

ANTIBODY PRODUCTION POLICIES AND PROCEDURES

POLYCLONAL ANTIBODY PRODUCTION

- **ADJUVANTS** - There are a number of adjuvants of interest to the IACUC. Freund's Complete Adjuvant (FCA) is of particular interest because it can cause severe inflammation and ulceration at the injection site if used incorrectly. FCA should be used only for the initial immunization, with Freund's Incomplete Adjuvant (FIA) used for subsequent booster injections. Other adjuvants should be considered, before FCA and FIA is used. FCA should only be used if no appropriate alternatives are available. **(1) It is necessary to provide the specific rationale for selection of species and adjuvant for use with particular antigens. Applications must be based on consideration of the amount of antibody required, the type of response required, and the nature of the antigen.**
- **FREUND'S ADJUVANT** - FIA consists of 85% mineral oil or paraffin oil and 15% mannide monooleate (Arlacel A) as emulsifier. With the addition of heat-killed mycobacteria (*M. butyricum* or *M. tuberculosis*) the mixture is termed FCA. FCA is known to commonly produce undesirable side effects. Negative effects routinely seen include granuloma formation, tissue necrosis and sloughing, abscessation, and fever. Other deleterious systemic effects, such as polyarthritis, have been reported. FCA is considered a human biohazard, with accidental self-innoculation, or eye splash have been shown to cause painful sequelae not readily amenable to treatment, as well as sensitization to tuberculin. (2)
- **OTHER ADJUVANTS** - Less inflammatory alternatives to Freund's adjuvant are available and should be considered. Ribi Adjuvant System® and TiterMax® are commonly cited as appropriate alternatives. Noninflammatory adsorptive adjuvants such as alum and aluminum hydroxide gel may also be considered. (3)
- **ROUTES OF ADMINISTRATION, VOLUME, SITES, AND SPECIES SELECTION**
Consideration and justification must be given in the animal use protocol for selection of the laboratory animal species, adjuvant, volume per injection site, site of administration, number of sites, and response required. Particularly with the use of Freund's adjuvant, it is important to note that the severity of potentially painful inflammatory reactions may be minimized by injection of a small volume of inoculum per site and the use of multiple, sufficiently separated, injection sites when appropriate. The table below lists the UVM requirements for maximum volumes of antigen when using Complete Freund's Adjuvant. These volumes are extracted from an article on the use of Freund's adjuvant by Harold F. Stills. (4)

Routes of Administration:

- Intramuscular
- Intraperitoneal (the intraperitoneal route of administration is only recommended in rodents and requires additional justification because of the generalized vs. localized inflammatory reaction initiated)
- Subcutaneous
- Intradermal

NOTE: Intravenous injections may be acceptable only if injecting soluble antigens without adjuvant. For example, rabbits have been immunized with soluble antigens (IV) of *C. jejuni* without problems.

Footpad injections are prohibited at the University of Vermont.

Routes of Administration, Volumes per site, Selected Species

Species	Subcutaneous	Intradermal	Intraperitoneal	Intramuscular
Mouse	< 0.1 ml	< 0.05 ml/ site**	< 0.2 ml	< 0.05 ml**
Rat	< 0.1 ml	< 0.05 ml	< 0.5 ml	< 0.1 ml**
Rabbit	< 0.25 ml	< 0.05 ml	*	< 0.25 ml***
Sheep/Goat	< 1.0 ml	< 0.1 ml	*	< 0.5 ml

* Not Recommended

** Must be justified

*** Only one limb recommended unless justified

- **FREQUENCY OF BOOSTERS** - The frequency of boosters must be addressed in the animal use protocol. Two to three weeks is generally considered the minimum time period between the initial and subsequent immunizations. Booster immunizations are sometimes delayed if significant inflammatory reactions are still present from the initial immunization. Booster immunizations can not use Complete Freund's adjuvant. (5)
- **EVALUATION OF PAIN AND DISTRESS** - It is the Principal Investigator's responsibility to ensure the animals are regularly checked. This is in addition to the daily checking done by caretakers. Investigators and veterinary staff should observe the animals for evidence of pain or distress, and for evidence of lesions such as swelling, abscess or fistula formation, and infection or ulceration at the immunization sites. The animal weight should periodically be compared to initial animal weights and this should be indicated in the protocol and documented. Veterinary follow-up, must include clinical observations and palpations of the injected sites and determination of the appropriate supportive therapy. Investigators should contact the Office of Animal Care Management if evidence of lesions at the immunization sites or evidence of pain/distress is identified in antibody production animals. (6)

Blood collection Guidelines. - The general guideline for collection of blood from any healthy research animal without causing anemia is 9 ml/kg of body weight once monthly. This amount is reduced to 6 ml/kg for collection every two weeks, and to 3 ml/kg for collection once every week. When multiple sequential blood collections are to be made, the animal's hematocrit must be checked at least once a month to evaluate the animal for the anemia development.

Maximum blood collection volumes and frequency for rabbits - Listed below are specific blood collection amounts recommended by the University of Vermont OACM to facilitate collection of safe amounts of blood in rabbits that have historically been shown to preclude the development of anemia in healthy research rabbits.

Weight (kg) of animal	Total Estimated Blood Volume (ml)	7.5%	10%	15%
		Once/week	Two weeks rest	Once/month
3.0	168	13 ml	17 ml	26 ml
3.5	196	15 ml	20 ml	30 ml
4.0	224	17 ml	22.5 ml	34 ml
4.5	252	19 ml	25 ml	38 ml
5.0	280	21 ml	28 ml	42 ml
5.5	308	23 ml	31 ml	46 ml
6.0	336	25 ml	34 ml	50 ml

MONOCLONAL ANTIBODY PRODUCTION IN MICE by ASCITES

The production of monoclonal antibodies is a two step process. First, an animal (usually a mouse) is immunized to generate antibody producing cells which are fused with a tumor cell line. The second step is to perpetuate the antibody secreting cells either in culture, or by injection into the peritoneum of mice to

yield ascites. (7) From an animal welfare standpoint *in vitro* methods of monoclonal antibody production are preferable. **Evidence must be presented in the protocol that *in vitro* methods are not acceptable for the production of the monoclonal antibodies required for the research study, before the use of the mouse ascites method can be approved.**

- **STEP ONE – IMMUNIZATION** - The guidelines listed in the preceding section for polyclonal antibody production apply to the immunization in the first step for production of sensitized cell colonies for monoclonal antibody production. These animals are euthanized and spleens are harvested for selection of specific cell colonies for hybridoma formation.
- **STEP TWO - IN VIVO MONOCLONAL ANTIBODY PRODUCTION**
 - **PRIMING AGENT** - Pristane is the agent most frequently used to "prime" the peritoneal cavity for successful growth of hybridomas as ascites producing tumors. The smallest volume of the priming agent causing minimal distress and yielding ascites producing tumors should be used. The priming agent selected and volume injected, or other methods or procedures used to enhance production of ascites fluid (e.g., irradiation), must be justified in the protocol.
 - **INOCULATION OF HYBRIDOMA CELLS** - Inoculation will be done by standard intraperitoneal injection. The standard hybridoma inoculum range is from 10^5 to 10^7 cells inoculated in a total volume from 0.1 to 0.5ml in PBS or basal cell culture media is the accepted normal range. This range varies with the cell line being used. Pilot studies are often required to determine optimal cell numbers for best response.
 - **ABDOMINAL PARACENTESIS** - Ascites pressure must be relieved by abdominal paracentesis when visible abdominal distention becomes evident, and prior to the development of marked abdominal distention with associated clinical signs of pain or distress. (8) It is recommended that anesthesia be used and that the needle insertion site is antiseptically prepared.

The smallest gauge needle feasible for the extraction of the viscous fluid should be used. The volume of ascites fluid removed should not exceed 3 ml/collection. (9) This volume is greater than the total blood volume of a mouse and physiologic distress from hypovolemia can result. To help prevent hypovolemic shock, 2-3 ml of warm saline or lactated ringers solution may be administered subcutaneously between the shoulder blades of the mouse immediately following paracentesis.

Collections of ascites should be limited to a maximum of 3 collections per animal, with the last one being a terminal procedure, with collection after euthanasia. Intervals of 1-3 days between taps are recommended depending upon the degree of abdominal distention.

Animals must be weighed daily. This should begin prior to tapping and continue until euthanasia. The tumor mass must not exceed 15% of the animal's body weight after the animal has been tapped.

- **CLINICAL OBSERVATION** - The person who will be checking the animals must be designated in the protocol. The PI is responsible for ensuring adequate training and oversight of all individuals delegated this task.

In addition to daily observation of animals to assure that abdominal paracentesis is performed before marked abdominal distention or discomfort becomes evident, included in the protocol should be provision for monitoring animals for hunched posture, roughened hair coat,

anorexia, dehydration, weight loss, loss of body condition, inactivity, difficulty in ambulation, tachypnea, and dyspnea. Animals with excessive abdominal distention not relieved by paracentesis should receive abdominal palpation to diagnose solid tumor growth in abdomen. Mice should be monitored for pale eyes, ears, and mucous membranes which may be indicative of anemia or shock. (See Footnote 10)

If animals exhibit severe clinical abnormalities or become moribund, they should be euthanized. Death is not considered an acceptable endpoint to the experiment.

References:

- 1: Institutional Animal Care and Use Committee Guidebook, OPRR and ARENA, page 127
- 2: AVAVMO Antibody Production Guidelines, Edited by Cindy Pekow and Mary Proctor, p.4
- 3: Institutional Policies and Guidelines on Adjuvant's and Antibody Production, Lynn R. Jackson and James G. Fox, ILAR Journal, Volume 37, Number 3
- 4: "The Use of Freund's Complete Adjuvant" by Harold F. Stills, Jr & Michael Q. Bailey, Lab. Animal. 20(4):25-30
- 5: Institutional Policies and Guidelines on Adjuvant's and Antibody Production, Lynn R. Jackson and James G. Fox, ILAR Journal, Volume 37, Number 3
- 6: Ibid.
- 7: Institutional Animal Care and Use Committee Guidebook, OPRR and ARENA, pages 128-129
- 8: ILAR Journal, p. 144
- 9: AVAVMO Antibody Production Guidelines, P. 15
- 10: ILAR Journal, p. 144