Canadian Council on Animal Care

guidelines on:

antibody production

This document, the CCAC *guidelines on: antibody production*, has been developed by the *ad hoc* subcommittee on immunological procedures of the Canadian Council on Animal Care (CCAC) Guidelines Committee:

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antibody production

A. PREFACE

The Canadian Council on Animal Care (CCAC) is responsible for overseeing animal use in research, teaching and testing. In addition to the *Guide to the Care and Use of Experimental Animals*,Vol. 1, 2nd Edn., 1993 and Vol. 2, 1984, which lay down general principles for the care and use of animals, the CCAC also publishes guidelines on issues of current and emerging concerns. The CCAC *guidelines on: antibody production* is the fifth of this series, and has been developed by the CCAC *ad hoc* subcommittee on immunological procedures.

The purpose of this document is to present guidelines for production of both polyclonal (pAb) and monoclonal antibodies (mAb) that assist investigators and research support personnel to achieve an acceptable immunological result with minimal discomfort for the animals involved. These guidelines are also provided to assist animal care committee (ACC) members to evaluate protocols involving the production of antibodies, to ensure that the highest standards of animal care and use are met. These guidelines supercede the guidance on antibody production given in the 1991 CCAC policy statement on *Acceptable Immunological Procedures*.

Many institutions already have excellent standard operating procedures (SOPs) in place for the production of both pAbs and mAbs. These CCAC guidelines borrow much from the experience of these institutions, and from the recent international initiatives to refine protocols for antibody production.

The refinement of animal use in research, teaching and testing is an ongoing process which is never complete. The CCAC recognizes that in moving towards implementation of these guidelines, considerable expenditure may be involved; for example, to establish central services for production of mAbs *in vitro*. As with the implementation of other CCAC guidelines, institutions must recognize such expenditures as responsibilities if sound humane research is to be carried out within their facilities. The CCAC is committed to assisting institutions by providing information on production of mAbs *in vitro* as well as on best practices for animal-based production for pAbs and mAbs where necessary.

B. INTRODUCTION

Antibodies are produced by the immune system of an animal in a specific response to a challenge by an immunogen. The immune system acts through two principal mechanisms: humoral type responses (production of antibodies) and cell-mediated responses. Immunogens (antigens) are molecules which can induce a specific immune response and are usually foreign proteins or carbohydrates, or sometimes lipids and nucleic acids. The immune systems of mammals are comprised of large numbers of lymphocytes, each characterized by its unique antigen-receptor specificity. This receptor diversity permits immune responses to a broad range of immunogens. The B lymphocytes, characterized by the presence of specific immunoglobulin receptors on their surface, are responsible for production of the humoral (antibody) response. Antibodies are secreted from plasma cells which have differentiated from B lymphocytes after appropriate stimulation by the foreign immunogen. Each antibody molecule is capable of recognizing a specific epitope (antigenic site), usually 5-6 amino acids or monosaccharide units which are either linear or topographically assembled, and is capable of binding to this epitope.

A polyclonal humoral response is comprised of antibodies, derived from various clonal populations having varying specificities (for different epitopes, even on the same molecule), affinities and classes, and hence provides an effective defense against pathogens. Polyclonal antisera are difficult to reproduce because of the variety of antibodies made in the polyclonal response. The level and quality of the antibodies produced will vary from animal to animal, and from a single animal over time. Therefore, pAbs have a finite availability and are subject to possible character change during the period of production. On the other hand, monoclonal antibodies (mAbs) are derived from a single clone and hence are specific for a single epitope and have a defined affinity for that epitope. Thus if the right mAb is obtained, it can be extremely specific for the relevant immunogen, and under appropriate conditions, an almost limitless production of a constant product is possible.

Polyclonal antisera can be obtained in a relatively short time frame (1-2 months), in contrast to standard mAb production procedures that can be tedious and require 3-6 months. (Newer procedures, however, can shorten the time for mAb derivation to as little as one month.) Polyclonal antibodies show different affinities for different epitopes and thus may demonstrate overall excellent binding achieved by adherence to a number of different sites on a complex immunogen or antigen. In contrast the single epitope specificity of mAbs may mean that a slight change in the structure of the epitope, for example by antigen denaturation (such as occurs in immunoblotting experiments or by sterilization of the immunogen prior to injection into the animal), can result in loss of antibody binding. For this reason two or three mAbs are sometimes combined.

Consideration should be given at the outset to procuring commercially produced antibodies from sources that comply with CCAC guidelines, or if international, are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). The Antibody Resource Site http://www.antibodyresource.com may assist in this regard as well as

provide background information on antibody production. A list of suppliers of mAbs produced *in vitro* has been compiled by the Focus on Alternatives Group, UK, and a second list of US suppliers has been compiled by the Alternatives Research and Development Foundation, US. Both lists can be found at http://www.frame.org.uk/Monoclonal_Suppliers.htm. Additional material, for example SOPs, are available on the CCAC website http://www.ccac.ca.

C. POLYCLONAL ANTIBODY PRODUCTION

Polyclonal antibodies (pAbs; antisera) have a number of uses in research, for example: for detection of molecules in ELISA-type assays; in Western blots; in immunohistochemical and immunoprecipitation procedures; and in immunofluorescence and immunoelectron microscopy.

Antisera are commonly produced by injection of the immunogen (antigen) of interest into an animal, often in combination with an adjuvant to increase the immune response. The antibody response can be enhanced by subsequent booster injections of the antigen with or without adjuvant. Blood samples are obtained from the animal to assess the level of antibodies produced, and once a sufficiently high titre has been reached, the antiserum is prepared by blood collection followed by serum preparation, with subsequent purification of antibodies from the serum if required.

Background knowledge of the mechanisms involved in generating a humoral immune response is essential in the development of the most appropriate SOPs for particular immunogens or antigens (Hanly, *et al.*, 1995). A number of references to suitable immunology texts are given in the reference list: Abbas, *et al.*, 2001; Anderson, 1999; Kuby, 2000; Roitt, *et al.*, 1998; Sharon, 1998; Sompayrac, 1999.

General guideline: In the production of polyclonal antibodies, the overriding consideration must be to minimize pain and distress for the animals used.

1. Animal Selection and Care

Guideline: Careful consideration should be given to the selection of the species to be used for polyclonal antibody production.

Careful consideration should be given to the appropriateness of the species and strain chosen. The investigator should consider the following factors: 1) the quantity of Ab or antiserum required (larger animals should be considered when larger quantities of Ab are required); 2) the phylogenetic relationship between the species from which the protein antigen originated and the species used to raise the Ab; 3) the effector function of the pAbs made by the species raising the Ab (such as complement fixing ability – see Hanly, *et al.*, 1995); and 4) the intended use of the antibodies (e.g., in an ELISA, the antibody which binds to the antigen may need to be derived from a different species than the secondary antibody used in the next step of the assay). Different strains of a species may also react differently, due to genetic variation in presentation of the antigen in major histocompatibility complex molecules and in immune response regulatory mechanisms (Hanly, *et al.*, 1995; Coligan, *et al.*, 1997).

The rabbit is the most commonly used animal for the production of pAbs, as it is easy to handle and to bleed, and for most applications will produce an adequate volume of high-titre, high affinity, antiserum (Stills, 1994). A typical bleed from a rabbit should yield approximately 250mg of pAb; a terminal bleed using saline displacement can yield approximately 1g of pAb. It is important to use disease-free rabbits for all immunological procedures to reduce the likelihood of *Pasteurella multocida* abscesses at injection sites, and to minimize the likelihood of cross-reactivity to other antigens the rabbits' immune systems may previously have encountered.

For protocols requiring the production of large volumes of pAbs, or where the animals are to be maintained as antibody producers for a long period of time, the use of whiffle balls or serum pockets for antibody collection may be considered (Whary, *et al.*, 1998). Investigators and ACCs should evaluate whether the subsequent ease of handling for serum sampling will outweigh the initial requirement for surgical intervention to implant the whiffle ball chamber.

The chicken is phylogenetically distant from mammals and, therefore, can be useful for raising pAbs to mammalian proteins, and in particular, to intracellular mammalian proteins, as the amino acid sequence of many intracellular proteins tends to be conserved between mammalian species. The product, IgY, is for almost all purposes equivalent to the mammalian IgG class of Abs. The chicken may be considered as a refinement of technique as pAbs can be extracted from the egg yolk, removing the necessity for blood collection. The use of the chicken can also represent a reduction in animal use as chickens produce larger quantities of antibodies than laboratory rodents (Schade, *et al.*, 1996; Bollen, *et al.*, 1995). Typically, a single egg will contain up to 250mg pAb in the egg yolk (Erhard, *et al.*, 2000). In general, chickens are potent antibody producers and their immunological responsiveness is similar to that of mammals. However, it is important to emphasize that the chicken is not suitable for all applications, and appropriate facilities for housing chickens must be available if they are to be used.

In general, rodents are used less frequently than rabbits for pAb production, but may be suitable when small volumes of pAbs are required. Blood volumes that can be collected from these animals are considerably smaller, and thus collection of a reasonable volume may require cardiac puncture under terminal anesthesia. The rat can be used when Abs of restricted specificity to mouse proteins are required, or for IgE production (Garvey, *et al.*, 1977). The hamster is used to produce anti-mouse protein Abs when such pAbs cannot be readily produced in the rat or when broader specificity of pAbs is required.

Historically, the guinea pig was commonly used for antibody production, in particular for use in insulin assays, but otherwise it does not appear to have any significant advantage over the use of rodents.

Larger species are used when large volumes of antisera are required, in particular for commercial production. Horses, sheep and goats, for example, have a long life span, are relatively easy to handle, and can be bled from the jugular vein. Requirement for large animal facilities and the expense of maintaining larger animals

has limited their use principally to commercial production of antisera. It should be noted that horses, which have been used to produce antisera for toxins and venoms for clinical applications, are particularly intolerant of oil-based adjuvants, such as Freund's Complete Adjuvant (FCA) and saponin (Quil A). Antibodies can also be harvested from the milk of cattle, sheep and goats and represent a non-invasive means to repeatedly acquire large volumes of pAbs (Brussow, *et al.*, 1987; Tacket, *et al.*, 1988; and Sarker, *et al.*, 1998).

Where species-specific considerations are not an issue, the species should be chosen to minimize pain and distress, bearing in mind ease of handling for injection and blood sampling. The expected requirement of the pAb will influence the volume of serum required and this will impact on the animal species and number of animals used. In general, young adult animals are better pAb producers than older animals, as immune function peaks at puberty and then slowly declines. In addition, females are preferred as they often produce a stronger immune response and tend to be more docile, easier to handle, and easier to pair or group house. For larger farm animal species, castrated males may also be suitable. Grossman (1989) has documented the enhanced production of pAbs by adult females.

Other factors which influence pAb production include: the nature of the immunogen to be used; the route and timing of administration; the type and quality of adjuvant; the nature of the immunogen/adjuvant formulation (i.e., emulsion, liposome formation, immunostimulatory complex [iscom] formation or adsorption); the method of blood collection; the strain, health and genetic status of the animals; the training, expertise and competence of the animal care staff; and the diet and housing. In addition, it should be noted that the presence of infectious agents and/or stress can suppress the immune response, and thus reduce the quantities of pAbs produced, or even result in no significant response.

The pre-immunization status of the animals, with respect to the immunogen, or other cross-reacting antigens to be administered, should be determined. Immunogen-specific pre-existing Abs can influence both the quality and the quantity of the Abs, especially where a monospecific pAb is required. Usually a pre-immunization (pre-bleed) sample of serum is prepared in sufficient quantity to use as a control throughout testing and subsequent use of the immune serum.

General guidance on appropriate care for animals to be used in Ab production is provided in other CCAC guidelines. Animals should be housed under conditions that best meet their social and behavioral needs to permit natural behavior, with group housing being preferred (CCAC, 1990). Where that is not possible, the decision should be justified to an ACC.

Guideline: Investigators, research support and animal care staff involved in polyclonal antibody production must have the appropriate training and competence.

The training and competence of the animal users and animal care staff are of great importance in minimizing pain and/or distress for the animals. In addition to having knowledge of animal care, the staff should understand basic immuno-

logical principles and be trained and competent in immunization procedures and in blood collection. For some species, this will require knowledge of anesthetic procedures. The staff must also be capable of recognizing pain and/or distress in the species used (CCAC, 1993, 1999).

2. Immunization Protocol

Guideline: Animal Care Committees are expected to evaluate immunization protocols with respect to animal welfare aspects; particular attention should be given to atypical immunogens and/or adjuvants likely to cause pain and/or distress.

The proposal for antibody production for each new immunogen or immunogen/adjuvant combination must be reviewed by the local ACC, and should include reasonable efforts to determine whether the product required is available commercially, or from another research group. The review can be brief if the type of immunogen has been used previously and SOPs are already in place. Protocols involving novel immunogens should be given a more careful review, and the advice of an immunologist should be sought in these instances. SOPs should be in place to address the routine procedures, but may need slight modification depending on the particular proposal. The use of FCA can cause considerable pain and distress for the animals concerned. Therefore, protocols which involve the use of FCA, or where the investigator anticipates that the animals may experience an adverse reaction, must receive particular attention and must be assigned to Category of Invasiveness level D (CCAC, 1991). This category may be changed to Category of Invasiveness level C following a favorable report on the condition of the animals by those responsible for their day to day care.

3. Standard Operating Procedures

Guideline: Each facility should establish Standard Operating Procedures for pAb production in each species.

The immunization protocol raises several significant issues that must be considered: 1) method of antigen preparation; 2) use of adjuvant – whether to use one and then which one; 3) route of injection, and number of sites to be used; 4) volume to be injected; and 5) schedule of injections. SOPs should also be implemented to limit the frequency of use of individual animals and to limit the length of time that an individual animal can be held for the purpose of antibody production.

4. Immunogen Preparation

Guideline: The immunogen should be prepared in such a manner to elicit an acceptable response without adversely affecting the well-being of the animals.

The quality of the immunogen preparation must be a prime consideration. The immunogen must be non-toxic and it must be prepared aseptically, or otherwise rendered sterile and free of toxins and pyrogens. In particular, any chemical residues, contaminating endotoxins or other toxic contaminants must be minimized (e.g., <1ng/ml for immunogens derived from gram -ve bacteria) and the pH must be adjusted within physiological limits. Most

protein immunogens can be filter sterilized through a microporous cellulose acetate filter (0.22μm pore size). In a recent survey of Canadian institutions that routinely prepare pAbs (Craig & Bayans, 1999), respondents stated that immunization with proteins in polyacrylamide gel was associated with adverse reactions at the site of injection.

The quantity of immunogen to be injected to elicit a good antibody response varies greatly depending on the species and strain of the animal to be immunized, the adjuvant used, the route and frequency of injection and the immunogenicity of the immunogen itself.

The quality and quantity of the pAb produced is dependent on the size and the state of the immunogen. Small polypeptides and non-protein molecules may require conjugation with a larger immunogenic carrier protein in order to provoke an immune response. Appropriate carrier molecules must be selected, so as not to induce suppression of the immune response to the antigen (carrier-induced epitopic suppression) due to pre-existing antibodies to the carrier (Schutz, *et al.*, 1985).

It should also be noted that the conformation of the immunogen may be important, as antibodies raised against native proteins may react best with native proteins, and those raised against denatured proteins may react best with denatured proteins. For example, if the pAbs are to be used to block an active site on a protein, the immunogen should not be denatured prior to injection. In this regard it should be noted that a preformed oil-in-water emulsion adjuvant with adsorbed immunogen will maintain a greater amount of native conformation than the immunogen prepared in a water-in-oil emulsion requiring vigorous shaking (Allison & Byars, 1991). Antigen adsorbed to alum can also give a good Ab response to native configuration (Chen, *et al.*, 1964; Dardiri & Delay, 1964; Butler, *et al.*, 1969; Cohen, *et al.*, 1970; Weeke, *et al.*, 1975; and Ziola, B., *pers. comm.*).

5. Choice of Adjuvant

Guideline: The decision as to whether an adjuvant is required should be carefully considered and justified. Investigators should seek the most appropriate adjuvant for the antigen of interest, bearing in mind the current state of knowledge on immunogen/adjuvant preparation. The advice of a knowledgeable immunologist should be obtained for each protocol involving a new adjuvant or novel immunogen. The use of Freund's Complete Adjuvant should be avoided where possible, and when necessary should only be used for the primary immunization, and should contain less than 0.5mg/ml mycobacteria.

Investigators and ACC members should not overlook the considerable pain and/or distress that adjuvants can cause for laboratory animals. Most of the undesirable side-effects of pAb production that range in severity and duration are caused by the adjuvant (Jennings, 1995). Therefore, it should be determined first whether an adjuvant is required. Adjuvants are used to enhance the immune response and when used should result in enhanced and sustained Ab levels.

Adjuvants work in several ways. They may form a depot of immunogen at the injection

site leading to a slow release over a period of time giving sustained stimulation to the immune system. The adjuvant can also help deliver the immunogen to the spleen and/or lymph nodes where many of the cell-cell interactions in the immune response occur. They may directly or indirectly activate the various cells, such as macrophages or T-helper lymphocytes, involved in the immune response. Adjuvants may also influence the duration, subclass and avidity of the antibodies produced and may affect cell-mediated immunity (Hunter, *et al.*, 1995).

The use of an adjuvant is usually necessary for soluble, relatively pure or pure immunogen preparations. If the immunogen is a small molecule, it usually does not induce an immune response by itself. The use of an adjuvant can also mean that less immunogen is required, which may be a consideration for molecules which are scarce or are particularly valuable. Highly aggregated antigens may not require the use of an adjuvant and may in fact induce a more authentic immune response in the absence of adjuvant.

The use of adjuvant with whiffle balls is not necessary as the whiffle ball itself serves as an immunogen depot, leading to prolonged release of the antigen (Clemons, *et al.*, 1992).

The immunogen/adjuvant mixture should be easily injectable in small volumes and should have low toxicity. The commonly used adjuvants include both Freund's Complete and Incomplete Adjuvants (FCA and FIA), Quil A, Ribi™ and TiterMax™, and mineral-based adjuvants – aluminium hydroxide, aluminium phosphate and calcium phosphate. However, all desirable characteristics of an adjuvant are not found together in any of the 100 or so known adjuvants. In addition to the brief descriptions of the commonly used alternatives to FCA/FIA used in Canada which are provided in Appendix A, see Leenars, *et al.* (1999); Jennings (1995); CEDARLANE Laboratories Limited "The Adjuvant Guide" (Hornby, Ontario), or the European Adjuvant Database (Stewart-Tull, 1995) for more detailed information.

The CCAC recognizes that many investigators have been reluctant to discontinue the use of FCA because it is used as a "gold standard" due to its well known effectiveness with a wide variety of antigens (Craig & Bayans, 1999). While FCA is one of the most effective adjuvants, it can cause a greater chronic inflammatory response, and therefore, should be used only when there is evidence that other adjuvants will not work (for example, when only small amounts of soluble immunogens are available, or when the antibody response is weak, e.g., Jennings, 1995). Broderson (1989) has shown that if the concentration of mycobacteria in the FCA preparation is less than 0.1mg/ml, less severe inflammatory reactions result. If large amounts of a particulate immunogen are available, or if the antigen is highly immunogenic, immunization without adjuvant or with other adjuvants should be used. If FCA must be used, it should be used only for the initial subcutaneous immunization, following the procedures detailed in Section C.7 – *Volume and Number of Injection Sites.* FIA should be used for subsequent booster immunizations, if an adjuvant is required. Secondary (booster) immunizations can sometimes be carried out using a saline buffer solution.

In general, an immunogen should be mixed with an equal volume of adjuvant and emulsified. Emulsification can best be achieved using two Luer-Lok syringes and a locking connector, passing the adjuvant and immunogen back and forth until it becomes paste-like. Sonication using a narrow probe is an alternative method of emulsification; however, care must be taken to avoid overheating by keeping the sonication mixture on ice. Proper preparation of the immunogen/adjuvant mixture is important as a major cause of immunization failure is due to inappropriate emulsification. Dispersal of the emulsion with an equal volume of 2% Tween 80 reduces the viscosity of the solution for easier injection (Herbert, 1965).

6. Route of Injection

Guideline: The route of injection must be selected with the objective of causing the least possible distress for the animal.

The route of injection may be subcutaneous (SC), intramuscular (IM), intradermal (ID), intraperitoneal (IP), or intravenous (IV), as detailed in Appendix B. **Footpad, intralymph node or intrasplenic routes are strongly discouraged and if required must be justified on a case by case basis. Injection into any closed space is painful, so even IM and ID can be questioned as a route of choice. No other routes are permitted**. Footpad injections have been used in the past to follow the immune response to a specific antigen by studying the cells of the immune system responsible for processing the antigen. Immunogens injected into the footpad are processed by the popliteal node, making it possible to routinely and accurately acquire the desired cells. However, this can be achieved with much less distress to the animal by injecting at the base of the tail, or in the popliteal area.

Intravenous administration can be the route of choice for small particulate immunogens because the immunogen is distributed throughout the body and hence, capture by lymphoid cells is high. The IV route should not be used for oil-based or viscous gel adjuvants or for large particulate immunogens due to the risk of pulmonary embolism (Herbert, 1978).

Subcutaneous injections should be used for oil or viscous gel adjuvants, in particular for FCA, in order to minimize the formation of sterile abscesses. The IM route should not be used for the injection of oil-based or viscous gel adjuvants in small animals such as mice or rats. Immunogen/adjuvant injected IM can spread along interfascial planes between muscle bundles and irritate the nerve bundles leading to serious pathology due to the inflammatory process.

The ID route should not be used in small animals, and should be restricted to cases where it is absolutely necessary. For rabbits or larger animals where the ID route is used, the volumes administered should be small (i.e., 25μl in rabbits) (Halliday, *et al.*, 2000).

Intraperitoneal injection of adjuvant mixtures is not recommended for pAb production, since it is known to induce inflammation, peritonitis, and behavioral changes (Leenars, *et al.*, 1999; Toth, *et al.*, 1989). See Section C.7 – *Volume and Number of Injection Sites*, and Appendix B for further recommendations, should the IP route be required.

Sites of injection should not interfere with subsequent handling of the animals for blood sampling, etc.

If large volumes of pAb are required, the use of whiffle balls should be considered. Whiffle balls used in rabbits are perforated plastic golf balls, available from a variety of commercial suppliers. The balls should be sterilized with ethylene oxide prior to implantation. Whiffle balls must be implanted using aseptic surgical techniques described by Hillam, *et al.* (1974) and Reid, *et al.* (1992). Strict asepsis must be maintained during surgery to avoid secondary infection of the implant. A minimum of four weeks post-surgery is required for healing prior to experimental use, during which time animals should be housed individually to avoid injury. Once prepared, the whiffle ball can be used both for primary and booster injections as well as for serum collection.

Immunization of chickens for pAb production is comparable to that of rabbits with respect to route of injection, the amount of antigen used and the kinetics of specific antibody generation (Haak-Frendscho, 1994; Song, *et al.*, 1985). Recommendations for chicken immunization (Schade, *et al.*, 1996), include the use of FCA, Specol, or preferably lipopeptide PCSL (Pam_3-Cys-ser-$[Lys]_4$; 250μg). Erhard, *et al.* (1997) found few side effects from the use of PCSL in comparison with multiple granulomas after FCA or FCA/FIA treatment. Immunogen should be administered in the 10ng-1mg range (preferably 10-100μg). Injection should be IM for young laboratory chickens (neck or breast muscle), and intramuscular injection in the leg should be avoided since it can lead to lameness. Chickens should be at least seven weeks of age. For older laboratory chickens, the SC route should be used with an injection volume <1ml; at least two immunizations are generally given. A primary vaccination and a booster should be given before the laying period with an interval of six weeks for emulsion-type adjuvants and four weeks for lipopeptide adjuvants. The stress induced by handling can have a negative effect on egg production; as can the nature of the antigen or antibody used. Yolk Ab titres should be checked 14 days after the last immunization, if Ab titres begin to decrease, boosters can be given during the laying period at 4-8 week intervals. Antibody contained in eggs can be collected throughout the laying period (about one year).

Other routes of administration of adjuvant include aerosol, oral and intranasal administration. These routes have advantages for stimulation of an IgA response, and also may be less stressful for the animals. For examples involving mice, see Shen, *et al.* (2000); Shen, *et al.* (2001); McCluskie and Davis (2001); McCluskie, *et al.* (2000); Coste, *et al.* (2000); Falero-Diaz, *et al.* (2000); and Holan, *et al.* (2000). Examples involving rats are found in Papp, *et al.* (1999); and Baxi, *et al.* (2000). For horses, see Nally, *et al.* (2001); for pigs, see Katinger, *et al.* (1999); and Tuboly & Nagy (2001); and for poultry, see Sharma (1999).

7. Volume and Number of Injection Sites

Guideline: The injection volume should be kept as small as possible and should be kept within the recommended limits.

Recommendations are given in the literature to limit the number of injection sites.

However, in terms of minimizing adverse reactions, it appears to be less stressful to the animals in the post-immunization phase, to minimize the injection volume and to use multiple sites. **Recommended injection volumes are given in Appendix B, both for immunogen plus adjuvant or immunogen alone**. It should be noted that in order to minimize immunization reactions, the volumes recommended per site for injection of immunogen with adjuvant are smaller than the generally recommended volumes for injection. It is necessary to use large gauge needles (20-21 gauge for the rabbit), as immunogen/adjuvant mixtures are quite viscous. Use of a double emulsification as described by Herbert (1965), reduces the viscosity of the solution enabling a smaller size needle (26-27 gauge) to be used.

Some species may require sedation prior to injection. This may be particularly important if multiple injection sites are to be used.

In all cases, the injection site should be aseptically prepared[1] and allowed to dry immediately prior to injection of the immunogen or immunogen/adjuvant mixture.

To minimize the chance of abscess formation when using FCA, it is important to ensure that all material is injected aseptically into the subcutaneous space, not ID or IM. In particular, care should be taken not to contaminate the needle track. Once the solution has been injected, and before it is withdrawn from the subcutaneous space, the plunger should be pulled back a few mm and the needle quickly removed from the subcutaneous space. In this manner, spillage into the dermal tissue will be minimized.

Where there is no indication of reaction to the initial injection, booster injections given in the vicinity of the initial site take advantage of the memory cells established in the draining lymph nodes. This may minimize the number of injections needed per animal and/or the number of animals required for an effective immune response. Injection sites should be sufficiently separated to prohibit the coalescing of the inflammatory lesions, which may result in disruption of blood supply to the area, with subsequent formation of draining abscesses, or occasionally tissue sloughing. However, if there are indications of local reactions, booster injections should be distant from previous injection sites and must never be given at the site of a granuloma or swelling induced by previous injections.

Animals must be closely monitored immediately following injection for any anaphylactic reactions, both after the primary injection and after the subsequent booster injections (see Section C.9 – *Monitoring of Animals*). Animals experiencing unrelievable pain and/or distress must be euthanized.

For whiffle balls, injection of immunogen should be directly into the whiffle ball chamber (Clemons, *et al.*, 1992).

The boosting protocol can have a significant effect on the result of the immunization. The time between two immunization steps can influence both the induction of B

[1] Aseptic skin preparation means clipping the fur and using a three-stage surgical preparation: surgical soap, alcohol rinse and surgical preparation solution.

memory cells and the class switch of B cells (from IgM to other antibody classes and subclasses). In general, a booster can be considered after the antibody titre has peaked or begun to decline, and when memory cells can be expected to be increasing. If the first immunization is performed without a depot-forming adjuvant, the antibody titre will usually peak 2-3 weeks after immunization. When a depot-forming adjuvant is used, a booster injection can follow at least four weeks after the first immunization. An adjuvant is not always necessary for booster immunizations.

In most cases, the endpoint of Ab production should be considered as the point when the Ab titre has reached an acceptable level (generally a maximum of two boosters). A long immunization schedule with repeated boosting is not generally useful as it may result not only in the production of Abs with increased affinity for the immunogen of interest, but also in production of more Abs specific for contaminants in the immunogen preparation (if the immunogen is not a purified protein or peptide) (Leenars, *et al.*, 1999). Multiple boosters should not require the use of adjuvant. In all cases the welfare of the animals must be taken into account.

Animals can be rested for long intervals between boosting. In addition, regular intervals of blood collection, once a sufficient serum titre has been reached, could facilitate the collection of adequate amounts of Abs for immunogens in limited supply. However, animals must not be kept in an Ab production program unnecessarily. Any animals kept long-term in an animal facility should be under long-term surveillance by a veterinarian.

8. Blood Collection

Guideline: The selection of blood collection procedure should aim to minimize stress for the animal and should follow current CCAC guidelines.

The SOP for each pAb preparation should include a section on blood collection. In this respect, the training and expertise of the staff carrying out the procedure is a significant factor (CCAC, 1999). The animals should be kept warm and away from environmentally-induced stresses such as noise. The use of organic solvents to induce vasodilation is not recommended. The use of general anesthesia is not necessary for blood collection in rabbits and larger animals. It is also not necessary for blood collection in rodents using the tail vein or saphenous vein. However, terminal anesthesia is required for cardiac puncture, which may be required to obtain sufficient volume. It may be useful to use local anesthetics, for example EMLA™ cream on rabbit ears, and/or parenteral analgesics. The needle used should be matched to blood vessel size. Rabbits should be bled by the ear vein using a needle rather than a scalpel blade as cutting may cause damage to the blood vessels and surrounding tissues, which may ultimately result in regional necrosis and sloughing of ear tissue. The maximum blood volume removed should not exceed 10% of the total blood volume of the animal (approximately 1% body weight), if collected every two weeks, and not more than 15% of the total blood volume if collected every four weeks (Morton, *et al.*, 1993; McGuill & Rowan, 1989; Diehl, *et al.*, 2001).

Serum collection from implanted whiffle

balls is relatively easy and does not require use of a local anesthetic. The skin over the whiffle ball chamber must be aseptically prepared. A sterile hypodermic needle with a cotton plug is inserted into the dorsal half of the chamber through one of the holes and the fluid withdrawn through a second needle connected to a syringe (Whary, *et al.*, 1998).

For pAb production in chickens, eggs are collected daily (usually one egg is laid per day) and marked for identification. They can be stored for up to one year at 4°C prior to antibody (IgY) purification (Haak-Frendscho, 1994; Svendsen, *et al.*, 1995).

Exsanguination must be performed under non-recovery general anesthesia, using a technique that results in a maximum collection of blood. After exsanguination is complete, the death of the animal must be assured following current CCAC guidelines for euthanasia.

9. Monitoring of Animals

Guideline: Animals must be monitored daily, and the Standard Operating Procedures should include a checklist for endpoints.

The SOP should include a requirement for record keeping. The immunization record should include the agent, route, site or sites, volume, date of injection and body weight of the animal.

Animals may have inflammatory responses to the injections of immunogen or immunogen/adjuvant mixtures and, therefore, must be monitored daily for responses at the injection sites in particular and for overall health or distress in general. Food and water intake, activity and general appearance should be monitored.

The staff taking care of the animals should have in their SOP a daily checklist for monitoring animals producing pAb with definitive endpoints for stopping the procedure (euthanasia) if the animal develops unrelievable distress due to the inflammatory response. SOPs should include a requirement for investigators to contact the veterinary staff (and for the veterinary staff to contact the principal investigator) if injection site lesions, or evidence of pain and/or distress, are identified in any animals. The veterinary staff must be responsible for the implementation of timely and appropriate assessment of the animals and for institution of therapy when required. For immunization induced lesions, supportive therapy may include topical cleansing, antibiotic administration, and/or analgesic administration. Fluid replacement or nutritional supplements may be required if animals have sustained anorexia or decreased fluid intake. Animals must also be carefully monitored following blood collection, in particular if the procedure is carried out under anesthetic (CCAC, 1998).

10. Disposition of the Animals

Disposition of the animals must be carried out in accordance with CCAC guidelines and local ACC regulations. It is the investigators' responsibility to inform the animal care staff when the collection of antibody has been completed so that animals can be disposed of as previously agreed in the protocol. This can include euthanasia,

or re-homing where relevant adoption programs are in place. It should be noted that if FCA is used in meat producing animals, they must not be resold for human consumption. The Canadian Food Inspection Agency[2] should be contacted for advice on disposition of animals where other adjuvants have been used.

D. MONOCLONAL ANTIBODY PRODUCTION

Monoclonal antibodies (mAb) are antibodies which have a single, selected specificity and which, usually, are secreted continuously by "immortalized" hybridoma cells. A hybridoma is a biologically constructed hybrid of an antibody-producing lymphoid cell and a malignant (immortal) myeloma cell. The development of hybridoma technology by Köhler & Milstein in 1975, provided a means for obtaining large quantities of highly specific antibodies which has had a profound impact on disease diagnosis, therapy, and on biomedical research in general.

There are essentially two major stages in the production of mAbs: 1) the induction of antibody producing lymphoid cells *in vivo* (immunization) and the selection of antibody-producing hybridoma cells *in vitro*; and 2) the propagation of selected hybridoma clones, either *in vitro* or *in vivo*. Appendix C contains a graphical representation of the stages of mAb production. Newer methods for production of mAbs, for example phage display techniques and expression as recombinant proteins, do not involve the use of animals and are therefore not discussed here.

Despite the original comments on the general methodology (*in vitro*) of hybridoma technology by Köhler and Milstein – "the manufacture of predefined specific antibodies by means of permanent tissue culture cell lines is of general interest" and "such cells can be grown *in vitro* in massive cultures" – the propagation of mAbs has, for almost all purposes, been carried out *in vivo*. It was apparent that mAbs could be produced by injecting the hybridoma cells into the abdominal cavities of different species of rodents. The subsequent propagation of the hybridoma cells in ascitic fluid offered an easy, economical route to the production of mAbs.

An alternative method of mAb production, although not routinely used, is the production of recombinant antibodies in the milk of genetically modified animals. As the need for larger quantities of antibodies increases, this approach may be increasingly adopted as it would appear that the expression of recombinant mAbs in the milk of transgenic mice and goats may be much greater than in cell culture systems (Young, *et al.*, 1998). The creation and use of animals as bioreactors for mAb production must be carried out in accordance with the CCAC *guidelines on: transgenic animals* (1997b).

General guideline: Every attempt should be made to obtain material already available or to use an *in vitro* method for production of mAbs. Therefore, any proposed production of monoclonal antibodies using the ascites method

[2] Canadian Food Inspection Agency, National Manager, Veterinary Biologics Section, Ottawa ON (tel.: 613-225-2342).

requires justification by the investigator to the animal care committee.

Commercially produced mAbs should be obtained from sources that comply with CCAC guidelines, or if international are accredited by AAALAC. For lists of suppliers of *in vitro* produced mAbs, see http://www.frame.org.uk/Monoclonal_Suppliers.htm.

The production of mAbs in mice by the ascites method raises several issues of concern regarding the potential for severe and unnecessary pain and suffering for the animals (Anon., 1989). In recognition of this fact, a number of *in vitro* replacements for the rodent ascites method of mAb production have been developed. A number of countries have instituted a ban on routine *in vivo* production of mAbs (Shavlev, 1998). A recent report prepared by a US National Research Council Committee, in response to a request from the National Institutes of Health, recommended that *in vitro* mAb production methods should be adopted as routine whenever practical, and that if the mouse ascites method is used, it must be justified to an institutional animal care and use committee (Peterson, 1999). It is now generally accepted that the *in vitro* techniques of mAb production have progressed to the point where these techniques can be used for more than 90% of mAb production (ILAR, 1999).

In this context, it should be noted that the CCAC *Ethics of Animal Investigation* (1989) requires investigators to follow the "Three Rs" of Russell & Burch (1959): Replacement (of animals with other, non-sentient material or with animals of lower sentience); Reduction (of numbers of animals used); and Refinement (of technique, to "reduce to an absolute minimum the amount of distress imposed on those animals that are still used").

The potential for severe pain and/or distress in the animals relates to: intraperitoneal (IP) injection of primer (e.g., Pristane or FIA); the effect of primer after administration; the IP inoculation of hybridoma cells; and the growth of the tumor cells in the abdominal cavity (primary effects) as solid peritoneal plaques that infiltrate the abdominal wall and/or abdominal organs. Specifically, IP injection is believed to cause moderate distress; ascites fluid production and tumor growth have been reported to cause suffering in human patients (Kuhlmann, *et al.*, 1989); and, in addition, the complex pathophysiological and pathological changes may cause severe distress for the animal involved (Hendriksen, 1998). While ascites fluid tapping is believed to relieve some of the pain and/or distress associated with abdominal distension, the immobilization and administration of anesthetic prior to ascitic fluid harvesting is an additional source of distress.

The CCAC recognizes that it has a responsibility to support ACCs in requiring that investigators provide evidence that the use of replacement methods are not appropriate (CCAC *guidelines on: animal use protocol review*, 1997a). Therefore, the CCAC will develop and maintain a repository of information on suitable *in vitro* techniques for the production of mAbs (Appendix D). It is also recognized that, in countries where a ban on the ascites method has been implemented, the use of *in vitro* methodology has been stimulated by the creation of Centres of Excellence, centralizing the production of mAbs *in vitro*, and providing training, expertise and resources for the *in*

vitro techniques. A number of organizations also offer to produce material by *in vitro* means on a contract basis.

Ideally, a replacement *in vitro* system should be equally or less expensive, not require special culture conditions, produce high concentrations of mAbs, be free from contamination, be re-usable, and require a reasonably short period of time to produce relatively pure mAbs in adequate quantities (Falkenberg, 1993; 1995). Currently, there are no *in vitro* systems that meet all of these criteria, but depending on the quality and quantity of mAbs required, it is possible for investigators to select an appropriate *in vitro* system (for references to various mAb production methods see Appendix D). If the required mAb is not commercially available from a reputable source, investigators should make all reasonable efforts to produce the required mAbs using an *in vitro* approach. In line with recommendations made in the US and elsewhere, prior to approval of a protocol for ascites production, the investigator should show that he or she has made a substantial effort to adapt the hybridoma to a culture system suitable for scale of mAb production required (e.g., tissue culture flasks, simple membrane systems, hollow fibre, fermentation systems) (Saxby, 1999; Smith & De Tolla, 1999). **The production of mAbs by growth of hybridomas as ascites tumors should be avoided unless absolutely necessary**.

The following guidelines have been elaborated to safeguard the well-being of animals (principally mice) that continue to be used in mAb production.

As up to 15% of hybridomas may produce little or no mAb *in vivo*, where *in vitro* methods cannot be used, investigators should be asked to test new hybridomas in a small pilot study involving 2-3 animals.

General guideline: Clearly defined endpoints and close monitoring of the condition of the animals are required to minimize the potential for pain and/or distress.

In accordance with CCAC *guidelines on: choosing an appropriate endpoint in experiments using animals for research, teaching and testing* (1998), clear endpoints must be developed which minimize pain and/or distress for the animal. A clear schedule for monitoring and reporting must be established, and SOPs must be established for each new immunogen/adjuvant combination.

1. Animal Selection and Care

Guideline: When selecting species and strain of animals for mAb production, the overall consideration must be to minimize pain and/or distress.

The mice (or rats) used should be the same strain (syngenic) both for immunization to produce the hybridoma clone and for subsequent mAb production, so that the tissue used is histocompatible. BALB/c mice are often the animal of choice, as many of the parental myeloma cells used in the fusion process are derived from BALB/c mice. Retired breeders may offer some advantages for ascites production due to the previously stretched abdominal musculature (Falkenburg, 1998).

SCID mice, although expensive, have been

reported to produce fewer non-specific murine antibodies with equivalent yields of specific mAb, thus minimizing interference in immunoassays, etc., (Pistillo, *et al.*, 1992). It should be noted that SCID mice require barrier facilities for housing and extensive (and expensive) procedures for appropriate care.

ACCs should ensure that the appropriate numbers of animals are used. This can be estimated by presuming that mice produce approximately 0.5-10mg/ml of mAb in ascites fluid, and that on average it is possible to harvest 2ml ascites fluid per tap (Smith & De Tolla, 1999). These are generalized estimates, and each hybridoma must be considered as a unique cell line.

2. Production of Hybridoma Clones

SOPs for immunization should be developed based on guidelines for *choice of adjuvant, route of injection* and *volume and number of injection sites* (see Sections C.5-C.7). Immunization procedures that do not require adjuvant and that can be performed in short time periods have been shown to be effective for hybridoma derivation (Chase, *et al.*, 2001). The procedures, which appear to cause less stress to the mice, require multiple boosts in a short time frame, eliminating the requirement for adjuvant. However, rapid immunization may not always yield mAbs of high affinity, a factor which should be considered in choice of immunization regimen.

As an alternative to *in vivo* immunization, *in vitro* immunization with antigen has been successfully used (Borrebaeck, 1989). This procedure is particularly useful when an antigen is in limited supply and previous attempts at *in vivo* immunization have yielded negative results due to similarity to self antigens or to the weak immunogenicity of antigen (Bunse & Henz, 1994). Other advantages of this approach are: fewer animals are required; the immunization period is 4-5 days, compared to several weeks for traditional *in vivo* immunization (Grimaldi & French, 1995); it is possible to monitor and control the immune response without the influence of the immune regulatory mechanisms that occur *in vivo*; and mAbs can be generated against agents that are toxic to animals.

For further details concerning production of hybridoma clones, see Grimaldi & French (1995). For a single-step selection and cloning of hybridomas, see Stebeck, *et al.* (1996); and Chase, *et al.* (2001).

It is important that prior to immunization, the methodology for detecting the specific antibody of interest in the mouse sera and tissue culture supernatant has been developed, otherwise significant time and resources may be wasted later in the mAb development phase. In addition, the use for the mAb should be carefully considered, so that mAbs that are effective in the final functional assay can be selected by appropriate screening.

3. Ascites Production

3.1 Priming

Guideline: Freund's Incomplete Adjuvant (FIA) or other less invasive adjuvant should be used as the intraperitoneal priming agent, with a maximum volume of 0.3ml (FIA) administered in one injec-

tion. If the use of Pristane is proposed, it should be justified to the institutional animal care committee and the volume should be limited to 0.2ml.

As originally described, the ascites method of mAb production included the use of Pristane (0.5ml injected intraperitoneally) as the chemical used to "prime" the peritoneal cavity of the mouse. The effect of the primer is two-fold; it suppresses the immune system so that the growth of the hybridoma cells in the abdominal cavity is not (strongly) impaired, and it causes a chemical irritation, which leads to peritonitis and the secretion of serous fluid (Kuhlmann, *et al.*, 1989). A hybridoma cell suspension is injected 7-10 days later.

Preliminary results indicate that the use of analgesics prior to priming with Pristane may lessen the pain and distress experience by the mouse (Fletch, A. & Delaney, K., *pers. comm.*).

Studies comparing Pristane (at several volumes) and other priming agents, have shown that the use of FIA has several advantages over Pristane, including: fewer indications of stress to the animals; injection of hybridoma cells can be performed as early as one day after priming; and fewer animals are required (Gillette, 1987; Mueller, *et al.*, 1986; and Jones, *et al.*, 1990). Trypan blue has also been used as a priming agent (Wu & Kearney, 1980). It should be noted that, prior to use, Trypan blue must be dialyzed in fresh glass-distilled water for 48 hours, replacing the water twice per day, to reduce low molecular weight impurities. Priming with Trypan blue is carried out 24 hours and again one hour prior to injection of the hybridoma cells.

3.2 Contamination

Guideline: Hybridomas should be tested for the presence of adventitious viral and mycoplasma agents prior to use for *in vivo* propagation of monoclonal antibodies.

Viral contamination is commonly found in murine specimens inoculated with a murine-derived hybridoma. This type of contamination may result in infection of the host animal(s) and in spread of the disease throughout an animal facility. In addition, lymphocytic choriomeningitis virus, which can lead to disease in humans, has also been isolated from murine tumor cell lines (Dykewicz, *et al.*, 1992). Mouse hepatitis virus (MHV) can provoke an immunomodulatory response, influencing the subsequent production of mAbs. It also spreads rapidly through an animal facility, affecting other work in progress. The typical testing method is the Mouse Antibody Production (MAP) test, which involves injecting a sample of tissue in question into mice, and, after an incubation period of 3-4 weeks, euthanizing the mice to run serological tests for a panel of known adventitious agents (Jackson, *et al.*, 1999). Molecular tests using polymerase chain reaction (PCR) and other related techniques will improve testing capabilities and turnaround times.

3.3 Hybridoma implantation

Guideline: Up to 3 x 10^6 hybridoma cells in a maximum volume of 1.0ml may be injected into the peritoneal cavity of a primed mouse.

Tumor cell lines prone to forming solid

tumors produce less ascitic fluid. They can be optimized for ascites production by performing serial passages of non-attached tumor cells that have been selected from mice with solid tumors by peritoneal lavage. Selection of hybridoma cell-lines which do not adhere to the tissue culture flask has been shown to reduce the likelihood of formation of solid tumors. For many hybridomas, 3 x 10^6 cells have been shown to be the maximum necessary to give good development of mAbs and ascitic fluid. In some circumstances (e.g., hybridomas produced in 1970s-1980s using first generation myeloma cells), a higher concentration of cells may be needed to produce any ascitic fluid. Some rapid proliferating, aggressive cell lines may require a lower concentration for inoculation as a rapidly developing tumor can lead to severe distress and death.

3.4 Monitoring the animals and endpoints

Following injection of hybridoma cells, routine care should include daily observations by appropriately trained staff for the first week (approximately) and before ascites fluid accumulation is evident (as indicated by the swelling of the abdomen). Any observations of unusual behavior or symptoms during this time should be addressed promptly. The CCAC *guidelines on: choosing an appropriate endpoint in experiments using animals for research, teaching and testing* (1998) should be used to develop monitoring procedures and an appropriate endpoint for the animal. Pertinent signs of distress include: decrease in activity; hunched appearance; ruffled hair coat; respiratory distress; and weight loss (which may be masked by the accumulating fluid in the abdomen). Once the ascites fluid accumulation has resulted in obvious abdominal swelling, usually 7-10 days, the condition of the animal must be assessed at least twice every 24 hours at regularly spaced intervals. It must be remembered that the rate of hybridoma cell propagation in the mouse can be quite variable.

3.5 Ascites tumor growth

Guideline: The increase in body weight due to the accumulation of ascitic fluid in the abdomen and/or tumor growth should not produce pain and/or distress to the animal; in no case should the increase in body weight exceed 20% of the normal body weight of age and sex matched animals of the same strain.

In accordance with the CCAC *guidelines on: choosing an appropriate endpoint in experiments using animals for research, teaching and testing* (1998), a chart for monitoring the animals is required with clearly defined endpoints and reporting lines. Once the endpoint has been reached, abdominal pressure must be relieved by harvesting of the ascitic fluid – or by humane killing followed by recovery of the ascitic fluid. Mice should be weighed daily, beginning four days after inoculation, to monitor progression of the ascites tumor by weight gain and to monitor the general health of the animals. A baseline weight measurement of the mouse should be taken on the day of inoculation and this value can then be used to assist in determining when the mouse reaches an increase of 20% body weight (Workman, *et al.*, 1998). Caution should be used when relying on weight, as mice are likely to lose body mass

at the same time as the tumor is growing. Body condition scores can also be used as an indicator of well-being (Ullman-Culleré & Foltz, 1999) and can assist in deciding when to terminate the procedure.

3.6 Ascites fluid collection

Guideline: Depending on the condition of the mouse, a maximum of two taps of the ascitic fluid are permitted, with the second tap being a terminal procedure. Training and experience in tapping or draining ascitic fluid is essential.

It should be noted that fluid removal carries the risk of hemorrhage, oedema and death. For more aggressive cell lines, known to cause significant morbidity, the number of taps should be limited to one terminal procedure under general anesthesia. Anesthesia, as recommended by current CCAC guidelines may be used for the first (non-terminal) tap. However, anesthesia leads to a decrease in blood pressure and respiratory suppression, which may be detrimental to the already compromised mouse. Therefore, it is recommended that mice are placed in an oxygen filled induction chamber for 5-10 minutes, followed by mild gaseous anesthesia (isoflurane) to give good immobilization of the animal for the procedure with rapid recovery. To minimize any bacterial contamination, the site of paracentesis should be aseptically prepared for the first tap.

It is recommended that a large gauge needle (21-22 gauge) be used for paracentesis. A large gauge needle permits rapid collection of the viscous ascitic fluid, reducing the period of restraint/anesthesia required; however, too large a needle can cause tissue damage. A maximum of 4-5ml of ascitic fluid may be collected at the first (survival) tap. The abdomen of the mouse should be palpated to determine whether an intra-abdominal solid tumor is present, the presence of a tumor being grounds for humane euthanasia of the animal. Administration of replacement fluids (1-2ml subcutaneous) should be considered when large volumes of ascitic fluid are harvested and the animals are not terminated (Jackson & Fox, 1995).

The animals should be closely monitored for the first 60 minutes and regularly for several hours following the first tap. Any signs of distress should result in euthanasia of the animal, according to current CCAC guidelines.

ACCs must ensure that the personnel responsible for carrying out these procedures (listed on the animal use protocol) have obtained the necessary training prior to conducting the procedure. CCAC *guidelines on: institutional animal user training* (1999) mandates the training of any personnel prior to the performance of animal-based procedures.

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Lists of suppliers of *in vitro* produced mAbs (http://www.frame.org.uk/Monoclonal_Suppliers.htm).

Nature Biotechnology Guide (http://www.guide.nature.com/).

University of California, Davis CA - Readings and Resources for mAbs (http://www.vetmed.ucdavis.edu/Animal_Alternatives/biblio~1.htm).

F. GLOSSARY

Adjuvant: A substance which increases the immune response to an antigen when it is administered at the same time and at the same site as the antigen. When adjuvants are used, smaller doses of the antigen are required and the antibody response persists for a longer period of time.

Affinity: The strength and stability of the bond between an epitope and antibody as measured by the amount of antibody-antigen complex found at equilibrium.

Antibody: A protein which is produced in response to a particular antigen and whose unique structure gives it the capacity to combine specifically with that antigen. Antibodies are secreted by lymphocytes and are divided into five classes: IgG, IgM, IgA, IgE, and IgD.

Monoclonal Antibody: antibody molecules which are produced by cells of one

clone and recognize only one epitope on an antigen.

Polyclonal Antibody: antibody molecules produced by different families of B lymphocytes and consequently recognize more than one epitope, even on a single antigen.

Antigen: Any substance which can bind to an antibody raised against it.

Antiserum: The serum, or non-cellular components of blood which remain after clotting, which results from the injection of antigens and contains the antibodies produced in response to those antigens.

Ascitic Fluid (also called ascites): An intraperitoneal fluid extracted from animals that have developed a peritoneal tumor.

Avidity: The strength of the bond between an antigen and antibody. Avidity depends on the affinity as well as the valences of the antigen and antibody.

Clone: Genetically identical cells derived from the same cell.

Monoclonal: derived from the same clone and therefore all genetically identical.

Polyclonal: derived from different clones.

Complement: A set of plasma proteins that act together to attack extracellular forms of pathogens. Complement can be activated spontaneously on certain pathogens, or by antibody binding to the pathogen.

ELISA (Enzyme Linked Immunosorbent Assay): Immunological analysis using antigens or antibodies marked by an enzyme.

Epitope: The part of an antigen which is recognized by an antibody. An epitope is capable of combining with only one antibody molecule, but a single antigen may have more than one epitope.

Granuloma: Chronic inflammatory response maintained by persistent stimulation.

Histocompatibility: The degree of genetic similarity between the donor and receiver of a graft.

Major Histocompatibility Complex: the genes responsible for the rejection of grafts between individuals.

Hybridoma: A biologically constructed hybrid of an antibody-producing lymphoid cell and a malignant (immortal) myeloma cell.

Immune Response: A humoral or cell-mediated response of the immune system to an antigen.

Humoral Response: the generation of antibodies in response to the presence of an antigen.

Cell-Mediated Response: the binding of a particular kind of T lymphocyte, known as a cytotoxic T lymphocytes, with foreign or infected cells, followed by lysis of these cells.

Immunoblotting: A technique used to identify characteristics of protein antigens. A Western blot is a blot of protein onto nitrocellulose paper followed by detection of specific proteins using labeled antibodies.

Immunogen: A substance which can stimulate the immune system.

Immunogen/Adjuvant Formulation:

Emulsion: stable oil-in-water or water-in-oil mixture.

Liposome Formation: formation of a

spherical particulate in an aqueous medium by a lipid bilayer enclosing an aqueous compartment.

Immunostimulatory Complex (iscom) Formation: complex of antigen held within a lipid matrix that acts as an adjuvant and enables the antigen to be taken up into the cytoplasm of a cell after fusion of the lipid within the plasma membrane.

Adsorption: attraction and retention of other material on the surface.

Immunogenicity: The ability to elicit an immune response.

Immunoglobulin: A member of a group of proteins having antibody properties, regardless of whether or not its binding target is known. (To be considered an antibody, the antigen with which it binds must be known.) In mammalian species there are antibodies to one of five immunoglobulin (Ig) classes: IgM; IgG; IgA; IgD; or IgE. In avian species they belong to one of three classes: IgM; IgY or IgA.

Immunohistochemistry, Immunoprecipitation, Immunofluorescence, Immunoelectron Microscopy: Techniques for detection or separation of molecules relying on binding of the molecules with specific antibodies, often labeled with fluorescent dyes, etc., in order to visualize the location of the molecules.

Lymphocytes: Cells which circulate through the lymph and blood and play a role in immunity. They are subdivided into B lymphocytes and T lymphocytes.

B Lymphocytes: produce antibodies and their precursors.

T Lymphocytes: act as the basis of cell-mediated immunity and assist B lymphocytes in the production of antibodies.

Memory Cells: Lymphocytes that are able to quickly and intensively respond to a new antigen due to previous exposure to the antigen.

Plasma Cells: Specialized cells that are derived from B lymphocytes and synthesize antibodies.

SCID: Severe Combined Immunodeficient.

Based on definitions from:

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For more information on these and other guidelines contact:

Canadian Council on Animal Care
315-350 Albert Street
Ottawa ON CANADA K1R 1B1
(Website: www.ccac.ca)

APPENDIX A

COMMON ADJUVANTS

This is a list of the more commonly-used adjuvants and their basic properties. It is not an endorsement of any specific product.

Freund's Complete Adjuvant (FCA) is a water-in-oil emulsion of mineral oil, mannide mono-oleate, and heat-killed *Mycobacterium tuberculosis* (or *M. butyricum*) or components of the organism. FCA is a potent adjuvant that stimulates both humoral and cell-mediated immunity. FCA is often strongly reactive as the mineral oil cannot be metabolized and the mycobacterial components can elicit severe granulomatous (inflammatory) reactions. The concentration of mycobacteria varies greatly among commercial preparations of FCA. In this regard, it should be noted that if the concentration of mycobacteria is less than 0.5mg/ml (0.1mg/ml in Broderson [1989]), less severe inflammatory reactions result.

Freund's Incomplete Adjuvant (FIA) is the same as FCA minus the mycobacterial cells or cellular components. FIA is less effective than FCA for inducing high antibody titres and enhancing cell-mediated immunity.

Ribi™ Adjuvants are oil-in-water emulsions in which the antigen is blended with a minimal volume of oil and then emulsified in a saline solution containing the surfactant Tween 80 (Ribi, *et al.*, 1975). Although oil-in-water emulsions are less viscous and easier to inject than water-in-oil emulsions, they are poor adjuvants alone, requiring immunostimulants to increase the immunogenicity. The immunostimulants used in the Ribi™ system include two refined mycobacterial products (trehalose 6,6'-dimycolate and cell wall skeleton) and a purified gram-negative bacterial product (monophosphoryl lipid A). For a further explanation of the biological activities of Ribi™ adjuvants see Jennings (1995). Ribi™ adjuvants are usually less potent than FCA, but they are also less toxic and have provided satisfactory adjuvant activity for many purposes.

Monophosphoryl lipid A (one of the components of the Ribi™ system) is by itself a potential adjuvant option. As with aluminum hydroxide, the safety profile of MPL is well documented and provides excellent adjuvant activity for some immunogens.

TiterMax™ Adjuvant relies on a microparticulate water-in-oil emulsion formed with a non-ionic block copolymer (CRL-8941) and squalene, a metabolizable oil. CRL-8941 is coated with silica particles which stabilize the emulsion. Stability is a key property of TiterMax™ as it enables the emulsion to contain a wide variety of antigens without the use of large amounts of toxic emulsifying agents. The copolymer enables more antigen to be carried on its surface than if the antigen were in solution. It also activates complement, assisting in retention of the antigen in the lymphoid tissues and activation of immunoreactive cells. TiterMax™ induces increased expression of class II (Ia) major histocompatibility complex molecules (MHC) on macrophages, and increases the presentation of Ag to T cells. When TiterMax™ works well, it can induce antibody titers as high or higher than FCA and exhibits less toxicity. However, adjuvantation with TiterMax™ is not always successful. In addition, TiterMax™ contains small quantities of an emulsifying agent and silica particles which

have been implicated in delayed-type vaccine reactions.

Quil A is a partially purified form of saponin or triterpene glycoside derived from the bark of the *Quila saponara* tree and purified to reduce the presence of components which cause adverse local reactions. In general, saponins should not be injected intraperitoneally or intravenously due to their hemolytic activity. As Quil A is only semi-purified, it can still induce significant pyrogenic and local reactions. Therefore, prior to using a formulation containing Quil A, it should be evaluated *in vitro* (i.e., for cell culture toxicity). The adjuvant properties of Quil A rely on the formation of iscoms (immune stimulating complexes) which are especially useful in inducing Abs to membrane antigens. The iscoms are 35nm clathrate bodies like micelles made of Quil A, cholesterol, phosphatidyl choline and antigen. The purified saponin component which has the highest adjuvant/toxicity ratio is called QS21. Saponin containing formulations should never be administered via mucosal surfaces, such as intranasal when induction of IgA is desired.

Mineral-Based Adjuvants. Three mineral compounds are generally used in adjuvants: aluminum hydroxide; aluminum phosphate; and calcium phosphate. Aluminium salts are claimed to be superior to all other adjuvants in their ability to increase immune responses against weak immunogens, including those for which FCA does not work. Alhydrogels are sterile aluminium hydroxide gels that are pyrogen-free and stable, they have a high adsorptive capacity. At pH <9 alhydrogels have a positive charge so they readily adsorb negatively charged molecules (e.g., proteins at neutral pH).

CpG DNA is a non-specific immune activator that can be used alone or in combination with other adjuvant components to augment immune responses. Commercial preparations of CpG DNA comprise short pieces of DNA (oligonucleotides) that contain unmethylated cytosine-guanine dinucleotides within a certain base contact. The mammalian immune system has evolved to recognize these sequences, which are found naturally in bacterial DNA, as a sign of infection. Different CpG DNA sequences activate the immune systems of different species, and the commercial preparations of this adjuvant are therefore species-specific.

Additional Information on Adjuvants

The Adjuvant Guide: CEDARLANE Laboratories Limited (http://www.cedarlanelabs.com).

Animal Welfare Information Center, Information Resources for Adjuvant and Antibody Production: Comparisons and Alternative Technologies (1990-97) (http://www.nal.usda.gov/awic/pubs/antibody/).

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APPENDIX B

IMMUNIZATION – RECOMMENDED ROUTES AND VOLUMES

(adapted from Leenars, *et al.*, 1999)

TABLE I SUGGESTED ROUTES OF INJECTION WITH OR WITH0UT ADJUVANT

Primary Injection		Booster Injection(s)	
with adjuvant	without adjuvant	with adjuvant	without adjuvant
SC	IV	SC	SC
IM+	SC	IM+	IM
ID‡	IM	ID‡	IV
IP*	IP		IP
	ID‡		ID‡

* Only in mice, and not recommended in general.

\+ Not to be used for viscous adjuvants in small animals.

‡ Not to be used in small animals, and not recommended in general.

TABLE II MAXIMUM VOLUMES FOR INJECTION OF IMMUNOGEN/ DEPOT FORMING ADJUVANT MIXTURES PER SITE OF INJECTION FOR DIFFERENT ANIMAL SPECIES

Species	Maximum Volume Per Site	Primary Injection	Subsequent Injections
Mice, hamsters	100μl (0.1ml)	SC	SC
Mice, hamsters	50μl (0.05ml)	IM+	IM+
Mice	500μl (0.5ml)	IP*	SC, IM+
Guinea pigs, rats	200μl (0.2ml)	SC, IM+	SC, IM+
Rabbits	250μl (0.25ml)	SC, IM	SC, IM
	25μl (0.025ml)	ID	SC, IM
Sheep, goats, donkeys, pigs	500μl (0.5ml) (if in multiple sites, 250μl [0.25ml]/site)	SC, IM	
Chickens	500μl	SC, IM	

* Not recommended in general for pAb production.

\+ Not recommended in general, in particular not for viscous adjuvants.

TABLE III RECOMMENDED MAXIMUM VOLUME OF INJECTION USED FOR ANTIGEN WITHOUT ADJUVANT FOR DIFFERENT ANIMAL SPECIES

	Maximum injection volume in mls				
	SC	**IM**	**ID**	**IP**	**IV**
Mice	0.5ml	0.05ml		1ml	0.2ml
Hamsters	1.0ml	0.1ml		2-3ml	0.3ml
Guinea pigs	1.0ml	0.1ml		10ml	0.5ml
Rats	1.0ml	0.1ml		5ml	0.5ml
Rabbits	1.5ml	0.5ml	0.05ml	20ml	1-5ml
Sheep or goats	5.0ml		0.05ml	10ml	30ml
Pigs (<50kg)	3.0ml	2.0ml	0.1ml	250ml	20ml
Chickens	4.0ml	0.5ml		10ml	0.5ml

These volumes are suggested maximum volumes, additional information can be found in the following texts:

BAUMANS, V., TENBERG, R.G.M., BERTENS, A.P.M.G., *et al.* (1993). Experimental Techniques. In: *Principles of Laboratory Animal Science* (eds. L.F.M. van Zutphen, V. Baumans & A.C. Beynen). Pp. 299-318. Amsterdam, The Netherlands: Elsevier.

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APPENDIX C

STAGES OF MONOCLONAL ANTIBODY PRODUCTION

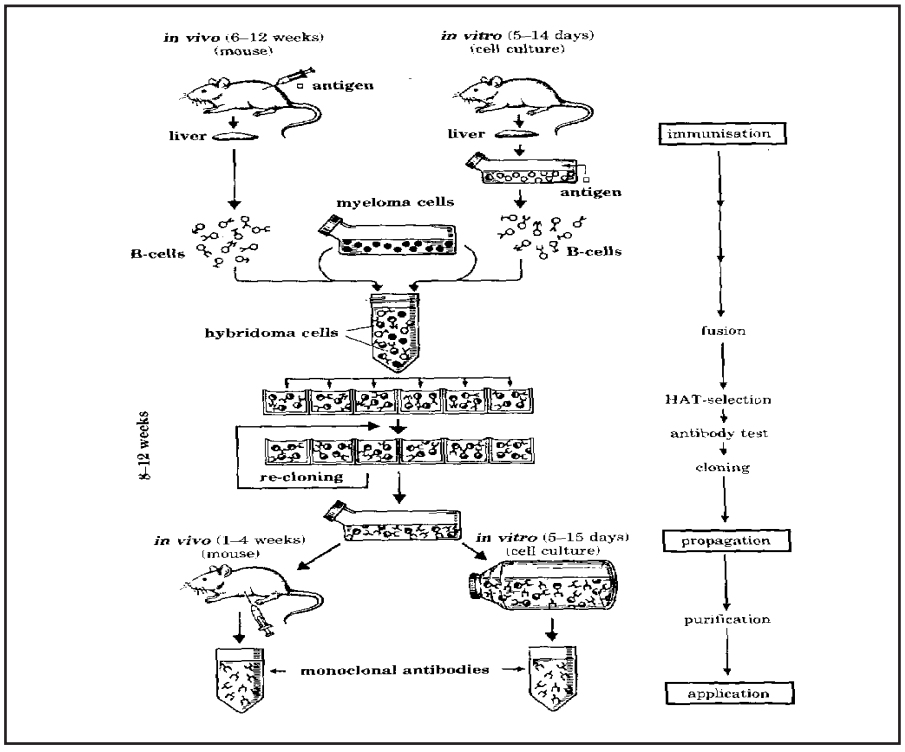
Formation and Selection of the Hybridoma Clone

The first stage (formation and selection of hybridoma clones) generally involves the use of one or more mice or rats. When using mice, it is recommended that they be 6-8 weeks of age and free of any concurrent infections.

1. The immunogen is injected into the animals, often, but not always, in combination with an adjuvant to enhance the immune response (see Sections C.2-C.7 – *Immunization Protocol, Standard Operating Procedures, Immunogen Preparation, Choice of Adjuvant, Route of Injection,* and *Volume and Number of Injection Sites*).

2. In general, the interval between booster doses of immunogen should be a minimum of 7-10 days, unless a rapid immunization protocol without adjuvant is used.

Production of monoclonal antibodies on a laboratory scale – *in vivo* (mouse) and *in vitro* (cell culture) methods



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3. Test bleeds should be performed three days after the last booster to ensure that there is an appropriate response to the antigen and that specific antibodies are being produced. Most immunologically-based assays for determining whether the desired Abs are being produced require less than 10μl of mouse serum. Once an appropriate response has been confirmed, the mouse should be boosted again; three days after the boost, the mouse should be euthanized and the spleen harvested. Lymphoid cells are then isolated from the spleen, and in some cases from the lymph nodes.

4. The lymphoid cells are fused with parental myeloma cells grown *in vitro*, by the addition of polyethylene glycol to promote membrane fusion. Only a small proportion of these cells fuse successfully.

5. The mixture of the two unfused cell types and the newly formed hybrids is cultured in a selective cell culture medium containing HAT. HAT is a mixture of hypoxanthine aminopterin and thymidine. One of the components of HAT, aminopterin, is a powerful toxin which blocks a metabolic pathway. This pathway can be bypassed if the cell is provided with the intermediate metabolites hypoxanthine and thymidine. While spleen cells can use this bypass pathway and grow in HAT medium, myeloma cells have a metabolic defect which prevents them from using the bypass pathway. Consequently, myeloma cells die in HAT medium. The spleen cells will later die naturally in culture after 1-2 weeks, but the fused cells will continue to survive as they have the immortality of the myeloma cells and the metabolic bypass of the spleen cells. Some of the fused cells will also have the antibody producing capacity of the spleen cells.

6. Immunoassay procedures are used to screen for hybridomas secreting the desired antibody. If positive, the cultures are cloned. This is accomplished by plating out the cells so that only one cell is in each well. This produces a clone of cells derived from a single progenitor which is both immortal and produces antibodies.

7. The selected hybridoma cells are often cloned a second time or third time *in vitro* to ensure cultures of truly monoclonal hybridomas with a single antibody specificity are produced (see Section D.3.2 – *Contamination*). At this stage, cells are grown to larger numbers in order to prepare for *cryopreservation*.

8. The propagation of cloned hybridoma cells can be accomplished either by continuing to grow the cells *in vitro*, or by propagating them *in vivo*. For information on *in vitro* production systems see Appendix D.

APPENDIX D

INFORMATION ON *IN VITRO* TECHNIQUES FOR MONOCLONAL ANTIBODY PRODUCTION

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DE GEUS, B. & HENDRIKSEN, C.F.M. *In vivo* and *in vitro* production of monoclonal antibodies: Current possibilities and future perspectives. 533-534.

FALKENBERG, F.W. Monoclonal antibody production: Problems and solutions. 542-547.

LIPSKI, L.A., WITZLEB, M.P. & REDDINGTON, G.M. Evaluation of small to moderate scale *in vitro* monoclonal antibody via the use of the i-mab™ gas-permeable bag system. 547-552.

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MARX, U. Membrane-based cell culture technologies: A scientifically and economically satisfactory alternative to malignant ascites production for monoclonal antibodies. 557-559.

FALKENBERG, F.W. Production of monoclonal antibodies in the miniperm™ bioreactor: Comparison with other hybridoma culture methods. 560-570.

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