Guidelines for the Use of Adjuvants in Research

The use of adjuvants in animal research requires careful consideration. While relatively nonspecific inflammation may elicit robust immunity, the investigator needs to evaluate the effect of associated local and/or systemic pain and distress of the research animal with the scientific benefit that may be gained from the experiment. The use of potent inflammatory agents, particularly Complete Freund's Adjuvant (CFA), can result in severe side effects. Although it is expected that alternatives to CFA should be used whenever possible (1, 8), the use of CFA is scientifically justified in many systems, such as the induction of autoimmune disease models for which no comparable alternatives exist (9).

When consistent with the scientific objectives, e.g. routine antibody collection, adjuvants known to produce less intense inflammatory responses should be considered as alternatives to CFA. These may include currently licensed adjuvants such as aluminum compounds (e.g. Alum), MF59, liposomes, and monophosphoryl lipid A (MPL); adjuvants in pre-clinical development [e.g. Montanides, polymeric microparticles, flagellin, saponins (Quil A and QS-21), RC529 (synthetic MPL + Alum), cytokines, and immunostimulatory nucleic acids (e.g. Iscom, CpG oligonucleotides); emerging adjuvants e.g. virus-like particles, nanoparticles, TLR agonists, muramyl dipeptides, tripeptides (MDP and MTP), and TDM (trehalose dimycolate), etc.]; and other procedures or emulsions such as subcutaneously- implanted chambers, TiterMax, EMULSIGENS, Syntex Adjuvant Formulation (SAF), and Specol (10, 11, 12, 16). In many situations, these alternatives are capable of eliciting cellular and humoral antibody responses sufficient for many scientific purposes with fewer side effects than those commonly seen with CFA. Information on alternative adjuvants is also available online (see references). All adjuvants used in animal research must be approved by the IC-ACUC, and adjuvants that could induce a severe reaction must be scientifically justified.

Complete Freund's Adjuvant

CFA, a water-in-oil emulsion containing heat-killed mycobacteria or mycobacterial cell wall components, is effective in potentiating cellular and humoral antibody responses to injected immunogens. Adjuvant activity is a result of sustained release of antigens from the oily deposit and stimulation of a local innate immune response, resulting in enhanced adaptive immunity. An essential component of this response is an intense inflammatory reaction at the site of antigen deposition, resulting from an influx of leukocytes and their interaction with the antigens. The use of CFA is an important biologic resource for investigators, which should be used responsibly and with care in order to avoid or minimize the adverse effects of excessive inflammation. CFA may result in local inflammation and granulomatous reactions at the site of injection, chronic inflammation, skin ulceration, local abscess or tissue sloughing, diffuse systemic granulomas secondary to migration of the oil emulsion, adjuvant-related arthritis, and very rarely, chronic wasting disease.

For most applications, CFA is usually only necessary for the initial immunization, while Incomplete Freund's Adjuvant (IFA), which lacks mycobacteria, is the adjuvant of choice for subsequent immunizations. Successive immunizations with CFA should be scientifically justified

and approved by the institutional ACUC. CFAs containing either *M. butyricum* or *M. tuberculosis* H37Ra (an avirulent strain) are commercially available. Additional information about CFA use is available online (see references).

Guidelines for Preparation and Injection

The following guidelines have proven effective in eliminating complications after immunization. Utilization of: a) sterile technique in the preparation of antigen-adjuvant emulsions; b) aseptic preparation of the injection site; c) appropriate injection technique; d) appropriate routes and sites of administration; e) adequate separation of injection sites; and f) use of smaller volumes at each injection site have all proven efficacious in the elimination of post-immunization complications.

1. Antigen preparations should be sterile and, ideally, isotonic, pH neutral, and free of urea, acetic acid, and other toxic solvents.
2. 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 prior to mixing it with the adjuvant is recommended to remove extraneous microbial contamination.
3. The mycobacteria in CFA is resuspended by vortexing or shaking the ampule or vial. The CFA is then removed from the ampule or vial using sterile technique.

   Although approaches may vary, one part or less of CFA to one part aqueous antigen solution (v/v) has been recommended (1). The CFA/antigen emulsion should be mixed deliberately and with care in order to avoid the introduction of air bubbles.
4. 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 necrosis observed with higher concentrations (2). 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 institutional ACUC.
5. 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.
6. 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 1) (3, 4).
7. Some routes of injection may potentially be less disruptive to the animal than other routes (e.g., subcutaneous injection vs. foot-pad administration).

Whenever possible, the least invasive methodology required to accomplish the experimental goal should be utilized. Intra-dermal, intramuscular, and footpad injections should be avoided unless scientifically justified.

8. It is necessary to separate multiple injection sites by a distance sufficient to avoid coalescence of inflammatory lesions.
9. A period of 2 weeks minimum between subsequent inoculations is recommended.
10. 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 scruff of the neck of a rabbit).

Routes of Administration Presenting Special Issues

1) Footpad Immunization:

Utilizing the footpad for immunizing small rodents may be necessary in studies where it is required to isolate a draining lymph node as a primary action site. Procedures to address the well-being of the subject animals should be used, e.g. limiting the quantity of adjuvant-antigen solution injected into the footpad, the use of only one foot per experimental animal, and housing on soft bedding rather than on screens. Footpad inoculation must not be used for routine immunization of rodents without specific scientific justification. Alternative sites with potential draining lymph node utility e.g. the hock (popliteal lymph node, 13) and cervical sites (auricular lymph node, 14; superficial cervical lymph node, 15) should be used in order to prevent the animal's locomotion from being affected. If scientific justification is provided, the recommended maximum footpad injection volumes are 0.01-0.05 ml in mice and 0.10 ml for rats (1). Rabbits must not be immunized in their feet because they lack a true footpad.

2) Peritoneal Exudate:

The production of rodent peritoneal exudate by the intraperitoneal administration of antigen and adjuvant is a recognized, valid scientific procedure for obtaining high-titer reagent. Undesirable side effects of painful abdominal distention and the resulting distress can be avoided by daily monitoring and relief of ascites pressure, or termination of the experiment. Intraperitoneal injections of CFA-antigen emulsions should normally be limited to less than 0.2 ml in mice (6).

Post-injection Observations and Treatments

Post-inoculation monitoring of animals for pain and distress or complications at the injection sites is essential, and should be done daily for a minimum of four weeks or until all lesions have healed. Supportive therapy may include topical cleansing, 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 and used under the direction of the veterinarian (taking into account the research objective). Steroidal or non-steroidal anti-inflammatory agents must be used with caution due to their known impacts on immunological processes.

Personnel Safety

Adjuvants that contain mycobacterial products can be an occupational hazard to laboratory personnel and should be handled with extreme care. Reports of accidental needle punctures in humans have been associated with clinical pain, inflammatory lesions, and abscess formation in tuberculin-positive individuals. Tuberculin-negative individuals have tested positive in subsequent tuberculin tests after accidental CFA exposure (7). Safety glasses should be worn in order to avoid accidental splashing of CFA in the eyes.

Other Considerations

Scientists preparing antigens for *in vivo* administration in conjunction with adjuvants should be aware of the potential presence of contaminating substances and other characteristics of the injectate which may have additive inflammatory effects. Care should be taken to consider and eliminate additional inflammatory stimuli whenever possible, e.g. excessive vehicle pH or the presence of by-products of purification such as polyacrylamide gel fragments. The preparation should be kept sterile.

Table 1. Recommended Volume of CFA-Antigen Emulsion (CFA-AE) per Site and Route of Administration

Species	**SubQ** (ml)	**Intradermal** (ml)	**Intraperitoneal** (ml)	**Footpad** (ml)	**Intramuscular** (ml)
Mouse	<0.1	*	<0.2	<0.05**	<0.05
Rat	<0.1	<0.05**	<0.5	<0.1	<0.1
Rabbit	<0.25	<0.05**	*	*	<0.25***

* Not recommended

** Only when justified

*** Only one limb recommended without justification

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http://www.ccac.ca/en/CCAC_Programs/Guidelines_Policies/GDLINES/Guidelis.htm (antibody production 2002)

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