Guidelines for Ascites Production

The mouse ascites method for monoclonal antibody production will be considered scientifically justified in cases where no other method provides the quantity and concentration of MAB required for an approved proposal. The principal concern of the ascites method is the pain and discomfort associated with the abdominal inflammatory reaction which not only involves the peritoneal lining (peritonitis) but fibrinous tags may adhere to the serosal surfaces of other abdominal organs such as intestine, spleen, and liver.

In proposals utilizing mouse ascites for MAB production every reasonable effort should be made to minimize discomfort and distress in the mice. This would include the following:

1. Immunization procedures performed by skilled, competent technical personnel;
2. Once daily observation by staff knowledgeable and capable of recognizing signs of distress once animals have demonstrated a 10% increase in body weight attributable to ascites;
3. Limiting the number of taps to three with the fourth being a terminal tap; and
4. Prompt euthanasia if signs of distress and moribundity are evident.

As tissue culture methods and facilities for the production of monoclonal antibodies are further developed, investigators are encouraged to consider in vitro alternatives to mouse ascites unless scientifically justified.

A listing of service laboratories that provide in vitro monoclonal antibodies can be found at http://altweb.jhsph.edu/mabs/where.html.

The following general considerations should be evaluated when preparing a monoclonal antibody production proposal:

1. Is the in vivo production of MAB scientifically justified? What are the goals of the research or project that requires antibodies?
2. What quantity of antibody is required; and over what period of time?
3. Have the hybridomas been viral tested?
4. Has there been an adequate attempt to expand the hybridoma in vitro?
5. Has use of a core facility or commercial source been considered?
6. Are the mouse strain and number of animals appropriate?
7. Is the priming appropriate in terms of type and amount and the length of time prior to inoculation of hybridoma? (see *Hybridoma Inoculation* below)
8. How will the animals be adequately monitored for clinical signs that constitute criteria for euthanasia? (see *Clinical Observation/Monitoring for Ascites* below)

Procedure for Production of Ascites:

The specific guidelines for consideration by Principal Investigators when developing animal study

proposals involving the mouse ascites method for review by the Frederick National Lab Animal Care and Use Committee are:

1. Priming: The volume of the priming agent should be reduced to as small a volume as necessary to elicit the growth of ascitic tumors and at the same time reduce the potential for distress caused by the irritant properties of the priming agents. Scientific justification is required for > 0.5 ml and multiple primings. The recommended dose volume for Pristane is 0.5 ml, based on the unpublished results of a scientific investigation conducted by Drs. James Kenny and Craig Reynolds in 1992 (Attachment A).
2. Hybridoma Inoculation: The time interval between priming and inoculation of hybridoma cells as well as the number of cells in the inoculum are determined empirically. Inocula up to 10^7 cells in volumes of 0.1-0.5 ml are usually administered 10-14 days after priming. Generally, very high concentrations are associated with greater mortality and concentrations < 1×10^5 elicit fewer ascitic tumors and these tend to have a smaller volume yield. Cell suspensions should be prepared under sterile conditions in physiological solutions.
3. MTBM (Molecular Testing of Biological Materials) Testing [viral screening]: Hybridomas should be viral screened before introduction into the animal host to prevent potential transmission of infectious agents from contaminated cell lines into facility mouse colonies and possibly to humans handling the animals. A copy of the MTBM test results must accompany the Frederick National Lab Animal Study Proposal form.
4. Clinical Observation/Monitoring for Ascites: Clinical observation of the individual animals should be performed by personnel familiar with clinical signs associated with ascites production. The animals should be monitored twice daily, including weekends and holidays, after inoculation. Daily observation allows the degree of abdominal distension to be frequently assessed so that abdominal paracentesis (tapping) can be performed as needed for each animal. Animals will be monitored twice daily for hunched posture, roughened hair coat, anorexia, dehydration, weight loss, loss of body condition, inactivity, difficulty in ambulating, tachypnea and dyspnea. Animals that exhibit severe clinical abnormalities, solid tumor growth, or become moribund before maximum ascites expansion (approximate doubling of the width of the abdomen) will be promptly euthanized. Death is not an acceptable endpoint. Endpoints should be clearly stated in the Frederick National Lab Animal Study Proposal form.
5. Abdominal Paracentesis/Harvesting of Ascites Fluid: Antibody production characteristics vary significantly among hybridoma cell lines. Correspondingly, clinicopathological changes vary as well. To maximize antibody yield and keep discomfort to a minimum, four imperatives should be kept in mind:

    a. Frequent assessment of abdominal distention for timely taps reduces adverse clinical symptoms and mortality;
    b. Mice should be monitored twice daily once animals have demonstrated a 10% increase in body weight attributable to ascites. Very close post-tap monitoring is critical to avoiding low volume shock. May be treated with 2-3 mls of warm saline subcutaneously;
    c. Mice should be tapped prior to 20% increase from day 0 (i.e., a 20g mouse should not exceed 24g) attributable to ascites production [considering normal weight gain]; and

d. Maximum of three survival taps (maximum antibody yield/minimum number of mice).

The Executive Summary of the ILAR Report (National Research Council, Monoclonal Antibody Production, National Academy Press, 1999) is included (Attachment B). A full text copy of the report can be found at http://grants.nih.gov/grants/policy/antibodies.pdf

Another useful alternatives reference for monoclonal antibody production can be found at http://altweb.jhsph.edu/pubs/

References:

1. Ascites Production in Mice. NIH Animal Research Advisory Committee Guideline. Revised March 27, 2002.
2. Special Section on Monoclonal Antibodies. Alternatives to Animal Testing on the Web (ALTWEB). http://altweb.jhsph.edu/pubs/
3. Behavioral, Clinical, and Physiological Analysis of Mice Used for Ascites Monoclonal Antibody Production (Attachment C). Norman C. Peterson. Comparative Medicine 50(5): 516-526, 2000.
4. Monoclonal Antibody Production in Murine Ascites I and II (Attachments D and E). Jackson LR, Trudel LJ, Fox JG, Lipman NS. Laboratory Animal Science 49(1): 70-86, 1999.
5. ILAR Journal Volume 37, Number 3, 141-152 (1995).
6. ILAR Report on Monoclonal Antibody Production. A Report of the Committee on Methods of Producing Monoclonal Antibodies. Institute for Laboratory Animal Research, National Research Council. 1999. http://grants.nih.gov/grants/policy/antibodies.pdf

Attachment A

Effect of Different Pristane Priming Protocols
On the Production of HPCG-14 MoAB in CD2F1 Mice

Pristane Dose	Tap	# of Mice		Yield (ml)		MoAB (mg)		Cumulative			
								ml		mg	
		Start	Tested	Per Mouse	Total	Concentration	Total	Per Mouse	Total	Total	Per Mouse
0.2 ml (1X)	1st	14	14	2.4	34	0.3	10.1	2.4	34	10.1	0.7
	2nd	--	6	2.3	14	<0.1	<0.1	3.4	48	10.2	0.7
0.5 ml (1X)	1st	14	8	3.0	24	1.6	38.4	3.0	24	38.4	4.8
	2nd	--	8	2.3	18	2.3	42.0	5.25	42	80.4	5.7
	3rd	--	2	2.1	4	0.6	2.5	5.76	46	82.9	5.9
0.2 ml (2X)	1st	18	14	4.3	60	0.6	37.7	4.3	60	37.7	2.7
	2nd	--	12	2.8	34	1.5	50.6	6.7	94	88.3	4.9
	3rd	--	3	2.3	7	3.2	22.2	7.2	101	110.5	6.1
0.5 ml (2X)	1st	20	19	4.3	81	0.6	47.4	4.3	81	47.4	2.5
	2nd	--	19	3.1	59	0.9	54.0	7.3	140	101.4	5.1
	3rd	--	10	2.0	20	1.4	27.4	8.0	160	128.8	6.4

James Kenney, Ph.D.
Craig Reynolds, Ph.D.
1992

Approved September 2000
Revised June 2012

Attachment B

Monoclonal Antibody Production
A Report of the Committee on Methods of Producing Monoclonal Antibodies
Institute for Laboratory Animal Research
National Research Council
1999
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 Commit tee 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 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 animals. Therefore, over the next several years, as tissue-culture systems are further developed, tissue-culture method 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 puri fication 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 µg/ml and 300 µg/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.