivation of Specific Antibody-Producing Tissue Culture and Tumor Lines by Cell Fusion. Eur J Immunol 6:511-519.

3. Goding JW. (1980) Antibody Production by Hybridomas. J Immunol Methods 39:285-308.

4. Chandler JP. (1987) Factors Influencing Monoclonal Antibody Production in Mouse Ascites Fluid. In Commercial Production of Monoclonal Antibodies: A Guide for Scale-Up. Seaver SS (Ed), Marcel Dekker, New York:75-92.

5. McGuill MW and Rowan AN. (1989) Refinement of Monoclonal Antibody Production and Animal Well-Being. ILAR News 31(1):7-11.

6. Leak LV, Potter M and Mayfield WJ. (1985) Response of the Peritoneal Mesothelium to the Mineral Oil Pristane. Current Topics Microbiol Immunol 122:221-233.

7. Amyx HL. (1987) Control of Animal Pain and Distress in Antibody Production and Infectious Disease Studies. J Am Vet Med Assoc 191(10):1287-1289.

8. Ettinger SJ. (1983) Ascites, Peritonitis, and Other Causes of Abdominal Enlargement. In Textbook of Veterinary Internal Medicine, Volume 1. Ettinger SJ (Ed), WB Saunders, Philadelphia:121-129.

9. Bender MD. (1985) Diseases of the Peritoneum. In Textbook of Medicine, 17th Edition, Volume 1. Wyngaarden JB and Smith LH (Eds), WB Saunders, Philadelphia:786-790.

10. Pockros PJ and Reynolds TB. (1986) Rapid Diuresis in Patients with Ascites from Chronic Liver Disease: The Importance of Peripheral Edema. Gastroenterology 90:1827-1833.

11. Kuhlmann I, Kurth W and Ruhdel I. (1985) Monoclonal Antibodies: In Vivo and In Vitro Production on a Laboratory Scale, with Consideration of the Legal Aspects of Animal Protection. ATLA 17:73-82.

12. Workman P, Balmain A, Hickman JA, et al. (1988) UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia. Lab Anim 22:195-201.

13. Lamont JT, Koff RS and Isselbacher KJ. (1980) Cirrhosis. In Harrison's Principles of Internal Medicine, 9th Edition. Isselbacher KJ, Adams RD, Braunwald E, Petersdorf RG, and Wilson JD. (Eds), McGraw-Hill, New York:1473-1482.

14. Ettinger SJ. (1983) Diseases of the Liver. In Textbook of Veterinary Internal Medicine, Volume 2. Ettinger SJ (Ed), WB Saunders, Philadelphia:1427-1429.

15. Truitt KE, Larrick JW, Raubitschek AA, Buck DE and Jacobson SW. (1984) Production of Human Monoclonal Antibody in Mouse Ascites. Hybridoma 3(2):195-199.

16. Misra DN, Noeman SA, Kunz HW and Gill TJ. (1986) Growth of Rat-Mouse Hybridomas in Nude Mice and Nude Rats. Methods Enzymol 121:386-411.

17. Weissman D, Parker DJ, Rothstein TL and Marshak-Rothstein A. (1985) Methods for the Production of Xenogenic Monoclonal Antibodies in Murine Ascites. J Immunol 135(2):1001-1003.

18. Noeman SA, Misra DN, Yankes RJ, Kunz HW and Gill TJ. (1982) Growth of Rat-Mouse Hybridomas in Nude Mice and Nude Rats. J Immunol Methods 55:319-326.

19. Ware CF, Donato NJ and Dorshkind K. (1985) Human, Rat or Mouse Hybridomas Secrete High Levels of Monoclonal Antibodies Following Transplantation into Mice with Severe Combined Immunodeficiency Disease (SCID). J Immunol Methods 85:353-361.

20. Boyd JE and James K. (1989) Human Monoclonal Antibodies: Their Potential, Problems, and Prospects. In Monoclonal Antibodies: Production and Application. Mizrahi (Ed), Alan R. Liss, New York:1-43.

21. Office of Biologics Research and Review, Center for Drugs and Biologics, FDA. (1987) Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use:1-20.

22. Reuveny S and Lazar A. (1989) Equipment and Procedures for Production of Monoclonal Antibodies in Culture. Monoclonal Antibodies: Production and Application. Mizrahi (Ed), Alan R. Liss, New York:45-80.

23. Manohar V and Hoffman T. (1992) Monoclonal and Engineered Antibodies for Human Parenteral Clinical Use: Regulatory Considerations. Trends Biotechnol 10:305-309.

24. Spicer SS, Spivey MA, Ito M and Schulte BA. (1994) Some Ascites Monoclonal Antibody Preparations Contain Contaminants that Bind to Selected Golgi Zones or Mast Cells. J Hisotchem Cytochem 42(2):213-221.

25. Garrett LR, Pascual DW, Clem LW and Cuchens MA. (1987) Conformational Changes in the DNA of Hybridoma Cells from Pristane Treated Mice. Chem-Biol Interactions 61:249-263.

26. Brown PC. (1986) Considerations in the Efficient Production of Secreted Proteins from Mammalian Cells in Large Scale Culture. Biotech 86. Online International, New York:1-11.

27. Patel TP, Parekh RB, Moellering BJ and Prior CP. (1992) Different Culture Methods Lead to Differences in Glycosylation of a Murine IgG Monoclonal Antibody. Biochem J 285:839-845.

28. Monica TJ, Goochee CF and Maiorella BL. (1993) Comparative Biochemical Characterization of a Human IgM Produced in Both Ascites and In Vitro Cell Culture. Bio/Technology 11:512-515.

29. Maiorella BL, Winkelhake J, Young J, Moyer B, Bauer R, Hora M, Andya J, Thomson J, Patel T and Parekh R. (1993) Effect of Culture Conditions on IgM Antibody Structure, Pharmacokinetics, and Activity. Bio/Technology 11:387-392.

30. Price BJ. (1986) Key Issues in Large-Scale Production of Monoclonal Antibodies for Human Health Care. Biotech 86. Online Publications, Pinner, UK:119-123.

31. Lord JM, Wang L, Epstein N, Griffin TW, Green BS, et al. (1989) Monoclonal Antibodies: Production and Application. Mizrahi A (Ed), Alan R. Liss, New York:194-386.

32. Von Wedel RJ. (1987) Mass Culture of Mouse and Human Hybridoma Cells in Hollow-Fiber Culture. In Commercial Production of Monoclonal Antibodies: A Guide for Scale-Up. Seaver SS (Ed), Marcel Dekker, New York:159-173.

33. Brodeur BR, Tsang P and Larose Y. (1984) Parameters Affecting Ascites Tumour Formation in Mice and Monoclonal Antibody Production. J Immunol Methods, 71:265-272.

34. Pannell R and Milstein C. (1992) An Oscillating Bubble Chamber for Laboratory Scale Production of Monoclonal Antibodies as an Alternative to Ascitic Tumours. J Immunol Methods 146:43-48.

35. Brodeur BR and Tsang PS. (1986) High Yield Monoclonal Antibody Production in Ascites. J Immunol Methods, 86:239-241.

36. Sjörgren-Jansson E and Jeanson S. (1990) Growing Hybridomas in Dialysis Tubing, Optimisation of Technique. In Laboratory Methods in Immunology. Zola E (Ed) CRC Press, Boca Raton:1:41.

37. Posillico EG. (1986) Microencapsulation for Large-Scale Antibody Production. Bio/Technology 4:114-117.

38. Himmler G, Palfi G, Ruker F and Katinger HW. (1985) A Laboratory Fermentor for Agarose Immobilized Hybridomas to Produce Monoclonal Antibodies. Dev Biol Stand 60:291-296.

39. Reuveny S, Velez D, Macmillan JD and Miller L. (1986) Factors Affecting Cell Growth and Monoclonal Antibody Production in Stirred Reactors. J Immunol Methods 86:53-59.

40. Shevitz J, Reuveny S, LaPorte TL and Cho GH. (1989) Stirred Tank Perfusion Reactors for Cell Propagation and Monoclonal Antibody Production. In Monoclonal Antibodies: Production and Applications. Mizrahi A (Ed), Alan R. Liss, New York:81-106.

41. Reuveny S, Velez D, Miller L, and Macmillan JD. (1986) Comparison of Cell Propagation Methods for Their Effect on Monoclonal Antibody Yield in Fermentors. J Immunol Methods 86:61-69.

42. Kurkela R, Fraune E and Vihko P. (1993) Pilot-Scale Production of Murine Monoclonal Antibodies in Agitated, Ceramic-Matrix, or Hollow-Fiber Cell Culture Systems. BioTechniques 15(4):674-683.

43. Knazek RA, Gullino PM, Kohler PO, and Dedrick RC. (1972) Cell Culture on Artificial Capillaries. Science 178:65-67.

44. Knazek RA. (1974) Solid Tissue Masses Formed In Vitro from Cells Cultured on Artificial Capillaries. Federation Proc 33(8):1978-1981.

45. Hopkinson J. (1985) Hollow Fiber Cell Culture Systems for Economical Cell-Product Manufacturing. Bio/Technology 3:225-230.

46. Altshuler GL, Dziewulski DM, Sowek JA and Belfort G. (1986) Continuous Hybridoma Growth and Monoclonal Antibody Production in Hollow Fiber Reactors-Separators. Biotechnol Bioeng 28:646-658.

47. Evans TL and Miller RA. (1988) Large-Scale Production of Murine Monoclonal Antibodies Using Hollow Fiber Bioreactors. BioTechniques 6(8):762-767.

48. Piret JM and Cooney CL. (1990) Mammalian Cell and Protein Distributions in Ultrafiltration Hollow Fiber Bioreactors. Biotechnol Bioeng 36:902-910.

49. Andersen BG and Gruenberg ML. (1987) Optimization Techniques for the Production of Monoclonal Antibodies Utilizing Hollow-Fiber Technology. In Commercial Production of Monoclonal Antibodies: A Guide for Scale-Up. Seaver SS (Ed), Marcel Dekker, New York:175-195.

50. Tiebout RF. (1989) Tissue Culture in Hollow-Fibre Systems: Implications for Downstream Processing and Stability Analysis. Dev Biol Stand 71:65-71.

51. Altshuler GL, Sowek JA, and Belfort G. (1987) IgG Production in Microporous Hollow Fiber Bioreactors. Proc 4th Eur Congr Biotechnol 1:127-129.

52. Kidwell WR. (1989) Filtering Out Inhibition Bio/Technology 7:462-463.

53. Handa-Corrigan A. (1991) Bioreactors for Mammalian Cells. In Mammalian Cell Biotechnology. Butler M (Ed) Oxford University Press, New York:139-158.

54. Potter M and MacCardle RC. (1964) Histology of Developing Plasma Cell Neoplasia Induced by Mineral Oil in BALB/c Mice. J Natl Cancer Inst 33:497-515.

55. Anderson PN and Potter M. (1969) Induction of Plasma Cell Tumors in BALB/c Mice with 2,6,10,14-Tetramethylpentadecane (Pristane) Nature 222:994-995.

56. Silverman J. (1987) Protocol Review. Monoclonal Antibody Production: In Vivo vs. In Vitro. Lab Animal 1987 (May/June):19-20.

57. Munoz J. (1957) Production in Mice of Large Volumes of Ascites Fluid Containing Antibodies. Proc Soc Exp Biol and Med 95:757-759.

58. Lieberman R, Mantel N, and Humphrey W Jr. (1961) Ascites Production in 17 Mouse Strains. Proc Soc Exp Biol and Med 107:163-165.

59. Harlow E and Lane DP. (1988) Monoclonal Antibodies. In Antibodies: A Laboratory Manual. Harlow E and Lane DP (Eds), Cold Spring Harbor Laboratory, Cold Spring Harbor:144-145.

60. Potter M. (1972) Immunoglobulin-Producing Tumors and Myeloma Proteins of Mice. Physiol Rev 52:631-719.

61. Stewart F, Callander A and Garwes DJ. (1989) Comparison of Ascites Production for Monoclonal Antibodies in BALB/c and BALB/c-Derived Cross-Bred Mice. J Immunol Methods 119:269-275.

62. Hoogenraad NJ and Wraight CJ. (1986) The Effect of Pristane on Ascites Tumor Formation and Monoclonal Antibody Production. Methods Enzymol 121:375-381.

63. Cranco M and Potter M. (1976) The Requirement of an Adherent Cell Substratum for the Growth of Developing Plasmacytoma Cells In Vivo. J Exp Med 144:1554-1567.

64. Kripke MC and Weiss DW. (1970) Studies on the Immune Responses of BALB/c Mice During Tumor Induction by Mineral Oil. Int J Cancer 6:422-430.

65. Freund YR and Blair PB. (1982) Depression of Natural Killer Activity and Mitogen Responsiveness in Mice Treated with Pristane. J Immunol 129(6):2826-2830.

66. Platica M, Bojko C, Clincea R and Hollander V. (1982) Lipopolysaccharide and Pristane-Induced Peritoneal Exudate Requirements for Plasma Tumor Cell Colony Formation. J Natl Cancer Inst 68(2):325-328.

67. Hoogenraad N, Helman T and Hoogenraad J. (1983) The Effect of Pre-Injection of Mice with Pristane on Ascites Tumour Formation and Monoclonal Antibody Production. J Immunol Methods 61:317-320.

68. Gillette RW. (1987) Alternatives to Pristane Priming for Ascitic Fluid and Monoclonal Antibody Production. J Immunol Methods 99:21-23.

69. Mueller UW, Hawes CS, and Jones WR. (1986) Monoclonal Antibody Production by Hybridoma Growth in Freund's Adjuvant Primed Mice. J Immunol Methods 87:193-196.

70. Jones SL, Cox JC and Pearson JE. Increased Monoclonal Antibody Ascites Production in Mice Primed with Freund's Incomplete Adjuvant. J Immunol Methods 129:227-231.

71. Galfre G and Milstein C. (1981) Preparation of Monoclonal Antibodies: Strategies and Procedures. Methods Enzymol 73:1-46.

72. Epstein N and Epstein M. (1986) The Hybridoma Technology: I. Production of Monoclonal Antibodies. Adv Biotechnol Processes 6:179-218.

73. Perlman D. (1979) Use of Antibiotics in Cell Culture Media. Methods Enzymol 108:110-116.

74. Mizrahi A and Lazar A. (1991) Media for Cultivation of Animal Cells: An Overview. In Animal Cell Culture and Production of Biologicals. Sasaki R and Ikura K (Eds), Kluwer Academic Publishers, Netherlands:159-180.

75. Zwitterionic Buffers: Their Function in Cell Culture. (1987) In Art to Science in Cell Culture. HyClone Laboratories, Logan, Utah 6(2):4-5.

76. Huang YL, Li SY, Dremel BA, Bilitewski U and Schmid RD. (1991) On-line Determination of Glucose Concentration Throughout Animal Cell Cultures Based on Chemiluminescent Detection of Hydrogen Peroxide Coupled with Flow-Injection Analysis. J Biotechnol 18:161-172.

77. Fleming JO and Pen LB. (1988) Measurement of the Concentration of Murine IgG Monoclonal Antibody in Hybridoma Supernatants and Ascites in Absolute Units by Sensitive and Reliable Enzyme-Linked Immunosorbent Assays (ELISA). J Immunol Methods 110:11-18.

78. Yelton DE, Desaymard C and Scharff MD. (1981) Use of Monoclonal Anti-Mouse Immunoglobulin to Detect Mouse Antibodies. Hybridoma 1(1):5-11.

79. Delaunay T, Louahed J and Bazin H. (1990) VIII. ELISA Measurement of Ig Production in Mouse Hybridoma Culture Supernatants. J Immunol Methods 131:33-39.

80. Hood L, Gray WR, Sanders BG and Dreyer WT. (1967) Light Chain Evolution. Cold Spring Harbor Sym Quant Biol 32:133-145.

81. Coniglio C, Pappas G, Gill WJ, Kashdan M and Maniscalco M. (1991) Clarification of Ascites Fluid. BioTechniques 10(4):457-460.

82. Andrews EJ, Bennett BT, Clark JD, et al. (1993) 1993 Report of the AVMA Panel on Euthanasia. J Am Vet Med Assoc 202(2):229-249.

83. Catty D and Raykundalia C. (1989) ELISA and Related Enzyme Immunoassays. In Antibodies Volume II. A Practical Approach. Catty D (Ed) IRL Press at Oxford University Press, Oxford, England:97-154.

84. Goding JW. (1986) Purification, Fragmentation and Isotopic Labelling. In Monoclonal Antibodies: Principles and Practice. Goding JW (Ed), Academic Press, New York:109-110.

85. Oi VT and Herzenberg LA. (1980) Immunoglobulin-Producing Cell Lines: Cloning by Limiting Dilution. In Selected Methods in Cellular Immunology. Mishell BB and Shiigi SM (Eds), WH Freeman and Company, San Francisco:366-367.

86. Tritsch GL and Moore GE. (1962) Spontaneous Decomposition of Glutamine in Cell Culture Media. Exp Cell Res 28:360-364.

87. Technical Information. BioWhittaker, Inc., Walkersville, MD.

88. Groopman JD, Trudel LJ, Donahue PR, Marshak-Rothstein A, and Wogan GN. (1984) High-Affinity Monoclonal Antibodies for Aflatoxins and Their Application to Solid-Phase Immunoassays. Proc Natl Acad Sci 81:7728-7731.

89. Turesky RJ, Forster CM, Aeschbacher HU, Würzner HP, Skipper PL, Trudel LJ, and Tannenbaum SR. (1989) Purification of Food-Borne Carcinogens 2-amino-3-methylimidazo[4,5-*f*]quinoline and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline in Heated Meat Products by Immunoaffinity Chromatography. Carcinogenesis 10(1):151-156.

90. Groopman JD, Skipper PL, Donahue PR, Trudel LJ, Wildschutte M, Kadlubar FF and Tannenbaum SR. (1992) Monoclonal Antibodies and Rabbit Antisera Recognizing 4-aminobiphenyl-DNA Adducts and Application to Immunoaffinity Chromatography. Carcinogenesis 13(6):917-922.

91. Bland JM and Altman DG. (1986) Statistical Methods for Assessing Agreement Between Two Methods of Clinical Measurement. Lancet (February 8, 1986):307-310.

92. Price HL and Ohnishi ST. (1980) Effects of Anesthetics on the Heart. Federation Proc 39(5):1575-1579.

93. Altura BM, Altura BT, Carella A, Turlapaty PD and Weinberg J. (1980) Vascular Smooth Muscle and General Anesthetics. Federation Proc 39(5):1584-1591.

94. Omasa T, Kobayashi M, Nishikawa T, Shioya S, Suga K, Uemura S and Imamura Y. (1991) Hybridoma Culture in the Hollow-Fiber System - The Effects of Growth Factors. In Animal Cell Culture and Production of Biologicals. Sasaki R and Ikura K (Eds), Kluwer Academic Publishers, Netherlands:229-236.

95. Omasa T, Kobayashi M, Nishikawa T, Shioya S, Suga K, Uemura S and Imamura Y. (1991) Effects of Growth Factors on Hybridoma Culture in the Perfusion System. In Animal Cell Culture and Production of Biologicals. Sasaki R and Ikura K (Eds), Kluwer Academic Publishers, Netherlands:237-243.