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Basic Concepts in the Application of Immunological Adjuvants

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The definition of immunological adjuvants goes back to the mid twenties, where Ramon demonstrated that it was possible to increase diphteria and tetanus antitoxin levels by adding different substances to the antigen.
These substances comprised lecithin, starch oil, agar and even bread crumbs (*Ramon* 1925).
What he actually observed was a positive correlation between a local abscess at the site of injection and high antibody levels. As a consequence of this Ramon defined adjuvant and immunity stimulating substances as products which, used in combination with specific antigen vaccines, enhance immunity levels above those, that the antigens are able to induce when injected alone (*Ramon* 1926).
Since the early work of Ramon several authors have redefined the concept of adjuvants in order to adjust it to the prevailing theories of their time; for example Munoz extented the definition to include substances with a stimulatory effect when injected separately within a period of time close to the injection of the antigen (*Munoz* 1964).
However, due to the broadness of the words "Combination" and "immunity levels" Ramons definition is still rather convenient.
Apart from the use of adjuvants, the result of any immunization procedure is a product of a number of parameters, including the nature of the antigen, the quantity of the injected antigen, the number and mode of immunizations and of course, the status and genetic constitution of the animal. Very often scientific papers dealing with imunizations leave the impression, that the choice of adjuvant has been more or less random and not subject to critical considerations.
Obviously, what should be done was to weigh the parameters mentioned above against the range of different adjuvants available and their characteristics, and it should all be wieved in the light of the overall purpose of the experiment.
A few examples may clarify this:
In one experiment the purpose is simply to raise rabbit antibodies against a given antigen for control purposes in another trial. The antigen in question is fairly immunogenic itself, easily obtainable and inexpensive. In such a case the advantage of using adjuvants may be rather limited. In this case there is no reason to cause unnecessary pain to the animal by choosing a powerfull adjuvant with severe side effects just to save a few injections of an inexpensive antigen.
In another experiment the task is to immunize mice in order to produce monoclonal antibodies against a purified antigen not commercially available. You have received a few microgrammes only from a colleague. Using a powerfull adjuvant may save you from spending two or three weeks of purifying some more antigen yourself.
A third experiment may be a pilot trial to develop a new vaccine for humans. Here you would like to use adjuvants to achieve the best possible protection, but significant adverse effects cannot be accepted. This restricts the choice of adjuvants to only a few.
Similar considerations could be made, for example with respect to the mode of immunization (e.g. food-pad injections are

very painful to the animal, and in many instances an adequate result could be achieved by another route of injection).
The most commonly used adjuvants and their characteristics are briefly rewieved below.

The characteristics of different groups of adjuvants

The most commonly used immunological adjuvants can be subdivided into four groups: oil adjuvants, mineral adjuvants, saponins and microbial products. Even several other reagents, including nucleic acids and vitamins, have been described as adjuvants (*Dresser* 1968), they are not commonly used as such, and hence not included in this review.

1) Oil adjuvants

The adjuvanticity of oil has been known for many years. Le Moignac and Pinoy probably made the first oil emulsion vaccine in 1916 (*Mc Kercher & Graves* 1977). Ramon started as already mentioned in 1925 with starch oil, but it was the pioneer work of Jules Freund and co-workers combining paraffin oil and killed mycobacteria for immune stimulation, that resulted in the comprehensive study of the effect of oil adjuvants and the development of Freunds adjuvants (*Freund* 1947, 1956).
Oil adjuvants are normally used as water-in-oil emulsions. The aqueous phase contains the antigen, if it is water soluble, and it is believed that amphiphilic antigens are embedded in the water-oil interphase in accordance with their polarity.
The stability of the emulsion is maintained by the addition of an emulsifier that contains polar and non-polar groups.
The emulsions are normally made by simply mixing the oil adjuvant and the antigen solution, followed by vigorous shaking. More stable emulsions are made in the vaccine industry by technical emulsifying devices.

The oil used in oil adjuvants is normally mineral oil, but several plant oils have also been utilized, e.g. safflower, sesame, peanut and poppy seed (*Stewart-Tull* 1985). The plant oils have fewer adverse effects compared to the mineral oils, but are also less efficient in raising and maintaining immunity levels.
The best known oil adjuvant is indisputedly Freunds adjuvant. It is basically a mixture of 85 % mineral oil and 15 % mannide monooleate (emulsifier "Arlacel A"), and it is available in two varieties: Freunds Incomplete Adjuvant, containing 85 % mineral oil and 15 % emulsifier only, and Freunds Complete Adjuvant, which in addition contains heat-killed mycobacteria (*Freund* 1956).
Freunds adjuvant is an extremely powerful adjuvant. It has been shown, that compounds originally thought not to be immunogenic in mice, are immunogenic when injected together with Frenuds adjuvants (*Janeway & Sela* 1967).
Hemocyanin in saline must be injected in very high doses (2 mg) to be immunogenic in rats, and under these circumstances only 19 S antibodies are produced. If, however, the same quantity is administered with Freunds adjuvant, both 19 S and 7 S antibodies are found (*Dixon et al.* 1966).
Apparently, in mice the use of Freunds adjuvant promotes the synthesis of IgG_1, and IgG_2 (*Warner et al.* 1968, *Bomford* 1980b) and has a certain regulatory effect on IgE (*Smith & Butchko* 1986). In rabbits injection of human serum albumin (HSA) together with Freunds adjuvant is able to elicit an antibody response in the newborn individual, which is normally immunologically immature and hence not able to raise an antibody response (*Jolles & Paraf* 1973).
When considering immunization procedures and the use of adjuvants, it is important to realize, that the pre-sensitization of the recipient organism with the

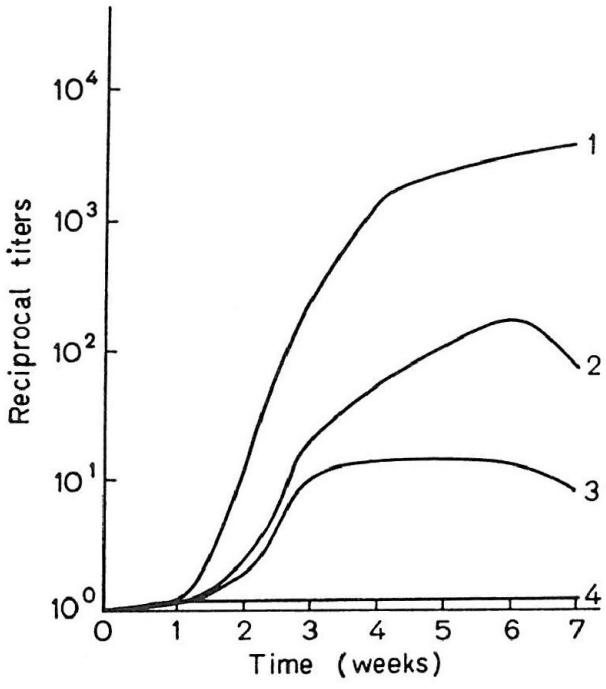


Fig. 1. Antibody synthesis inhibited by previous injections of HSA in saline. Mice that had received *1*: 100 μg HSA + FA. Mice that had received *2*: 1 mg HSA in saline, and 5 days later 100 μg HSA + FA. Mice that had received *3*: 1 mg HSA in saline, and 10 days later 100μg HSA + FA. Mice that had received *4*: 10 mg HSA in saline, and 10 days later 100 μg HSA + FA. FA = Freund's adjuvant. (Fig. 1 from *Jolles & Paraf*, 1973, p. 69).

antigen may not always promote the antibody response, but may in fact suppres it. For instance, Jolles and Paraf described that when the antigen (HSA in saline) is injected alone prior to immunization of the same antigen with Freunds adjuvant, the antibody response is suppressed (Fig. 1) (*Jolles & Paraf* 1973).

Unfortunately, the extreme efficacy of Freunds adjuvants is also reflected in a vast array of severe side effects, which restrict the utilization of the adjuvants. The most significant of these side effects are listed in Table 1.

2) Mineral adjuvants

Mineral salts have been used as immunological adjuvants for about sixty years, starting with the initial work of Glenny, who observed that mixing diphteria toxoid with potassium alum led to the formation of a precipitate, which had a strongly increased imunogenicity compared to the toxoid alone (*Glenny et al.* 1926).

Today three mineral salt adjuvants are regularly used, i.e. aluminium hydroxide, aluminium phosphate and calcium phosphate.

These three adjuvants even enjoy a reputation for safety in man, and are recommended by WHO (WHO technical report 595, 1976). A brief description of the three will follow below.

Aluminium hydroxide and aluminium phosphate

Aluminium hydroxide adjuvant is used in the form of a hydrated colloid gel having the ability to adsorb porteins.

The net charge of the gel is zero at pH 9.1–9.2 (*Hem & White* 1984), which

Table 1. Side effects observed following the use of Freunds Adjuvants.

Nodules and abscesses	Pittman, 1967
Polyarthritis	Pearson & Wood, 1963 Pearson & Wood, 1964
Amyloidosis	Tal & Laufer, 1960
Granulomas	Steiner et al., 1960
Glomerulonephritis	Steblay, 1962 Steblay, 1963
Allergic reactions	Becker et al., 1961
Plasmacytomas in Balb/C mice following i.p. injections	Potter & Robertson, 1962

means, that at pH below 9, the gel is positively charged and thus able to adsorb proteins, which are mostly negatively charged at physiological pH values. The attraction of opposite charges is assumed to be the major cause responsible for the binding capacity of the gel.

Normally proteins are simply adsorbed onto the gel by incubating it with the antigen solution. A 2 % aluminium hydroxide gel is incubated with the antigen solution in the ratio 1:5 at approx. pH 6 over night at 4 °C as a general guideline. The amount of antigen adsorbed may be subject to considerable variation, depending on the antigen and the pH during the adsorption, not to mention the structure and the internal surface of the colloid gel. Obviously, one should choose a pH value at which the antigen is sufficiently negative without running the risk of denaturation. Pilot trials should, however, be performed in each case to determine the adsorption.

As an example, the degree of adsorption ranges from 15–25 mg/ml for a 2 % gel, when HSA is used as a model antigen.

It is important to notice, that multivalent negatively charged ions present, like phosphate and sulphate, interfere and bind to the gel in competition with the relevant antigens. Hence in order to assure optimum binding to the gel, phosphate buffered saline, for example, should not be added.

Aluminium phosphate gel has a much lower protein binding capacity (approx. 1 mg HSA/ml of 2 % "Adju-phos", Superfos a.s.), apparently reflecting the influence of bound phosphate.

Aluminium compounds in themselves seem to be able to stimulate the immune system, and there is reason to believe, that aluminium hydroxide gel is effective in at least two ways: gradual release of adsorbed antigen i.e. a depot effect, as well as the unspecific stimulation of the lymphoid system by aluminium. Aluminium phosphate, having less adsorption capacity, cannot provide a similar depot effect.

It has been demonstrated, that immunization with aluminium hydroxide stimulates the production of IgG_1 (*Warner et al.* 1968) and that aluminium adjuvants in general enhance the production of IgE and homocytotropic antibodies (*Vassilev* 1978, *Vijay et al.* 1979, *Fujimaki et al.* 1984).

The amount af aluminium hydroxide in a vaccine may exceed an upper limit, beyond which the degree of immunization and protection will be declining. If injections are made using equal amounts of antigen, examplified by influenza virus, with increasing quantities of aluminium hydroxide, the dose-response curve will show a distinct optimum (*Schmidt* 1967). The protection following a challenge-infection, measured as LD_{50} as a function of the percentage of aluminium hydroxide will decline beyond this point (Fig. 2). Thus in every case, determination of the optimum combniation of antigen and adjuvant will require critical considerations.

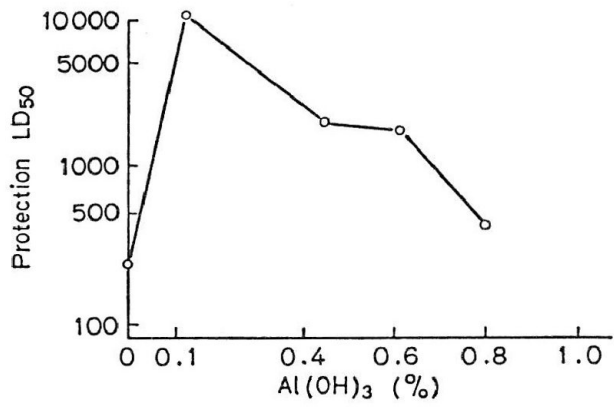


Fig. 2. Dose-response curve: mouse protection dependent on the increasing amount of $Al(OH)_3$ adjuvant. (From *Schmidt*, 1967).

Calcium phosphate

Calcium phosphate was developed as an adjuvant at the Pasteur Institute, primarily by Relyveld and Raynaud, and its aplica-

tion has been rewieved (*Relyveld & Raynaud* 1967, *Relyveld et al.* 1970). Calcium phosphate has characteristics quite different from those of the aluminium gels.
Apparently calcium phosphate does not enhance the production of IgE and homocytotropic antibodies, like the aluminium compounds (*Vassilev* 1978), which makes it interesting as adjuvant and carrier of allergen extracts in the hyposensitization of allergic patients and also as adjuvant in human vaccines.
The protein adsorption capacity of calcium phosphate is of the same magnitude as that of aluminium phosphate. The sedimentation of calcium phosphate is more pronounced compared to the aluminium gels.
Calcium phosphate, being a natural constituent of the organism, does not seem to cause any side effects.

3) Saponins
A third class of adjuvants belongs to the socalled saponins, which is a complex group of molecules, that can be extracted from a number of plant families.
Saponin preparations have been used as adjuvants regularly since the work of Espinet from the early fifties (*Espinet* 1951), but some of the plant extracts used as adjuvants by Ramon in the twenties belonged to this group.
Saponin is now included as a constituent in an increasing number of licensed vaccines for veterinary use.
Originally crude extracts of saponins were used as adjuvants, but the lack of purity and homogeneity of the preparations led to unpredictable results.
In 1972 *Kristian Dalsgaard* isolated a pure saponin fraction from the cortex of the south american tree Quillaja Saponaria Molina (*Dalsgaard* 1972). The adjuvant properties of this fraction in foot-and-mouth disease vaccines were further described in subsequent publications (*Dalsgaard* 1974, 1977), and he named it "Quil-A". At present "Quil-A" has taken over as the most commonly used among the saponin adjuvants.
The molecular structure of "Quil-A" is not fully elucidated. It is a water-soluble fraction known to consist of a triterpenoid quillaic acid (Fig. 3) bound to an oligosaccharide moiety by a glycosidic linkage. Only little is known, however, about the sugar moiety.

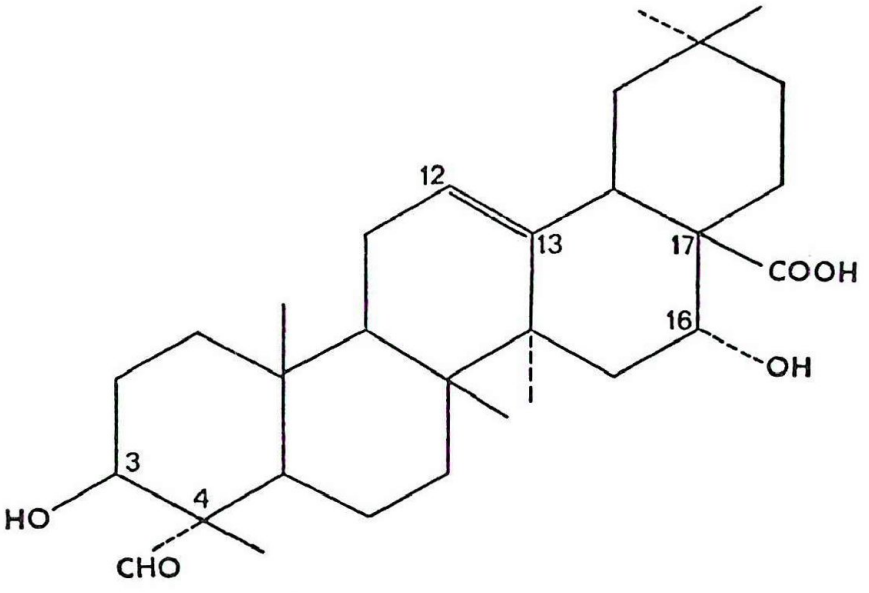


Fig. 3. Quillaic acid.

The mode of action of saponin adjuvants is not understood in details either. It has been shown, that saponins are able to form complexes with membrane bound cholesterol (*Bangham & Horne* 1962, *Bomford* 1980a). But independant of this saponin has an immunostimulatory effect which is demonstrated when saponin and antigen is injected separately. Hence, prior attachment of saponin to the antigen (exampli fied by sheep red blood cells) does not seem to be a strict necessity for achievement of an adjuvant effect (*Bomford* 1982).

Bomford also compared the adjuvant effect of "Quil-A" in combination with serum albumin and in combination with SRBC (*Bomford* 1980b). He found, that the saponin had little effect in raising antibody titers against albumin, but had a pronounced effect in combination with SRBC. Thus, if generalisations should be made, it appears that "Quil-A" is an efficient adjuvant in combination with amphipatic/membrane bound protein antigens, but less efficient in combination with water-soluble proteins. These observations suggest, that interactions between polar/non-polar groups of the surface active saponin and the amphipatic cell membrane- or virus envelope antigens are involved in the adjuvanticity.

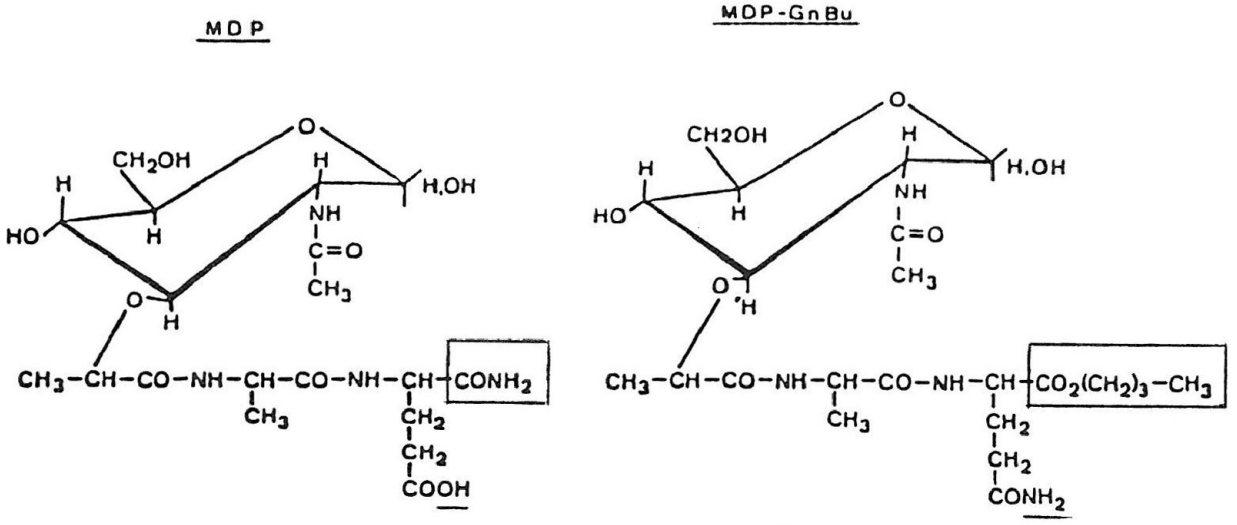
The surface activity of saponin may lead to local necrotic reactions at the site of injection, when injected subcutaneously in too large quantities. Intravenous injections will cause hemolysis. These side effects both reflect the detergent effect of the saponin.

It has been shown, that the optimum dose for immunization is 10 microgrammes per vaccine dose for mice and 50 microgrammes for guinea pigs (*Dalsgaard* 1984). Given in these doses, "Quil-A" does not seem to cause significant adverse effects.

4) Microbial products

A number of microbial products have been shown to possess a non-specific immunostimulatory potential. These include fractions of Corynebacterium parvum, mycobacterial vaccine BCG (Bacillus Calmette Guerin), lipopolysaccharides from E. coli and extracts from Bordetella pertussis (*Bomford* 1980b, *Dresser et al.* 1970).

A most interesting approach, which may provide a better understanding of adjuvanticity in general, however, is the work



A. B.

Fig. 4. (from *L. Chedid*, 1983).
A. Muramyl dipeptide. B. Murabutide.

of identifying the active subunit from mycobacterial cell walls in Freunds Complete Adjuvant.

In 1975 this subunit was identified and synthesized independently by two groups of investigators, i.e. dr. Lederers group in France (*Ellouz et al.* 1974) and dr. Stewart-Tull in Glasgow in collaboration with a japanese group (*Stewart-Tull et al.* 1975, *Kotani et al.* 1975).

This molecule was identified as N-acetyl-muramyl-L-analyl-D-isoglutamine (Fig. 4a), a dipeptide with a carbohydrate moiety called muramyl dipeptide or simply MDP.

A very comprehensive study of this compound has been undertaken by Louis Chedid and co-workers at the Pasteur Institute in Paris and was rewieved in 1983 (*Chedid* 1983).

A large number of MDP derivatives have been produced, in which the main structure of MDP is preserved. By inducing slight alterations in the chemical structure it has been possible to change its functional characteristics and the profile of the adjuvant. For instance, the original MDP is highly pyrogenic, whereas a butylated derivative: MDP (Gln)-On Bu (Fig. 4b) called murabutide is not (*Chedid et al.* 1982).

MDP derivatives have been shown to affect a large number of functional parameters of the monocyte-macrophage system, including increased in vitro antitumorcell activity, increased phagocytic activity, increased production of prostaglandin and cyclic AMP (*Chedid* 1983).

When MDP is used as an adjuvant, the adverse effects which are seen after administration of Freunds Complete Adjuvant are less severe or even absent. Here it is worthwhile noting, that MDP injected in saline is rapidly cleared through the kidneys (*Parant et al.* 1979). However, granulomas and adjuvant arthritis have been observed following injection of MDP (*Emori & Tanaka* 1978, *Kohashi et al.* 1980). Nevertheless the work with MDP and its derivatives is extremely promising and may for the first time provide a tool for specific triggering of certain parts of the immune system, and there is little doubt that the intensive research in MDP will yield major progress.

Conclusion

In experimental immunology the scientist often may use immunological adjuvants which are not subject to the same restrictions, that are seen in therapy or veterinary profylaxis. This lack of restrictions leaves the choice of adjuvants more open, and it is strongly recommended – before choosing – to take the special characteristics of the adjuvants into consideration, since not all adjuvants would be equally suitable for the overall purpose of a given experiment.

There are several practical reasons why immunization procedures accompanied by severe side effects should be avoided when possible. For example, the presence of an abscess or necrosis at the site of injection may render the animal more susceptible to infections, thus introducing unforeseen antigens or even death before the experiment is completed.

An association between psycological stress and the immune system has also been postulated. Severe side effects, altered behaviour as is seen in foot-pad immunizations, and local irritation may introduce a significant stress factor, which is not introduced to the control group of the experiment having often received injections of saline only.

Obviously, the more we learn about the mechanisms participating in the immune response, the better we may manipulate it, but right now, the study of adjuvants is still a science of applied empirical immunology.

Resumé

De mest anvendte immunologiske adjuvantia opdeles i fire overordnede grupper, hver med deres karakteristika. Disse karakteristika, samt de eventuelle bivirkninger, som måtte ledsage anvendelsen af de enkelte reagenser, omtales i hovedtræk.

Artiklen taler for det synspunkt, at eksperimentator bør anlægge en kritisk vurdering over for hvilke adjuvantia, der inddrages ved en given immunisering, idet valget af adjuvant er væsentligt for forløbet af recipientens reaktion.

Yhteenveto / K. Pelkonen

Eniten käytetyt immunologiset adjuvantit jaetaan neljään pääryhmään ominaisuuksiensa perusteella. Artikelissa käsitellään pääpiirteittäin näitä ominaisuuksia sekä kunkin aineen käyttöön liittyviä mahdollisia sivuvaikutuksia. Kirjoittajat suosittavat, etta tutkija suorittaisi immunisoinnin yhteydessä käytettävän adjuvantin valinnassa kriittistä arviointia, koska adjuvantin valinnalla on olennainen vaikutus vastaanottajan reaktioihin.

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