Medical Microbiology Volume 2

Immunization Against Bacterial Disease

edited by

C. S. F. EASMON J. JELJASZEWICZ

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C. S. F. EASMON
Wright Fleming Institute
St Mary's Hospital Medical School
London, UK

J. JELJASZEWICZ
National Institute of Hygiene
Warsaw
Poland

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Contributors

J. La Brooy
Department of Medicine
University of Adelaide, Adelaide
South Australia 5000

B. Cohen
Department of Dental Science
Royal College of Surgeons
of England, Downe
Orpington, Kent BR6 7JJ

C. E. Frasch
Bureau of Biologics
Food and Drug Administration
Bethesda, MD 20205, USA

J. E. Heckels
Department of Microbiology
Southampton General Hospital
Tremona Rd
Southampton SO9 4XY

R. J. Jones
MRC Vaccine Research Laboratories
Clinical Research Block
Medical School
Vincent Drive
Birmingham B15 2TJ

R. Parton
Microbiology Department
Glasgow University
Glasgow G11 6NU

S. L. Peach
Department of Dental Science
Royal College of Surgeons
of England, Downe
Orpington, Kent BR6 7JJ

G. Peter
Division of Biology and Medicine
Brown University
Providence, Rhode Island

G. A. W. Rook
Department of Microbiology
School of Pathology
Middlesex Hospital Medical School
London

D. Rowley
Department of Microbiology and
Immunology, University
of Adelaide, Adelaide
South Australia 5000

R. R. B. Russell
Department of Dental Science,
Royal College of Surgeons
of England, Downe
Orpington, Kent BR6 7JJ

G. Schiffman
Department of Microbiology and
Immunology, Downstate
Medical Center, State University
of New York
New York 11203

J. L. Stanford
Department of Microbiology
School of Pathology
Middlesex Hospital Medical School
London

D. E. S. Stewart-Tull
Department of Microbiology
Alexander Stone Building
Garscube Estate, Bearsden
Glasgow G61 1QH

A. C. Wardlaw
Microbiology Department
Glasgow University
Glasgow G11 6NU

P. J. Watt
Department of Microbiology
Southampton General Hospital
Tremona Rd
Southampton SO9 4XY

S. H. Zinner
Department of Medicine
Roger Williams General
Hospital, Rhode Island, USA

Preface

The chapters in this second volume, although covering a wide variety of infections, are linked by the theme of immunization. The increase in resistance to antimicrobial agents and the realization that there is never going to be a final solution to this problem, coupled with the increase in our knowledge of cellular immunology and pathogenic mechanisms, have led to renewed interest in immunization. Prevention is better than cure and may be significantly more cost effective.

In selecting these ten topics we have tried to bridge the gap between the clinical and the experimental. Some of the vaccines are in clinical use, others are still at the developmental stage. The first four chapters deal as much with the theoretical basis for future clinical developments as with current practical exploitation. With pertussis and cholera there is room for improvement in terms of toxicity and efficacy respectively while both cholera and dental caries involve new problems of antigen presentation and the stimulation of local immunity. Meningococcal, pneumococcal and pseudomonas vaccines have proved to be very effective, but there are still problems with group B meningococci, with very young children and with the immunocompromised.

Charles Easmon *Janusz Jeljaszewicz*
June 1983

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1 Immunopotentiating products of bacteria

DUNCAN E. S. STEWART-TULL

I. INTRODUCTION

A. Terminology

An adjuvant substance was defined by early research workers as one which stimulated the production of increased levels of antibody against protein antigens. This simplistic connotation was changed when it was demonstrated that, in addition, adjuvants could stimulate cell-mediated (delayed-type hypersensitivity) or allergic responses against protein antigens and autoimmune reactions against homologous or heterologous tissue antigens.

In recent years, adjuvant substances have been shown to cause either an increase in the immune response (immunopotentiation) or a decrease in the immune response (immunodepression). The latter effect diminishes the philological value of the term adjuvant, from the Latin adiuvare (to help), and more frequently, these substances are being referred to as immunomodulating agents.

Recent discussions (World Health Organization, 1976; Edelman *et al.*, 1980; Edelman, 1980) indicate that the criteria for safe immunopotentiating agents requires the addition of numerous factors to control the harmful effects of an otherwise active substance. An immunopotentiating agent should be able to increase the immunogenic potency of vaccines, possibly containing poorly immunogenic or low relative molecular mass or genetically manipulated antigens, administered in small quantities and number of doses. The immunopotentiating agent must not (a) induce hypersensitivity reactions to the host's own tissues or to itself; (b) contain cross-reactive

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antigens with human tissue; (c) be carcinogenic, teratogenic or abortogenic; (d) be contaminated with substances that might stimulate other immunological events not involved in the specific response; (e) be non-biodegradable in the human body; (f) produce harmful nodules or abscesses if administered intramuscularly (i.m.) or subcutaneously (s.c.) and (g) be unstable in the manufactured vaccine for a period of at least 2 years.

Ideal immunopotentiating agents would be those fulfilling these criteria and, in addition, those capable of triggering the most suitable arm of the immune response, i.e. either humoral immunity or cell-mediated immunity.

These are indeed harsh conditions to impose on the adjuvant researchers and in the search it will be necessary to be cognizant of the biological activities of established, if unacceptable, adjuvants. Therefore, this aspect will form the predominant theme in this chapter so that in the long-term new immunopotentiating substances, e.g. derivatives of muramyl dipeptide, will be rigorously tested to ensure that any harmful activity associated with adjuvanticity is removed. In this respect, the biological activities of the natural, polymeric substances isolated from bacteria will provide the foundation and justification for the commercial use of their synthetic counterparts.

It will be impossible to cover all bacteria used as adjuvants, so consideration will be given predominantly to those species where there is major documentation on biological activity and to active substances derived from such bacteria, especially mycobacteria (e.g. the classical Freund's complete adjuvant). Unfortunately, the mode of action of these immunopotentiating substances is too complex to include here, but there are some reviews that lead into this area (Paraf, 1970; Jollès and Paraf, 1973; Allison, 1973; Dresser and Phillips, 1973; White, 1973, 1976; Frost and Lance, 1973, 1978; Chedid *et al.*, 1978; Waksman, 1979; Stewart-Tull, 1983).

B. Adjuvants from non-bacterial sources

1. Adsorbents

Initial studies showed that antigens adsorbed on particulate matter stimulated the production of antibodies more efficiently than an injection of the same amount of unadsorbed antigen (Ramon, 1925; Glenny *et al.*, 1926). The addition of 0·4% potassium alum to 2·3 g formal diphtheria toxoid produced a precipitate (0·24 g) which increased the antitoxin response in guinea-pigs (Glenny *et al.*, 1926). When 0·1 ml of the alum precipitate

containing 4 Lf units of diphtheria toxoid was injected, guinea-pigs became Schick-negative 12–14 days later. Hektoen and Welker (1933) found similar results after a single i.m. injection of aluminium hydroxide containing 0·1% protein antigen. For example 203 days after injection the anti-ovalbumin precipitin titre was 1·0 $\log_{10}$ 4–5. Relyveld (1980) injected mice with 0·5 ml calcium phosphate—tetanus toxoid (6·1 IU ml^{-1}) or aluminium hydroxide—tetanus toxoid (15 IU ml^{-1}) and detected 5–15 antitoxic units in 1·0 ml serum.

Various other adsorbents have been used to produce an adjuvant effect, among them 4% phosphorylated hesperidin (a glycoside, $C_{22}H_{26}O_{12}$) (Moss *et al.*, 1956), silica particles (Vigliani and Pernis, 1959; Antweiler, 1959; Pernis and Paronetto, 1962; Mancino and Bevilacqua, 1977, 1979; Mancino and Ovary, 1980), sodium alginate (Scherr *et al.*, 1965), calcium phosphate (Relyveld and Raynaud, 1967), and chrome alum, $KCr(SO_4)_2.12H_2O$ (Ando, 1953; Ando and Shimojo, 1957).

Alginic acid (polymerized mannuronic acid) is isolated from seaweed in the form of sodium alginate. In the presence of a calcium salt, a coagulum of insoluble calcium alginate is formed. Formol diphtheria toxoid (5 Lf) was injected into guinea-pigs in 1·0% sodium alginate, the animals were boosted 14 days and 28 days after the primary injection. A mean of 0·51 serum antitoxin units ml^{-1} was found compared to the group of animals given toxoid in saline, 0·03 units ml^{-1}. In another experiment in which animals were given two doses containing 2·8 Lf diphtheria toxoid, the mean antitoxin levels were 8·3 and 15·0 units ml^{-1} for alginate and aluminium phosphate respectively (Amies, 1959).

At present, the only safe, non-toxic chemical adjuvants used as human vaccine additives are aluminium hydroxide gel, aluminium phosphate and calcium phosphate, although these are not effective with all antigens. Further information on the earlier use of aluminium compounds in vaccines for human beings can be found in reviews by Aprile and Wardlaw (1966) and Joó (1972).

2. *Oil and water mixtures*

Ramon *et al.* (1935) showed that vaseline oil, lanolin and cholesterol were effective adjuvants in mixtures containing tetanus toxoid. Guinea-pigs injected with tetanus toxoid in vaseline oil containing 2·5% cholesterol produced 20 IU antitoxin 6 weeks after the primary injection. Rabbits produced similar levels of antitoxin after injection of the toxoid in vaseline oil containing 1·5% cholesterol. After injection of toxoid alone, the levels of antitoxin produced in guinea pigs and rabbits were 3·0 and 0·33 IU respectively. Incorporation of diphtheria toxoid into a mixture of equal parts of lanolin and vaseline oil stimulated a twenty-fold increase in the

production of antitoxin, 40·0 IU, compared with animals injected with diphtheria toxoid alone.

3. *Saponin*

This is a glycoside of quillaiac acid containing D-glucose, which is soluble in water, forms emulsions with oils and prevents the deposition of small particulate matter. It complexes the cholesterol in cell membranes and causes haemolysis of erythrocytes. Because of toxicity, its adjuvant use is confined to animal vaccines. Guinea-pigs or rabbits injected with a mixture of diphtheria toxoid containing 3·0% saponin (20 saponin in 100 ml water, w/v) produced 30 IU and 7 IU diphtheria antitoxin respectively. Similar experiments with tetanus toxoid showed antitoxoid levels of 30 IU and 20 IU respectively (Ramon *et al.*, 1935). It is a more efficient adjuvant than alminium hydroxide gel in mice for parasite antigens. With DNP-keyhole limpet haemocyanin, it stimulates the production of IgG_{2a} and IgG_{2b} in mice (R. Bomford, personal communication).

The current Wellcome tetanus vaccine is a suspension of highly purified toxoid prepared by formalin detoxification of *Clostridium tetani* exotoxin adsorbed onto aluminium hydroxide. This vaccine should be injected deep s.c. or i.m. and the manufacturer's warn that the intradermal (i.d.) route should not be used because of the formation of a persistent skin nodule at the injection site. Although uncommon, headache, lethargy, malaise, myalgia, pyrexia, anaphylaxis, urticaria, angioneurotic oedema, serum sickness and peripheral neuropathy *may* occur.

II. IMMUNOPOTENTIATING PROPERTIES OF BACTERIAL CELLS OR THEIR PRODUCTS

A. Early studies

The presence of a variety of whole organisms, from different bacterial strains, in a vaccine may stimulate increased levels of antibodies against unrelated antigens. Clark *et al.* (1922) found that intravenous (i.v.) injections of heat-killed Gram-positive cocci had a stimulatory effect on the production of agglutinins to *Salmonella typhi.* Schroeder (1923) showed that higher haemolysin titres were obtained from the sera of rabbits which developed abscesses at the site of immunization; a similar effect was observed in animals infected with *Streptococcus pneumoniae.*

Schultz and Swift (1934) sensitized rabbits with either intracutaneous (i.c.) or i.v. injections of non-haemolytic or haemolytic streptococci before

the injection i.c. of horse serum. The animals reacted more strongly and the precipitin titre increased to $1{\cdot}0 \log_{10}4$ after 9 days, although skin hypersensitivity was induced against the streptococci.

Burky (1934) and Swift and Schultz (1936a,b) repeated these observations with staphylococcal toxin instead of the streptococci. Weakly antigenic, ragweed pollen induced a marked immune response in rabbits when injected with staphylococcal toxin (Burky, 1934). A similar increase in precipitin titre was found after the i.c. injection of beef lens extract and a non-toxic dose of staphylococcal crude toxin (Swift and Schultz, 1936a); a delayed-type hypersensitivity to the lens antigen was also stimulated (1936b). Animals injected with killed *Pseudomonas aeruginosa*, *Escherichia coli* or *Salmonella paratyphi* B prior to the injection of *Salmonella enteritidis* produced a ten-fold increase in the agglutinin titre (Khanolkar, 1924).

Ramon and Zoeller (1926, 1927) and Ramon (1931, 1936, 1937) found that *Salmonella* TAB vaccine increased the production of antitoxin against tetanus or diphtheria toxoid in both animals and man. The TAB vaccine did not act as an adsorbent of the tetanus toxoid since no loss in potency of the toxoid was detected if it was tested for several days after the addition of TAB vaccine. The tetanus antitoxin response in guinea-pigs was much higher if the two doses were injected at the same site compared to the response obtained if the two doses were injected at different sites (Barr, 1956). Ramon *et al.* (1950) also found that *Brucella abortus* cells had a stimulatory effect on the production of diphtheria and tetanus antitoxins. The immunopotentiating activity associated with these whole cells may appear to be unrelated, but they do contain surface polymers such as lipopolysaccharide and peptidoglycan, subsequently shown to be adjuvant-active.

B. *Bordetella pertussis*

Pertussis vaccine appears to contain three distinct adjuvant substances: (a) the peptidoglycan, (b) the lipopolysaccharide (LPS) which is common to all Gram-negative bacteria and is heat (100°C) stable and (c) a unique heat-labile (80°C) component associated with histamine-sensitizing factor (HSF) and leukocytosis promoting factor (LPF).

B. pertussis vaccine mixed with diphtheria toxoid stimulated antitoxin production, as monitored by the change of the patients from Schick-positive to -negative (Greenberg and Fleming, 1947, 1948; Fleming *et al.*, 1948). As the number of *B. pertussis* organisms increased in the injection mixture, up to $1{\cdot}6 \times 10^{11}$, with 40 Lf doses of diphtheria toxoid, there was

an associated stimulation of the immune response. With 3·5 Lf doses of diphtheria toxoid, Barnes and Holt (1955) found a 4·4-fold increase in antitoxin response with 1×10^9 *B. pertussis*, but only a 1·2-fold increase with 2×10^9 organisms.

In 1957, Kind showed that the intraperitoneal (i.p.) injection of female white mice with 0·1 ml *B. pertussis* vaccine plus 0·05 ml of a 5% suspension of chicken erythrocytes stimulated the production of low levels of agglutinins, maximum titre 128. This adjuvant response persisted for at least 85 days. No agglutinins were detected when the chicken erythrocytes were injected alone. In addition, 40% of the mice produced symptoms of anaphylactic shock after i.p. challenge with 1·0 ml of 5% chicken erythrocytes and of these animals, some 20% died. The adjuvant and anaphylaxis stimulation were not necessarily linked because in some experiments, no agglutinins were detected but 18/37 animals died after challenge. This sensitization required the injection of *B. pertussis* i.p.: no effect was observed after s.c. injection of *B. pertussis* vaccine and chicken erythrocytes.

The relationship between either *B. pertussis* whole cells, LPS, or lipid A and an adjuvant response to diphtheria toxoid was examined by Farthing (1961). Antitoxin levels were increased forty-fold in guinea-pigs injected with $5–10 \times 10^8$ *B.pertussis* organisms mixed with 5 Lf doses of diphtheria toxoid. If the whole organisms were replaced by 1·75 μg phenol-water extracted LPS (equivalent to 1×10^9 bacteria) a similar increase in antitoxin production was noted. The lipid A (50 μg) which accounted for 19% of the LPS, stimulated a nine-fold increase in the antitoxin response. Although these results indicated the importance of LPS (Section IIF) Pittman (1957) had previously found the adjuvant activity of *B. pertussis* whole cells to be greater than that expected from their LPS content alone. None would now dispute this, in view of the studies with peptidoglycan adjuvants (Section IIG). Further evidence that the activity of *B. pertussis* was not solely due to the presence of the LPS moiety was provided by Finger *et al.* (1971) who showed that LPS-mediated immunosuppression could be overcome by *B. pertussis* cells.

Farthing (1961) found no reduction in the guinea-pig adjuvant response with 5 Lf doses of diphtheria toxoid if the *B. pertussis* vaccine was heated at 100°C for 1 h. On the other hand, Pieroni and Levine (1966), after heating the vaccine at 100°C for 40 min, showed that mice did not withstand a challenge with 15 times the minimal lethal dose of tetanus toxin. They concluded that the HSF, protective antigen and heat-labile adjuvant substance, were identical (Pieroni and Levine, 1967). These authors noted the increase in antitoxin levels with 0·8–6·4 Lf doses of tetanus toxoid and unheated *B. pertussis* vaccine, but less protection was afforded to those

mice given toxoid s.c. plus heated vaccine i.p. than toxoid alone. These contradictory results may be complicated by the guinea-pig's lack of sensitivity to HSF (Stronk and Pittman, 1955) and the extreme sensitivity shown by the mouse. In addition, Finger (1975) pointed out that the timing of the *B. pertussis* vaccine in relation to the antigen was crucial; the organisms had to be injected together with the antigen if an adjuvant response was to be stimulated by the i.v. or i.p. routes. A similar observation was made by Murgo and Athanassiades (1975) who found that the haemagglutinin response to sheep erythrocytes was stimulated when the *B. pertussis* vaccine was injected at the same site (Fig. 1). Munoz

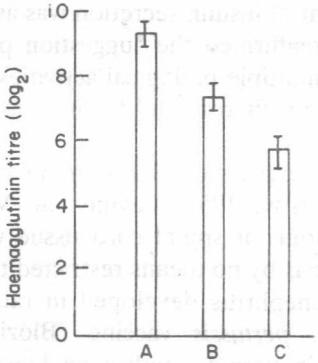

Fig. 1 The effect of heated *B. pertussis* vaccine on the production of haemagglutinins to sheep erythrocytes. Each point represents the mean titre of 11 C57Bl mice bled 3 weeks after injection. Vertical lines indicate ± standard error of the mean. A, Pertussis vaccine + SRBC; B, Heated pertussis vaccine + SRBC; C, SRBC alone (Murgo and Athanassiades, 1975; reproduced by kind permission of the authors and the American Society for Microbiology, publishers of Infection and Immunity).

(1973) found that 4·0 mg ovalbumin and 2×10^9 *B. pertussis* cells were required to stimulate a titre of 8000 anti-ovalbumin antibody in a passive haemagglutination test. By comparison, 0·1 mg ovalbumin was sufficient to stimulate a titre of 90 000 in the presence of a water-in-oil emulsion containing *Mycobacterium phlei*. The adjuvant activity and susceptibility of mice to anaphylaxis was examined after the i.p. injection 1·0 mg of ovalbumin combined with 2×10^9 *B. pertussis* cells. Thirty days later, all animals died of anaphylactic shock after i.v. challenge with 0·5 mg ovalbumin in 0·2 ml saline. No deaths occurred in mice challenged after 4 days, the optimum period for the development of histamine sensitivity, so there appeared to be little relation betwwen the adjuvant factor and the HSF. However, this conclusion was challenged by the experimental work of

Wardlaw *et al.* (1979). The *B. pertussis* C mode vaccine, which lacks HSF and LPF was found to be poorly active in either the elicitation of experimental allergic encephalomyelitis (EAE) in Lewis rats with 25 mg heterologous spinal cord or in the stimulation of IgE antibodies against 200 μg ovalbumin. By contrast, the protective X-mode vaccine stimulated IgE in 17/20 mice and a pronounced encephalomyelitis in rats. An extracellular protein with a relative molecular mass of 77 000 was isolated by Mizushima *et al.* (1979) from the culture fluid of *B. pertussis*. This protein possessed islet-activating activity (Yajima *et al.*, 1978), HSF, LDF and adjuvant activity. The protein was effective in stimulating IgE antibodies against 1·0 mg DHP-Ascaris antigen at a dosage of 25 μg protein/kg rat, but the enhancement of insulin secretion was associated with hypoglycaemia. These results reaffirmed the suggestion previously made that a single component with multiple biological activities existed in *B. pertussis* (Levine and Pieroni, 1966; Pieroni and Levine, 1966; Lehrer *et al.*, 1975, 1976; Munoz, 1976).

The stimulation of an experimental autoimmune disease was originally observed (Lee and Olitsky, 1955; Levine and Wenk, 1961) when *B. pertussis* vaccine and brain or spinal cord tissue were injected i.p. into mice. This characteristic is by no means restricted to *B. pertussis* adjuvant (see p. 30). Glomerulonephritis developed in rats injected with homologous kidney and *B. pertussis* vaccine (Blozis *et al.*, 1962). The vaccine was also used in various studies on hyperacute EAE with the antigen either in aqueous mixture (Levine and Wenk, 1965; Levine *et al.*, 1965) or in water-in-oil emulsions (Wiener *et al.*, 1959; Shaw *et al.*, 1964; Levine and Wenk, 1967). Rats injected with guinea-pig spinal cord plus *B. pertussis* vaccine or Freund's complete adjuvant, also developed EAE (Lennon *et al.*, 1976). It would seem unlikely that the ability to stimulate experimental allergic encephalomyelitis is related to the infrequent brain damage associated with *B. pertussis* vaccine in infants as the latter effect occurs too quickly and is non-immunological in nature (A. C. Wardlaw, personal communication).

Farthing and Holt (1962) demonstrated that the adjuvant effect was important in the primary immune response and also that the presence of *B. pertussis* vaccine in the injection mixture decreased the minimal stimulating dose of antigen. *B. pertussis* stimulated the secondary immune response only when the organisms were omitted from the primary injection. This was an important finding because many research workers have found that the dose of adjuvant in experimental animal vaccines can greatly influence the modulation of the immune response between potentiation and depression.

After the injection of 5×10^6 sheep erythrocytes into mice, Finger *et al.*

(1969) found few direct or indirect spleen plaque-forming cells. However, if animals were given 3×10^9 *B. pertussis* cells at the same time, there was a 9-fold increase in direct and a 17-fold increase in indirect plaque forming cells after 23 days. In this study, it was reported that the effect on the secondary response was insignficant when *B. pertussis* was used as the adjuvant. Previously, it was proposed that the adjuvant effect was due to an accelerated and prolonged multiplication of B cells (Finger *et al.*, 1967). Subsequently, Finger *et al.* (1973) showed that B-T cell cooperation was required to produce an adjuvant effect because the pronounced increase in IgG-producing cells was lacking in mice with congenital aplasia of the thymus. This was not entirely surprising since, in this instance, a T-dependent antigen was used.

Further detail of the relationship of adjuvant activity to other known biological activities can be found in reviews by Finger (1975), Olson (1975), Morse (1976) and Wardlaw and Parton (1982).

C. *Corynebacterium parvum*

C. parvum has been shown to have similar properties to *C. acnes* and taxonomically shows a close homology to the *Propionibacteria* and *P. acnes* (Johnson and Cummins, 1972; Cummins and Johnson, 1974).

In 1964, Halpern and his colleagues injected animals with the heat-killed anaerobic diphtheroid, *Corynebacterium parvum*, and found splenomegaly and hepatomegaly due to proliferation of macrophages and lymphocytes. O'Neill *et al.* (1973) also found that an intraperitoneal injection of 250 μg *C. parvum* caused an increase in the spleen and liver weight and this was associated with an increase in the rate of phagocytic uptake of carbon particles. Essentially similar conclusions were reached by Warr and Sljivić (1974a,b). Cummins *et al.* (1981) exposed *C. parvum* to various chemical and enzymatic treatments and concluded that the capacity to cause splenomegaly was due to polysaccharide determinants on the surface of the organism; possibly amino sugars with free-NH_2 groups. The growth phase was important, usually late logarithmic phase. Pringle and Cummins (1982) showed that the addition of chloramphenicol to a culture accelerated the ability to induce splenomegaly, and penicillin reduced the activity.

Howard *et al.* (1973) suggested that the adjuvant effect of *C. parvum* noted by Neveu *et al.* (1964) and by Biozzi *et al.* (1966) was mediated by activated macrophages. Watson and Sljivić (1976) confirmed this since there were 7200 plaque-forming cells to sheep erythrocytes in unfractionated mouse spleen tissue cultured in Marbrook chambers, but the number dropped to 290 if the adherent cells were removed. The latter were also

resistant to 600R X-irradiation *in vitro*, both observations indicated the importance of the macrophage.

O'Neill *et al.* (1973) examined the adjuvant effect of *C. parvum* in mice, guinea-pigs and chickens. In mice, the injection of 250 μg human serum albumin (HSA) and 250 μg *C. parvum* heat-killed cells in saline or water-in-oil emulsion did not stimulate an increased production of antibodies, in either the primary or secondary immune responses. However, when guinea-pigs were injected with 1·0 mg ovalbumin in a water-in-oil emulsion containing 200 μg *C. parvum*, there was stimulation of the levels of precipitating antibodies produced. It appeared that the IgG_2 class of antibody, typically stimulated with a Freund complete adjuvant was not produced.

These authors found no evidence that the *C. parvum* caused the stimulation of an associated cell-mediated immune response to the protein antigen. This is quite unlike the situation found with mycobacterial products, in the same species, where characteristically one expects both arms of the immune response to be stimulated, see p. 16. When chickens were injected into the breast muscle with 500 μg heat-killed *C. parvum*, either in saline or water-in-oil emulsion containing 1·0 mg HSA, there was no evidence of an adjuvant effect (Fig. 2A) as judged by a comparison with antigen in a water-in-oil emulsion. There was, however, a strong adjuvant

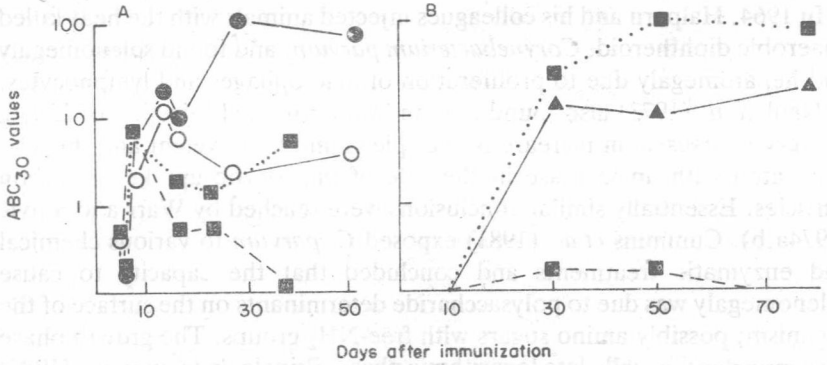

Fig. 2 A. Comparison of serum antibody responses in the chicken after an intramuscular injection of 1·0 mg HSA with (■ · · · · · · ■) *C. parvum* in water-in-oil emulsion (■ – – ■) *C. parvum* in saline, (● ——— ●) *M. avium* in water-in-oil emulsion, or (○ ——— ○) water in oil emulsion alone (O'Neill *et al.*, 1973; published by kind permission of Blackwell Scientific Publications Ltd., publishers of *Immunology*). B. Comparison of serum antibody responses in mice after a subcutaneous injection of 50 μg BSA with (■ · · · · · · ■) *C. parvum* in water in oil emulsion, (■ – – – ■) *C. parvum* in saline or (▲ ——— ▲) FCA containing *M. butyricum*. (Bomford, 1980; published by kind permission of Blackwell Scientific Publications Ltd., publishers of *Immunology*).