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Report of a WHO Scientific Group

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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

administration of *C. parvum* in cancer immunotherapy, they may not be relevant to its possible use as an adjuvant.

Further assessment is required of *C. parvum* as a non-oil-based adjuvant given locally together with antigen.

2.3 Calmette-Guérin bacillus

BCG, a potent immunostimulant, is at the present time the most commonly used nonspecific immunostimulating substance in human therapy. As an immunostimulant BCG increases resistance in mice to several unrelated bacterial infections and markedly enhances the clearance of particulate materials by the reticuloendothelial system; such stimulation persists for a very long period. BCG enhances the production of antibodies and increases the number of spleen antibody-producing cells. Also, it increases delayed-type hypersensitivity for particulate or soluble antigens, accelerates the rejection of skin allografts and transplanted tumours in mice, and inhibits carcinogenesis. BCG tends to modify the action of chemotherapeutic drugs such as cyclophosphamide (*27*), so that in respect of toxicity BCG may act synergistically or antagonistically with certain chemotherapeutic drugs.

BCG could help to promote the immunological rejection of tumours or augment resistance to infection in two ways. The first depends on the potentiating effect that mycobacteria have on the immune response in general. The second consists in the inducing of reticuloendothelial hyperplasia and hyperactivity. This is seen not only in animals injected with BCG but also when subjects with cell-mediated immunity are deliberately or inadvertently exposed to the specific antigen. The first effect of BCG would serve to enhance the host's capacity to make a specifically directed attack on the tumour cells or the parasite; the second would help in the final expression of resistance by creating a more efficient scavenging system. The latter seems at present to be as crucial in promoting antitumour immunity as in augmenting resistance to some infectious diseases (*26*).

Stimulation of cell-mediated immunity is best achieved with a freshly prepared culture of BCG containing a high proportion of live bacilli: the mode of preparation and preservation of bacterial suspensions and the strains used are of importance for the efficiency of BCG adjuvanticity. There is evidence that optimum activity depends upon the use of bacteria from the pellicle growth rather than from submerged culture. Killed bacilli act mainly to increase circulating antibodies.

In order to avoid any possible hazards arising from the use of strains of live, though attenuated, bacilli in human subjects, efforts have been made to substitute saprophytic bacteria or to use subcellular fractions of mycobacteria (see section 3.1).

2.4 Bordetella pertussis

The addition of killed bacteria from cultures of smooth or phase I *B. pertussis* causes in rodents a marked increase in antibody response to otherwise nonimmunogenic amounts of a variety of antigens injected simultaneously, and elicits increased and prolonged T- and B-cell memory. When minimal amounts of a protein antigen are given to rodents in combination with the organisms, the production of IgGl and IgE is selectively stimulated.[a] However, if the antigen is given 2 days after the administration of *B. pertussis* the antibody response may be decreased.

B. pertussis is known to activate macrophages and thus to increase the effectiveness of macrophage-associated antigens. It also causes a marked shift of lymphocytes from T- and B-cell areas of lymphoid tissues into the circulation, by preventing the normal passage of lymphocytes through high endothelial postcapillary venules, which persists for a week or more. This is attributed to a particulate lymphocytosis-promoting factor (LPF) present on the organisms, which has been shown to attach to the lymphocyte surface. Other effects are transient hypoglycaemia, and increased susceptibility to histamine and 5-hydroxytryptamine that reaches a maximum 4 days after injection but persists for several weeks.

The activity of lipopolysaccharide (LPS) extracted from *B. pertussis* is similar to that of other forms of LPS (see section 3.2) and may be an additional component of the effects of *B. pertussis*.

There is evidence that *B. pertussis* increases the numbers of helper T-cells and the degree of proliferation of B-cells, as well as activating macrophages. It is not known which components or properties of the organisms are responsible, or how these interact. However, the histamine sensitization effect is probably not relevant. It seems probable that the action of LPF on the redistribution of lymphocytes would have important effects on immunity, but it is not clear how an immuno-

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[a] There are differing nomenclatures for mouse immunoglobulins. In this report we have used a nomenclature similar to that employed for human immunoglobulins. This does not imply homology between the Ig classes of the mouse and man denominated herein by the same symbol.

potentiating effect would result. *B. pertussis* also has profound β-adrenergic activity, a consequence of which is hypersecretion of insulin, which results in hypoglycaemia. If prolonged this may lead to severe cerebral damage.

Pertussis vaccine given simultaneously with other agents (tetanus toxoid, diphtheria toxoid) in man also increases the antibody response to the latter (*15*). However, the tendency of pertussis vaccine to cause febrile responses and convulsions in some children may limit its future acceptability as an adjuvant for use in man.

3. ADJUVANTS USED IN LABORATORY ANIMALS

3.1 Water-in-oil emulsions with added mycobacterial or nocardial organisms

Freund's complete adjuvant now holds an established place in the laboratory as a means of attaining in animals very high levels of avid antibody against protein (or thymus-dependent) antigens (see section 5.3). This type of adjuvant mixture is made by the addition of various mycobacterial or nocardial species to the simple water-in-oil emulsions previously discussed. Mycobacteria that are effective in such mixtures include *M. tuberculosis* (human and bovine strains), *M. avium*, *M. phlei* (saprophytic strains), *M. smegmatis*, and *M. kansasii* (atypical strains). Several nocardial species—*N. asteroides*, *N. brasiliensis* and *N. rhodochronus*—are also active. The last-named is apparently identical with "*Corynebacterium rubrum*", which has also been used extensively as an alternative for the two first-named organisms of this group. However, the true *Corynebacterium* strains, such as *C. diphtheriae* or *C. xerosis*, are inactive.

This adjuvant effect, as judged by results in the guineapig, consists of a substantial increase in levels of serum antibody and a prolongation of such elevated antibody levels over many months. Other effects include a prolonged increase in delayed hypersensitivity to the various incorporated protein antigens (including those associated with the mycobacteria), and formation of a persistent and often necrotic granuloma at the site of injection. Mycobacteria have also been added to water-in-oil emulsions for the production of acute disseminated encephalomyelitis using homologous brain components as the immunogen; analogous autoimmune disorders can be induced against a range of tissue antigens. The use of mycobacteria also entails dramatic qualitative

changes in the antibody response. Thus the sera of guineapigs immunized 3–4 weeks previously with ovalbumin in Freund's complete adjuvant contain considerable antiovalbumin antibody in the form of IgG2, whereas controls contain mainly IgG1 antibody. In this case mycobacteria potentiate the production of antibodies with biologically important properties such as complement activation (IgG2) and affinity for macrophages.

Early studies to define the active principle for these adjuvant effects centred on extracts from whole mycobacteria, which could be obtained by neutral organic solvents, particularly chloroform. Such chloroform extracts, known as purified wax fraction (*2*), wax D (*5*) and, in a later purified form, peptidoglycolipid (peptide-containing lipid) (*23*, *43*) were shown to reproduce the various adjuvant effects of whole mycobacteria in the guineapig. However, it became clear that only the extracts from human-type mycobacteria resulted in a high yield of biologically effective adjuvant and that this effectiveness correlated with the presence in the extracts from the human strains of peptidoglycolipids containing amino-acids, D- and L-alanine, D-glutamic acid and *meso*-diaminopimelic acid, as well as aminosugars that included muramic acid. All these elements indicated an analogy to the murein or peptidoglycan of the bacterial cell wall. More recent developments include the isolation from the cell walls and from whole bacteria of the species *M. smegmatis* of " water-soluble adjuvants " (designated WSA and neo-WSA respectively) by a simple procedure involving treatment with lysozyme (*1*) or even water (*29*). These extracts, when incorporated in an emulsion of water in mineral oil, proved effective in generating humoral and cell-mediated responses to protein antigens in the guineapig, but did not show a very significant increase in activity, measured on a weight basis, over that of cell walls or of whole mycobacteria. Large yields of such active peptidoglycan can be obtained from the crude culture filtrates of mycobacteria. Similar adjuvant-active peptidoglycans have been extracted from a wide range of Gram-negative and Gram-positive bacterial species (*30*). The minimal molecular structure required for such adjuvant activity has been investigated by the use of synthetic compounds and it is recorded that *N*-acetyl muramyl-L-alanyl-isoglutamine (but not the muramyl monopeptide) possesses adjuvant activity in the guineapig (*37*). The anomaly remains that such water-soluble adjuvants require to be injected in a mineral-oil vehicle, which suggests that further insight into their activity may depend upon an understanding of the physico-chemical factors involved in their presentation to immunocompetent cells.

It is necessary to add that even the renowned Freund's complete adjuvant can depress rather than enhance an immune response. Thus a potent operational immunosuppression may occur when a second immunogen is administered after a previous injection of a different antigen in Freund's complete adjuvant. This may result in the virtual elimination of antibody and delayed hypersensitivity responses to the second antigen. Also, increasing the dose of mycobacteria in Freund's complete adjuvant above an optimum may suppress the development of experimental allergic encephalomyelitis (*36*).

The following cellular mechanisms may account for the main effects of water-in-oil and mycobacterial or nocardial adjuvant mixtures:

(1) a depot-effect of the antigen in water-in-oil emulsion (*12*);

(2) a general activation of macrophages by specific increase of T-cell activity and by direct effect of oil and mycobacterial components;

(3) the trapping of T-cell populations in the lymph nodes regional to the site of injection of Freund's complete adjuvant (*38*);

(4) interference with the normal segregation of B-lymphocytes into germinal centres acting against normal homeostasis of the antibody response by 7S antibody product—a hypothesis based on experiments in chickens (*41*);

(5) diversion of the specific response to an antigen from tolerance induction to antibody synthesis (discussed in section 5.1).

3.2 Bacterial lipopolysaccharides (endotoxins)

Lipopolysaccharides (LPSs) from a wide variety of Gram-negative bacteria, when administered by various routes in microgram amounts to laboratory animals, have been shown to elicit large early IgM antibody responses (and later IgG responses in some species) against the polysaccharide components of the LPSs. The amount of antibody is much greater than that elicited by corresponding amounts of the polysaccharide component alone. When some other antigens are administered in small amounts simultaneously with LPSs the antibody response against them is also greatly increased. LPSs may therefore be regarded as adjuvants. They also have a wide variety of biological activities that are not directly related to their effect on the immune response. These include pyrogenicity, activation of plasminogen, adrenal cortical stimulation, adherence of polymorphs and platelets to vascular endothelium (followed by leucocytosis), stimulation of acute phase protein synthesis (including

properdin) by the liver, interferon release and stimulation, and activation of the alternate complement pathway. LPS is thought to act on a variety of different cells, of which polymorphs and macrophages may be the most important, since these are the probable source of secondarily active factors (e.g., interferon, lysosomal enzymes, plasminogen activator and other neutral proteases, and possibly lymphocyte-activating factors).

The biological activities are wholly attributable to the presence of lipid A, which is common to all LPSs. Lipid A is composed of two molecules of $\beta\,(1 \rightarrow 6)$-linked glucosamine phosphate, to the amino groups of which are attached myristic acid; additional molecules of lauric and palmitic acid are attached at positions 3 and 4 to one of the glucosamine molecules. Modification of this structure by chemical means is possible. Pure lipid A aggregates to a variable extent in the presence of divalent cations, and can only be reliably studied in the monomeric form (conveniently as a trimethylamine salt prepared by electrodialysis), or as a complex with an inert protein, such as bovine serum albumin.

Pure lipid A is pyrogenic in primates at a dose of 0.01 µg per kg of bodyweight, but very much larger quantities can be administered without proving fatal. Tolerance to the pyrogenic action is rapidly produced by repeated administration. Lipid A is normally not immunogenic although antibodies can be elicited by injection of killed " super-rough " salmonella organisms treated so as to be coated exclusively with lipid A. Detectable antibodies are not present in normal animals or man even after recovery from enterobacterial infection.

Although its wide spectrum of biological activities make it unlikely that lipid A would normally be useful as an adjuvant, it is of interest because it has been shown to be a potent activator of a subset of B-lymphocytes (detected in mouse, rabbit or human spleen, but with a variable distribution in other tissues), causing them to differentiate and to divide and to secrete IgM. This results in nonspecific activation of the susceptible B-lymphocytes irrespective of their antibody specificity, and increases the proliferation of B-lymphocytes already specifically stimulated by a separate antigen administered simultaneously. It has been shown *in vitro* that when a thymus-independent antigen is itself linked to lipid A or LPS the latter becomes focused on to specific antigen-sensitive lymphocytes, and at low doses these are specifically stimulated to secrete IgM antibody. Whether a similar process can account for the adjuvant effect of LPS *in vivo* is, however, unproved. LPS, by activating macrophages (see section 6) and in other ways already mentioned, could produce different indirect effects on the immune response. Interpreta-

tion is complicated when near-toxic doses of LPS are used *in vivo* by the consequences of adrenocortical stimulation, which causes lysis and temporary depletion of lymphocytes. LPS also causes profound temporary changes in the circulation within lymphoid tissues, characterized by greatly increased microvascularity, resembling in some respects the changes caused by lymphocyte-activation products. LPS has been found to suppress in mice the antibody response to some thymus-dependent antigens administered one or two days later. The mechanism of this is not known, nor is it known whether or to what extent T-lymphocytes are affected.

A distinct application of LPS has been its administration to tumour-bearing animals, either intravenously or directly into the tumour tissue. This results *inter alia* in necrosis of the endothelium lining the fragile venule within the tumour and in activation of macrophages; it may also directly or indirectly result in necrosis of tumour cells. Whether specific immunity mediated by antitumour T-cells is also stimulated is not clear.

3.3 Lysolecithin analogues

Lysolecithin analogues (LLAs) have been synthesized (*3*) which are not metabolized in cell membranes via the normal phospholipid cycle that reconverts lysolecithin to lecithin (*9*). Such analogues not only have a greatly prolonged half-life but can retain the surface-active properties of lysolecithin. This material has been shown in low concentrations to be a potent activator of macrophages, to facilitate intercellular bridging and even to cause cell fusion (*35*), although in higher concentrations it brings about cell lysis. LLAs such as C_{18} ether hydroxylysolecithin or C_{12} or C_{16} ether deoxylysolecithin can be administered as a single injection intraperitoneally or intravenously into mice without causing gross toxic effects, and when small amounts of antigens such as bovine serum albumin or sheep erythrocytes are injected intraperitoneally 1–4 days later a much increased primary antibody response ensues. Secondary responses may also be much increased, especially when the initial dose of LLA was small (1 µg). For optimum adjuvant action, however, the doses of LLA and of antigen, as well as the timing of the injections, must be carefully evaluated.[a]

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[a] Munder, P. G. et al. Effects of lysolecithin and synthetic analogues on the immune response (unpublished WHO document IMM/SG15/WP/75.5, 1975).

A single intraperitoneal injection of some long-chain LLA 4 days before inoculation of 10^4 Ehrlich ascites tumour cells protected mice against the tumour. Following inoculation of lethal numbers of a variety of tumour cells, daily injections of 10 µg of LLA, given intravenously (or even intracutaneously at a site removed from the tumour-cell inoculation) for 16–20 days gave complete protection against some tumours and very significant protection against others. Mice that had been protected were specifically resistant to subsequent challenge with larger amounts of inocula of the same tumour. Direct injection of LLA into an experimental tumour mass has been shown to cause lysis of many of the tumour cells and subsequent disappearance of the remainder (perhaps because the LLA increased the immunogenicity of the lysed cell membranes).

Tests in guineapigs indicate that single intravenous doses far in excess of those required for adjuvant activity are not significantly toxic, and preliminary tests in baboons also indicate that LLAs are well tolerated.

The LLAs, which can be prepared in pure form, and which have many potentialities for further chemical modification, provide a promising new class of adjuvants that deserve wider investigation, especially in relation to the immunotherapy of tumours. Their mode of action is not yet understood.

3.4 Substances that labilize lysosomes

Several substances have the common property of causing lysosomes to leak the enzymes contained in them. These include vitamin A in toxic doses, beryllium salts, and toxic forms of silica, which have all been shown to act as adjuvants in rodents when administered simultaneously with a variety of antigens. Certain alkyl quaternary ammonium compounds of optimum chain length act similarly.

All are potent activators of macrophages in doses insufficient to kill them, and it is presumed (and has in some instances been demonstrated) that this is responsible for their adjuvant action. Because of their general toxicity they are not of practical interest but they illustrate the importance of macrophages as regulators of the immune response.

3.5 Polyanions

Polyanions (*40*), including double-stranded natural and synthetic polyribonucleic acids (*7*), have an adjuvant effect shortening the induction period and enhancing the level of the humoral immune response.

For example, the immunopotentiating effect of polyanions such as dextran sulfate, poly I : C or poly A : U has been demonstrated for a variety of thymus-dependent (but not thymus-independent) antigens, when the antigens were administered in suboptimum doses. However, if the antigen was injected 2–3 days after administration of polyanions the immune response was suppressed. It has also been shown that polyanions increase the number of memory cells *in vivo*. The adjuvant activity seems to be related to the molecular size of the polymeric backbone as well as to the chemical nature of the anionic groups, such as sulfate, carboxylate and phosphate. The fact that polyanions are able to restore the capacity of mice depleted in T-cells to respond to thymus-dependent antigens *in vivo* as well as *in vitro* indicates that they may act by replacing or stimulating such T-cell function. Concerning the target cell of their action, it has been demonstrated that polyanions are generally mitogenic for B-lymphocytes and probably also for such primitive lymphoid stem cells and even some nonlymphoid cells. Activation of helper T-cells and of macrophages has been achieved using poly I : C or poly A : U, and potentiation of the reactivity of thymocytes to phytohaemagglutinin has been demonstrated by pretreatment of the cells with sulfated polymers. One of the points that remains to be clarified is whether polyanions affect cell-mediated immunity due to T-cells or K (killer)-cells or act exclusively on the humoral response. Polyanions are highly reactive compounds showing a variety of biological activities, including interferon induction, inhibition of some complement factors (C1q), activation of C3, and effect on the blood-clotting system. These properties do not seem to be related to the adjuvant activity of the compounds. It seems unlikely that polyanions will be generally used in man, since they are pyrogenic and have been found to produce certain effects in animals that may cause concern with regard to safety, especially the potentiation of autoimmune disease in NZB/W mice; moreover, most of them in themselves have profound toxic side effects.

3.6 Fungal polysaccharides

Japanese workers have investigated the antitumour activity of several polysaccharides of fungal origin. Lentinan, a (1 → 3)-β-D-glucan (glucose polymer; molecular weight approximately 10^6) was found to inhibit the growth of sarcoma 180 in intact but not in thymectomized mice (*28*). This indication of a possible potentiation of T-lymphocytes in tumour immunity has been extended to a potentiation of the T-

lymphocyte helper function. Although lentinan has no effect on allograft immunity or delayed hypersensitivity and stimulates the production of large quantities of an as yet unidentified serum protein, it is nontoxic in mice in relatively large doses. It is reasonable that further investigations of the potential adjuvant activity of lentinan and related compounds should be carried out.

3.7 Levamisole

It has been shown that levamisole, an imidazole derivative, extensively used as an anthelmintic drug, stimulates antibody formation and tumour regression in animals (*34*) and some aspects of cell-mediated immunity in man (*39*). The mode of action of levamisole is still unknown. Its potential adjuvant activity, as well as that of related compounds, requires further investigation.

4. ADJUVANTS IN RELATION TO POSSIBLE IMMUNOTHERAPY OF TUMOURS

A number of adjuvants including BCG, methanol-extraction residue, anaerobic coryneforms and LLAs can induce substantially increased resistance to grafts of allogeneic and syngeneic tumours in rodents. Earlier studies were concerned with the effect of treatment with adjuvant prior to the introduction of tumour cells and were of clearly restricted relevance to therapeutic application. More recently, a number of studies have established that injection of adjuvant into the region of the tumour (e.g., anaerobic coryneforms) or systemically (LLAs) is capable of bringing about substantial degrees of tumour regression. Another observation relevant to possible cancer immunotherapy is that treatment by BCG and anaerobic coryneforms around the time when tumour cells are injected intravenously can totally suppress the subsequent development of tumour nodules in the lungs. This adjuvant therapy might provide antimetastatic cover when a neoplasm is surgically resected.

More than one mechanism seems to underlie the various experimental models. The first of these represents the nonspecific effect of adjuvant-activated macrophages that can be shown to be highly cytostatic for tumour cells *in vitro*. A second involves the development of tumour-specific immunity elicited by T-cells. This can arise when anaerobic coryneforms are injected locally either around a tumour or together

with lethally irradiated tumour cells. Tumour-specific immunity also develops following the regression induced by LLA.

Currently, a substantial number of organized clinical trials of cancer immunotherapy involving BCG and anaerobic coryneforms are in progress in several countries. In addition, the experimental use of adjuvants as antitumour agents in mice is under intensive study in numerous laboratories, which it is hoped will provide some guidelines for their rational use in man.

5. THE POSSIBLE MODE OF ACTION OF ADJUVANTS ON THE IMMUNE RESPONSE

5.1 Adjuvant action at the cellular level

It has become clear during the last decade that the immune response usually depends on a complex interaction between antigen and one or more of several cell types. The possibility that contact with antigen can elicit in various lymphocyte categories one or other of two mutually exclusive immunological states (immunity and tolerance) is outlined in Table 1. It is envisaged that adjuvants can act on any one or more

TABLE 1. IMMUNITY OR TOLERANCE ELICITED BY ANTIGEN IN DIFFERENT CELL TYPES

Consequence of administration of antigen	Cells involved in response or "silenced" in tolerance	
	Reticuloendothelial system (nonspecific)	Lymphocytes (specific)
(1) Response	Macrophages (various)	T-lymphocytes (cell-mediated immunity, helper, suppressor)
(2) No response (*a*) null (*b*) tolerance	—	B-lymphocytes (of different classes)

of the cell types listed and that in different situations, where operational immunopotentiation is observed, there may be stimulation of different combinations of these cell types.

Various substances with empirically defined adjuvant properties may stimulate macrophages with reference to any one of their several roles in immune responses. These include :

(1) the production of processed antigen with increased immunogenicity;

(2) the removal of excess antigen, thereby protecting the animal against tolerance induction;

(3) improved and/or prolonged presentation to lymphocytes of antigen held on their surface membrane;

(4) action as cellular reservoirs of antigen that can be released over an extended period (it is implicit here that repeated stimulation by antigen over an extended period will produce higher antibody titres than a single exposure); and

(5) the synthesis and release of (nonspecific) lymphocyte-activating and/or -inhibiting factor.

As with Freund's complete adjuvant (see section 3.1), many adjuvants and immune complexes potentiate the trapping of circulating lymphocytes in draining lymphoid organs. This mechanism, which may be mediated by stimulation of certain specialized macrophages (littoral cells), increases the probability of successful contact between all the necessary cellular protagonists in the immune response and antigen.

Antigen, either directly or after macrophage handling, can trigger events in lymphocyte populations that lead either to a state of tolerance or to a state of immunity. There are a few reasonably well defined model systems where an important aspect of adjuvant action is to switch cells from a pathway leading towards tolerance to one leading to immune differentiations. Both adjuvant action and tolerance induction can be at the level of any one or any combination of the lymphocyte subtypes listed in Table 2.

TABLE 2. SELECTIVE ACTION OF SOME ADJUVANTS ON DIFFERENT CELL TYPES

Cell type (primary effect)		Adjuvants
T cell-mediated immunity	delayed hypersensitivity	levamisole
	allograft	BCG, *C. parvum* BCG, *C. parvum*
	"immunity"	BCG, *C. parvum*
T (helper)		poly A, poly U; lentinan; *B. pertussis*
T (suppressor)		*B. pertussis*
T (killer)		uncertain
B (μ class)		small effect } LPS; polyanions
B (γ class)		large effect } (incl. polynucleotides)
Macrophage		*C. parvum*; *B. pertussis*, lysosome labilizers, aluminium salts, polynucleotides
Killer cells (antibody-dependent cytolysis)		uncertain

Cell differentiation is a time-dependent phenomenon and it is unequivocal that at least some elements of its mechanisms are involved in immune differentiation. Consequently it is of some interest and relevance that the stimulatory and suppressive effects of both adjuvants and immunosuppressive procedures are dependent on the timing of their administration (and their dose) in relation to that of antigen. For example, LPS injected 24 hours prior to antigen can have a profoundly suppressive effect, whereas if it is administered concomitantly with antigen or very shortly afterwards it can be an immunopotentiator. A theoretically possible mechanism for certain adjuvants that awaits exploration is that they act as selective suppressors of the regulatory T (suppressor)-cells that are normally elicited by an administered antigen. Regulatory mechanisms involving B-cell products (antibody), which are stimulated by Freund's adjuvant, have been described in chickens (*41*) (see section 3.1).

In brief, it is envisaged that adjuvants can act at the cellular level in one or more of the following three ways:

(1) by helping lymphocytes to " decide " to enter a pathway of immune rather than tolerance differentiation;

(2) by amplifying " post-decision " proliferation of immune-induced lymphocytes, either directly or via the release of stimulatory factors from macrophages: thus, the size of the eventual clones of antibody-producing cells would be increased; and

(3) by increasing the efficiency of macrophages for storage and preservation of antigen to the extent that the specific stimulus is prolonged, thereby encouraging " post-decision " proliferation in a way other than that noted in paragraph (2) above.

This last point serves to emphasize that intermediary macrophages may be an important factor in many—if not most—examples of immune induction.

5.2 Effect of adjuvants on antibody classes

Many adjuvants induce a preferential synthesis of one or more antibody classes. The triggering of IgE antibody class is particularly dependent on the type of adjuvant used to stimulate the primary response. Both *B. pertussis* and particles of Al $(OH)_3$ on which antigen is adsorbed

are very efficient adjuvants for enhancing the synthesis of IgE antibody in rabbits and rodents. On the other hand, Freund's complete adjuvant favours the synthesis of IgG antibody in most species. Mice immunized with a combination of thymus-dependent antigen such as human serum albumin and *B. pertussis* produce more IgG1 and IgG2b than IgG2a. Guineapigs immunized with Freund's complete adjuvant produce IgG1 and IgG2 antibodies, whereas when these animals are stimulated with Freund's incomplete adjuvant they produce almost entirely IgG1 antibody. Intraperitoneal immunization of rabbits with alum-precipitated antigen enhances the production of IgE antibody, whereas immunization with Freund's complete adjuvant enhances IgG.

5.3 Effect of adjuvants on antibody affinity

It has been reported that small amounts of antigen acting over a prolonged period of time (as in the case of Freund's complete adjuvant) stimulate the production of antibodies against any given determinant whose average affinity increases with time (*10*). When the antigen is a complex molecule possessing a variety of determinants, the antibodies become more avid, as measured, for example, by the capacity of an antiserum to form indissociable complexes with the antigen. Increase in avidity is explained in part by the production of antibodies against more determinants as stimulation is prolonged. The changes in antibody affinity occur in antibodies of the IgG class, and only to a minor extent in those of the IgM class (*8*).

Increased average antibody affinity is due to preferential stimulation of those lymphocytes in the antigen-sensitive B-cell population with the highest affinity for the antigenic determinants. Such stimulation becomes increasingly selective once high-affinity antibody has been elicited that can form a complex with residual antigen so as to prevent it from acting as a stimulus to B-cells with receptors of lower affinity. When large doses of antigen, with or without adjuvant, are used to elicit a primary antibody response there is minimal selection of high-affinity cells, and low-affinity antibody predominates; restimulation also elicits mainly low-affinity antibody. Small doses of antigen, without adjuvant, may fail to stimulate an ongoing response of sufficient intensity or duration for a selective process to operate. Thus, when the production of high-affinity antibody is required, this is most likely to be achieved by the administration of minimal amounts of antigen in a persistent form together with a persistent potent adjuvant.

5.4 Adjuvanticity

It is a matter of common experience that foreign antigens administered in their native state to laboratory animals or to man differ greatly in the readiness with which they elicit antibody formation. Some tend to induce specific immunological unresponsiveness, while others may elicit antibody responses of variable duration and intensity, involving different antibody classes, and a greater or lesser degree of immunological memory, and others still are virtually ineffective. The term adjuvanticity has been coined to describe the properties of an antigen which, in addition to the presence of foreign immunological determinant groups (epitopes), enable it to elicit an effective antibody response. The term is convenient, even though it is more descriptive of some built-in adjuvant activity than explanatory of how this may operate. Consideration of some antigens that have been found to be potent immunogens suggests that adjuvanticity may include a number of different attributes, such as those listed below, whose relevance to the complex factors regulating the immune response may be apparent by reference to section 5.1.

(1) Possession of many different epitopes with stable configurations, so that the population of potentially responsive T- and B-cells is relatively large. Suitable distribution of epitopes for multipoint attachment to lymphocyte receptors. Presence of epitopes cross-reactive with antigens with which the recipient has already been immunized, so that there exists an increased pool of potentially reactive helper T- and/or B-cells.

(2) Possession of receptors for determinants on lymphocytes and/or macrophages that enable the antigen to bind to responsive lymphocytes by ligands additional to those provided by specific receptors for the antigenic determinants. Examples are provided by the bacterial exotoxins, such as cholera and tetanus, that bind to gangliosides ; molecules that bind C3b or expose Fc determinants ; complexes containing lipid A ; and plant lectins and toxins.

(3) Ability to stimulate T- and/or B-lymphocytes to differentiate and divide whether or not the antigens interact with these cells via specific receptors (polyclonal activation).

(4) Ability to be taken up by macrophages and prolonged retention of the antigen molecules at the surface, in a form accessible to lymphocytes.

(5) Capacity to cause macrophages to release lymphocyte-activating factors (see section 6).

(6) Chemical stability in the presence of enzymes of body fluids.

(7) Adequate molecular size to avoid rapid excretion from the lymphoid organs of the animal. Molecular size is also relevant to multiplicity of epitopes, to uptake by macrophages, and to other factors not readily defined.

In so far as the features responsible for adjuvanticity can be identified it is theoretically possible to add them to a chosen antigen and thereby increase its immunogenicity. Examples of instances where this has been done are the covalent attachment of small peptide hormones or carbohydrate groups to synthetic polypeptides or to other carrier molecules such as tetanus toxoid, so as to elicit antibody against the attached groups, and the formation of non-covalent ionic complexes of poor immunogens with methylated bovine serum albumin.

5.5 Adjuvants in the induction of cell-mediated immunity

Strong cell-mediated immunity usually develops in the course of chronic infections, characteristically those with intracellular pathogens, or following the transplantation of normal or malignant allogeneic tissues. By contrast, cellular immunity is usually not detectable following conventional active immunization with soluble or particulate antigens alone, although it does develop, even in response to sheep erythrocytes injected intravenously, when small enough doses are used (*25*). The response is only transient, however, and is highly sensitive to antigen excess. The failure of cell-mediated immunity to persist after immunization without adjuvant is thought to involve feedback inhibition by antigen/antibody complexes. Thus, suppression of the humoral antibody component of the response by cyclophosphamide or splenectomy substantially augments and prolongs the cellular immunity to sheep erythrocytes (*25*). An alternative interpretation would attribute the evanescence of cell-mediated immunity following higher doses of antigen to suppressor-cell generation, and cases have been made out for both T- and B-cell populations in this role (*4*, *24*).

The effective production of cellular immunity by active immunization still largely involves the use of a restricted range of adjuvants—strains of mycobacteria and anaerobic coryneforms (or fractions thereof) either incorporated in an oil emulsion of the Freund type or administered alone. The special attribute of these materials in amplifying cell-mediated immunity is by no means clear, for they usually potentiate

humoral responses as well. Mackaness et al. (*27*) concluded from studies with viable *M. tuberculosis* (strain BCG) that the mechanism was probably compounded of efficient clearance of antigen/antibody complexes by reticuloendothelial stimulation and an increased output of activated T-cells. (BCG and *C. parvum* are both powerful and prolonged reticuloendothelial stimulants, which probably act directly on macrophages initially and subsequently via interaction with specifically activated T-cells.)

5.5.1 *Water-in-oil adjuvants*

Delayed-type hypersensitivity is most commonly induced by the incorporation of antigen in Freund's complete adjuvant. A number of other bacteria may be substituted for *M. tuberculosis* (see section 3.1) and so may microgram amounts of bacterial peptidoglycan even in monometric form. The possibility of using Adjuvant 65 for the induction of experimental cell-mediated immunity has not so far been explored.

5.5.2 *Mycobacterial and coryneform adjuvants without oil*

Augmented cell-mediated responses can usually be induced in rodents that have been injected systemically 7–14 days previously with viable *M. tuberculosis* (BCG) or its methanol-extracted cell wall fraction. This has been demonstrated with the induction of delayed-type hypersensitivity to proteins and their hapten conjugates, specific immunity to tumour cells and isografts, and rejection of skin allografts. The formation of cytotoxic T-lymphocytes under these conditions has been demonstrated.

The local injection of very small doses of *C. parvum* (even 1% of the amount given systemically) together with irradiated tumour cells has now been found to induce strong, protective, specific cell-mediated immunity. The potentiality of this approach to the use of *C. parvum* as an adjuvant is currently being explored for other expressions of cellular immunity. It does obviate undesirable sequelae of the high-dose approach—granulomata, lymphoid hyperplasia, increased sensitivity to endotoxins and the possible risk of autoimmune disease.

5.5.3 *Immunosuppressive effects of adjuvants on cell-mediated immunity*

One attribute which seems to be shared by Freund's complete adjuvant, BCG, methanol-extraction residue and the anaerobic coryneforms, and which continually recurs in a variety of different situations,

is the tendency to suppress rather than augment cell-mediated immunity. The following examples may be cited :

(1) Prior injection of Freund's complete adjuvant alone has depressed the subsequent induction of delayed-type hypersensitivity and augmented the growth of syngeneic hepatoma cells. When antigen has been incorporated in Freund's complete adjuvant for immunization, both skin and tumour allograft rejection have been delayed.

(2) BCG pretreatment, when repeated, delays rather than accelerates skin allograft rejection. BCG-treated F_1 hybrid mice show increased resistance to graft-versus-host reaction. Injection of BCG into rats with an established syngeneic tumour has facilitated the growth of the neoplasm.

(3) Pretreatment with methanol-extraction residue in high doses may facilitate, rather than suppress, the growth of transplanted tumours. This does not seem to occur when such pretreatment is combined with specific antitumour immunization.

(4) There are many reports of the suppressive effects of the anaerobic coryneforms. Pretreatment of mice with the vaccine will prolong retention of H-2 histo-incompatible skin graft, increase resistance to graft-versus-host reaction, depress phytohaemagglutinin and mixed lymphocyte culture reactivity, depress induction of cell-mediated immunity by use of tumour-specific antigens and inhibit induction of delayed hypersensitivity by picryl chloride.

It seems unlikely that all these observations are expressions of a single mechanism. Enhancement by serum blocking factors has been demonstrated in some cases, whereas in others it does not seem to be the explanation for a depression of cell-mediated cytotoxicity demonstrable *in vitro*. Analysis of some of the *C. parvum*-induced phenomena *in vitro* has revealed that the T-cell functions are reversibly suppressed by the presence of highly activated macrophages. To what extent this explanation may be more general is not known. The practical indications are clear enough, however. In the case of all the adjuvants, most of the examples of the suppression of cell-mediated immunity have involved either the use of relatively high doses or the administration of the adjuvants several days or even weeks before the introduction of the antigen stimulus, or a combination of both procedures. Immunization schedules that avoid these practices should be sought.

6. MACROPHAGE ACTIVATION

The access of foreign particles to the tissues is followed by their engulfment by both microphages (granulocytes) and macrophages. Since the latter cell is far more persistent than the granulocyte in the reactions at the site of a continuing infection or invasion by allogeneic or syngeneic cells (neoplasm or normal allograft) it commands much greater attention from the immunologist. During a local inflammatory reaction, created, for example, by an adjuvant, macrophages arise mainly from invading blood monocytes, although local multiplication of tissue macrophages can also occur. The bulk of ingested materials, including bacteria, are rapidly broken down in the phagolysosomes, though significant amounts of materials derived from them may be retained in macromolecular form at the macrophage surface and there function as potent immunogens. The macrophages obtained from recently injected animals exhibit enhanced ability to kill and digest such ingested bacilli. The various cellular adaptations involved are referred to as " activation " and entail the synthesis of large quantities of lysosomal and other enzymes by an energy-requiring process, with a variety of end results. *In vitro* comparison with unstimulated peritoneal macrophages from normal guineapigs yields the following indicators of the activation process :

(1) increased ability to adhere to glass and plastic and to flatten and spread as a thin cytoplasmic layer over this surface ;

(2) increased ruffled membrane movement ;

(3) increase of glucose oxidation by the hexose monophosphate shunt ;

(4) increase in the number of intracytoplasmic granules—the lysosomes—, with a coincidental increase of acid hydrolases (β-glucuronidase, cathepsin D, lysozyme acid phosphatase, etc.) ; and

(5) liberation of factors that can inhibit cell division, and of other factors that stimulate mitosis of lymphocytes.

Macrophages can be activated to develop these attributes by nonspecific and specific processes. The latter include the specific antigenic recognition by and stimulation of T-lymphocytes, liberation of lymphokines (macrophage activation factor) and resulting macrophage stimulation. When this process of activation occurs by adding sensitized lymphocytes and homologous antigen on a monolayer of macrophages, the process of activation can be completed within 24 hours.

7. SPECIAL FACTORS TO BE CONSIDERED IN ASSESSING THE SUITABILITY AND SAFETY OF ADJUVANTS PROPOSED FOR USE IN MAN

Unfortunately there are no specific guidelines for assessing the safety of adjuvant preparations considered for use in man. Routine acute and chronic toxicity assays employed for assessing the safety of drugs for tests in man do not suffice. Instead, assessment of safety lies mainly in the area of judgement using as guidelines the kinds of adverse effects that have been observed during trials of the repository adjuvants (see section 2.1) and in the course of ordinary acute and chronic toxicity tests, such as are ordinarily carried out for drugs in laboratory animals. Listed below are several factors deemed worthy of experimental investigation prior to consideration of the use of adjuvants in the human subject.

7.1 Balancing the advantages of adjuvants with their adverse effects

Perhaps the most important initial observation of any potential candidate adjuvant is the degree of immunopotentiation that it produces. Immunopotentiation, such as that produced by Freund's complete adjuvant, may be intolerably excessive and cause hypersensitivity responses directed against the host's own tissues, possibly with induction of amyloidosis. Conversely, insufficient immunopotentiation would render a candidate adjuvant unworthy of serious consideration. Useful adjuvants, in the practical sense, would avoid both extremes, being maximally effective in immunopotentiation but not overstimulatory to the point of causing serious adverse effects.

7.2 Composition

Substances used for adjuvant purposes should be chemically pure, of defined chemical composition, or at least reproducible. They must be free of chemical carcinogens and of contaminating substances that may bring about adverse reactions. Thus, for example, polycyclic hydrocarbons must be eliminated from the light mineral oil used in Freund's incomplete adjuvant, and the aqueous vaccine incorporated into the emulsified oil adjuvants must be free from bacteria or bacterial endotoxins and enzymes. Additionally, the antigens used and the adjuvant itself ought not to contain antigens cross-reactive with those of human origin or cross-reactive antigens found in human tissues that might give rise to autoimmune phenomena such as glomerulonephritis and

allergic encephalomyelitis. Finally, the substances used for adjuvants must not of themselves cause allergic hypersensitization to the adjuvant components.

7.3 Biodegradability

Ideally, the adjuvant components should be readily biodegradable —i.e., their elimination from the injection site and from the body as a whole should occur within a period of months so that the following conditions can be avoided:

(1) long-term irritancy that may lead to tissue damage;

(2) overstimulation of the immune system;

(3) excessive accumulation of products, such as occurs when pyran copolymer collects in megakaryocytes and leads to platelet depletion; and

(4) possible induction of cancer by long-term "irritancy" effects.

7.4 Carcinogenicity

The chemicals used in the adjuvant must not be carcinogenic themselves and should not be cocarcinogenic promoters, as has been found for certain of the surfactants. Carcinogenicity is not to be confused with the increased occurrence of tumours induced in rodents by a wide variety of injectibles that has been described in section 2.1.

7.5 "Cascade" effects

Satisfactory adjuvants for general use ought ideally to give the least possible immunostimulatory effects to bring about appropriate immunopotentiation without the sequence of diverse immunological events that may attend, for example, the use of poly I : C. Poly I : C stimulates not only the production of antibodies but also that of factors such as interferon released by lymphocyte activation; it is pyrogenic, and possibly causes a cascade of events leading to unacceptable adverse effects.

7.6 Definition of action

The kinds of immunostimulation produced by adjuvants should be measured and judged in relation to the sort of use for which they are intended. Antibody response against certain tumour antigens, for example, may block the action of lymphocytes in cell-mediated immunity, and may actually cause enhancement of tumours rather than suppression.

7.7 Pathology

Acute and chronic toxicity assays of new candidate immunological adjuvants should be performed in controlled studies with attention to both the local and the systemic sites and following both single and multiple injections of the adjuvant. Further, the adjuvant should be tested for possible teratogenic effects if it is to be used in females of child-bearing age.

7.8 Adverse pharmacological effects

Tests in animals should be carried out to detect, so far as possible, any adverse pharmacological effects that might occur. A case in point is the reported β-adrenergic action of *B. pertussis* adjuvant that leads to hyperinsulinaemia and hypoglycaemia, as noted in section 2.4.

7.9 Stability

To be clinically useful, vaccines containing adjuvants ought to have a shelf-life of at least 12–18 months, retaining useful adjuvant activity during that period and without developing properties that would increase adverse reaction. Additionally, such preparations must be free of viable microbial contaminants.

8. SUGGESTIONS FOR FURTHER RESEARCH

Future research with adjuvants falls into two areas governed mainly by time factors. The one is *technical*: what existing knowledge and technology can advantageously be applied now or in the foreseeable future? The other is *basic*: what knowledge and understanding of the immune mechanisms can be evolved so that future applications can be more precisely directed to the particular effects desired and so that safer and more effective materials can be developed?

With regard to practical considerations, it should be noted that safe and effective repository adjuvants are in existence and their application can be extended. Central to the matter of application is the need for a greater guarantee of safety and this, in turn, rests largely on the kind or kinds of immunogens for which one wishes to use the adjuvant. It may be very desirable, for example, to employ subunit antigens of bacteria and viruses directing the immune responses to particular antigens, since the eliciting of immune reactions against certain of the antigens in the whole organism may actually be detrimental. Critical to the

future application of the emulsified oil adjuvant to bacterial vaccines is the need to separate out or eliminate enzymes that can degrade fatty esters to release fatty acids, and to remove endotoxins that may potentiate a more severe response. In this connexion, the need to free viral vaccines of bacteria and pharmacological or bacterial products is critical to the use of the adjuvants with killed viral vaccines.

As regards theoretical and basic concerns, the central theme for progress may lie in the precise definition of the actions and interactions of the three kinds of cells involved in the immune response—viz, macrophages, B-lymphocytes, and T-lymphocytes. These, in turn, may depend upon: (*a*) elucidation of the mechanism for T- and B-cell differentiation and the means for maintaining differentiation of lymphocytes by the central regulatory organs (thymus and bursal equivalent); and (*b*) the discovery and definition of all of the products of the B- and T-cells themselves through which they may communicate and regulate their interreactions and by which they may direct the activities of other kinds of cells. Thus it should, for example, be instructive to isolate, purify and define all important actions of such factors as thymic hormone, and T-cell lymphokines, especially transfer factor, interferon, and migration inhibitory factor. For B-cells, it may be important to elucidate the factors and mechanisms concerned with feedback control, antibody production and with regulation of B-cell function by T-cells. Isolated purified control factors (e.g., thymic factor, interferon, and transfer factor) could actually find immediate clinical application, and sequencing and synthesis of the purified active substances will be essential to practical application in clinical medicine.

One very practical consideration of great importance in basic research studies would be to learn how to separate and to direct the responses to antigens specifically to T-cells and not to B-cells, and *vice versa*. In cancer, for instance, blocking antibody or antigen/antibody complex may obviate the effect of T-cell immunity. Another very important consideration would be to develop methods, perhaps by immunological procedures, to remove blocking antibodies or to eliminate antigen/antibody complexes that may be important in cancer immunity and in the immune-complex diseases, such as, for example, arthritis, and diseases of the liver, of the cardiovascular system (endothelial lining deposition) and of the kidney (glomerulonephritis).

It is important to observe here that one significant means of altering the reactivity of the immune system is by the administration of antigens (vaccines), and central to the effectiveness of many of these is the use of immunological adjuvants.

More specific suggestions for future work on adjuvants are given below.

(1) Many adjuvant materials act by stimulating macrophages and the effectiveness of repository adjuvants is correlated with granuloma formation. Activated macrophages are known to release enzymes, interferon and other factors that can stimulate or inhibit the mitosis of lymphocytes. The process of macrophage activation and the biochemical activities of macrophages in various forms (e.g., free macrophages, tissue histiocytes, and epithelioid cells) require more intensive study, and the factors active upon lymphocytes and macrophages themselves should be characterized in order to understand the part played by macrophage activation in immunopotentiation.

(2) There is now convincing evidence that, in specific immune responses to thymus-dependent antigens, T-lymphocytes may act either as helper cells, recognizing the " carrier " and cooperating with B-lymphocytes to stimulate antibody formation against particular epitopes, or as suppressor cells, which specifically prevent such B-lymphocyte stimulation. If suppressor cells predominate, the result is effectively to cause specific unresponsiveness. It is not known whether adjuvant materials selectively alter the balance between suppressor and helper T-cells. In the mouse these can be differentiated by possession respectively of Ly 1 or Ly 2, 3 surface antigens, and are therefore amenable to study. It would be of great value if markers for suppressor and helper T-cells could be discovered in man.

(3) Encouragement should be given to research aimed at attaching materials with known adjuvant activity to specific antigens in the hope that the adjuvant would be focused by the antigen so as selectively to magnify the response of antigen-responsive lymphocytes, either for antibody production or for cell-mediated immunity.

(4) Though biodegradable oil-in-water depot emulsions are preferable to nondegradable materials, they may be hydrolysed by enzymes present in the added antigen, thus causing the release of free extracellular fatty acids that produce unacceptable local inflammation. Work should therefore be encouraged with the following aims : (*a*) to discover a means of eliminating or inactivating the relevant enzymes (e.g., lipases) without loss of the desired antigenic properties ; and (*b*) to discover alternative biodegradable oils, emulsifiers and stabilizers whose breakdown products would not produce unacceptable inflammatory changes.

(5) The potentialities of administering antigens in liposomes of varying composition and containing adjuvant additives (e.g., peptidoglycan) should be defined and efficacy assessed by direct comparison with their administration in water in biodegradable oil emulsion.

(6) More information is required on the use in animals of small doses of coryneform bacteria injected together with antigen locally without oil. Their potency as an adjuvant in this way should be compared with that of the methanol-extracted fraction from mycobacteria and the water-soluble fraction from nocardial organisms.

(7) It is proposed that various immunodefective mice, such as " nude " and NZB mice, should be used to investigate the potentiality for adjuvant abrogation of specific immunodefective states. The metastasis of tumours may be amenable to investigation in such an experimental system.

(8) It is suggested that an experimental exploration could be made into the use of adjuvants for immunopotentiation in the various clinical forms (lepromatous and tuberculoid) of leprosy. Thus an initial experiment might be to use anaerobic coryneform organisms, which have been found to possess specific ability to stimulate chemotaxis in macrophages, and test their effects after local injection into the skin lesions of leprosy patients. The experiment would provide a test of the ability of anaerobic coryneforms to resolve the lesion by invasion of activated macrophages and possibly by a stimulation of cell-mediated immunity.

(9) It would be useful to carry out the following activities: (*a*) to introduce nontoxic and nonimmunogenic drugs with known adjuvant activity—e.g., SP 54^{R}, pentosan sulfate—into water-in-oil emulsion, for instance to achieve potentiation of antiviral vaccination; (*b*) to search for adjuvants acting exclusively (or at least preferentially) on either cell-mediated or humoral immunity; (*c*) to search for conditions in which known adjuvants combined with drugs may preferentially enhance either cell-mediated or humoral immunity; (*d*) to show whether adjuvants may act at the level of regulatory T-cells; and (*e*) to search for compounds that may modulate the intracellular " signal " mechanism leading to the maturation of thymus-derived cells to functionally mature T-cells (acting either in humoral or in cell-mediated responses).

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