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 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. Twice daily observation by staff knowledgeable and capable of recognizing signs of distress;
3. Limiting the number of taps to three with the fourth being a terminal tap; and
4. Prompt euthanasia if signs of distress and morbundity 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/topics/mabs/producers.htm

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

1. Is the 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 MAP 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)

<u>**Procedure for Production of Ascites**</u>:

The specific guidelines for consideration by Principal Investigators when developing animal study proposals involving the mouse ascites method for review by the NCI-Frederick Animal Care and Use Committee are:

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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. Although 0.5 ml of Pristane has been used for adult mice, 0.1-0.2 ml has been found to be as effective for many hybridomas. Scientific justification is required for > 0.2 ml and multiple primings. The recommended dose volume for Pristane is 0.1 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 hybridma cells as well as the number of cells in the inoculum are determined empirically. Inocula range from 10^5-10^7 cells in volumes of 0.1-0.5 ml and are usually administered 10-14 days after priming. Generally, very high concentrations are associated with greater mortality and concentrations $< 1 \times 10^5$ 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. MAP Testing: Hybridomas should be MAP (mouse antibody production) tested 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 MAP test results must accompany the NCI-Frederick 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 NCI-Frederick 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. 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); and
   d. Maximum of three 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

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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/topics/mabs/mabs.htm

References:

1. Ascites Production in Mice. NIH Animal Research Advisory Committee Guideline. Revised March 27, 2002. http://oacu.od.nih.gov/ARAC/ascites.pdf
2. Special Section on Monoclonal Antibodies. Alternatives to Animal Testing on the Web (ALTWEB). http://altweb.jhsph.edu/topics/mabs/mabs.htm
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

**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

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.

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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.

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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.

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Behavioral, Clinical, and Physiologic Analysis of Mice Used for Ascites Monoclonal Antibody Production

Norman C. Peterson

Background and Purpose: **The effects of pristane inoculation, ascites accumulation, peritoneocentesis, and analgesics on the well-being of mice used in monoclonal antibody (MAb) production protocols were investigated.**

Methods: **Four experiments, each containing 17 to 21, 6- to 8-week-old male Balb/c mice, were conducted. Each experiment involved a period in which baseline data were collected, followed by intraperitoneal injections of pristane or phosphate-buffered saline (PBS) inoculations into each mouse. One week later mice received intraperitoneal inoculations of either hybridoma cells or PBS. Parameters used to assess well-being throughout each of these periods included: wheel-running activity, food and water consumption, open-field box activity, clinical observation, and plasma corticosterone concentration.**

Results: **Compared to controls, pristane inoculation had slight to no affect on mice. There was no evidence of distress in cell-inoculated mice prior to their gaining 25% of their baseline body weight. The number of times (up to three) that peritoneocentesis was performed did not have a significant impact on mice's well-being, but ascites yields were greater when multiple harvests were performed. Cell-inoculated mice that gained weight slowly or developed high-particulate ascites were at higher risk of being distressed.**

Conclusion: **Ascites yields can be maximized by performing multiple harvests; however, the well-being of mice used in such protocols should be closely monitored, as suggested here.**

As a result of recent measures to ban use of the ascites method for production of monoclonal antibodies (MAb) in several European countries (1) and a petition for similar action to be taken in the United States (2), several workshops and forums have been conducted to evaluate the current technology of in vitro methods of MAb production and to assess whether use of mice in this application is still justified (3–6). Findings at each of these meetings concluded that the in vitro methods can adequately provide the antibodies that are necessary for most experimental situations and that the in vitro techniques should be adapted as the method of choice for routine production of MAbs. However, the National Research Council Report on MAb Production and others indicate that, in a small number of cases (< 10%), the in vitro methods may be insufficient and use of the ascites method may be justified (4, 5).

If situations exist in which animals are required for the production of MAbs, efforts must be directed at implementing procedures targeted at reducing pain and distress whenever possible (7, 8). It can be assumed that the in vivo method of MAb production is potentially distressful, because, if allowed to progress without intervention, death will ensue. Clinical and pathologic observations of mice being used for MAb production by Jackson et al. (9, 10) provide evidence that this procedure is distressful to mice. Additionally, information gathered from human cases indicate that ascites accumulation negatively affects cardiovascular function and impedes normal respiratory tract function (11–13). However, there is little information available to define when and under what conditions mice being used for ascites production become distressed (5).

The study presented here was conducted to analyze the effects of commonly used practices in ascites production on the well-being of mice. The information provided will be useful for researchers, veterinarians, and institutional animal care committees in developing practical guidelines aimed at minimizing pain and distress in protocols where the use of the ascites method has been justified.

Materials and Methods

Animals: Seventeen to 21, six-week-old male BALB/c mice per experiment were obtained from Charles Rivers Laboratories (Wilmington, MA). Mice were certified to be free of all commonly tested rodent pathogens by the vender, and health status was monitored by serologic testing of sentinel animals on a quarterly basis in this facility. On arrival, mice were group housed in polycarbonate shoebox cages with filter tops and containing laboratory grade pine shaving bedding. Each cage contained a small rodent running wheel. Four days later, mice were individually and randomly distributed to similar cages with mouse wheels connected to a monitor. Mice had ad libitum access to rodent chow (Purina, St. Louis, MO) and water.

Food, water, and bedding of individually housed mice were changed weekly. Mice were housed in a room at temperature of 20°C and 55% humidity. A 12/12-hour light/dark cycle was maintained. No other animals were housed in the same room with these experimental groups.

Cells: Three hybridoma cell lines, which were produced in this laboratory and reactive against Signal Regulatory Protein (SiRP), were used to produce ascites. Syngeneic hybridomas were produced from the fusion of a single laboratory stock myeloma cell-line (SP2/0) and splenocytes harvested from anti-

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gen immunized BALB/c mice. Two of the hybridomas used produce IgG1s (MAb 77.2 and MAb 122.1) and one cell line produce IgM (MAb 49.7).

Cells were grown in 10% fetal bovine serum (FBS; Hyclone, Logan, UT) in Dulbecco's modified Eagle's medium with glucose (4.5 g/L; Biowhittaker, Walkersville, MD) supplemented with 200 m*M*, L-glutamine (Gibco BRL, Gaithersburg, MD) and 1% penicillin-streptomycin (Gibco BRL). Prior to inoculation, cells were washed two times in PBS, and viability and concentration were determined by use of trypan blue dye exclusion and hemocytometer count. Cells were then resuspended to the appropriate concentration in PBS.

Experimental design and procedures: Four experiments involving four groups of mice at various times were performed as outlined (Table 1). Each of the experiments involved a pristane phase (7 days) and an ascites phase (8 to 12 days). At the start of the pristane phase, mice received either 0.2 ml of pristane (2,6,10,14-tetramethylpentadecane; Sigma Chemical Co., St. Louis, MO) or 0.2 ml of filter-sterilized PBS by intraperitoneal (i.p.) inoculation, using a 22-gauge, 1-in. needle. The ascites phase began 7 days after pristane injection, with one group of pristane-primed mice receiving i.p. inoculations of either 1 to 4 million hybridoma cells resuspended in 0.5 ml of sterile PBS (designated "cell" group) or 0.5 ml of PBS alone (designated "pristane" group). Mice that received PBS during the pristane phase also received 0.5 ml of PBS at the start of the ascites phase (designated "PBS" group).

Non-terminal ascites harvesting was performed on healthy-appearing mice according to their experimental groupings, using a 20-gauge, 1.5-in. needle. Mice were designated to either cell group 1, 2, or 3 dependent on the number of times ascites was harvested. The first peritoneocentesis was targeted for when the mouse's body weight increased 20 to 30% (cell group 3), 30 to 40% (cell group 2), or 40 to 55% (cell group 1), and was performed at least 9 days after cell inoculation. Multiple harvests were performed two days apart, except that the second and third harvests were done on consecutive days. If mice were manifesting signs of pain or it was time for their final harvest, they were euthanized by exsanguination under anesthesia (Ketamine: 42 mg/kg of body weight; Fort Dodge; Fort Dodge, Iowa, and Xylazine: 24 mg/kg; Phoenix Pharmaceuticals, St. Joseph, MO, given subcutaneously) and ascites was collected.

Harvested ascites was placed in a 37°C water bath for 30 minutes, and then was placed on ice for 1 to 4 hours. The liquid portion of the ascites was separated from any particulate matter or clot by centrifugation (1,000 X g, 10 minutes). The amount of sediment formed was calculated by subtracting the initial harvested ascites volume from the postcentrifugation supernatant. The ascites supernatant was frozen at –20°C for further analysis. Antibody titering was performed by use of an enzyme linked immunosorbant assay and goat anti-mouse immunoglobulin (Sigma Chemical Co.). The gastrointestinal tract, from the stomach to the distal portion of the colon also was collected at euthanasia to determine the increase of gastrointestinal weights as estimates of tumor growth.

Food and water consumption was determined on a weekly basis, beginning with the initiation of each phase, with a final measurement taken when the animals were euthanized. Baseline mouse body weight for calculating percentage gain was obtained immediately prior to cell inoculation. Mouse weight and abdominal distention were measured at least weekly and more

frequently during the ascites phase (Figure 1). Abdominal distention was determined by caliper measurement at the widest point along the horizontal plane of the mice's abdomen as indicated. On days when ascites harvests were performed, mouse weight and abdominal distention were recorded prior to peritoneocentesis. Mice were monitored daily for signs of clinical abnormality by the study director and, in experiments 3 and 4, a health score ranging from 0 to 3 was assigned to each mouse. Health scores were assigned as follows: 0 = normal, healthy appearance, 1 = slight roughness in coat around the head and neck area only, 2 = unkempt area extended to mid-thorax and/or eyes having decreased luster, 3 = generalized rough- and/or greasy-appearing coat, hunched posture, decreased activity and/or dehydration. Animals that appeared distressed or in pain on the basis of poor health score (3) and/or low wheel-running activity (< 20 revolutions per day) were euthanized. The University of Pennsylvania Institutional Animal Care and Use Committee approved all experimental procedures.

During the later part of the cell phase in experiment 4, two groups of mice (cell-inoculated and PBS-inoculated) received subcutaneous injections of 0.1 mg of buprenorphine/kg (Reckitt & Colman Pharmaceuticals Inc., Richmond, VA) twice each day, beginning 9 days after cell inoculation. Two analogous groups of mice received equal volumes of subcutaneous injections of PBS at the same times.

Mouse wheels: Mouse wheel-running activity was monitored daily as described (14). Briefly, galvanized wire was used to mount one metal mouse wheel (Petco retail stores, Haverford, PA) to the wire top of each mouse cage. Magnetic reed switches (Radio Shack) were also wired to the wheel and an activating point on the top of the mouse cage. Reed switch activity was conveyed to a Mac SE 120 computer via telephone wire cable, which was soldered to the keyboard letter terminals. Hence, each rotation of the wheel resulted in the typing of a designated letter. The Mouse-O-Meter software to tabulate the data was downloaded from a University of Minnesota website (no longer posted, but available from author on request). A maximum of 20 mouse cages could be monitored simultaneously by use of this method.

Mice were acclimated to the wheel for one week (4 days in group housing and 3 days individually housed) prior to recording. Between the end of this acclimation period and the start of the pristane phase, wheel-running activity was monitored in all mice for one week. This information was used as a baseline covariate in statistical analyses.

Behavioral analysis: Mice were placed in a 14 x 8 x 9.5-cm. black plastic box with a 3.5 x 3.0-cm square exit hole. The mice were allowed a maximum of two minutes to exit the box to an open-field box. If a mouse exited the box in fewer than two minutes, the time was recorded and the open-field box time was started. Mice that remained in the black box for the duration of the two minutes were removed to the open-field box and a time of two minutes was recorded. Incidences of head pokes, as defined by extension of the mouse's head beyond the base of the pinna and contraction or retreat back into the black box, also were recorded. Once the mouse was in the open-field box (30 x 19.5 x 8.5 cm; Tupperware container bottom with fifteen 5.2 x 5.2- cm demarcated squares), the black box was withdrawn and the number of squares the mouse traversed in three minutes was tabulated. The number of times the mouse stood on its hind limbs along the edges of the open-field box also were monitored (rear-

Table 1. Experimental design: experiments were performed, using four groups of mice and various hybridoma cell inoculations as indicated. The pristane phase was initiated immediately after mice were inoculated with 0.2 ml of pristane or phosphate-buffered saline (PBS), and the procedure was similar for all four groups. At the beginning of the cell phase, pristane-primed mice were inoculated with hybridoma cells (i.p.) to induce ascites or PBS (i.p.) (second control). Mice that were inoculated with cells were further allotted to subgroups according to the number of harvests or analgesics administered.

Exp. #	Objective	Cell-line	Cell Dose (x10⁶)	Pristane Phase Treatment	n	Cell Phase Inoculum	Harvests	n	Treatment Analgesics***	Health *** Scores	Abdominal*** Distention
1	Abdominal Distention	77.2a*	4	PBS	4	PBS	1	4	N	N	Y
				Pristane	17	PBS	1	4			
						Cells	1	13			
2	Multiple Harvests	49.1	2	PBS	4	PBS	1	4	N	N	Y
				Pristane	15	PBS	1	3			
						Cells	1/2/3	4/4/4			
3	Multiple Harvests	122.1	1.2	PBS	0	PBS	0	0	N	Y	Y
				Pristane	17	PBS	1	4			
						Cells	1/2/3	5/4/4			
4	Analgesia	77.2b*	1	PBS	8	PBS	2	4	N	Y	N
						PBS	2	4	Y		
				Pristane	10	Cells	2	5	N		
						Cells	2	5	Y		

*Monoclonal antibodies 77.2a and 77.2b are two subclones that produce the same antibody; ** parameters that were not observed in all experiments, all other parameters described in the text were consistently monitored, ***N = observation or treatment not performed, Y = observation or treatment performed.

ing) during that period. All equipment was sprayed with 70% ethanol, wiped, and allowed to dry before each test. Individuals who conducted the behavioral observations were required to review a detailed description of criteria used to score the analyses, and each individual performed at least two practice runs in the presence of the study director to ensure continuity of data collection.

Corticosterone measurement: Base-line plasma corticosterone concentration was obtained from mice a minimum of seven days after they were individually housed. Mice were anesthetized by i.p. injection of a ketamine (35 mg/kg)-xylazine (20 mg/kg) mixture, and approximately 100 μl of blood was taken from the retro-orbital sinus, using a heparinized mini- capillary tube. Final blood samples were taken by cardiac puncture of mice, which were anesthetized with the ketamine-xylazine (42–24 mg/kg) mixture given subcutaneously in the dorsal thoracic region. Plasma was separated from blood by centrifugation (1,000 X g) and stored at –20℃ for further analysis. Corticosterone concentrations in plasma and ascites were determined by use of a radioimmunoassay (ICN, Costa Mesa, CA) as described by the manufacturer.

Statistics: Daily wheel-running activity and behavioral data were analyzed, using an analysis of covariance (ANCOVA) where the covariate was baseline activity. Baseline activity data were collected from each mouse the week prior to experimental manipulation. An analysis of variance (ANOVA) was used for health scores, food and water consumption, and ascites comparisons. These analyses were followed by calculation of the least square means to determine individual group differences. A regression analysis was performed to evaluate possible correlations between plasma corticosterone concentration and abdominal width, weight gain, intestinal weight, abdominal distention, ascites volume, ascites residue, and ascites corticosterone concentration. Statistical significance was set at $P = 0.05$. Data are presented as mean ± SEM.

Results

Wheel-running activity: Mouse wheel-running activity data were effectively and inexpensively collected, using a home-made computer-monitored system. Of the 2,017 data entries that were collected, only 28 (1.4%) were lost, principally due to reed switches that occasionally became misaligned. After mice were acclimated to the wheel and individual housing, baseline or normal daily running activity was determined for a period of

one week prior to experimental manipulation. The average wheel-running activity of normal unaffected individually housed mice of all four experiments ranged from 128 to 26,202 revolutions per day, with a mean of 7,683 revolutions per day. To accurately compare the treatment effects on each group in the face of this variability, statistical analyses were performed using normal baseline running activity data from each group as a covariate in statistical calculations.

Analysis of wheel-running behavior of all pristane-inoculated mice revealed an initial decrease in activity followed by a gradual increase for most of the remaining week (Figure 1A). Although this profile may be reflective of a response to a mild inflammatory stimulus, significant differences between the running activities of PBS- and pristane-inoculated mice during the pristane phase were not evident.

Running activity in cell-inoculated mice began to decrease between four and six days after inoculation (Figure 1 B-E), with groups inoculated at higher doses initiating the decrease sooner (Figures 1B and 1C). However, once initiated, the rate of decrease in activity of cell-inoculated mice appeared to be similar for all cell lines analyzed. The running activity of the controls did not change significantly during that period. The point at which differences in wheel- running activity reached significance also varied among the different experiments (cell lines). Despite the variability among experiments, this point appeared to be at the time when the mice were approximately 25% over their baseline body weight and had abdominal distention of about 30% above baseline (Figure 1 C-E). The estimated average mouse weight and abdominal distention at this point in experiment 1 appear to be higher (Figure 1B). However, considering that if it were not for a less active pristane-inoculated group, significant difference in activity would have been reached one day earlier, and at that point (seven days after inoculation), measurements would more likely approximate those of the other experiments. During that period, control mice also gained approximately 5% of their body weight.

Analysis of wheel-running activity beyond the first 9 days of the cell phase was not informative because of the smaller group sizes introduced by increasing the number of treatments, large variation of activity within groups, and the short time interval (0 to 4 days) in which significant change could be detected.

The effects of harvesting ascites on wheel-running activity also were investigated in ascitic mice of experiments 2 and 3. To

include several mice in this analysis (n = 25), the change in running activity of harvested mice from the "day before" to "day of" peritoneocentisis was compared with that of the nonharvested cohorts during the same period.

Mice that underwent peritoneocentesis had a larger decrease in activity (mean: 5,145; SEM:1,190) than did nonharvested mice (mean: 2,346, SEM:1,005) when the changes in harvest day to previous day activities were compared; however, the difference was not significant ($P = 0.087$). Similar results were observed when the statistical analysis was repeated comparing harvest day with baseline/normal activity changes among harvested and unharvested groups ($P = 0.17$). Additionally, the volume of ascites removed did not influence the magnitude of this decrease.

Food and water consumption: Another indicator of distress in man and animals is decrease in food and/or water consumption. These parameters were monitored weekly during the pristane (1 week) and cell (1 complete week and 1 partial remaining week) phases of four experiments (Figure 2). There was no difference in food ($P = 0.91$) or water ($P = 0.15$) consumption in mice primed with PBS or pristane during the pristane phase. However, during the first week of ascites progression, food consumption was significantly different among the three groups analyzed (PBS, pristane, and cell: $P = 0.004$). Analysis by use of least squares means revealed that this difference was largely attributed to decreased appetite in the cell group, compared with the PBS group ($P = 0.002$), but was not significant when compared with the pristane group ($P = 0.08$). There was no difference in water consumption among the three groups during the cell phase ($P = 0.56$).

The effects of peritoneocentesis on food and water consumption were analyzed in the final three and four days of experiments 2 and 3, respectively. At that point ascites harvesting had been initiated and the cell group was now subdivided according to the number of harvests performed in each group. In contrast to the earlier findings of the first week of the cell phase, the amount of food consumed per day during that period did not differ among all groups tested including controls ($P = 0.50$). One possible explanation for this observation could have been that the harvesting of ascites relieved some of the intra-abdominal gastric pressure, thus increasing appetite and easing the stomach's ability to expand; however, food consumption of nonharvested ascitic mice was not different from that of the controls or multiharvested groups ($P > 0.31$). Water consumption during the final days of the cell phase also was not significantly different among the five groups tested when analyzed by ANCOVA ($P = 0.08$). However, because the level of significance was slight and water consumption in cell groups 1, 2, and 3 appeared somewhat higher, a least square means calculation was performed. This analysis indicated that water consumption was significantly higher in mice that underwent three harvests (cell group 3), compared with controls or cell group 2 group ($P = 0.03$), but not cell group 1 ($P = 0.11$).

In summary, pristane priming had no effect on food or water consumption, whereas food consumption in mice decreased within one week of hybridoma induction. During the second week of ascites progression, a significant difference in food and water consumption among all groups was not observed; however, water consumption in mice that underwent harvest three times was increased in the late cell phase.

Health status: Of the 77 mice used for this study, only one mouse was found dead and six mice were described as mori-
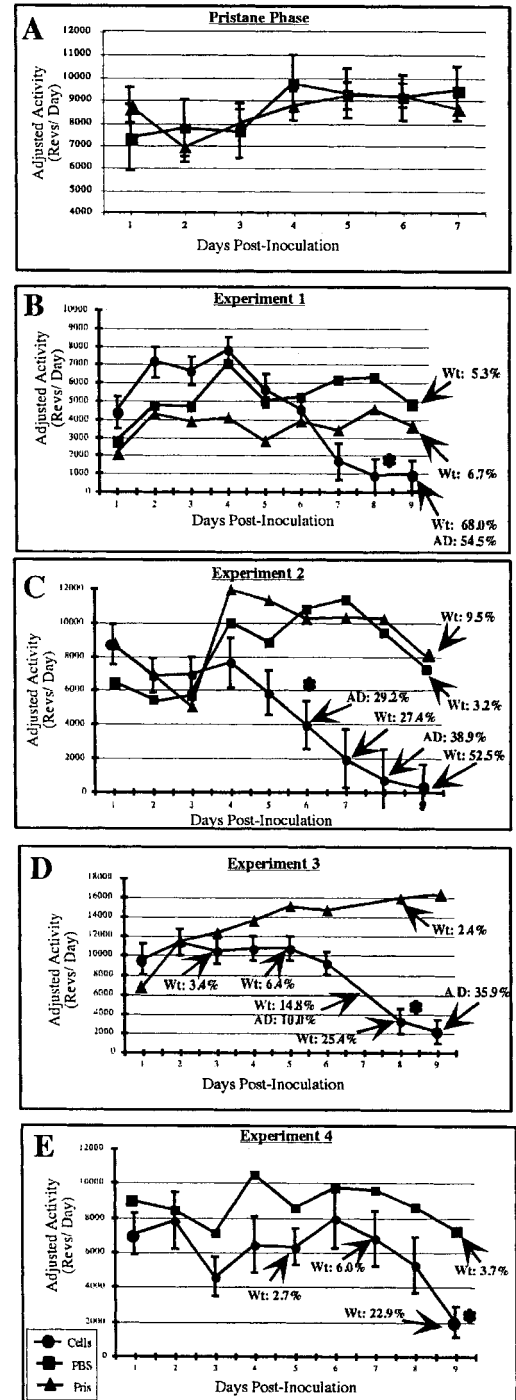

Figure 1. Wheel-running activity was continuously monitored, using a Mac SE 120 computer as described in Materials and Methods. Daily mean wheel-running activity of each group was adjusted for pre-experimental baseline activity values. **(A)** Pristane phase adjusted daily wheel- running activity. Each mouse received an intraperitoneal (i.p.) inoculation of 0.2 ml of pristane or phosphate-buffered saline (PBS) at the beginning of day 1. **(B–E)** Hybridoma cells (as indicated in Table 1) or PBS was inoculated at the start of day 1. The mean percentage of increase in weight (Wt) and abdominal distention (AD) from day 1 are designated. Standard error of the mean bars are presented for cell-inoculated mice only for clarity. Legend at bottom of panel E: *indicates first point at which wheel-running activity becomes significantly different from that of controls (analysis of covariate, with baseline as the covariate).

bund (reluctant to move and clinically assessed as likely to die within 12 hours) during the cell phase of all experiments. Clinical assessment of mice during the pristane and cell phases of experiments 3 and 4 was recorded as a health score index ranging from 0 to 3, with 0 representing no abnormal signs and 3 indicating unkemptness, dehydration, and/or sluggishness (see Materials and Methods for more details).

During the initial three days of the pristane phase, 15 of the 25 pristane-inoculated mice received a health score of 0 (normal) whereas the remaining 10 mice received a score of 1 to 3. All control mice in the PBS group (n = 12) received a 0 score, except for one mouse, which received a score of 1. All health scores returned to 0 within four days of pristane inoculation; however, scores progressively increased in cell-inoculated mice beginning around eight days after inoculation.

To determine whether poor health assessments were reflected by decrease in activity levels, wheel-running activities of mice grouped according to their health status were compared (Figure 3A). When analyzed at two days after pristane injection or eight days after cell inoculation, mice that appeared clinically worse (higher health score) tended to be less active (Figure 3A); however, the differences did not reach significance ($P = 0.13$ and $P = 0.18$, respectively). An increase of weight in cell-inoculated mice as a result of ascites accumulation may also have contributed to this decreased activity, as there was a negative correlation between the amount of weight the mice had gained and wheel-running activity $r = -0.66$; data not shown). Health status also was related to weight gain, as healthy appearing mice gained significantly less weight than did mice that were given a health score of 1 ($P = 0.05$). Interestingly, the weight gain of mice given health score ≥ 1 did not significantly vary (Figure 3B).

Corticosterone analysis: To determine whether in vivo MAb production procedures resulted in changes in plasma corticosterone concentration, blood samples were collected from acclimated mice prior to pristane inoculation (baseline) and at euthanasia. Baseline plasma corticosterone concentrations that were measured before any experimental manipulation were highly variable among all mice (mean: 294 ng/ml; SD: 170 ng/ml) and when compared with concentrations in samples taken from all mice at euthanasia (mean: 170 ng/ml; SD: 170 ng/ml), baseline values appeared increased. This difference might have been attributed to the means by which blood samples were obtained in each case. Baseline samples were taken by retro-orbital puncture of anesthetized mice, whereas final samples were taken by cardiac puncture of more deeply anesthetized animals. Evidence suggesting that terminal plasma corticosterone values were reflective of distress was obtained from two cell-inoculated mice (of two) that were found to be moribund, but not ascitic, and having corticosterone values of 400 and 500 ng/ml. Because baseline corticosterone data appeared not to be representative of the status of normal, undisturbed mice, it was not used in these analyses.

There were no significant differences in terminal plasma corticosterone values among groups—PBS, pristane, and cell—for any of the four experiments conducted ($P > 0.12$) (Figure 4). However, in experiments 2 and 3, the single harvest group (cell group 1) consistently had the highest plasma concentration of corticosterone. This is in accordance with my observation that, in several cases, the health status of mice in the single harvest groups deteriorated slightly faster than that of mice in the two and three harvest groups, requiring them to be euthanized a day
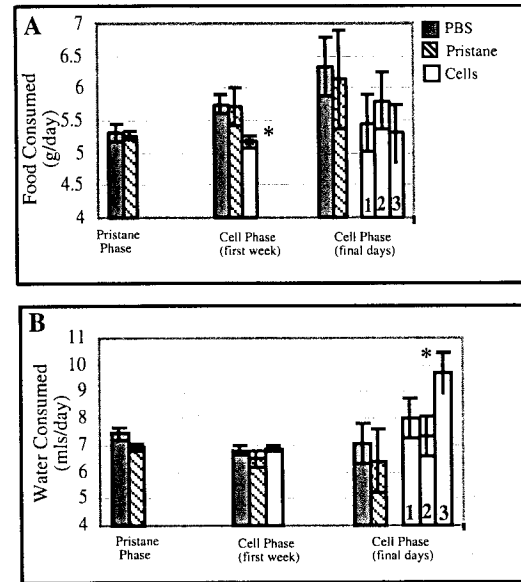

Figure 2: Food **(A)** and water **(B)** consumption of each mouse monitored at weekly intervals, or less during the final days. Mice from all experiments are included, except those given buprenorphine in experiment 4. Number of harvests performed are designated in final days. *Significant difference within grouping.

earlier. Unrelieved pressure from ascites accumulation could explain these observations; however, there was no correlation between mice's abdominal width ($r = 0.29$, $P = 0.26$) or the volume of ascites removed from single harvested mice ($r = 0.016$, $P = 0.95$). Surprisingly, there was a significant negative correlation between plasma corticosterone concentration and weight gain in single harvested mice of experiment 1 ($r = 0.55$, $P = 0.023$). Dilution of plasma corticosterone concentration by increased extravascular fluid accumulation seems unlikely, as plasma and ascites corticosterone concentrations were not correlated ($r = 0.18$, $P = 0.47$).

To estimate the amount of tumor cell growth on the serosal surface of gastrointestinal tract organs, the gastrointestinal tract from the stomach to the colon of each euthanized mouse was removed and weighed. The gastrointestinal tract of cell-inoculated mice was significantly heavier than that of controls ($P = 0.0001$) and tended to increase with the number of harvests. However, there was no correlation between intestinal weights and corticosterone values ($r = 0.02$, $P = 0.94$). When tumor cell growth resulted in increased sediment in the ascites fluid, corticosterone values increased accordingly ($r = 0.69$, $P = 0.002$). However, there was no relationship between the animal health status score and amount of sediment in its ascites ($P = 0.68$).

Production characteristics: As expected, larger quantities of ascites were obtained by harvesting multiple times, and this resulted in greater MAb yields (Figure 5A). The increase in volume and quantity obtained with multiple harvests was slightly less than the factorial (2x, 3x) amount of the one harvest yields. The amount of ascites obtained from each cell line were similar when groups of equal harvest numbers were compared (Figure 5A). However, the MAb concentrations obtained from each cell line were significantly different and this impacted the MAb yield (Figure 5 B and C). Additionally, the MAb concentration of subsequent harvests was not affected by prior harvests (Figure 5C). The low
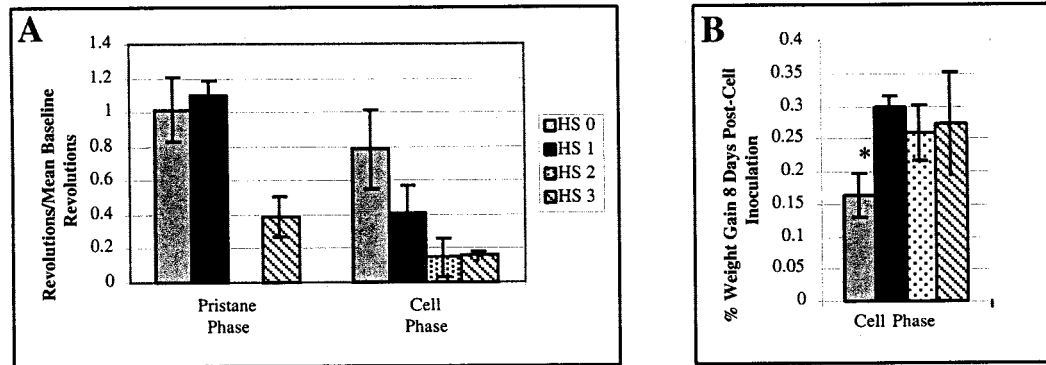
Figure 3: Mice from experiments 3 and 4 grouped according to health status score and compared according to: **(A)** wheel-running activity two days after pristane inoculation and eight days after cell inoculation, and **(B)** percentage of weight gained from day of cell inoculation to eight days after cell inoculation. *Significantly different from HS 1 group.

yield from mice harvested three times in experiment 2 was attributed to an unexplained null yield during the third harvest of two mice. Although the MAb (77.2) produced in experiment 1 and 4 is the same, a different subclone was used to produce the ascites in each case. The difference in MAb yield between these two experiments emphasizes the need to appropriately screen cell lines for high levels of production before introducing them into mice.

Behavioral analysis: To assess whether the ascites method of producing MAb induced anxiety or other behavioral changes in the mice, black box and open-field analyses were conducted. Pristane injection had no effect on any of the behavioral parameters that were analyzed at three or six days after reagent administration in any of the four experiments ($P > 0.30$; data not shown). When the open-field analysis was performed nine days after cell inoculation (prior to harvesting), the exploratory behavior was reduced in the cell-inoculated, compared with the pristane-inoculated group ($P = 0.04$), but the reduction in activity was not significant when compared with that of the PBS-inoculated group ($P = 0.10$; Figure 6C).

The cell-inoculated mice also were more reluctant to come out of the black box and the number of times the mice poked their heads out was reduced; however, these difference were not significant ($P = 0.34$ and $P = 0.10$, respectively; Figure 6 A and B).

Analgesia: To determine whether analgesics could improve the well-being of mice, ascites was allowed to accumulate in two groups of mice until they reached a 25% increase in weight, at which point one group received subcutaneous injections of 0.1 mg of buprenorphine/kg twice each day for three days while the other group received PBS. Two parallel control groups that were not inoculated with hybridoma cells were also included in this study (experiment 4). There were no significant differences in plasma corticosterone concentrations (Figure 4D), wheel-running activity, or health status among the four groups compared (data not shown). Interestingly, use of buprenorphrine may have resulted in decreased corticosterone concentration in control mice, although the differences were not significant.

Discussion

The assessment of well-being in animals requires analysis of a combination of several criteria, which include performance, clinical state, neurochemistry, endocrinology, behavior, immunology, and ethology (15). Several of these criteria were analyzed in this report to provide the most accurate interpretation of the state of mice being used in ascites-generating protocols. In accordance with the

AVMA Panel on Euthanasia, the term "pain" used here refers to the perception of noxious stimuli via the nociceptive pathways (16). In the case of ascites, pain could potentially be caused by abdominal distention or soft tissue tumor infiltration. "Distress" refers to the effect of physical, physiologic, or emotional factors that interfere with an animal's homeostasis (16). Potential causes of distress in the ascitic mouse could be chronic visceral pain or fear derived from actions associated with pain, such as peritoneocentesis. The well-being of an animal is best met when its pain and distress are minimized. For a more comprehensive definition and discussion, the reader is referred to the following references (15, 17–19).

To place this study in the proper perspective, its limitations should be addressed first. Ascites progression and response to stressful stimuli may be influenced by the strain of mouse selected, and these differences were not addressed in this report which involved only 6- to 8-week-old male BALB/c mice. Additionally, the SP2/0 myeloma fusion partner used to create the hybridoma cell-lines of this study was derived from the same laboratory cell stock. If characteristics of this fusion partner predominate, the hybridoma cell lines may share common characteristics, such as growth and invasive properties. Variability may have been introduced by using different hybridoma cell lines and different inoculating doses for each experiment; however, if results from interexperimental subgroups were found to be significantly different, they were not combined for further analysis.

The environmental conditions of the mice may also have influenced results. Rodents are highly social, and individual housing, such as used in this study, may cause some distress. To minimize this effect, mice were allowed a minimum of three days to adapt to individual housing conditions before data collection began. Access to mouse wheels also may have influenced the progression of tumor growth and ascites accumulation. Human studies suggest that exercise has a negative effect on tumor growth (20). Furthermore, there is some debate as to whether exercise modulates the response to pain by stimulating the release of endorphins (21). It would be of further interest to determine whether wheel-running activity can improve the well-being of mice used for ascites production. Although these limitations should be considered in interpretation, the results of this study are logical and consistent among themselves and with those of other recent studies (9, 10).

Clinical observation of pristane-inoculated mice indicated that 60% of the mice may have experienced some mild transient dis-
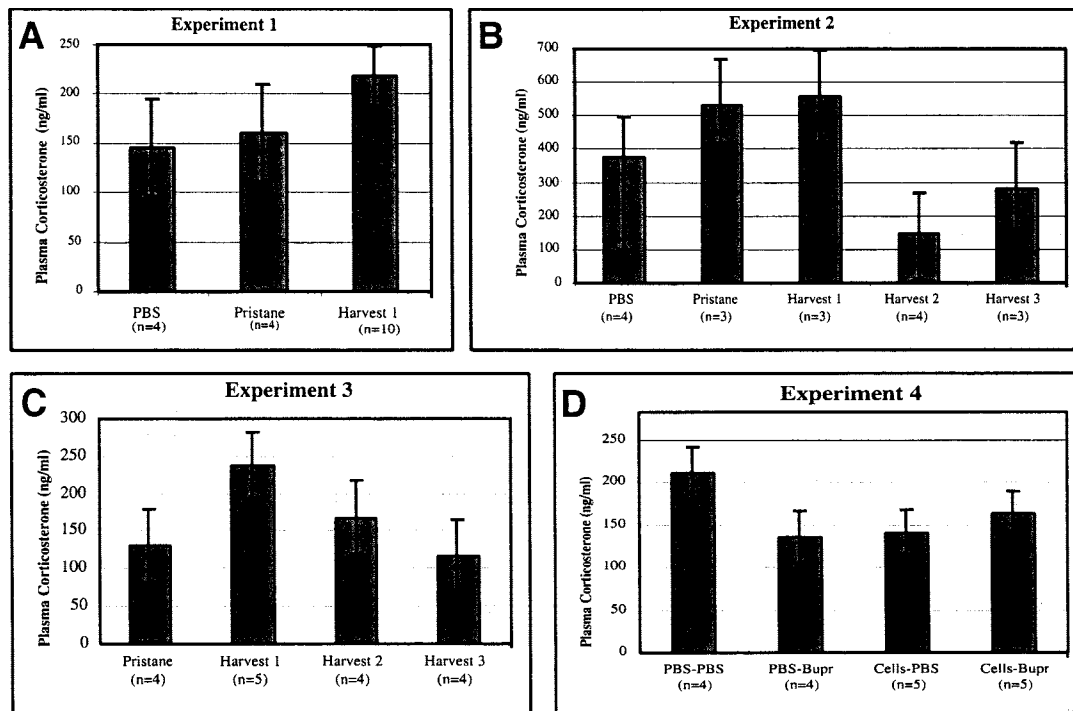
Figure 4: Plasma corticosterone concentrations determined by use of a radioimmunoassay of blood taken at euthanasia.

tress within the first three days of inoculation. Signs were largely restricted to mild unkemptness around the cervical and upper thoracic region (health score of 1). Wheel-running behavior was slightly depressed the second day after pristane inoculation; however, activity levels never reached significance and quickly returned to normal. Body weight, food and water consumption, and behavior analyses were unchanged by pristane inoculation in this study. Amyx (22) suggested that inoculation of a higher dose (0.5 ml) of pristane resulted in weight loss, hunched posture, and reduced activity of mice. However, clinical abnormalities or weight loss were not detected in 0.5-ml pristane-inoculated mice in a study performed by Jackson et al. (10), and in an unpublished blinded study, Gebhart was unable to separate pristan-inoculated mice from PBS-inoculated mice (5).

The dose of 0.2 ml of pristane was sufficient to prime the mice for ascites production. Others have reported no significant difference in ascites production when mice were primed with 0.1, 0.2, or 0.5 ml of pristane (23, 24). Freund's incomplete adjuvant (0.3 ml) has been proposed as a superior priming agent in that it induces fewer indications of distress, and injection of hybridoma cells can be performed earlier (25–27). Regardless of the agent used, care must be taken, as there are some risks associated with incorrect intraperitoneal inoculation that could result in additional abdominal organ lesions and/or hemorrhage (28). In summary, i.p. inoculation of 0.2 ml of pristane induced little or no signs of distress; Freund's incomplete adjuvant may be considered as an alternative priming agent; and the health status of inoculated animals should be well monitored.

Human studies of patients with a variety of diseases (cirrhosis, liver transplants, cancer) indicate that abdominal distention with ascites resulted in adverse effects on the respiratory and cardiovascular systems and negatively affected prognosis (11, 12, 29). In this study, mouse wheels were used as a convenient means

to continuously monitor animal-well being. Change in wheel-running has been used previously as a behavioral indicator of pain associated with subtle visceral organ damage as a result of sublethal explosive blast pressure in rats (30). A disadvantage to this approach is that the increased effort and discomfort associated with abdominal ascites accumulation may also negatively influence running performance. Although weight gain was negatively correlated with running activity in this study, several other factors need to be considered, as weight gain and disease progression also are tightly associated here. The running activity of one pristane-inoculated mouse and two cell-inoculated non-ascitic mice sharply decreased at least 2 days prior to their reaching a morbid state. Additionally, running performance appeared to decrease as clinical assessments became less favorable (Figure 3A). If weight were a predominate factor in determining running activity, one would expect activity levels to increase after harvesting approximately 10% of a mouse's body weight as ascites. This was not the case; in fact, wheel-running activity appeared to decrease more markedly after harvesting. Taken together, along with results of a previous study (30), these observations suggest that decreased wheel-running activity may be associated with distress and/or pain in rodents.

Current recommendations and several guidelines limit the amount of weight gain in an ascites-producing mouse to 20% of its normal body weight (31–33). Significant changes in wheel-running activity in cell-inoculated mice occurred at the point when they gained approximately 25% of their body weight. Some of this weight gain may be attributed to normal growth of the young mice used in these procedures, as controls gained an average of 4% body weight during the ascites phase. Taking this into consideration, the point of significant change in wheel-running behavior approximates current acceptable standards. A decrease in food consumption also suggests that mice were affected near,
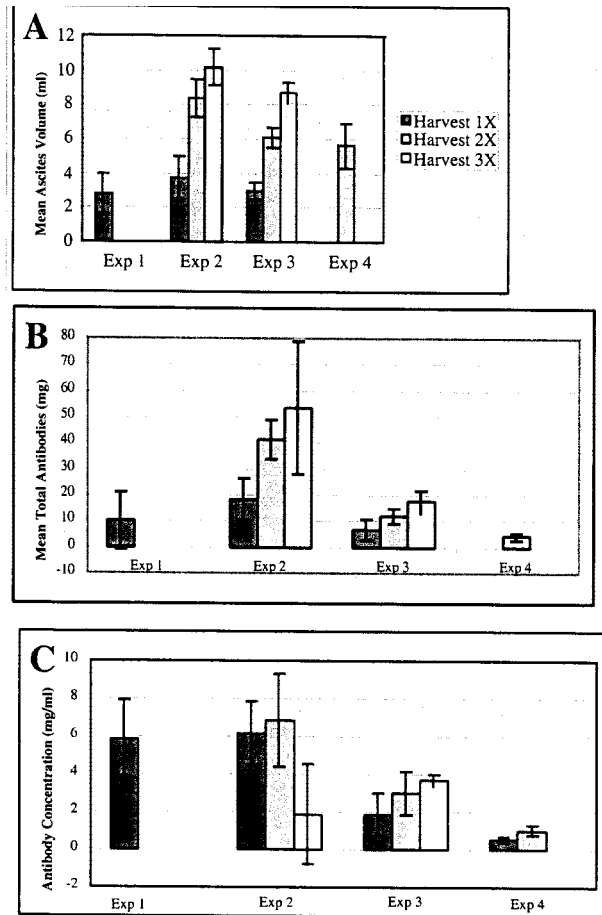
Figure 5: Ascites and antibody quantification. **(A)** Each bar indicates mean cumulative volume of ascites obtained from mice in the specified group. **(B)** Each bar indicates mean cumulative quantity of antibodies obtained from mice in the specified group. **(C)** Each bar represents concentration of the specified harvest of all mice in the designated experiment.

Figure 6: Behavioral analysis. **(A)** Mice were placed in a black box with an opening on one side. The number of times the mouse extended at least its entire head out of the box within a two-minute period was tabulated. *Significantly different from PBS-inoculated group. **(B)** Amount of time, up to two minutes, that it required the mouse to leave the black box was tabulated. **(C)** On leaving the black box, mice enter the open-field box with 15 demarcated squares. The number of squares the mice passed through during a three-minute interval was tabulated. *Significantly different from pristane-inoculated group.

or prior to, this point (Figure 2A). Additionally, clinical signs of distress were significantly more apparent in mice that were 25 to 30% over their baseline body weight (Figure 3B). However, once mice were identified as being clinically affected (health score > 0) at eight days after inoculation, body weight did not differ significantly among mice grouped according to health status scores. This may be because mouse weight was affected by several factors, including tumor cell growth, ascites accumulation, and body mass. It is likely that mice that remain healthy and not distressed more readily gain weight and provide a better environment that is conducive to hybridoma cell growth and ascites production. This postulate is supported by the observation that plasma corticosterone concentration was negatively correlated to weight gain.

Although we did not objectively investigate the minimal point of weight gain at which ascites could be harvested, previous experience indicates that efficient peritoneocentesis before mice reached a 20% increase in weight may be difficult. When the abdomen is not sufficiently swollen, it is more likely that repeated needle sticks or additional probing may be necessary to locate ascites-rich pockets. As a result, animals may incur additional distress and the risk of puncturing visceral organs or blood vessels is increased. To obtain optimal yields with minimal distress, my
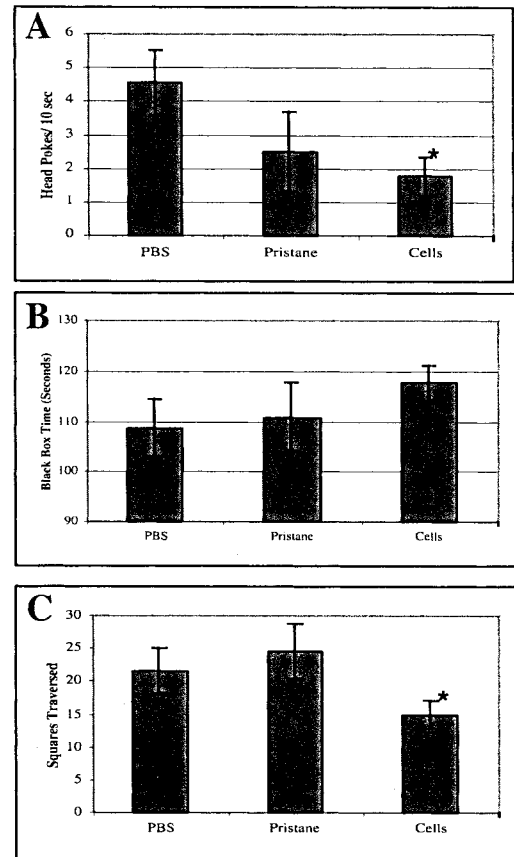
experience indicates that the first harvest in growing mice should be performed when body weight becomes 20 to 25% of baseline body weight. Assuming that approximately 5% of this increase may be due to normal growth, the point at which the first harvest should occur in adult mice may need to be adjusted to 15 to 20% above normal body weight.

To determine whether and when mice used in ascites protocols become distressed, several mice in these studies were allowed to gain substantially more weight than recommended in current guidelines. Although the mice tolerated increases in body weight up to 60%, we do not recommend that this procedure be adopted as a standard for several reasons. As discussed previously, access to exercise and use of less offensive cell lines may have resulted in better health in these mice. Additionally, ascites yields were not markedly improved by delaying the time of the initial harvest, as indicated by comparing respective values of later harvests (experiments 1 and 2) with earlier harvests (experiments 3 and 4). By using 20 to 25% weight gain as a marker to initiate ascites harvesting, comparable ascites yields can be obtained, and mice are placed at lower risk of developing later complications, which may lead to distress.

Institutional guidelines vary according to the number of as-

cites harvests that are recommended, with most ranging from 1 to 3 (5). Results of this study indicate that there were no differences in health score, food consumption, or corticosterone concentration when multiple ascites harvest groups were compared. Human clinical studies indicate that peritoneocentesis provides relief from pain associated with abdominal distention and improves breathing efficiency (11, 12). The fact that mice with unrelieved abdominal distention (cell group 1) often required euthanasia earlier than did those of the other harvest groups suggests that peritoneocentesis benefits mouse survival time. Although comparison of plasma corticosterone concentrations did not reveal any significant difference among groups, some notable trends were observed. Single harvest groups consistently had the highest corticosterone concentrations and multiple harvest groups always had lower values than those of this group, often lower than those of the controls (Figure 4). These results could be explained under the conditions that corticosterone concentration had adjusted to the presence of chronic distress caused by abdominal distention in unharvested animals. Once peritoneocentesis was performed, some of the stressor was removed and glucocorticoid values decreased. Further work is needed to confirm this hypothesis.

The major advantage of harvesting ascites from mice multiple times is that potentially greater MAb yields can be obtained. If, for example, in experiment 2 or 3 (Figure 5), the number of harvests were restricted to one, as opposed to three, approximately 2.5 additional mice would be needed to produce the same amount of MAb. However, in some instances, there may not be a substantial increase in yield on the third harvest (versus two harvests), as in experiment 2, (Figure 5B) and for one of five clones analyzed by Jackson et al. (9). In these situations prior experience with a hybridoma cell line may be necessary to determine whether the number of harvests should be limited to two. Additionally, as implied earlier, optimization of hybridoma cell line clones by selection of high MAb secretors may also reduce the numbers of animals needed.

Although peritoneocentesis may have some benefit to the animal's health, multiple harvests in the ascitic mouse also raise some additional concerns. The increased longevity that multiple harvests may afford the mice is accompanied by a prolonged opportunity in which the ascites-producing tumor cells can grow and invade tissues. This is evidenced by my findings that gastrointestinal tract organ weight increased as the number of harvests increased. Jackson et al. (10) also found that mouse survival decreased with each harvest. On the basis of my experience, here and elsewhere, we found that typically, a four-day regimen (harvest-rest-harvest-harvest) worked best for providing maximal yield and meeting the welfare needs of the animal. During that period, mouse weight may continue to increase by approximately 10% due largely to tumor cell growth (data not shown).

Hypovolemia also should be considered as a potentially harmful effect when multiple harvests are performed. Jackson et al. (10) observed that mice tended to have the most pronounced clinical signs of distress within 30 minutes of harvesting and mortality was highest at that point. Although the mice of the study reported here were not monitored as closely, abnormal clinical signs were not worse at that point; however, mice compensated for bodily fluid loss as evidenced by an increased water consumption in mice that were harvested three times (Figure 2B). Collectively, these results indicate that the well-being of mice

can be positively and negatively affected by peritoneocentesis, and when performed in mice, health status should be monitored closely. Under these circumstances and as long as mice continue to be robust with no signs of pain or distress, it would seem most appropriate to maximize yields by performing three harvests.

Although there was no relationship between health scores in mice and ascites sedimentation in this study, personal experience supports the assumption that mice with clear ascites tend to be healthier than those with bloody or particulate-laden ascites. A negative correlation between the amount of sediment found in ascites harvests and plasma corticosterone concentration upholds this postulation. Additionally, in studies performed by Jackson et al. (9, 10), the group of mice that had the lowest survival rate also had hemorrhagic ascites. Of additional interest, this same group of mice also had the lowest weight gain and poorest MAb yield of the five groups analyzed, which coincides with my observation that healthier mice gain more weight. These results indicate that special attention should be directed to mice that produce hemorrhagic or cloudy ascites, especially if they are not gaining weight.

A change in exploratory behavior or locomotion, as measured in the open-field box, may be an indicator of pain and/or distress in animals (34–36). Technicians also commonly rely upon locomotion and behavioral responses (to prodding) to assess the well-being of animals (personal observation). Thus, this study may provide a somewhat analogous means to quantitatively evaluate these crude measures for assessing the well-being of ascitic mice. Again, increase in body weight of cell-inoculated mice may also affect locomotion and the animal's risk/fear assessment of escaping potential harm. However, a modest weight gain of 7.5% in rats with hepatomegaly induced by a high cholesterol diet had no effect on exploratory behavior (37). In another study performed in lymphoma-inoculated mice by van Loo (38), exploratory behavior decreased as tumors formed. Results for each of the behavioral parameters observed suggest that exploratory behavior decreased in the cell-inoculated group; however, significance was never reached. Perhaps longer intervals of behavioral analysis may have lead to more concrete observations. These observations may suggest that to fully assess the well-being of mice used in MAb producing protocols, short-interval behavioral examination may be insufficient.

Use of buprenorphine had no effect upon the parameters that were analyzed in this assessment of animal well-being. It is likely that the analgesic was ineffective under these circumstances. Van Loo (38) also found that buprenorphine did not sufficiently relieve pain associated with SL2 lymphoma inoculated mice. In a recent study by Gades et al. (39), the longevity of buprenorphine administered to mice was 3 to 5 hours (39). The dosage regimen used in the study reported here may not have been sufficient to significantly reduce pain or cover the period of peak running activity in the middle of the night. Additionally, buprenorphine was administered when mice had already reached a 23% increase in weight. Perhaps additional benefit would have been gained if dosing had started earlier. An interesting relevant finding of Kanarek et al. (21) was that running activity of rats decreased the analgesic properties of morphine, supposedly due to the presence of increased endogenous β-endorphins. This would suggest that endogenous β-endorphins may serve as an agonist-antagonist and that their release, as a result of wheel-running, may have some analgesic properties.

Administration of buprenorphine could potentially be harm-

ful in this situation through its suppressive effects on the cardiovascular system, and these side effects should be considered before analgesics are administered to an ascitic mouse. However, additional studies using different agents, regimines, or doses may lead to development of more suitable pain-relieving approaches for in vivo MAb production protocols.

This study has taken a refinement approach to producing MAbs in mice, as mentioned previously, these approaches should find use only if the in vitro methods have proven to be unsuccessful in the production of a specific MAb. If the use of animals for ascites production has been justified, the following recommendations, based on the findings presented here, are suggested: 1) procedures should be performed by skilled individuals, who are familiar with the recognition of pain in mice, and preferably an institutional veterinarian should be involved; 2) a maximum of 0.2 ml of pristane should be sufficient to prime mice; 3) animals should be monitored and weighed on the day of cell inoculation and at least daily, beginning no later than the fifth day after inoculation; 4) healthy robust mice can be harvested up to three times, as long as the initial harvest occurs when body weight is between 20 and 25% of baseline (if adult mice are used, a 20% gain above normal weight may be more appropriate), thereafter, body weight should not exceed 30%, and a four-day period from the time of first to last harvest should be targeted; 5) mice that are slow at gaining weight or have hemorrhagic/cloudy ascites should be monitored closely and considered for euthanasia.

Acknowledgments

References

1. **Hendriksen, C. F. M.** 1998. A call for a European prohibition of monoclonal antibody production by the ascites procedure in laboratory animals. ATLA **26**:523–540.
2. **American Anti-Vivisection Society.** Petition requesting the National Institutes of Health to prohibit the use of animals and implement non-animal alternatives in the production and use of monoclonal antibodies. April 23, 1997.
3. **Marx, U., M. J. Embleton, R. Fischer, *et al*.** 1997. Monoclonal antibody production: The report and recommendations of ECVAM workshop 23. Alternative to Laboratory Animals: ATLA **25**: 121–137.
4. Alternatives in monoclonal antibody production. A Workshop of The John Hopkins Center for Alternatives to Animal Testing and the Office of Protection from Research Risks, NIH. September 24, 1997. Baltimore.
5. **National Research Council.** 1999. Monoclonal antibody production. Proceedings of the Production of Monoclonal Antibodies Workshop. National Academy Press, Washington.
6. **Proceedings of the Production of Monoclonal Antibodies Workshop.** 1999. Bologna, Italy, August 29, 1999.
7. **Animal Welfare Act.** 1970. Public Law 91–579.
8. **National Research Council.** 1996. Guide for the Care and Use of Laboratory Animals. National Academy Press, Washington.
9. **Jackson, L., L. Trudel, J. Fox, *et al*.** 1999. Monoclonal antibody production in murine ascites II. Production characteristics. Lab. Anim. Sci. **49**:81–86.
10. **Jackson, L., L. Trudel, J. Fox, *et al*.** 1999. Monoclonal antibody production in murine ascites. I. Clinical and pathologic features. Lab. Anim. Sci. **49**:70–80.
11. **Gotleib, W., B. Feldman, O. Feldman-Moran, *et al*.** 1998. Intraperitoneal pressure and clinical parameters of total palliation of symptomatic ascites in ovarian cancer. Gynecol. Oncol. **71**:381–385.
12. **Chang, S., H. Chang, F. Chen, *et al*.** 1997. Therapeutic effects of diuretics and paracentesis on lung function in patients with non-alcoholic cirrhosis and tense ascites. J. Hepatol. **26**: 833–838.
13. **Angueira, C., and S. Kadakia.** 1994. Effects of large-volume paracentesis on pulmonary function in patients with tense cirrhotic ascites. Hepatology **4**:825–828.
14. **Horton, R., P. Karachunski, S.-A. Kellerman, *et al*.** 1995. Simple, inexpensive computerized rodent activity meters. BioTechniques **19**:594–597.
15. **Clark, J., D. Rager, and J. Calpin.** 1997. Animal well-being III. An overview of assessment. Lab. Anim. Sci.. **47**:580–585.
16. 1993. Report of the AVMA Panel on Euthanasia. J. Am. Vet. Med. Assoc. **202**:229–249.
17. **Clark, J., D. Rager, and J. Calpin.** 1997. Animal well-being I. General considerations. Lab. Anim. Sci. **47**:564–570.
18. **Clark, J., D. Rager, and J. Calpin.** 1997. Animal well-being II. Stress and distress. Lab. Anim. Sci. **47**:571–579.
19. **Clark, J., D. Rager, and J. Calpin.** 1997. Animal-well being IV. Specific assessment criteria. Lab. Anim. Sci. **47**:586–597.
20. **Woods, J.** 1998. Exercise and resistance to neoplasia. Can. J. Physiol. Pharmacol. **76**: 581–588.
21. **Kanarek, R., A. Gerstein, R. Wildman, *et al*.** 1998. Chronic running-wheel activity decreases sensitivity to morphine-induced analgesia in male and female rats. Pharm. Biochem. Behav. **61**:19–27.
22. **Amyx, H. L.** 1987. Control of animal pain and distress in antibody production and infectious disease studies. J. Am. Vet. Med. Assoc. **191**:1287–1290.
23. **Brodeur, B., P. Tsang, and Y. Larose.** 1984. Parameters affecting ascites tumour formation in mice and monoclonal antibody production. J. Immunol. Methods **71**:265–272.
24. **Hoogenraad, H., T. Helman, and J. Hoogenraad.** 1983. The effect of pre-injection of mice with pristane on ascites tumor formation and monoclonal antibody production. J. Immunol. Methods **61**:317–320.
25. **Gillette, R.** 1987. Alternatives to pristane priming for ascitic fluid and monoclonal antibody production. J. Immunol. Methods **99**: 21–23.
26. **Mueller, U., C. Hawes, and W. Jones.** 1986. Monoclonal antibody production by hybridoma growth in Freund's adjuvant primed mice. J. Immunol. Methods **87**:193–196.
27. **Jones, S., J. Cos, and J. Pearson.** 1990. Increased monoclonal antibody ascites production in mice primed with Freund's incomplete adjuvant. J. Immunol. Methods **129**:227–231.
28. **Walvoort, N.** 1991. Assessment of distress through pathological examination. p. 265–271. *In* C. F. M. Hendriksen and H. B. W. M. Koeter (ed.), Replacement, Reduction, and Refinement: Present Possibilities and Future Prospectives, Elsevier Publications, Amsterdam.
29. **Cirera, I., M. Navasa, A. Rimola, *et al*.** 2000. Ascites after liver transplantation. Liver Transpl. **6**:157–162.
30. **Bauman, R., N. Elsayed, J. Petras, *et al*.** 1997. Exposure to sublethal blast overpressure reduces the food intake and exercise performance of rats. Toxicology **121**:65–79.
31. **Canadian Council on Animal Care.** 1986. Guidelines on: Antibody production.
32. **The Animals (Scientific Procedures) Act of l986.** Home Office, United Kingdom.
33. **Smith, J., and L. DeTolla.** 1999. An IACUC Guide to Reviewing Protocols Calling for Monoclonal Antibody Production by Mouse Ascites. Lab Animal: Special Supplement—Small Scale Monoclonal Antibody Production. Autumn: 12–17.
34. **Aloisi, A., M. Albonetti, and G. Carli.** 1994. Behavioral effects of different intensities of formalin pain in rats. Physiol. Behav. **58**:603–610.
35. **Van Dijken, H., J. Van der Heyden, J. Mos, *et al*.** 1992. Inescapable footshocks induce progressive and long-lasting behavioural changes in male rats. Physiol Behav. **51**:787–794.

36. **Arnsten, A., C. Berridge, and D. Segal.** 1985. Stress produces opioid-like effects on investigatory behavior. Pharmacol. Biochem. Behav. **22**:803–809.

37. **Beynen, A., V. Baumans, A. Bertens,** *et al.* 1988. Assessment of discomfort in rats with hepatomegaly. Lab. Animals **22**: 320–325.

38. **van Loo, P., L. Everse, M. Bernsen,** *et al.* 1997. Analgesics in mice used in cancer research: Reduction of discomfort? Lab. Animals **31**:318–325.

39. **Gades, N., P. Danneman, S. Wixson,** *et al.* 2000. The magnitude and duration of the analgesic effect of morphine, butorphanol, and buprenorphine in rats and mice. Contemp. Topics Lab. Anim. Sci. **39**:8–13.

Monoclonal Antibody Production in Murine Ascites
I. Clinical and Pathologic Features

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Background and Purpose: **Murine ascites production has been associated with appreciable morbidity and mortality, thus raising animal-welfare concerns. To address these concerns, the clinicopathologic changes associated with in vivo production of monoclonal antibodies in mice were characterized, and results were compared among cell lines.**

Methods: **Five hybridoma cell lines were grown in groups of 20 mice. Fourteen days prior to inoculation with 10^6 hybridoma cells, mice were primed with 0.5 ml of pristane given intraperitoneally; 12 mice were sham treated (controls). Ascites fluid was collected a maximum of three times by abdominal paracentesis. Clinical observations and pre- and postabdominal tap body weights were recorded. Necropsies were performed on all mice.**

Results: **For all groups combined, overall survival to tap 1 was 98%, to tap 2 was 96%, and to tap 3 was 79%; survival among groups ranged from 90 to 100% for tap 1, 85 to 100% for tap 2, and 35 to 100% for tap 3. Disseminated intra-abdominal seeding with irregular soft tissue and/or solid tumor masses was observed at necropsy.**

Conclusions: **Significant clinicopathologic changes were associated with monoclonal antibody production in mice, and differences between various hybridoma cell lines were apparent.**

Many developments have emerged in the production and application of monoclonal antibodies since Köhler and Milstein first described the technique for producing hybridoma cells that secrete monoclonal antibodies (MAbs) (1, 2). Murine ascites production has been the time-honored technique for producing small-scale, research laboratory quantities of MAbs (3–5). An ascitogenic priming agent is administered intraperitoneally to mice, followed by inoculation with hybridoma cells. Ascitic tumors form in the abdominal cavity of the mouse and produce ascites fluid that contains a high concentration of MAb secreted by the hybridoma cells. This antibody-rich ascitic fluid is harvested by paracentesis, processed, and purified to obtain the antibody.

Appreciable morbidity and mortality may be associated with this procedure (4), and animal-welfare concerns have been raised regarding the potential for various aspects of this procedure to induce pain and/or distress in the animals (5–7). As a result of these concerns, several European countries have established guidelines or regulations that restrict or prohibit ascites production in rodents (6, 8, 9), and attention from animal-welfare organizations, the scientific community, and regulatory agencies is increasing

in the United States as well (10, 11). Although information in the literature regarding antibody production is considerable, little information addresses the clinicopathologic changes associated with this procedure or correlation of these changes with production parameters in mice. As veterinarians and others concerned with the welfare and humane care of animals used in research, we sought to characterize the clinicopathologic changes in mice associated with ascites production, and to correlate specific experimental manipulations with observed morbidity and mortality. Our intent was to develop recommendations for handling these animals in a manner that would minimize the potential for pain and/or distress associated with this procedure.

Materials and Methods

Hybridoma cell lines: Five hybridomas were evaluated (Table 1); five mice groups (n = 20 each) were named for the hybridoma cell line with which they were inoculated. These cell lines were selected in part because of the need for particular MAbs for experimental use, but also in an attempt to choose hybridomas representative of varied plasmacytoma fusion partners, varied isotypes of secreted antibody to include IgM and IgG, different subclasses of IgG, and mouse x mouse and rat x mouse hybridomas. Hybridomas 2B11 (12), 3C9 (13), 2C6D9, and 3D6 (14) were generously provided (Dr. Gerald Wogan, MIT Division of Toxicology, Cambridge, Mass.), as was hybridoma RMK (Ann Marshak-Rothstein, Boston University, Boston, Mass.).

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Table 1. Hybridoma cell lines

Cell line	Antibody	Plasmacytoma	Isotype
2B11	Anti-aflatoxin	Sp2/0-Ag14	IgM
3C9	Anti-MeIQx	P3-X63Ag8	IgG_{2b}
2C6D9	Anti-BSA	Sp2/0-Ag14	IgG_1
3D6	Anti-4-ABP	Sp2/0-Ag14	IgG_1
RMK	Rat anti-mouse K	Sp2/0-Ag14	IgG_{2a}

Cell line designation, antibody specificity, plasmacytoma fusion partner, and antibody isotype and subclass are presented for each cell line. All cell lines are BALB/c mouse x mouse hybridomas except RMK, which is a rat x mouse heterohybridoma.

MeIQx = 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; BSA = bovine serum albumin; 4-ABP = 4-aminobiphenyl; K = kappa light chain of mouse immunoglobulin

Animals: Each mouse x mouse hybridoma was grown as ascitic tumors in 20 CAF1/J male pathogen-free mice (The Jackson Laboratory, Bar Harbor, Maine). An additional 12 CAF1/J mice served as sham controls. The rat x mouse heterohybridoma was grown as ascitic tumors in 20 Fox Chase SCID, C.B-17/IcrTac-scidDF male pathogen-free mice (Taconic Farms, Germantown, N.Y.). An additional 12 SCID mice served as sham controls.

Animals were maintained in a facility that has animal care and use programs approved by AAALAC, International. Animals were 7 weeks of age at receipt and were housed four per cage in polycarbonate shoebox cages with wire bar lids and filter tops (Micro-barrier; Allentown Caging, Allentown, N.J.). Laboratory-grade pine shavings (Northeastern Products Corp., Warrensburg, N.Y.) were used for bedding. The CAF1 mice were fed pelleted rodent chow (Prolab 3000; Agway, Syracuse, N.Y.), and fresh distilled water in bottles was available ad libitum. The SCID mice were fed autoclaved pelleted rodent chow (Prolab 3500; Agway), and autoclaved distilled water in bottles was available ad libitum. Cages and bedding were also autoclaved prior to use for SCID mice. A 12-h light/dark cycle was maintained.

Animal husbandry was performed according to the standard operating procedures of the institution and in conformance with applicable guidelines. Husbandry procedures and experimental manipulations with SCID mice were conducted in a positive-flow horizontal laminar mass air displacement unit (EdgeGARD; Baker Co., Sanford, Maine), using aseptic technique.

All mice were held for a minimum of 6 days after arrival to allow for acclimation. Animals were then randomized into groups by body weight and were identified by ear punch. The protocol for animal use was approved by the MIT Committee on Animal Care.

Pristane priming: On study day –14, each test mouse received a single intraperitoneally (i.p.) administered 0.5-ml dose of pristane (2,6,10,14-tetramethylpentadecane; Sigma Chemical Co., St. Louis, Mo.). Animals in the control groups received a single i.p. administered 0.5-ml dose of saline (0.9% NaCl, USP; Abbott Laboratories, North Chicago, Ill.). Injections of pristane or saline were administered into the caudal left quadrant of the ventral portion of the abdomen, using sterile syringes and 25-gauge 5/8-in needles.

Bacterial culture of pristane: Bottles of pristane maintained under routine use and storage conditions were evaluated for sterility. Samples were obtained, using aseptic technique, from four bottles of pristane that were currently in use in the animal facilities. Aerobic and anaerobic bacterial cultures were performed. For aerobic culture, 1 ml of pristane was inoculated into 5 ml of tryptic soy broth (Remel, Lenexa, Kans.) and was incubated for 18 to 24 h at 35°C. Broth samples were then plated on blood agar/MacConkey agar biplates (Remel) and incubated for 18 to 24 h at 35°C.

For anaerobic culture, 1 ml of pristane was plated on blood agar/MacConkey agar biplates and incubated for 46 to 48 h at 35°C in an anaerobic jar with a gas pack (BBL Gas Pak Plus; Becton Dickinson, Cockeysville, Md). All samples were protected from light.

Hybridoma cell inoculation: Hybridoma cells were maintained frozen in liquid nitrogen in a solution composed of 95% fetal bovine serum (Endlo; JRH Biosciences, Lenexa, Kans.) and 5% dimethyl sulfoxide (Sigma Chemical Co). Cells were defrosted and grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g of glucose/L (GIBCO, Grand Island, N.Y.), supplemented (10 ml/L) with 10 mM MEM non-essential amino acid solution and 200 mM L-glutamine (GIBCO) for a final concentration of 6 mM L-glutamine. Fetal bovine serum was added to 20% (vol:vol) final concentration. Cells were expanded in static culture to provide >20 x 10^6 cells in logarithmic growth phase for each group of mice. Prior to inoculation in mice, antibody secretion into the cell culture supernatant was verified for each hybridoma cell line by use of an enzyme-linked immunosorbent assay (ELISA [15]).

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

Abdominal paracentesis: Abdominal paracentesis was performed when moderate abdominal distention was visible. Paracentesis was performed before exceeding an approximate 20% increase in body weight from day 0, in accordance with institutional and United Kingdom Coordinating Committee on Cancer Research (UKCCCR) guidelines (8, 16). Abdominal taps were performed every 1 to 3 days on the basis of clinical appearance of each mouse and the rate of ascites production, as assessed by degree of abdominal distention. Abdominal paracentesis was performed a maximum of three times for each mouse.

The animals were manually restrained for abdominal paracentesis, which was performed aseptically, using a sterile 18-gauge, 1.5-in needle inserted into the peritoneal cavity through the left lateral abdominal wall. Ascites fluid was collected via gravity flow by permitting the fluid to drip from the hub of the needle, and directly from the paracentesis site, into a sterile centrifuge tube. Gentle digital pressure was applied to the abdomen, and the position of the mouse was altered as needed to facilitate removal of

the ascites fluid. The total volume of fluid collected and the day of collection were recorded for each tap for each mouse.

Abdominal paracentesis was also performed for mice in the control groups. Paracentesis was performed for all control mice three times during a time interval approximating that for the abdominal taps performed for mice of the test groups (days 15, 17, and 19 for CAF1 mice; days 14, 17, and 19 for SCID mice). Abdominal paracentesis was performed as previously described. The mice were restrained in a manner similar to and for a period approximately equal to that necessary to collect ascites fluid from test mice.

Clinical observations: All mice were observed at least once daily beginning on day −14 after administration of pristane. During the time interval when abdominal taps were performed, mice were observed at least twice daily (morning and night), to include observations immediately after abdominal taps. Clinical parameters evaluated included general appearance, character of the coat, posture, activity level, and assessment of the degree of abdominal distention.

Body weight measurements: Body weight was recorded for each mouse on study day −14 prior to pristane injection; on day −7; on day 0 prior to hybridoma cell inoculation; on days 2, 4, 7, and 9; and daily thereafter until study completion. On days when paracentesis was performed on test mice, before- and after-tap body weight measurements were recorded.

Euthanasia: During the study, any mouse with persistent, severe clinical abnormalities that were interpreted to be indicative of pain or distress or were suggestive that the mouse might not survive to the next observation period was euthanized. Mice surviving to the third abdominal tap were euthanized just prior to the paracentesis procedure. Six mice from each control group were euthanized at the time the last test mice were euthanized. Euthanasia was performed, using CO_2 in accordance with accepted guidelines (17).

Necropsy and histologic examination: Complete gross necropsy was performed on all mice of test groups and on six mice of each control group. Organs and tissues were examined in situ, then were dissected free and fixed in neutral-buffered 10% formalin. Representative tissues from selected animals were processed for microscopic examination. Organs and tissues were trimmed, processed by standard methods, and sectioned at 5-μm thickness. Slides were prepared, stained with hematoxylin and eosin, and examined microscopically.

Statistical analyses: Unpaired t tests were used to compare mean body weights between pristane-treated and control mice on days −14, −7, and 0. Differences among mean body weight changes and mean percentage changes in body weight between day 0 and the first abdominal tap for mice of the five test groups were tested for statistical significance, using a one-way repeated measures analysis of variance (ANOVA), followed by pairwise comparisons among group means, using the Newman-Keuls test (18). Differences among means for abdominal tap days were tested for significance, using a one-way ANOVA, followed by pairwise comparisons among group means, using the Newman-Keuls test.

For comparison of mean body weights among test and control mice for each tap, control mean weights used were those for the mean study day on which the tap was performed for the test group. Control group weights were compared with the post-tap weights for the test groups because these weights were considered to more accurately reflect actual body weights of the mice. Mean body weights were tested for significance at each tap, using unpaired t tests. Mean and percentage changes in pre- and post-tap body weights among treated groups were tested for significance, using a one-way ANOVA, followed by pairwise comparisons among group means, using the Newman-Keuls test. Comparisons of mean and mean percentage changes in body weight among taps within groups were made, using a one-way repeated measures ANOVA, followed by pairwise comparisons between taps, using the Newman-Keuls test. Statistical significance was set at $\alpha = 0.05$. Data are presented as mean, with SD as a measure of dispersion.

Results

Bacterial culture of pristane: Aerobic and anaerobic culture of samples submitted from the four bottles of pristane did not yield bacterial growth.

Time intervals for abdominal taps: The time intervals for abdominal taps are presented in Figure 1. Significant differences were observed within and between groups in onset and rate of development of ascites and, consequently, the range of days during which abdominal taps were performed. For groups 2B11 and 2C6D9, development of ascites was relatively synchronous among mice within each group, and consequently, the days on which abdominal taps 1, 2, and 3 were performed were nearly the same for all mice in each group. The range of days during which each tap was performed was progressively greater for groups 3C9, 3D6, and RMK, respectively, indicating greater variability among mice in the time of ascites development and, consequently, the time of abdominal taps. Comparison of mean tap days between groups for taps 1, 2, and 3 revealed significant differences, with the exception of values for groups 3C9 and RMK, which were not significantly different from each other.

Clinical observations: Incidence of clinical abnormalities in all groups of mice are presented in Table 2. All mice of the control groups remained clinically normal throughout the study. (One SCID control mouse was euthanized after accidental trauma during the acclimation period.) Clinical abnormalities were not observed in any test mice during the 2-week period after administration of pristane. One mouse of group 2B11 was euthanized (day 9), and one mouse of group 2C6D9 was found dead (day 11) after hybridoma cell inoculation and prior to any abdominal taps. Both mice had palpable caudal abdominal masses. Clinical abnormalities observed in mice during ascites production were similar among groups, yet differed in rapidity of progression, severity, and incidence of specific abnormalities.

The onset of clinical abnormalities in all test groups was generally related to the development of ascites, as determined by visible abdominal distention and increasing body
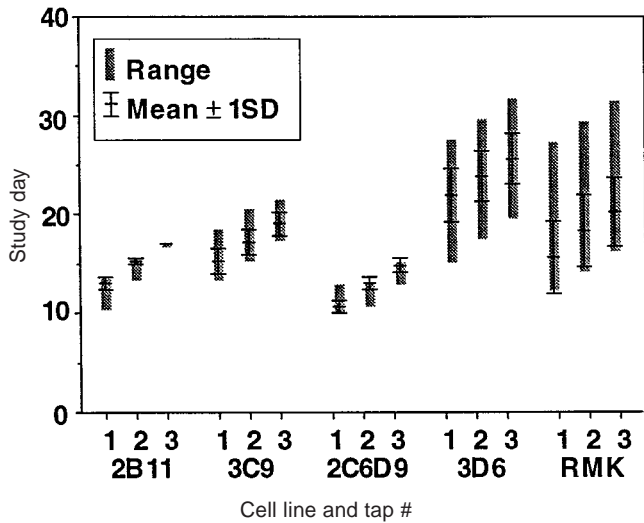
Figure 1. Time intervals for abdominal tap, represented by the range of days during which abdominal taps 1, 2, and 3 were performed for each group. Mean ± 1 SD for each tap interval are presented.

weight. The range of days over which the initial abdominal tap was performed reflected the time of initial ascites development for mice in each group (Figure 1). Initial clinical abnormalities in all groups included rough coat, hunched posture, and progressively increasing abdominal distention. The severity of clinical abnormalities increased progressively over time, and as the number of abdominal taps increased, for mice of all groups. Decrease in activity, palpable abdominal masses, thin appearance, and evidence of dehydration, as determined by decreased skin turgor and enophthalmos, were most often observed later in the course of study.

Appreciable clinical abnormalities were observed during the period immediately after abdominal paracentesis (generally ≤30 min from the time of the tap). These abnormalities were observed in some mice of all groups and included rough coat, hunched posture, decreased activity often associated with the observation of mice huddling together in the cage, tachypnea, and in some instances dyspnea, and pallor that was most evident on the muzzle and ears. These signs were most often transient and mild to moderate in severity, but in 19 mice from four of the five test groups, these signs were persistent and severe, leading to death (n = 5) or euthanasia (n = 14) of mice after the first or second abdominal tap. Twelve of these 19 mice were from group 2C6D9.

Although there was variability between mice and for different taps within groups, the ascites fluid obtained from mice of group 2C6D9 was often markedly hemorrhagic. Ascites fluid collected from mice of group RMK was generally clear. Ascites fluid collected from other groups was generally mildly to moderately hemorrhagic. Persistent leakage of ascites fluid from the abdominal tap site was observed only in SCID mice from group RMK. The leakage of ascites fluid usually resolved within 2 days after abdominal tap.

Small subcutaneous soft tissue nodules were visible, and

palpable in some mice of all groups, at the caudal left ventral portion of the abdomen, the site of i.p. administration of pristane on day −14, and hybridoma cells on day 0. Nodules observed after pristane administration were apparent in eight mice of four groups, and were first observed on study day 0 prior to hybridoma cell administration. The nodules were raised, and size ranged from 2 to 6 mm in diameter. In five of the eight mice, size of nodules decreased or nodules became inapparent within 7 to 14 days from the initial observation. In two mice, size of the nodules slightly increased, and in one mouse, size of the nodule remained unchanged. Nodules observed after hybridoma cell administration were first observed 13 to 20 days later in 14 mice of three groups. The nodules were raised, and size ranged from 2 to 5 mm in diameter. Nodule size increased over time in 9 of 14 mice. Change in nodule size over time was not observed for the remaining five mice.

Noticeable differences observed between groups included high incidence of subcutaneous soft tissue masses at the hybridoma injection site in group-3C9 mice; rapid onset of ascites production and rapidly progressive and severe clinical abnormalities in group-2C6D9 mice, as indicated by the highest incidence of mortality and euthanasia prior to the third paracentesis; slowly progressive and mild clinical abnormalities in group-RMK mice, as indicated by 100% survival to the third paracentesis; persistent leakage of ascites fluid from the abdominal paracentesis site in group-RMK mice; and failure of ascites to develop in two group-3D6 mice, which remained clinically normal throughout the study.

The number and percentage of mice that were tapped, as an index of survival, for all groups for taps 1, 2, and 3 ranged from 90 to 100%, 85 to 100%, and 35 to 100%, respectively. Overall survival to tap 1 was 98%, to tap 2 was 96%, and to tap 3 was 79%. Detailed data can be found in an accompanying report (15).

Body weight measurements: *Days −14 through 0:* Differences in mean body weights were not significant for the CAF1 control versus pristane-treated CAF1 mice or the SCID control versus pristane-treated SCID mice at days −14, −7, and 0.

Day 0: Comparisons of group mean body weights at day 0 indicated that mean body weight for the SCID mice was significantly lower than mean body weight for the CAF1 mice ($P = 0.0001$); therefore, body weight comparisons were not made between CAF1 and SCID mice.

Day 0 to initial tap: Mean body weight and mean percentage increase in body weight between day 0 and initial abdominal tap for each group are presented in Table 3. Mean percentage increase in body weight was significantly greater for mice of group RMK ($P = 0.0001$), and was significantly less for mice of group 2C6D9 ($P = 0.0001$) in comparison with values for other groups. Mean percentage increase in body weight between day 0 and the before tap weight at initial abdominal tap for all groups combined was 17.14%.

Day 0 through the end of study: Comparisons of mean body weights between test and control groups at each tap and percentage decrease in body weight for test groups at each tap are presented in Table 4.

Group 2B11: Because development of ascites and, conse-

quently, days of abdominal taps were relatively synchronous among mice of this group, mean body weights for this test group and the corresponding control group have been graphed over time, to include mean body weights before and after tap for the test group at taps 1, 2, and 3 (Figure 2). Body weights for the test group, relative to the control weights, began to increase on day 10, continued to increase, then reached a plateau between day 11 and initial abdominal tap on day 13. Mean percentage decrease in body weight was significantly greater at initial tap in comparison with taps 2 and 3 ($P = 0.0002$). There was no significant difference between post-tap 1 weight for the test group and control weight. Post-tap weights for the test group were significantly lower than the control weights after taps 2 ($P = 0.0040$) and 3 ($P = 0.0111$). Likewise, post-tap

Table 2. Incidence of clinical abnormalities in mice with ascites

| | CAF1 mice | | | | | SCID mice | |
	Control	2B11	3C9	2C6D9	3D6	Control	RMK
No. of mice	12	20	20	20	20	11	20
Daily observations							
Clinically normal	12	0	0	0	2[a]	11	0
Subcutaneous mass at injection site after pristane	0	4	1	2	0	0	1
Subcutaneous mass at injection site after hybridoma	0	0	10	0	3	0	1
Rough coat	0	20	20	20	18	0	20
Hunched posture	0	20	20	20	18	0	20
Decreased activity	0	3	8	18	12	0	5
Palpable abdominal mass	0	1	6	0	6	0	0
Thin appearance	0	4	3	1	2	0	1
Dehydration	0	3	0	18	1	0	0
Animals euthanized prior to taps	0	1	0	0	0	0	0
Animals died prior to taps	0	0	0	1	0	0	0
Abdominal distention	0	19	20	19	18	0	20
Animals tapped	0	19	20	19	18	0	20
Animals euthanized posttap 1	0	0	0	1	0[a]	0	0
Animals died posttap 1[b]	0	0	0	1	0	0	0
Animals euthanized posttap 2	0	4	1	7	1	0	0
Animals died posttap 2[b]	0	0	1	3	0	0	0
Animals euthanized at tap 3	0	15	18	7	17	0	20
Observations during posttap period							
Rough coat	0	19	10	19	5	0	0
Hunched posture	0	19	17	19	6	0	10
Decreased activity	0	19	17	19	8	0	9
Tachypnea	0	19	4	19	3	0	11
Pallor	0	19	7	19	10	0	3
Ascites leak from tap site	0	0	0	0	0	0	9

For each clinical observation listed, incidence is presented as the total number of animals in each group that were observed to have the indicated clinical sign. Detailed descriptions of each clinical observation are provided in the text.
[a]Two mice of group 3D6 did not develop ascites and remained clinically normal.
[b]Mice died within 30 min after abdominal tap.

weights in group-2B11 mice were significantly lower after taps 2 and 3, compared with tap 1 ($P = 0.0001$).

Group 3C9: Mean percentage decreases in body weight were not significantly different for taps 1, 2, and 3. There were no significant differences between post-tap weights for the test group and the corresponding control weights; however, post-tap body weights significantly and progressively decreased in group-3C9 mice from taps 1 through 3 ($P = 0.0001$).

Group 2C6D9: Mean percentage decrease in body weight decreased significantly from the first through the third abdominal tap ($P = 0.0001$). There was no significant difference between post-tap 1 weight for the test group and control group weight. Post-tap weights for the test group were significantly lower than control weights after taps 2 ($P = 0.0011$) and 3 ($P = 0.0038$). Likewise, post-tap weights in group-2C6D9 mice were significantly lower after taps 2 and 3 in comparison with weights after tap 1 ($P = 0.0001$).

Group 3D6: Mean percentage decreases in body weight were not significantly different for taps 1, 2, and 3. There were no significant differences among post-tap weights for the test group and the corresponding control weights; however, post-tap body weights significantly decreased within the test group from tap 1 to tap 2 ($P = 0.0003$).

Group RMK: Mean percentage decreases in body weight were significantly greater for taps 1 ($P = 0.0004$) and 3 ($P = 0.0004$) in comparison with tap 2. There were no signifi-

cant differences between post-tap weights for the test group and the corresponding control weights after tap 1; however, post-tap body weights for the test group were significantly greater than the corresponding control weights after taps 2 ($P = 0.0040$) and 3 ($P = 0.0047$). Likewise, post-tap weights in group-RMK mice were significantly greater at taps 2 ($P = 0.0001$) and 3 ($P = 0.0001$) in comparison with weights at tap 1.

Correlation between body weights and clinical observations: In examination of individual mice data, definitive correlation could not be made between the magnitude of the percentage decrease in body weight at the time of abdominal taps and presence or absence of post-tap clinical abnormalities, such as pallor, tachypnea, hunched posture, decreased activity, or death.

Gross and microscopic lesions: Incidence of gross lesions observed in all groups of mice at necropsy are presented in Table 5. Significant gross lesions were not observed in CAF1 or SCID control mice.

Lesions in the abdominal cavity: Hemoperitoneum was frequently observed in all groups of CAF1 mice. Hemoperitoneum was observed in only one SCID mouse of group RMK, and an additional three mice from this group had clear ascitic fluid in the abdominal cavity at the time of necropsy.

With the exception of one mouse from group 2B11 and one mouse from group 2C6D9, all test mice examined from all groups had disseminated tumor and/or solid tumor

Table 3. Change in body weight from study day 0 to tap 1

Group	Day 0	Tap 1	% Increase
2B11	30.26 ± 1.73	34.79 ± 2.27	15.00 ± 3.46
3C9	29.71 ± 2.00	35.39 ± 2.11	19.24 ± 4.27
2C6D9	30.75 ± 2.35	33.86 ± 3.36	10.11 ± 7.66
3D6	29.65 ± 1.93	34.85 ± 2.78	17.59 ± 6.36
RMK	24.57 ± 2.07	30.41 ± 3.04	23.77 ± 6.27

Mean (± 1 SD) body weights (g) and mean (± 1 SD) percentage increase in body weight between day 0 and the before-tap weight at initial abdominal tap are presented for each group.

masses in the abdominal cavity. The disseminated tumor was characterized by intra-abdominal seeding with multi-lobulated, nodular to irregular, reddish to tan-white soft tissue, principally involving the mesentery from stomach to rectum, and involving dorsal lumbar, perirenal, peritesticular, and caudal abdominal/cranial pelvic areas with an apparent tropism for abdominal fat (Figure 3). Solid tumors (Figure 4) were characterized as distinct and generally larger soft tissue masses, and often were associated with the diffusely seeded tumor. Size of solid tumor masses ranged from approximately 0.5 x 0.5 x 0.5 cm to 3.5 x 2.2 x 1.5 cm. The most common location of solid tumors was the left caudal, ventrolateral portion of the abdomen, which was the site of hybridoma cell inoculation. Tumors were seldom attached to the peritoneum. Solid tumor masses in the peritesticular fat also were frequently observed.

Microscopic examination of tissues from representative mice demonstrated accumulations of tumor cells on the serosal surfaces of organs and tumor cell invasion of mesenteric fat and lymphoid tissues in the peritoneal cavity. Tumor cell invasion into parenchymal tissue of the diaphragm, liver, spleen, pancreas, kidney, and accessory sex glands was observed in some mice. Tumor masses were composed of viable and necrotic tumor cells.

Abdominal adhesions, observed in some mice from all test groups, were characterized by fibrous tissue attachments between abdominal structures that included the tumor, abdominal wall, diaphragm, liver, gastrointestinal tract, kidneys, ureters, bladder, seminal vesicles, and testicles.

Singular or multiple tumor nodules, approximately 0.1 to 0.5 cm³, were observed attached to the hepatic surface of

Table 4. Posttap body weights and percentage decrease in body weight at taps 1, 2, and 3

Group	Tap 1 body wt	Tap 1 % drop	Tap 2 body wt	Tap 2 % drop	Tap 3 body wt	Tap 3 % drop
2B11	30.79 ± 2.15	11.50	28.27 ± 2.56	6.04	28.89 ± 2.79	5.24
Control	31.13 ± 2.47		31.21 ± 2.53		31.68 ± 2.39	
3C9	32.84 ± 2.11	7.18	30.98 ± 2.75	7.12	29.87 ± 3.02	5.75
Control	31.39 ± 2.37		31.88 ± 2.53		31.78 ± 2.43	
2C6D9	30.36 ± 2.91	10.23	27.79 ± 2.38	5.61	27.61 ± 1.62	3.47
Control	30.61 ± 2.11		31.13 ± 2.47		31.21 ± 2.53	
3D6	32.38 ± 2.39	6.99	31.21 ± 2.08	5.40	30.57 ± 2.14	5.21
Control	31.92 ± 2.56		31.96 ± 2.54		32.19 ± 2.78	
RMK	27.80 ± 2.46	8.45	29.66 ± 3.21	4.31	30.36 ± 3.45	6.99
Control	27.03 ± 1.49		27.01 ± 1.49		27.61 ± 1.51	

Mean (± 1 SD) posttap body weights for the test and control groups (g) at taps 1, 2, and 3 are presented. Control group body weight for each test group reflects mean body weight of the control group on the mean study day on which the specific tap was performed for the test group. Mean percentage decrease in body weight for test groups is based on before- and after-tap weights (% change) at taps 1, 2, and 3.
Data are expressed as mean ± SD body weight and mean decrease (%) in weight.

the diaphragm and/or the hepatic-diaphragmatic ligament in mice from four of the five test groups. Incidence was greater in SCID mice of group RMK (85%) than in CAF1 mice of other groups. Plaques or linear streaks in the diaphragm were observed in mice of groups 3C9 and 3D6. Plaques were characterized as raised, usually multiple, white to tan foci ≤ 0.3 cm² in diameter. Linear streaks also were white to tan and raised. Microscopic examination of diaphragmatic lesions revealed variable degrees of tumor cell infiltration into the diaphragm musculature or accumulation of tumor cells on the serosal surface.

Incidence of hepatomegaly, splenomegaly, and renomegaly was variable. Ureteral dilatation, bladder distention, and intestinal dilatation were infrequently observed among test groups. Organ dilatation or distention was generally related to distal constrictive lesions created by tumor growth and/or adhesions.

Edema of subcutaneous tissues with or without muscle of the ventral abdominal wall was observed in 35% of SCID mice of group RMK. In some mice, edema of the ventral neck region, thoracic wall, and/or hind limbs also was observed.

Lesions in the thoracic cavity: Enlarged mediastinal lymph nodes were frequently observed in mice from four of the five test groups. Mediastinal masses were observed in three SCID mice of group RMK and one CAF1 mouse of group 3D6. Masses were located in the cranial mediastinum or at the base of the heart. Microscopic examination of affected tissue revealed accumulations of tumor cells in mediastinal lymph nodes.

Lesions at the injection site: Gross lesions were observed at the site of pristane and hybridoma cell injection in mice of all test groups. Soft tissue masses in subcutaneous tissues and in the abdominal wall musculature were observed most frequently in mice of group 3C9. Masses were reddish to tan and ranged in size from approximately 0.2 cm³ to 0.6 x 0.6 x 0.3 cm. Intra-abdominal masses adhering to the peritoneum at the injection site were observed in mice of three test groups. These masses ranged in size from 0.3 x 0.2 x 0.1 cm to 0.6 x 0.6 x 0.1 cm. Microscopic examination of affected tissues indicated that these soft tissue masses were composed of tumor cells proliferating at the injection site.

Lesions at the abdominal tap site: Gross lesions were observed at the abdominal tap site in mice from all test groups. Subcutaneous hemorrhage was frequently observed, and muscle hemorrhage was infrequently observed.

Subcutaneous soft tissue masses were observed at the abdominal tap site only in SCID mice of group RMK (60%). These lesions were characterized by raised, tan to white soft tissue masses ranging from 0.2 to 1.0 cm² in diameter. Microscopic examination indicated that the tissue was composed of tumor cells. Edema was observed in the subcutaneous tissues and abdominal wall musculature at the tap site only in SCID mice of group RMK (20%).

Necropsy observations for selected mice: One mouse of group 2B11 was euthanized on day 9.

At necropsy, bilateral renomegaly, right hydroureter, and a soft tissue mass at the neck of the bladder were observed. On cross section the bladder wall was thick. On microscopic examination, the bladder wall was diffusely invaded by tumor cells, resulting in destruction of the normal architecture and luminal occlusion.

One mouse of group 2C6D9 was found dead on day 11. At necropsy, a distended bladder and an irregular soft tissue mass at the neck of the bladder were observed. On cross section the bladder wall was thick, suggesting presence of an intramural mass. Autolysis precluded histologic evaluation of tissues.

Two mice of group 3D6 remained clinically normal and did not produce ascites. Lesions observed at necropsy in one mouse included a large, thin-walled, cystic mass in the left caudolateral portion of the abdomen, and a second mass attached to the cystic mass. The other mouse had two soft tissue masses in the mesentery of the descending colon, hepatomegaly, renomegaly, multiple plaques within the diaphragm, and a soft tissue mass at the base of the heart.

Discussion

Results of this study indicate appreciable clinicopathologic changes in mice associated with MAb production via growth of ascitic tumors. A variety of pathologic processes were identified in association with various aspects of the production technique.

Pristane, a hydrocarbon derived from mineral oil, is the ascitogenic agent most frequently used to "prime" the peritoneal cavity for successful growth of hybridomas as ascitic tumors. In mice that do not receive a priming agent prior to hybridoma cell inoculation, solid tumors may form, but little or no ascites fluid is produced (4, 19–21). The reported biologic effects of pristane given i.p. in mice include induction of granulomatous inflammation in the peritoneum (22, 23), immunosuppression (24, 25), induction of growth factors (22, 26), lymphatic obstruction by oil-laden neutrophils and macrophages (23), and reduced clearance of particulate materials and cells from the peritoneal cavity (27).

Peritonitis is known to cause abdominal pain in animals and in humans (28, 29); therefore, it is reasonable to speculate that induction of granulomatous peritonitis subsequent to i.p. administration of pristane may cause pain or distress in mice. A transient decrease in food intake and exploratory behavior lasting 12 h has been reported in mice after injection of pristane (30). Injection of large volumes of pristane has been associated with weight loss, hunched appearance, and inactivity (7). Recommended dose volumes for pristane and alternative ascitogenic priming agents have been reviewed (31).

Clinical abnormalities and significant differences in body weights between test and control mice were not observed during the 2 weeks after injection of pristane. These findings suggest that i.p. injection of 0.5 ml of pristane may not cause clinically detectable morbidity. However, body weights were recorded once weekly during this period, so the possibility that there could have been a transient decrease in body weights within the first or second week after pristane injection cannot be definitely ruled out. Re-
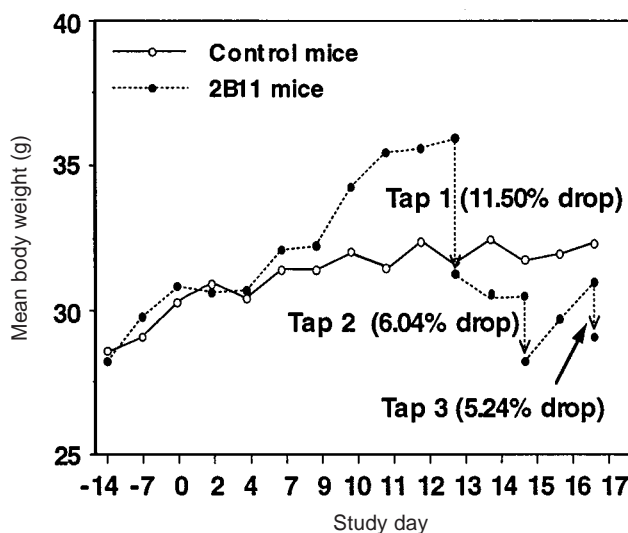

Figure 2. Body weight of mice with ascites over time for hybridoma cell line 2B11.

sults of this study also suggest that pristane is unlikely to support bacterial growth under routine storage and use conditions. This information is of particular importance with regard to pristane use in SCID or other immunodeficient mice.

Subcutaneous nodules at the site of pristane injection were observed in 8% of the mice. These nodules decreased in size or became clinically inapparent over time in greater than half of the mice. On the basis of observations of granulomatous inflammation induced by pristane (22, 23), it is suspected that these nodules were granulomas induced by deposition of a small amount of viscous pristane along the needle track during i.p. injections. Subsequent resolution of the granulomas may have accounted for the reduction in nodule size over time in some mice.

Subcutaneous nodules were also observed at the site of hybridoma cell inoculation. These nodules increased in size over time in greater than half of the affected animals, and histologic examination revealed subcutaneous foci of tumor growth. Hybridomas have been previously reported to grow in subcutaneous sites (2, 32–34). Tumor growth within the abdominal wall musculature at the injection site also was observed. Tumor growth at these sites likely resulted from the deposition of hybridoma cells along the needle track during i.p. injections. The greater prevalence of tumor growth at the injection site in mice of group 3C9 suggests that this hybridoma may have a greater propensity for growth in subcutaneous or intramuscular sites.

Solid tumor masses were observed with greatest frequency in the left caudal ventrolateral portion of the abdomen, and some tumors were attached to the peritoneum at the injection site. These findings suggest that solid tumor growth begins locally, at the site of hybridoma cell injection. Dividing the hybridoma inoculum for administration at more than one site may result in the growth of multiple smaller tumors instead of the large solitary tumors most frequently observed in this study. The potential benefit to the mouse, if any, and impact on ascites production needs

Table 5. Incidence of gross lesions at necropsy in mice with ascites

	CAF1 mice					SCID mice	
	Control	2B11	3C9	2C6D9	3D6	Control	RMK
No. of mice	6	20	20	20	20	6	20
Mean day of necropsy	31	16	19	13	26	32	20
Abdominal cavity							
Ascites	0	0	0	0	0	0	3
Hemoperitoneum	0	9	16	14	12	0	1
Disseminated tumor	0	19	20	18	17	0	19
Solid tumor mass	0	13	13	10	19	0	3
Peritesticular mass	0	3	11	0	5	0	1
Adhesions	0	12	2	9	12	0	1
Diaphragm-nodules	0	0	1	4	1	0	17
Diaphragm-plaques	0	0	10	0	12	0	0
Hepatomegaly	0	4	1	2	7	0	2
Splenomegaly	0	2	5	0	7	0	6
Renomegaly	0	1	0	0	3	0	1
Ureter dilated	0	1	1	0	0	0	1
Bladder distended	0	0	0	2	1	0	1
Bowel dilated	0	0	0	0	5	0	0
Ventral edema	0	0	0	0	0	0	7
Thoracic cavity							
Enlarged lymph nodes	0	12	9	0	11	0	9
Mediastinal mass	0	0	0	0	1	0	3
Injection site							
Subcutaneous mass	0	2	10	1	2	0	4
Abdominal wall mass	0	1	10	0	1	0	2
Peritoneal mass	0	3	1	0	0	0	2
Abdominal tap site							
Subcutaneous hemorrhage	0	18	20	16	17	0	18
Muscle hemorrhage	0	3	0	3	2	0	0
Subcutaneous mass	0	0	0	0	0	0	12
Edema	0	0	0	0	0	0	4

For each gross lesion, incidence is presented as the total number of mice in each group that had the indicated lesion. Detailed descriptions of each gross lesion are provided in the text.

to be determined.

Necropsy and histologic findings for the two mice that died or were euthanized after hybridoma cell inoculation (2 of 100 mice or 2%) indicated intramural tumor within the bladder wall. Inadvertent injection of hybridoma cells into the wall of the bladder could potentially account for the apparent rapid infiltrative tumor growth at this site.

The onset of clinical abnormalities, such as rough coat and hunched posture, was associated with initial development of ascites, and probably tumor growth, as determined by the progressive increase in abdominal distention and body weight. In humans, mild ascites is often asymptomatic and generally not painful, but large amounts of ascites can cause severe abdominal discomfort, respiratory distress, anorexia, nausea, heartburn, and difficulty in ambulation (29, 35). Ascites may also cause abdominal pain in animals (28). Increased abdominal pressure created by ascites may result in cranial displacement of the diaphragm, which decreases ventilatory capacity and compromises gas exchange. Additionally, increased abdominal pressure can lead to abdominal venous stasis and decreased arterial blood pressure and renal blood flow (28). Respiratory distress and difficulty in ambulation were not observed in our mice with ascites, suggesting that abdominal taps were performed prior to development of excessive accumulations of ascites fluid. Presence or absence of anorexia in the mice could not be determined because animals were group housed and food consumption was not monitored. The relationship of the observed clinical signs to discomfort or pain, if any, is subjective.

Mean percentage increase in body weight between day 0 and pre-tap weight at initial abdominal tap, for all groups of test mice combined, was 17.4%. This figure correlates well with the UKCCCR guidelines (8), which state that ascites volumes should not usually exceed 20% of normal body weight. The plateau in mean body weight in the 2 days immediately preceding tap 1, as illustrated graphically for group 2B11 (Figure 2), suggests that additional delay in paracentesis would not have resulted in collection of greater ascites volumes. The plateau in body weight may indicate that there was little further distensibility in the abdominal wall, and consequently, the pressure created by the accumulated fluid may have prohibited further ascites production. The timing of abdominal taps has generally been based on subjective assessment of the degree of abdominal distention (20, 21, 36–38). Monitoring body weight provides an additional and objective means to assist determination of the appropriate timing for abdominal taps.

Although differences between groups were significant, the general progression of clinical abnormalities over time among groups was similar. The differences that were observed between groups in incidence, severity, and rapidity of progression of clinical abnormalities during the study did have significant impact on survival, and ultimately, on the amount of antibody produced (15).

The clinical abnormalities that appeared to have the greatest impact on survival were those observed immediately after abdominal paracentesis. These abnormalities included rough coat, hunched posture, decreased activity, tachypnea, dyspnea, and pallor. These abnormalities were not observed in control animals after sham paracentesis, suggesting that these signs were associated with the removal of ascitic fluid, rather than with stress resulting from restraint and paracentesis. Definitive correlation could not be made, however, between the magnitude of the percentage decrease in body weight at the time of abdominal taps and presence or absence of clinical abnormalities after paracentesis.

The clinical abnormalities observed after paracentesis were compatible with signs of circulatory shock. Rapid or repeated removal of ascitic fluid in humans has been associated with hypotension (29), hypovolemia from compensatory fluid shifts (29, 35, 39), and protein depletion (39). Similar complications have been observed in animals after removal of ascitic fluid (40). In this study these signs were most often transient, suggesting that the mice were able to compensate via physiologic mechanisms. The recommendation for use of general anesthetics during abdominal
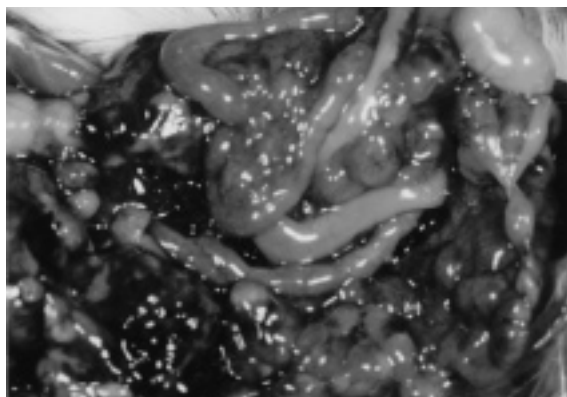
Figure 3. Disseminated tumor in the abdominal cavity of a mouse used for monoclonal antibody production.

Figure 4. Solid tumor mass in the abdominal cavity of a mouse used for monoclonal antibody production.

paracentesis in mice might be questioned in view of these findings, because many general anesthetic agents cause myocardial depression (41) and hypotension (42), and could potentially exacerbate existing cardiovascular deficits. General anesthetics are not routinely used for abdominal paracentesis in human or veterinary clinical practice, although local anesthetic agents are sometimes used. Supportive therapy, such as supplemental oxygen and warm fluids for volume expansion, could be considered but may be of questionable practicality.

All mice of this study that died did so within 30 min after abdominal tap, with the exception of one mouse that died early in the study. These findings point to the need for careful observation of animals for at least 30 min after abdominal paracentesis to permit identification of animals with severe or persistent posttap clinical abnormalities that warrant humane euthanasia. Careful clinical monitoring and appropriate use of euthanasia were successful in preventing mortality at other time points during this study, and were essential for providing humane animal care.

Overall survival to tap 3 for all groups combined was 79%, which is significantly higher than the reported 10 to 25% survival (4). This further supports the value of frequent and careful clinical monitoring, assessment of the degree of abdominal distention, and timely performance of abdominal paracentesis in reducing mortality. It should also be noted that clinical observations were made and abdominal taps were performed as necessary 7 days a week, including weekends and holidays. Greater survival also positively impacts total antibody production (15).

Six of 100 mice died during this study, for an overall mortality of 6%. Mortality ranged from 0 to 25% among groups. These findings suggest that, even with frequent and careful clinical observations, some mortality may occur, and significant differences in mortality may be observed between various cell lines. The most severe and rapidly progressive clinical abnormalities, shortest duration of survival, and highest mortality were observed in mice of group 2C6D9. The ascites fluid obtained from mice of this group often was markedly hemorrhagic. Observation of hemorrhagic ascites with subsequent anemia and death of animals has been reported (43). Necropsy observations for
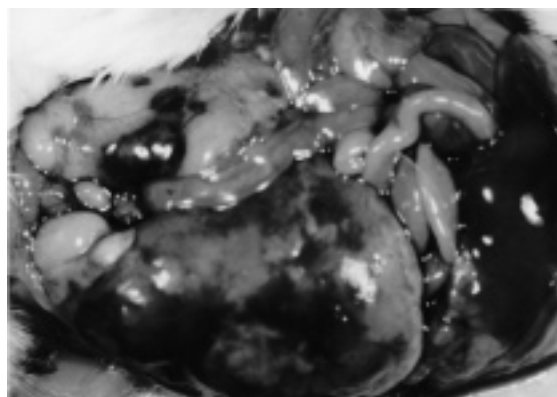
animals of this group did not indicate pathologic changes that were significantly different from those in mice of other groups. Extensive histologic examination of tissues from these mice might have provided more insight into pathologic mechanisms. A consideration for use of cell lines such as this one that induce rapid onset and severe clinical abnormalities would be to use a smaller cell inoculum with the intent of trying to slow the onset and progression of ascites production and tumor pathology.

Clinical abnormalities in mice of group RMK were the least severe and most slowly progressive, and all mice of this group survived to the end of the study. The ascites fluid collected from these animals was generally clear, and hemoperitoneum was not observed at necropsy. It was the impression of the first author that a number of the mice in this group could have had one additional tap without unduly compromising their clinical condition.

Results of clinical observations and abdominal tap intervals observed in this study indicate variability among cell lines and among individual animals within groups; therefore, decisions regarding the timing of abdominal taps, the total number of abdominal taps performed, and the time of euthanasia need to be made for each individual animal on the basis of an ongoing assessment of the animal's clinical condition.

Gross lesions observed at necropsy clearly indicated appreciable pathologic changes in these mice associated with MAb production from ascitic tumors. Hemoperitoneum could have resulted from trauma associated with paracentesis, or may have resulted from erosion of blood vessels, which has been documented for invasive abdominal plasmacytomas with associated hemoperitoneum (44, 45). Abdominal adhesions were also commonly observed. All mice had disseminated intra-abdominal tumor and/or solid tumor masses. The sites of tumor growth suggested apparent tropism for abdominal fat, particularly mesenteric fat. Previous studies have indicated that some murine ascites tumors secrete vascular permeability factor, which renders peritoneal lining microvessels hyperpermeable to plasma fibrinogen. Extravasation of fibrinogen into the peritoneal cavity and subsequent formation of extravascular fibrin deposits provided a matrix that trapped tumor cells and

favored their attachment to peritoneal lining tissues, such as the mesentery, peritoneal wall, and diaphragm (46–48).

Presence of subcutaneous tumor at the tap site of mice in group RMK suggests that tumor cells seeded the subcutaneous tissues during paracentesis. The relationship, if any, between presence of subcutaneous tumor and persistent leakage of ascites from the tap site, observed only in this group, is unknown. Chronic leakage of ascites from paracentesis sites has been observed after repeated paracentesis in people with cirrhosis (39). Dependent subcutaneous edema was observed only in SCID mice of group RMK. This finding may be related to a greater tendency for development of hypoproteinemia in SCID mice resulting from the combination of normally lower serum total protein concentration related to agammaglobulinemia, and the loss of large quantities of albumin in ascites. Albumin concentration in mouse ascites fluid has been reported to be 1.1 to 1.8 g/dl (49), and hyperpermeability of peritoneal lining microvessels with extravasation of plasma proteins into the peritoneal cavity has been documented for some ascites tumors in mice (46, 47).

Histologic evaluation of enlarged mediastinal lymph nodes revealed tumor cells within these nodes. Metastasis of tumor cells from the abdominal cavity to the thoracic lymph nodes, presumably via the lymphatics, has been reported for plasmacytomas (44, 45).

Gross necropsy observations for the two mice of group 3D6 that failed to develop ascites indicated that despite appreciable pathologic changes in the abdomen and thorax, mice can continue to appear clinically normal. Development of nonsecreting solid tumors rather than ascitic tumors (21) and descriptions of <100% development of ascites in mice after injection of pristane and hybridoma cells (6) have been described. The overall incidence of mice that did not produce ascites was 2% for those that survived to the time of ascites development.

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

Abdominal carcinomatosis in humans and animals is generally associated with progressive weakness, debilitation, and cachexia (28, 29). Abdominal carcinomatosis in humans has also been reported to be painful (29), and the potential for abdominal distention and pain induced by an increasing abdominal tumor mass in mice has been addressed (5, 6, 8) and should be considered in these animals. Although abdominal pressure may be at least temporarily reduced by removal of ascitic fluid, the tumor remains and continues to increase in mass over time. In view of all these findings, it is clear that MAb production in murine ascites is not a benign process, and the authors have to assume that there must be distress associated with this process. On the basis of the clinical appearance of most animals by tap 3, the authors suggest that, as a general recommendation, three abdominal taps per animal should be considered maximum, but that additional taps could be considered for cell lines with clinical effects similar to those observed for group-RMK mice.

Others have reported significant differences in the behavior of different hybridoma cell lines in mice (4), and differences between cell lines were observed in this study. Because only five cell lines were evaluated in this study, these results likely do not encompass the range of biologic behaviors possible among different hybridoma cell lines. Differences observed between groups of CAF1 mice and SCID mice cannot be differentiated solely on the basis of difference in the strain of mouse or difference in the hybridoma cell line.

In conclusion, results of this study indicate significant clinicopathologic changes in mice associated with MAb production in ascites. Procedural parameters affecting MAb production in murine ascites and in vitro alternatives available for laboratory scale growth of hybridoma cells have been reviewed (15, 31). Every attempt should be made to improve procedural protocols to reduce the potential for pain and distress in the animals and to maximize production so that the smallest number of animals can be used without compromising humane care. In vitro alternatives for MAb production should be used whenever feasible.

References

1. **Köhler, G., and C. Milstein.** 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature **256:**495–497.
2. **Köhler, G., and C. Milstein.** 1976. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. Eur. J. Immunol. **6:**511–519.
3. **Goding, J. W.** 1980. Antibody production by hybridomas. J. Immunol. Methods **39:**285–308.
4. **Chandler, J. P.** 1987. Factors influencing monoclonal antibody production in mouse ascites fluid, p. 75–92. *In* S. S. Seaver (ed.), Commercial production of monoclonal antibodies: a guide for scale-up. Marcel Dekker, Inc., New York.
5. **McGuill, M. W., and A. N. Rowan.** 1989. Refinement of monoclonal antibody production and animal well-being. ILAR News **31:**7–11.
6. **Kuhlmann, I., W. Kurth, and I. Ruhdel.** 1985. Monoclonal antibodies: in vivo and in vitro production on a laboratory scale, with consideration of the legal aspects of animal protection. ATLA **17:**73–82.
7. **Amyx, H. L.** 1987. Control of animal pain and distress in antibody production and infectious disease studies. J. Am. Vet. Med. Assoc. **191:**1287–1289.

8. **Workman, P., A. Balmain, J. A. Hickman,** *et al.* 1988. UKCCCR guidelines for the welfare of animals in experimental neoplasia. Lab. Animals **22:**195–201.

9. **Marx, U., M. J. Embleton, R. Fischer,** *et al.* 1997. Monoclonal antibody production: the report and recommendations of ECVAM workshop 23. ATLA **25:**121–137.

10. Workshop sponsored by the Johns Hopkins Center for Alternatives to Animal Testing and the Office for Protection from Research Risks, National Institutes of Health. 1997. Alternatives in monoclonal antibody production.

11. **National Institutes of Health.** 1997. Production of monoclonal antibodies using mouse ascites method. OPRR Reports, Animal welfare number 98-01.

12. **Groopman, J. D., L. J. Trudel, P. R. Donahue,** *et al.* 1984. High-affinity monoclonal antibodies for aflatoxins and their application to solid-phase immunoassays. Proc. Natl. Acad. Sci. USA **81:**7728–7731.

13. **Turesky, R. J., C. M. Forster, H. U. Aeschbacher,** *et al.* 1989. Purification of food-borne carcinogens 2-amino-3-methylimidazo[4,5-*f*]quinoline and 2-amino-3,8- dimethylimidazo[4,5-*f*]quinoxaline in heated meat products by immunoaffinity chromatography. Carcinogenesis **10:**151–156.

14. **Groopman, J. D., P. L. Skipper, P. R. Donahue,** *et al.* 1992. Monoclonal antibodies and rabbit antisera recognizing 4-aminobiphenyl-DNA adducts and application to immunoaffinity chromatography. Carcinogenesis **13:**917–922.

15. **Jackson, L. R., L. J. Trudel, J. G. Fox,** *et al.* 1999. Monoclonal antibody production in murine ascites. II. Production characteristics. Lab. Anim. Sci. **49:**81–86.

16. Guidelines for the utilization of rodents in experimental neoplasia and ascites production. 1992. Committee on Animal Care, MIT.

17. **Andrews, E. J., B. T. Bennett, J. D. Clark,** *et al.* 1993. Report of the AVMA panel on euthanasia. J. Am. Vet. Med. Assoc. **202:**229–249.

18. **Gerrold, H. Zar.** 1984. Biostatistical analysis, p. 190–191. Multiple comparisons. Prentice Hall, Englewood Cliffs, N.J.

19. **Garrett, L. R., D. W. Pascual, L. W. Clem,** *et al.* 1987. Conformational changes in the DNA of hybridoma cells from pristane treated mice. Chem-Biol. Interactions **61:**249–263.

20. **Brodeur, B. R., P. Tsang, and Y. Larose.** 1984. Parameters affecting ascites tumour formation in mice and monoclonal antibody production. J. Immunol. Methods **71:**265–272.

21. **Hoogenraad, N. J., and C. J. Wraight.** 1986. The effect of pristane on ascites tumor formation and monoclonal antibody production. Methods Enzymol. **121:**375–381.

22. **Cranco, M., and M. Potter.** 1976. The requirement of an adherent cell substratum for the growth of developing plasmacytoma cells in vivo. J. Exp. Med. **144:**1554–1567.

23. **Leak, L. V., M. Potter, and W. J. Mayfield.** 1985. Response of the peritoneal mesothelium to the mineral oil pristane. Curr. Top. Microbiol. Immunol. **122:**221–233.

24. **Kripke, M. C., and D. W. Weiss.** 1970. Studies on the immune responses of BALB/c mice during tumor induction by mineral oil. Int. J. Cancer **6:**422–430.

25. **Freund, Y. R., and P. B. Blair.** 1982. Depression of natural killer activity and mitogen responsiveness in mice treated with pristane. J. Immunol. **129:**2826–2830.

26. **Platica, M., C. Bojko, and V. Hollander.** 1982. Lipopolysaccharide and pristane-induced peritoneal exudate requirements for plasma tumor cell colony formation. J. Natl. Cancer Inst. **68:**325–328.

27. **Moore, J. M., and T. V. Rajan.** 1994. Pristane retards clearance of particulate materials from the peritoneal cavity of laboratory mice. J. Immunol. Methods **173:**273–278.

28. **Ettinger, S. J.** 1983. Ascites, peritonitis, and other causes of abdominal enlargement, p. 121–129. *In* S. J. Ettinger (ed.), Textbook of veterinary internal medicine, vol. 1. W. B. Saunders Co., Philadelphia.

29. **Bender, M. D.** 1985. Diseases of the peritoneum, p. 786–790. *In* J. B. Wyngaarden and L. H. Smith (ed.), Textbook of medicine, 17th ed., vol. 1. W. B. Saunders Co., Philadelphia.

30. **Silverman, J.** 1987. Protocol review. Monoclonal antibody production: in vivo vs. in vitro. Lab Anim. **16:**19–20.

31. **Jackson, L. R., and J. G. Fox.** 1995. Institutional policies and guidelines on adjuvants and antibody production. ILAR J. **37:**141–152.

32. **Truitt, K. E., J. W. Larrick, A. A. Raubitschek,** *et al.* 1984. Production of human monoclonal antibody in mouse ascites. Hybridoma **3:**195–199.

33. **Misra, D. N., S. A. Noeman, H. W. Kunz,** *et al.* 1986. Growth of rat-mouse hybridomas in nude mice and nude rats. Methods Enzymol. **121:**386–411.

34. **Noeman, S. A., D. N. Misra, R. J. Yankes,** *et al.* 1982. Growth of rat-mouse hybridomas in nude mice and nude rats. J. Immunol. Methods **55:**319–326.

35. **Pockros, P. J., and T. B. Reynolds.** 1986. Rapid diuresis in patients with ascites from chronic liver disease: the importance of peripheral edema. Gastroenterology **90:**1827–1833.

36. **Hoogenraad, N., T. Helman, and J. Hoogenraad.** 1983. The effect of pre-injection of mice with pristane on ascites tumor formation and monoclonal antibody production. J. Immunol. Methods **61:**317–320.

37. **Mueller, U. W., C. S. Hawes, and W. R. Jones.** 1986. Monoclonal antibody production by hybridoma growth in Freund's adjuvant primed mice. J. Immunol. Methods **87:**193–196.

38. **Jones, S. L., J. C. Cox, and J. E. Pearson.** 1990. Increased monoclonal antibody ascites production in mice primed with Freund's incomplete adjuvant. J. Immunol. Methods **129:**227–231.

39. **Lamont, J. T., R. S. Koff, and K. J. Isselbacher.** 1980. Cirrhosis, p. 1473–1482. *In* K. J. Isselbacher, R. D. Adams, E. Braunwald, *et al.* (ed.), Harrison's principles of internal medicine. McGraw-Hill, New York.

40. **Ettinger, S. J.** 1983. Diseases of the liver, p. 1427–1429. *In* S. J. Ettinger (ed.), Textbook of veterinary internal medicine, vol. 2. W. B. Saunders Co., Philadelphia.

41. **Price, H. L., and S. T. Ohnishi.** 1980. Effects of anesthetics on the heart. Fed. Proc. **39:**1575–1579.

42. **Altura, B. M., B. T. Altura, A. Carella,** *et al.* 1980 Vascular smooth muscle and general anesthetics. Fed. Proc. **39:**1584–1591.

43. **De Deken, R., J. Brandt, F. Ceulemans,** *et al.* 1994. Influence of priming and inoculation dose on the production of monoclonal antibodies in two age groups of BALB/c mice. Hybridoma **12:**53–57.

44. **Potter, M., and R. C. MacCardle.** 1964. Histology of developing plasma cell neoplasia induced by mineral oil in BALB/c mice. J. Natl. Cancer Inst. **33:**497–515.

45. **Potter, M.** 1972. Immunoglobulin-producing tumors and myeloma proteins of mice. Physiol. Rev. **52:**631–719.

46. **Nagy, J. A., E. M. Massee, K. T. Herzberg,** *et al.* 1995. Pathogenesis of ascites tumor growth: vascular permeability factor, vascular hyperpermeability, and ascites fluid accumulation. Cancer Res. **55:**360–368.

47. **Nagy, J. A., M. S. Meyers, E. M. Masse,** *et al.* 1995. Pathogenesis of ascites tumor growth: fibrinogen influx and fibrin accumulation in tissues lining the peritoneal cavity. Cancer Res. **55:**369–375.

48. **Nagy, J. A., E. S. Morgan, K. T. Herzber,** *et al.* 1995. Pathogenesis of ascites tumor growth: angiogenesis, vascular remodeling, and stroma formation in the peritoneal lining. Cancer Res. **55:**376–385.

49. **Coniglio, C., G. Pappas, W. J. Gill,** *et al.* 1991. Clarification of ascites fluid. BioTechniques **10:**457–460.

Monoclonal Antibody Production in Murine Ascites
II. Production Characteristics

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Objective: To characterize monoclonal antibody production parameters of five hybridoma cell lines in murine ascites for correlation with clinicopathologic changes in mice.

Methods: Five hybridoma cell lines were grown in groups of 20 mice. Fourteen days prior to inoculation with 10^6 hybridoma cells, mice were primed with 0.5 ml of pristane given intraperitoneally. Ascites fluid was collected a maximum of three times by abdominal paracentesis; volume was measured and antibody concentration was determined by ELISA for each sample.

Results: Trends differed among cell lines when comparing ascites volumes and antibody concentrations over time from the first to the third tap. Antibody production was greatest at tap 1 for Groups 2B11 and 2C6D9; tap 2 for Group 3C9; and tap 3 for Groups RMK and 3D6. Total antibody production ranged from 422.90 to 996.64 mg; total ascites fluid volume ranged from 74.2 to 115.7 ml; and mean antibody concentration for taps 1, 2, and 3 ranged from 2.50 to 15.03 mg/ml among cell lines.

Conclusion: Production characteristics were significantly different among hybridoma cell lines. Determination of production characteristics of hybridomas and correlation with clinicopathologic changes in mice may be valuable in making recommendations for managing mice with ascites.

Published literature is replete with information regarding in vivo production of monoclonal antibodies (MAbs) in murine ascites. Numerous parameters have been identified that may affect MAb production and/or the likelihood of causing severe clinical abnormalities, pain, distress, or death in the animals as a result of the procedures used. These parameters have been reviewed (1, 2) and include hybridoma cell line used (3); stock or strain (3–5), sex, and age (3, 6–8) of mouse selected; volume of pristane (2, 7, 9–11) or other ascitogenic priming agent administered (8, 12–14), and timing of pristane or other ascitogenic priming agent administration in relation to hybridoma cell inoculation (3, 7–9, 12–16); hybridoma cell inoculum used (3, 7, 8, 17); frequency and total number of abdominal taps performed to collect the ascitic fluid (3, 6, 18–20); method used to perform the abdominal taps (3, 7, 12); and frequency of clinical observations and criteria for euthanizing animals (20).

Unfortunately, there is lack of consensus regarding optimization of many procedural parameters for producing MAbs in murine ascites, and it is difficult to make meaningful comparisons between studies because of variations in the aforementioned procedural parameters, known variability in the biological behavior and production characteristics of various hybridoma cell lines, and differences in the specific production parameters evaluated. The experimental regimen used in this study was selected based on the following criteria: that antibody production be maximized, that the regimen be representative of current practices in use in academic and industrial settings, and that the selected procedures be in compliance with guidelines established by the Institutional Animal Care and Use Committee.

Published production characteristics of hybridoma cell lines are generally limited to total ascites fluid volume, mean volume of ascites/mouse, mean antibody concentration or titer, and total antibody produced. Rarely have differences in production parameters of cell lines been compared over time from the first to the last abdominal tap (3, 8), and to the authors' knowledge, there has been no comprehensive study to evaluate clinicopathologic changes in mice and associated production characteristics of individual hybridoma cell lines. With the view that such information may be valuable in formulating recommendations for managing mice with ascites in a manner to maximize antibody production while minimizing potential for pain and distress associated with the procedure, we sought to compare production characteristics and associated clinicopathologic changes in the mice over time among five hybridoma cell lines, using a standardized protocol.

Materials and Methods

Materials and methods for this study have been described in detail in a companion article (20). In brief:

Hybridoma cell lines: Five hybridomas were evaluated (20). Hybridomas representative of varied plasmacytoma

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fusion partners, varied isotypes of secreted antibody to include IgM and IgG, different subclasses of IgG, and mouse x mouse and rat x mouse hybridomas were used.

Animals: The mouse x mouse hybridomas were grown as ascitic tumors in 20 CAF1/J male pathogen-free mice (The Jackson Laboratory, Bar Harbor, Maine). The rat x mouse heterohybridoma was grown as ascitic tumors in 20 Fox Chase SCID, C.B-17/IcrTac-scidDF male pathogen-free mice (Taconic Farms, Germantown, N.Y.). The protocol for animal use was approved by the MIT Committee on Animal Care.

Pristane priming: On study day –14, each mouse received a single i.p. injection of 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane; Sigma Chemical Co., St. Louis, Mo.) given in the caudal left quadrant of the ventral portion of the abdomen.

Hybridoma cell inoculation: Storage conditions, culture media, expansion of hybridoma cells, and preparation for inoculation have been described (20). Briefly, cells were expanded in static culture to provide >20 x 10^6 cells in logarithmic growth phase for each group of mice. Antibody secretion into the cell culture supernatant was verified by use of an antigen-specific enzyme-linked immunosorbent assay (ELISA) for each hybridoma cell line prior to inoculation into mice.

On study day 0, a hybridoma cell suspension containing 2 x 10^6 live cells/ml of basal cell culture medium was prepared. Each mouse received a single i.p. injection of 10^6 live cells in a total volume of 0.5 ml of basal culture medium. All injections were administered in the left caudal quadrant of the ventral portion of the abdomen.

Abdominal paracentesis: Abdominal paracentesis was performed when moderate abdominal distention was visible. Abdominal taps were performed every 1 to 3 days on the basis of clinical appearance of each mouse and the rate of ascites production as assessed by the degree of abdominal distention. The procedure was performed on each mouse a maximum of three times.

Paracentesis was performed aseptically, using a sterile 18-gauge, 1.5-in needle inserted into the peritoneal cavity through the left lateral abdominal wall. Ascites fluid was collected via gravity flow by permitting the fluid to drip from the hub of the needle, and directly from the paracentesis site, into a sterile centrifuge tube. Gentle digital pressure was applied to the abdomen, and the position of the mouse was altered as needed to facilitate removal of the ascites fluid.

The total volume of ascites fluid collected and the day of collection were recorded for each tap for each mouse. Samples were centrifuged at 550 X g for 10 min, and the resultant volume of the supernatant was recorded for each tap for each mouse. Samples were frozen at -20°C until analysis.

Euthanasia: During the study, any animal with persistent, severe clinical abnormalities that were interpreted to be indicative of pain or distress or were suggestive that the animal might not survive to the next observation period was euthanized. Mice surviving to the third abdominal tap were euthanized just prior to paracentesis. Euthanasia was performed by use of CO_2 in accordance with ac-

Table 1. Number and percentage of mice that were tapped

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

[a]Two mice in group 3D6 did not produce ascites and were not tapped.

cepted guidelines (21).

Antibody quantitation: Indirect antibody ELISAs were used to screen the cell culture supernatant of hybridoma cells for secreted antibody prior to inoculation of cells into mice and to quantitate in absolute units the antigen-specific antibody in each ascites sample.

Preparation of antibody standards: The IgG MAb standards were purified from ascites fluid, using Affi-Gel Protein A MAPS II Kit (Bio-Rad Laboratories, Richmond, Calif.) per manufacturer's instructions. The IgM MAb standards were purified, using 40% ammonium sulfate precipitation of ascites fluid dialyzed against phosphate-buffered saline (PBS), followed by reprecipitation by dialysis against 2% boric acid, pH 6.0. Antibody concentrations were determined by measuring optical density (OD) at 280 nm, using the extinction coefficients for IgG (1.4) and IgM (1.2) OD units equal to 1.0 mg/ml. Antibody was diluted in Tris-2% bovine serum albumin (BSA; Boehringer Mannheim, Indianapolis, Ind.) buffer with 0.02% sodium azide and was stored at 4°C until used.

Quantitative indirect ELISA: Assays were developed, using techniques described by Catty and Raykundalia (22). Briefly, checkerboard titrations were used to determine optimal antigen concentration. Antigens in PBS were adsorbed overnight at 4°C onto 96-well polyvinyl chloride plates (Dynatech, Alexandria, Va.). The plates were washed with deionized water three times and dried. Plates were blocked with 0.2% BSA in PBS for 1 h at 20 to 22°C (room temperature) to control nonspecific binding. The plates were washed and dried, covered with parafilm, and stored at –20°C. Appropriate concentrations of alkaline phosphatase-conjugated goat anti-mouse IgG and IgM and mouse anti-rat IgG (H&L) (Boehringer Mannheim) were determined for each assay by testing various concentrations of the standard curve dilutions and selecting the concentration that provided the highest signal with the lowest background noise.

Construction of standard curves: Standard curves were constructed, using concentrations of purified antibodies, starting at 1 μg/ml with twofold dilutions to 25 ng/ml in 10% horse serum/PBS (JRH Biosciences, Lenexa, Kans.). One hundred microliter samples were applied to the plate, which was incubated at 37°C for 2 h. Plates were washed with 0.05% Tween 20 (Sigma Chemical Co.) in PBS three times and dried. Specifically, 100 μl of the enzyme antibody conjugate dilution was added to each well, and plates were incubated for 1 h at 37°C. The plate was washed and dried as described previously, and 100 μl of substrate (1 mg of *p*- nitrophenyl phosphate/ml [Sigma Chemical Co.] in 0.1 M diethanolamine buffer, pH 9.8 [Sigma Chemical Co.]) was added to the plate and incubated for 15 to 45 min at room temperature until OD for the lowest dilution on

the standard curve was 1 to 1.5 at 405 nm by use of a Dynatech MR 7000 automatic ELISA Reader (Dynatech, Alexandria, Va.). The ELISA reader was programmed to calculate the curve fit for the standards and to determine the mean and SD of the duplicates, and concentration of antibody.

Statistical analyses: Differences among means for ascites fluid volumes and antibody quantitation were tested for statistical significance, using a one-way repeated measures analysis of variance (ANOVA) followed by pairwise comparisons among group means, using the Newman-Keuls test (23). The value for determining statistical significance was set at $\alpha = 0.05$. Data are presented as mean, with SD as a measure of dispersion.

Results

Number of mice tapped: The number and percentage of mice in each group on which taps 1, 2, and 3 were performed are presented in Table 1. These data reflect the survival of mice with ascites in each group over time. It should be noted, however, that two mice of group 3D6 did not produce ascites and so were not tapped, but survived to tap 3. For all groups combined, overall survival to tap 1 was 98%, to tap 2 was 96%, and to tap 3 was 79%. Percentage of mice in all groups combined that underwent taps 1, 2, and 3 ranged from 90 to 100%, 85 to 100%, and 35 to 100%, respectively. Four of the five groups had ≥75% of the possible third taps performed. For group RMK, no mice died or were euthanized during the study; therefore, survival and percentage of mice that underwent taps was 100% at all time points. The lowest percentage was 35% for group-2C6D9 mice at tap 3.

Ascites fluid volumes: Total ascites fluid volumes (after centrifugation) collected at each tap for each group and mean ± 1 SD volume per mouse are presented in Table 2. Different trends were observed among groups when mean ascites fluid volume per mouse was compared over time for taps 1 through 3. For group 2B11, mean fluid volume per mouse obtained at tap 1 (3.2 ml) was significantly greater than mean volumes obtained at taps 2 and 3 (1.5 and 1.2 ml, respectively; $P = 0.0001$). For group RMK, mean volumes were significantly greater at taps 1 and 3 (2.5 and 2.1 ml, respectively), compared with tap 2 (1.2 ml; $P = 0.0094$). For group 2C6D9, significant and progressive decreases in mean volume were observed from taps 1 through 3 (2.6, 1.2, and 0.6 ml, respectively; $P = 0.0001$). Significant differences were not observed in mean volumes over time for groups 3C9 (2.0, 2.1, and 1.6 ml, respectively) and 3D6 (2.1, 1.6, and 1.4 ml, respectively).

Ascites fluid volume summary data are presented in Table 3. Group-RMK mice, which had 100% survival and underwent 100% of possible taps, produced the largest total volume of ascites fluid. Group-2C6D9 mice, which had the lowest survival and underwent the lowest percentage of possible taps (72%), produced the lowest total volume of ascites fluid. Mean volume per tap for taps 1, 2, and 3 com-

Table 2. Ascites fluid volumes and antibody quantitation

Cell line	Tap no.	No. of mice	Total volume[a] (ml)	Volume (ml/mouse)[a]	Antibody conc (mg/ml)	Total antibody (mg)	Antibody (mg/mouse)
2B11	1	19	61.4	3.2 ± 0.73	3.61 ± 1.18	229.30	12.07 ± 5.13
	2	19	27.7	1.5 ± 0.74	4.32 ± 1.30	116.93	6.15 ± 3.60
	3	15	18.6	1.2 ± 0.84	6.36 ± 1.99	108.27	7.22 ± 4.97
	Totals		107.7			454.50	
3C9	1	20	40.5	2.0 ± 0.80	2.50 ± 1.20	104.14	5.21 ± 3.29
	2	20	41.0	2.1 ± 0.72	6.12 ± 1.56	237.36	11.87 ± 3.57
	3	18	28.0	1.6 ± 0.87	3.84 ± 1.01	104.07	5.78 ± 3.48
	Totals		109.5			445.57	
2C6D9	1	19	49.5	2.6 ± 0.75	5.17 ± 1.85	264.27	13.91 ± 5.95
	2	17	20.2	1.2 ± 0.45	6.82 ± 1.96	132.67	7.80 ± 3.31
	3	7	4.5	0.6 ± 0.27	6.53 ± 2.74	25.96	3.71 ± 1.14
	Totals		74.2			422.90	
3D6	1	18	37.5	2.1 ± 1.28	3.83 ± 1.58	121.56	6.75 ± 4.07
	2	18	28.3	1.6 ± 0.89	6.25 ± 1.69	163.17	9.06 ± 4.86
	3	17	24.5	1.4 ± 1.06	7.56 ± 2.38	204.63	12.04 ± 9.11
	Totals		90.3			489.36	
RMK	1	20	50.2	2.5 ± 0.92	4.39 ± 2.97	213.05	10.65 ± 7.56
	2	20	24.0	1.2 ± 0.88	5.69 ± 2.75	135.87	6.79 ± 5.56
	3	20	41.5	2.1 ± 1.10	15.03 ± 10.94	617.72	30.89 ± 20.83
	Totals		115.7			996.64	

[a]Volumes are postcentrifugation volumes.
Data are presented as mean ± 1 SD where applicable.

Table 3. Ascites volume summary data

Cell line	No. of taps	Percentage of taps[a]	Total volume (ml)	Mean volume/tap (ml)
2B11	53	88	107.7	2.0
3C9	58	97	109.5	1.9
2C6D9	43	72	74.2	1.7
3D6	53	88	90.3	1.7
RMK	60	100	115.7	1.9

[a]Percentage of taps performed/possible maximum of 60

bined was similar among groups, ranging from 1.7 to 2.0 ml. For taps 1, 2, and 3, the percentage of the total volume of ascites fluid collected that was discarded as cell pellets after centrifugation ranged from 20 to 26% for group 2C6D9, 13 to 16% for group 2B11, 12 to 17% for group 3C9, 12 to 14% for group 3D6, and 7 to 8% for group RMK.

Antibody concentration: Mean ± 1 SD antibody concentration for each tap for each group is presented in Table 2. Trends differed among groups in comparison of antibody concentrations in ascites fluid over time from taps 1 through 3. For groups 2B11 and RMK, there were no significant differences in antibody concentrations at taps 1 and 2; however, the concentrations were significantly greater at tap 3 ($P = 0.0001$, $P = 0.0044$, respectively). Mean concentrations were 3.61, 4.32, and 6.36 mg/ml for group 2B11 and 4.39, 5.69, and 15.03 mg/ml for group RMK at taps 1, 2, and 3, respectively. The large SD value (± 10.94 mg/ml) for mean antibody concentration for group RMK mice at tap 3 reflects the marked variability in antibody concentrations among individual mice, ranging from 0 to 44.45 mg/ml. For group-3C9 mice, the mean concentration of antibody was significantly greater at tap 2 (6.12 mg/ml; $P = 0.0001$), compared with taps 1 (2.50 mg/ml) and 3 (3.84 mg/ml). For group-3D6 mice, there was a significant and progressive increase in mean antibody concentration from taps 1 through 3 (3.83, 6.25, and 7.56 mg/ml, respectively; $P = 0.0001$). For group-2C6D9 mice, mean concentrations at tap 2 (6.82 mg/ml) and 3 (6.53 mg/ml) were significantly ($P = 0.0003$) greater than
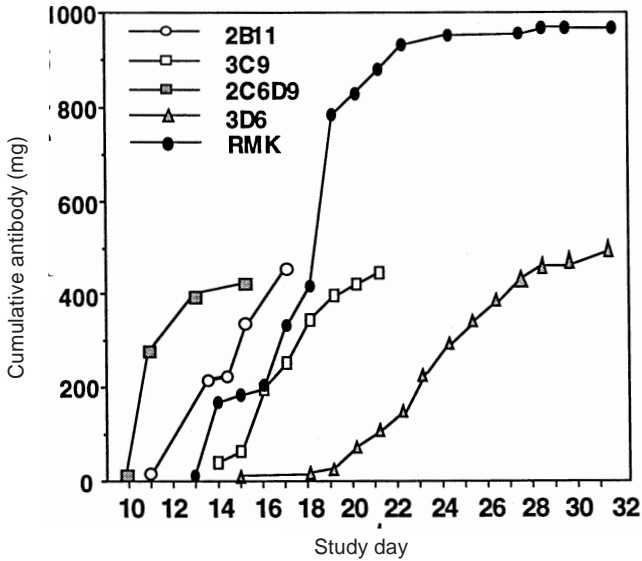
Figure 1. Cumulative antibody production over time.

mean concentration at tap 1 (5.17 mg/ml).

Total antibody production and mean production per mouse: Total antibody production and mean antibody production (milligrams per mouse) for each tap and group (Table 2) reflect combined contributions of ascites fluid volumes and antibody concentrations. For groups 2B11 and 2C6D9, total antibody production was significantly ($P = 0.0015$ and 0.0017, respectively) greater at tap 1. For group 2C6D9, production at tap 2 was significantly ($P = 0.0017$) greater than that at tap 3. For group 3C9, production was significantly greater at tap 2 ($P = 0.0037$), and for groups RMK and 3D6, production was significantly ($P = 0.0047$ and $P = 0.0116$, respectively) greater at tap 3. For group 3D6, production at tap 3 was significantly ($P = 0.0116$) greater than that at tap 1, but production at taps 2 and 3 were not significantly different.

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

Cumulative antibody production in ascites: Cumulative antibody production over time for each group is presented in Figure 1. Production began for each group at the time the first mouse was tapped. Considerable variability in production time was observed among groups. Production was complete by day 15 for groups 2B11 and 2C6D9, whereas production for group 3D6 was just beginning on day 15 and extended until day 31. Production began on day 14 for group-3C9 mice and was complete on day 21. Production began on day 13 for group-RMK mice and was not complete until day 31. The flat slope of the production line for group-RMK mice from approximately day 22 through day 31 indicates that mice producing ascites late in time (after day 22) contributed little to total antibody production.

Discussion

Results of this study indicate that duration of survival

of the mice, number of abdominal paracentesis procedures, volume of ascites fluid collected, and antibody concentration of ascites fluid impact total antibody production.

Increased survival of mice positively impacts total antibody production; direct correlation was observed between percentage of possible taps performed and total ascites fluid volumes for the five hybridomas studied. The greater the percentage of possible taps performed, the greater the total volume of ascites fluid collected. The percentage of possible taps performed as a measure of survivability was generally higher in this study, compared with that of another published report (3).

Significant variability in the onset and rate of development of ascites in individual animals within a single hybridoma cell line, as well as between different hybridoma cell lines, has been documented (20); it is important to emphasize that clinical assessment must be individualized for each mouse. Frequent and careful clinical monitoring, assessment of the degree of abdominal distention, and timely performance of abdominal paracentesis are valuable in reducing mortality (20).

An inverse relationship between number of hybridoma cells inoculated and time interval between cell inoculation and onset of ascites production, as well as duration of survival of the mice, has been reported (3, 7, 8). These findings suggest that, for smaller cell inocula, tumor burden and ascites production may increase more slowly, permitting the animal more time for physiologic adaptation and hence increased survivability, and presumably decreased potential for pain and/or distress, with more slowly progressive pathologic changes. Literature recommendations for optimal hybridoma cell inocula vary from $5 \times 10^5 (3)$ to 0.6 to 3.2×10^6 (7) to 10^7 (17) cells. The disparity in these results suggests that optimal cell inocula likely vary among cell lines. Higher cell inocula result in earlier onset of ascites production and earlier mortality; lower cell inocula result in fewer mice developing ascites and lower ascites fluid volume yields. Decreasing the inocula for cell line 2C6D9, which caused rapid and progressive lesions and high mortality prior to tap 3 (20), would be one consideration for increasing survivability of mice inoculated with this hybridoma. The optimal cell inoculum would be one for which all mice develop ascites and which has good survivability and ascites yields, thereby positively impacting antibody production with appropriate consideration for animal welfare. The volume in which the hybridoma cells are suspended may also affect production because cells in 0.5 ml of medium result in increased production, compared with the same number of cells in a smaller volume of 0.2 to 0.3 ml (8).

The number of abdominal taps permitted per mouse affects total antibody production. A maximum of three taps per mouse was performed. All mice of group RMK survived to the third abdominal tap. In addition to maintaining good clinical condition and body weight over time without evidence of pain or distress (20), these mice had high concentrations of antibody in ascites fluid from the third abdominal tap. Approximately two-thirds of the total antibody produced by this group was obtained from ascites fluid from tap 3. If concentrations remained high, subsequent taps, if

performed, may have yielded considerably more antibody. These findings should be taken into consideration by Institutional Animal Care and Use Committees. If, for example, the number of abdominal taps had been restricted to a single procedure, 93 mice would have been needed to produce the equivalent amount of antibody obtained from 20 mice in three taps. Increasing antibody concentration in ascites fluid from successive abdominal taps has been reported by others (3, 7, 8).

Contrary to the example provided by group RMK, there may be cell lines in which antibody production is sufficiently greater at early taps, or progression of pathologic changes is sufficiently rapid or severe to warrant euthanasia at the first or second tap. For cell line 2C6D9, 94% of the antibody produced was obtained from ascites fluid at taps 1 and 2. Only 35% of the mice in this group survived to tap 3. Euthanasia at tap 2 would be a consideration for future use of this cell line.

These results suggest that decisions on limitation of the number of abdominal taps performed would best be made in view of the clinical condition of the mice and antibody production parameters, to include ascites fluid volumes and antibody concentration for taps over time. Although lot-to-lot variation in murine ascites production parameters has been observed within the same cell line (3), the potential usefulness of quantitating production by tap is clearly apparent and, taken together with clinical observations, may be useful in assisting management decisions regarding the number of abdominal taps to be performed, particularly for cell lines that are used repeatedly. Although limiting the number of abdominal taps may provide greater assurance that the potential for pain and/or distress are minimized, larger numbers of animals will likely need to be used to satisfy antibody production requirements. On the basis of the clinical appearance of most animals by tap 3, it is the authors' opinion that, as a general recommendation, three abdominal taps should be considered maximum, but that additional taps could be considered for cell lines with clinical effects and production parameters similar to those for group-RMK mice. It should be emphasized, however, that regardless of the maximal number of permissible taps, decisions for the timing of euthanasia should be made on the basis of ongoing clinical assessment of individual animals.

Results of this study indicate that the trends in ascites fluid volume and antibody concentration over time from the first to the third abdominal tap may differ significantly for different hybridomas. This factor contributes to the difficulties in formulating general recommendations for managing mice with ascites. Although it is difficult to make meaningful comparisons with literature regarding antibody production parameters because of the significant variability in many of the procedural parameters used in those studies and the known variability among hybridoma cell lines, the production parameters reported here, including ascites fluid volume, antibody concentration, and total antibody production, compare favorably to those of other published reports (3, 7, 8, 24). The volume of ascites fluid collected per tap per mouse is, however, in some instances smaller in our report than in other published reports. This may have re-

sulted from the care taken to ensure that ascites fluid accumulation in the animals did not become excessive.

In this study, abdominal paracentesis was performed via gravity flow. A vacuum aspiration technique also has been described and has been considered advantageous on the basis of more thorough removal of ascites fluid in a shorter period, with potentially less stress and trauma on the animals (3). More thorough removal of ascites may contribute to increased volume of ascites fluid collected, and hence increased production.

Many alternatives are available for laboratory-scale growth of hybridoma cells in vitro, and the available methods have been reviewed (25–27). In vitro methods include stationary cultures in T-flasks and suspension cultures in roller bottles and spinner flasks (24, 28, 29). Other techniques include growth of cells in dialysis tubing in a culture bottle (30), a roller bottle-like apparatus (31) or tumbling chamber (32), and use of oscillating bubble dialysis chambers (33) or gas-permeable tissue culture bags (34). Laboratory-scale stirred tank reactors (35, 36), fermentors (33, 37), ceramic-matrix bioreactors (38), packed-bed bioreactors (39), and hollow fiber bioreactors (38, 40–42) are also in use. Three of the hybridomas evaluated in this study were successfully grown in vitro in hollow fiber bioreactor systems, and comparisons have been made between antibody production in the bioreactor systems and in mice (42). Antibody produced in the bioreactor systems in 65 days was equivalent to production in 4 to 48 mice, dependent on hybridoma cell line and bioreactor system used.

Every attempt should be made to improve procedural protocols to increase survival of the mice in an attempt to maximize production so that the smallest number of mice can be used without compromising humane animal care. Because hybridomas generally grow well and secrete large amounts of antibody in the microenvironment of the pristane-primed mouse peritoneal cavity, future studies should be designed to identify the specific factors involved in the enhancement of cell growth and secretion at this site, and to explore potential applications of this information to enhance antibody production in in vitro production systems. Refinements in procedural techniques for monoclonal antibody production in murine ascites have been reviewed (1, 2) and should be applied as long as mice continue to be used for antibody production. In vitro alternatives for MAb production should be used whenever feasible.

References

1. **Jackson, L. R., and J. G. Fox.** 1995. Institutional policies and guidelines on adjuvants and antibody production. ILAR J. **37:**141–152.

2. **McGuill, M. W., and A. N. Rowan.** 1989. Refinement of monoclonal antibody production and animal well-being. ILAR News **31:**7–11.

3. **Chandler, J. P.** 1987. Factors influencing monoclonal antibody production in mouse ascites fluid, p. 75–92. *In* S. S. Seaver (ed.), Commercial production of monoclonal antibodies: a guide for scale-up. Marcel Dekker, Inc., New York.

4. **Brodeur, B. R., and P. S. Tsang.** 1986. High yield monoclonal antibody production in ascites. J. Immunol. Methods **86:**239–241.

5. **Stewart, F., A. Callander, and D. J. Garwes.** 1989. Comparison of ascites production for monoclonal antibodies in BALB/c and BALB/c-derived cross-bred mice. J. Immunol. Methods **119:**269–275.

6. **Workman, P., A. Balmain, J. A. Hickman, *et al.*** 1988. UKCCCR guidelines for the welfare of animals in experimental neoplasia. Lab. Animals **22:**195–201.

7. **Brodeur, B. R., P. Tsang, and Y. Larose.** 1984. Parameters affecting ascites tumor formation in mice and monoclonal antibody production. J. Immunol. Methods **71:**265–272.

8. **De Deken, R., J. Brandt, F. Ceulemans, *et al.*** 1994. Influence of priming and inoculation dose on the production of monoclonal antibodies in two age groups of BALB/c mice. Hybridoma **12:**53–57.

9. **Hoogenraad, N. J., and C. J. Wraight.** 1986. The effect of pristane on ascites tumor formation and monoclonal antibody production. Methods Enzymol. **121:**375–381.

10. **Amyx, H. L.** 1987. Control of animal pain and distress in antibody production and infectious disease studies. J. Am. Vet. Med. Assoc. **191:**1287–1289.

11. **Silverman, J.** 1987. Protocol review. Monoclonal antibody production: in vivo vs. in vitro. Lab. Anim. **16:**19–20.

12. **Gillette, R. W.** 1987. Alternatives to pristane priming for ascitic fluid and monoclonal antibody production. J. Immunol. Methods **99:**21–23.

13. **Mueller, U. W., C. S. Hawes, and W. R. Jones.** 1986. Monoclonal antibody production by hybridoma growth in Freund's adjuvant primed mice. J. Immunol. Methods **87:**193–196.

14. **Jones, S. L., J. C. Cox, and J. E. Pearson.** 1990. Increased monoclonal antibody ascites production in mice primed with Freund's incomplete adjuvant. J. Immunol. Methods **129:**227–231.

15. **Cranco, M., and M. Potter.** 1976. The requirement of an adherent cell substratum for the growth of developing plasmacytoma cells in vivo. J. Exp. Med. **144:**1554–1567.

16. **Hoogenraad, N., T. Helman, and J. Hoogenraad.** 1983. The effect of pre-injection of mice with pristane on ascites tumor formation and monoclonal antibody production. J. Immunol. Methods **61:**317–320.

17. **Zola, H.** 1995. Conventional mouse monoclonal antibodies, p. 29. *In* H. Zola (ed.), Monoclonal antibodies: the second generation. BIOS, Oxford, England.

18. **Galfre, G., and C. Milstein.** 1981. Preparation of monoclonal antibodies: strategies and procedures. Methods Enzymol. **73:**1–46.

19. **Epstein, N., and M. Epstein.** 1986. The hybridoma technology: production of monoclonal antibodies. Adv. Biotechnol. Processes **6:**179–218.

20. **Jackson, L. R., L. J. Trudel, J. G. Fox, *et al.*** 1999. Monoclonal antibody production in murine ascites. I. Clinical and pathologic features. Lab. Anim. Sci. **49:**70–80

21. **Andrews, E. J., B. T. Bennett, J. D. Clark, *et al.*** 1993. Report of the AVMA panel on euthanasia. J. Am. Vet. Med. Assoc. **202:**229–249.

22. **Catty, D., and C. Raykundalia.** 1989. ELISA and related enzyme immunoassays, p. 97–154. *In* D. Catty (ed.), Antibodies volume II. A practical approach. IRL Press at Oxford University Press, Oxford, England.

23. **Gerrold, H. Zar.** 1984. Biostatistical analysis, p. 190–191. Multiple comparisons. Prentis Hall, Englewood Cliffs, N.J.

24. **Kuhlmann, I., W. Kurth, and I. Ruhdel.** 1985. Monoclonal antibodies: in vivo and in vitro production on a laboratory scale, with consideration of the legal aspects of animal protection. ATLA **17:**73–82.

25. **Marx, U., M. J. Embleton, R. Fischer, *et al.*** 1997. Monoclonal antibody production: the report and recommendations of ECVAM workshop 23. ATLA **25:**121–137.

26. **McArdle, J.** 1997/1998. Alternatives to ascites production of monoclonal antibodies. Animal Welfare Information Center Newsletter, vol. 8, no. 34.

27. **C., Petrie, D. Jensen, T. Allen, *et al.* (ed.),** 1997. Information resources for adjuvants and antibody production:comparisons and alternative technologies, p. 67–117. Agricultural research service national agricultural library animal welfare information center, USDA, Beltsville, Md.

28. **Boyd, J. E., and K. James.** 1989. Human monoclonal antibodies: their potential, problems, and prospects, p. 1–43. *In* A. Mizrahi (ed.), Monoclonal antibodies: production and application. Alan R. Liss, New York.

29. **Reuveny, S., and A. Lazar.** 1989. Equipment and procedures for production of monoclonal antibodies in culture, p. 45–80. *In* A. Mizrahi (ed.), Monoclonal antibodies: production and application. Alan R. Liss, New York.

30. **Sjörgren-Jansson, E., and S. Jeanson.** 1990. Growing hybridomas in dialysis tubing, optimization of technique, p. 41. *In* E. Zola (ed.), Laboratory methods in immunology. CRC Press, Boca Raton, Fla.

31. **Falkenberg, F. W., T. Hengelage, M. Krane, *et al.*** 1993. A simple and inexpensive high density dialysis tubing cell culture system for the in vitro production of monoclonal antibodies in high concentration. J. Immunol. Methods **165:**193–206.

32. **Jaspert, R., T. Geske, A. Teichmann, *et al.*** 1995. Laboratory scale production of monoclonal antibodies in a tumbling chamber. J. Immunol. Methods **178:**77–87.

33. **Pannell, R., and C. Milstein.** 1992. An oscillating bubble chamber for laboratory scale production of monoclonal antibodies as an alternative to ascitic tumors. J. Immunol. Methods **146:**43–48.

34. **McArdle, J., J. Reddington, G. Reddington, *et al.*** 1996. Further development of a unique, simple, inexpensive, efficient in vitro method for small, medium and large scale production of monoclonal antibodies. ATLA **24:**318.

35. **Reuveny, S., D. Velez, L. Miller, *et al.*** 1986. Comparison of cell propagation methods for their effect on monoclonal antibody yield in fermentors. J. Immunol. Methods **86:**61–69.

36. **Shevitz, J., S. Reuveny, T. L. LaPorte, *et al.*** 1989. Stirred tank perfusion reactors for cell propagation and monoclonal antibody production, p. 81–106. *In* A. Mizrahi (ed.), Monoclonal antibodies: production and applications. Alan R. Liss, New York.

37. **Falkenberg, F. W., H. Weichert, M. Krane, *et al.*** 1995. In vitro production of monoclonal antibodies in high concentration in a new and easy to handle modular minifermenter. J. Immunol. Methods **179:**13–29.

38. **Kurkela, R., E. Fraune, and P. Vihko.** 1993. Pilot-scale production of murine monoclonal antibodies in agitated, ceramic-matrix, or hollow-fiber cell culture systems. BioTechniques **12:**674–683.

39. **Moro, A. M., M. T. A. Rodrigues, N. M. Gouvea, *et al.*** 1994. Multiparametric analyses of hybridoma growth on cylinders in a packed-bed bioreactor system with internal aeration. Serum-supplementation and serum-free media comparison for MAb production. J. Immunol. Methods **176:**67–77.

40. **Hopkinson, J.** 1985. Hollow fiber cell culture systems for economical cell-product manufacturing. Bio/Technology **3:**225–230.

41. **Alsthuler, G. L., D. M. Dziewulski, J. A. Sowek, *et al.*** 1986. Continuous hybridoma growth and monoclonal antibody production in hollow fiber reactors-separators. Biotechnol. Bioeng. **28:**646–658.

42. **Jackson, L. R., L. J. Trudel, J. G. Fox, *et al.*** 1996. Evaluation of hollow fiber bioreactors as an alternative to murine ascites production for small scale monoclonal antibody production. J. Immunol. Methods **189:**217–231.