Guidelines on Adjuvant Use in Rabbits Rats and Mice

Last Updated 15 April 2016

1. **Purpose**
   
   To outline available adjuvants and select procedures and techniques recommended by ULAM Veterinary Staff for use in rabbits, rats and mice.

2. **Responsibility**

   1. It is the responsibility of the Principal Investigator (PI) to choose an appropriate adjuvant and route for their species of interest that minimizes possible adverse effects to the animal.
   2. It is the responsibility of husbandry technicians to report adverse effects seen in the animals after adjuvant administration.
   3. Veterinary staff is responsible for consultation with the PI if needed and follow-up care of any animals that show adverse effects.

3. **Definitions**

   1. **Adjuvant**: pharmacological or immunological agent that modifies the effect of other agents (e.g., drugs, vaccines) while having few if any direct effects when given by itself.
   2. **Immunologic Adjuvant**: agents that non-specifically increase immune responses to specific antigens that are weakly immunogenic.
   3. **Viscous Adjuvant**: an adjuvant that includes oil in its makeup.
   4. **Aqueous Adjuvant**: an adjuvant that uses water or physiologic saline as its background.

4. **Procedures**

   1. **Adjuvant Selection**
      
      1. When choosing an adjuvant, the PI needs to consider several factors: the size and composition of the antigen of interest, the animal species being injected, and the route of administration.
      2. It is the role of the investigator to consider the toxicity of the antigen preparation due to contamination with endotoxins or chemical residues and to ensure appropriate pH adjusted within physiological limits.
      3. The following is a list of some of the most common adjuvant preparations, however many options exist. Consulting with the literature or immunologists may provide help in choosing the appropriate adjuvant.

   2. **Viscous Adjuvants**
      
      1. Freund's Complete Adjuvant (FCA): one of the most commonly used adjuvants. FCA is a water-in-oil emulsion of mineral oil, mannide monooleate (a surfactant), and heat killed or avirulent *Mycobacterium tuberculosis* or *M. butyricum*. It is effective in potentiating cellular and humoral responses to immunogens. Its activity is a result of sustained release of antigens from the oily deposit and stimulation of local innate immune responses.
         1. The use of FCA is associated with necrotizing dermatitis. Refer to Freund's Incomplete Adjuvant (FIA) for further guidelines.
      2. Freund's Incomplete Adjuvant (FIA): the same as FCA minus the mycobacterial cells or cellular components and thus, is less effective than FCA in inducing high antibody titers and enhancing cell-mediated immunity.
         1. The use of FCA/FIA is associated with necrotizing dermatitis, tissue sloughing, localized injection site granulomas, as well as diffuse systemic granulomas secondary to migration of the oil emulsion. More recent formulations of FCA are using less virulent strains of Mycobacterium, which may reduce the occurrence and severity of these lesions. Because of the potential for pain and distress, it is recommended that an alternative to FCA/FIA be used if possible.
2. If FCA/FIA is scientifically justified, a few guidelines must be followed.
   1. FCA cannot be used more than once in the same animal as doing so may cause severe tissue damage.
   2. FCA with mycobacterial concentrations <0.1 mg/ml are recommended although mycobacterial concentrations of up to 0.5 mg/ml may be used without the requirement to provide scientific justification.
   3. FCA is usually necessary only for the initial immunization and subsequent immunizations are completed with FIA
   4. Unless otherwise scientifically justified, routes of administration, sites of administration, volumes, and concentrations must follow the guidelines for FCA/FIA in this document.

3. TiterMax®: a microparticulate water-in-oil emulsion that uses the metabolizable oil squalene. Silica particles stabilize the emulsion and enables the emulsion to carry a wide variety of antigens without using large amounts of toxic emulsifying agents thus, minimizing lesions at the injection site. TiterMax is reported to produce antibody titer levels similar to FCA/FIA through activation of the complement system and inducing increased expression of HMC class II. The manufacturer recommends intramuscular injections, but other routes are available for species for which intramuscular injections are not recommended such as rodents.

4. The Ribi® Adjuvant System: an oil-in-water emulsion whereby antigens are emulsified with a minimal amount of oil and surfactant (Tween 80). Oil-in-water emulsions are less viscous and easier to inject than water-in-oil emulsions. However, this attribute also makes oil-in-water emulsions poor adjuvants alone and require immunostimulants such as refined mycobacterial products (trehalose 6,6'-dimycolate and cell wall skeleton) or purified gram negative bacterial products (monophosphoryl lipid A). The optimal formulation will depend on the nature of the antigen immunostimulants.

5. Other less common examples of viscous adjuvants are Specol, EMULSIGENS, and Syntex Adjuvant Formulation (SAF).

3. Aqueous (Mineral-based) Adjuvants
   1. Aluminum salts are claimed to be particularly effective at eliciting an immune response with weakly immunogenic antigens. Granulomas are common when subcutaneous or intradermal routes are used.
      1. Aluminum hydroxide: alhydrogels are sterile aluminum hydroxide gels which are pyrogen-free, stable and have a high adsorptive capacity. At pH <9, alhydrogels are positively charged which then readily adsorb negatively charged molecules such as proteins at neutral pH.
      2. Aluminum phosphate
      3. Calcium phosphate.

4. Less commonly used adjuvants
   1. Montanide
   2. Gerbu

5. Route of Administration
   1. Possible routes of adjuvant administration are subcutaneous (SC), intramuscular (IM), intraperitoneal (IP), intravenous (IV), or intradermal (ID). The appropriate route for use depends on the adjuvant and the animal species. When using oil or viscous gel adjuvant (e.g., FCA) in rabbits, mice or rats, SC is the recommended route. Viscous adjuvants should never be given IV due to the risk of thrombosis and pulmonary embolism. Aqueous adjuvants (or antigens without adjuvant) are usually given SC, IP, or IV to rats and mice, or SC, IM, or IV in rabbits. For any adjuvant preparation not listed on this document, refer to the manufacturer and literature for recommendations on route of administration.
      1. SC: Subcutaneous injections allow for easy monitoring of the injection site and use the antigen-processing dendritic cells in the dermis (Langerhans cells). Multiple injections should be spaced apart so resulting inflammatory lesions do not coalesce or join into a larger affected area.
      2. IM: Intramuscular injections allow relatively large volumes per injection compared to SC and ID injections and are easy to administer in larger species. However, the site of injection is difficult to monitor and is potentially painful. IM injections should not be used in mice and rats due to their limited muscle mass and the ability of the adjuvant to travel along fascial planes and irritate inflamed nerves and vasculature.
      3. IP: Aqueous adjuvants can be administered intraperitoneally in rats and mice but it is not recommended for viscous adjuvants. IP injections of viscous adjuvants have been reported to induce peritonitis, acute inflammatory reactions, and behavioral changes and are often deemed to be inappropriate.
      4. IV: Intravenous is the preferred route for initial administration of small, particulate immunogens in aqueous adjuvants. The rapid, systemic absorption quickly delivers the antigens to the lymphatic system. FCA and FIA cannot be given IV, as they will cause pulmonary emboli and granuloma formation.
      5. ID: Intradermal immunizations also utilize local Langerhans cells and the site of injection is easily monitored. However, there is a significant potential for pain and ulceration, so justification is required and is only an option for rabbits, as it has been shown to cause significant pain in rodents due to the small closed space of the site.

6. Site of Administration
   1. The antigen must be prepared in an aseptic manner including mixing.
   2. All subcutaneous and intradermal injection sites in all animals should be clipped and aseptically scrubbed with betadine or noivasan. This will reduce the likelihood of abscess formation and infection as well as make it easier to monitor the sites post-injection. Sterile needles, syringes, and aseptic technique should be used for all injections to minimize the risk of infection and abscess.
   3. Injection sites should be chosen that do not interfere with handling or restraint of the animal, blood draws, or the animal's ability to move or bear weight. When administering multiple concurrent injections, sites should be clustered but distant enough from each other to prevent merging of resultant lesions or infections, should they occur.
4. If large amounts of antibody need to be collected, the wiffle ball method in the rabbit is an alternative option. This involves the aseptic, subcutaneous implantation of a plastic, perforated, hollow ball, which acts as a depot for the injected antigen. Once the animal has been given sufficient recovery time, initial and booster injections may be given directly into the wiffle ball, and subsequent fluid draws taken from it.

7. Age
1. Age is important to consider depending on the outcome of the the immunization. The immune response in young adults tends to be robust and less affected by previous immune challenges than older animals. Recommendations for age are given in Table 1 (Adapted and modified from Leenaars and Hendriksen, 2005; CCAC 2002).

8. Blood collection volumes
1. Please refer to Guidelines on Blood Collection.

9. Injection volume
1. There are no universal recommendations for volumes of adjuvant to be administered. The goal is to use the smallest volume possible in order to elicit the desired antibody response. Where a range of volumes is suggested, start with the smallest recommended volume and modify as needed. It may be possible to use less than the minimum recommended volume per injection. NIH volume recommendations for immunizations using FCA or FIA are list below in Table 1. Suggested volumes, sites, and numbers of initial injections are outlined in Tables 2, 3 and 4.
2. With questions regarding volumes, please refer to Guidelines on Administration of Substances to Laboratory Animals.

10. Boosters
1. Booster injections should be considered when antibody production has plateaued or begun to decline. If the initial immunization was given with a viscous (depot forming) adjuvant, one should wait at least 4 weeks before administering a booster. If the first immunization was with an aqueous adjuvant, or no adjuvant, boosters can be given after 2-3 weeks. Titers should be checked after the first booster to see if subsequent boosters are needed. FCA should not be used as a booster, due to severe tissue reactions with more than one injection in an animal. Instead, FIA can be used for a booster. The maximum recommended number of boosters is 3. Adjuvants are not always necessary in a booster injection.
2. As long as there is no (or an insignificantly-sized) lesion, booster injections should be given near to the initial site, but not so close that the resulting inflammatory lesions can coalesce into one lesion. This takes advantage of memory cells in the lymph node(s) associated with that anatomical region. If there is a local lesion due to the initial immunization, the booster should be given at a different location. Booster injections must never be given into a granuloma or swelling. Boosters should not be given IV or IP due to a high risk for inducing anaphylactic shock. If the initial injection was IV or IP, boosters should be SC (preferred) or IM (not recommended).

11. Ascites production of monoclonal antibodies
1. Collection of monoclonal antibodies via ascites has largely been replaced by \textit{in vitro} methods using hybridomas. The necessity for utilizing the ascites method must be scientifically justified and proof provided that \textit{in vitro} methods have failed for all appropriate cell lines.
2. The total number of abdominal taps (withdrawal of fluid with a needle) is recommended to be three or less.

\textbullet\ Table 1. Recommended age of animals for polyclonal antibody production

<table>
<thead>
<tr>
<th>Animal</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>6 wk</td>
</tr>
<tr>
<td>Rats</td>
<td>6 wk</td>
</tr>
<tr>
<td>Rabbits</td>
<td>3 mo</td>
</tr>
</tbody>
</table>

\textbullet\ Table 2. Recommended volumes (mL) of Freund’s Complete or Incomplete Adjuvant by species and routes

<table>
<thead>
<tr>
<th></th>
<th>(SQ)</th>
<th>(ID)</th>
<th>(IP)</th>
<th>Footpad</th>
<th>(IM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>&lt;0.1</td>
<td></td>
<td></td>
<td>&lt;0.05**</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Rat</td>
<td>&lt;0.1</td>
<td>&lt;0.02**</td>
<td></td>
<td>&lt;0.1**</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Rabbit</td>
<td>&lt;0.25</td>
<td>&lt;0.05**</td>
<td></td>
<td></td>
<td>&lt;0.25</td>
</tr>
</tbody>
</table>

*Not recommended
**Only when justified
***Only one limb recommended without justification

Adapted and modified from \url{http://oacu.od.nih.gov/ARAC/documents/Adjuvants.pdf}
### Table 3. Recommendations of volume (mL) and number of injections per route for viscous adjuvant administration in rabbits, rats, and mice

<table>
<thead>
<tr>
<th>Route</th>
<th>Site</th>
<th>Volume</th>
<th># Injections Site</th>
<th>Volume</th>
<th># Injections Site</th>
<th>Volume</th>
<th># Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td>Over each shoulder and quadriceps</td>
<td>0.25</td>
<td>4</td>
<td>Base of tail</td>
<td>0.2</td>
<td>1</td>
<td>Base of tail</td>
</tr>
<tr>
<td>IM</td>
<td>Each quadriceps</td>
<td>0.25</td>
<td>2</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>ID</td>
<td>Dorsum</td>
<td>0.025</td>
<td>4-10</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

* Not recommended

### Table 4. Recommendations of volume (mL) and number of injections per route for aqueous or no adjuvant administration in rabbits, rats, and mice

<table>
<thead>
<tr>
<th>Route</th>
<th>Site</th>
<th>Volume</th>
<th># Injection Site</th>
<th>Volume</th>
<th># Injection Site</th>
<th>Volume</th>
<th># Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td>Over each shoulder and quadriceps</td>
<td>1.5</td>
<td>1</td>
<td>Base of tail</td>
<td>0.5</td>
<td>1</td>
<td>Base of tail</td>
</tr>
<tr>
<td>IM</td>
<td>Into each quadriceps</td>
<td>0.2-0.5</td>
<td>2</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>IP</td>
<td>*</td>
<td>*</td>
<td>Abdomen</td>
<td>5</td>
<td>1</td>
<td>Abdomen</td>
<td>1</td>
</tr>
<tr>
<td>ID</td>
<td>Dorsum</td>
<td>0.05</td>
<td>4-10</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>IV</td>
<td>Saphenous, cephalic, or auricular vein</td>
<td>1.5</td>
<td>1</td>
<td>Tail vein</td>
<td>0.5</td>
<td>1</td>
<td>Tail vein</td>
</tr>
</tbody>
</table>

*Not recommended in general for pAb production
a-Adapted and modified from Leenaars and Hendriksen, 2005; CCAC 2002

5. Related Documents

1. Guidelines on Administration of Substances to Laboratory Animals
2. Guidelines on Blood Collection

6. References