Immunological Adjuvants

Report of a
WHO Scientific Group

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World Health Organization, Geneva 1976

This report contains the collective views of an international group of experts and does not necessarily represent the decisions or the stated policy of the World Health Organization.

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WHO SCIENTIFIC GROUP ON IMMUNOLOGICAL ADJUVANTS

Geneva, 6–10 October 1975

Members : *

Professor T. Diamantstein, Steglitz Clinic, The Free University, Berlin

Dr M. R. Hilleman, Director, Virus and Cell Biology Research, Merck Institute of Therapeutic Research, West Point, PA, USA

Dr J. H. Humphrey, Division of Immunology, National Institute for Medical Research, London, England (*Chairman*)

Dr P. Lagrange, Hôpital Saint-Joseph, Paris, France

Dr I. Mota, Head, PAHO/WHO Immunology Research and Training Centre, Instituto Butantan, São Paulo, Brazil

Professor O. Westphal, Director, Max Planck Institute for Immunology, Freiburg im Breisgau, Federal Republic of Germany

Professor R. G. White, Department of Bacteriology and Immunology, University of Glasgow, Western Infirmary, Glasgow, Scotland (*Rapporteur*)

Secretariat :

Dr D. Dresser, National Institute for Medical Research, London, England (*Temporary Adviser*)

Dr J. G. Howard, Department of Experimental Immunobiology, The Wellcome Research Laboratories, Beckenham, Kent, England (*Temporary Adviser*)

Dr G. Torrigiani, Acting Chief, Immunology, WHO, Geneva, Switzerland (*Secretary*)

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* Unable to attend : Professor R. M. Fauve, Département de Biologie moléculaire, Institut Pasteur, Paris, France.

IMMUNOLOGICAL ADJUVANTS

Report of a WHO Scientific Group

A WHO Scientific Group on Immunological Adjuvants met in Geneva from 6 to 10 October 1975. The meeting was opened by Dr D. Tejada-de-Rivero, Assistant Director-General, on behalf of the Director-General.

1. INTRODUCTION

Immunization of man and other animals by artificial means is carried out for several distinct purposes. The main aim, whose achievement has already produced enormous benefit to mankind, is prophylactic immunization against infectious diseases so that long-lasting effective immunity results from controlled stimulation of the immune system by administration of a harmless vaccine rather than from uncontrolled stimulation by natural clinical infections. For prophylactic immunization to prevent clinical infection by those microbes or parasites susceptible to inhibition by antibody, it is sufficient that an adequate concentration of antibody should be maintained in the circulation and/or at mucous surfaces, and that a rapid increase in antibody formation should be possible if subclinical infection occurs later. The effects of prophylactic immunization must therefore be long-lasting. For practical and economic reasons, prophylactic immunization needs to be obtained with a minimum number of administrations, and preferably by a single injection employing the least amount of antigen compatible with efficient immunization. When non-living agents are used, some special means, of which the use of adjuvants is the main example, are needed to ensure the fulfilment of this requirement. Adjuvants, by definition, are substances that are incorporated into, or injected simultaneously with, an antigen and that potentiate nonspecifically the ensuing immune responses.

When clinical infection has occurred by organisms that are not susceptible to inhibition by circulating antibody (either by direct interaction or by opsonization and destruction by phagocytes)—for example, when the causative agents are established intracellularly, or extracellularly, as in the case of certain parasites—elimination of the infection requires the participation of specific cell-mediated immunity brought about by lymphocytes. Thus vaccines eliciting mainly or exclusively antibody production may be ineffective, and it is now becoming recog-

nized that effective prophylactic immunization may require immunization procedures aimed at eliciting prolonged potential cell-mediated immunity.

A second purpose of artificial immunization procedures is to elicit large quantities of specific antibodies for the preparation of therapeutic antisera, or as diagnostic and quantitative reagents. Here, too, adjuvants are widely used as a means of effecting the necessary increase in the antibody response elicited by the proteins and other antigens.

A third purpose is to increase the effective immune response against tumour cells or cells infected with intracellular agents (e.g., *Mycobacterium leprae*) that are already present in the body and are not being adequately checked by the naturally elicited immune response. The immunostimulation needed for therapeutic purposes in such cases may involve increasing both the nonspecific killing power of macrophages and the stimulation of specific cell-mediated immunity. Adjuvant materials are again employed but the aim is short-term therapy, although long-term prophylactic protection could also result.

This report discusses the principles underlying the use of adjuvants for each of these purposes, the mechanisms by which it is assumed they work, and the possible practical uses of existing adjuvant materials, as well as the restrictions imposed by potential adverse reactions. A final section contains proposals for further work that the Scientific Group considers to be profitable. For purposes of understanding, it should be noted that the immune processes here discussed are concerned primarily with the functions and interrelationships of three kinds of cells—namely, the cells derived from the thymus (T-cells), which are responsible for recognition of antigen; the lymphocytes derived from the bone marrow (B-cells), which cooperate with the T-cells and develop into a family of antibody-producing plasmacytes; and the macrophages.

2. ADJUVANT PREPARATIONS SUITABLE FOR MAN

2.1 Repository adjuvants

Immunological adjuvants are generally considered to be materials that are added to vaccines with the intent of potentiating the immune response so that a greater amount of antibody is produced, a lesser quantity of antigen is required, and fewer doses need to be given. There are two basic kinds of adjuvant among those commonly called *repository adjuvants*. These are (1) aluminium and calcium compounds (including

aluminium phosphate, aluminium hydroxide, aluminium oxide and calcium phosphate), and (2) the emulsified water-in-oil adjuvants. Principal among the water-in-oil adjuvants are mixtures of water in mineral oil (Freund's incomplete adjuvant) and water in peanut oil (Adjuvant 65).

The aluminium phosphate and hydroxide adjuvants have been used the most widely and for the longest period. These compounds enjoy a reputation for safety in man, although sterile abscesses and persistent nodules may follow their use. Antibody levels against antigens in these vaccines are clearly, though moderately, elevated above those obtained with the corresponding aqueous vaccine. Such serum antibody responses are short-lived, although they can be made to endure in a satisfactory way by the administration of multiple doses.

2.1.1 *Aluminium adjuvants*

When a small dose of radioactively labelled protein is injected subcutaneously into the paw of a mouse, it is found that 98–99% of these foreign macromolecules leave the limb within 24 hours, only a tiny fraction remaining in the draining lymph node (*32*). The immunogenic stimulus can be greatly enhanced by slowing down the escape of antigen from the injection site and by lengthening the period of contact of antigen with macrophages or other antigen-receptive cells. Alum-precipitated antigens retain the antigen in high concentration locally at the site of injection and release it slowly. Antibody-producing plasmacytes develop in the draining lymph node in greater numbers and over a much longer period of time when the antigen is injected in the alum-precipitated state than when the same dose of antigen is injected in simple solution (*42*). The increased stimulation of plasmacytes derives presumably from the fact that macrophages engulf the antigen-bearing aluminium salt and thereby increase the immunogenic effect beyond that of the same quantity of soluble antigen. Substantial dispersion of macrophages containing alum to the regional lymph nodes also occurs. It is further the case that a local granuloma develops after the use of the adjuvant and that this comes to be the site of large numbers of antibody-producing plasmacytes, itself contributing to the overall synthesis of antibody.

It is characteristic that the antibody response to antigens employing aluminium-containing substances is relatively short-lived : thus, antibody levels decrease rapidly at 3–4 weeks after injection. It is clear from the work of Holt (*21*) that although the antigen persists locally, it rapidly fails to act as a stimulus to the antibody-producing mechanism, in a

way that contrasts with the much more enduring effect of water-in-oil adjuvant mixtures. This may be overcome, in part at least, by giving repeat injections of vaccine. For example, two injections of alum-precipitated antigens were adequate for creating a satisfactory and enduring level of potential immunity in human prophylaxis of diphtheria.

To form an effective adjuvant mixture with the immunogen it is essential that careful attention should be paid to ensuring that the antigen is actually associated with the aluminium compound. Sometimes the aluminium salt is formed in the presence of antigen, securing occlusion of the antigen in the adjuvant but necessitating a careful check that the pH remains within acceptable levels. Alternatively, the preformed aluminium compound is added to the antigen. Effective adjuvanticity here depends on actual adsorption of the antigen to the adjuvant and it is necessary to assure that adsorption has actually occurred.

The formation of a small granuloma is inevitable with alum-precipitated vaccines and should be considered as a necessary requirement for effective adjuvant action. Care should be taken to see that alum-precipitated vaccines are injected intramuscularly, since the granuloma that develops after subcutaneous injection can undergo necrotic breakdown and can cause cyst and abscess formation.

2.1.2 *Emulsified water-in-oil adjuvants*

The emulsified water-in-oil adjuvant vaccines have been of more limited use than the alum adjuvants. They are, however, of special importance for the future since they provide the means of obtaining very greatly elevated antibody titres using smaller doses of antigen and with retention of elevated titre for periods of years. Further, there is generally a marked broadening of antigenic response, which, in the case of killed influenza virus vaccine, reduces the importance of the continuing change in antigenic composition of the prevalent influenza virus strains that render vaccines prepared from older strains less efficacious or even worthless.

Freund's incomplete adjuvant consists of an emulsion of the aqueous vaccine in light mineral oil using Arlacel A (impure form of the ester of mannitol and oleic acid) as the emulsifier. This is distinguished from Freund's complete adjuvant, to which killed mycobacteria have been added to increase the inflammatory response (*11*, *13*) and which is far too reactive to permit its use in man. Adjuvant 65 as used at present consists of an emulsion of aqueous vaccine in highly refined peanut

oil using chemically pure mannide monooleate as the emulsifier and chemically pure aluminium monostearate as the stabilizer (*17–20*).

The emulsified water-in-oil adjuvants appear to act in three ways. First, there is a slow-releasing repository of the emulsified antigen at the injection site. Second, the emulsion serves to carry the antigen to multiple focal sites throughout the lymphatic system. Third, granulomatous reactions occur at the injection site and at focal sites throughout the body. These consist mainly of oil emulsion surrounded by mononuclear cells (macrophages, lymphocytes, and plasma cells) that form highly effective " organelles " for antibody synthesis. The role of macrophages is especially important. The macrophages ingest these oily emulsions both locally and throughout the regionally relevant reticuloendothelial system, and the kinetics of the endocytosis and digestion of the emulsion are crucial for the outcome of the immunization process and for any manifestations of toxicity that might occur.

There is little doubt as to the need for the very substantial immunological potentiation that both of the water-in-oil vaccines afford. The principal consideration in comparing the mineral-oil with the peanut-oil adjuvant is the question of relative safety. A variety of criticisms of mineral-oil adjuvant have been raised, among which are allegations that its use causes excessive systemic pathology, induces various autoimmune disorders, and potentiates allergic responses; moreover, attention has been drawn to the paucity of experimental data essential to judging its safety (metabolic fate of the adjuvant components and long-term toxicity and pathological studies in animals). It must be noted that many of the data on adverse reactions were derived from studies carried out for different purposes and are not necessarily relevant to judging the vaccine. No really important adverse effects have been found in long-term studies in man. However, the mineral-oil adjuvant even when properly prepared does cause occasional sterile abscesses, and there is long-term, probably lifelong, retention of the oil in the tissues. This is regarded by some workers as unacceptable.

In viewing the adjuvant picture as a whole, there appear to be few qualitative differences in host responses to foreign substances. Instead, there are quantitative differences in kind, duration, and extent of individual host reactions. These range from a minimal and transient reaction induced by aqueous vaccines to a severe and long-term reaction resulting from the administration of Freund's complete adjuvant with added mycobacteria. A worthy objective would be to select a formulation that provides for ample potentiation of the immune response to antigens (adjuvant action) while avoiding the overstimulation and long-term

persistence of components of the adjuvant that may produce a harmful effect. Adjuvant 65, prepared using refined peanut oil, has certain obvious advantages in that chemically pure reagents are used, including highly refined arachis oil, and all the components are readily metabolizable. Tests have shown that the adjuvant is almost completely metabolized within 2 months of injection, thereby reducing the chance of immunological overstimulation and eliminating the problem of long-term persistence that might cause harmful effects. The stimulation of antibody levels in the blood with Adjuvant 65 closely approximates to that of mineral-oil preparations. Very extensive short-term (acute) and long-term (chronic) toxicity tests have been carried out in guineapigs, mice and monkeys after a few or many injections of this preparation without important adverse effects, and the preparation has not been found to be teratogenic in rabbits. No sensitization to the components of the adjuvant, including peanut oil, occurred. The components of Adjuvant 65 and of mineral-oil adjuvant, as well as many other medical products, can cause an increase in the occurrence of neoplasms in certain strains of mice—particularly male specimens—when injected subcutaneously. Extensive pathological studies showed that none of the substances was carcinogenic, but that the effect was due to well recognized physiochemical alterations that commonly promote tumours in certain rodents and are without relevance to man (*14*). Injection of distilled water or even repeated puncturing of the skin of mice has been found to produce these effects. Follow-up studies in man for 18 years after the injection of mineral-oil adjuvant and for 10 years after that of peanut-oil adjuvant showed that there had been no increase in the occurrence of neoplasms or other clinically important adverse effects.

It has been found extremely important in the case of both mineral-oil adjuvant and peanut-oil adjuvant that their components should be devoid of free fatty acids and that the aqueous vaccine component should be totally free of esterases and lipases that degrade mannide oleates and peanut oil to release fatty acids. These are toxic and, when present in adjuvant, can cause severe local reaction in the muscle leading to fluctuant nodule and abscess formation. This problem has limited the application of the oil adjuvants to killed purified viral vaccines and has hitherto largely prevented their effective application to bacterial vaccines.

The preparation of emulsified oil adjuvants requires very careful control of the adjuvant components and of the quality of the emulsions. In the case of peanut-oil adjuvant, highly refined peanut oil that is free of peanut proteins is used. It is tested for freedom from aflatoxin, a hepatic carcinogen commonly present in peanuts on which mould has

grown. The chemical purity of the mannide monooleate and aluminium monostearate is tested and the absence of free fatty acid is established. Assays for polycyclic aromatic hydrocarbons (carcinogens) are carried out for all three components. They are also tested for irritancy in appropriate assays in guineapigs and mice. The aqueous vaccine is tested for microbial sterility and for freedom from enzymes capable of releasing fatty acids from the peanut oil, the aluminium monostearate, and the mannide monooleate. It may be noted that the mineral-oil adjuvant has been used in studies in man with cholera, typhoid and tetanus toxoid vaccines; marked adverse reactions occurred, including the formation of cysts and draining abscesses. These effects were found to be due to the release of oleic acid from the mannitol in the Arlacel A emulsifier but may also have been due in part to the presence of endotoxin in the bacteria.

Emulsification is carried out and the product is assayed for viscosity and stability on storage at 4°C and at elevated temperatures. Too thick and stiff emulsions may restrain release of the material from the site of deposition and this may reduce the adjuvant effect. Emulsions that are too fluid may function badly as adjuvants. Breakage of emulsions in the body, especially when allergens are employed, may be extremely dangerous to the subject. The final product is tested for microbial sterility, for the absence of free fatty acids, and for its ability to provoke an inflammatory response in rabbits—for example, by injection into the sacrospinalis muscle and evaluation by histopathological examination. Finally, the product is checked by comparing its ability to produce elevated serum antibody levels by injection into animals, generally guineapigs, with that of a like amount of antigen in aqueous solution. The increase in the amount of antibody is found to be at least fourfold —usually far greater.

It is important that all emulsified oil adjuvants should be given by deep intramuscular injection, since there is a far greater chance of adverse effects when they are deposited subcutaneously. As the administration of emulsified oil adjuvants into the subcutaneous tissue can cause severe adverse reactions it is necessary that physicians and nurses administering such vaccines should be trained in the art of giving accurate deep muscular injection and should learn to appreciate the need for it in this context.

The tests to date to control mineral-oil adjuvant have been less thorough than those for Adjuvant 65. It would seem necessary that, where appropriate, tests designed to measure the same effects should be carried out for mineral-oil adjuvant.

Modification of mineral-oil emulsions that aim to improve the keeping qualities, ease of injection, and homogeneity of vaccines have been developed. In one such preparation, the water-in-oil emulsion is dispersed in saline solution using a suitable oil-in-water emulsifying agent (Tween 80) (*16*) to give a so-called water-in-oil-in-water emulsion. While of obvious potential importance, this adjuvant has not been extensively tested to date.

2.2 Anaerobic coryneforms

A number of bacterial strains which have been known by the general designation "*Corynebacterium parvum*" have shown unusually potent stimulatory activity for lymphoreticular tissue. These bacteria have proved difficult to classify, although it is clear that their inclusion in the genus *Corynebacterium* is inappropriate. The general designation "anaerobic coryneforms" has been adopted provisionally (*33*) for organisms that may eventually be formally classified as belonging to the genus *Propionibacterium* (*22*) and identified with organisms that bear the designations "*Propionibacterium acnes*" and "*P. avidum*". Such bacteria can be used as an adjuvant in three ways:

(1) They can be added to the emulsified water in mineral oil. In this form potentiation of the serum antibody response and delayed-type hypersensitivity in the skin was reported in early studies (*31*). However, later work, which used different strains of anaerobic coryneforms, while confirming the effect on the serum antibody response, recorded no potentiation of delayed-type hypersensitivity.

(2) Intravenous injection of a high dose (e.g., 350 μg dry-weight of bacteria) in saline in mice induces an intense activation and proliferation of macrophages lasting several weeks. These have increased ability to kill bacteria (e.g., *Bordetella pertussis*) and protozoa (*Plasmodium berghei* and *Trypanosoma cruzi*). The mice also develop increased resistance to a wide range of syngeneic tumours, which is particularly notable in relation to suppression of lung nodules when malignant cells are introduced intravenously (*6*). This phenomenon has been associated with the cytostatic activity of activated macrophages.

When the organisms are injected in mineral oil or saline a local granuloma develops at the site of injection and in draining nodes. The development of miliary disseminated granulomata follows intravenous injection. The macrophage is the principal cell in such granulomata and, in comparison with the cellular reactions to mycobacteria, necrosis is rarely found.

Antigens injected several days after, but not together with, *C. parvum* mostly induce augmented antibody responses. This is found with optimum immunizing doses of thymus-independent antigens, which are refractory to other adjuvants. In the case of particulate thymus-dependent antigens, such as heterologous erythrocytes, immunoglobulin M (IgM) responses are augmented when minimal immunizing doses are used, whereas IgG potentiation is found with higher doses only. Adjuvant activity is not found with some soluble protein antigens. At the same time as immunostimulation is present for antibody responses, a state of immunosuppression is found for many aspects of cell-mediated immunity. Thus, skin allograft rejection is prolonged, phytohaemagglutinin and mixed lymphocyte culture reactivity are depressed, resistance to graft-versus-host reaction is increased and induction of T-cell immunity by tumour-specific antigens and picryl chloride is depressed. Depression of T-cell function appears to be reversible and, in some instances at least, under the influence of activated macrophages.

(3) Recently, injection of a small dose of the bacteria (e.g., 5 μg) in combination with irradiated tumour cells or into the vicinity of a growing neoplasm was found to lead to the induction of specific T-cell immunity in the regional lymph node. The extent to which this approach could be exploited for prophylactic immunization with nontumour antigens is currently being studied.

The molecular basis for the potent activity of this group of bacteria is being sought. Lipid-free cell walls retain all the properties of the intact organisms, with the exception of mitogenicity. Killed *C. parvum* possess an unusual capacity to persist within macrophages; such persistence could potentiate the effect of an active component even when present in a relatively inactive strain.

Many anaerobic coryneform bacteria have chemotactic properties that attract mononuclear phagocytes. This property is demonstrable *in vitro* in serum or plasma-free medium and is specific for macrophages and, according to recent reports, for transformed lymphocytes. The ability to produce this chemotactic factor is correlated with the activity of the bacteria in enhancing carbon clearance in mice, and possibly relates to their other immunostimulant effects.

Many organized trials of *C. parvum* as an immunotherapeutic agent in human cancer patients are currently in progress. These are based on higher dosages than would be envisaged for its use as an adjuvant for immunization against infectious or parasitic diseases. Although these studies will provide useful information as to the sequelae of the