University of Cincinnati
Animal Care and Use Program

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

This document provides guidance on the use of potent inflammatory agents, particularly Complete Freund's Adjuvant, in animals. These guidelines are adopted from NIH Animal Research Advisory Committee Guidelines for the Use of Adjuvants in Research.

Questions – send email to LAMS-Veterinary@uc.edu

Background

The use of adjuvants in animal research requires careful consideration. While relatively nonspecific inflammation may promote 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 may be scientifically justified for the induction of autoimmune disease models for which currently no comparable alternatives are known to exist.[1,2,3,4,5,9]

Recommendations

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 (Table 1). In many situations, these alternatives are capable of eliciting robust cellular and humoral local or systemic immune responses with fewer side effects than those commonly seen with CFA. Extensive information on alternative adjuvants is also available online (see references). All adjuvants used in animal research must be approved by the Institutional Animal Care and Use Committee (IACUC), and use of adjuvants that could induce a severe reaction must be scientifically justified.

Table 1: Alternatives to CFA

Licensed adjuvants	Examples
Aluminum Compounds	Alum
Squalene-in-water Emulsions	MF59, AS03
Monophosphoryl Lipid A (MPL)	
Ribi adjuvants combined with alum	AS04
Montanides	
Polymeric Microparticles	
Saponins	Quil A QS-21, ISCOMS, ISCOMATRIX
Immunostimulatory Nucleic Acids	(CpG oligodeoxynucleotides, poly IC:LC
Toll-like receptor-agonists	Flagellin, Imidazoquinolines, Small Molecules
Cationic Liposome Formulations (CAF)	
Trehalose Dibehenate (TDB)	
Virus-like Particles, Nanoparticles[19,20,21]	
Oligonucleotide Complexes[22]	
Proprietary formulations	(TiterMax, EMULSIGENS, Syntex Adjuvant Formulation (SAF), and Specol[10,11,12,16,17,18]

Considerations when using Complete Freund's Adjuvant

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.[4,9]

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 Institute/Center (IC) Animal Care and Use Committee (ACUC).[37] 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).

CFA preparation

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.[1] 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 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.[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 IACUC.
4. 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.
5. 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 2).[3,4]

CFA Guidelines for Preparation and Injection

The following guidelines have proven effective in significantly alleviating complications after immunization with adjuvants. 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. 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.
2. 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.
3. It is necessary to separate multiple injection sites by a distance sufficient to avoid coalescence of inflammatory lesions.
    a) A minimum period of 2 weeks between subsequent inoculations is recommended.
4. 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).

Routes of Administration Presenting Special Issues

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], cervical sites, auricular lymph node[14], and 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.

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 and the resulting distress can be avoided by daily monitoring and relief of ascites pressure, or termination of the experiment. The *Guide for the Care and Use of Laboratory Animals* 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 IACUC 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, Incomplete Freund's Adjuvant (IFA) has also been shown to be an effective "priming" agent. Concern has been expressed about the potential for discomfort and distress that may be associated with "priming" agents, particularly Pristane.[32] Due to this concern, many guidelines suggest a lower 0.1 to 0.2 mL dose of Pristane.[1,30,31,32,33] It is also recognized, as an ILAR report states, "in some strains of mice, 0.2 mL might not be sufficient to produce ascites and that as much as 0.5 mL might be required."[32] A maximum dose of 0.3 mL is recommended for IFA.[31] Consideration for using the lowest does

of “priming” agents is strongly encouraged 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, 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 (taking into account the research objective). Steroidal or non-steroidal anti-inflammatory agents must be used with caution due to their known impact 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 2: Recommended Volume of CFA-Antigen Emulsion (CFA-AE) per Site and Route of Administration

Species	SubQ (mL)	Intradermal (mL)	Intraperitoneal (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*

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