Preface

Vaccine Adjuvants: Preparation Methods and Research Protocols was developed to promote the optimal use of immunological adjuvants in preclinical studies. The book's primary focus is on the use of adjuvants in vaccination studies in order to induce potent immune responses against either antigens derived from infectious organisms or cancer-associated antigens. In general, our work should be of interest and significant value to researchers who need to induce potent immune responses against their respective antigens, including those involved in the development of vaccines for infectious diseases, cancers, fertility regulation, and autoimmune disorders. In addition, the book should also be valuable for those involved in the selective manipulation of the immune response, including virologists, bacteriologists, parasitologists, and immunologists. Each chapter describes a single approach, but includes suggestions as to why the specific adjuvant might be preferred for a given antigen, depending on which type of immune response is desired. Alternative adjuvant approaches are presented in detail in such a manner as to permit researchers to choose those most efficacious for their specific indications.

The main focus of *Vaccine Adjuvants: Preparation Methods and Research Protocols* is on the use of adjuvants in vaccines, since it is already clear that the new generation of vaccines—based on recombinant proteins, synthetic peptides, or DNA— will require adjuvants for optimal efficacy. Each chapter describes in detail the preparation and characterization of an adjuvant or an adjuvant formulation, including recommended protocols for its in vivo evaluation in preclinical studies. Whenever possible, detailed adjuvant preparation and characterization methods are presented in each chapter by the individuals who originally invented or developed the approaches, including specific examples for guidance. The preparation methods described range from simple mixing of an antigen with a preformed adjuvant, to a complex formulation process requiring the antigen to be physically associated within, or entrapped within, an adjuvant formulation. In all chapters, practical advice and guidance is provided to allow optimal adjuvant preparation. Each chapter also includes detailed notes, which highlight important practical points, and warns against potential pitfalls and problems. Following adjuvant preparation, steps are of-

ten necessary to characterize the vaccine/adjuvant formulation, to ensure that the preparation was successful, and to allow quantitative estimation of important parameters, including antigen incorporation or association, and antigen integrity. Whenever necessary, these steps are described in detail, with full practical guidance and examples of the expected results. In addition, an overview chapter describing the evaluation of novel adjuvants in clinical studies is included. Also included is a chapter describing recommended guidelines to evaluate the safety of novel adjuvants and adjuvant formulations.

Derek T. O'Hagan

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Harmful and Beneficial Activities of Immunological Adjuvants

Duncan E. S. Stewart-Tull

1. Introduction

There are no officially recognized regulations for the design and toxicity testing of adjuvants or adjuvant formulations; the former are also referred to as immunomodulators and immunopotentiators. At the "Immunological Adjuvants and Vaccines" meeting held in Greece in 1988, however, immunoadjuvant researchers discussed experimental toxicological tests that might be used to monitor new immunomodulators ***(1)***. The usefulness of these tests for the range of immunomodulators and adjuvant formulations was examined over a 2-yr period and subsequently, at the next NATO meeting in 1990, further recommendations were made ***(2)***. Although as yet, no final agreement has been reached and a variety of tests are still in use.

At the "Harmonization of regulatory procedures for Veterinary Biologicals" meeting in Ploufragan, Brittany, a number of scientists and administrators from the regulatory bodies of the United States of America and the European Community indicated that "adjuvants are too reactive for inclusion in vaccines" ***(3)***. This viewpoint was challenged before discussions about new harmonized quality assurance, and quality control regulations were instigated, otherwise the development and release of new vaccines would be delayed. In addition, there has been a degree of lobbying against one or another immunomodulator in order to substantiate the efficacy claims for a particular substance or adjuvant formulation. Eventually, agreement will be reached among adjuvant researchers, vaccine producers, and licencing authorities in regard to the most suitable biological and toxicity tests for new immunomodulators or adjuvant

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formulations, but until then it would seem profitable to monitor the tests which are currently being appraised by adjuvant researchers. It should also be stressed that the battery of recommended tests may include some that would be specific for a particular group of immunomodulators; for example, the capacity of aluminum compounds to adsorb the vaccine antigen is an essential test.

In addition, comments expressed about the unsuitable and inadmissible use of adjuvants in vaccines contradict the *status quo*. Whole-cell vaccines of Gram-negative bacteria contain peptidoglycan and lipopolysaccharide (LPS), long established as efficient immunomodulators. Some 2.5 billion doses of BCG vaccine have been administered in the fight against tuberculosis and each dose contains approximately 3.0–5.0 mg of peptidoglycan, a good adjuvant. A course of three injections of the whole-cell pertussis vaccine would contain between 6.5–50.0 mg peptidoglycan and 6.0–35.0 mg of LPS ***(4)***. Would the critics really expect all whole-cell vaccines to be withdrawn irrespective of their efficacy because they contained an adjuvant? I suggest the answer would be No. Robbins ***(5)*** expressed the opinion that "any toxicity that we accept is a compromise." Such a compromise must become an accepted principle in the search for adjuvants suitable for use in human vaccines because one of their functions is to stimulate antigen-presenting cells (particularly dendritic cells and macrophages). It is doubtful whether this stimulation would occur if adjuvants were completely innocuous substances, lacking any cellular aggravation activity. This does not mean that an adjuvant should be designed to include low-level toxicity. The compromise adjuvant researchers seek is the design of adjuvant molecules with the insertion, substitution, or removal of chemical groups which will increase their immunopotentiating activity, while at the same time, reducing significantly their tissue reactivity, hence the array of MDP derivatives that the chemists have produced—of which very few have been shown to be acceptable.

An adjuvant or immunopotentiator should stimulate high antibody titres, but in the process it should have low toxicity and not induce harmful side effects after injection into either animals or human beings. The main function of an adjuvant is to stimulate antibody production against a range of antigens, even with small quantities of poorly antigenic substances, preferably in a small number of injections or administrations. These objectives would seem to be easy to achieve, but after much research the perfect adjuvant is still elusive to vaccinologists. Indeed, it is unlikely that such a universal adjuvant will be found as different vaccines will require different adjuvants. More than 100 adjuvants have been described ***(6)***, but many of these would not be routinely included in vaccines because of a variety of reasons, e.g., cost and the complex preparation of the injection mixture, and many are too reactive in toxicology tests.

The design of an adjuvant depends to a certain extent on the arm of the immune response one is attempting to enhance. But a high priority should be placed on the overall welfare of and possible stress caused to animals during the evaluation of a new prototype formulation to be included in the injection mixture. Furthermore, the nature of the adjuvant should be reflected in the route, protocols of injection, and the type of vaccine, e.g., "routine" preventative vaccines vs cancer vaccines.

2. Materials

2.1. Hemolysis Test

1. A New Zealand white (NZW) rabbit or equivalent.
2. Heparinized bleeding set, e.g., Vacutainer (Becton and Dickinson, Rutherford, NJ).
3. Sodium chloride (0.85% (w/v); Sigma, St. Louis, MO).
4. Saponin (Sigma).
5. Cyanmethemoglobin standard, Merck Ltd., Darmstadt-Mannheim, Germany, Cyanmethemoglobin standard for photometric determinations of hemoglobin, 1.0 mL in 200 mL distilled water. Cat. 36210P or Hemoglobin standard, Sigma, Cat. 525-A.
6. Hematocrit and bench centrifuge.
7. Phosphate buffer pH 7.5.
8. Drabkin's reagent: (Merck): this reagent is stable if stored in the dark.

Potassium hexacyanoferrate	200 mg
Potassium cyanide	50 mg
Potassium dihydrogen orthophosphate	140 mg
Colorless nonionic surfactant in distilled water, e.g., Nonidet P40	1.0 mL
Distilled water to 1 L	

9. Matburn blood cell suspension mixer (Matburn Surgical Equipment Ltd, Portsmouth, U.K.).
10. Spectrophotometer capable of reading from A540 nm to A592 nm with 1.0 cm lightpath cuvets.
11. Microhematocrit tubes (Volac; J. Poulten Ltd., Barking, Essex, U.K.).

2.2. Rabbit Pyrogenicity Test

1. NZW rabbits, 2–3 kg.
2. Pyrogen-free glassware, needles, and syringes, as well as pyrogen-free physiological saline.
3. Rectal thermometer.

2.3. Limulus Lysate Assay

1. Commercial *Limulus polyphemus* amoebocyte lysate (LAL) test kits, e.g., either Sigma E-Toxate, multiple test vial system sensitive to 0.005–0.5 endotoxin units (EU)/mL Cat. 210-2, or M.A. Bioproducts' LAL test system with a reagent which

will detect 0.25 ng/mL of FDA reference endotoxin with the addition of 1.0 ng/mL of *Escherichia coli* O 111:B4. Sigma, Cat. 50-505U.
2. Pyrogen-free water for making dilutions of the standard, e.g., Endotoxin-free water Sigma Cat. 210-7.
3. All glassware must be pyrogen-free, autoclave at 121°C for 1 h followed by 3 h in the drying oven at 175°C or use commercially available pyrogen-free disposables.

2.4. Toxicity Assays

2.4.1. Cytoxicity Assay

1. Tissue-culture flasks (80 cm^2; Falcon, Los Angeles, CA) and tissue-culture plates, 24-well, Greiner.
2. Tissue-culture cell lines (European Collection of Cell cultures).
3. Minimal essential medium, Eagles' (Cat. 32360-026), fetal bovine serum (Cat. 10084-069), L-glutamine 200 m*M* (Cat. 25030-024), Fungizone (Cat. 152-018), penicillin/streptomycin (Cat. 15140-114), trypsin-EDTA (x1, Cat. 45300-019), all from Gibco, Gaithersburg, MD.
4. MEM nonessential amino acids (100x; Cat. 11140-035, Gibco) and Insulin-transferrin-selenium-G.
5. Supplement (Cat. 41400-045; Gibco) are required for the CaCO-2 cell-line growth.
6. Incubator (37°C and 5%CO_2).
7. Phosphate buffered physiological saline (BDH Merck).
8. Dialysis tubing (Medicell Int. Ltd.).
9. Millipore filters 0.22-µm pore size.
10. MTT, (3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide, thiazolyl blue) Sigma Cat. M2128).
11. DMSO (dimethyl sulfoxide; Sigma, Cat. D5879).
12. Triton-X 100 (t-octylphenoxy polyethoxy-ethanol) Sigma Cat. T9284.

2.4.2. Single and Multiple Dose and Systemic Toxicity Tests

1. Adjuvant in physiological saline as polar solvent.
2. Adjuvant in sesame oil (Sigma) as nonpolar solvent.
3. Needles and syringes.
4. Two mammalian species, e.g., rabbits and mice.

2.5. Induction of Allergy to Food Proteins

1. Ovalbumin and lactalbumin (Sigma).
2. Gelatin capsules.
3. Ascorbic acid.
4. Physiological saline.
5. 1.0 mL syringes.
6. Evans blue dye.
7. ELISA plates, coating buffer, washing buffer, peroxidase-labeled secondary antibody, substrate and reader.

3. Methods

3.1. Hemolysis Test

At very low concentrations, adjuvants should not be hemolytic. This is particularly relevant for the crude, triterpenoid plant saponins, which reportedly destroy erythrocytes if injected intravenously, although this effect may be owing to contaminatory substances ***(7)***. The immune stimulating complexes (ISCOMs; see Chapter 14) contain a saponin, which is also used to produce the positive 100% lysis of erythrocytes in the method below. It is often stated that such complexes cannot be used in vaccines because of the hemolytic properties of this component, however, no such drastic hemolysis has been detected in the numerous successful studies with ISCOM vaccines in animals. Nevertheless, it is wise to check for the hemolytic activity of a new adjuvant compound either separately, or chemically conjugated to antigen, or in combination with an antigen in the final vaccine formulation. It is obviously very important to check new adjuvant preparations for hemolytic activity against erythrocytes from different sources and species, for example, if the vaccine is to be used in sheep, then a sheep hemolysis assay would be essential.

This procedure is based on the British Standard 5736: Part 11: ***(8)***.

1. Bleed (10.0 mL) a NZW rabbit from the ear vein into a heparinized tube. Centrifuge at 2000*g* for 10 min and wash the cells twice in physiological saline. Resuspend the packed cells in a small quantity of saline and determine the percentage of erythrocytes in the suspension by the haematocrit method. Dilute an aliquot to 2.0% for use.
2. Tests and controls are set up as shown :

	Test	Test	Positive control	Negative control
Tube	1	2	3	4
Heparinized blood	2.0 mL	2.0 mL	2.0 mL	2.0 mL
Adjuvant in sterile physiological saline	0.1 mL	0.1 mL	—	—
Sterile physiological saline	—	—	0.1 mL	0.1 mL
White saponin 125 mg in sterile physiological saline	—	—	0.1 mL	—

3. Incubate the tubes at 37°C ± 2°C on a Matburn cell suspension mixer for 4 h. Centrifuge the tubes at 2000*g* for 10 min and determine the percentage hemolysis in each tube. This can be done by measurement of the A_{540nm} or more accurately by measurement of the hemoglobin concentration in the supernatant fluid by the cyanmethemoglobin conversion method.
4. Measurement of the total hemoglobin in blood as cyanmethemoglobin
   a. Spectrophotometric measurement at A_{540nm} in 1.0 cm-pathway cuvets. Add 20 µL of the positive or negative controls or test adjuvant samples to 4.0 mL

Drabkin's reagent and mix. Measure the A_{540nm} of these mixtures and the reference cyanmethemoglobin solution against a distilled water blank.

The hemoglobin concentration is calculated from:

$$\text{Hemoglobin concentration (g L}^{-1}) = \frac{A_{540} \text{ of test blood}}{A_{540} \text{ of reference cyanmethemoglobin}} \times \frac{200C}{1000}$$

where C = concentration of cyanmethemoglobin in the reference solution, expressed in mg/L.

b. Lyse freshly obtained heparinized blood from the NZW rabbit with distilled water. Measure the hemoglobin concentration by the cyanmethemoglobin conversion method described above. Prepare a series of reference hemoglobin solutions from 0.05 g/L to 0.75 g/L by diluting the lysed blood stock solution with a phosphate buffer, pH 7.5. Measure the A_{560nm}, A_{576nm}, and A_{592nm} against a distilled water blank with 1.0-cm path length. Measure test samples at the same wavelengths.
   i. Calculate the function for each reference hemoglobin solution from $2y - (x + z)$

      i.e., $2(A_{576nm}$ value$) - (A_{560nm}$ value $+ A_{592nm}$ value$)$

   ii. Plot the values from $2y - (x + y)$ against the hemoglobin concentration.
   iii. Repeat with each of the test samples and read the hemoglobin concentrations off the graph. Express the results in g/L and calculate the percentage hemolysis by comparison with the reference.

Calculate and record the percentage hemolysis by:

$$\% \text{ hemolysis} = \frac{\text{Test hemoglobin concentration}}{\text{Total hemoglobin concentration in positive control}} \times 100$$

Tube	1	2	3	4
% Hemolysis				

The test is invalid if either the % hemolysis for the negative control is >5.0 or the % hemolysis for the positive control is <1.0 or >20.0. Hemolytic activity in the final adjuvanted vaccine formulation would preclude its general use.

3.2. Pyrogenicity Tests

3.2.1. Rabbit Pyrogenicity Test (9,10)

The pyrogen test is designed to limit to an acceptable level the risks of febrile reactions that might occur after the injection of a product containing adjuvant. This method is a modification of the British Standard 5736: Part 5: ***(10)***. All glassware, solutions for washing or rinsing apparatus and diluents

must be pyrogen-free, either heat at 250°C for not less than 30 min or use Toxaclean (Sigma) in washing solutions as applicable before use.

1. The adjuvant should be dissolved/suspended in pyrogen-free physiological saline and warmed to 38.5 ± 1.0°C.
2. Rabbits should be housed at a temperature of 20–23°C. Before use in a pyrogen test the rabbits should be sham-tested with an injection of 10.0 mL/kg physiological saline into a marginal ear vein 7 d before use. Withhold food from the rabbits the night before any test and until the completion of the test. Weigh the rabbits and record their temperature, with an accurate thermocouple or thermistor probe thermometer (+ 0.1°C) inserted into the rectum 50–75 mm, at 30-min intervals, beginning 90 min before injection of the saline solution, and at 30 min intervals for 3 h after injection. Exclude rabbits before the injection of the test adjuvant solution/suspension if:
   a. the difference between any two consecutive readings is >0.2°C.
   b. the range of temperature readings exceeds 0.4°C.
   c. the initial temperature is not in the range 38.0–39.8°C.
3. This procedure is repeated with each dose of adjuvant. Although it is more time-consuming, from the point of view of animal welfare it is reasonable to proceed with one animal at a time for each dose of adjuvant. Inject 10 ml/kg of the adjuvant preparation into the marginal vein of one ear of rabbit 1 within a period of 4.0 min. Record the temperature at 30-min intervals for 3 h after injection. If rabbit 1 passes the test, repeat with rabbits 2 and 3.
4. The adjuvant solution/suspension is deemed to be nonpyrogenic if either no rabbit showed an increase of 0.6°C above its respective control temperature before the injection of the adjuvant, or the sum of the three individual maximum temperature increases of rabbits 1–3 does not exceed 1.4°C.
5. If neither of the above criteria are met, it has been suggested that the test should be repeated with 5 other rabbits, although if there is excessive fever it may be deemed politic to reject the new adjuvant and save the needless use of animals. If the test is carried out, the adjuvant solution/ suspension is deemed to be nonpyrogenic if either 3 of the 8 do not show an average increase of 0.6°C, or the sum of the eight individual maximum temperature increases of the rabbits 1–8 does not exceed 3.7°C.

3.2.2. Limulus polyphemus Amoebocyte Lysate (LAL) Assay of Diluents and Adjuvants

The LAL assay for endotoxin, reviewed by McCartney and Wardlaw ***(11)***, is very sensitive and can detect as little as 0.1 ng/mL endotoxin activity. Nonspecificity may be a result of contaminatory endotoxin, so it is very important to ensure that all equipment and glassware used in the assay are endotoxin-free. The LAL assay was adopted by the U.S. Food and Drug Administration ***(12)*** for routine testing of biological products and medical devices, but as yet has not been accepted as an alternative for the rabbit pyrogenicity test by the

European Pharmacopoeia. One way forward would be to use the rabbit pyrogenicity test and the LAL assays in tandem during the development phase of a new vaccine containing an adjuvant and show negative results. Subsequently, it might be possible to persuade authorities to accept the LAL assay for routine batch-testing during manufacture of a new vaccine (*see* **Note 1**).

1. The precise procedure for this assay, which should include a series of control endotoxin dilutions from 4–0.005 ng/mL, is described by the manufacturer, but it is important that a minimum of 4 tests are set up for each sample.
2. A positive result is seen where a solid gel is formed in the test tubes and a negative result is where there is no solidification of the clottable protein extracted from the circulating amoebocytes of *L. polyphemus*.

3.3. Measurement of the Toxicity of Adjuvants

3.3.1. Cytotoxicity Assay in Cultured Monolayers of Human or Animal Cell Lines

This type of assay has the great merit of reducing the number of animals which must be used to comply with standardized toxicity tests. The MTT assay ***(13)*** was adapted for determining cell survival and proliferation by a number of workers ***(14–16)*** with different cell types and toxins.The assay compares favorably with other similar systems ***(17)***, is less time-consuming and objective than microscopic examination of cells, and eliminates the risks associated with assays involving radioisotope release. As examples, the protocols for three different cell lines have been described, but these are not exclusive.

1. Tissue culture cells and growth conditions. The cells should be checked for the absence of virus contamination. This is confirmed by electron microscopy and for the presence of contaminating mycoplasmas by a specific staining technique before use. Human colon adenocarcinoma (CaCO-2) cells are grown in Eagle's MEM medium with Earle's salts and 25 m*M* HEPES (Gibco) in 80-cm^2 tissue-culture flasks (Falcon). Non-essential amino acids (1.0% w/v), glutamine (2 mmol/L), 100 µg/mL penicillin/streptomycin, 1.0% v/v of growth promoter, insulin-transferrin-selenium, and 10% v/v fetal calf serum are added and the cells are incubated at 37°C in 5.0% carbon dioxide atmosphere. The cells are routinely split 1 in 5 by rinsing with 5.0 mL sterile PBS followed by 2.0 mL of 0.25% w/v trypsin/EDTA. A confluent monolayer of CaCO-2 cells in an 80.0-cm^2 flask is obtained usually within 5–7 d, with regular changes of medium. A cell suspension of cells prepared by trypsinization is used to inoculate wells in a 24-well plate.

   African green monkey kidney (VERO) or HeLa cells, are grown in Eagle's MEM medium (Gibco) containing 100 µg/mL penicillin/streptomycin and 5.0% v/v fetal calf serum. The cells are incubated at 37°C in an atmosphere of 5% CO_2. VERO and HeLa cells are routinely split in the same manner as the CaCO-2 cells.

2. HeLa or VERO cells are harvested with trypsin/EDTA and resuspended in growth medium to a density of 5×10^4 cells/mL. Each well of a flat-bottomed 24-well plate is loaded with 200 µL of the cell suspension and incubated at 37°C overnight. The growth medium (100 µL) is discarded and 100 µL of twofold dilutions of the adjuvant in tissue-culture medium is added to each well: duplicate wells of each dilution are set up. Cells with PBS only or 1.0% v/v Triton X-100 in PBS serve as the 100% and 0% live controls, respectively. After 24 h incubation at 37°C, 20 µL of MTT solution (5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (Sigma) in PBS, filter-sterilized are added to each well and incubation continued for 4 h. After emptying the wells the resultant formazan crystals are solubilized by the addition to each well of 100 µL 0.4 mol/L HCl in dimethyl sulphoxide v/v (DMSO; Sigma) and the absorbance measured at 540 nm in the Anthos 2001 plate reader. The percentage of cell deaths, adopted to take account of the variable growth of the cells, was calculated with the formula:

$$1 - \frac{[A_{540nm}\ \text{test} - A_{540nm}\text{Triton X +ve Control}]}{[A_{540nm}\ \text{PBS –ve control} - A_{540nm}\text{Triton X +ve Control}]} \times 100$$

3.3.2. Intracutaneous Toxicity Test

This procedure assesses any skin irritation at the site of injection and is based on the British Standard 5736: Part 7 ***(18)***. This test may be relevant with some adjuvants, which are to be included in vaccines injected by the intradermal or subcutaneous routes. For example, Kensil et al. ***(19)*** fractionated Quil A from *Quillaja saponaria*, the South American soap tree, and one preparation QS-7 was nontoxic at an intradermal dose of 500 µg whereas QS-18 was lethal at a dose of 25.0 µg. The latter preparation would not be acceptable either as a saponin adjuvant nor as part of any other adjuvant formulation. This type of test may involve single-dose toxicity or repeated dose toxicity reactions of the adjuvant formulation. The test is usually done by intraperitoneal (ip) or subcutaneous (sc) injection into two mammalian species, but the number of animals in the test groups is being questioned and some authorities may well invoke the 3 Rs, namely replacement, reduction, and refinement for the sake of animal welfare.

3.3.2.1. Single-Dose Toxicity Test

This is a qualitative and quantitative study of the possible toxic reactions, which may result from a single administration of the active substance, in this instance the adjuvant, in an acute toxicity test. As with other tests, it is important to use the adjuvant alone or in the injectable form. The test should be done in two mammalian species, with equal numbers of males and females, if a vet-

erinary product the intended animal should be included. There should be at least two routes of administration, for example by ip and sc injection. After injection, the animals should be examined at regular intervals, at least three times daily, for not less than 7 d, and any animal with obvious signs of ill health or in a moribund state should be killed.

3.3.2.2. Repeated-Dose Toxicity Test

This is intended to monitor the effect of repeated administration of vaccines containing an adjuvant component. It is the responsibility of the investigator to give valid reasons for the extent and duration of the trials and the dosages chosen. However, the maximum dose should be selected so as to indicate potential harmful effects and lower doses will enable the animal's tolerance to the new adjuvant. The repeated-dose toxicity test should be done in two mammalian species (1 nonrodent).

Animals that are mentioned in European rules governing medicinal products ***(28)*** for use in these two tests are: mouse (*Mus musculus*), rat (*Rattus norvegicus*), guinea-pig (*Cavia porcellus*), golden hamster (*Mesocricetus auratus*), rabbit (*Oryctolagus cuniculus*), nonhuman primates, dog (*Canis familiaris*), cat (*Felis catus*), quail (*Coturnix coturnix*).

Evaluation of the adjuvant may be done by a variety of means: monitoring the behavior and weight gain of the animals, hematological, and physiological tests. If an animal dies, an autopsy and histological examination of tissues, including the sites of injection, should be done.

1. The adjuvant is dissolved/suspended in either a polar solvent, sterile physiological saline, or a nonpolar solvent, sesame oil (Ph. Eur), usually heated at 180°C for 60 min.
2. Preparation of animals. The fur is clipped on the back of each animal, e.g., rabbits, before injection.
3. Rabbit 1. (a) inject four sites on the left-hand side of the body with 0.1 mL of the test mixture subcutaneously or (b) inject four sites on the right-hand side with 0.01 mL intradermally with: (i) adjuvant in polar solvent; (ii) polar solvent alone; (iii) adjuvant in nonpolar solvent; iv) nonpolar solvent alone. The injection sites are examined for 5 d and the size of any skin reactions measured with precision calipers, for example, Mecanic in nylon-asbestos (Camlab, U.K.).
4. Rabbits 2, 3, and 4 should only be injected if the rabbit 1 test is negative.
5. The injection sites are examined for erythema (redness at the site of injection), eschar (scab formation at the site of injection), or edema (swelling at the injection site) (**Table 1**).

3.3.3. Systemic Toxicity Test

The aim of this procedure is to measure undesirable effect(s) at sites distant from the injection site, which may become apparent after the administration of

Table 1
Classification System for Skin Reactions

Reaction	Numerical grading
Erythema and eschar formation:	
No erythema	0
Very slight erythema	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet-redness) to slight eschar formation	4
Edema formation:	
No edema	0
Very slight edema	1
Well-defined edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond exposure area)	4

NOTE: Other adverse changes at the skin sites should be recorded and reported.

the adjuvant alone or the adjuvant formulation. The adjuvant is injected intraperitoneally in polar or nonpolar diluents or intravenously in a nonpolar solvent with appropriate controls. These are tests which the regulatory bodies may require with groups of five mice, but there may be moves to reduce the number of animals to be tested. It is feasible that these tests could eventually be phased out when there are sufficient experimental results accumulated to allow the validation of alternative toxicity tests. The method is based on British Standard 5736: Part 3 ***(20)***.

1. Groups of five weanling mice, 3–4-wk old are weighed and injected, either intraperitoneally with 0.5-mL volumes of graded doses of the adjuvant mixtures:
   Group 1. Adjuvant in sesame oil
   Group 2. Sesame oil alone
   Group 3. Adjuvant in physiological saline
   Group 4. Physiological saline alone
   Or, intravenously with 1.0 mL of:
   Group 5. Adjuvant in physiological saline
   Group 6. Physiological saline alone
2. The animals are observed for 14 d, frequently during the 4 h immediately following injection and at least three times a day thereafter.
3. Record any visible signs of reaction after injection of the adjuvant preparation, for example, time of onset after injection, their duration, and intensity. Weigh all

the animals daily for 7 d, refer to the "weight-gain test" below, and kill all surviving animals and record the appearance of the animals. Record any deaths if they occur on the respective day after injection. Postmortem all animals at the end of the experiment and record the appearance of organs and the histological examination of tissues of interest including: heart, lungs, gastrointestinal tract, liver, spleen, kidneys, and gonads. The report could be produced in the format shown in **Table 2**.

3.3.4. Mouse Weight-Gain Test

The 7-d mouse weight-gain test is still a standard and reliable method. After injection of a substance containing endotoxin, the animal may show a decrease in weight during 24 h if endotoxin is present ***(21)***. If this is followed by a steady increase in the animal's weight over 7 d, it is assumed that the product is acceptable. If, on the other hand, the product is highly toxic the animal may steadily continue to lose weight or in extreme cases become moribund and is killed. The tests in **Subheading 3.3.2.** and **3.3.3.** may provide evidence of unacceptable levels of toxicity in which case it may be unnecessary to proceed with a weight-gain test as the adjuvant is probably too reactive.

The protocols for these laboratory assays should not be regarded as alternatives to statutory tests required for licensed medical or veterinary products, however, they will show whether financial investment in a new immunopotentiator or adjuvant formulation is warranted. Invariably, if a new adjuvant formulation gives a positive reaction in one of the tests described above, it is highly unlikely that the preparation will be suitable for routine vaccine use (*see* **Note 2**).

3.4. Induction of Allergy to Nonvaccine or Food Proteins

This is a particularly important test when examining the suitability of an adjuvant for inclusion in an oral vaccine, as there could be a reaction to food proteins ***(23)***. With the interest in the oral route as a means of stimulating mucosal immunity, there is a possibility that an adjuvant could induce an allergic response to dietary proteins. In this study, both lactalbumin and gluten failed to elicit an IgE response in the presence of the original Freund's Complete or Incomplete Adjuvants (FCA or FIA) in HAM1/CR mice or Dunkin Hartley guinea pigs. On the other hand, the guinea pigs showed increased IgE production after oral administration of ovalbumin or soy bean protein, both unusual proteins in their normal pellet diet. Such tests are valid only if all of the previous toxicity tests are negative.

1. Groups of mice or guinea pigs are fed freely with moistened ovalbumin or lactalbumin as the main food supply for 24 h. This does not affect their normal weight gain or health. Subsequently, the mice are dosed orally with 0.2 mL of the

Table 2
Style of Report for the Toxicity Tests

	Mouse groups					
	1	2	3	4	5	6
Weight (g) at the start of the test						
Weight (g) at the end of the test						
Difference in weight (g)						
Deaths						
Autopsy report						
Histology report						
Assessment. P = Pass; F = Fail						

adjuvanted formulation or with physiological saline, as the control, containing 2.0 mg of the test protein. The dose in guinea pigs is 0.3 mL of adjuvanted formulation, administered in a gelatin capsule, whereas the saline is delivered from a syringe without a needle at the back of the oral cavity.

2. All animals are fed on the protein diet for a further 24 h after adjuvant dosing and then returned to their normal pellet diet and water. The guinea pigs are also given ascorbic acid to prevent vitamin C deficiency. All animals were bled out on day 21.
3. Passive cutaneous anaphylaxis (PCA) reactions are measured in hairless mice, *hrhr*, injected intradermally with 0.05 mL of serum diluted 1 in 2 at four sites on the dorsal surface. For IgG PCA tests, the sera are heated for 2 h at 56°C to inactivate IgE. After 2 h for IgG and 48 h for IgE, 1.0 mg of the respective protein in 0.2 mL saline containing 0.5% (w/v) Evans Blue dye is injected into the caudal vein, and after 30 min the areas of blueing on the skin are measured. The hair is clipped from the dorsal surface of the guinea pig, injected with 0.1 mL of the serum and after 4 h for IgG and 12 d for IgE injected with 0.1 mL saline containing 1.0% dye and 1.0 mg of the respective protein. The zones of blueing are measured after 2 h, intensively staining zones of >0.5 cm^2 are indicative of a positive reaction, although the positive zones appear more diffuse with the IgE response in the guinea pig.
4. The sera may also be examined for the presence of antibodies by a standard ELISA.

3.5. Standard Adjuvants and Antigens, Routes, and Volumes of Injection Mixtures for Use with New Adjuvant Formulations in Tests to Measure the Stimulation of Humoral and Cell-Mediated Responses

3.5.1. Standard Adjuvants

Those recommended were Alhydrogel and the FCA produced by the Statens Serum Institute, Copenhagen ***(1)***. A suitable alternative for the latter is a "Non-

Ulcerative Freund's Complete Adjuvant (NUFCA)" which contains BCG vaccine BP, BNF intradermal (*see* **Note 3**). The BCG vaccine for sc injection should not be used as this will cause local ulceration. The id BCG vaccine is reconstituted according to the manufacturer's instructions and 0.1 mL is added to 0.9 mL of the aqueous phase-containing antigen. Note that this is a major difference between FCA and NUFCA as the *Mycobacterium tuberculosis* in FCA was suspended in the oil phase. The aqueous phase is emulsified with the oil phase before use. The manufacturers indicate that this NUFCA can be administered by id, im, or sc routes and agree with the WHO ***(22)*** that the im route produces fewer adverse reactions and creates a longer-lived slow-release depot which tends to provide a better immune response.

3.5.2. Standard Antigens

For the comparative biological testing of immunomodulators, the antigens chosen were ovalbumin (Ovalbumin,grade V crystallized and lyophilized, Sigma), and influenza H3N2 type A hemagglutinin ***(1)***, however, it was pointed out that the latter antigen is an unsuitable standard for guinea pigs ***(2)*** (*see* **Note 5**).

3.5.3. Animals for Standard Antibody Production Tests

The guinea pig was the animal of choice for biological tests. In regard to mice, the influence of the animal's genetic background and MHC haplotype must also be considered. For this reason, animals with either similar genetic background and variable H-2 haplotype (e.g., C3H H-2^k and C3H.B10 H-2^b) or variable genetic background and similar H-2 haplotype (e.g., Balb/c H-2^d and DBA/c H-2^d) should be included in comparative tests.

3.5.4. Route of Injection

In most instances, researchers have their own preferences in regard to the site of injection of an adjuvant-formulated, experimental vaccine, however, consideration should be given to whether the vaccine is for human or veterinary use. It is doubtful whether patients would be willing to accept ip or iv injections as a routine vaccination procedure. Consequently, it is advisable to give the injections either subcutaneously or intramuscularly. Similarly, in no circumtances should an oil or alum-adjuvanted veterinary vaccine be injected intravenously nor booster injections administered iv or ip as there is a danger of inducing anaphylactic shock in the animals. Intraperitoneal injection of adjuvanted mixtures into some animals may result in decreased weight gain over 7 d. This inflammation may resolve itself after 7 d, but later postmortem

Table 3
Some Recommended Routes and Volumes for Injection Doses

Species	Maximum volume per injection site	Injection Sites: Primary	Injection Sites: Secondary
Mice or hamsters	50 µL	sc; im	sc; im
	200 µL		oral
Guinea pigs or rats	200 µL	sc; im. into one hindlimb	sc; im into one hindlimb
	300 µL		oral
Rabbit	250 µL (if in multiple sites <25 µL/site*)	sc; im. into one thigh muscle; id*	sc; im into one thigh muscle; id*
Large animal	500 µL (if in multiple sites <250 µL/site*)	sc; im into one hindlimb; id*	sc; im into one hindlimb; id*
Chicken	250 µL	sc; im	sc; im

sc: subcutaneous; im: intramuscular.
* If the intradermal (id) multiple injection site schedule is used.

examination may reveal macroscopic evidence of such a response. There may be specific tests where there is a requirement for another route, for example, id injections.

3.5.5. Volume of Injection Mixture

For animals the maximum volumes per site of injection are shown in **Table 3**. These dosages are based on the use of an adjuvanted vaccine which has already been shown to be nontoxic and nonpyrogenic.

3.5.6. Dose of Adjuvant

The upper limit of adjuvant per dose may be dictated by the results obtained in the toxicity and pyrogenicity tests, although it may be preferable for economic reasons to determine a lower dose at which an adjuvant response is obtained. In general however, the weight in the injection mixture should not exceed 25 µg for a mouse and 200 µg for guinea pigs, rats, or rabbits. If the dose is to be spread among multiple injection sites in larger animals, the volume should be not more than 250 µL per site and preferably as little as 25 µL. If a new adjuvant is being developed for veterinary use, it is important that, if possible, the animal species ear-marked for the vaccine is tested during the development phase.

3.6. Discussion

Manufacturers are expected to provide safety data sheets for their products and these will confirm the lack of toxicity of the product (*see* **Note 4**), but the addition of the vaccine candidate antigen may also alter the overall reactivity of the complete vaccine. Therefore, it is unwise to rely completely on manufacturer's specifications alone. Before evaluating the possible toxicity of a new adjuvant formulation, it is assumed that the investigator will have checked for either the innate toxicity of the antigen preparation, e.g., for lipopolysaccharide (endotoxin), or for chemicals used in antigen preparation, e.g., formaldehyde, glutaraldehyde, sodium azide. The inoculation mixture should be prepared aseptically to minimize contamination with endotoxins. In addition, the immunogen itself may have innate adjuvant activity and this should be checked before preparing complex adjuvant formulations. Conjugation of the immunogen to a carrier may impart innate adjuvant activity (*see* **Note 5**).

Because of necessity, many of these tests require the use of living animals to obtain standard values for the various tests, however, some attempts could be made to extrapolate from data in published records of adjuvant research. It does appear that the criteria required for animal vaccines are not applicable to human vaccines. In the Rules Governing Medicinal Products in the European Community (Volume 5) under veterinary medicinal products it states "it is recognized that for veterinary medicinal products a degree of toxicity and hazard for the animal are acceptable, provided that such toxicity has no consequences for man." (*See* **Note 4**.) Although much effort has gone into the development of alternative toxicological tests to recommended animal tests it is important to remember that the mammalian system is a complex series of interrelated physiological reactions, which are impossible to mimic in the test tube. Therefore, it is doubtful whether it will be possible to eliminate completely all use of experimental animals. It is more likely that in vitro tests could be developed to eliminate highly toxic test substances before animal tests must be considered. The debate will continue about the tests required to ensure that adjuvants, (immunopotentiators or immunomodulators) will be safe for use as vaccine additives in human vaccines.

4. Notes

1. Limulus assay. This requires very careful preparation and experience in reading the tests, therefore it is wise to run the tests with more than one person to check any differences from one operator to another. It should be borne in mind that false-positive results can be produced by substances other than endotoxin, lipopolysaccharide of Gram-negative bacteria.

2. Creatine kinase assay. The creatine kinase (CK) assay is measured in the serum of the test animal 3 d after the injection of the adjuvant. The test is not difficult to do as there is a standardized kit available from Sigma, Creatine kinase diagnostic kit, Cat. 520 or 520-C, but there are difficulties in regulating the standard values for each animal species. In our laboratory, a narrow range of CK values was not obtained with the guinea pig and one suggestion was that pain following injection or the mere handling of timid animals could lead to elevation of their normal CK levels for a period of time.
3. Freund's Complete Adjuvant. It is now time to commit to history FCA. Few laboratories are in a position to make the original FCA because it was formulated with heat-killed, whole cells of *Mycobacterium tuberculosis* in a mineral oil manufactured before 1969, the antigen in the aqueous phase and both of these emulsified with mannide monooleate ***(24)***. Today, adjuvant immunologists retain the use of FCA manufactured by the State Serum Institute in Copenhagen as a "gold" standard against which new adjuvants are compared. There may also be specific examples where FCA is required to stimulate a cell-mediated immune response but in general it is unnecessary to produce routine antisera with this material.

   There are some modern formulations that can achieve the same effect. A ready-to-use preparation at a ratio of 70 parts Montanide ISA 720 to 30 parts aqueous phase-containing antigen did not cause adverse reactions in human volunteers (Montanide ISA 720. Seppic, Paris, France). Similarly, NUFCA antigen in saline plus the BCG vaccine (BCG vaccine prepared by Evans Medical for intradermal use from John Bell & Croydon, 52-54 Wigmore Street, London, W1H 0AU, UK) in oil that conforms to the United States and EC Pharmacopoeias and comply with FDA regulations 21 CFR 172.878 and 178.3620a (Guildhay Ltd., 6, Riverside Business Centre, Walnut Tree Close, Guildford, Surrey, GU1 4UG, UK) may work just as well.
4. Mineral oil adjuvants. Reference to adverse effects of mineral oils from the petroleum industry in experiments completed prior to the late 1960s should not be seen as relevant to current experimental results. During earlier studies with FCA and FIA, the mineral oil produced by the acid treatment or oleum method was obtained as an intermediate stage in the refining process with an estimate of the hydrocarbon chain length but with no quality control. Published reports that oil emulsions can never be used in human vaccines ignore the fact that an oil-adjuvanted influenza vaccine was administered to many American servicemen more than 50 years ago. Surveillance of these vaccinees showed no evidence of increased disease states when compared to the incidences in the normal population ***(25)***.

   Since the 1970s, white mineral oil has been produced by the single or double hydrogenation procedure and far superior grades of oil have been obtained. A number of oils and emulsifiers are available now which lack the adverse effects of old crude mineral oils and conform to the requirements of the US and EC

Pharmacopoeias and comply with the FDA regulations 21 CFR 172.878 and 178.3620a. Manufacturers safety data sheets should provide QA data with new oils, e.g., mass spectrometer and gas liquid chromatography analyses,which may be used in adjuvanted vaccine oil formulations ***(26)***. Many adverse reactions attributed to oil-adjuvanted vaccines were caused by the poor quality control of the original oils obtained from the petroleum industry. Unacceptable footpad reactions were obtained with mineral oils containing short-chain hydro-carbons (C_8-C_{12}), but medium-chain length hydrocarbons (C_{16}-C_{18}) showed minimal toxicity. The toxicity test must be relevant to the use of oils in vaccines: a 90-d feeding study does not necessarily equate with either oral administration or a parenteral injection of a vaccine dose containing ~0.5 mL oil ***(24)***.

The induction of adjuvant arthritis test in Lewis rats was mainly required for oils produced prior to 1970 which were commonly used in experimental vaccines ***(27)***. It is doubtful if it is required as a routine test for adjuvants, but may prove useful in some QA/QC procedures for selective adjuvants.

5. Sometimes an antigen alone or conjugated to another molecule will possess innate adjuvanticity ***(2)***, therefore the addition of another separate adjuvant to the new vaccine may lead to a depression of the antibody response. Therefore, it is advisable to test whether the antigen requires the presence of an adjuvant. This can be done by testing the antigen as an adjuvant with the standard antigen ovalbumin. If the antibody response is greater than that obtained with ovalbumin in saline alone, it is reasonable to assume that the antigen has innate adjuvanticity.

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