The UNIVERSITY of NEWCASTLE

ANIMAL CARE AND ETHICS COMMITTEE

USE OF ADJUVANTS IN THE PRODUCTION
OF POLYCLONAL ANTIBODIES

INTRODUCTION

In recent years the production of polyclonal antibodies in laboratory animals has become an essential part of many research projects. Investigators preparing to produce antibodies are confronted with a number of complex choices, some of which may be critical for success. The purpose of this document is to assist investigators in designing experimental protocols which result in the production of high-titre, high-affinity antisera in a manner consistent with the welfare of the animals being immunised.

The document is divided into 4 sections:

Part 1: Summary of recommendations
Part 2: Preferred immunisation protocols
Part 3: Background Paper
Part 4: Alternatives to Freund’s Complete Adjuvant

PART 1: SUMMARY OF RECOMMENDATIONS

(For further information, please consult Part 3 “Background Paper”.)

1. Choose animal species that yield large amounts of antibodies (eg. sheep, chickens).
2. Choose an adjuvant with low toxicity.
3. Before using Freund’s Complete Adjuvant (FCA), consider the use of Freund’s Incomplete or another adjuvant.
4. Inject the smallest possible volume of adjuvant (use concentrated antigen).
5. Do not use FCA more than once.
6. Use the subcutaneous route whenever possible.
7. Choose an injection site where local pathological responses cause least pain, and can be readily observed and treated if necessary.
8. Use widely scattered, multiple injection sites (if applicable).
9. Ensure that the inoculum is as free as possible of extraneous microbial or chemical contamination.
10. Minimise microbial contamination during injection procedure by aseptic preparation of the site.
11. Allow a minimum of 3 weeks between initial and subsequent immunisations.
12. Use long booster intervals (at least four weeks).
13. Take advantage of the antibody memory response.
14. Minimise animal numbers by consecutive rather than simultaneous immunisations of animals.
15. Monitor animals carefully for signs of adverse effects. Monitor injection sites for the development of lesions. Monitor 3 times weekly for 4 weeks after each injection, or until lesions heal.
16. Handle Freund’s Adjuvants as hazardous substances.
PART 2: PREFERRED IMMUNISATION PROTOCOLS

For species commonly used for the production of polyclonal antibodies at the University of Newcastle, the preferred immunisation schedules are listed below. Departure from these guidelines will require adequate justification to the Animal Care and Ethics Committee.

1. RABBIT

Adjuvants (Initial and Booster): To be determined by the Investigator.

Antigen dose: To be determined by the Investigator.

Route: Subcutaneous, using a 22 G needle.

Site: Multiple sites along the dorsum of the back.

Number of sites: Maximum of 4 sites

Total volume: 0.4 - 0.8 mls

Injection volume per site: 0.1 - 0.2 mls.

Immunisation frequency: Once established, every 4 weeks - if required.

Test bleed volume: Not more than 0.6% of animal’s body weight from the lateral ear vein (eg. for a 3 kg rabbit, not more than 18 mls).

Test bleed frequency: Every 4 weeks, approximately 2-3 weeks after Booster immunisation.

Method of bleed-out under anaesthesia: Cardiac puncture, non-survival.

Total length of time an individual animal will be used: 6 months.

Regime:

Week 1-2: Acclimatisation of animal to experimental situation.

Week 3: Initial non-immune bleed

Week 4: Initial immunisation

Week 7: Booster immunisation

Week 11: Booster immunisation

Week 13: Test bleed

> Week 13: If required: booster immunisations at intervals of at least 4 weeks. Test bleed 2 weeks after immunisation.

2. SHEEP

Adjuvants (Initial and Booster): To be determined by the Investigator.

Antigen dose: To be determined by the Investigator.

Route: Intramuscular, using a 21G needle.

Site: Anterior muscles of hind leg (biceps femoris).

Number of sites: 2 sites (1 site per hindleg)

Total volume: 1 ml

Injection volume per site: 0.5 mls.

Immunisation frequency: Once established, every 4 weeks - if required.

Test bleed volume: Not more than 0.6% of animal’s body weight from the jugular vein (eg. for a 40 kg sheep, not more than 240 mls).
Test bleed frequency: Every 4 weeks, approximately 2 weeks after Booster immunisation.

Total length of time an individual animal will be used: 6 months.

Regime:

Week 1-2: Acclimatisation of animal to experimental situation situation (not necessary if housed on a farm).
Week 3: Initial non-immune bleed
Week 4: Initial immunisation
Week 7: Booster immunisation
Week 11: Booster immunisation
Week 13: Test bleed

> Week 13: If required: booster immunisations at intervals of at least 4 weeks. Test bleed 2 weeks after immunisation.

3. CHICKEN (Schade et al, 1996)

Adjuvants (Initial and Booster): 1. FIA. 2. Specol®. 3. Lipopeptide (PAM₃-Cys-[Lys]₄) 250 µg

Antigen dose: 10 ng - 1 mg (preferably 10 - 100 µg)

Route: Intramuscular (young laboratory chickens, field studies)

Subcutaneous (older laboratory chickens).


Number of sites: 2 (intramuscular) - 4 (subcutaneous)

Total volume: 1 ml

Injection volume per site: 0.25 ml (subcutaneous) - 0.5 ml (intramuscular).

Immunisation frequency: Initial and booster immunisations should be given prior to laying period, with interval of at least 4 weeks (lipopeptide adjuvants) or 6 weeks (emulsion-type adjuvants). If antibody titres are low, boosters to be given during the laying period.

Total length of time an individual animal will be used: Entire laying period (approximately 12 months).

Regime:

Week 1-2: Acclimatisation of animal to experimental situation.
Week 3: Initial immunisation
Week 7-9: Booster immunisation (time interval dependent upon the nature of adjuvant)
Week 9-11: Egg collection for check of yolk antibody titres.

> Week 9-11: If necessary, booster immunisations at intervals of at least 4-8 weeks.

4. WALLABY

Adjuvants (Initial and Booster): To be determined by the Investigator.

Antigen dose: To be determined by the Investigator.

Route: Subcutaneous, using a 22G needle.

Site: Multiple sites on the side of the thorax

Number of sites: Maximum of 4 sites.

Total volume: 0.4 - 0.8 mls.
Injection volume per site: 0.1 - 0.2 mls.

Immunisation frequency: Once established, every 4 weeks - if required.

Test bleed volume: Not more than 0.6% of animal’s body weight from the lateral tail vein (eg. for a 5 kg wallaby more than 30 mls).

Test bleed frequency: Every 4 weeks, approximately 2-3 weeks after Booster immunisation.

Total length of time an individual animal will be used: 6 months.

Regime:
- Week 1-2: Acclimatisation of animal to experimental situation.
- Week 3: Initial non-immune bleed
- Week 4: Initial immunisation
- Week 7: Booster immunisation
- Week 11: Booster immunisation
- Week 13: Test bleed
- >Week 13: If required: booster immunisations at intervals of at least 4 weeks. Test bleed 2 weeks after immunisation.

**PART 3: BACKGROUND PAPER**

**1.0 BACKGROUND**

The production of polyclonal antibodies involves the administration of a specific foreign substance (antigen) to an animal in order to stimulate its immune system to produce large quantities of antibodies to that substance. Such antibodies are of value in biological research, where they serve as essential components in a variety of diagnostic systems used for the qualitative and quantitative determination of a wide range of substances.

In order to ensure the necessary stimulation of the immune system, adjuvants are normally mixed with the antigen. This is particularly important if the antigen itself is only weakly recognised as foreign. In general, adjuvants contain substances which have a depot effect. This results in slow degradation of the antigen with a prolonged period for antigen stimulation of the immune system. The most widely used adjuvants cause local inflammation at the injection site and often result in painful lesions.

Polyclonal antibody production involves two major steps:

- the immunisation itself; and
- collection of blood, which is a prerequisite for antibody preparation.

Both procedures are performed on multiple occasions, and each can cause pain or distress to the animals involved.

**2.0 CHOICES AND DECISIONS IN MAKING POLYCLONAL ANTIBODIES**

Major decisions in making polyclonal antibodies center on choice of the species to be used for immunisation, and on design of an immunisation protocol. The immunisation protocol requires decisions regarding the form of the antigen, the quantity of antigen, the particular adjuvant, the quantity of adjuvant, the ratio of antigen to adjuvant, the route of injection, number and distribution of injection sites, and the frequency of antigen injections. Investigators must always consider the potential of adjuvants to cause pain and distress to the animal, and must maintain a concern for the welfare of the animal in designing their immunisation protocols.
3.0 CHOICE OF ADJUVANT

Adjuvants may possess activity in one or more of three broad categories:

- antigen presentation (the way in which individual antigen molecules are presented to the immune system);
- antigen targeting (the efficiency and mechanism by which the antigen is delivered to the appropriate effector cells of the immune system);
- immune modulation (modifying the processing of antigens by immune effector cells such that either the magnitude or the nature of the specific response is modified)

When selecting an adjuvant, the Investigator must consider the following:

- what does the adjuvant do?
- how does it act?
- what is the toxicity of the adjuvant?
- is a less toxic adjuvant suitable?
- what is to be achieved by its use?
- the nature of the antigen;
- the animal species to be used.

Freund’s Adjuvants are the most commonly used adjuvants to date.

Freund’s Incomplete Adjuvant (FIA) is the most common example of a water-in-oil adjuvant. It contains mineral oil (85-90%) and manninide monooleate (10-15%) (Cox, 1995).

Freund’s Complete Adjuvant (FCA) (Freund and McDermott, 1942) is a “combination” adjuvant containing FIA (a water-in-oil emulsion) and dried, heat-killed Mycobacteria species. Inclusion of mycobacterium in this emulsion increases local inflammatory reactions, which give rise to markedly elevated immune responses to the included antigen as well as to the mycobacterial antigen. These reactions tend to be long-lasting and tissue injury is often a result. FCA was first used in the late 1930s, and still remains the “gold standard” for polyclonal antibody production.

Alternatives to Freund’s Adjuvant

Dissatisfaction with Freund’s Adjuvant has resulted, not from its performance, but from adverse site reactions. Most modifications have attempted to retain efficacy but reduce reactogenicity. Less inflammatory alternatives to Freund’s adjuvant are available and should be considered; eg. Ribi Adjuvant System® and TiterMax®. Noninflammatory adsorptive adjuvants such as alum and aluminium hydroxide gel may also be considered.


4.0 ADVERSE EFFECTS OF ADJUVANTS

Given the widespread use of Freund's Adjuvants, it is important that Investigators be familiar with their side-effects. Lesions associated with their use include granulomatous inflammation, focal necrosis, ulceration of skin, fistulous tracts, muscle atrophy, self-induced trauma, hypersensitivity reactions, and weight loss.

Local inflammatory lesions invariably occur following injection of FCA or FIA. They are usually caused by the installation of too much inoculum at the injection site and by the use of injection sites that are too close together. Lesions may coalesce, the blood supply becomes disrupted, and local cell death occurs, often creating draining abscesses and occasional tissue sloughs.
Subcutaneous injections produce a granulomatous swelling that may show reddening, alopecia and infrequently ulceration. Those lesions which do not resolve (approximately 25%) increase in size. The inoculum may migrate to more ventral and superficial locations via fistulous tracks, and result in cutaneous ulceration at remote sites. As a rule, granulomas should not be painful on palpation. Painful or severely inflamed site reactions associated with severe reddening of the site and surrounding area, or mutilation of the site by chewing and scratching, are usually associated with bacterial contamination of the inoculum.

Intramuscular injection results in a granuloma similar to that produced by subcutaneous injection. However, it is less visible and more difficult to palpate. As with subcutaneous deposition, the dose tends to migrate following the fascial planes ventrally, and may eventually drain some distance from the site of injection. In animals inoculated with FCA and IFA intramuscularly, lesions have been found at the injection sites up to six months post-injection (Broderson, 1989).

Both subcutaneous and intramuscular injections can cause "metastatic" granulomas in lymph nodes, lung, kidney and other organs, depending on the distribution of the emulsion via the lymphatics and circulatory system.

Intradermal injection consistently results in local ulceration regardless of the dose.

Intraperitoneal injection in the mouse has been shown to cause granulomatous inflammation of the serosal surfaces of the abdomen, adhesion between surfaces and lymphoid hyperplasia. Animals may exhibit weight loss, decreased activity, hunched posture and mild diarrhoea. Excessive abdominal distension from the development of ascites can have a significant impact on the well-being of the animal.

Following intravenous injection of FCA or FIA in rabbits, adjuvant emboli were identified in the subpleural regions of the lung and multiple granulomas were formed.

Severe and acute hypersensitivity reactions can be seen after administration of booster doses of FCA, presenting as acute respiratory distress and death. Such reactions result from the re-exposure of the animal to mycobactrium.

5.0 MECHANISMS TO MINIMISE ADVERSE EFFECTS OF ADJUVANTS

Minimise dose-volume

Carefully limiting the dose of antigen and adjuvant injected at each site, and ensuring adequate barriers of normal skin between sites, prevents conditions that lead to lesions and limits the pathology of inflammatory reactions. This can be achieved by:

- using concentrated antigen and
- using widely scattered, multiple injection sites.

Selection of the proper site to limit the potential for pain and distress.

Use toxin-free preparations

Use of commercial preparations of FCA and FIA, which are free of toxic substances, and antigens free of microbial contaminants and endotoxin will minimise any adverse effects.

FCA must be used only once, usually for the initial immunisation, in all species. Freund’s Incomplete Adjuvant (FIA), without killed mycobacteria, is commonly recommended for booster immunisations to avoid hypersensitivity reactions resulting from re-exposure of the animal to mycobacteria.
Mycobacterium concentration

Formulations of FCA not exceeding 0.1 mg dry mycobacteria cell mass/ml are recommended to avoid proportionally increased inflammation and necrosis observed with higher concentrations of mycobacteria (Broderson, 1989).

Use experienced staff

Staff should be skilled, competent, and experienced in the handling of the species being used and in performing the techniques. They must be knowledgeable and capable of recognising signs of distress in the experimental animals, and be responsible for taking action when necessary.

Monitoring

All animals must be examined regularly following immunisation for any changes which might indicate pain, distress or suffering (see Section 12)

6.0 ANTIGEN PREPARATION

The antigen preparation should be free of extraneous microbial contamination. Sterilisation by filtration through a 0.22 micron filter should be performed routinely. However, bacterial endotoxin may not be removed by this process. The presence of byproducts (eg. polyacrylamide gel) should be avoided because of their inflammatory properties. Extremes of pH, particulate matter and contamination with chemicals such as urea, acetic acid, or other solvents or potentially toxic agents should also be avoided. Special precautions may be necessary if the antigen itself is a viable microbe.

The recommendation for the ratio of solution to adjuvant is one part antigen to one part (or less) of Freund's Adjuvant (range 1:1 to 3:1). Information on the quantity of antigen to be used is provided by Hanly et al (1995).

Investigators should be aware that there are multiple means of making the emulsions for injection, and that the appropriate method for the antigen-adjuvant to be used should be researched.

7.0 SELECTION OF ANIMALS

Species

Selection of an appropriate species for polyclonal antibody production is described in detail by Hanly et al (1995). The species most frequently used are rabbits, sheep, goats, chickens mice and rats.

An important aspect in the choice of species is the amount of antibody that can be harvested from the animal. When a large amount of antibody can be harvested, it is unlikely that the experiment will have to be repeated at a later date. Hence, the number of experimental animals used in the long-term will be minimised. During a period of four weeks, only 300 mg of IgG can be obtained from a rabbit. However, 1-2 gms of IgY can be obtained from a chicken and up to 4 gms from a sheep (Palmer and Masters, 1996).

Rabbits are the most commonly used laboratory animal species. The use of specific-pathogen free (SPF) rabbits is recommended. Their use dramatically reduces morbidity and mortality which is frequently documented in rabbits infected with microbial pathogens, particularly (Pasteurella multocida). All rabbits should be vaccinated against Rabbit Calicivirus Disease (RCD) at least 2 weeks prior to their use.

Antibody production in chickens offers the advantage of providing a non-invasive means to obtain antibody that is recovered from the egg yolk. Indications and procedural methods for this technique are described by Hanly et al (1995) and Schade et al(1996). Advantages include:
- Purification of chicken egg yolk immunoglobulins Y (IgY) does not require animal bleeding.
- A single egg contains as much antibody as an average bleed from a rabbit.
- Eggs from immunised chickens provide a continual, daily source of polyclonal antibody.
- Due to the phylogenetic distance between birds and mammals, there is greater potential of producing a higher percentage of specific antibody against mammalian antigens when using chickens.
- Since chicken IgY does not cross-react with mammalian IgG and does not bind bacterial mammalian Fc receptors, non-specific binding is reduced, and the need for cross-species immunoabsorptions is also eliminated.

Although the strains of chickens used commercially for egg production give an acceptable response, higher antibody responses are usually achieved using inbred strains of chickens. It should be noted that immunostimulators which are effective in the chickens are different from those used in many mammals.

The use of FCA is specifically discouraged for use in food animals and nonhuman primates because of subsequent interference with routine tuberculin skin tests used for diagnostic purposes. FCA must not be used in horses or dogs.

**Age and Sex**

In general, young adult animals are better antibody producers than older animals. Although either sex may be used quite satisfactorily, adult females have been reported to mount more vigorous immune responses, both cellular and humoral, than males.

**8.0 NUMBER OF ANIMALS**

For most antigens, the immunisation of two animals will be sufficient for the production of useful antibodies (Palmers and Masters, 1996). The number of animals required may be influenced by many factors including the gender of the animals, and whether they are inbred or outbred animals (Hanly et al, 1996). When the use of more than two animals may be necessary (eg. for antigens conserved across species), adequate justification must be provided.

**9.0 ROUTES, VOLUMES, AND SITES OF ADMINISTRATION**

Ideally, for optimum antibody response, antigen and adjuvant should be broadly distributed to lymphoid tissue. The effectiveness of various routes apparently relates to the efficiency of delivery of antigen to the lymphoid tissues. The use of multiple sites will assist in the distribution of antigen to more lymphoid tissue.

*No single route can be regarded as universally superior for the initial immunisation. Hence the choice of route is usually based upon the choices of species and adjuvant. For most practical applications however, the protocol to follow is the one which causes the least harm to the animals.*

The following recommendations apply primarily to antigen solutions in FCA or FIA. Guidelines have been summarised in Appendix 1 (ANZCCART 1995, CCAC 1991, Hanly et al 1995, Jackson and Fox 1995). For adjuvants other than Freund’s adjuvants, it is recommended that Investigators follow manufacturers’ recommendations.

When selecting injection sites, avoid those that may be prone to self-mutilation, or those that may interfere with ambulation such as footpads. Anatomic sites used for grasping, handling, or restraint such as the dorsal cervical/scapular areas and rump in rabbits and dorsal cervical/scapular areas and tail base in rodents should be avoided when possible. Care should be taken when making injections near the dorsal spinal column because of the proximity of spinal nerves and the lack of muscle mass.

Booster immunisations do not need to be administered by the same route as that used for the Initial immunisation. Booster injection sites should be distanced from previous injection sites.
**Subcutaneous**

In general, this route provides for slow absorption of antigen, which occurs primarily through the lymphatics to local lymph nodes.

_The subcutaneous route should be used whenever possible._ It offers the advantage of easy access for visualisation and palpation to monitor for post immunisation complications. Most Investigators find this route easy to use in most species. While relatively large volumes may be accommodated, the administration of large quantities of antigen or large volumes of inflammatory adjuvants to single sites is not recommended.

Subcutaneous injections are usually administered over the back (rabbit, mouse) or the neck (chicken, mouse).

**Subcutaneous Chambers**

An alternative method for production of polyclonal antibodies is via placement of a subcutaneous “whiffle” ball chamber, which has been described in rabbits (Clemons et al. 1992) and chickens (Ermeling et al. 1992). Immunisations are made directly into the chamber and antibody-rich fluid is harvested from the chamber. Advantages cited for this technique include greater flexibility in preparation of the antigen, minimal discomfort and minimal tissue reaction in the animal, ease of immunisation and collection of fluid from the chamber, and recovery of large volumes of antibody-rich fluid with low cellularity and absence of lipids. This procedure does require surgical placement of the chamber.

**Intramuscular**

Intramuscular injections are usually made in the muscle mass of the anterior thigh (_biceps femoris_), and are generally larger volumes of 0.25 ml or more. Care must be exercised to avoid adjacent nerves and blood vessels as well as fascial planes. Intramuscular injections are usually painful.

Because of their relatively large muscle mass, the intramuscular route is often the preferred route in livestock species such as sheep, goats, and cattle. It is not recommended in rodents.

Disagreement exists as to the appropriateness of intramuscular injection of Freund’s adjuvant. A significant disadvantage is that, while effective, lesions are difficult to evaluate unless loss of limb function or muscle atrophy occurs. In addition, comparable results may be achieved using the subcutaneous route.

**Intraperitoneal**

The intraperitoneal route is permitted for immunisation of small rodents only, where it is usually used when volumes over 100 µl are involved. Antibodies are recovered from the resultant ascitic fluid. The production of polyclonal antibodies using this method must be in accordance with the guidelines produced by the Animal Care and Ethics Committee (ACEC, 1993).

**Intradermal**

The intradermal route is commonly used to take advantage of dendritic cells present within the dermis which are able to carry intact antigen, as well as processed antigen, to draining lymph nodes in a short period of time. Because of the frequent ulceration and infections that occur at the site of such injections, the use of the intradermal route may be justified only when sound scientific evidence is available, and when the purpose is to induce cell-mediated response (CCAC, 1991).

Investigators wishing to use intradermal immunisation must provide evidence of their proficiency with the technique.
In rabbits, injection volumes should not exceed 0.05 mls per site. The location of the site(s) should be carefully selected so as to prevent mutilation. A minimum of five sites should be selected, and the distance between each site should be maximised. Injections are most commonly placed on the back and can be performed using a small 25 to 27 gauge needle.

The intradermal route is not recommended in rodents.

**Footpad**

Where scientific justification is provided, footpad injections may be permitted in rodents, but only in one hind foot, and with the animals housed on soft bedding. “Footpad” injections in rabbits are prohibited. The lack of anatomically defined footpads, plus the weight-bearing function of the rabbit’s feet, preclude this site for immunisation. Furthermore, significant antibody titres are routinely produced by immunisation at other sites.

**Intranodal and intrasplenic**

Both of these routes have been successfully used for direct delivery of small doses of antigen to the lymphoid tissue. With practice, lymph nodes (eg. popliteal lymph node) can often be palpated and the injection performed percutaneously, thus obviating the need to perform a surgical incision and dissection to locate the node for injection. With the intranodal procedure, care must be taken not to inject excess volumes of material into the node (Jarvis, *pers comm*).

**Intravenous**

Intravenous use of FCA or FIA is not permitted. Intravenous route may be used for booster immunisation of *aqueous antigen* after primary immunisation with the antigen associated with an effective adjuvant. However the risk of anaphylaxis must always be considered.

**10.0 FREQUENCY**

For an animal to sustain an antibody response, a continual or intermittent supply of antigen is needed. One way an adjuvant may aid the immune response is by forming a depot of antigen at the injection site resulting in the sustained release of small quantities of antigen over a long period of time. This approach gives sustained stimulation while minimising suppressive effects. Even with an adjuvant that forms a depot of antigen, at some point in time the quantity of antigen is diminished and the antibody titer declines. At this time a second injection of antigen (a booster dose) may be given. When an animal that has responded maximally is given a booster dose of antigen too soon, suppression rather than enhancement of the immune response may ensue. *Ideally, one follows the serum antibody titer in a hyperimmunised animal and gives a booster injection of antigen only after the antibody titer has begun to decline.* However, when an animal has responded less than maximally (which is the more usual situation for small doses of antigen), a booster dose of antigen given at 3 to 6 weeks after the first antigen dose will usually increase the serum antibody titer. Booster doses of antigen are typically equal to or less than (approximately $\frac{1}{2}$) the priming dose. The advantage of using a smaller antigen dose is that only the higher affinity clones of B cells are stimulated, thus improving the quality of the antibody produced.

The protocol to follow for frequency of immunisations is the one which causes the least harm to the animals. The trade-off for minimising the stress to the animals may be that it takes longer to achieve good antibody titres.

A minimum period of three weeks should be allowed between the initial and subsequent immunisations. Booster immunisations should be delayed if significant inflammatory reactions are still present from the initial immunisation. Where excessive inflammatory reactions are seen with the initial immunisation, due consideration should be given to using lesser concentrations, smaller volumes per injections site, or both for subsequent immunisations.
Intervals between subsequent booster injections should be long, at least 4 weeks. Initial immunisation with 2-3 booster injections followed by a longer rest for 3-4 months before a final booster injection without an adjuvant, often results in a “memory response”, i.e., a dramatic increase in antibody titre with high affinity (better quality) (Hanly et al, 1995). Decreasing the amounts of antigen used with subsequent boosters will also increase the antibody affinity.

11.0 INJECTION SITE PREPARATION

To minimise microbial contamination of injected tissue, aseptic preparation of all injection sites is essential. Following clipping of the hair, the skin should be cleaned, and then disinfected using either chlorhexidine gluconate (Hibitane®) or povidone-iodine solution. This practice not only reduces the potential for the development of infection and abscess formation, but also facilitates visualisation of the injection sites to permit appropriate treatment of lesions if they develop.

The use of sterile needles and syringes is also mandatory.

12.0 POST-INJECTION OBSERVATIONS

Frequency

Observations must be made at least 3 times weekly for a period of 4 weeks after each injection, or until all lesions have healed.

Monitoring criteria

Investigators should monitor:

- the general appearance and behaviour of the animals for evidence of pain or distress;
- injection sites for evidence of lesions such as swelling, abscess or fistula formation, and infection or ulceration.

A direct measure of the animal's continuing well-being is maintenance of its body weight.

Depending on the species and observational skills of the investigator, pain and distress is usually manifested by changes in the following parameters:

- personality
- mobility and posture - guarded movement, bracing, hunched
- frequency of normal behaviours - self or mutual grooming, foraging, resting
- vocalization
- body temperature - increase or decrease
- locomotion - reluctance to move or abnormalities in movement
- loss of appetite, with resultant changes in body weight
- appearance of coat - piloerection, sweating, hydration of subcutis
- extremities - temperature and/or colour
- fluid balance - dehydration or oedema - local or general
- colour of mucous membranes
- consistency and colour of faeces, frequency of elimination
- urine - colour, frequency of urination.

Biochemical markers of stress and muscle damage may also be useful. Among those used are cortisol and creatine phosphokinase (CPK).

Although lesser in severity and frequency by comparison to Freund’s adjuvant, inflammatory lesions can occasionally be observed following immunisation with alternative adjuvants such as TiterMax® (Check et al, 1990) or Ribi adjuvants. Hence, similar post-injection observations should be performed for animals receiving alternative adjuvants.
Records

An “immunisation clinical record” is helpful in maintaining careful data on individual animals. This record should include the agent, route, site or sites, volume, date of injection and the body weight of the animal on the injection date.

Problems

Investigators must contact the Animal Technician of the facility, or the Veterinary Manager (Animal Services Unit) if injection site lesions or evidence of pain or distress are identified in any animal. This will permit timely and appropriate assessment and institution of therapy when required. Supportive therapy may include topical cleansing, antibiotic administration, analgesic administration, or all three.

13.0 BLOOD COLLECTION

Up to 10% of the total blood volume of the animal (approximately 0.6% of body weight) can be taken on a single occasion. Collection of this volume may be repeated after 3-4 weeks (UFAW, 1993). Investigators proposing to exceed these recommendations are required to perform additional monitoring procedures (eg. periodically evaluating hematocrit and total protein) and provide appropriate fluid or blood replacement therapy.

To ensure that the safe blood volume is not exceeded, the animal’s body weight must be determined prior to blood collection. This is especially important in small animals where the margin for error is potentially greater.

Survival test bleeding is recommended via tail vein or retro-orbital sinus from rodents and via the marginal ear vein in rabbits. Blood is generally collected from large animal species via jugular venipuncture.

*Blood collection from rabbit ears by transecting the vein with a razor cut is not permitted.* During venipuncture, vasodilation can be enhanced by the use of heat lamps. Because of the risk of cardiac tamponade, pulmonary haemorrhage, and pneumothorax, intracardiac blood collection in both rodents and rabbits is limited to terminal procedures, and is performed under general anaesthesia.

Because of its irritant properties, and potential mutagenic and carcinogenic activity, the use of xylene for vasodilation is also *not permitted*.

Arrangements may be made through the Manager, Animal Services Unit (telephone: 4921 6220), for regular test bleeds, and the terminal bleed, in rabbits to be performed by appropriately qualified staff of the Unit.

14.0 RESTRAINT

Proper methods for restraint must be used during immunisation and blood collection procedures to reduce stress, enhance vasodilation, and prevent injury to the animal and personnel. It is helpful to acclimatise animal to handling and restraint procedures prior to the initiation of any experimental procedure. With rabbits and chickens, the practice of wrapping the animal in a towel during the procedures usually results in a relaxed animal without the need for sedation.

15.0 PERSONAL SAFETY

Freund's Adjuvant should be handled as a hazardous substance. Accidental needle sticks in humans have been associated with chronic, painful inflammatory lesions and abscesses, particularly in individuals that were already sensitised to mycobacteria. Other severe side effects have also been reported. As stated above, the use of xylene for vasodilation is not permitted because of its potential mutagenic and carcinogenic activity.
Recommendations:

- The use of protective clothing, including lab coat, safety glasses, and gloves.
- The use of luer-lock syringes in mixing antigen-adjuvant emulsions.
- Avoidance of re-capping needles.
- The use of appropriate restraint, sedation, or anaesthesia of animals to prevent injury to animals or personnel during immunisations.
- Special care to be taken during intradermal immunisation since the viscosity of the emulsion and the nature of the injection site increase the chances of accidental injection.

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**Subcutaneous Ball**


**Production of polyclonal antibodies in chickens**


**Blood collection**

APPENDIX 1: GUIDELINES FOR THE USE OF FREUND’S ADJUVANTS

PART A

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>ADMINISTRATION ROUTE AND MAXIMUM VOLUME (in mls)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Footpad #1</td>
</tr>
<tr>
<td></td>
<td>per site</td>
</tr>
<tr>
<td>Mouse</td>
<td>0.025</td>
</tr>
<tr>
<td>Rat</td>
<td>0.05</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>-</td>
</tr>
</tbody>
</table>

PART B

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>ADMINISTRATION ROUTE AND MAXIMUM VOLUME (in mls)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intradermal #3</td>
</tr>
<tr>
<td></td>
<td>per site</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.05</td>
</tr>
<tr>
<td>Sheep/goat</td>
<td>-</td>
</tr>
<tr>
<td>Cattle</td>
<td>-</td>
</tr>
<tr>
<td>Pig</td>
<td>-</td>
</tr>
<tr>
<td>Poultry</td>
<td>-</td>
</tr>
</tbody>
</table>

Freund's adjuvants must **not** be given intravenously, because the oily vehicle will cause multiple organ failure. Complete Freund's Adjuvant must **not** be used for booster injection.

#1   Footpad inoculation on one hind leg only. Footpad injection is not acceptable for the rabbit.
#2   Intraperitoneal administration, with the exception of mice, requires special justification (e.g. stimulation of a local immune response in the gastrointestinal tract or other mucosal surfaces).
#3   Requires special justification. Intramuscular and intradermal administration must not be applied simultaneously, but rotation between boosters is acceptable.
#4   Intramuscular administration in the rabbit requires special justification. Intramuscular administration is not recommended in the mouse, rat or guinea pig.
PART 4: ALTERNATIVES TO FREUND'S COMPLETE ADJUVANT (FCA)

The scientific literature contains few impartial evaluations of adjuvants for specific practical applications. There are some limited studies that make some comparisons. However, they do not examine all variables, generally only compare a couple of adjuvants to each other, and are often not published in peer-reviewed journals. The following list is not comprehensive and does not represent endorsement of specific commercial products. However, the ACEC does endorse continual efforts to use refinements in animal research techniques and hopes that this information will be useful to investigators.

Ribi Adjuvant System (RAS)

Ribi markets a wide variety of immunologic products. The primary product marketed as an alternative to FCA is a oil-in-water emulsion containing detoxified endotoxin (MPL) and mycobacterial cell wall components (TDW, CWS) in 2% squalene. It is as convenient to use as FCA (or more so because of its lower viscosity) and it has very low toxicity, having been used in humans. In some circumstances, especially in mice, it compares well with FCA. Preparation of the adjuvant-antigen emulsion in a tissue grinder with a teflon pestle (Potter-Elvehjem type) is encouraged for optimal results but it is not essential. Available from:
Sigma-Aldrich Pty. Ltd.
Unit 2, 14 Anella Ave, Castle Hill NSW, Australia 2154
Phone (toll free): 800-800-097  Fax (toll free): 800-800-096
Tel:  (02)-9841-0555   Fax:  (02)-9841-0500

TiterMax

TiterMax is a stable, metabolizable water-in-oil adjuvant, and has been marketed specifically as a replacement for FCA. The manufacturer has shown that a single injection of TiterMax will induce antibody titres greater than 2 injections of Freund’s (FCA followed by Freund's Incomplete Adjuvant). Available from:
Bio Scientific Pty Ltd.
Ph: 1-800-251-437 (freecall)  Sydney metro: (02) 9521 2177  Fax: (02) 9542 3100
Web:  http://www.biosci.com.au   Email:  Daryn@biosci.com.au or  TechServ@biosci.com.au

Syntex Adjuvant Formulation (SAF)

Has been developed as an alternative to FCA. It is a preformed oil-in-water emulsion stabilised by Tween 80 and pluronic poloxyethylene/polyoxypropylene block copolymer L121. SAF activates complement by the alternate pathway and is said to bias the humoral immune response to IgG2a in the mouse. Like other oil-in-water adjuvants, it works better with proteins that have some hydrophobic aspect to promote their adherence to oil droplets. Available from:
Syntex Corp. Palo Alto CA 94304 USA.
Ph: 415-852-1887 Fax: 415-852-1784.

Freund's Incomplete Adjuvant (FIA)

The most inflammatory component of FCA, killed mycobacteria, is not included in FIA. FIA is routinely used for boosting immunisations subsequent to FCA. It can also be used for the initial immunisation, particularly when a strong antigen is used or moderate antibody levels are sufficient. As with FCA, efficacy is dependent upon vigorous mixing of the adjuvant and antigen until a stable emulsion has formed. Available from: Sigma Chemical Co. or Difco Agents throughout Australia.

ALUM - aluminum hydroxide; Al(OH)

Aluminum hydroxide is a widely used adjuvant, especially in commercial products such as vaccines. It is very well suited for strong antigens. Many sources of aluminum hydroxide are available:
a) Reheis “Hydrogel HPA” from AS Harrison & Co. Brookvale NSW 2100. Ph: 02 9938 1066 Fax: 02 9905 0065
b) Superfos “Alhydrogel” from Superfos Biosector DK 2950 Denmark. Ph: 454-289-3111 Fax: 454-289-1595

**SuperCarrier**

This and some similar products are convenient kits for coupling haptens, such as peptides and small proteins, to larger carrier molecules to enhance immunogenicity. The coupled proteins can be combined with other adjuvants. Available from: Syntex Research 3401 Hillview Ave. P.O. Box 10850 Palo Alto, CA 94303

**Elvax 40W**

This is an ethylene-vinyl acetate copolymer. While the production process is somewhat involved, the resulting immune response can surpass that induced by FCA. Sold in 25 gram amounts, however small gratis amounts are available. Available from: DuPont Chemical Co. Wilmington, DE (800)-441-9494; (request Elvax customer representative).

**L-tyrosine**

A co-precipitate of the amino acid and antigen has been shown to have excellent adjuvant properties, even surpassing FCA in some circumstances. While the co-precipitation procedure is not difficult, it requires some manipulation. The cost is negligible. Available from: Numerous chemical companies.

**Montanide**

A manide-oleate compound that has been shown to produce antibody levels equivalent to FCA. Small aliquots are available upon request from the producer. Available from: Seppic 75321 Paris. Ph: 331-4062-5720 Fax: 331-4062-5252.

**AdjuPrime**

This is a carbohydrate polymer marketed as an alternative to FCA. Independent studies comparing its efficacy to other adjuvants do not appear to be available. The carbohydrate is thought to create both a "depot" effect and an enhancement of the interaction between the antigen and antigen-presenting cells.

**Nitrocellulose-absorbed protein**

Nitrocellulose-absorbed protein will give a desirable slow release of antigen over a period of 2 weeks to 2 months. The nitrocellulose is inert and causes minimal inflammatory response.

**Gerbu adjuvant**

This is an aqueous phase adjuvant that does not have a depot effect. It therefore requires frequent boosting to achieve a high-titer response, but produces minimal inflammatory response. Available from: Scimar, Templestowe, Victoria 3106. Ph: 03 842 3386 Fax: 03 842 3407.

**Immune-stimulating complexes (ISCOMS)**

ISCOMS are Ag-modified saponin/cholesterol micelles that form stable cage-like structures. ISCOM-associated antigen molecules do not form a depot site but are transported to the draining lymph nodes. Quantities of antigen as low as 1 µg have elicited a significant immune response. ISCOMS can be successfully prepared in the laboratory (Coligan et al, 1995).