Guidelines for the Use of Rodents in Experimental Neoplasia and Production of Polyclonal and Monoclonal Ascites[1]

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Summary — Guidelines for the Use of Rodents in Experimental Neoplasia

1. OLAC and Environmental Health and Safety must be consulted when developing protocols involving inoculation with cells that may harbor viruses or transmissible agents. Appropriate arrangements must be made for isolating the animals. Xenografts of human tumors implanted in immunodeficient hosts may be contaminated with human pathogens. These require appropriate biohazard containment procedures. Tumors from rodent cell lines or that have been passaged through rodents need special testing prior to inoculation into animals in order to avoid inadvertent introduction of potential rodent pathogens. OLAC must be contacted prior to use of all rodent-derived biological materials in live rodents.

2. Endpoints of experimental neoplasia must be determined and justified in the experimental protocol.

3. Tumor implantation sites should be chosen to minimize interference with normal body functions such as ambulation, eating, drinking, defecation, or urination. Subcutaneous implantation in the flank is considered the least painful. All tumor injection sites must be justified and approved in the Master Animal Use Protocol (MAUP).

4. Tumors must not exceed 1.5 cm diameter in mice and 2.5 cm diameter in rats. If there are multiple tumors, the diameters of all tumors totaled must not exceed 1.5 cm (mice) or 2.5 cm (rats). These should be considered maximum size, earlier size end-points should be used If possible.

5. Animals must be observed with sufficient frequency to ensure that they can be euthanized according to established end-points. All animals should be inspected at least three times a week until the tumor development becomes evident. Observations (and measurements) must be daily as tumors approach maximum size.

6. Animals with tumors exceeding 1.5 cm (mice) or 2.5 cm (rats), ulcerated tumors, or with tumors that interfere with normal activity must be euthanized.

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[1] Sections of this document were developed or excerpted from guidelines approved in 1993 by the Division of Comparative Medicine at the Massachusetts Institute of Technology, and the U.S. Veterans' Administration. These guidelines also include contributions from Professors J. Allison and S. Nandi of the UCB Cancer Research Laboratory and School of Public Health, and Drs. H. Diggs, D. Berger and P. Collins of the UCB OLAC Veterinary staff.

Summary — Guidelines for the Use of Rodents in Ascites Production

1. The justification for ascites production must be included in the MAUP and reviewed and approved by the ACUC.

2. Rodents should be primed once i.p. with 0.2 ml of Pristane, 10 to 14 days before hybridoma cells are injected.

3. After inoculation with an ascites-producing tumor line, mice should be observed at least three times in the first 5 days, and daily thereafter to monitor the amount of abdominal distention and signs of illness or distress.

4. Ascites fluid should be removed by peritoneal tap before abdominal distention is great enough to cause discomfort or interfere with normal activity. Mice should be euthanized if the ascites becomes bloody or infected, or when the mice show signs of poor condition such as huddling, ruffled coat, or inability to take food and water. No more than three taps per mice may be performed; animals should be euthanized immediately following the third tap.

5. Trained personnel may remove peritoneal fluid with an 18 gauge or smaller needle from unanesthetized mice. Students and new personnel should be trained using anesthetized mice.

1. OBJECTIVE

These guidelines are general in scope. They have been defined to accommodate a wide range of experimental protocols while minimizing the number of animals needed, duration of animal use, the procedures on each animal, the pain, distress, and invasiveness of the procedures, and the risk to the animal, animal colony, and personnel.

Tumor and ascites development in rodents evokes a range of effects that depend on the experiment, the tumor line, and the response of the individual animal. This document provides guidelines for designing and conducting procedures that will accomplish the experimental objectives without causing unnecessary distress or debility. ACUC will consider alternative procedures, provided they can be justified or documented as more likely to succeed.

2. GENERAL CONSIDERATIONS

Understanding the biology of the tumor or system you intend to employ is critical to the design of your protocol. For example, tumors that metastasize have entirely different effects on the animal than tumors that develop locally. Replication times differ with tumor type, and determine the duration of the study and the frequency with which animals must be observed. Tumors induced by carcinogens or viruses may pose additional problems in predicting the onset, duration, and severity of the effects. A thorough literature search and consultation with persons who are doing similar research should be conducted before an animal use protocol is written for this type of experiment. Excellent starting references include Kallman, et al. (1985), Kallman (1987); Martin, et al. (1986) and Workman, et al. (1988).

Tumors may secrete factors that cause severe morbidity independent of tumor size, location or other aspects of their biology. Lymphomas and tumors of the internal organs such as liver or lung may cause morbidity not evident by external examination. The host animal may become severely debilitated before this type of tumor becomes very large. Body weight, behaviors such as lethargy and difficulty breathing, and biochemical parameters such as blood chemistry should be monitored when tumors with these properties are involved. The termination of the experiment should be based on the changes in these parameters.

Transmissible Pathogens

Investigators must notify OLAC before animals are to be inoculated with cells that may harbor viruses or transmissible agents, and appropriate arrangements must be made for isolating the animals. Tumor-producing and ascites-producing cell lines, especially those that have been passed in animals, ideally should be tested to demonstrate that they are free of murine viruses and other transmissible agents that could contaminate the animal colony, infect humans, and introduce unwanted experimental variables. In particular, xenografts of human tumors which are typically grown in immunodeficient hosts may be contaminated with human pathogens.

These require appropriate biohazard containment procedures. OLAC and Environmental Health and Safety must be consulted when developing protocols of this type.

We recognize that many studies are done with established cell lines that appear to be free of pathogens and have a long history of use with no adverse reactions in the host. Because testing of such lines is very difficult or impractical to do, investigators are not required to demonstrate that established rodent cell lines are free of these agents.

End points
Experiments using rodents in experimental neoplasia and ascites production must have definable end points. Death is NOT considered to be an acceptable end point, except in unusual circumstances. Endpoints and guidelines for euthanasia should be specified when the animal protocol is designed. Whether the end point is based on time post-implantation or on tumor size, animals must be observed frequently enough to minimize distress and morbidity. All animals should be inspected at least three times a week until the tumor or ascites development becomes evident, and the animals must be observed daily thereafter, until they are to be euthanized. Attention should be given to the animal's over-all appearance, weight, respiratory rate and pattern, color, fecal and urinary output, size of the tumor, etc. Appropriate supportive and/or analgesic therapy may be proposed to allow some experiments to continue humanely.

Pilot Studies
In many experiments the end point or the range of effective experimental parameters such as number of cells, amount of DNA, or dosage of chemicals or radiation may not be precisely known at the outset. Investigators are strongly encouraged to propose smaller scale pilot experiments that would use the fewest animals possible to establish the optimum conditions or estimate the morbidity or mortality before undertaking a full-scale study that would require more animals.

3. INOCULATION WITH SOLID TUMORS
The site of tumor implantation is an important factor that determines potential pain and distress. Subcutaneous or intradermal implantation in the flank is the least painful, preferred method. Sites should be chosen to minimize damage to adjacent normal structures and interference with normal bodily functions such as ambulation, eating, drinking, defecation, or urination. Sites involving the sensory functions, such as the eye, should be avoided. Intramuscular implantation should be avoided if possible, because a growing tumor causes muscle distention that could be painful.

Tumor burden is one of the most important factors affecting the animal's health and well being. It is extremely difficult to provide guidelines for the upper limit (endpoint) of tumor burden, because it depends on several factors that include (but are not limited to) the tumor biology, growth rate, implantation site, and condition of the host. When animals develop difficulty ambulating, eating, drinking, defecating, or urinating, they should be humanely euthanized. Mice inoculated with cells producing solid tumors must be observed with sufficient frequency (a minimum of three times per week) to ensure that they can be euthanized before the tumors ulcerate or become large enough to interfere with normal activity. The mass of a tumor is proportionate to the cube of its diameter. As a general guideline, tumors should not exceed 10% of the animal's normal body weight or 1.5 cm diameter in mice and 2.5 cm diameter in rats. However, it is important to recognize that animals with smaller tumor burdens may require euthanasia. The checklist appended to these guidelines should be used to ensure that your Animal Use Protocol includes all relevant information for an approvable neoplasia experiment.

4. OPTIONS FOR PRODUCING ANTIBODIES AS ASCITES IN MICE
The most frequent reason for ascites production is the growth of hybridoma lines as ascites to obtain large amounts of monoclonal antibody. However, it is also possible to produce substantial amounts of polyclonal antibodies by inducing ascites in mice that have raised antibodies to a particular immunogen. In this case, the ascites is induced by intraperitoneal injection of a sarcoma cell line, and the desired antibodies are secreted into the ascitic fluid by the host's B-lymphocytes. Mice cost much less than rabbits to purchase and maintain, they require much less space, are easier to handle, generally respond to less antigen, and their genetics of immunoresponsiveness offers more options. A high-titer ascites from two to four mice may give the user about as much antibody as all of the serum from a

rabbit. The sarcoma cells used to generate polyclonal ascites can be stored indefinitely in liquid nitrogen.

Situations for which polyclonal ascites antibodies in mice are advantageous include:

- Projects in which the investigator has a one-time need for an immunoreagent, and it is unnecessary or undesirable to maintain and boost rabbits or other animals.
- Projects involving several different candidate immunogens, and it is not known beforehand which will be best. Examples include peptide and small molecule haptens.
- Projects that may lead to hybridoma production.
- Making use of the greater sensitivity of mice to microgram or smaller amounts of immunogen.

Key steps in ascites production
Polyclonal ascites is induced by inoculating immunized, antibody-producing mice with a rapidly growing sarcoma. No “priming” step is used for polyclonal ascites production. Production of monoclonal ascites from hybridomas requires a priming step, and it may also require irradiation to immunosuppress the host, as described below. Procedures for harvesting the ascites, monitoring the health of the animals, and euthanasia are the same for polyclonal and monoclonal ascites.

Polyclonal ascites
Details of the preferred procedure have been published (Karu, 1993, p. 264) and may be found in the Appendix to these guidelines. Other methods which we do not recommend have also been published for inducing polyclonal ascites by intraperitoneal injection of Freund adjuvant, or using one of the mouse myeloma lines commonly used for hybridoma production (Tung, Ju, et al., 1976; Lacy and Voss, 1986; Harlow and Lane, 1988). Induction with Freund adjuvant is unpredictable and may even occur before the animal has developed a usable response (it is a good argument against the use of Freund adjuvants for i.p. injections). The myeloma lines are of the BALB/c genotype and may grow poorly or be rejected in other strains of mice.

Mice must be euthanized at the end of polyclonal ascites production. Ascites formation is not induced until the mice develop serum titers of 10^4 or greater against the immunogen. The yield per mouse is 5 to 20 mL of ascites at approximately the same titer as the serum. Generally this is sufficient for hundreds or thousands of assays (ELISA, Western blot, immunofluorescence, etc.). The IgG antibodies may be affinity purified from polyclonal ascites if necessary.

Monoclonal ascites
Hybridoma cells can induce ascites into which they secrete concentrated monoclonal antibody in properly sensitized mice. The concentration, amount, and purity of the antibody will vary between cell lines and individual mice. Because all of the mouse cell lines commonly used as hybridoma fusion partners were derived from BALB/c mice, hybridomas are often made with BALB/c splenocytes. Ascites may then be made in BALB/c mice, because the resulting hybridomas are fully histocompatible. However, many laboratories have found that it is preferable to immunize mice of different strains and prepare the hybridomas from splenocytes of the best-responding mice. These inter-strain hybridomas will be rejected by BALB/c mice, but ascites can be made in immunodeficient or immunosuppressed hosts. As with any procedure for scale-up of a product from a cell line, investigators are strongly advised to use a stable subclone to prepare monoclonal ascites.

"Priming" for monoclonal ascites production

For monoclonal ascites production the mice must be "primed" with an intraperitoneal injection of a substance that stimulates macrophages and makes the peritoneum better accommodate growth of the hybridomas. The most commonly used and most effective priming agent is the branched-chain alkane 2,6,10,14,-tetramethylpentadecane, known as Pristane. Pristane (available from Aldrich Chemical Co. and most other organic chemical manufacturers) is the component of mineral oil (and hence of Freund adjuvants) that promotes growth of plasmacytomas. It is an irritant and a potential carcinogen, it depresses the mouse's normal immune response, and it interferes with peritoneal lymphatic drainage, increasing fluid yields. Pristane is toxic at doses somewhat higher than those used to prime mice. It should be handled with gloves and skin contact and self-inoculation should be avoided.

Because of its adverse effects, pristane should be used at the smallest dose needed to ensure ascites formation in 100% of the mice. A summary of the properties of pristane and the optimum amounts and priming intervals for ascites production in mice may be found in Hoogenraad and Wraight (1986). Our guidelines conform to their findings. A single dose of 0.2 ml i.p. is recommended for adult mice (20 to 30 gm). Larger amounts up to 0.5 ml may be justified in larger mice, proportionate to their weight, if investigators have experienced a reduced percentage of ascites-producers, a smaller volume of ascites, or an increased percentage of mice developing solid tumors. Additional priming doses may harm the animal, and there is no conclusive published evidence that more than one dose improves the efficiency of ascites tumor formation or the yield of antibody. The pristane should be administered 10 to 14 days before the hybridomas are injected. Mice should be observed the day after pristane is administered and any animals that show ill effects should be euthanized. Excess pristane is secreted through the skin, giving the fur an oily appearance.

Incomplete Freund Adjuvant (IFA; mineral oil) has also been used as a priming agent. The active substance in IFA is probably pristane or similar alkanes. Some investigators have observed fewer adverse side effects with IFA than with pristane, and it has been reported that hybridomas can be injected as early as 24 hr after introduction of IFA. One disadvantage of

IFA is that it is not as rapidly cleared as pristane from the peritoneum and may be recovered in the ascitic fluid. A second disadvantage is the potential for inducing a "mineral oil plasmacytoma" in mice, even if they receive no exogenous cells. Microbiological media such as proteose-peptone and thioglycollate broth that were used in the past to induce peritoneal macrophage proliferation were reported to be much less efficient than pristane for priming (Gillette, 1987).

Immunosuppression by irradiation

Female Swiss Webster retired breeders or any other large mice (as well as BALB/c mice) may be used to make ascites from any hybridoma if the mice are immunosuppressed. This is most easily done by giving the mice 500 rads of whole-body gamma radiation 12 days after the pristane is injected. The mice should be brought to the irradiation site and subsequently maintained in cages with filter bonnets (isolator cages). Investigators who plan to irradiate mice must be trained and certified by the appropriate radiation safety personnel.

The 500 rads of whole-body g-radiation that the mice receive are well below the LD_{50}, which is on the order of 1,000 rads. This dosage is sufficient to immunosuppress but should cause no symptoms of radiation sickness or distress. Symptoms of radiation sickness in mice are diarrhea, redness and irritation that cause the mice to scratch their eyes, ears, and noses, loss of hair, and cessation of eating, in that order of severity. Mice that show such symptoms should be euthanized.

Alternatives to irradiation are the use of immunodeficient mice (nude or SCID), chemical immunosuppression, or the i.p. administration of anti-mouse thymocyte serum. Immunodeficient mice

are smaller and more expensive to purchase and maintain than normal mice. Chemical or antibody-caused immunosuppression is less consistent and may have other effects on the animal that influence ascites production.

Inoculation with a hybridoma

The hybridoma suspension (approximately 5 x 10^5 to 2 x 10^6 cells in 0.5 ml sterile serum-free cell culture balanced salt solution) is administered i.p. 24 to 36 hr after irradiation, or 10 to 14 days after pristane injection in unirradiated mice. Inoculation with fewer cells may lead to a long delay or failure in ascites production. Inoculation with more cells may lead to solid tumor formation and little or no ascites fluid. The mice should be monitored for adverse effects the day after the cells are injected, and for ascites formation from the fifth day. Ascites production most commonly occurs between the 7th and 14th days after the cells are injected.

Harvesting ascites

The ability to judge when and how to harvest ascites and when to euthanize the mice should be learned from experienced personnel. New personnel and students should be trained using anesthetized mice.

Ascites fluid should be removed by peritoneal tap with a needle of 18 gauge or smaller before abdominal distention is great enough to cause obvious discomfort or interfere with normal activity. Unanesthetized mice may be held by properly trained personnel during the procedure, or the animal may be anesthetized[2]. The abdominal area should be disinfected with 70% ethanol or Betadine and gently dried before puncture with the needle. In general, the volume collected should not exceed 20% of normal body weight (about 5 to 8 ml). The fluid is allowed to drip through the needle hub into a collection tube. When the ascites is viscous collection is more efficient if the needle is removed and the fluid is allowed to drip from the puncture into a tube. A good flow is about one drop every 3 or 4 seconds. When collection is complete, the puncture site should be disinfected again before the animal is returned to its cage.

The progress of ascites tumors is different in each mouse. It is mandatory that mice be examined every day from 5 days post-injection, including weekends and holidays, and tapped or euthanized as needed. There is no prescribed schedule for tapping. In general mice will have to be tapped every third day during active ascites production, and distress or debility becomes evident after the third tap. In keeping with animal care guidelines adopted by the U.S. Veterans' Administration and many academic and research institutions, ascites collection at Berkeley is limited to a maximum of three taps, with the mouse being euthanized immediately after the third tap. Exceptions to this policy will be considered, but require (a) adequate scientific justification and (b) evidence that mice subjected to additional tapping do not show signs of distress or debility. Mice that fail to produce ascites within 25 days after hybridoma injection should be euthanized. Mice that form solid tumors should be euthanized if the tumor mass exceeds 10% of the average body weight. Mice that show signs of distress, cachexia (loss of weight), failure to eat and drink, abnormal respiration, or any signs of abdominal hemorrhaging or infection should be euthanized. Glazing of the eyes and a translucent, pale appearance of the ears (due to reduced blood flow) are signs of debility.

Problems encountered in ascites production.

Normal ascites has the straw-like color of serum. It may be viscous because the cells have grown to high density, and a clot may form because fibrin is present. A thick, milky appearance indicates possible infection. The ascites may contain some red blood cells or hemoglobin from ruptured capillaries, but if it resembles blood in color there may be internal hemorrhaging. Mice should be euthanized when they show signs of poor condition, such as huddling, ruffled coat, inability to take food

[2] Use of ether is discouraged because it is flammable and explosive. The Office of Environment, Health and Safety requires ether to be used and stored in a properly ventilated fume hood or safety cabinet.

or water, or the occurrence of bloody or thick, milky ascites. The most commonly encountered difficulties are summarized in the table below:

Problem	Cause and Corrective Action
Failure to produce ascites	Cells had low viability when introduced, or they were rejected. Hybridomas should be in mid-log growth (approx. 5 x 10^5 cells/ml) when inoculated. If rejection is the likely cause, use immunosuppressed mice.
Solid tumor formation	Too many cells (more than 2 x 10^6) were inoculated. Use fewer cells. Some hybridoma lines tend to form solid tumors.
Bloody or infected ascites	Some hybridoma lines may cause hemorrhaging. The ascites probably has usable antibody titer. Harvest the ascites tap in progress and euthanize the mouse. Pus or milky material in ascites indicates non-sterile inoculation technique or damage to intestine. Harvest the ascites tap in progress and euthanize the mouse.

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APPENDIX

Procedure for Generating Polyclonal Ascites Antibodies in Mice

The T-180 mouse sarcoma is the cell line of choice for highly predictable ascites induction. A vial can be obtained from the American Type Culture Collection (Cat. # TIB 66). Initially the investigator should propagate his/her own stock of cell suspension in mice that have not been immunized. Inoculation of five mice with aliquots of the cell suspension will yield enough ascites to perform the procedures in 50 to 75 immunized mice.

Swiss Webster female retired breeders are preferred for this procedure, because they are outbred, respond well to most immunogens, produce more ascites than smaller mice, and they are histocompatible with the T-180 sarcoma. The cell line also may be propagated and induces ascites in other mouse strains.

To propagate the cell stock, it is rapidly thawed at 37• C and aliquots of 0.2 ml are injected i.p. in five mice. The mice are monitored daily beginning five days after inoculation, and ascites is harvested into sterile tubes as it develops. Aliquots of 1 ml of the ascites (containing about 107 suspended cells) are transferred to sterile cryogenic vials and frozen by the procedures commonly used to freeze cell cultures. The vials may be stored in liquid nitrogen until needed. Alternatively, the cells may be harvested from the ascites by centrifugation (approx. 1,000 x g, 10 min), counted by hemacytometer, resuspended at approx. 107 cells/ml in serum-free cell culture medium containing 10% dimethylsulfoxide (as cryoprotectant), and frozen.

To generate polyclonal ascites, young or older mice are immunized according to any approvable protocol, and the serum antibody titer is monitored by any convenient immunoassay (for recommended immunization and bleeding schedules, see the ACUC Guidelines for Antibody Production). Additional boosts may be necessary for weak antigens. When a satisfactory titer has developed, the mice are given a final boost. Three to five days later, the mice are given an intraperitoneal injection of 106 to 107 T-180 sarcoma cells in 1 ml of saline or serum-free culture medium. From the 7th day after the cells are injected, the mice are monitored daily for ascites development, and ascites fluid is harvested as necessary by peritoneal tap with an 18-gauge needle. The mice are euthanized by cervical dislocation when they show signs of distress such as huddling, ruffled coat, or difficulty moving or taking food or water.

OPTIONS FOR RAISING ANTIBODIES IN MICE