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# Monoclonal Antibody Production

*A Report of the*
Committee on Methods of Producing Monoclonal Antibodies
Institute for Laboratory Animal Research
National Research Council

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Monoclonal Antibody Production: A Report of the Committee on Methods of Producing Monoclonal Antibodies, Institute for Laboratory Animal Research, National Research Council.

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# Preface

Monoclonal antibodies (mAb) are used extensively in basic biomedical research, in diagnosis of disease, and in treatment of illnesses, such as infections and cancer. Antibodies are important tools used by many investigators in their research and have led to many medical advances.

Producing mAb requires immunizing an animal, usually a mouse; obtaining immune cells from its spleen; and fusing the cells with a cancer cell (such as cells from a myeloma) to make them immortal, which means that they will grow and divide indefinitely. A tumor of the fused cells is called a hybridoma, and these cells secrete mAb. The development of the immortal hybridoma requires the use of animals; no commonly accepted nonanimal alternatives are available.

An investigator who wishes to study a particular protein or other molecule selects a hybridoma cell line that secretes mAb that reacts strongly with that protein or molecule. The cells must grow and multiply to form a clone that will produce the desired mAb. There are two methods for growing these cells: injecting them into the peritoneal cavity of a mouse or using in vitro cell-culture techniques. When injected into a mouse, the hybridoma cells multiply and produce fluid (ascites) in its abdomen; this fluid contains a high concentration of antibody. The mouse ascites method is inexpensive, easy to use, and familiar.

However, if too much fluid accumulates or if the hybridoma is an aggressive cancer, the mouse will likely experience pain or distress. If a procedure produces pain or distress in animals, regulations call for a search for alternatives. One alternative is to grow hybridoma cells in a tissue-culture medium; this technique requires some expertise, requires special media, and can be expensive and time-consuming. There has been considerable research on in vitro methods for growing hybridomas and these newer methods are less expensive, are faster, and produce antibodies in higher concentration than has been the case in the past. The existence of alternatives to the mouse ascites method raises the question: Is there a scientific need for the mouse ascites method of producing mAb?

The American Anti-Vivisection Society (AAVS) petitioned the National Institutes of Health (NIH) on April 23, 1997, to prohibit the use of animals in the production of mAb. On September 18, 1997, NIH declined to prohibit the use of mice in mAb production, stating that "the ascites method of mAb production is scientifically appropriate for some research projects and cannot be replaced." On March 26, 1998, AAVS submitted a second petition, stating that "NIH failed to provide valid scientific reasons for not supporting a proposed ban." The office of the NIH director asked the National Research Council to conduct a study of methods of producing mAb.

In response to that request, the Research Council appointed the Committee on Methods of Producing Monoclonal Antibodies, to act on behalf of the Institute for Laboratory Animal Research of the Commission on Life Sciences, to conduct the study. The 11 expert members of the committee had extensive experience in biomedical research, laboratory animal medicine, animal welfare, pain research, and patient advocacy (Appendix B). The committee was asked to determine whether there was a scientific necessity for the mouse ascites method; if so, whether the method caused pain or distress; and, if so, what could be done to minimize the pain or distress. The committee was also asked to comment on available in vitro methods; to suggest what acceptable scientific rationale, if any, there was for using the mouse ascites method; and to identify regulatory requirements for the continued use of the mouse ascites method.

The committee held an open data-gathering meeting during which its members summarized data bearing on those questions. A 1-day workshop was attended by 34 participants, 14 of whom made formal presentations (see agenda, Appendix A). A second meeting was held to finalize the report. The present report was written on the basis of information in the literature and information presented at the meeting and the workshop.

This report has been reviewed by persons chosen for their diverse perspectives and technical expertise in accordance with procedures approved by the National Research Council's Report Review Committee. The purposes of the independent review are to provide candid and critical comments that will assist the authors and the Research Council in making the published report as sound as possible and to ensure that the report meets institutional standards of objectivity, evidence, and responsiveness to the study charge. The contents of the review comments and the draft manuscript remain confidential to protect the integrity of the deliberative process. We wish to thank the following persons for their participation in the review of this report:

J. Donald Capra, Oklahoma Medical Research Foundation, Oklahoma City
Philip Carter, North Carolina State University, Raleigh
Joseph Chandler, Maine Biotechnology Services, Inc., Portland
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The list shows the diversity and background of the reviewers, again attesting to the rigor of the process of producing this report. Although the persons listed have provided many constructive comments and suggestions, responsibility for the final content of this report rests solely with the author committee and the National Research Council.

To the committee members, reviewers, and staff, I extend my deepest appreciation. Members of the committee devoted precious weekends, evenings, and work hours and endless energy to meet short deadlines. The reviewers also worked under short deadlines, and their efforts greatly improved the logic, coherence, and comprehensibility of our report.

I am grateful for the guidance and support provided by the Institute for Laboratory Animal Research staff throughout the process. Kathleen Beil provided timely and important communications to the committee in arranging travel and lodging and in report production. Ralph Dell's focus on the topic and his management of the review and publication were of inestimable value. Norman Grossblatt's editing made the report eminently more readable—a feature that will be appreciated by readers.

Peter A. Ward, Chair
Committee on Methods of Producing Monoclonal Antibodies

# CONTENTS

# Executive Summary

Monoclonal antibodies (mAb) are important reagents used in biomedical research, in diagnosis of diseases, and in treatment of such diseases as infections and cancer. These antibodies are produced by cell lines or clones obtained from animals that have been immunized with the substance that is the subject of study. To produce the desired mAb, the cells must be grown in either of two ways: by injection into the abdominal cavity of a suitably prepared mouse or by tissue culturing cells in plastic flasks. Further processing of the mouse ascitic fluid and of the tissue culture supernatant might be required to obtain mAb with the required purity and concentration. The mouse method is generally familiar, well understood, and widely available in many laboratories; but the mice require careful watching to minimize the pain or distress that some cell lines induce by excessive accumulation of fluid (ascites) in the abdomen or by invasion of the viscera. The tissue-culture method would be widely adopted if it were as familiar and well understood as the mouse method and if it produced the required amount of antibody with every cell line; but culture methods have been expensive and time-consuming and often failed to produce the required amount of antibody without considerable skilled manipulation. However, culture methods are now becoming less expensive, more familiar, and more widely available.

The American Anti-Vivisection Society (AAVS) petitioned the National Institutes of Health (NIH) in early 1997 to prohibit the use of an animal in the production of mAb. NIH responded late in 1997, asserting that continued use of the mouse method for producing mAb was scientifically required. In a second petition, in early 1998, AAVS did not accept the NIH response. NIH asked the National Research Council to form a committee to study this issue. The Committee on Methods of Producing Monoclonal Antibodies was composed of 11 experts with extensive experience in biomedical research, laboratory animal medicine, pain research, animal welfare, and patient advocacy. The committee was asked to determine whether there is a scientific necessity for producing mAb by the mouse method and, if so, to recommend ways to minimize any pain or distress that might be associated with the method. The committee was also to determine whether there are regulatory requirements for the mouse method and to summarize the current stage of development of tissue-culture methods.

On the basis of relevant literature, material submitted to the committee, the experience of members of the committee, and presentations at a 1-day workshop attended by 14 speakers and 20 additional observers, as well as two separate working committee meetings, the committee came to specific conclusions and made recommendations.

We believe that choosing the method of producing monoclonal antibodies should be consistent with other recommendations in the *Guide for the Care and Use of Laboratory Animals*. One such recommendation pertains to multiple survival surgery; the *Guide* states (page 12) that this practice "should be *discouraged* but permitted if scientifically justified by the user and approved by the Institutional Animal Care and Use Committee (IACUC)" [emphasis added]. Similarly, we recommend that mAb production by the mouse ascites method be permitted if scientifically justified and approved by the relevant IACUC. We further believe that tissue-culture methods should be used routinely for mAb production, especially for most large-scale production of mAb. When hybridomas fail to grow or fail to achieve a product consistent with scientific goals, the investigator is obliged show that a good-faith effort was made to adapt the hybridoma to in vitro growth conditions before using the mouse ascites method.

**Recommendation 1: There is a need for the scientific community to avoid or minimize pain and suffering by animals. Therefore, over the next several years, as tissue-culture systems are further**

**developed, tissue-culture methods for the production of monoclonal antibodies should be adopted as the routine method unless there is a clear reason why they cannot be used or why their use would represent an unreasonable barrier to obtaining the product at a cost consistent with the realities of funding of biomedical research programs in government, academe, and industry. This could be accomplished by establishing tissue-culture production facilities in institutions.**

There are several reasons why the mouse method of producing mAb cannot be abandoned: some cell lines do not adapt well to tissue-culture conditions; in applications where several different mouse mAb at high concentrations are required for injection into mice, the in vitro method can be inefficient; rat cell lines usually do not efficiently generate mAb in rats and adapt poorly to tissue-culture conditions but do produce mAb in immunocompromised mice; downstream purification or concentration from in vitro systems can lead to protein denaturation and decreased antibody activity; tissue-culture methods can yield mAb that do not reflect the normal modification of proteins with sugars, and this abnormality might influence binding capacity and other critical biologic functions of mAb; contamination of valuable cell lines with fungi or bacteria requires prompt passage through a mouse to save the cell line; and inability of some cell lines that do adapt to tissue-culture conditions to maintain adequate production of mAb poses a serious problem. For these reasons, the committee concludes that there is a scientific necessity to permit the continuation of the mouse ascites method of producing mAb. However, note that over time, as in vitro methods improve, the need for the mouse ascites method will decrease.

**Recommendation 2: The mouse ascites method of producing monoclonal antibodies should not be banned, because there is and will continue to be scientific necessity for this method.**

There does not appear to be convincing evidence that significant pain or distress is associated with the injection into the mouse of pristane (a chemical that promotes the growth of the tumor cells), but during the accumulation of ascites there is likely to be pain or distress, particularly when some cell lines that are tissue-invasive are used and in situations of significant ascites development. Therefore, after injection of hybridoma cells, mice should be evaluated at least daily, including weekends and holidays, after development of visible ascites and should be tapped before fluid accumulation becomes distressful. A limit should be placed on the number of taps and multiple taps should be allowed only if the animal does not exhibit signs of distress.

**Recommendation 3: When the mouse ascites method for producing mAb is used, every reasonable effort should be made to minimize pain or distress, including frequent observation, limiting the numbers of taps, and prompt euthanasia if signs of distress appear.**

Two of 13 mAb approved by the Food and Drug Administration for therapeutic use cannot be produced by in vitro means, or converting to an in vitro system for their production would require (because of federal regulations) proof of bioequivalence, which would be unacceptably expensive. Furthermore, many commercially available mAb are routinely produced by mouse methods, particularly when the amount to be produced is less than 10 g, another situation where it would be prohibitively expensive to convert to tissue-culture conditions. However, with further refinement of technologies, media, and practices, production of mAb in tissue culture for research and therapeutic needs will probably become comparable with the costs of the mouse ascites method and could replace the ascites method.

**Recommendation 4: mAb now being commercially produced by the mouse ascites method should continue to be so produced, but industry should continue to move toward the use of tissue-culture methods.**

In a few circumstances, the use of the mouse ascites method for the production of mAb might be required. We suggest the following as examples of criteria to be used by an IACUC in establishing guidelines for the production of mAb in mice by the ascites method.

1. When a supernatant of a dense hybridoma culture grown for 7–10 days (stationary batch method) yields an mAb concentration of less than 5 μg/ml. If hollow-fiber reactors or semipermeable-membrane systems are used, 500 mg/ml and 300 mg/ml, respectively, are considered low mAb concentrations.
2. When more than 5 mg of mAb produced by each of five or more different hybridoma cell lines is needed simultaneously. It is technically difficult to produce this amount of mAb since it requires more monitoring and processing capability than the average laboratory can achieve.
3. When analysis of mAb produced in tissue culture reveals that a desired antibody function is diminished or lost.
4. When a hybridoma cell line grows and is productive only in mice.
5. When more than 50 mg of functional mAb is needed, and previous poor performance of the cell line indicates that hollow-fiber reactors, small-volume membrane-based fermentors, or other techniques cannot meet this need during optimal growth and production.

We emphasize that those criteria are not all-inclusive and that it is the responsibility of the IACUCs to determine whether animal use is required for scientific or regulatory reasons. Criteria have not been developed to define a cell line that is low-producing or when tissue-culture methods are no longer a useful means of producing mAb.

# Introduction

Monoclonal antibodies (mAb) are important reagents used in biomedical research, in diagnosis of diseases, and in treatment of such diseases as infections and cancer. These antibodies are produced by cell lines or clones obtained from animals that have been immunized with the substance that is the subject of study. The cell lines are produced by fusing B cells from the immunized animal with myeloma cells (Köhler and Milstein 1975). To produce the desired mAb, the cells must be grown in either of two ways: by injection into the peritoneal cavity of a suitably prepared mouse (the in vivo, or mouse ascites, method) or by in vitro tissue culture. Further processing of the mouse ascitic fluid and of the tissue-culture supernatant might be required to obtain mAb with the required purity and concentration. The mouse ascites method is generally familiar, well understood, and widely available in many laboratories; but the mice require careful watching to minimize the pain or distress induced by excessive accumulation of fluid in the abdomen or by invasion of the viscera. The in vitro tissue-culture method would be widely adopted if it were as familiar and well understood as the mouse ascites method and if it produced the required amount of antibody with every cell line; but in vitro methods have been expensive and time-consuming relative to the costs and time required by the mouse ascites method and often failed to produce the required amount of antibody even with skilled manipulation. Modern in vitro methods have increased the success rate to over 90% and have reduced costs.

The anticipated use of the mAb will determine the amount required (Marx and others 1997). Only small amounts of mAb (less than 0.1 g) are required for most research projects and many analytic purposes. Medium-scale quantities (0.1-1 g) are used for production of diagnostic kits and reagents and for efficacy testing of new mAb in animals. Large-scale production of mAb is defined, in this context, as over 1 g. These larger quantities are used for routine diagnostic procedures and for therapeutic purposes.

The use of monoclonal antibodies (mAb) in biomedical research has been and will continue to be important for the identification of proteins, carbohydrates, and nucleic acids. Their use has led to the elucidation of many molecules that control cell replication and differentiation, advancing our knowledge of the relationship between molecular structure and function. These advances in basic biologic sciences have improved our understanding of the host response to infectious-disease agents and toxins produced by these agents, to transplanted organs and tissues, to spontaneously transformed cells (tumors), and to endogenous antigens (involved in autoimmunity). In addition, the exquisite specificity of mAb allows them to be used in humans and animals for disease diagnosis and treatment. Under the appropriate conditions, mAb-producing hybridomas survive indefinitely, so continued production of mAb is associated with the use of fewer animals, especially when production involves the use of in vitro methods. Despite all those benefits associated with production of mAb with the mouse ascites method, it can be distressful to the host animal.

The *U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training* (IRAC 1983) states that “animals selected for the procedure should be of appropriate species and quality and the minimum number required to obtain valid results. Methods such as mathematical models, computer simulation, and in vitro biological systems should be considered. Proper use of animals, including the avoidance or minimization of discomfort, distress, and pain when consistent with sound scientific practices, is imperative.” The *Guide for the Care and Use of Laboratory Animals* (NRC 1996, page 10) specifically addresses excessive tumor burden in animals and states, "occasionally, protocols include procedures that have not been previously encountered or that have the potential to cause pain or distress that cannot be reliably controlled. . . . Relevant objective information regarding the procedures and the purpose of the study should be sought from the literature, veterinarians, investigators and others knowledgeable about the effects in animals.” The *Public Health Service Policy on Humane Care and Use of*

*Laboratory Animals* (NIH 1996, page 7) requires IACUCs to ensure that approved protocols conform with the PHS requirement that "procedures with animals . . . avoid or minimize discomfort, distress and pain to animals (in a way that is) consistent with sound research design." It is therefore incumbent on the scientist to consider first the use of in vitro methods for the production of mAb. If in vitro production of mAb is not reasonable or practical, the scientist may request permission to use the mouse ascites method. However, "prior to approval of proposals which include the mouse ascites method, IACUCs must determine that (i) the proposed use is scientifically justified, (ii) methods that avoid or minimize discomfort, distress and pain (including in vitro methods) have been considered, and (iii) the latter [refers to in vitro methods] have been found unsuitable" (NIH 1997). The charge to the present committee excluded evaluation of steps needed to produce an antibody secreting cell line.

# 1

# Generation of Hybridomas: Permanent Cell Lines Secreting Monoclonal Antibodies

Production of monoclonal antibodies involves in vivo or in vitro procedures or combinations thereof. Before production of antibodies by either method, hybrid cells that will produce the antibodies are generated. The steps in producing those cells are outlined below (figure 1). The generation of mAb-producing cells requires the use of animals, usually mice. The procedure yields a cell line capable of producing one type of antibody protein for a long period. A tumor from this "immortal" cell line is called a hybridoma.

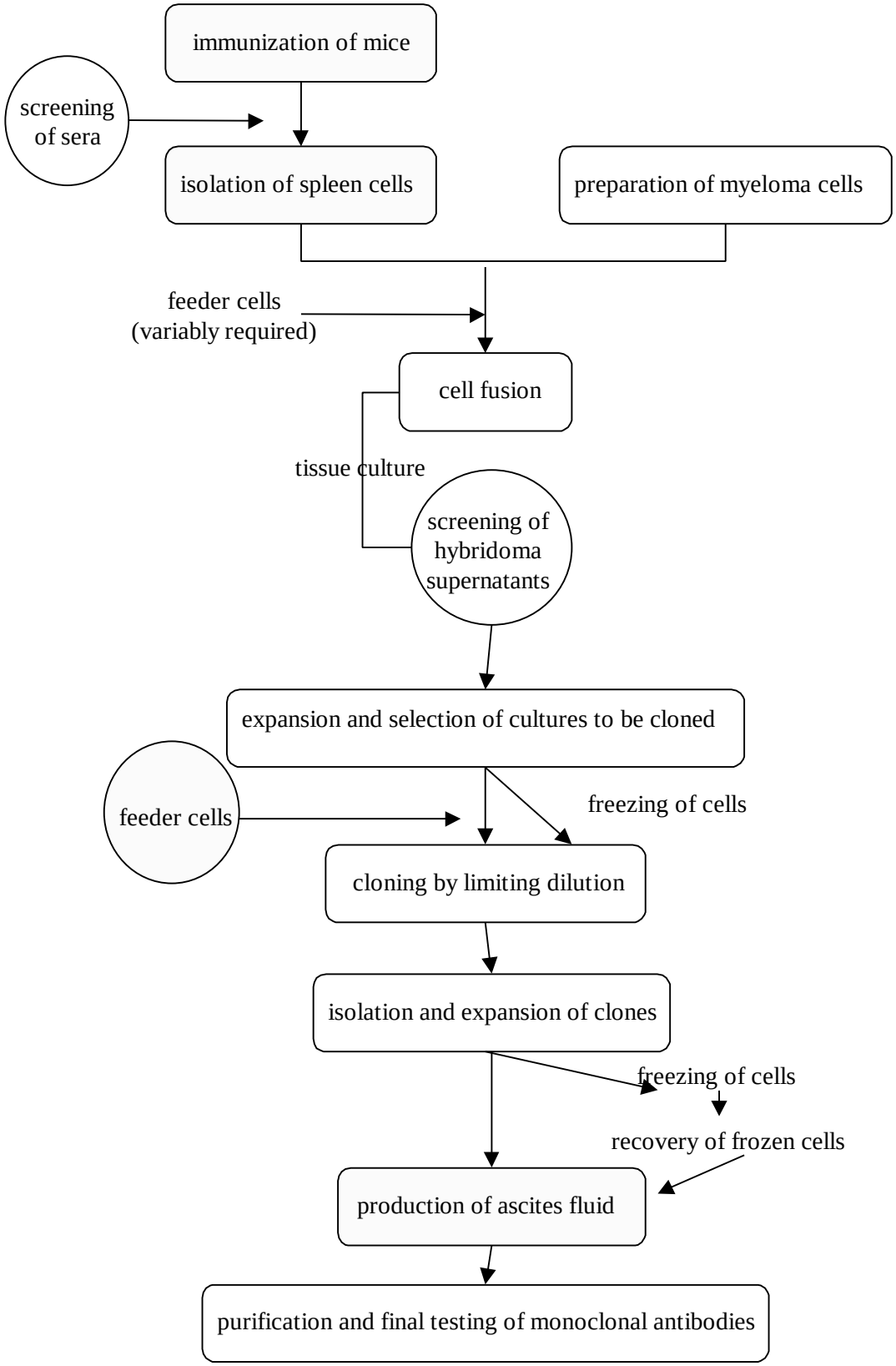
No method of generating a hybridoma that avoids the use of animals has been found. Recent in vitro techniques allow the intracellular production of antigen-binding antibody fragments, but such techniques are still experimental and have an uncertain yield, efficacy, and antibody function (Frenken and others 1998). It has also been possible to genetically replace much of the mouse mAb-producing genes with human sequences, reducing the immunogenicity of mAb destined for clinical use in humans. Before the advent of the hybridoma method, investigators could produce only polyclonal serum antibodies; this required large numbers of immunized animals and did not immortalize the antibody-producing cells, so it required repeated animal use to obtain more antibodies. Development of the hybridoma technology has reduced the number of animals (mice, rabbits, and so on) required to produce a given antibody but with a decrease in animal welfare when the ascites method is used.

Step 1: Immunization of Mice and Selection of Mouse Donors for Generation of Hybridoma Cells

Mice are immunized with an antigen that is prepared for injection either by emulsifying the antigen with Freund's adjuvant or other adjuvants or by homogenizing a gel slice that contains the antigen. Intact cells, whole membranes, and microorganisms are sometimes used as immunogens. In almost all laboratories, mice are used to produce the desired antibodies. In general, mice are immunized every 2-3 weeks but the immunization protocols vary among investigators. When a sufficient antibody titer is reached in serum, immunized mice are euthanized and the spleen removed to use as a source of cells for fusion with myeloma cells.

Step 2: Screening of Mice for Antibody Production

After several weeks of immunization, blood samples are obtained from mice for measurement of serum antibodies. Several humane techniques have been developed for collection of small volumes of blood from mice (Loeb and Quimby 1999). Serum antibody titer is determined with various techniques, such as enzyme-linked immunosorbent assay (ELISA) and flow cytometry. If the antibody titer is high, cell fusion can be performed. If the titer is too low, mice can be boosted until an adequate response is achieved, as determined by repeated blood sampling. When the antibody titer is high enough, mice are commonly boosted by injecting antigen without adjuvant intraperitoneally or intravenously (via the tail veins) 3 days before fusion but 2 weeks after the previous immunization. Then the mice are euthanized and their spleens removed for in vitro hybridoma cell production.




Shaded areas represent mouse use.

In part, prepared from Current Protocols in Molecular Biology. Ed: Frederick M. Ausubel, 1998.

Step 3: Preparation of Myeloma Cells

Fusing antibody-producing spleen cells, which have a limited life span, with cells derived from an immortal tumor of lymphocytes (myeloma) results in a hybridoma that is capable of unlimited growth. Myeloma cells are immortalized cells that are cultured with 8-azaguanine to ensure their sensitivity to the hypoxanthine-aminopterin-thymidine (HAT) selection medium used after cell fusion.[1] A week before cell fusion, myeloma cells are grown in 8-azaguanine. Cells must have high viability and rapid growth. The HAT medium allows only the fused cells to survive in culture.

Step 4: Fusion of Myeloma Cells with Immune Spleen Cells

Single spleen cells from the immunized mouse are fused with the previously prepared myeloma cells. Fusion is accomplished by co-centrifuging freshly harvested spleen cells and myeloma cells in polyethylene glycol, a substance that causes cell membranes to fuse. As noted in step 3, only fused cells will grow in the special selection medium. The cells are then distributed to 96 well plates containing feeder cells derived from saline peritoneal washes of mice. Feeder cells are believed to supply growth factors that promote growth of the hybridoma cells (Quinlan and Kennedy 1994). Commercial preparations that result from the collection of media supporting the growth of cultured cells and contain growth factors are available that can be used in lieu of mouse-derived feeder cells. It is also possible to use murine bone marrow-derived macrophages as feeder cells (Hoffman and others 1996).

Step 5: Cloning of Hybridoma Cell Lines by "Limiting Dilution" or Expansion and Stabilization of Clones by Ascites Production

At this step new, small clusters of hybridoma cells from the 96 well plates can be grown in tissue culture followed by selection for antigen binding or grown by the mouse ascites method with cloning at a later time. Cloning by "limiting dilution" at this time ensures that a majority of wells each contain at most a single clone. Considerable judgment is necessary at this stage to select hybridomas capable of expansion versus total loss of the cell fusion product due to underpopulation or inadequate in vitro growth at high dilution. In some instances, the secreted antibodies are toxic to fragile cells maintained in vitro. Optimizing the mouse ascites expansion method at this stage can save the cells. Also, it is the experience of many that a brief period of growth by the mouse ascites method produces cell lines that at later in vitro and in vivo stages show enhanced hardiness and optimal antibody production (Ishaque and Al-Rubeai 1998). Guidelines have been published to assist investigators in using the mouse ascites methods in these ways (Jackson and Fox 1995).

[1] The selection growth medium contains the inhibitor aminopterin, which blocks synthetic pathways by which nucleotides are made. Therefore, the cells must use a bypass pathway to synthesize nucleic acids, a pathway that is defective in the myeloma cell line to which the normal antibody-producing cells are fused. Because neither the myeloma nor the antibody-producing cell will grow on its own, only hybrid cells grow.

# 2

# In Vitro Production of Monoclonal Antibody

A major advantage of using mAb rather than polyclonal antiserum is the potential availability of almost infinite quantities of a specific monoclonal antibody directed toward a single epitope (the part of an antigen molecule that is responsible for specific antigen-antibody interaction). In general, mAb are found either in the medium supporting the growth of a hybridoma in vitro or in ascitic fluid from a mouse inoculated with the hybridoma. mAb can be purified from either of the two sources but are often used as is in media or in ascitic fluid. In vitro methods should be used for final production of mAb when this is reasonable and practical. Many commercially available devices have been developed for in vitro cultivation. These devices vary in the facilities required for their operation, the amount of operator training required, the complexity of operating procedures, final concentration of antibody achieved, cost, and fluid volume accommodated. The cost of additional equipment should be considered in the cost of in vitro production methods.

Each hybridoma cell line responds differently to a given in vitro production environment. This section describes in vitro production methods that are available and discusses the usefulness and limitations of each method.

## Batch Tissue-Culture Methods

The simplest approach for producing mAb in vitro is to grow the hybridoma cultures in batches and purify the mAb from the culture medium. Fetal bovine serum is used in most tissue-culture media and contains bovine immunoglobulin at about 50 µg/ml. The use of such serum in hybridoma culture medium can account for a substantial fraction of the immunoglobulins present in the culture fluids (Darby and others 1993). To avoid contamination with bovine immunoglobulin, several companies have developed serum-free media specifically formulated to support the growth of hybridoma cell lines (Federspiel and others 1991; Tarleton and Beyer 1991; Velez and others 1986). In most cases, hybridomas growing in 10% fetal calf serum (FCS) can be adapted within four passages (8-12 days) to grow in less than 1% FCS or in FCS-free media. However, this adaptation can take much longer and in 3-5% of the cases the hybridoma will never adapt to the low FCS media. After this adaptation, cell cultures are allowed to incubate in commonly used tissue-culture flasks under standard growth conditions for about 10 days; mAb is then harvested from the medium.

The above approach yields mAb at concentrations that are typically below 20 µg/ml. Methods that increase the concentration of dissolved oxygen in the medium may increase cell viability and the density at which the cells grow and thus increase mAb concentration (Boraston and others 1984; Miller and others 1987). Some of those methods use spinner flasks and roller bottles that keep the culture medium in constant circulation and thus permit nutrients and gases to distribute more evenly in large volumes of cell-culture medium (Reuveny and others 1986; Tarleton and Beyer 1991). The gas-permeable bag (available through Baxter and Diagnostic Chemicals), a fairly recent development, increases concentrations of dissolved gas by

allowing gases to pass through the wall of the culture container. All these methods can increase productivity substantially, but antibody concentrations remain in the range of a few micrograms per milliliter (Heidel 1997; Peterson and Peavey 1998; Vachula and others 1995).

Most research applications require mAb concentration of 0.1-10 mg/ml, much higher than mAb concentrations in batch tissue-culture media (Coligan and others). If unpurified antibodies are sufficient for the research application, low-molecular-weight cutoff filtration devices that rely on centrifugation or gas pressure can be used to increase mAb concentration. Alternatively, tissue-culture supernatants can be purified by passage over a protein A or protein G affinity column, and mAb can then be eluted from the column at concentrations suitable for most applications (Akerstrom and others 1985; Peterson and Peavey 1998). However, bovine or other immunoglobulin present in the culture medium will contaminate the monoclonal antibody preparation. Either concentration step can be performed in a day or less with minimal hands-on time.

In short, batch tissue-culture methods are technically relatively easy to perform, have relatively low startup costs, have a start-to-finish time (about 3 weeks) that is similar to that of the ascites method, and make it possible to produce quantities of mAb comparable with those produced by the mouse ascites method. The disadvantages of these methods are that large volumes of tissue-culture media must be processed, the mAb concentration achieved will be low (around a few micrograms per milliliter), and some mAb are denatured during concentration or purification (Lullau and others 1996). In fact, a random screen of mAb revealed that activity was decreased in 42% by one or another of the standard concentration or purification processes (Underwood and Bean 1985).

## Semipermeable-Membrane-Based Systems

As mentioned above, growth of hybridoma cells to higher densities in culture results in larger amounts of mAb that can be harvested from the media. The use of a barrier, either a hollow fiber or a membrane, with a low-molecular-weight cutoff (10,000-30,000 kD), has been implemented in several devices to permit cells to grow at high densities (Evans and Miller 1988; Falkenberg and others 1995; Jackson and others 1996). These devices are called semipermeable-membrane-based systems. The objective of these systems is to isolate the cells and mAb produced in a small chamber separated by a barrier from a larger compartment that contains the culture media. Culture can be supplemented with numerous factors that help optimize growth of the hybridoma (Jaspert and others 1995). Nutrient and cell waste products readily diffuse across the barrier and are at equilibrium with a large volume, but cells and mAb are retained in a smaller volume (1-15 ml in a typical membrane system or small hollow-fiber cartridge). Expended medium in the larger reservoir can be replaced without losing cells or mAb; similarly, cells and mAb can be harvested independently of the growth medium. This compartmentalization makes it possible to achieve mAb concentrations comparable with those in mouse ascites.

Two membrane-based systems are available: the mini-PERM® (Unisyn Technologies, Hopkinton, MA) and the CELLine® (Integra Bioscience, Ijamsville, MD). The Celline has the appearance of and is handled similarly to a standard T Flask but is separated into two chambers by a semi-permeable membrane and a gas-permeable membrane is on its underside next to the cell chamber. The mini-PERM has a similar design but is cylindrical and comes with a motor unit that functions to roll the fermentor continuously to allow gas and nutrient distribution. Startup for these units costs about $300-800 and requires a $CO_2$ incubator. The advantage of membrane-based systems is that high concentrations of mAb can be produced in relatively low volumes and fetal calf serum can be present in the media reservoir with only insignificant

crossover of bovine immunoglobulins into the cell chamber. A disadvantage is that the mAb may be contaminated with dead cell products. Technical difficulty is slightly more than that of the batch tissue-culture methods but should not present a problem for laboratories that are already doing tissue culture. The total mAb yield from a membrane system ranges from 10-160 mg according to Unisyn literature.

In the hollow-fiber bioreactor, medium is continuously pumped through a circuit that consists of a hollow-fiber cartridge, gas-permeable tubing that oxygenates the media, and a medium reservoir. The hollow-fiber cartridge is composed of multiple fibers that run through a chamber that contains hybridoma cells growing at high density. These fibers are semipermeable and serve a purpose similar to that of membrane-based systems. The hollow-fiber bioreactor is technically the most difficult of in vitro systems, partly because of the susceptibility of cells grown at extremely high density to environmental changes and toxic metabolic-byproduct buildup. The hollow-fiber bioreactor is designed to provide total yields of 500 mg mAb or more. Startup of this kind of system usually costs more than $1,200. For those reasons, hollow-fiber reactors are used only if large quantities of mAb are needed. The hollow-fiber reactor has been successfully used in many independent laboratories (Jackson and others 1996; Knazek and others 1972; Peterson and Peavey 1998). If investigators are unable to invest the time or material costs, several institutional core facilities and government and commercial contract laboratories produce mAb from a hybridoma. For example, commercial contract laboratories typically charge $11/mg to produce 1,000 mg with hollow-fiber reactors (Chandler, 1998).

Recently, several workshops, forums, and publications have discussed the use of the alternative methods to replace mice for production of mAb (Center for Alternatives to Animal Testing and OPRR/NIH 1997; Marx and others 1997; de Geus and Hendriksen, eds 1998). Their conclusions indicate that alternative methods can often provide an adequate means of generating most of the mAb needed by the research community. In vitro methods for producing mAb are appropriate in numerous situations, and it is the responsibility of the researcher to produce scientific justification for using the mouse ascites method. It is the responsibility of the IACUC to evaluate researchers' scientific justification and to approve or disapprove the use of mouse ascites methods.

# 3

## Scientific Needs for Mouse Ascites Production of mAb

Although in vitro techniques can be used for more than 90% of mAb production, it must be recognized that there are situations in which in vitro methods will be ineffective. Because hybridoma characteristics vary and mAb production needs are diverse, in vitro techniques are not suitable in all situations, and requiring their use might impede research, especially if large numbers of mAb have to be screened for efficacy or specificity in the treatment of disease. In some cases, in vitro production of mAb has not met the scientific aims of a project. The National Institutes of Health (NIH) has identified many of these in its response to the American Anti-Vivisection Society (AAVS), as shown in appendix C of the NIH response (Varmus 1997). The committee reviewed appendix C and offers the following explanation for the items listed in the appendix based on the collective experience of its own members.

**1. Some hybridoma cell lines do not adapt well to in vitro conditions.** Although in vitro methods produce mAb from over 90% of hybridomas, there is a finite and significant failure rate. The NIH response to the first AAVS petition suggested that the failure rate is 4% (Varmus 1997). That is consistent with the 3% failure rate observed by Dutch scientists (Hendriksen and others 1996). A recent European workshop discussed the effects of restrictions on the ascites method in various European countries; each country's laws provide for an exception based on the inability of a hybridoma to grow and produce mAb in vitro. Countries that maintain data banks on requests for exceptions continue to issue such exemptions (Marx and others 1997). Although in vitro conditions are used initially to select mAb-producing hybridomas, the initial culture contains many normal spleen cells that can act as feeder cells. In some instances, continued in vitro culture does not support hybridoma growth; in these instances, the rising concentration of antibody might adversely affect hybridoma growth or secretion. Transfectomas—myeloma lines transfected with mutated antibody sequences, which are often used to determine structure-function relationships—are notoriously low antibody producers. In general, the only way to obtain adequate amounts of antibody for experimental study from such lines is to use the ascites method.

**2. mAb from mouse ascitic fluids might be essential for experiments in which mAb are used in mice.** There are, in the committee members' experience, numerous examples to support this observation. The need for the mouse ascites method arises when small volumes of concentrated antibody are needed for a rapid screening in mice in order to select hybridomas with the desired bioreactivity. In vivo studies often examine the ability of an antibody to block a receptor-ligand interaction, to inhibit some aspect of microbial pathogenesis, or to induce the lysis or apoptosis of a particular cell type. To assess antibody function in these situations fully, high concentrations of mAb are often necessary. The mouse ascites method is also required when foreign (nonmouse) proteins could confound results. Halder and others (1998) have stated that mAb produced with an in vitro method should be equally suitable and that ascites contains other factors, such as cytokines, which could render the use of ascites fluids "scientifically wrong". Although mAb can be produced in vitro, the time required to adapt a hybridoma to media containing 1% or less FCS (which can take several weeks and does not include downstream purification) would severely retard progress directed at

selecting a hybridoma that is active in vivo. Because mAb concentration is high in ascitic fluid, only a small volume of the fluid needs to be injected into the mouse to test for effect. Although this small volume might contain small amounts of other factors, such as cytokines, no biologic effect due to these factors is noted. There are three reasons for this observation: the project is not affected by small amounts of contaminants, the contaminant is diluted in the body fluids, and the biologic half life of the contaminant is short (hours) relative to mAb half-life (days). Contaminating antibodies can be avoided by using mice with severe combined immunodeficiency disease syndrome. Semipermeable-membrane-based systems have been developed in which several hybridomas could be grown simultaneously. More experience is needed with this technique to determine whether it will meet the need for rapid screening of many hybridomas to find a cell line that produces a therapeutically effective mAb.

The mouse ascites method for mAb production might be the only choice when contamination of antibody with other mouse proteins does not interfere with the intended scientific goals (especially when the negative controls are also ascites-based). Similarly, the small-scale production of mAb for initial screening as potential diagnostic reagents when several different mAb need to be screened simultaneously would be hampered if the mouse ascites method could not be used.

Studies can be seriously confounded by purification procedures that alter the native structure of mAb and result in a loss of reactivity with antigen or loss of ability to bind components of the complement system. In many cases, denatured antibodies copurify with active antibodies and interfere with the in vivo function of the active antibodies. Denatured antibodies are more likely to be taken up by phagocytic cells or removed from the circulation by other clearance mechanisms; denaturation can lead to enhanced immunogenicity of the antibody preparation and thus result in a shortening of antibody retention time after in vivo administration. We recognize, however, that when hybridoma selection has been made and large-scale production of pure antibody is needed, in vitro cultures are preferable.

**3. Rat hybridoma cell lines do not generate ascites efficiently in rats, usually adapt poorly to in vitro conditions, but usually generate ascites in immunocompromised mice.** In some situations mAb to mouse epitopes are required, necessitating the use of another species (usually rat) for immunization. Although some rat hybridomas adapt to in vitro conditions, this often requires tedious manipulation of the culture. When small volumes of concentrated rat mAb are needed and the hybridoma does not easily adapt to culture conditions, the mouse ascites method using immunocompromised mice is required (Wolf 1998). However, if large-scale production (especially of purified antibody) is required, attempts should be made to adapt the rat hybridoma cells to in vitro growth. Other investigators have found that rat-mouse or hamster-mouse fusions yield heterohybridomas that are less stable than rat-rat hybridomas and for that reason have selected the mouse ascites method to obtain high-concentration mAb quickly for testing before extensive recloning procedures are used in preparation for large-scale in vitro production (Ohlin and Borrebaeck 1994).

**4. Downstream purification can lead to protein denaturation and decreased antibody activity.** When a pure product is not necessary for research goals but maintenance of high affinity and biologic activity is necessary, the mouse ascites method often offers the best option. There are many laboratory situations in which the concentration of antibody obtainable by current in vitro methods is not high enough for experimental studies and absolute purity of the antibody reagent is not essential. Other situations that require the mouse ascites method of producing mAb are related to the need for high binding affinities, the presence of complement-fixing activities, and mAb that are naturally glycosylated. Many of the in vitro-produced antibodies cannot be readily concentrated from culture supernatant, because standard procedures result in losses of antigen binding activity or other antigen-antibody features (Underwood and Bean 1985;

Lullau and others 1996), although such a concentration step might not be required with semipermeable-membrane-based systems. For example, immunoglobulin M (IgM) and immunoglobulin G3 (IgG3) antibodies often undergo denaturation during in vitro purification techniques, resulting in the loss of complement-binding activity (Roggenbuck and others 1994). Random antibodies of other isotypes exhibit similar quirks. OKT3 is an excellent example of a mAb with substantial therapeutic application; it cannot be adequately purified from culture fluids and retain full function, so it must be produced by the ascites method (Stein 1998).

Downstream purification is particularly difficult for immunoglobulin A (IgA) mAb, in which monomeric IgA (with poor antigen-binding abilities) must be separated from dimeric and polymeric IgA (Lullau and others 1996). This problem is alleviated by the mouse ascites method of IgA production.

**5. Serum-free or low-serum conditions cannot provide sufficient amounts of mAb for some purposes, such as the evaluation of new vaccines against infectious organisms.** Some cell lines can be readily adapted to low-serum or serum-free conditions, but others cannot (Stein 1998; Chandler 1998). More important, it has been noted (Chandler 1998) that some cell lines that appear to be maintained adequately in serum-free or low-serum media, as assessed by viability, but they make less than 10% as much antibody under these conditions compared to their being maintained in higher-serum media. If media with 1% serum result in 10% as much antibody production as media with 10% serum, nothing is gained in purity or yield that warrants the expense and time needed to adapt the cells to the modified culture conditions. The quality of serum can vary from batch to batch and manufacturer to manufacturer, and adapting a cell line to 1% of a particular batch of serum does not guarantee that the same cell line will grow comparably in 1% FCS obtained from another batch.

Those observations are related to manufacturing quality-assurance issues that are especially important to the Food and Drug Administration. Adapting hybridoma cell lines, initially approved for ascites-generated mAb, to serum-free conditions requires the hybridoma owner to demonstrate analytic comparability. Alterations in mAb binding affinity or other biologic functions could result in expenditure of millions of dollars (Maxim 1998).

Some investigators report difficulty in adapting hybridomas that produce IgM or IgA antibodies to serum-free conditions (Varmus 1997). The reason for emphasis on IgM and IgA mAb production is that IgM is a potent complement-fixing antibody generated early in the human immune response in many infectious diseases. IgA is associated with a variety of human diseases (such as Berger's IgA nephropathy, now one of the most common types of glomerulonephritis and Henoch-Schönlein vasculitis and glomerulitis), in none of which cases is the pathogenesis understood that could lead to effective clinical treatment. These observations indicate the need for production of IgA and IgM isotypes that are biologically active and exhibit high affinity. The committee recognizes that some success has been obtained in the in vitro production of IgA mAb; however, very few IgA-secreting hybridomas have been tested in vitro, and a high concentration of antibody generally depends on the addition of FCS to the culture medium (Stoll and others 1995; Stoll and others 1996), prolonged incubation, and critical attention to antibody concentration to avoid production of inactive IgA molecules (Stoll and others 1997). Although Roggenbuck and others (1994) produced milligram quantities of polyreactive IgM mAb with in vitro methods, 1% FCS in the media was required, and reactions between the IgM mAb and other components of the media led to impaired solubility of the antibody and poor reproducibility of purification results. Their two-step purification technique was capable of recovering only 30% of the immunoreactive IgM. Others have observed a loss of up to 99.9% of reactivity during purification of in vitro-produced IgM (Poncet and others 1988).

The mouse ascites method might be required when mAb to infectious agents or tumor antigens are being tested for toxicity and efficacy in mouse models of human diseases. Such testing is usually needed to establish a proof of principle (that is, showing that the mAb in fact is effective therapeutically) or for the preclinical studies required by federal agencies. In those situations, large numbers of mAb of different isotypes and specificities often have to be tested in dose escalation studies before a candidate is chosen for more detailed analysis, and this requires initial production of large amounts of mAb so that enough subjects can be challenged to establish a statistically significant result. Unexpected toxicities or questions of efficacy sometimes require additional batches; in these cases, the presence of nonmouse contaminating proteins and the immune responses to them can distort the results.

**6. Culture methods sometimes yield populations of IgG mAb that are glycosylated at positions different from those harvested from mouse ascites fluid, thereby influencing antigen-binding capacity and important biologic functions.** Leibiger and others (1995) describe in vitro production of IgG mAb that contained terminal mannose moieties at all glycosylation sites. In some cases, such glycosylation of mAb substantially affected mAb function; in other cases, it was irrelevant. The authors attribute this unusual property to the in vitro culture conditions and speculate that the increased in vivo clearance of such antibodies was due to binding to mannose receptors. It is claimed that culture conditions can be adjusted to achieve the desired terminal sialic acid during glycosylation (Marx and others 1997), but we are unaware of any publication demonstrating this phenomenon. Indeed, manipulating the expression of glycosylation enzymes to achieve the correct in vitro placement of sugars, sialic acids, and so on, on the IgG molecule is a formidable task, extremely expensive, and often not attainable with present technology (Wright and Morrison 1994, 1997, 1998; Matsuuchi and others 1981). In vitro glycosylation patterns might yield Mab with preferred pharmacokinetic characteristics for in vivo applications (Maiorella and others 1993; Monica and others 1993; Patel and others 1992).

**7. When hybridoma cells producing mAb are contaminated with infectious agents, such as yeasts or fungi, the cells often must be passed through mice.** Yeast, fungal, or mycoplasma contamination of in vitro cultures of hybridoma can be removed by passing cells from the culture through mice. Removal of the organisms cannot be accomplished by current antimicrobial drugs. Thus, one mouse may save a valuable hybridoma which would necessitate more mice to be used to make new hybridomas and, in addition, months of lost time and money. Stein (1998) has independently verified the success of this technique.

# 4

# Summary of Advantages and Disadvantages of In Vitro and In Vivo Methods

Advantages and disadvantages of in vitro and mouse ascites methods for producing mAb are highlighted in this section. It should be noted that it is likely that in vitro methods will meet more than 90% of the needs for mAb. Some of the advantages and disadvantages are concerned with animal-welfare issues. Others deal with the economics of producing mAb. As noted below under the section titled "In Vivo and In Vitro Methods for Commercial Production of mAb", in vitro methods can cost ½ to 6 times the mouse ascites method. Some of the factors that cause in vitro production to be expensive are labor and equipment costs that are usually due to poor hybridoma production of mAb in vitro. If the investigator must use several types of media or different equipment, as happens occasionally, labor costs rise and research is delayed (Moro and others 1994; Stoll and others 1996; Butler and Huzel 1995).

## Advantages of In Vitro Methods

- In vitro methods reduce the use of mice at the antibody-production stage (but can use mice as a source of feeder cells when antibody generation is under way).
- In vitro methods are usually the methods of choice for large-scale production by the pharmaceutical industry because of the ease of culture for production, compared with use of animals, and because of economic considerations.
- In vitro methods avoid the need to submit animal protocols to IACUCs.
- In vitro methods avoid or decrease the need for laboratory personnel experienced in animal handling.
- In vitro methods using semipermeable-membrane-based systems produce mAb in concentrations often as high as those found in ascitic fluid and are free of mouse ascitic fluid contaminants.

## Disadvantages of In Vitro Methods

It should be noted that each of the items below pertains to only a fraction (3- 5%) of hybridomas, but they indicate some of the difficulties associated with in vitro methods.

- Some hybridomas do not grow well in culture or are lost in culture.
- In vitro methods generally require the use of FCS, which limits some antibody uses. The use of in vitro methods for mAb production generally requires the use of FCS, which is a concern from the animal-welfare perspective.

- The loss of proper glycosylation of the antibody (in contrast with in vivo production) might make the antibody product unsuitable for in vivo experiments because of increased immunogenicity, reduced binding affinity, changes in biologic functions, or accelerated clearance in vivo.
- In general, batch-culture supernatants contain less mAb (typically 0.002-0.01) per milliliter of medium than the mouse ascites method. Note that semipermeable-membrane-based systems have been developed that can produce concentrations of mAb comparable with concentrations observed in mouse ascites fluid.
- In batch tissue-culture methods, mAb concentration tends to be low in the supernatant; this necessitates concentrating steps that can change antibody affinity, denature the antibody, and add time and expense. Adequate concentrations of mAb might be obtained in semipermeable-membrane-based systems.
- Most batches of mAb produced by membrane-based in vitro methods are contaminated with dead hybridoma cells and dead hybridoma-cell products, thus requiring early and expensive purification before study.
- MAb produced in vitro might yield poorer binding affinity than those obtained by the ascites method.
- In vitro culture methods are generally more expensive than the ascites method for small-scale or medium-scale production of mAb (Hendriksen and de Leeuw1998; Jackson and others 1996; Peterson Peavey 1998; Marx 1998; Lipman 1997).
- The number of mAb produced by in vitro methods is limited by the amount of equipment that it is practical to have available.
- The Food and Drug Administration (FDA) estimates that proving the equivalence of an mAb produced by in vitro methods to an mAb previously produced by the mouse ascites method would cost the sponsor $2-10 million (Stein 1998; Maxim 1998)

## Advantages of Mouse Ascites Method

The mouse ascites method usually produces very high mAb concentrations that often do not require further concentration procedures that can denature antibody and decrease effectiveness.

- The high concentration of the desired mAb in mouse ascites fluid avoids the effects of contaminants in in vitro batch-culture fluid when comparable quantities of mAb are used.
- The mouse ascites method avoids the need to teach the antibody producer tissue-culture methods.

## Disadvantages of Mouse Ascites Methods

The mouse ascites method involves the continued use of mice requiring daily observation.

- MAb produced by in vivo methods can contain various mouse proteins and other contaminants that might require purification.
- The mouse ascites method can be expensive if immunodeficient mice in a barrier facility must be used.
- In vivo methods can cause significant pain or distress in mice.

# 5

# Large-Scale Production of Monoclonal Antibodies

About 25,000 mAb are listed in *Linscott's Directory* (1998-9). Most are produced in small quantities (less than 0.1 g) for bench-related research purposes (de Geus and Hendriksen 1998). However, some have become commercially successful and so require a scale of production different from that usually experienced in research facilities. Commercial interests consider production scales of 0.1-10 g as small, 10-100 g as medium, and over 100 g as large. Commercial-scale production is generally performed to produce mAb for three purposes: diagnosis, therapy, and research on and development of new therapeutic agents.

## Monoclonal Antibody Production for Diagnostic and Therapeutic Purposes

The amount of mAb needed and the importance of such factors as cost, turnaround time, and regulatory compliance depends on the purpose. The very competitive diagnostic industry is concerned with cost, turnaround time, and regulatory requirements. The diagnostic-industry scale of mAb production is usually small to medium and seldom large. The therapeutic industry is considerably less concerned than the diagnostic industry with cost and turn-around time, and its production scale is medium to large. The therapeutic industry is highly regulated and sensitive to regulatory structure and to the very high regulatory cost of any procedural change. The biotechnology industry that develops therapeutic agents produces mAb on a small to medium scale; it is less concerned with cost of production than the diagnostic industry and much more concerned than the therapeutic industry, but turnaround time is very important. Therefore, it requires rapid turnaround to increase the chance of being first in the marketplace with a product that will probably have a short life span.

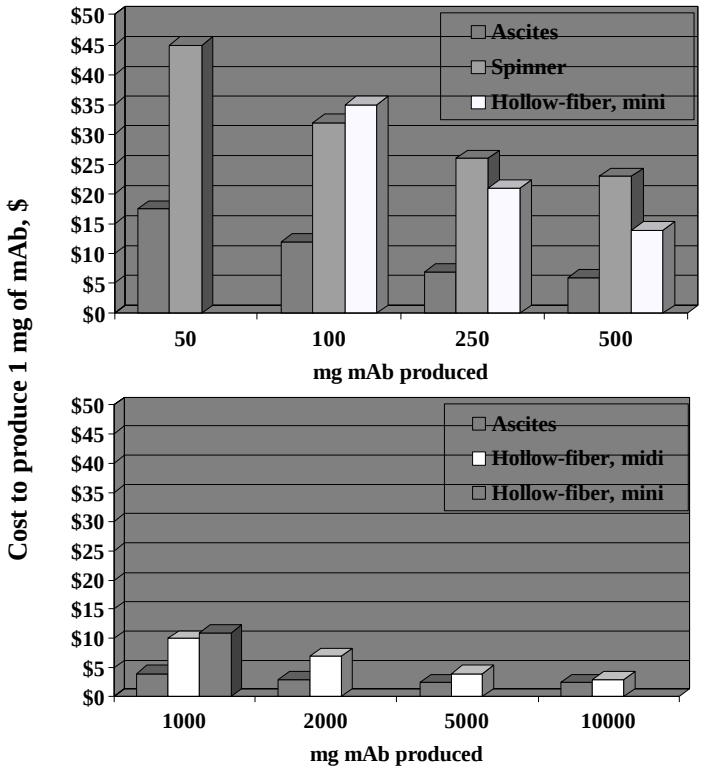
Commercial mAb production requires more than the culturing of large batches of cells or their injection into large numbers of mice. It requires considerable preproduction effort to ensure that the cell line is stable, can produce commercially appropriate quantities of a stable antibody, and can produce an uncontaminated product. Commercial production also involves building a high-quality facility for in vivo and in vitro production and for processing of the antibody. There is a need for quality control and quality assurance departments to meet the requirements of good manufacturing practices that are required for commercial products. Product-lot testing is necessary to ensure product reproducibility. Production-process verification and documentation are necessary to protect the consumer and are required by FDA in its regulatory "Points to Consider in the Manufacture and Testing of mAb Products for Human Use" (FDA 1997).

# In Vivo and In Vitro Methods for Commercial Production of mAb

Commercial mAb production uses both the mouse ascites method and in vitro methods. Cost is usually the major consideration in determining the method except for marketed therapeutic products.

When all fully-loaded production and pre-production and post-production costs are considered for a commercially viable line, economics usually favor in vivo production. However, as the amount of mAb increases, existing in vitro production technology can become more economical because high, fixed optimization costs (costs associated with selecting a subclone with the best growth and mAb production characteristics and grow in low-serum or serum-free conditions) associated with in vitro production are spread over a larger production amount, making cost per gram competitive with in vivo production, which has a higher and more variable cost structure (figure 2). When production costs are compared for small-scale production, in vitro methods are ½ to 6 times higher, depending on the cell line (Hendriksen and de Leeuw 1998; Jackson and others 1996; Peterson and Peavey 1998; Marx 1998; Lipman 1997). However, these costs might not include all factors, such as animal housing costs and technician time. In large-scale production runs, in vitro systems are economically competitive and are usually selected because they reduce animal use and decrease the presence of contaminating foreign antigens if serum-free media can be used. When the time of mAb production is critical and small amounts are required, in vivo production is selected because it takes only 6 weeks. For in vitro systems, time requirements vary considerably. Production time depends on the amount of time required to optimize the hybridoma to the system being used and on the quantity of mAb needed. Commercial-quantity in vitro production of mAb requires more time than in vivo production because of the lengthy optimization process and the increased time for producing a given quantity of mAb (Butler and Huzel 1995; Moro and others 1994; Stoll and others 1996).

The therapeutic industry uses primarily serum-free in vitro technology because of a concern for treatment-related allergic responses caused by repeated foreign-antigen exposure. Immune responses are of concern here because mice are the source of the cell lines used in most mAb production methods. The human immune system tends to reject mouse-derived antibodies, which can lead to allergies or decreased effectiveness of injected mAb. Therefore, techniques that replace most of the mouse's antibody genes with human DNA have been developed. Humanizing antibodies and producing antibody in SCID mice or in an in vitro system have alleviated this problem (Boyd and James 1989; Reuveny and Lazar 1989). In the therapeutic industry, early work to determine whether the mAb will have the desired effect is usually done with in vivo-derived mAb because turnaround time is shorter and production costs are lower. During the same period, the company will develop its final in vitro manufacturing process. When in vitro optimization and product development are completed, the company will develop its final product-effectiveness information and file a final proof-of-process document with FDA.




In the diagnostic industry, keen competition leads to overriding cost considerations, whereas the presence of foreign antigens is less important. As a result, in vivo-derived products are commonly used. In vivo procedures are optimized to increase productivity by reducing hybridoma invasiveness and increasing mAb secretion (Harlow and Lane 1988, p 274-275). This optimization can result in a reduction in animal use by a factor of 2-10 that greatly reduces production costs. Ascites production costs are important because ascites production has a high-variable cost component. However, the research industry—that is, industry concerned with research on and development of new therapeutic agents—is most concerned with production time and binding affinity of the mAb. Therefore, whether in vivo or in vitro methods are used depends on the purpose of the project and on the quality of mAb produced by the cell line in that system. For very-small-scale production, ascites production is often used because it is a much more forgiving procedure than in vitro production and can be done without optimizing cell lines in an in vitro culture.

## In Vivo Production

Biologic behavior of a hybridoma cell line is very important in determining whether in vitro culture will be successful or the ascites method must be used. Biologic behavior also affects the concentration of mAb produced and, for the mouse ascites method, the quantity of ascites produced. Researchers and production facility personnel can optimize production results of both in vitro and in vivo methods by adjusting production variables and selecting appropriate clones. Experience has shown that when cost is

important to a client, system optimization often favors even more the economics of in vivo production for the client's cell line. In vivo optimization is necessary only for cell lines that will go into continuous production, but in vitro optimization is necessary for all cell lines to produce acceptable growth and mAb production (Capiaumont and others 1995; Chua and others 1994; Shacter 1989; Trampler and others 1994). It is an important expense factor for in vitro production because it requires much labor by highly paid, highly trained employees. The optimization process also requires large quantities of disposable supplies, an important factor in the increased costs associated with in vitro production.

The variables affecting in vivo production and optimization (Hendriksen and de Leeuw 1998; Chandler 1987) include age, sex, strain of the host, size of the hybridoma-cell inoculum, number of taps, and type and volume of primer. Those variables can be manipulated to affect ascites yields and mAb concentration. For instance, low-ascites-producing subclones usually form only a few large tumors in the peritoneal cavity, whereas high-producing subclones form numerous colonies of small tumors that grow extensively throughout the mesentery (Cancro and Potter 1976). Therefore, clones that form less-invasive small soft tumors should be selected. Sequential tapping provides the highest yields and greatest mAb concentration from a group of mice (Chandler 1987). Except for very invasive cell lines that allow for only one needle tap, sequential tapping usually reduces the number of mice needed per gram of mAb by a factor of 2-3. The number of needle taps allowed should therefore be based on the clinical condition of the mice, and the maximum, in general, should be three taps (Jackson and others 1999b).

Optimal in vivo production requires reduction of the invasive nature of a cell line so that all of the mice survive completion of a production run. Selecting appropriate clones and altering hybridoma cell concentration injected into the peritoneal cavity of the mice are two ways to optimize production. The volume and concentration of mAb produced depend on the clone selected, and this makes systematic comparisons difficult. Therefore, the best way to achieve maximal in vivo yields is to screen clones in mice and to use the clone that provides the best yield. Cell growth conditions are optimal in vivo, so almost all cell lines will produce antibody, even when they are not optimized (Hendriksen and de Leeuw 1998). That is why injecting into mice usually saves cell lines that are difficult to grow in vitro.

Ascites production is a simple procedure, once proper technique is learned. Daily observation of the mice requires skilled observers to determine the optimal time for tapping the fluid and to determine when the mouse should be euthanized. It is quicker, is more forgiving, is more economical for small-scale and medium-scale production, produces a higher concentration of mAb, and is easier to scale up in production (Chandler 1987). For most cell lines, purification costs are the same as in vitro methods. The major problems associated with in vivo production are the use of animals, the possibility that the animal could be harmed if technicians are not properly trained and procedures are not followed properly, the presence of endogenous mouse immunoglobulin contamination except when immunodeficient mice are used (Ware and others 1985), and the possibility of contamination with murine pathogens, which requires the use of high-quality animals and a high-quality program for health assurance. High-speed centrifugation of the ascitic fluid brings pristane to the top, where it can be removed easily.

## In Vitro Production

Variables affecting in vitro production and optimization are presented in several papers (de Geus and Hendriksen 1998; CAAT 1997; Jackson and others 1996; Beck and others 1987; Seaver 1987; Reuveny and others 1985). Numerous in vitro commercial systems meet the different needs and requirements of users. These systems are of two types: single-compartment systems that allow only low-density cell culture and double-compartment systems that allow high-density cell culture, which results in increased mAb

concentration. For very-small-scale production (less than 10 g), the simple low-density cell-culture systems—such as culture flasks, roller bottles, gas permeable bags, and hollow-fiber bioreactors—are used. For small-scale and medium-scale production (10-100 g), double-compartment, high-density cell-culture systems, such as hollow-fiber systems, are used, as well as spinner flasks and roller bottles. High-scale production (over 100 g) is performed in large capital-intensive systems, such as homogeneous suspension culture in deep-tank stirred fermenters, perfusion-tank systems, airlift reactors, and continuous-culture systems.

An antigen-free product can be obtained by adapting the cell line to low-serum or serum-free media, with generally minor inhibitory effects on the cell line (Kurkela and others 1993). Benefits of in vitro production are the absence of live-animal use, although some products in the culture media come from animals; the possibility of low-serum or serum-free media production (Klerx and others 1988); and the absence of host-contributed immunoglobulin or antigens. As the cost of disposable materials decreases further and technologic changes increase production efficiency and decrease equipment costs, the cost of in vitro production should decrease further, so it should become the preferred method of commercial production.

Problems associated with in vitro systems today are as follows (note that the items in the following list do not necessarily apply to all the numerous in vitro systems mentioned above): material, labor, and equipment costs are higher than for the in vivo method (Jackson and others 1996; Peterson and Peavey 1998; Brodeur and Tsang 1986; Lipman 1997); characteristics of the hybridoma are more critical than in vivo; about 3-5% of all clones cannot be maintained in existing in vitro systems (de Geus and Hendriksen 1998; Hendriksen and de Leeuw 1998); the great potential for microbial contamination, poor growth, and mechanical failure of the system or supporting systems requires constant monitoring and attention every day (Lebherz 1987); production of large quantities of mAb is slower because of low mAb concentration, compared with the ascites method; the increased employee technical capabilities and educational background required by increased training time and system manipulations increase labor expense; the design of downstream processing is emphasized because large volumes of media are required to obtain large quantities of mAb and to ensure product economy and purity (Stang and others 1998); and residual endotoxin, residual DNA from cell death, and bovine IgG contamination with cell lines that require some serum all complicate the process.

It is difficult for a user to choose a particular in vitro system on the basis of manufacturers' claims because of how costs are calculated and because the amount of antibody secreted by different hybridoma lines in identical medium and culture conditions can vary by a factor of as much as 100 (Seaver 1987). Therefore, it is important to compare the productivity of several systems by using several cell lines and to include optimization costs of each system in calculating the overall cost per gram. Numerous commercial-volume systems are available, and none is inexpensive. In the near future, as new technologies (recombinant, transgenic, and so on) are developed and in vitro systems become more economical and efficient, most commercial mAb production will undoubtedly use in vitro systems.

One of the most common causes of failure of in vitro methods is poor adherence to basic tissue-culture techniques, such as sterilization of cultureware, equipment, and media and humidity and temperature control in the system. In large-scale and medium-scale production, it is important to have tight procedural and environmental controls to minimize losses due to system microbial contamination. To help avoid a major economic effect of such losses in commercial production, expensive facilities and tightly controlled procedures are implemented, all of which add to the high fixed cost of in vitro mAb production.

# Regulatory Requirements

Regulatory requirements in the United States for in vitro and in vivo manufacturing of mAb have not changed considerably in the last two revisions of the "Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use" (FDA 1997). The required differences for the two methods of production focus on the testing and monitoring of mice used for ascites and the testing of the final product for the 16 adventitious viruses that can come from the animals used in production. Specific guidelines in the "Points to Consider" (FDA 1997) recommend an intense health-monitoring program for the animals, including complete, routine health monitoring of the animal stock. Monitoring also covers mouse antibody production, mycoplasma testing, and complete physiologic and physical examination of the animals. There should also be a surveillance system that uses animal sentinels for health and serologic screening. These programs need to be continually updated as other adventitious viruses are identified by the FDA.

Protocols for ascites production require specifics on the animals used for manufacturing—such information as sex, age, and species. There are requirements for volume of pristane, cell concentration of the inoculum, and timing for priming, inoculation, and harvesting of ascites. Other requirements are strictly related to the well-being of the animals, such as bedding, feeding schedules, and general housing conditions of the facility involved in manufacturing.

FDA has approved 13 mAb for clinical use, two of which must be produced by the ascites method. Most new-drug applications to FDA are for mAb that are produced in vitro. It is likely that large-scale manufacturing of mAb will use in vitro methods as systems and technology are optimized to reduce the final cost per unit. FDA is encouraging using in vitro methods for producing mAb. In Europe, Germany, the Netherlands, Sweden, Switzerland, and the UK have restricted or prohibited the use of mice for production of ascites and more countries will probably join them.

More important than regulatory differences between the two modes of manufacturing of mAb are requirements that must be met when the mode of manufacturing is changed during product development before licensing. Changes in manufacturing often occur in clinical development of a product. The FDA requires a plan for demonstrating that the products made in different ways are similar. The requirement also applies if there is a scaleup without substantial changes in the manufacturing process during or after completion of phase 3 trials. As presented by the FDA Center for Biologics Evaluation and Research Division (Stein 1998), it could take 3-8 years to obtain data needed to approve a product formerly produced by the ascites method and later produced in vitro. There have been cases in which the two methods of production have yielded similar antibodies that are not comparable. As stated in Stein's presentation, the earlier in the development of the process the changes are made, the better the success of the product. She stated that investigators should adapt the hybridoma to in vitro conditions early when developing mAb for clinical applications.

# 6

# Animal-Welfare Issues Related to the Ascites Method for Producing Monoclonal Antibodies

## Availability of Data

Few published data are available on animal-welfare issues related to the ascites method for producing mAb. Most references simply state that animal-welfare problems are related to this method but do not provide supporting data. For instance, a report on mAb production published by the European Centre for the Validation of Alternative Methods (ECVAM) (Marx and others 1997) states that "the main disadvantage of the ascites method is that it is extremely painful for the animals used, due to the following: a) the injection of primer; b) the resulting peritonitis caused by the primer; c) abdominal tension; d) the invasive tumors which result." Such statements are generally not supported in the literature. Most of the cited papers contain scant data and tend to be circular and to cite each other or to cite a review of the earlier literature by McGuill and Rowan (1989) or a short paper by Amyx (1987) that contains only opinion and no data.

Almost 10 years ago, McGuill and Rowan (1989) concluded their article on the refinement of mAb production and animal well-being with a statement that “research is necessary to provide a better understanding of the stresses endured by animals under protocols designed to maximize production of mAb. The effects of large intraperitoneal tumor masses, of frequent and large-volume paracentesis, and of pristane priming must be studied to provide a scientific basis for developing procedures that minimize animal distress and suffering.” Very little has been done to develop such data since then.

## Animal-Welfare Issues Related to Ascites Method

Some cell lines, especially unoptimized or aggressive cell lines, produce signs in mice that can be interpreted as indicating pain, but it is not clear from the literature that ascites production itself is necessarily associated with pain. Gebhart (personal communication) evaluated pain in ascites production and found that possible pain associated with intraperitoneal administration of pristane in mice is no more significant than that observed with the administration of intraperitoneal saline.

In humans, intraperitoneal administration of drugs is generally perceived as mildly uncomfortable but not distressful or painful. In clinical experience in humans, abdominal tumors are rarely painful unless they invade the intestinal organs; and ascites fluid accumulation is not painful, although a large accumulation can cause distress and discomfort because it interferes with respiration. When ascites fluid accumulation does become uncomfortable, tapping of the fluid is perceived as a welcome relief—not painful. Therefore, in mice it is probably more important to perform needle taps frequently to avoid ascites fluid accumulation sufficient to cause distress.

However, some cell lines produce clinical signs in mice indicating distress, including anorexia, rapid breathing, hunched posture, hypothermia, and decreased activity. Those signs can be observed in any sick mouse, regardless of the cause, and are usually seen with poor mAb-producing, more-aggressive cell lines. (It is important to note that this depends on the cell line.) Also, cell lines can produce various pathologic changes in mice that vary with the cell line and may be associated with pain or distress (Jackson and others 1999a).

## Methods for Measuring Pain or Distress in Laboratory Rodents

Clearly, data are required if one is to determine objectively whether the ascites method for producing mAb causes pain or distress in mice, but it is unclear what such data would have to be. There is no reliable scientific measurement of pain or distress in rodents. Although that statement is true with regard to our ability to know with certainty the levels of pain or distress that an animal is experiencing, there is some information on what might or might not cause pain or distress (NRC 1992). Current guidelines suggest that if a procedure might be expected to cause pain in humans, we should expect it to cause pain in animals. Very little other guidance is given, and, as stated above, there are no broadly accepted techniques for assessing the degrees of pain or distress in animals. In most instances, the standard approach is to assume that an animal is in distress if it is experiencing significant weight loss (such as a weight loss of greater than 15 or 20%) or if it stops taking food and water. Those measures might be useful as indicators of severe distress, but there is a shortage of data on weight gain and loss in rodents that are undergoing different procedures. In any case, weight loss is not useful for assessing mice with ascitic fluid, in that they all gain weight regardless of degree of distress.

More subtle behavioral indices are needed to provide an indication whether the animals are experiencing mild to moderate pain or distress. Such subtle methods might include observing an animal under red light. Rodents are generally more active under red light, and declines in activity will be easier to identify than when the animals are relatively quiescent under bright light.

Another possibility might be the use of a formal behavioral index, such as the disturbance index of Barclay and others (1988), which permits measurement of relatively subtle changes in behavior that appear to be related to an animal's level of disturbance. In developing the disturbance index, the investigators noted that departures from normal behavior could be produced by relatively minor procedures. They assumed that the extent of behavioral change is related directly to the severity of pain or distress. They found that the amount of exploratory behavior of a rat or a mouse placed in a strange cage was very predictable. Increases or decreases in an animal’s exploratory behavior during a period of 1½ -3 hours after a test treatment could be converted into a disturbance index that quantified the departure from normal exploratory behavior. In one of the tests conducted on the animals, the investigators found that intraperitoneal injections of mineral oil resulted in a statistically significant increase in the disturbance index after 6 days (Barclay and others 1988).

No further work on the disturbance index has been done, and the index has not been correlated with other signs of pain or distress, such as physiologic or clinical changes. Therefore, it remains unclear whether the information gathered with the index can provide a means of classifying mild, moderate, and severe pain or distress. In recent years, a few investigators, such as David Morton (University of Birmingham, UK) have begun to explore other approaches to assessing the distress experienced by laboratory animals. In 1985, Morton and Griffith published a paper outlining how one might use behavioral and clinical signs to assess animal distress. The ideas in the paper have been applied in a number of cases and can be reduced to five types of observations:

At-a-distance-observations:
Behavior
Appearance
Observations requiring handling:
Body weight, temperature, and so on
Clinical signs
Provoked behavior

Morton has promoted the use of score sheets individualized for specific experimental procedures or projects. Preliminary data from those score sheets and sheets developed by other investigators (reported at the recent Conference on Humane Endpoints in Zeist, the Netherlands, November 23-25, 1998) are beginning to provide stronger evidence of the experience of pain or distress in laboratory rodents and rabbits. The sheets provide a significant amount of data that have proved to be of benefit to both animal care staff and investigators.

In summary, biomedical scientists have not recorded much evidence of pain or distress in laboratory rodents undergoing the ascites method of mAb production. That could be because they have not looked carefully or because sensitive techniques have not been developed to measure signs that might indicate the presence of pain or distress. The committee concludes that the possibility of distress in the mouse ascites method, particularly around the time of removing ascitic fluid (Jackson and others 1999; Detolla 1998), should limit the use of the mouse ascites method as a routine method for producing mAb.

## Priming

Priming of the peritoneal cavity is often accomplished through an intraperitoneal injection of pristane. Pristane is believed to act by inducing granulomatous reactions and interfering with peritoneal fluid drainage (Amyx 1987). Amyx (1987) suggested that large volumes of pristane injected intraperitoneally into mice are associated with weight loss, a hunched appearance, and lack of activity and that these clinical signs can be minimized by lowering the dose of pristane while achieving the desired effect. In spoken comments at a colloquium on recognition and alleviation of animal pain or distress (Amyx 1987), Amyx reported that the usual dose of 0.5 ml of pristane produces strong distress symptoms and that a smaller dose of 0.2 ml produces milder symptoms.

Research on the optimal amount of pristane that would produce the greatest yields of ascites showed no statistically significant differences in the volumes produced in mice given preinjections of 0.1, 0.2, and 0.5 ml of pristane (Brodeur and others 1984; Hoogenraad and Wraight 1986). Hoogenraad and Wraight (1986) found that mice given 0.5 ml of pristane yielded an average of 9.72 ml of ascites fluid, and mice given 0.1 ml yielded an average of 9.65 ml. Alternatives to pristane have been evaluated. Gillette (1987) reported that of seven agents studied as primers, only incomplete Freund's adjuvant (IFA) produced results comparable or superior to those of pristane (it is suggested in some of the literature that IFA substantially reduces the 2-week waiting period between priming and hybridoma-cell injection). Although stress caused by these priming agents was not evaluated, mice primed with IFA survived for more taps once ascitic fluid was produced than did mice primed with pristane.

In addition to the clinical signs described by Amyx (1987) and the Netherlands Code of Practice for the Production of Monoclonal Antibodies (Anon. 1989), two papers by Jackson and others (1999a) address the clinical and pathologic features of mAb production in mouse ascites. These papers also provide some data on the effects (or lack of apparent effects) of pristane priming on an animal. Reports from Kuijpers in

the Netherlands (Anon 1989) state that mice treated with pristane showed various stages of peritonitis 10-20 days after treatment, with various amounts of abdominal fluid. There appears to be little dispute that pristane causes inflammation and blocks lymph flow in the peritoneal cavity, but there is some dispute about whether it causes animal distress. Pristane does induce a lupus-like syndrome in mice 4-8 weeks after intraperitoneal injection, which affects animal welfare (Satoh and Reeves 1994; Richards and others 1998).

Jackson and others (1999a) note that peritonitis is known to cause abdominal pain in animals and in humans; they therefore find it reasonable to speculate that the induction of granulomatous peritonitis after intraperitoneal injection of pristane might cause pain or distress in mice. However, Jackson and others (1999a) fail to provide any clinical data to support the speculation. Clinical abnormalities were not observed in any of their test animals during the 2-week period after an injection of 0.5 ml of pristane. In unpublished observations from a blinded study, Gebhart observed intraperitoneal injections of 0.2 ml of pristane or 0.2 ml of saline into 12-week-old male BALB/c mice (Charles River) and was unable to distinguish by observation mice receiving pristane from those receiving saline. No writhing was produced by either pristane or saline, and there was no discernable nociceptive effect of either injection other than what would be attributed to penetration of the peritoneal cavity by a 25-gauge needle. As the data from the disturbance index paper (Barclay and others 1988) suggest, however, discomfort can develop slowly and not be apparent for several days (it took 6 days for mineral oil to produce an observable change in the disturbance index). When observed a week after injection of either pristane or saline, BALB/c mice appeared normal and healthy. Their coats were clean and smooth, and activity was normal (mice were housed two per cage). In previous experience (Ness and Gebhart 1990; Ness and Gebhart 1988), abdominal visceral discomfort in rodents is typically associated with a guarded posture and reduced activity. A disturbance index was not used, but it was not possible to distinguish mice that received pristane and mice that received saline a week after the injections. Perhaps a more provocative test or index would reveal some differences, but no differences were apparent in this mouse strain. In this regard, there are likely to be differences in effect and response between different strains of mice. The BALB/c mice used here are relatively docile and easy to handle. In future studies attempting to assess disturbances or distress in mice, the strain should be considered. Those observations suggest that injection of pristane does not produce pain or cause distress.

## Ascites

After priming, a hybridoma cell-suspension is injected into the peritoneal cavity of the research animal. This leads to the development of a tumor, to the accumulation of ascitic fluid, and to abdominal distension. Factors that affect animal welfare during this step of mAb production include the number of hybridoma cells injected, the type of tumor that develops (disseminated tumor or solid, invasive mass), and the effects of the ascites itself.

Brodeur and others (1984) reported that survival times of mice reflect the number of hybridoma cells injected intraperitoneally. Mice given $2.5 \times 10^7$ cells survived an average of 8.5 days; mice given $3.2 \times 10^6$ cells survived an average 12.7 days. In this experiment, ascites formed sooner with larger cell inocula, but larger cell inocula shortened survival times. Current standard protocols usually call for the injection of $10^6$ cells, which leads to longer survival times. In the study by Jackson and others (1999a), most of the animals survived 17-20 days.

As indicated above, mild ascites in humans causes discomfort and distress but is generally painless (Burnside and McGlynn 1987). However, patients with massive ascites that causes a tense abdomen are frequently unable to walk, and they experience abdominal discomfort, indigestion, and heartburn (Mauch

and Ultmann 1985; Pockros and Reynolds, 1986). Mauch and Ultmann (1985) reported that elevation of the diaphragm due to ascites is associated with dyspnea, orthopnea, or tachypnea. It therefore seems reasonable to assume that mice with large accumulations of ascitic fluid experience discomfort and distress. Clinical and pathologic data of Jackson and others (1999a) and reports by Kuijpers in the Netherlands (Anon 1989) support that assumption.

Kuijpers reported clinical signs in animals that include weakness, dehydration, anemia, and apathy. Kuijpers reported that without intervention animals die within 2-4 weeks from cachexia, dehydration, and complications associated with the tumor. Kuijpers also stated that “the abdominal wall is stretched in a relatively short time, which causes pain." Pathologic findings reported by Kuijpers include fluid in the thorax and abdominal cavity, adhesions of the abdominal organs, empty digestive tract, expanded spleen, and pale kidneys and liver. In addition, tumors filled the abdominal cavity, particularly near the lymph nodes in the mesentery. It should be stressed that these mice had particularly aggressive hybridomas, which invaded the peritoneal surfaces and intestinal walls.

Jackson and others (1999a) monitored animals daily and reported clinical signs that included roughened hair coat, hunched posture, progressive increase in abdominal distension, decrease in activity, palpable abdominal masses, thin appearance, and dehydration (see table 1). The onset of those signs was related to the accumulation of ascitic fluid, which was determined by visible abdominal distension and increasing body weight, and the severity of the abnormalities increased with time. No such abnormalities were observed in the control mice. Necropsy revealed such pathologic findings as gross lesions, hemoperitoneum, abdominal adhesions, and disseminated intra-abdominal tumor or solid tumor masses, which might interfere with normal organ function. Although there were statistically significant differences among the hybridoma cell lines in the incidence, severity, and rate of progression of the abnormalities, the general progression was similar. The different rates of ascites development had a statistically significant effect on survival and, ultimately, on the amount of antibody produced.

# Harvesting Ascitic Fluid

Timing of harvesting, frequency of harvesting, and the effect of fluid harvesting on the animal all appear to be critical for the welfare of the animal. Brodeur and others (1984) reported that draining ascitic fluid as soon as it accumulates is necessary to reduce mortality. The UKCCCR guidelines (Workman and others 1998) recommend that ascitic-fluid volumes should not exceed 20% of the baseline body weight before abdominal paracentesis. Clinical data from Jackson and others (1999a) provide insight into the animal-welfare aspects of harvesting ascites. In their study, five different hybridomas were each injected into 20 mice. Abdominal paracentesis was performed when moderate abdominal distension was visible (the mean weight gain for all test mice was 17.4%). Each mouse was tapped a maximum of three times. However, any animal with persistent, severe clinical abnormalities was euthanized. During the 30 minutes immediately after the tap, the following clinical abnormalities were noted in some animals in each group: roughened hair coat, hunched posture, decreased activity, tachypnea, dyspnea, and pallor most evident on the muzzle and ears (see table 1). Generally, those signs were transient and mild to moderate. However, in 19 of the animals from four of the five groups, after the first or second tap, symptoms were persistent and severe; five died, and the remaining 14 were euthanized (see table 1). Survival rates of the mice for tap 1 were 90-100%; for tap 2, 85-100%; and for tap 3, 35-100%. No clinical abnormalities were observed in control animals after a sham paracentesis. Jackson and others (1999a) conclude that the abnormal clinical signs observed after paracentesis were compatible with circulatory shock.

Some IACUCs have established policies (see table 2) that require tapping of abdominal fluid to be performed under anesthesia and to be followed by injection of 1-2 ml of warmed saline to minimize posttapping hypovolemic shock. It is not clear that anesthesia use does reduce overall distress (for example, anesthetic administration causes handling stress), and it is possible that hypovolemic reactions can be minimized if the fluid is drawn off slowly (as is recommended for removing ascitic fluid from human patients—generally, up to 5% of body weight over 4 hours). However, if more than one tapping is to be permitted, the clinical signs observed by Jackson and others (1999a) after tapping indicate that some level of fluid replacement might alleviate some distress.

There was some discussion at the ILAR working-group meeting of whether it was preferable for animal welfare to conduct fewer taps, which would mean that more mice would be needed to produce the required amount of antibody, or whether one should perform several taps and reduce mouse use by 50-75%. It is evident from the Jackson and others (1999a) paper that the clinical signs and evidence of animal discomfort and distress increase sharply once ascites has developed to the point where fluid can be harvested. Thus, one can reduce animal use; but if distress is high and increasing steadily in the 4-6 days from the first to the third and terminal tapping, then the extra taps are likely to be accompanied by substantial distress. If the animals are tapped just once under terminal anesthesia, the extent of distress will be minimized, but more animals will be put through the procedure. The overriding criterion should be animal distress. Therefore, the number of taps should be limited and varied according to animal welfare and characteristics of the hybridoma being used. Some hybridomas seem to cause little distress (Jackson 1999a), and multiple taps could be allowed. Determining the number of taps depends on careful monitoring of the animal; if signs of distress appear, a terminal tap should be performed.

A number of IACUCs have adopted a 0.2 ml volume limit on the pristane injection of based on recommendations made by McGuill and Rowan (1989) (table 2). Members of the committee did not object to this as a guideline, but several reported that, in some strains of mice, 0.2 ml might not be sufficient to produce ascites and that as much as 0.5 ml might be required. There are some questions about the suitability

of IFA as a substitute for pristane, and the committee recommends that IACUCs determine the reaction of animals to IFA before permitting its routine use. Most IACUCs with public policies recommend that animals be monitored daily after inoculation of the hybridoma cells. The committee believes that this is an appropriate standard with the proviso that IACUCs require that investigators pay special attention to animals after ascites develops and abdominal distension occurs.

Most IACUCs limit the number of taps to two or three (with the final tap being terminal). The number of taps is a critical issue for animal welfare, and the investigator should pay particular attention to the condition of the animals. If signs of distress, such as hunched posture and roughened coat, are observed, the animals should be euthanized immediately; the committee believes that the primary task of the IACUC should be to limit animal pain and distress rather than animal numbers. There are too few data to develop a formal recommendation on either the use of anesthesia during the tap or the administration of saline replacement after the tap. The committee urges IACUCs to collect data on their experience with mAb production and animal welfare and make them available to others at meetings and through appropriate publications and discussion groups.

## Feeder Cell Harvesting and Serum Supplements for In vitro Hybridoma Culture

The use of fetal bovine serum is accompanied by animal-welfare costs. The harvesting of such fetal serum has raised concerns about the welfare of the animals from which it is obtained, but there are few descriptions in the published literature of such operations. It has been suggested that hybridoma cell cultures require feeder cells harvested from mice peritoneal cavities or mouse embryoblast serum (Harlow and Lane 1988, p 220-221). The prevalence of the use of feeder cells is unknown, and the extent to which serum and feeder-cell supplements are required was disputed during the 1-day workshop held by the committee.

## Summary of Animal Welfare Issues

Data from the study by Jackson and others (1999a) suggest that the clinical condition associated with the production of ascites generally worsens in association with progressive tumor growth, continuing ascites production, and repeated abdominal paracentesis. Clearly, there is a lack of data on animal-welfare issues related to mAb production by the ascites method. The article by Jackson and others (1999a) is the only published study in the peer-reviewed literature of the last decade that provides clinical data on how this procedure affects the experimental animal. Lack of conclusive data is evident when one looks at university policies for this procedure (see table 2). There are no best-practice guidelines regarding animal welfare; variations are apparent from university to university for the procedures of priming, number of taps, monitoring of animals, anesthesia, and use of replacement fluid.

An additional animal-welfare concern, although not directly related to the ascites method, is the use of fetal bovine serum during in vitro mAb production. It should not be assumed that in vitro procedures are inherently more humane; the use of fetal bovine serum raises questions with regard to the methods for collecting the serum, in that the serum might be obtained under circumstances that may lead to distress for the animals (McGuill and Rowan 1989).

# 7

# Conclusions and Recommendations

On the basis of relevant literature, material submitted to the committee, the experience of members of the committee, and presentations at a 1-day workshop attended by 14 speakers and 20 additional observers, followed by 2 days of committee meetings, the committee came to specific conclusions and made recommendations.

We believe that choosing the method of producing monoclonal antibodies should be consistent with other recommendations in the *Guide for the Care and Use of Laboratory Animals*. One such recommendation pertains to multiple survival surgery; the *Guide* states (page 12) that this practice "should be *discouraged* but permitted if scientifically justified by the user and approved by the IACUC" [emphasis added]. Similarly, we recommend that mAb production by the mouse ascites method be permitted if scientifically justified and approved by the relevant IACUC. We further believe that in vitro methods should be used routinely for mAb production, especially for most large-scale production of mAb. When hybridomas fail to grow or fail to achieve a product consistent with scientific goals, the investigator is obliged to show that a good-faith effort was made to adapt the hybridoma to in vitro growth conditions before using the mouse ascites method.

> **Recommendation 1: There is a need for the scientific community to avoid or minimize pain and suffering by the animals. Therefore, over the next several years, as in vitro systems are further developed, in vitro methods for the production of monoclonal antibodies should be adopted as the routine method unless there is a clear reason why they cannot be used or why their use would represent an unreasonable barrier to obtaining the product at a cost consistent with the realities of funding of biomedical research programs in government, academe, and industry. This could be accomplished by establishing in vitro production facilities in institutions.**

There are several scientifically based reasons why the mouse ascites method for producing mAb should not be abandoned: some hybridoma cell lines do not adapt well to in vitro conditions; when small volumes of mouse mAb at high concentrations are required for injection into mice, the in vitro method often does not yield an acceptable product; rat hybridoma cell lines usually do not efficiently generate ascites in rats and adapt poorly to in vitro conditions but do produce mAb in immunocompromised mice; concentrating mAb from in vitro culture supernatant can lead to protein denaturation and decreased antibody activity; in vitro culture methods can yield mAb that do not reflect normal glycosylation patterns, in contrast with mAb generated by the mouse ascites method, and the lack of natural glycosylation might influence antigen-binding capacity and critical biologic functions; contamination of valuable in vitro clones with fungi or bacteria requires prompt passage through a mouse to save the cell line; and inability of in vitro-adapted cell lines to maintain adequate production of mAb poses a serious problem.

**Recommendation 2: The mouse ascites method of producing monoclonal antibodies should not be banned, because there is and will continue to be scientific necessity for this method.**

There is no convincing evidence that significant pain or distress is associated with the priming of a mouse with pristane. During the development of ascites, there is likely to be pain or distress, particularly with some cell lines that are tissue-invasive and in situations of significant ascites fluid accumulation. Therefore, after injection of hybridoma cells, mice should be evaluated at least daily after development of visible ascites and should be tapped before fluid accumulation becomes distressful. A limit should be placed on the number of taps, and multiple taps should be allowed only if the animal does not exhibit signs of distress. It is incumbent on the IACUC to ensure that those directly responsible for using the animals be well trained and experienced in all phases of the procedure, including observation, handling, injection, and tapping of the animals.

**Recommendation 3: When the mouse ascites method for producing mAb is used, every reasonable effort should be made to minimize pain or distress, including frequent observation, limiting the numbers of taps, and prompt euthanasia if signs of distress appear.**

It is clear that some mAb used therapeutically cannot be produced by in vitro means or that converting to an in vitro system for their production would require proof of bioequivalence, which would be unacceptably expensive. Furthermore, many commercially available mAb are routinely produced by mouse ascites methods, particularly when the amount to be produced is less than 10 g, another situation where it would be prohibitively expensive to convert to in vitro conditions. However, with further refinement of technologies, media, and practices, in vitro production of mAb for research and therapeutic needs will probably become comparable in costs to the mouse ascites method.

**Recommendation 4: mAb now being commercially produced by the mouse ascites method should continue to be so produced, but industry should continue to move toward the use of in vitro methods.**

In some circumstances, the use of the mouse ascites method for the production of mAb might be required. Examples of criteria to be used by an IACUC in establishing guidelines for the production of mAb in mice by the ascites method are:

1. When a supernatant of a dense hybridoma culture grown for 7-10 days (stationary-batch method) yields an mAb concentration of less than 5 μg/ml. If hollow-fiber reactors or semipermeable-membrane systems are used, 500 mg/ml and 300 mg/ml, respectively, are considered low mAb concentrations.
2. When more than 5 mg of mAb produced by each of five or more different hybridoma cell lines is needed simultaneously. It is technically difficult to produce this amount of mAb since it requires more monitoring and processing capability than the average laboratory can achieve.
3. Cell lines will not grow and secrete in vitro, or analysis of mAb produced in vitro reveals that a necessary biologic activity is reduced or absent.
4. More than 50 mg of functional mAb is needed, and previous poor performance of the hybridoma indicates that hollow-fiber reactors, small-volume membrane-based fermentors, other high-density cell systems, or other techniques cannot meet this need during optimal growth and production.
5. Hybridoma cells producing mAb, contaminated with infectious agents, often must be passed through mice.

We emphasize that the listed criteria are not all-inclusive and that it is the responsibility of the IACUC to determine whether animal use is required for scientific or regulatory reasons. Criteria have not been developed to define a low-producing hybridoma cell line or when in vitro methods are no longer a useful means of producing mAb.

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**Table 1. Incidence of Clinical Abnormalities in Ascitic Mice**

| | No. $CAF_1$ Mice[a] | | | | | No. SCID Mice | |
|---|---|---|---|---|---|---|---|
| | **Control** | **A** | **B** | **C** | **D[b]** | **Control** | **E** |
| **Number of Animals** | 12 | 20 | 20 | 20 | 20 | 11 | 20 |
| **Daily observations** | | | | | | | |
| Rough hair coat | 0 | 20 | 20 | 20 | 18 | 0 | 20 |
| Hunched posture | 0 | 20 | 20 | 20 | 18 | 0 | 20 |
| Animal died after tap 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| Animal died after tap 2 | 0 | 0 | 1 | 3 | 0 | 0 | 0 |
| **Observations during posttapping period** | | | | | | | |
| Hunched posture | 0 | 19 | 17 | 19 | 6 | 0 | 10 |
| Decreased activity | 0 | 19 | 17 | 19 | 8 | 0 | 9 |
| Tachypnea | 0 | 19 | 4 | 19 | 3 | 0 | 11 |
| Pallor | 0 | 19 | 7 | 19 | 10 | 0 | 3 |

[a] A, B, C, D, and E designate different hybridoma cell line groups.
[b] Two mice in Group D produced no ascites and remained clinically normal.

**Source:** Adapted from Jackson and others, 1999a.

# Table 2. University Policies for Production of Monoclonal Antibodies

| | Columbia | Penn State | Stanford | U. Arizona | UC Davis | U. Iowa | UM |
|---|---|---|---|---|---|---|---|
| Test Cell Lines for Murine Virus | -- | X | X | -- | -- | X | |
| Priming | If pristane used, not to exceed 0.25 ml | Pristane as low as 0.1 ml or IFA = 0.5 ml | -- | Minimum necessary CFA or pristane to produce ascites | Pristane not to exceed 0.2 ml; agents other than pristane must be justified | Pristane not to exceed 0.2 ml; IFA as good or better | Pris IFA pref 0.1- max 0.5 |
| Needle size for tap | -- | 18-22 gauge | 18 gauge or smaller | 21 gauge or smaller | 18-22 gauge | 18-20 gauge | 18 g sma |
| No. tappings | 3, then sacrifice | Maximum 3, last terminal | -- | -- | 3, last terminal | 2, last after euthanasia | max pref last euth |
| Monitoring after inoculation of tumor cell line | Daily | Daily | 3 times/week for first week, then daily | Daily | 3 times/week for first week, then daily | # daily | At l |
| Replacement fluid after ascites harvesting | -- | -- | -- | Administration of saline to help prevent shock | -- | 1-2 ml of saline subcutaneous | |
| Anesthesia during tapping | -- | Used to minimize P&D from tapping (methoxyflurane) | Anesthesia used for training of new personnel or students | Anesthesia preferable | Anesthesia used for new personnel | -- | |

Figure 1. Generation of mAb. Flowchart illustrating steps needed to produce mAb by mouse ascites method. Note that all steps up to production of ascites fluid are required for either in vivo or in vitro production of mAb.

Figure 2. Cost of producing 1 mg of mAb in mouse ascites, spinner flask (1 to 8L Belco spinners), and mini (Spectrum CellMax) and midi (Cellex Accusyst Jr.) hollow-fiber bioreactors (Chandler 1998). Note that absence of a bar does not mean that cost of technique is virtually zero, but rather the in vitro system was not used. Thus, spinner flask is not used above 1,000 mg, mini hollow-fiber reactor is used only in range of 100 - 1,000 mg, and midi hollow-fiber reactor is used for production needs of 1,000 mg and above.

# Appendix A

**Workshop on Methods of Producing Monoclonal Antibodies**

**November 10, 1998**

**Agenda**

| | | |
|---|---|---|
| 9:00 – 9:30 | Is the mouse ascites method painful? – Via Telephone | Gebhart |

9:30 – 3:00 Presentation by workshop participants:
9:30 – Mr. Tim DeSutter
9:45 – Dr. James Valdes
10:00 – Dr. John Reddington
10:15 – Break
10:30 – Dr. Simon Saxby
10:45 -- Dr. Vahe Bedian
11:00 – Dr. Joseph Chandler
11:15 – Ms. Tracie Letterman
11:30 – Dr. Paul E. Thomas
11:45 – Questions and Answers
12:00 – Lunch for Committee and Workshop Participants
1:00 – Dr. Kathryn Stein
1:15 – Dr. Peter Maxim
1:30 – Dr. Dennis Dixon
1:45 – Dr. Arturo Casadevall
2:00 – Dr. John McArdle
2:15 – Dr. Louis Detolla

3:00 Adjourn

**Workshop Participants**

1. Dr.Vahe Bedian, Principal Research Investigator, Pfizer Central Research
2. Dr. Arturo Casadevall, Department of Microbiology & Immunology, Albert Einstein College of Medicine
3. Dr. Joseph Chandler, President, Maine Biotechnology Services, Inc.
4. Mr. Tim DeSutter, Vice President, Integra Biosciences, Inc.
5. Dr. Louis Detolla, Chairman, Comparative Medicine Program, University of Maryland
6. Dr. Dennis Dixon, Chief, Bacteriology and Mycology Branch, Division of Microbiology and Infectious Diseases, NIH/NIAID
7. Ms. Tracie Letterman, Esquire, International Center for Technology Assessment
8. Dr. Peter Maxim, FDA/CDRH

9. Dr. John McArdle, Director, Alternatives Research and Development Foundation
10. Dr. John Reddington, DiagXotics, Inc.
11. Dr. Simon Saxby, Director, Contract Operations, Unisyn Technologies
12. Dr. Kathryn Stein, Director, Division of Monoclonal Antibodies, FDA/CBER
13. Dr. Paul E. Thomas, Professor, Department of Chemical Biology, Rutgers the State University
14. Dr. James Valdes, Edgewood Research Development & Engineering Center

# Appendix B

## Biographical Sketches of Authoring Committee

**Peter A. Ward, MD, Chairman**
Dr. Ward is Professor and Chairman of the Department of Pathology of the University of Michigan Medical School. Dr. Ward received his MD from the University of Michigan in 1960 and has worked at Scripps, the Armed Forces Institute of Pathology and the University of Connecticut Health Center. He has an extensive background in immunopathology, inflammation, and the biological role of complement and mechanisms of antibody formation. Dr. Ward is also a member of the Institute of Medicine.

**Jane Adams, BA**
Ms. Adams is Associate Director for Public Affair at the Juvenile Diabetes Foundation. Ms. Adams received her BA from University of Vermont. She has an extensive background in policy work and biomedical research. Ms. Adams has had type 1 diabetes since the age of 12.

**Denise Faustman, MD, PhD**
Dr. Faustman is Associate Professor of Medicine at Harvard Medical School and Director of Immunology Laboratories, Massachusetts General Hospital. Dr. Faustman received her MD and PhD from Washington University School of Medicine. She has an extensive background in the role of major histocompatibility complex in transplant rejection and role of defective antigen processing in autoimmunity.

**Gerald F. Gebhart, PhD**
Dr. Gebhart is Professor and Head of the Department of Pharmacology at the University of Iowa College of Medicine. Dr. Gebhart received his PhD from the University of Iowa. He has an extensive background in pain and pain modulation and mechanisms of visceral pain and visceral hyperalgesia. Dr. Gebhart is also a member of ILAR Council.

**James G. Geistfeld, DVM**
Dr. Geistfeld is Director of Laboratory Animal Medicine at Taconic Farms and Vice President of Taconic Ventures. Dr. Geistfeld received his DVM from the University of Minnesota School of Veterinary Medicine. He has an extensive background in methods of producing monoclonal antibodies and methods of producing laboratory animals.

**John W. Imbaratto, BS**
Mr. Imbaratto is Manager of Cell Culture Manufacturing at Covance Biotechnology Services, Inc. in Research Triangle Park. Mr. Imbaratto received his BS from Kent State University in Ohio. He has worked in Quality Control and manufacturing since graduation, first as Head of Bacterial Fermentation and Toxoid Manufacturing for Lederle Laboratories, then as Manager of cGMP manufacturing of monoclonal antibodies

using the mouse ascites method with Charles River Laboratories. Currently, he is with Covance Biotechnology Services as the Manager of Cell Culture Manufacturing providing in-vitro cGMP contract manufacturing services.

**Norman C. Peterson, PhD, DVM**
Dr. Peterson is a Research Associate in the Department of Clinical Studies at the University of Pennsylvania. Dr. Peterson received his DVM from the University of Illinois and his PhD from the University of Pennsylvania. His research involves investigations of oncogenic cell-surface receptor interactions and he has an extensive background in methods of producing monoclonal antibodies.

**Fred Quimby, PhD, DVM**
Dr. Quimby is Director of the Center for Laboratory Animal Resources at Cornell University, Ithica. Dr. Quimby received his VMD and PhD from the University of Pennsylvania. He has an extensive background in immunology, differentiation antigens on canine lymphoctes, and immunologic abnormalities in autoimmune disease.

**Ann Marshak-Rothstein, PhD**
Dr. Rothstein is Professor of Microbiology and Director of Immunology Training Program at Boston University School of Medicine. Dr. Marshak-Rothstein received her PhD from University of Pennsylvania School of Medicine. She has an extensive background in cellular immunology, murine models of autoimmunity, autoantibody-medicated pathogenesis, and regulation of Fas/FasL medicated apoptosis.

**Andrew N. Rowan, DPhil**
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Dr. Scharff is Professor of Cell Biology at Albert Einstein College of Medicine. Dr. Scharff received his MD from New York University College of Medicine. He has an extensive background in the basis for immunoglobulin variable region somatic hypermutation and isotope switching to discover better methods of producing monoclonal antibodies for treatment and prevention of disease. He is Co-Chair of the Board of Scientific Councilors and a member of the Executive Committee of the National Cancer Institute. Dr. Scharff is also a member of the National Academy of Sciences.