

Guidelines for the Use of Adjuvants

Introduction

Adjuvants help antigens to stimulate the immune system. They have few direct effects when given by themselves, but when given with a vaccine or drug, they have the ability to stimulate cell-mediated immunity and/or humoral antibody formation.

Although many adjuvants are non-pharmaceutical grade agents, the IACUC requires that pharmaceutical-grade components be used when available and that methods to preclude bacterial contamination be employed (e.g., antigen filtration prior to mixing with the adjuvant). Some adjuvants such as Freund's complete adjuvant can result in significant pain and distress including painful ulcerative sores. Consequently, investigators are expected to consider alternatives such as Ribi Adjuvant System®, and Hunter's TiterMax®.

Selection of the adjuvant

1. Freund's Mineral Oil Adjuvant Emulsions

Freund's complete adjuvant (FCA) is a mixture of a non-metabolizable oil (mineral oil), a surfactant (mannide monooleate), and killed mycobacteria (*Mycobacterium tuberculosis* or *M. butyricum*). Freund's incomplete adjuvant (FIA) is the same as FCA except that it does not contain killed mycobacteria. Freund's adjuvants are prepared as water-in-oil emulsions by combining approximately equal volumes of adjuvant and aqueous antigen solution in such a way that the oil becomes the continuous phase. If properly mixed, the antigen will be distributed over a large surface area, which increases the potential for interaction with relevant antigen presenting cells in vivo. Moreover, like other water-in-oil adjuvants, FCA and FIA exert an antigenic depot effect and therefore sequester and release the antigen over long periods of time, thus resulting in a prolonged antibody response. Both formulations can result in adverse reactions resulting from the stimulation of cell-mediated immune responses. FCA is particularly toxic when injected into laboratory animals because the host response to the non-metabolizable oil and the mycobacteria can result in both local and disseminated granulomatous reactions. Less severe inflammatory reactions result when: a) the concentration of mycobacteria in FCA is less than 0.5 mg/ml; b) more concentrated aqueous antigen solutions are added, resulting in an antigen-rich emulsion and reduction in the quantity of emulsion injected; c) multiple injection sites with minimal volumes of emulsion are injected at any one site; and d) the injection sites are separated to avoid fusion of inflammatory lesions.

In addition, FCA is to be used only for weakly immunogenic antigens and only for initial immunizations. If booster immunizations are necessary, FIA must be used instead of FCA. Animals receiving FCA are normally placed in Pain/Distress Category of E (pain without relief). Humane endpoints, scoring sheets and intervention plans should be included in the protocol.

The recommended volumes per of CFA-Antigen Emulsion per site and route are as follows:

Species	Subcutaneous	Intramuscular	Footpad	Intradermal	Intraperitoneal
Mouse	0.1 ml/site 4 sites max	Not Permitted	0.05 ml max *Justification Required	0.05 ml/site 4 sites max	0.25 ml max
Rat	0.2 ml/site 4 sites max	Not Permitted	0.1 ml max *Justification Required	0.05 ml/site 4 sites max	0.5 ml max
Rabbit	0.25 ml/site 4 sites max	0.25 ml/site 4 sites max Justification Required	**Not Recommended	0.05 ml/site 1.0 ml total	Not Permitted

*Justification required for use; only one foot; only hind foot

** Rabbits do not have a true footpad

2. Ribi Adjuvant Systems (RAS)

The manufacturer offers different mixtures of oil, detergent, and immunostimulator(s) from which to choose. Three RAS formulations, each containing different detoxified bacterial products as immunostimulators, are commercially available: a) mycobacterial trehalose dimycolate (TDM) emulsion recommended for use with strong immunogens in mice, guinea pigs, and rats; b) Gram-negative bacterial monophosphoryl lipid A (MPL®) + TDM emulsion recommended for use in mice, guinea pigs, and rats; and c) MPL® + TDM + mycobacterial cell wall skeleton (CWS) emulsion recommended for use in rabbits and large animals (e.g., goats). Adding aqueous antigen to the adjuvant and vortexing the mixture forms stable oil-in-water emulsions. In general, Ribi's adjuvant emulsions, like other oil-in-water emulsions, are more suitable for hydrophobic or amphipathic protein antigens than for hydrophilic proteins since the effectiveness of RAS is dependent on antigen absorption to the oil droplets in the oil-in-water emulsion. The inflammatory response associated with adjuvant use is minimized by utilizing metabolizable oil (squalene) and detoxified bacterial components as immunostimulators. Furthermore, since significantly less oil is needed to form stable oil-in-water emulsions (e.g., RAS) than for stable water-in-oil emulsions (e.g., Freund's-type adjuvants), Ribi's adjuvant causes less tissue damage when injected into animals. It should be noted, however, that since RAS has less of an antigenic depot effect than water-in-oil emulsions, more frequent booster injections are usually needed for an adequate antibody response. Immunization protocols recommended by the RAS manufacturer are:

*MPL® +TDM Emulsion, R-700 or TDM, R-760**

Mouse: 0.2 ml dose, 0.1 ml administered subcutaneously in two sites

Guinea Pig, Rat: 0.4 ml dose, 0.2 ml administered subcutaneously in two sites

*Use with strong immunogens

Inject on day 0, boost on day 21, and test bleed on day 26 or 28. If necessary, booster injections may be given every 21 days using the same formulation. For monoclonal antibodies, boost intravenously (or intraperitoneally) with saline/antigen only 14 days after last adjuvant/antigen injection. Remove spleen for fusion three days following intravenous injection.

MPL® + TDM + CWS Emulsion, R-730

Rabbit: 1.0 ml dose, 0.05 ml administered intradermally in six sites, 0.3 ml administered intramuscularly into each hind leg, and 0.1 ml administered subcutaneously in the neck region. Inject on day 0, boost on day 28, and test bleed on day 38 and 42. If necessary, booster injections may be given every 28 days using the same formulation. Note: Injecting more frequently than every 28 days may result in a reduction in the immune response.

3. Hunter's TiterMax®

TiterMax® is a water-in-oil emulsion, similar to Freund's adjuvants, containing metabolizable oil (squalene) and a non-ionic surfactant (copolymer of polyoxyethylene and polyoxypropylene). Most adjuvant activity is attributed to the surfactant's ability to activate and bind certain complement components, which putatively target the antigen to follicular dendritic cells in the spleen and lymph nodes.

Some studies suggest that TiterMax® is superior to Freund's adjuvants with some protein antigens, particularly in rabbits and mice. Compared with FCA, TiterMax® can be used in smaller quantities for initial injection, which minimizes the inflammatory reaction at the injection site(s), and less frequent booster injections are required.

Recommended routes of administration and injection:

Species	Injection Route	Total Injections	Volume per Injection
Mouse	IM	2	0.02 ml
	SubQ	1	0.04 ml
Rat	IM	2	0.05 ml
Guinea Pig	IM	2	0.05 ml
	SubQ	4	0.05 ml
Rabbit	IM	2	0.04 ml
	SubQ	4	0.10 ml
	ID	10	0.04 ml

4. Montanide ISA Adjuvants®

A group of oil/surfactant-based adjuvants where surfactants are combined with a non-metabolizable and/or metabolizable oil. Components undergo quality control to protect against contaminants yielding excessive inflammation. Performance of the ISA 50 and ISA 70 were found to be similar to Freund's incomplete adjuvant for antibody production, but with less inflammatory response. The surfactant in ISA 50 and ISA 70 is a major component of the Freund's adjuvant surfactant, mannide oleate.

5. Syntex Adjuvant Formulation (SAF)®

Recently developed as a Freund's complete adjuvant alternative, this pre-formed oil-in-water emulsion is stabilized by Tween 80 and pluronic polyoxyethylene/polyoxypropylene block copolymer L121. SAF uses a low toxicity, high stimulatory derivative of muramyl dipeptide, thymopentin (Thymopentin), and the metabolizable oil squalene.

Antigen Preparation

Antigen preparations should be free of extraneous microbial contamination. Millipore filtration

of the antigen prior to mixing with the adjuvant is recommended. The presence of byproducts, such as polyacrylamide gel, should be avoided because of their inflammatory or toxic properties. Avoid pH extremes, particulate matter, and contamination with chemicals such as SDS, urea, acetic acid, or other solvents or potentially toxic agents. Special precautions may be necessary if the antigen is a viable microbe.

Antigen-Adjuvant Emulsions

If FCA is used, the mycobacteria should first be resuspended by vortexing or shaking. One part or less of Freund's adjuvant to one part antigen (v/v) is recommended. Two sterile luer-lock syringes, one containing Freund's adjuvant and one containing the antigen, preferably in sterile saline, are used for these purposes. Glass syringes or plastic disposable syringes without rubber plungers are preferred as the oil reacts with the rubber plunger on plastic disposable syringes. The antigen solution is injected into the adjuvant through a 3-way stopcock or mixing cannula, and the emulsion is prepared by pushing the solution back and forth between the syringes for several minutes. An emulsion is properly prepared when it becomes thick, is difficult to inject back and forth through the cannula, and will not separate on standing; a droplet placed into a saline solution should not disperse. Emulsification is enhanced by using cold (4°C) adjuvant. Failure of the preparation to emulsify may be due to antigen contamination with SDS or organic solvents. For antigen-adjuvant preparations using adjuvants other than Freund's, manufacturer's instructions must be followed.