



## Administering Complete Freund's Adjuvant (CFA) and other Adjuvants

**367.1 Policy:** Emory University permits antibody production using CFA according to the specific requirements in this policy. Non-inflammatory adjuvants or adjuvants that produce less intense responses but still produce a humoral antibody response should be used if possible. Requests for deviations from the policy must be scientifically justified and will be considered on a case by case basis by the IACUC at the time of protocol review or in response to a request for protocol modification.

### 367.2 Definitions of Key Terms Specific to this Policy

- 1) Adjuvant: An immunological agent that increases the antigenic response
- 2) Antibody: A protein that is secreted in response to an antigenic stimulus that neutralizes the antigen by binding to it
- 3) Complete Freund's Adjuvant: It is a water in oil emulsion which is composed of inactivated and dried mycobacteria, usually *Mycobacterium tuberculosis* (the pathogenic agent of tuberculosis)
- 4) Incomplete Freund's Adjuvant: Freund's adjuvant without the mycobacteria component

**367.3 The Use of CFA:** Complete Freund's Adjuvant (CFA) is widely used and considered to be the most effective adjuvant available for consistently producing high titer antibodies to diverse antigens. It is irreplaceable and vital for immunology research and antibody production at the present time. CFA used improperly or excessively can cause undesirable side effects in animals. It may produce severe chronic local inflammation causing skin ulcerations and draining sinuses with granulomas. It may cause lymph node structural changes. The oil droplet may disseminate and produce systemic granulomas and chronic wasting disease. It may also induce an autoimmune disease, especially arthritis, which is debilitating to the animal host. These adverse effects can be minimized or eliminated by adhering to the following protocol.

- 1) The concentration of Mycobacteria in the CFA antigen-adjuvant emulsion should be 0.5 mg/ml or less. When sufficient for study objectives, concentrations of less than 0.1mg/ml are recommended to minimize inflammation and necrosis.
- 2) CFA is generally only necessary for the initial immunization with Incomplete Freund's Adjuvant (IFA) used in subsequent immunizations. However, in the induction of experimental allergic encephalomyelitis (EAE) in mice, any number of strains and mutants may be used and administered nerve tissue antigen in combination with CFA **once** (Levine and Sowinski, 1973) or **twice** 3-7 days apart (Contarini, et al, 2018; Stromnes and Goverman, 2006; Muller, et al, 2000) - sometimes in association with IP or IV 200-500 ng pertussis toxin (PTX). PTX enhances EAE development by providing additional adjuvant and facilitating entrance of autoimmune T cells into the

CNS (Contarini, et al, 2018). The CFA-antigen emulsion is administered at 0.2 ml/mouse divided between 2-4 sites (Stromnes and Goverman, 2006). Repeating the CFA use increases the incidence and severity of EAE, necessary for some studies (Stromnes and Goverman, 2006).

- 3) The priming dose with CFA for antibody production should be followed by boosters given either (1) as a response to confirmed waning antibody titers or (2) empirically once between 4-8 weeks after priming and once again 2-3 weeks after the first booster. Except for instances separate from polyclonal antibody production (i.e., specific T cell stimulation and recruitment), this regimen should produce a good titer (> 1 mg/ml serum) for immunogenic material. If additional boosters are necessary, such should be requested via a request for protocol modification.
- 4) The investigator should know the characteristics of the antigen and avoid factors that will excessively stimulate or inhibit the inflammatory effect. Extraneous microbial contamination, protein contaminants, pH extremes in the antigen preparation, the presence of chromatographic by-products, such as polyacrylamide gel, or chemical contamination (i.e., SDS, urea, acetic acid, solvents) may lead to a low titer of the desired antibody. Sterilization by filtration through a low binding 0.22 micron filter (i.e., cellulose acetate) should be done whenever possible. Antigen prepared by gel electrophoresis should be either (1) eluted, lyophilized, ground to a fine powder, and resuspended in sterile saline, or (2) transferred to nitrocellulose paper, trimmed and cut into fine pieces.
- 5) The adjuvant should be injected subcutaneously in small doses (see Table 1). For optimal immunostimulation it is recommended that the injections to non-rodents be distributed over the 4 quadrants of the animal (i.e., bilaterally, on the side behind the shoulder and in front of the hind leg). Bilateral injections at the base of the tail in rats and mice gives optimal immunological response. Intradermal and intramuscular injections are discouraged and must be specifically justified. The maximum total injection dose and amount at each site is shown in Appendix 1.

#### Post-injection observation

The lab must have a post-injection schedule of monitoring in place to observe animals for the development of pain and distress. Observation three times per week is recommended for the first three weeks. With the exception of intraperitoneal CFA, post-injection pain or distress is not well documented in rodents thus analgesics are not routinely required. However, if overt pain or distress is observed, the use of an opioid analgesic such as buprenorphine must be considered. Additional supportive therapy such as cleansing agents or antibiotics may also be appropriate.

#### Limitations of Foot Pad Immunization

Foot pad immunization of rodents or other species should not be used for routine immunization due to the potential for subsequent impairment of animal mobility. It may be used in particular studies where isolation of a draining lymph node as primary action site is required. An alternative method whereby the rodent is injected in the hock, the lateral tarsal region just above the ankle, has been

shown to act at the same primary lymph node without similar impact on animal mobility. Foot pad immunization requires specific justification and approval by the IACUC. In these cases:

- 1) The use of inflammatory adjuvants is strongly discouraged;
- 2) only one rear foot pad can be used per experimental animal;
- 3) the quantity of the antigen-adjuvant emulsion injected should be kept to a minimum (i.e., 0.05 ml); and
- 4) the animals should be housed on soft bedding rather than screens.
- 5) Rabbits should not be immunized in their "foot pads," because they do not have true foot pads.

#### Peritoneal Exudate

Intraperitoneal administration of antigen and adjuvant is often used in rodents to obtain high titered reagent or monoclonal antibodies. The undesirable side effects of painful abdominal distention associated with development of the peritoneal exudate can be readily avoided by daily monitoring and relieving ascites pressure as appropriate. Please refer to the *IACUC Guidelines on Monoclonal Antibody Production* for methodology for this procedure.

**367.4 Some Other Adjuvants:** Most adjuvants incorporate two components. One component forms a deposit to protect the antigen from catabolism. The 2 traditional methods for deposit formation are mineral oils or aluminum hydroxide precipitates (Alum). Liposomes and synthetic surfactants are alternate deposit-forming systems (Hunter et. al., 1981, Atkinson et. al., 1988). The second component is a nonspecific stimulant which acts by increasing the amount of lymphokines present. Lymphokines directly stimulate the activity of antigen processing cells, causing a local inflammatory reaction at the injection site. Heat-killed bacteria (using *Bordetella pertussis* or *Mycobacterium tuberculosis*) or lipopolysaccharide are used as nonspecific stimulants.

As a general suggestion, CFA should be used for weakly immunogenic compounds or for small amounts of immunogen. Several other synthetic, non-inflammatory adjuvants are available which may offer advantages in some situations. Some examples include: (1) RAS (Ribi Adjuvant System, Ribi Immunochemical Research, Inc., P.O. Box 1409, Hamilton, Montana 59840), (2) Hunter's TiterMax or TiterMax Gold (without silica) (CytRx, 154 Technology Parkway, N.W., Norcross, GA 30092) and (3) Quil A, a saponin-type, surface-active adjuvant (Accurate Chemical Scientific Corporation, Westbury, NY 11590).

**367.5 Recommendations for Enhanced Antibody Production with Freund's Adjuvants:**

The following are *suggestions* for antigen and emulsion preparation and handling which should enhance antibody production:

- 1) Antisera to be used for screening bacterial expression cDNA libraries or for immunoblots are best made against denatured protein, whereas those to be used for

screening cDNAs expressed in eukaryotic transfection systems or for immunoprecipitation of native-cell- synthesized structures might be best made against native protein.

- 2) An antigen dose range of 50-1000 micrograms is recommended for rabbits and 10-200 micrograms for a mouse.
- 3) As a general rule, the greatest immunogenicity is associated with the largest antigens given in the greatest quantity. Cross-linking antigen or binding to a carrier protein should be considered for nonprotein antigens and for polypeptides < 10 kD.
- 4) Booster doses should use half to an equivalent quantity of antigen as that which was used for priming.
- 5) Do not use Tris-based buffers for generating CFA or IFA emulsions. Phosphate-buffered saline is recommended for preparing antigen in solution.
- 6) Use glass syringes when preparing and injecting Freund's adjuvant-antigen emulsions.
- 7) Test for emulsion stability by extruding a small drop onto the surface of 50 ml cold water in a 100 ml beaker. An adequate emulsion will retain droplet form on the water surface.
- 8) Discard unused immunogen as protein denaturation will occur over time.
- 9) For small quantities of rare antigen, consideration should be given to direct injection of pure antigen (without adjuvant) into the spleen or lymph nodes under surgical conditions and proper anesthesia.

**367.6 Potential Hazards to Research Personnel:** Special care must be taken to avoid parenteral exposure of personnel involved in the preparation and administration of CFA. Accidental intradermal or intramuscular inoculation of the mycobacterial-in-oil suspensions may result in tuberculin sensitization of tuberculin negative individuals and moderate to severe local, regional, or systemic hypersensitivity reactions in individuals who are sensitized to tuberculin. Persons who have had tuberculosis may develop chronic ulcerating granulomas following injection of very small amounts of CFA. Inadvertent ocular exposure can lead to blindness. The following procedures are recommended for the safe use of CFA:

- 1) For non-rodents, sedate or properly restraint the animal and shave the proposed injection sites prior to administration of the antigen-adjuvant.
- 2) Use two sterile luer-lock syringes joined by a stopcock to prepare the emulsion.
- 3) Wear safety glasses.

### 367.7 References

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**367.8 Document Properties:**

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**APPENDIX 1:**

**The Maximum Total Volume/Animal and Maximum Amount of Adjuvant- Antigen Emulsion at Each Subcutaneous Injection Site.**

<b>SPECIES</b>	<b>MAXIMUM TOTAL INJECTION (ML)</b>	<b>MAXIMUM AMOUNT (ML)/SUBCUTANEOUS SITE</b>
Mice	0.3	0.05
Rat	0.5	0.1
Chicken	0.5	0.1
Guinea Pig	1.0	0.1
Rabbit	1.0	0.1
Goat/Sheep*	2.0	0.2
Primates **		

\* Deep intramuscular injections at a maximal volume of 0.5 ml antigen-adjuvant emulsion per site is permitted in large domestic animals.

\*\* Freund's Complete Adjuvant is not recommended for use in primates. In many cases it causes an excessive inflammatory response and would negate any TB testing in treated animals. Should an investigator feel that he must use this adjuvant in nonhuman primates, they should confer with the attending clinical veterinarian and seek IACUC approval.