The Biology of the Mycobacteria

Volume 2
Immunological and Environmental Aspects

Edited by
COLIN RATLEDGE and JOHN STANFORD

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Preface

After a volume on the inate complexities of the mycobacteria themselves must come that on the complexities of their relationships with their environment, animate and inanimate. For this second volume perhaps a title of "Natural History" would be better than "Biology", for in it is given an account of the ecology of mycobacteria, the effect they have on their surroundings and how these surroundings effect them. Of course the mycobacteria present the usual conundrum of the chicken and the egg; did they evolve, by natural selection, a system for overcoming immunological defences, or are they the evolutionary pressure to which our immunity is still measuring up? Arguments in support of either contention might be put forward.

The last chapter on diseases of possible mycobacterial aetiology underlines the amount we still have to find out. A growing number of mycobacteriologists have a suspicion that there is a form of mycobacterium that is not readily recognized, but which is present, together with normal forms of the bacilli, in the tissues of patients with the accepted mycobacterioses. These much less obvious forms may be the prime aetiological agents of several ideopathic diseases, such as sarcoidosis and Crohn's disease. Our knowledge of these diseases is approaching the position of Villemin, in the last century, with respect to tuberculosis, but we still await the new Koch to convincingly demonstrate the causative organisms.

The philosophy underlying this volume differs from that of the first, since our lack of knowledge in the fields covered have different effects. In most of the aspects of mycobacteria considered in Volume 1, that which is "known" about them is likely to be true, and that which is not known may be discovered, whereas in this voume, that which is "thought" about them may be fifty per cent or more wrong, and tomorrow's views may be diametrically opposed to those of today. Thus the authors of this volume were asked to express personal rather than consensus opinions and although some of them have done their best to hide their opinions, in most cases the discerning reader will discover them. Most of the chapters cover rapidly expanding fields of research and there is no doubt that in the event of the publication of a second edition some radical changes can be expected. Yet if the best that can be said of this book in the future is that it represented the views of a few workers in the early 1980s, let us also remember that those of us in this centenary year of the discovery of the

tubercle bacilli, who have looked back on the views of 100 years ago, have been staggered by what old Koch knew!

The personal opinions requested of the authors have led to a little, but necessary, repetition from chapter to chapter as different views are expressed on the same topics. Since at this time there is no way of knowing whose opinions are the right ones, the editors did not consider that over rigorous editing was appropriate in order to present a unified opinion.

With any multi-author book the greatest difficulty is experienced in getting all the typescripts in on time, and in this case several deadlines came and went before all chapters were received. The editors beg the forgiveness of those who met the first deadline and will forbear from mentioning the names of those who met the last. However, the editors wish to thank those who updated their chapters after they reasonably thought that they had finished with them.

No less than for the first volume is it right that we should crave indulgence from our wives and children for the time spent with papers spread everywhere and for the frayed tempers that arose. This we humbly do.

During the later stages of this volume being prepared we were very gratified to receive enthusiastic response to our first volume which appeared in the Spring of 1982. Strong representations were made to us to consider editing a third volume which would be devoted to the clinical aspects of mycobacterial disease. This has met with considerable encouragement from our publishers, so much so, that as this volume finally goes to press, we can say we hope this can be done. Given co-operative authors, the concluding volume of this trilogy should appear in the next two or three years.

Spring 1983

John Stanford
Colin Ratledge

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Immunological Aspects

1

Immunologically Important Constituents of Mycobacteria: Adjuvants

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I. Introduction

The first half of the 1970s has witnessed the completion of a further stage in the complex pattern of mycobacterial adjuvant research. Consequently it is a most opportune moment to consider this important facet of the mycobacterial organism. The term adjuvant is derived from *adiuvare* Latin "to help". Munoz (1964) in a general review defined an adjuvant as "a substance which enhances the antibody response to antigens injected simultaneously with it or within a period of time closely spaced to the injection of antigen". This definition could be extended to include the stimulation of cell-mediated responses against protein antigens and the production of allergic or autoimmune reactions.

II. Early Developments

The initial event, like so many in science, which founded adjuvant research was an accident. Tuberculous guinea pigs were inoculated with sheep erythrocytes and higher levels of sheep haemolysin antibodies were obtained. This preliminary observation of Lewis and Loomis (1924) was repeated under controlled conditions. Guinea pigs were inoculated with 10 μg living *Mycobacterium bovis* intraperitoneally and subsequently with 5.0 ml of a 20% suspension of sheep erythrocytes intraperitoneally and the same amount subcutaneously 21 days later. A secondary injection of erythrocytes was given to surviving animals 33 days after the primary injection. A small peak of haemolysin production was evident after about 9 days which was followed by a sharp fall. However, instead of a continued decline in the level of haemolysin there was a second increase beginning about day 12 and continuing to a second peak at 22 days which was followed by a further sharp decline (Fig. 1). In tuberculous guinea pigs there was a 20-fold increase in the production of haemolysin.

The curve of the haemolysin production in normal guinea pigs when plotted on an expanded scale showed apical points which were coincident with similar points in the curve from tuberculous animals (Fig. 2). After a second antigen

injection tuberculous guinea pigs reacted similarly to the normal animals but on a much higher level. The authors concluded that the effect of tuberculosis was to stimulate a normal mechanism of antibody production. A similar effect was achieved in guinea pigs given tubercle organisms killed by heating at 60°C for 1 hour. An intraperitoneal injection of 2.0 mg *M. bovis*, 2 days before sheep erythrocytes, stimulated an increased production of haemolysin antibodies although the effect was lower than that found in tuberculous guinea pigs.

In a succeeding paper Lewis and Loomis (1926) showed that tuberculous rabbits infected with 50 μg *M. bovis* were also stimulated to produce higher levels of haemolysin. The establishment of an infection of considerable, but not of overwhelming, severity was required before the adjuvant effect was observed in rabbits.

These experiments were expanded by Dienes and Schoenheit (1926, 1927a,b) during their studies on hypersensitivity reactions in tuberculosis.

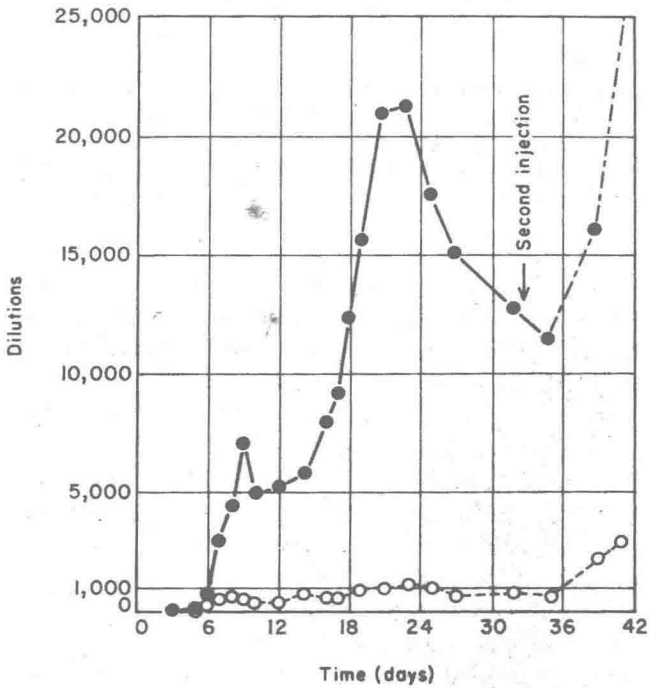

FIG. 1. Anti-sheep erythrocyte serum titres in normal (- - - -) and tuberculous (——) guinea pigs. Tuberculous guinea pigs were infected 21 days prior to the zero point of the chart. At zero days all animals were given 5 ml of 20% sheep erythrocyte suspension intraperitoneally and the same amount subcutaneously; a booster injection was given on day 33 (Lewis and Loomis, 1924). Reproduced by kind permission of the Rockefeller University Press publishers of the *Journal of Experimental Medicine*.

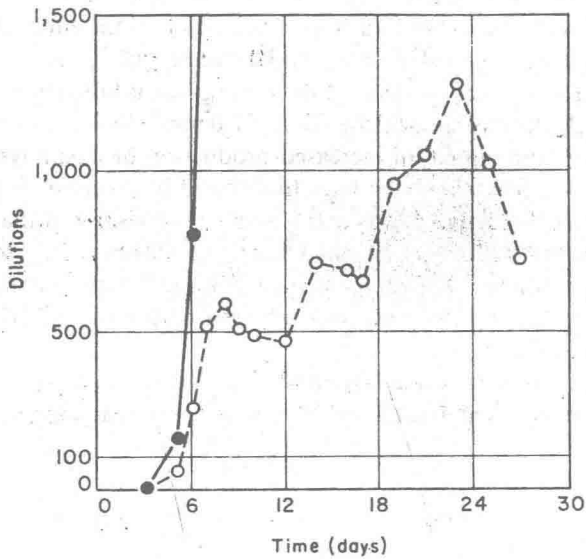

FIG. 2. Same observations as in Fig. 1 using an expanded scale to show that there are significant fluctuations in the curve of anti-sheep haemolysin for normal (- - - -) animals and that the apical points are coincident with similar points in the tuberculous (——) curve. (Lewis and Loomis, 1924). Reproduced by kind permission of the Rockefeller University Press publishers of the *Journal of Experimental Medicine*.

Guinea pigs were injected with various amounts (0.03–50 mg) of egg-white and higher antibody levels were detected in tuberculous than in non-tuberculous animals. There was no complement fixation with the crystalline albumin component of egg-white. Sera from tuberculous animals contained high levels of precipitating antibodies (1 in 10^6 titre) against egg-white, crystalline egg-albumin and egg-globulin. The precipitation with the sera of animals treated with globulin and crystalline egg albumin was strongly specific to the globulin and albumin. At the same time, it was noticed that animals with high levels of serum-precipitating antibodies showed hypersensitivity reactions to egg-white. Non-tuberculous guinea pigs sensitized with egg-white produced the usual oedematous reactions of the Arthus type which were maximal within a few hours and disappeared in 24–48 hours. This reaction corresponded to the *immediate* hypersensitivity reaction described by Zinsser (1921), and could be passively transferred to normal animals by means of serum (Dienes, 1927). In sensitized tuberculous guinea pigs the hypersensitivity initially resembled that of the non-tuberculous animals but later gave rise to a persistent necrotic reaction. Although this hypersensitivity induced by ordinary protein antigens was similar to the tuberculin or *delayed* type of hypersensitive response it differed in essential characteristics. In the

majority of experiments the guinea pigs had precipitins against egg-white in the serum. The hypersensitive animals were not killed by the subcutaneous injection of 0.1–0.5 mg egg-white although an extensive necrosis developed at the site of injection, but intravenous injection of egg-white was fatal because of anaphylaxis.

Hanks (1935) noted that both the *immediate* and *delayed* (tuberculin type) hypersensitivity reactions occurred successively in the same skin test site and generally a delayed necrosis was required to confirm the latter reaction.

A strong delayed reaction against egg-white was induced in guinea pigs which were given repeated daily injections of 1–2 mg *M. tuberculosis* strain 05 into the groin to form a firm nodule of tuberculous tissue and three injections of egg-white (0.05, 0.2 and 0.2 mg) mixed with tubercle bacilli at 2-day intervals into this nodule to sensitize the animals (Dienes, 1928a). The injection of the antigen directly into the tuberculous lesion stimulated a better immune response than either a subcutaneous or intravenous injection into a tuberculous animal (Dienes, 1928b). The author initially concluded that proliferation of cells producing antibody and inflammatory reactions in the tuberculous lesion was directly responsible for the adjuvant effect. Subsequently, however, he reversed this idea (Dienes, 1929) because the antigen was found to stimulate a response without the lesion.

III. Freund's Adjuvant

From 1937 to the time of his death in 1960 Jules Freund and his colleagues were among the leaders in mycobacterial adjuvant research. Dienes was once his colleague so he was conversant with the earlier work and the parallel investigations pursued in France by Coulaud and Saenz on the induction of tuberculin hypersensitivity by killed tubercle bacilli mixed with paraffin oil. Both lines of research were amalgamated by Freund in a series of studies on delayed-type hypersensitivity to protein antigens which ultimately led to the formulation of Freund's incomplete and Freund's complete adjuvant mixtures. Such water-in-oil emulsions are widely used now in experimental immunization procedures and as they vary in composition it is basically incorrect to designate them all as Freund's adjuvant. It is important to qualify the use of this term with the precise formulation of the emulsion.

A. FREUND'S INCOMPLETE ADJUVANT

As early as 1916 Le Moignic and Pinoy described the beneficial effect of preparing bacterial vaccines in water-in-oil emulsions; vaseline oil and lanolin from camphor were used. The inclusion of the derivative from camphor prevented the formation of an abscess at the site of injection. Ramon *et al.*

(1937) introduced the term *adjuvant* in their studies on active immunity in diphtheria and tetanus. A variety of substances was incorporated into the vaccines and efficient mixtures contained vaseline or olive oil mixed with cholesterol or lanolin. Subsequently, the use of water-in-oil emulsions in active immunization procedures against influenza virus by Friedewald (1944a,b) and Henle and Henle (1945) led to greater attention being directed towards the oil components and the emulsifier. Halbert *et al.* (1945, 1946) successfully used Atreol 9, a light mineral oil, and Falba, a lanolin derivative, in a *Shigella* vaccine. From the studies on influenza immunization Salk *et al.* (1952) selected Drakeol 6-VR* as the most suitable naturally occurring oil for use in emulsified vaccines. The use of Bayol F† caused development of persistent local reactions at the site of injection in human beings which took a year or more to resolve (Philip *et al.*, 1954). These reactions were attributed to the variability in the toxicity of different batches of oil due to their complex nature (Sachanen, 1954; Franks, 1961). The crude oils contain paraffins (alkanes, saturated non-cyclic hydrocarbon chains), cycloparaffins (naphthenes, saturated cyclic hydrocarbon rings), olefins (unsaturated hydrocarbon chains) and aromatic hydrocarbons. Himmelweit (1960) found that vaccines of low viscosity gave as good an antibody response as those of higher viscosity and were less likely to produce local reactions. Wilner *et al.* (1963) rejected the use of straight-chain hydrocarbons with low carbon number because of their irritating properties as measured by guinea-pig skin tests. As the carbon number increased, skin irritation decreased and this was associated with the increase in melting point. Straight-chain hydrocarbons ($n C_{15}H_{32}$–$n C_{20}H_{42}$) could be substituted for the mineral oil in Freud's adjuvants. The short-chain solvent hydrocarbons ($n C_6H_{14}$–$n C_{13}H_{28}$) were not effective and produced local inflammatory reactions, and long-chain hydrocarbons ($n C_{22}H_{46}$–$n C_{24}H_{50}$) which are solid at body temperature were also ineffective (Shaw *et al.*, 1964). Stewart-Tull *et al.* (1976) showed that the most effective pure, fully saturated hydrocarbons in water-in-oil emulsions were $n C_{16}H_{34}$–$n C_{19}H_{40}$. Mineral oils containing predominantly short-chain hydrocarbons instead of showing adjuvant activity with the protein antigen, caused immunosuppression.

Woodhour *et al.* (1961) compared the effectiveness of peanut, chaulmoogra, safflower, sesame and poppyseed oils but found that mineral oils were more effective. Freund (1951) had shown that plant oils were degraded

* Drakeol 6-VR mineral oil is composed of a complex mixture of normal, cyclic and branched-chain hydrocarbons and aromatics. No polynuclear aromatics which are known carcinogens were found. Monobutyl and dibutylphthalate were present in small quantities (Murray *et al.*, 1972).

† Bayol F (Bayol 55) is a highly refined mineral oil, free of unsaturated components and asphaltenes, consisting of 42.5% paraffins, 31.4% monocyclic naphthenes and 26.1% polycyclic naphthenes.

by animal tissue lipases although mineral oils were not and this may account for the lack of activity generally found with vegetable oils. Some stress has been laid upon the nature of the emulsion in Freund's incomplete adjuvants but as will be noticed in Section VII.A (pg. 47) this is an important factor in the control of biological activity and toxicity of water-in-oil emulsions.

The practicalities of preparing mineral-oil adjuvants were described in detail by Herbert (1978).

B. FREUND'S COMPLETE ADJUVANT

Vallée (1924) used vaseline in vaccines against bovine tuberculosis and enteritis. However, Thompson (1922) was the first person to investigate the influence of tuberculin on the formation of antibodies against sheep erythrocytes in rabbits. He found that the injection of tuberculin prior to the injection of erythrocytes stimulated an increased production of haemolysin. The synergistic stimulating effect of melted paraffin was recorded by Coulaud (1934) in the sensitization of rabbits to the tubercle bacillus. This effect was compared with the synergic action of a *Schlepper* with lipoidal partial antigens by Swift and Schultz (1936). Saenz (1935a) continued this work and stimulated an intense tuberculin hypersensitivity in guinea pigs. The animals were given three oral administrations of 100 mg of dead *M. bovis* "Vallée" mixed in 1.0 ml of vaseline. Some of the emulsion entered the trachea and sensitization occurred by contact with the mucous membrane in the lungs. Similar levels of sensitization were achieved by subcutaneous injection of 1.0 mg of *M. bovis* or intramuscular and intratesticular injections of 100 mg of *M. bovis* in vaseline or paraffin oil (Saenz, 1935b, 1937). Sensitization was less with vegetable oils and animal fats such as lanolin. With this background, Freund recognized the opportunity to study delayed-type hypersensitivity and antibody production to protein antigens and commenced a series of investigations which dominated the study of mycobacterial adjuvants from 1937 to 1959.

Freund *et al.* (1937) recognized the immunopotentiating effect of paraffin oil on antibody production by the chance use of sera from guinea pigs sensitized with killed mycobacteria as a source of complement in a tuberculin complement fixation test. Mycobacterial antibodies were found to be present in higher titres which persisted for 20 months in the sera of these animals. Sensitization to picryl chloride was achieved by Landsteiner and Chase (1940) using one or two intraperitoneal injections of killed tubercle bacilli suspended in paraffin oil followed by an injection of picryl chloride soon afterwards.

Freund and McDermott (1942) overcame the difficulty of influencing sensitization to protein antigen (horse serum) with killed tubercle bacilli suspended in paraffin-oil by combining the antigen in the aqueous phase with