University of Louisville
Institutional Animal Care and Use Committee
Policies and Procedures

Use of Adjuvants in Rodents and Rabbits

Policy: The use of adjuvants in research animals must be reviewed and approved by the IACUC. Alternatives to adjuvants, particularly Complete Freund's Adjuvant (CFA), must be considered and utilized if and when possible. If adequate alternatives are not feasible, undesirable side effects should be reduced or eliminated through the use of appropriate routes of administration, adequate separation of injection sites, and the use of a small amount of inoculum per site.

Rationale: The use of CFA and other potent adjuvants is associated with the potential for pain or distress in research animals. Utilizing alternative adjuvants and administration techniques that are associated with fewer side effects may improve animal welfare. The NIH's Office of Animal Care and Use (OACU) maintains guidelines to ensure adjuvants are used only when necessary and that researchers employ current recommendations for refinement when consistent with scientific objectives. Because of the potential for complications from certain routes of administration of immunizations, the following guidelines have been established.

Procedures, Guidelines, and Exceptions:

1. **Complete Freund's Adjuvant (CFA)**: CFA is a water-in-oil emulsion containing mineral oil, a surfactant, and heat-killed mycobacteria that potentiates cellular and humoral antibody responses to immunogens. CFA creates an intense inflammatory reaction and may result in side effects such as granulomatous reactions, necrotizing dermatitis, and adjuvant-related arthritis. Accordingly, it should only be used if scientifically justified and no comparative alternatives exist. CFA may be used only for the first (priming) dose. Subsequent immunizations should be performed using Incomplete Freund's Adjuvant (IFA) or another adjuvant unless explicitly justified. Re-immunization with CFA is rarely warranted. If approved by the IACUC, an interval of at least three weeks should be given between doses.
    a. **Preparation**:
        i. The mycobacteria in CFA is re-suspended by vortexing or shaking the ampule or vial. The CFA is then removed from the ampule or vial using sterile technique.
        ii. Although approaches may vary, one part or less of CFA to one part aqueous antigen solution (v/v) has been recommended. The CFA/antigen emulsion should be mixed deliberately and with care in order to avoid the introduction of air bubbles.
        iii. Formulations of CFA containing 0.5 mg/ml of mycobacterial components are commercially available and have been successfully used by many researchers. Concentrations of <0.1 mg/ml are recommended in order to minimize the inflammation and focal necrosis observed with higher concentrations. Some protocols, such as autoimmune disease induction protocols, may require the use of greater concentrations than those available commercially, and must be scientifically justified and approved by the IACUC *Proposal*.
        iv. The use of preparations containing disrupted mycobacterial cells rather than preparations containing whole, intact bacilli may be preferred, since it is difficult to histologically distinguish the latter from live, acid-fast cells.
        v. For favorable results while minimizing undesirable side effects, use the recommended injection volumes and sites appropriate for the species, size of the animal, and experimental goal (Table below).

2. **Alternatives to CFA**: Alternatives to CFA must be used whenever possible. In many situations, these alternatives produce a robust immune response with fewer side effects than CFA. IFA has a similar

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composition to CFA but lacks the mycobacterial component making it less inflammatory and able to be administered multiple times. Some other alternatives include TiterMax®, the RIBI Adjuvant System®, and aluminum compounds. For more information, see OACU, 2022.

3. **Preparation and Injection**
    a. Antigen preparations should be sterile and, ideally, isotonic, pH neutral, and free of urea, aceticacid, and other toxic solvents. Antigens separated using polyacrylamide gels should be further purified whenever possible in order to minimize the amount of secondary inflammation/irritation from gel fragments. If further purification is not possible, then the amount of polyacrylamide contaminant should be minimized by careful trimming. Millipore ultrafiltration of the antigen, for example, prior to mixing it with the adjuvant, is recommended to remove extraneous microbial contamination.
    b. Some routes of injection may potentially be less disruptive to the animal than other routes (e.g., subcutaneous injection vs. footpad administration). Whenever possible, the least invasive methodology required to accomplish the experimental goal should be utilized. More invasive injection routes should be avoided unless scientifically justified.
    c. It is necessary to separate multiple injection sites by a distance sufficient to avoid coalescence of inflammatory lesions.
    d. A minimum period of 2 weeks between subsequent inoculations is recommended.
    e. In addition to the route of administration, the site of injection should be chosen with care in order to avoid areas that may compromise the normal movement or handling of the animal (e.g., intradermal injections in the neck scruff of a rabbit).

4. **Routes and Volume of Administration**
    a. The least invasive route of administration should be used and injection sites should be chosen that do not interfere with locomotion or handling. Subcutaneous (SC) administration is the preferred route for CFA and most other adjuvants.
    b. If routes other than SC *must* be used for CFA, the *Proposal* must contain a strong justification. Intradermal (ID) injections frequently result in skin necrosis and sloughing; intramuscular (IM) injections can result in temporary or permanent lameness; intravenous (IV) injections can cause pulmonary lipid embolism; and intraperitoneal (IP) injections can cause peritonitis.
    c. The recommended volumes of CFA-Antigen Emulsion are summarized in the table below (adapted from OACU 2022):

Species	**SC** (ml) **PREFERRED ROUTE**	**ID** (ml)	**IP** (ml)	**Footpad** (ml)
Mouse	<0.1	*	<0.2	<0.05**
Rat	<0.1	<0.05**	<0.5	<0.1**
Rabbit	<0.25	<0.05 **	*	*

**Not recommended*
***Only when justified*

    d. Footpad injection of CFA in rodents is discouraged because animals may develop arthritis, chronic pain and lameness, and secondary infections in the inflamed areas. If this procedure is to be used, it must be described and its use scientifically justified, including documentation that injections in other sites do not produce adequate antibody titers for the specific antigen being used. If used, only one hind foot may be injected and injections must be spaced at 2 week intervals. Animals that have received foot pad injections must be housed on contact bedding rather than wire-bottomed cages.

5. **Post-injection Observations and Treatments**: Animals must be observed daily for adverse reactions for at least four (4) weeks or until any associated lesions have resolved. The *Proposal* must specifically detail

clinical signs or behaviors indicating a need for intervention, such as the use of analgesics. Examples may include significant erythema, reluctance to bear weight, inappetence, and body weight loss. If IP administration of adjuvant and antigen is scientifically justified and approved, animals must be monitored daily for ascites. Abdominal distention is painful and necessitates relief of ascites pressure and clear humane endpoints in the *Proposal*. Supportive therapy may include topical cleansing, application of sterile petroleum jelly and/or sterile normal saline, antibiotics and analgesics. If overt pain or distress is anticipated or observed, the use of narcotic agonists, mixed agonist-antagonists, or other species-appropriate agents should be considered. Any lesions or side effects resulting from adjuvants not listed in the IACUC *Proposal* must be reported to CMRU veterinary staff immediately.

6. **Personnel Safety**: Accidental inoculation of personnel with adjuvants that contain mycobacterial products (e.g. CFA) can result in sensitization to tuberculin as well as chronic, local inflammation which is poorly responsive to antibiotic therapy. Use caution when handling and administering these products.

References

Office of Animal Care and Use (OACU). 2022. Guidelines for the Use of Adjuvants in Research: Special Emphasis on Freund's Adjuvant. Animal Research Advisory Committee Guidelines (available at: https://oacu.oir.nih.gov/animal-research-advisory-committee-guidelines, last access 18 October 2022).

Office of Laboratory Animal Welfare (OLAW). 2002. Institutional Animal Care and Use Committee Guidebook, 2nd edition (available at: http://grants.nih.gov/grants/olaw/guidebook.pdf, last access 7 February 2020).

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