P. Jollès · A. Paraf

# Chemical and Biological Basis of Adjuvants

P. Jollès · A. Paraf

# Chemical and Biological Basis of Adjuvants

With 24 Figures

Chapman & Hall Limited · London
Springer-Verlag Berlin · Heidelberg · New York
1973

*Professor Dr. Pierre Jollès*

Directeur de Recherche, Centre National de la Recherche Scientifique (C.N.R.S.),
Laboratoire de Biochimie, Université de Paris VI, Paris, France

*Professor Dr. Alain Paraf*

Directeur de Recherche, Laboratoire de Virologie et d'Immunologie,
Institut National de la Recherche Agronomique, Paris, France

ISBN 0-412-10180-7 Chapman & Hall Ltd. London

ISBN 3-540-06308-0 Springer-Verlag Berlin Heidelberg New York
ISBN 0-387-06308-0 Springer-Verlag New York Heidelberg Berlin

# Contents

# Introduction and Historical Notes

What are adjuvants? As WHITE [1967 (2)] said, "the list of them reads like a medieval alchemist's shopping list." We would like first to give a clear and simple definition which yet takes into account the complexities of a field where many authors have used the same words for completely different concepts. RAMON (1926), whose primary goal was the enhancement of antibody synthesis against diphtheria or tetanus toxoid, called "adjuvant and immunity stimulating substances" products which, used in combination with specific antigen vaccines, enhance immunity levels above those that the vaccines are capable of developing when injected alone. Even at that early date, he concluded: "Si intéressant que soit ce procédé du point de vue pratique, il ne l'est pas moins du point de vue théorique, à cause des recherches qu'il peut susciter pour essayer de pénétrer le mécanisme intime, soit de l'augmentation d'antitoxine ainsi provoquée, soit de l'élaboration des antitoxines au sein de l'organisme". In other words, RAMON thought that adjuvants could be used as a tool to gain new insights into the machanism of antibody response. FREUND (1956), without giving any definition, emphasized the different manifestations of adjuvant effect:

(a) enhancement of antibody formation and alteration of sensitization to proteins;

(b) sensitization to simple chemical compounds;

(c) allergy.

Munoz (1964) defined an "adjuvant" as a substance that enhances the antibody response to antigen injected either simultaneously with it or within a period of time close to the injection of the antigen. This meaning is extended to all substances that enhance hypersentitivity reactions that are directly related to antibodies, or suspected to be associated with the antibody response. WHITE [1967 (1)] applied the term "adjuvant" (L. adjuvare: to help) only to substances which, when injected together with antigen (i) convert an apparently nonantigenic substance to an effective antigen, (ii) increase levels of circulating antibody, (iii) lead to the production of delayed hypersentitivity or to its increase, and (iv) lead to the production of certain disease states such as thyroiditis, aspermatogenesis, allergic encephalomyelitis, adrenalitis or arthritis and iridocyclitis.

As we have seen from the different definitions, the manifestations of adjuvant effects are numerous, and to the antibody response, delayed hypersensitivity and allergic diseases we could add homograft reactions, some growth processes, inducement of plasmocytoma, ascites, and interferon synthesis.

Although an adjuvant may literally help the immune response, we feel compelled to discuss opposite effects too, since such very well-known adjuvants as Freund's adjuvant have been observed to lower the immune response (JANKOVIC, 1963). More recently extensive work has been done on phytohemagglutinin adjuvant activity; some authors have concluded that these heterogeneous substances exert an enhancing effect on the immune response [GAMBLE, 1966 (2); SINGHAL *et al.*, 1967],

while others have observed an opposite effect (MARKLEY *et al.*, 1967; JASIN and ZIFF, 1968).

Therefore the definition of an adjuvant must have two aspects. From a practical point of view, as pointed out by WHITE [1967 (1)], it refers to substances enhancing the immune response, whatever this may be.

From a theoretical point of view it should refer to any substance which acts on (i) the nonspecific part of the antigen, called "adjuvanticity" by DRESSER (1961), and (ii) the nonspecific activity of the cells involved in the immune response (mainly macrophages and lymphocytes) by enhancing cell multiplication or by stimulating cell transformation.

It is obviously difficult to make a clear distinction between such products as (i) phytohemagglutinin, which is able to enhance DNA, RNA and protein synthesis besides possessing blastogenic capacity; (ii) Freund's adjuvant, which stimulates hormonal secretions in addition to its activity on macrophages and lymphocytes; (iii) such hormones as somatotropin or folliculin, which enhance protein synthesis (including synthesis of globulin); and (iv) mitotic drugs, which will not be included as adjuvants. Thus we could define as adjuvants or immunity-stimulating substances *any product which acts (i) on a hapten or an antigen by enhancing its antigenic properties, or (ii) on the cells involved in the immune response (this being understood as including antibody synthesis, anaphylaxis, delayed hypersensitivity, allergic diseases, and graft reactions immunized).*

RAMON [1925 (1)] noticed a correlation in immunized horses between a local abscess at the site of antigen injection and a high level of antibodies; he demonstrated that it was possible to artificially increase diphtheria or tetanus antitoxin levels by adding substances such as bread crumbs, aleurone seeds, agar, tapioca, starch oil, lecithin.

SORDELLI and SERPA (1925) reported the antigenic value of the precipitate that occurs when diphtheria toxin and specific antitoxin are mixed together. HARTLEY (1952) and GLENNY (1926) pointed out that such a precipitate was antigenic and had a higher immunizing value than the supernatant liquid. Lipovaccines were discovered by LE MOIGNIC and PINOY [1916 (1, 2)]. LEWIS and LOOMIS (1924) observed that antibody formation against various antigens was remarkably intense in guinea pigs which had received an injection of living virulent tubercle bacilli into the peritoneal cavity a few days before they were given antigens. These lipovaccines were also used by RAMON and ZOELLER (1927), WALSH and FRAZER (1934), COULAUD (1935), and SAENZ (1937),

With these observations as a foundation, FREUND (see FREUND *et al.*, 1937) started his classic experimentation on the production of delayed hypersensitivity and on antibody synthesis which showed that the two effects of the allergic irritability due to the tuberculous infection, namely the enhancement of antibody production and the alteration of sensitization, can be reproduced in the absence of tuberculosis (FREUND and MCDERMOTT, 1942). FREUND reviewed these observations in 1947 and 1956.

The powerful antibody-stimulating effect of Freund's adjuvant has not been equalled by any other adjuvant. This mixture has made it possible to produce antibodies even in animals considered poor producers, such as rats (HAVAS and ANDRE, 1955) or mice (ANACKER and MUNOZ, 1961; MUNOZ, 1963), and to induce autoallergic diseases (KABAT *et al.*, 1946; MORGAN, 1946). The next step of the investi-

gations on adjuvant activity was the isolation of active material from various bacteria: *B. pertussis* (ELDERING, 1942), Salmonella (RIBI *et al.*, 1959), and Mycobacteria (RAFFEL, 1948, 1950; WHITE *et al.*, 1955; LEDERER, 1959). Finally for 10 years scientists have tried to understand the activity of adjuvants on nonantigenic molecules (DRESSER, 1961; SELA *et al.*, 1962) or the activity of adjuvants on the immunocompetent cells (UNANUE *et al.*, 1969; NOWELL, 1960).

The plan of this book is as follows:

I Study of the substances exhibiting adjuvant activity with special reference to the preparation of a crude material from the Mycobacteria.
II Detailed study of the adjuvant-active waxes D of Mycobacteria.
III Correlation between chemical structure and adjuvant activity.
IV Biological activity of adjuvants.
V Mechanism of adjuvant activity.
VI Practical use of adjuvants.

# List of Abbreviations

| | |
|---|---|
| Ala | Alanine |
| Glu | Glutamic acid |
| Gly | Glycine |
| DAP | Meso-$\alpha$, $\alpha'$-diaminopimelic acid |
| Ara | Arabinose |
| Gal | Galactose |
| Glc | Glucose |
| Lip | Lipid, lipidic moiety |
| Myc | Mycolic acid |
| PA | "Nitrogen-containing" moiety (peptide + amino sugar) |
| Poly | Polysaccharide moiety |
| HSA | Human serum albumin |
| BSA | Bovine serum albumin |
| HGG | Human gamma-globulin |
| BGG | Bovine gamma-globulin |
| OA | Ovalbumin |
| PHA | Phytohemagglutinin |
| BCG | Calmette and Guérin bacillus (non pathogenic Mycobacteria) |
| PFC | Plaque-forming cells |
| EAE | Experimental allergic encephalomyelitis |
| Poly A:U | Polyadenylic-polyuridylic acid |

Chapter I

# Substances Exhibiting an Adjuvant Effect: Preparation of "Crude Material"

Many adjuvants, or components of adjuvants, occur among the pharmaceutical emulsifying agents, which are surface-active substances capable of stabilizing an oil-water interface. This seems to be a property common to many adjuvants. They are very often made up of *separate lipophilic and hydrophilic moieties* which is what makes them surface-active. Mycobacteria are among the best adjuvants used and have been widely studied. We deal first with some of their components.

## A. Cell Walls of Mycobacteria

Mycobacterial cell walls are known to be adjuvants and to induce delayed hypersensitivity [KOTANI *et al.*, 1960; LARSON *et al.*, 1963 (1)]. They seem chemically and antigenically more complex than other bacterial walls so far studied. The characteristic feature of their chemical composition is their high lipid content, as shown by KOTANI *et al.*, (1959); significant amounts of free lipids can be removed by treatment with hot acetone before chemical disintegration of the cells.

### 1. Preparation of Cell Walls

Many techniques have been employed for isolating cell walls from *Mycobacterium tuberculosis*. Most of these are based on mechanical disintegration followed by differential centrifugation and treatment with proteolytic enzymes (CUMMINS and HARRIS, 1958; RIBI *et al.*, 1958; RIBI *et al.*, 1959; KOTANI *et al.*, 1959; KANAI and YOUMANS, 1960; BELKNAP, CAMIEN and DUNN, 1961). The preparation of cell walls from the following strains has also been described: *M. phlei* (TAKEYA and HISATSUNE, 1963; DE WIJS and JOLLÈS, 1964), *M. butyricum* (LARSON *et al.*, 1963(1)], *M. tuberculosis*, BCG [LARSON *et al.*, 1963 (1); MISAKI *et al.*, 1966], *M. fortuitum* and *M. kansasii* (DE WIJS and JOLLÈS, 1964), *M. smegmatis* (PETIT *et al.*, 1969).

### 2. Composition and Structure of the Walls

(DE WIJS and JOLLÈS, 1964; MISAKI *et al.*, 1966; PETIT *et al.*, 1969)

The analyses of different cell wall preparations are presented in Table 1. Walls from undefatted cells had high lipid (48.7%) and low nitrogen (5.36%) contents compared with those from defatted cells.

1. *Amino acids.* The main components of purified cell walls are: Ala, Glu and DAP (8 to 12%); for most cell walls the molar ratios are approximately 3:2:2,

respectively (Table 2). They are very similar to those found in different waxes D of Mycobacteria.

2. *Amino sugars.* The major amino sugars found in the mycobacterial cell walls are N-acetylglucosamine and N-acetylmuramic acid; the molar ratios are nearly 1:1 (Table 2). Traces of galactosamine have been detected in the cell walls of *M. kansasii.*

3. *Reducing neutral sugars.* Chromatography of the hydrolyzed cell walls revealed that the main sugar constituents were arabinose and galactose (Table 1). In some cases, glucose and traces of mannose could be detected.

4. *Chemical structure studies.* (See for comparison Chap. II, part D). PETIT *et al.* (1969) studied the structure of the cell walls of *M. smegmatis.* A tripeptide Ala-Glu-

Table 1. Composition of cell walls of several Mycobacteria (DE WIJS and JOLLÈS, 1964; MISAKI *et al.*, 1966)

| | Bound lipids | Neutral reducing sugars[a] | Amino[b] sugars | Amino acids | |
|---|---|---|---|---|---|
| | | | | Ala, Glu, DAP | Others |
| | % | % | % | % | % |
| *M. tuberculosis var. bovis* BCG | 31.0 | 31.2[c] | 2.58 | [d] | |
| *M. phlei* | 14.0 | 9.8 | 3.39 | 8.00 | 2.05 |
| *M. smegmatis* | 33.0 | 30.0[e] | 8.5 | 12.4 | |
| *M. fortuitum* | 24.0 | 11.9 | 1.67 | 3.65 | 1.32 |
| *M. kansasii* No. 4 | 31.3 | 25.3 | 4.65 | 10.28 | 1.38 |

[a] Calculated as galactose.
[b] Calculated as glucosamine, HCl.
[c] Molar ratios: Gal (1.00); Glc (0.71); Ara (2.12).
[d] Nitrogen content of amino acids present in the walls: 3.71%.
[e] Molar ratios: Gal (1); Ara (2).

DAP, a tetrapeptide Ala-Glu-DAP-Ala, and also a tetrasaccharide and a disaccharide which gave equimolecular amounts of glucosamine and muramic acid after hydrolysis, were obtained. The disaccharide can be distinguished from the typical $\beta$-1,4-N-acetylglucosaminyl-N-acetylmuramic acid by its $R_f$ value in different solvents, as the muramic acid residue is N-glycolylated, instead of N-acetylated (ADAM *et al.*, 1969). The peptide subunits of mycobacterial cell walls were identified by mass spectrometry by WIETZERBIN-FALSZPAN *et al.* (1970).

## 3. Digestion of Cell Walls by Enzymes

Lysozymes (EC 3.2.1.17) of different origins (bird egg-whites, human milk) are able to digest mycobacterial cell walls [KOTANI *et al.*, 1963 (1); DE WIJS and JOLLÈS, 1964; ADAM *et al.*, 1972]. Other enzymes, such as the $L_{11}$ enzyme separated from culture supernatants of *Flavobacterium* spp. and pronase, also attack these cells. (See also preparation of adjuvant active "bound wax D", Section 5.)

Table 2. Content of major amino acids and amino sugars of mycobacterial cell walls (DE WIJS and JOLLÈS, 1964)

| Amino acid or amino sugar | *M. phlei* | | *M. fortuitum* | | *M. kansasii* | | *M. smegmatis* |
|---|---|---|---|---|---|---|---|
| | µmole/mg | Molar[a] ratio | µmole/mg | Molar[a] ratio | µmole/mg | Molar[a] ratio | Molar[a] ratio |
| Ala | 0.263 | 3.0 | 0.10 | 3.0 | 0.327 | 3.0 | 1.6 |
| Glu | 0.19 | 2.2 | 0.10 | 3.0 | 0.251 | 2.3 | 1.0 |
| DAP | 0.142 | 1.65 | 0.055 | 1.65 | 0.179 | 1.65 | 1.0 |
| Gly | 0.027 | 0.31 | 0.032 | 0.9 | 0.038 | 0.35 | tr. |
| Glucosamine | 0.076 | 0.87 | 0.035 | 0.98 | 0.079 | 0.71 | 1.0 |
| Muramic acid | 0.06 | 0.70 | 0.032 | 0.90 | 0.095 | 0.87 | 1.0 |
| Galactosamine | 0 | 0 | 0 | 0 | 0.01 | 0.09 | — |

[a] Based on alanine = 3.0.

### 4. Biological Properties

WHITE *et al.* (1958) showed that delipidated bacterial cells of bovine, avian and saprophytic strains of Mycobacteria have high adjuvant activity. Similar observations were made by different authors and especially by MISAKI *et al.* (1966); the BCG-cell wall preparations were able to increase the resistance of mice to staphylococcal infection to a value as high as that of the intact BCG cells. Both the periodate-degraded walls and the mucopeptide fraction elicit in mice a resistance to staphylococcal infection similar to that produced by the original cell walls. This observation of MISAKI *et al.*, (1966) clearly indicates that the mucopeptide is responsible for the protective effect of BCG cells. In another experiment, the mucopeptide fraction was shown to produce tuberculin-type hypersensitivity when injected intradermally into guinea pigs sensitized with killed BCG. However, skin tests in guinea pigs showed that the mucopeptide fraction failed to induce the delayed hypersensitivity. This result, together with analytical data concerning the amino acid composition, suggested to MISAKI *et al.* (1966) that the delayed hypersensitivity elicited by the cell walls is due mainly to a protein moiety associated with the mucopeptide. This suggestion is not in accordance with the demonstration that "bound wax D" is a cell wall constituent which can produce a delayed type of hypersensitivity [KOTANI *et al.*, 1963 (1)]. BONHOMME *et al.* (1969) were able to induce experimental arthritis (termed adjuvant arthritis) in rats with cell walls of *M. tuberculosis var. hominis*, strain Test, of *M. tuberculosis var. bovis*, strain BCG and of *M. avium*.

### 5. "Bound Wax D"

[KOTANI *et al.*, 1963 (2)]

BCG cells were extracted with neutral organic solvents at room temperature to obtain "delipidated" BCG cells; these latter were submitted to successive treatments with hen egg white lysozyme and the $L_{11}$ enzyme produced by *Flavobacterium* spp. These enzyme treatments rendered about 40% of the cell wall material soluble. Solubility tests in organic solvents and various physical and chemical determinations (see Chap. II, Part E) demonstrated that the insoluble residue consisted mainly of materials essentially identical to the wax D fractions isolated from human type *M. tuberculosis*. The residue was therefore designated "bound wax D", and its adjuvant activity was tested. It was shown that the "bound wax D" fraction exhibits a marked enhancing effect on both the production of circulating antibody and the development of a delayed type of hypersensitivity when injected with egg white albumin or hen egg white lysozyme into guinea pigs.

## B. Wax D Fractions of Mycobacteria

There are two possible interpretations of the chemical nature of the adjuvant component: (1) the adjuvant activity is related to a chemically well-defined substance which is present in different amounts in different bacterial cells; (2) the difference in the adjuvant activity of the assayed bacteria is due to differences in the chemical structure of the active principle. Only Mycobacteria have been intensively studied as regards point (2). We shall now try to establish whether the adjuvant activities of different strains of Mycobacteria can be related to a well-defined compound.

## 1. Paraffin Oil Extracts

COULAUD (1934) and SAENZ (1939) observed that paraffin oil extracts of dead cells of Mycobacteria possess some adjuvant activity. It was thus suggested that some lipids may be involved in this activity. The main work in this area was done by CHOUCROUN (1939, 1946, 1947, 1948), who described the preparation of a biologically active fraction called "Pmko"; according to ASSELINEAU, CHOUCROUN and LEDERER (1950), this fraction was a mixture containing mycolic acids (45%) and nitrogen- and phosphorus-rich lipopolysaccharide (55%).

Paraffin oil extraction of BCG cell walls also gave [LARSON *et al.*, 1963 (2)] biologically active extracts.

RAFFEL (1948) found that bacilli which had the lipids removed by chloroform extraction lost the ability to induce tuberculin hypersensitivity, although the protein antigenicity of the treated bacillary bodies remained intact. By adding extracted constituents to these defatted bacilli, it was found that a fatty substance obtained by ANDERSON (1941) as "purified wax fraction" was essential for the sensitizing process.

## 2. Extraction of Wax Fractions from Mycobacteria: the Biologically Active Wax D Fraction

Recent studies intended to define the active principle of Mycobacteria have used materials prepared by prolonged extraction with neutral organic solvents of cultures usually 4 to 6 weeks old. Extraction with an ethanol-ether mixture followed by chloroform, in accordance with the procedure developed by ANDERSON (1927, 1929, 1941), yields a series of waxy materials. ASSELINEAU and LEDERER (1953) described the preparation of four waxy substances referred to as waxes A, B, C, and D (Table 3). Wax A, which contains mainly phtiocerol dimycocerosate, is soluble in a mixture of alcohol-ether (1:1, v/v) and can be separated from waxes B, C and D, which are soluble in chloroform. Wax B can be separated as indicated in Table 3; it is also soluble in cold acetone. It is mainly composed of phtiocerol, glycerol and esters of fatty and mycolic acids. As wax D remains insoluble in boiling acetone, it can finally be separated from wax C, which is soluble in this solvent; wax C contains a high proportion of "cord factor", which is trehalose dimycolate (NOLL *et al.*, 1956) (Fig. 1).

RAFFEL (1950) demonstrated that wax D from the human strain Test of *M. tuberculosis*, when mixed with tuberculo-protein, induces tuberculin hypersensitivity. When wax D was isolated, it was hoped that the adjuvant active principle of Mycobacteria had been obtained. However, WHITE *et al.* (1958) established that only wax D fractions from human strains of *M. tuberculosis* possessed this activity, *i.e.* the ability to induce delayed hypersensitivity to a protein antigen (ovalbumin) and to produce allergic encephalitis. Many mycobacterial extracts, other than wax D, were tested and found inactive.

These observations made it necessary to further purify wax D from Mycobacteria, called at this stage "crude wax D" (Chap. II, part A). Later, wax D from other strains of *M. tuberculosis* (var. bovis) [MIGLIORE and JOLLÈS, unpublished data, 1969 (2); MIGLIORE *et al.*, 1971] and other Mycobacteria *(M. kansasii)* (WHITE *et al.*, 1964) were also found to be active (see Chap. III).

Table 3. Extraction of waxes A, B, C und D based on the work of ASSELINEAU and LEDERER (1953)