Guidelines for the Use of Adjuvants in Research
Special Emphasis on Freund's Adjuvant

Introduction: Due to their ability to promote robust inflammation, the use of adjuvants in research may be beneficial for those studying the immune system, modeling autoimmune disease, developing vaccines, or producing antibodies.(1-11) Regardless of the scientific justification, investigators using adjuvants should carefully consider their selection of adjuvants and aim to utilize an adjuvant that meets the scientific needs while introducing the least possible risk to the animals. No matter what adjuvant is selected, all adjuvant use must be reviewed and approved by the Institute/Center (IC) Animal Care and Use Committee (ACUC).

Adjuvant Selection:
Adjuvants can be useful in various types of studies. No matter the aims of the research, the investigator needs to evaluate the effect of associated local and/or systemic pain and distress of the research animal compared to the scientific benefit that may be gained from the use of adjuvants in the experiments.(5, 12, 13)

Complete Freund's Adjuvant (CFA) is a potent inflammatory agent but can result in severe side effects.(5, 13, 14) The use of CFA may be scientifically justified for the induction of autoimmune disease models for which currently no comparable alternatives are known to exist, but it is important to understand its potential side effects.(5, 12, 14, 15) CFA, a mineral oil containing a suspension of whole or pulverized heat-killed mycobacteria which is emulsified together with a solution of the antigen of interest to form a water-in-oil emulsion, 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, lymph node structural changes, 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.(5, 12, 13, 15)

Given these findings, alternatives to CFA should be used whenever possible. 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. If CFA will be used more than once it must be scientifically justified and approved by the IC ACUC.(5, 8, 16) 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).

When consistent with the scientific objectives, e.g., routine antibody production, 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), squalene-in-water emulsions (MF59 and AS03), monophosphoryl lipid A (MPL), Ribi adjuvants, combined with alum (AS04); adjuvants in pre-clinical development (e.g., Montanides), polymeric microparticles, saponins (e.g., Quil A QS-21, ISCOMS, ISCOMATRIX), immunostimulatory nucleic acids (e.g., CpG oligodeoxynucleotides, poly IC:LC), other toll-like receptor-agonists (e.g., flagellin, imidazoquinolines, small molecules), cationic

liposome formulations (CAF) combined with immune stimulators such as trehalose dibehenate (TDB) virus-like particles, virosomes, nanoparticles, and oligonucleotide complexes, mucosal adjuvants (e.g., cholera toxin, LTK3, LTR72, chitosan); and other procedures or emulsions such as subcutaneously- implanted chambers, TiterMax, EMULSIGENS, Syntex Adjuvant Formulation (SAF), and Specol.(4-7, 17-24) In many situations, these alternatives are capable of eliciting robust cellular and humorallocal or systemic immune responses with fewer side effects than those commonly seen with CFA. Extensive information on alternative adjuvants is also available online and in publications.

Guidelines for the Preparation of Complete Freund's Adjuvant:

1. 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.
2. Although approaches may vary, one part or less of CFA to one part aqueous antigen solution (v/v) has been recommended.[12] The CFA/antigen emulsion should be mixed deliberately and with care in order to avoid the introduction of air bubbles.
3. Formulations of CFA containing 0.5 mg/ml of mycobacterial components are commercially availableand 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.[14] 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 IC ACUC.
4. The use of preparations containing disrupted mycobacterial cells rather than preparations containingwhole, intact bacilli may be preferred, since it is difficult to histologically distinguish the latter from live, acid-fast cells.
5. Antigen preparations should be sterile and, ideally, isotonic, pH neutral, and free of urea, acetic acid, and other toxic solvents. Antigens separated using polyacrylamide gels should be further purified whenever possible 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 bycareful trimming. Millipore ultrafiltration of the antigen, for example, prior to mixing it with the adjuvant, is recommended to remove extraneous microbial contamination.

Guidelines for Injecting Adjuvants:

The following guidelines have proven effective in significantly alleviating complications after immunization with adjuvants. In all situations, injections should be prepared considering the following criteria that have all proven efficacious in the elimination of post-immunization complications:

1. Reduce contamination of the solution for injection.
    a. 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.
    b. Care should be taken to consider and eliminate additional inflammatory stimuli whenever possible.
    c. Reduce the presence of by-products of purification such as polyacrylamide gel fragments.
    d. Use aseptic technique in the preparation of antigen-adjuvant emulsions and keep the solution sterile.
2. Control the pH of the solution for injection.
    a. pH should be appropriate for injection. Ideally pH should be physiologic (7.3-7.4) A small range outside of neutral (pH 4-9) may be tolerated for intramuscular and intravenous

injections. Intraperitoneal injections should aim for that physiologic pH.(25)

3. Perform aseptic preparation of the injection site and utilize appropriate injection techniques(26).
    a. Clipping and cleaning the injection site may be beneficial to reduce contamination.
    b. Users should be able to perform all injections without complications or unwanted side effects. Short-term anesthesia can be beneficial in some situations.
4. Utilize appropriate routes and sites of administration.
    a. Some routes of injection may potentially be less disruptive to the animal than other routes. For example, subcutaneous injection may have benefits over footpad administration.
    b. 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. In addition to the route of administration, the site of injection should be chosen with care 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 or rodent may make handling difficult and painful).
5. Separate injection sites adequately.
    a. It is necessary to separate multiple injection sites by a distance sufficient to avoid coalescence of inflammatory lesions.
6. Use smaller volumes at each injection site.
    a. 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. Publications describe appropriate injection volumes based on route for various species(26, 27) but special considerations may be necessary if CFA is used (Table 1).
7. A minimum period of 2 weeks between subsequent inoculations is recommended.

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

Species	**SubQ** (mL)	**Intradermal** (mL)	**Intraperitoneal** (mL)	**Footpad** (mL)
Mouse	≤ 0.1	≤ 0.05**	≤ 0.2	≤ 0.05**
Rat	≤ 0.1	≤ 0.05**	≤ 0.5	≤ 0.1**
Rabbit	≤ 0.25	≤ 0.05**	*	*
Nonhuman Primate**	Freund's Adjuvant is not generally recommended for use in Nonhuman primates, as it may interfere with TB testing results and cause excessive inflammation. Nevertheless, it is recognized that some models may require use of CFA. If used, the recommended volumes should not exceed those used in rabbits and should be scientifically justified.			

* Not recommended ** Only when scientifically justified

<u>Routes of Administration Presenting Special Issues</u>:

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 ofthe 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.

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, cervical sites, auricularlymph node, and superficial cervical lymph node, should be used in order to prevent the animal's locomotion from being affected.(19, 28, 29) If scientific justification is provided for footpad injection, the volume should be kept as small as possible and not above the volume shown in Table 1.(12) Rabbits must not be immunized in their feet because they lack a true footpad.

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 such as painful abdominal distention may occur. The resulting distress can be avoided by daily monitoring and relief of ascites pressure, or termination of the experiment. The *Guide* and the Public Health Service Policy on Humane Care of Laboratory Animals both require that *in vitro* methods be considered prior to the use of *in vivo* methods for monoclonal antibody production. The use of the mouse ascites method must be scientifically justified and approved by the IC ACUC and methods to avoid or alleviate pain and distress (including *in vitro* methods) must also be considered. In addition, generation of ascites fluid typically requires the use of a "priming" agent. Pristane is a commonly used "priming" agent, however, IFA has also been shown to be an effective "priming" agent.(16, 30) Concern has been expressed about the potential for discomfort and distress that may be associated with "priming" agents, particularly Pristane. Due to this concern, consideration for using the lowest dose of "priming" agents is strongly encouraged. Despite the fact that higher doses may sometimes be required to produce a large amount of ascites, many guidelines suggest a lower 0.1 to 0.2 mL dose of Pristane.(16) Lower doses of IFA should also be considered and a maximum dose of 0.3 ml is recommended by some authors for IFA. Investigators and ACUCs should consider the need of using peritoneal exudate to collect antibodies when other methods exist and, if required scientifically, whether analgesics, periodic abdominal tapping, reducing doses, using alternative methods, using alternative adjuvants, or other methods can be considered to alleviate discomfort.(8, 16, 22, 31-39)

Post-injection Observations and Treatments:
Post-inoculation monitoring of animals for pain and distress or complications at the injection sites is essential. Animals should be monitored daily for the first several days after administration and then at a frequency agreed to by the laboratory and ACUC. If there are any injection site reactions, monitoring should be done daily until all lesions have healed. If lesions develop, supportive therapy may be provided and 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 and used under the direction of the attending veterinarian (considering the research objective). Steroidal ornon-steroidal anti-inflammatory agents must be used with caution due to their known impacts on immunological processes.

Personnel Safety:
At the time of ASP review, the NIH Division of Health and Safety will add appropriate guidelines for staff working with adjuvants and these guidelines must be followed.

Adjuvants that contain mycobacterial products, such as CFA, 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.(40)

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