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# The Role of Anaerobic Coryneforms on Specific and Non-specific Immunological Reactions

## I. EFFECT ON PARTICLE CLEARANCE AND HUMORAL AND CELL-MEDIATED IMMUNOLOGICAL RESPONSES

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**Summary.** A wide range of different strains of anaerobic coryneforms and classical propionibacteria have been surveyed for some of their macrocytostimulant effects (included under this term are the ability to increase the rate of phagocytic uptake of carbon after intravenous injection of the latter into mice, ability to stimulate an increase in lysosomal hydrolases and ability to exert directly a chemotactic stimulus on macrophages) and their ability to increase humoral and cellular immunity when admixed with an immunogen.

Of twenty-one strains of anaerobic coryneforms tested, fifteen strains were able to produce an increase of the phagocytic index in mice of at least 50 per cent. Micro-organisms that were effective in these tests occurred in each of the four main serological groups. Although no single serological group showed outright superiority in causing an increase of *K*, three of the five strains which had highest activity belonged to serological group I. Four organisms representing the 'classical' propionibacteria were also tested, but none of these caused an increase of phagocytic index in mice.

Many of the strains that stimulated a rise in phagocytic index also caused an increase in the weight of the spleen measured at 10 days after injection and a moderately good correlation was apparent between the magnitude of the two effects.

Tests in mice and chickens for an adjuvant action on serum levels of antibody failed to show any enhancement of primary responses, although a barely significant elevation of the secondary response in the mouse was observed. In the guinea-pig, a clear adjuvant effect on the levels of serum antibody in a primary response was observed. However, no evidence could be obtained that any of several anaerobic coryneforms was able to enhance cell-mediated hypersensitivity, as shown by delayed-type skin tests or corneal reactions.

A study of the histological responses to a footpad injection of various anaerobic coryneforms (in water-oil emulsion with ovalbumin) showed that the regional (popliteal) node underwent a considerable expansion of the lymphoid-cell content of the paracortical (thymus-dependent) area. This was accompanied by extensive sinus plugging by lymphocytes and endothelial hypertrophy of post-capillary venules.

## INTRODUCTION

In recent years a micro-organism designated *Corynebacterium parvum* has been used as an adjuvant for stimulating the phagocytes of the reticuloendothelial system (Halpern, Prévot, Biozzi, Stiffel, Mouton, Bouthillier and Décreusefond, 1964) for the inhibition of tumour growth (Halpern, Biozzi, Stiffel and Mouton, 1966; Woodruff and Boak, 1966; Smith and Woodruff, 1968; Fisher, Grace and Mannick, 1970), and for the production of autoimmunization (Halpern and Fray, 1969; McCracken, McBride and Weir, 1971). However, further study of these phenomena is hampered by a lack of bacteriological criteria for the identification of the biologically active organism as *C. parvum*, and the lack of evidence for a taxonomic identity or relationship between any of the variety of organisms used by the above authors. Johnson and Cummins (1972) have questioned whether or not there is an organism which should be referred to as *C. parvum*.

In this communication a wide range of anaerobic coryneform organisms and classical propionibacteria have been submitted for comparative tests of biological activity in stimulating a rise of systemic phagocytic particle-clearance activity, in causing an increase in mass of lymph nodes and spleen, and in increasing humoral and cell-mediated immunity in a variety of animal species.

## MATERIALS AND METHODS

*Sources of various micro-organisms*

The original bacterial strains which were used in a study of macrocytostimulant activity (ability to increase the clearance of carbon in mice) (White, Eslami and Aiyedun, 1971) were kindly provided by Burroughs Wellcome Laboratories (strains A, B and C *C. parvum*). Dr Adlam (personal communication) states that these three strains were all derived from an N.C.T.C. culture with the designation *C. parvum* 10387.

Many further strains (tabulated in Table 1) were kindly provided by Professor C. S. Cummins, Anaerobe Laboratory, College of Agriculture and Life Sciences, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, U.S.A. and by Professor Sebald, Institut Pasteur, Paris. Examples of 'classical propionibacteria' were obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland.

*Culture methods for micro-organisms*

Organisms which in general were received as lyophilized cultures were grown on solid medium (brain-heart infusion) for 5 days in an anaerobic jar in order to check purity. Organisms for continuation cultures were selected from a single colony by sub-culture in thioglycollate medium (U.S.P.) (Oxoid, London). Cultures were maintained at 37° for 10 days. The culture was sterilized by heat at 56° for 30 minutes; the cells were harvested by centrifugation and washed thrice in 0·15 M saline. Finally they were freeze dried and stored at room temperature.

*Method for determination of phagocytic index in mice*

Mice (Porton, females at 3–4 months old) were injected intravenously with a gelatin-stabilized suspension of carbon (obtained from Gunther Wagner, Hannover) in a dose of 16 mg/100 g. The kinetics of disappearance of the carbon from the blood were determined by a series of bleedings from the retro-orbital venous plexus. Each sample of 0·025 ml

obtained by pipette was diluted and haemolysed in 2 ml of distilled water, before estimation of optical density in a colorimeter (Gallenkamp, London).

$K$ (phagocytic index) was determined as the slope of the straight line derived by plotting the optical density values expressed as logarithms against time in minutes, according to the equation $K = \frac{\log_{10} \frac{C_t}{C_o}}{t}$ where $C_t$ = concentration of carbon at time $t$, $C_o$ is the starting concentration of carbon at time 0. The average value for a group of four or five mice was determined.

TABLE 1

CULTURE COLLECTION NUMBERS AND SOURCES OF STRAINS OF ANAEROBIC CORYNEFORMS AND CLASSICAL PROPIONIBACTERIA

| Strains used | No. in culture collection | Source* | Alternative strain numbers | | | |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 |
| *C. parvum* | 10390 | 1 | 10390 | 5936 | — | 643-C |
| *C. parvum* | 10387 | 1 | — | 5888 | — | — |
| *C. parvum* | A | 2 | — | 5888 | — | — |
| *C. parvum* | B | 2 | — | 5888 | — | — |
| *C. parvum* | C | 2 | — | 5888 | — | — |
| *C. parvum* | 0208 | 3 | — | — | 0208 | 2683 |
| *C. parvum* | 3085 | 4 | — | — | — | 3085 |
| *C. parvum* | 1383 | 4 | — | — | 0207 | 1383 |
| *C. parvum* | 6134 | 2 | — | 6134 | — | Not divulged |
| *C. acnes* | 737 | 1 | 737 | — | 0389 | — |
| *C. acnes* | 6280 | 2 | — | 6280 | — | — |
| *C. liquefaciens* | 814 | 4 | — | — | 0200 | 814 |
| *C. diphtheroides* | 2764 | 4 | — | 6295 | 0186 | 2764 |
| *C. liquefaciens* | 6290 | 2 | — | 6290 | — | 3044-B |
| *C. granulosum* | 6290 | 2 | — | 6290 | — | 3024-B |
| *C. lymphophilum* | 6294 | 2 | — | 6294 | — | — |
| *C. anaerobium* | 578 | 4 | — | — | 0162 | 578 |
| *P. granulosum* | 0507 | 3 | — | — | 0507 | — |
| *P. avidum* | 0575 | 3 | — | — | 0575 | — |
| *P. avidum* | 0589 | 3 | — | — | 0589 | — |
| *P. avidum* | 4982 | 3 | — | — | 4982 | — |
| *P. freudenreichii* | 10470 | 1 | 10470 | — | — | — |
| *P. jensenii* | 5960 | 5 | — | — | — | — |
| *P. arabinosum* | 8901 | 5 | — | — | — | — |
| *P. rubrum* | 5958 | 5 | — | — | — | — |

* Source.
1. National Collection of Type Cultures, Colindale Avenue, London.
2. Burroughs Wellcome Laboratories, Beckenham, Kent.
3. Professor C. S. Cummins, Virginia Polytechnic and State University, Blacksburg, Virginia, U.S.A.
4. Professor Sebald, Pasteur Institute, Paris.
5. National Collection of Industrial Bacteria, Aberdeen, Scotland.

Organisms for test were injected, as heat-killed organisms in saline suspension, intraperitoneally in 0·2 ml of pyrogen-free saline (0·15 M) at a dose of 250 $\mu$g (dry weight) ten days before the injection of carbon suspension. The mice were maintained on a pellet diet in a thermostatically controlled environment at 21°.

*Measurement of hypertrophy of lymphoid tissue*

After the completion of each carbon-clearance test, the mice were killed by ether inhalation and dissected to provide the liver and spleen which were quickly dried on filter paper and weighed as individual organs.

*Tests of adjuvant activity in mice*

The tests in mice made use of female, pure strain BALB/c mice which were derived from a pathogen-free nucleus but maintained under clean, conventional conditions. Animals were generally injected intraperitoneally with 250 μg of each sample of heat-killed micro-organism (dry weight) in 0·2 ml of saline (0·15 M NaCl), together with 250 μg of human serum albumin (HSA: Reinste, Behringwerke, Germany).

Certain groups of animals were injected with both micro-organisms and HSA combined in water-in-oil emulsion. This was prepared by mixing to a white cream an equal volume of a saline solution of HSA with Arlacel (mannide mono-oleate)/Drakeol mixture (9 parts Arlacel to 1 part by volume Drakeol). The dose of HSA per mouse was 250 μg and the volume of water-in-oil emulsion was 0·2 ml. Drakeol was obtained from Pennsylvania Refining Company and Arlacel from Honeywill and Stein, Ltd.

Serum samples (0·25 ml) were obtained by bleeding from the retro-orbital venous plexus at various intervals after a single immunizing injection. Some groups of mice were boosted after 16 days or, later in the series, 3 weeks, with an injection of 250 μg HSA in 0·2 ml saline (0·15 M NaCl) intraperitoneally, and bled 10 days later.

Anti-HSA levels in serum were determined by the haemagglutination technique as described by Herbert (1973)

*Tests of adjuvant activity in guinea-pigs*

Batches of three to five guinea-pigs (usually female) were selected to form a test group for an individual bacillary sample. A similar number of animals of the same age and sex formed the control group. Animals belonging to test and control groups were allotted randomly to individual cages. Control guinea-pigs were injected into the left foot-pad with 1 mg of ovalbumin (thrice-crystallized, Armour) in 0·2 ml water-in-oil emulsion. The water-in-oil emulsion was prepared in the proportions by volume of saline solution of antigen : Arlacel A : Drakeol = 1 : 1 : 3. Test animals were injected similarly into the foot-pad with the same water-in-oil emulsion with added 200 μg, 1 mg or 2·5 mg dose of bacilli to the Drakeol.

Guinea-pigs were fed a pellet diet with added daily supplement of fresh cabbage and carrots. At 18 days after injection they were tested by an injection of 10 μg, 50 μg or 200 μg ovalbumin in 0·1 ml saline intradermally into the clipped skin of the flank. Swelling of the skin was measured as the double-skin thickness at 3 hours afterwards by means of a Schnell-taster dial calliper gauge fitted with circular flat end pieces (System Kröplin Type AO2T, H. C. Kröplin, Schüchtem, Hessen, West Germany).

At 18 days guinea-pigs were tested by an intracorneal injection of ovalbumin into the left eye under local anaesthesia by means of 2 per cent cocaine hydrochloride (Herbert, Horne and White, 1970). Readings were made 24 and 48 hours afterwards.

Guinea-pigs were killed 21 days after injection. In some instances the left foot-pad, left popliteal lymph node, a left iliac lymph node, spleen and liver were taken for histological examination after fixation in 10 per cent formalin in saline.

Immunoelectrophoretic analysis of guinea-pig sera (21 days after injection) was set up in 0·8 per cent Ionagar No. 2 (Oxoid) in barbitone buffer, 0·05 M, pH 8·4. After electrophoresis, the trough on one side of the separated serum protein bands was filled with ovalbumin at a strength of 100 μg/ml in phosphate buffered saline (0·15 M NaCl, 0·01 M $PO_4'''$, pH 7·2). The trough on the other side was filled with rabbit anti-guinea-pig

globulin serum. Precipitin arcs were allowed to develop for 36 hours and the plates then washed and stained.

*Tests for adjuvant activity in chickens or hens*

Birds were chosen for these tests since mycobacteria have been shown previously to provoke a new and unique delayed peak of antibody (French, Stark and White, 1970) in hens. Earlier tests used white leghorn female birds 5–6 weeks old (chickens). Later tests used older birds, 12–15 weeks old (hens).

In an initial experiment five groups of chicken (three birds, 23 day old, per group) were injected with 0·2 ml of a water-in-oil emulsion containing 1 mg HSA intramuscularly into the left breast. The emulsion consisted of Drakeol : Arlacel : saline solution of HSA in the proportions (by volume) of 3 : 1 : 1.

The groups received (i) heat-killed *C. parvum* 0208 (Serol. group 1) 500 μg in 0·2 of the above antigen mixture; (ii) heat-killed *C. parvum* 10390 (Serol. group 2) 500 μg in 0·2 of the above antigen mixture; (iii) heat-killed *C. parvum* 10387 (Serol. group 3) 500 μg in 0·2 of the above antigen mixture; (iv) heat-killed *Propionibacterium granulosum* 0507 (Serol. group 3) 500 μg in 0·2 of the above antigen mixture and (v) no addition of micro-organisms (controls).

In the second experiment two groups of chickens were used in a test for possible adjuvant effect by *C. parvum* 0208 when administered as a saline suspension of 1 mg in 1·0 ml, containing 2 mg HSA, intravenously. The control group of chickens was treated at the same time with injections which were similar but lacking *C. parvum* organisms.

In the third experiment, two groups of chickens were used: one was injected intramuscularly into a breast muscle with 0·2 ml of a water-in-oil emulsion, made up in the proportions (by volume) of 1 : 1 : 3; the other (control groups) received the same volume of water-in-oil emulsion containing 2 mg HSA but lacking *C. parvum* organisms.

In the fourth experiment, a comparison was planned between the effect of a mycobacterium (*M. avium*) and an anaerobic coryneform (*C. anaerobium* 578) in order to determine whether the latter could stimulate the production of a delayed peak of anti-HSA. Groups of hens were injected intramuscularly with (i) 0·2 ml water-in-oil emulsion containing 40 μg HSA and 1 mg of heat-killed *M. avium* in the proportions (by volume) 1 : 1 : 3 of saline solution of HSA : Arlacel A (mannide mono-oleate) : Drakeol (containing the *M. avium*). (ii) The same injection mixture minus *M. avium*; (iii) 0·2 ml saline containing 40 μg HSA.

These chickens were bled at regular intervals up to 50 days and anti-HSA levels estimated by Farr test.

A further group of hens was injected intravenously with (1) 0·5 ml of a water-in-oil mixture containing 40 μg HSA and 5 mg of heat-killed *M. avium* (Herbert, 1965). This was prepared by first mixing a water-in-oil emulsion in proportions (by volume) containing Drakeol with *M. avium* (mineral oil) : Arlacel : saline solution of HSA = 9 : 0·1 : 1·0 and then re-emulsifying 0·25 ml of the above water-in-oil emulsion in 0·5 ml of 2 per cent Tween 80 (Honeywill and Stein, Ltd). (ii) 0·5 ml of a water-in-oil-in-water emulsion prepared as above but lacking *M. avium* (controls). (iii) 0·5 ml of a water-in-oil-in-water emulsion prepared as above but containing 5 mg of heat-killed *C. anaerobium* 578 instead of the *M. avium*.

These hens were bled at regular intervals up to 41 days and anti-HSA levels estimated by Farr test (Farr, 1958).

## RESULTS

### METHODS USED FOR THE CLASSIFICATION OF MICROBIAL STRAINS

Micro-organisms, received from various sources (see Materials and Methods) were, in general, subcultured in thioglycollate broth and submitted to tests for biochemical activity (ability to ferment with acid production solutions of the sugars glucose, sucrose, maltose, trehalose, mannose and melezitose, ability to produce indole from a culture in peptone water, ability to grow in a liquefied gelatine, and ability to grow in and reduce nitrate) and to tests for ability to grow in and reduce nitrate) and to tests for ability to agglutinate with specific antisera prepared against *Corynebacterium acnes*, type I, *C. acnes*, type II and *C. granulosum* (type III). The latter results were kindly provided by Professor C. S. Cummins.

TABLE 2

BIOCHEMICAL REACTIONS OF GROUPS 1–4 OF ANAEROBIC CORYNEFORMS AND 'CLASSICAL' PROPIONIBACTERIA

| Organism | Indole | Nitrate | Gelatin | Glucose | Mannose | Trehalose | Maltose | Sucrose | Melezitose |
|---|---|---|---|---|---|---|---|---|---|
| Corynebacterium acnes | | | | | | | | | |
| Groups I and II | | | | | | | | | |
| *C. parvum* 10390 | + | − | + | + | + | − | − | − | − |
| *C. parvum* 0208 | + | + | + | − | + | − | − | − | − |
| *C. parvum* 3085 | + | − | − | − | + | − | − | − | − |
| *C. parvum* 1383 | + | + | + | − | + | − | − | − | − |
| *C. liquefaciens* 814 | + | − | + | − | + | − | − | − | − |
| *C. liquefaciens* 6290 | + | + | + | − | + | − | − | − | − |
| *C. diphtheroides* 2764 | + | + | + | − | + | − | − | − | − |
| *C. anaerobium* 578 | + | + | + | + | + | − | − | − | − |
| *C. granulosum* 6292 | − | + | + | − | + | − | − | − | − |
| *C. lymphophilum* 6294 | + | − | + | + | + | − | − | − | − |
| *C. parvum* 6134 | Only obtained as killed suspension | | | | | | | | |
| *C. acnes* 737 | + | + | + | + | + | − | − | − | − |
| Group III granulosum | | | | | | | | | |
| *P. granulosum* | − | − | − | + | + | + | + | + | + |
| *C. parvum* 10387 | − | − | − | + | + | + | + | + | + |
| *C. parvum* A | − | − | − | + | + | + | + | + | + |
| *C. parvum* B | − | − | − | + | + | + | + | + | + |
| *C. parvum* C | − | − | − | + | + | + | + | + | + |
| Group IV Avidum | | | | | | | | | |
| *P. avidum* 0575 | − | − | + | + | + | + | + | + | + |
| *P. avidum* 0589 | − | − | + | + | + | + | + | + | + |
| *P. avidum* 4982 | − | + | + | + | + | + | + | + | + |
| 'Classical' propionibacteria | | | | | | | | | |
| *P. freudenreichii* 10470 | − | + | − | + | + | − | − | − | − |
| *P. arabinosum* 5958 | − | + | − | + | + | + | + | + | − |
| *P. jensenii* 5960 | − | − | − | + | + | + | + | + | − |
| *P. rubrum* 8901 | − | − | − | + | + | + | + | + | − |

+ Positive biochemical result. − No reaction observed.

The results of these tests are given in Table 2. It can be seen that the results allow a provisional classification of strains into four distinct groups of organisms, which for purposes of reference are designated anaerobic corynebacteria, types I, II, III and IV.

For much of the subsequent biological testing, organism 0208 has been used as typical of anaerobic coryneforms, type I; organisms 10390 and 578 have been used as typical of anaerobic coryneforms, type II; organisms 10387, 0507 have been used as typical of anaerobic coryneforms, type III (*P. granulosum* group) and organisms 0575 and 4982 have been used as typical of anaerobic coryneforms type IV (P. *avidum* group).

EFFECT OF VARIOUS STRAINS OF ANAEROBIC CORYNEFORMS ON CARBON CLEARANCE IN THE MOUSE

It is apparent from Table 3 that representative organisms which fall into each of the serological groups I, II, III and IV produced substantial increases in the phagocytic index of groups of mice. These results contrast with those obtained with representative organisms of the 'classical' propionibacteria. None of the four strains tested (*P. freudenreichii*, *P. arabinosum*, *P. jensenii* and *P. rubrum*) caused a significant rise in phagocytic index.

TABLE 3

VALUES OF PHAGOCYTIC INDICES AND PERCENTAGE INCREASES IN SPLEEN WEIGHTS IN FOUR SEROLOGICAL GROUPS OF ANAEROBIC CORYNEFORMS AND IN A GROUP OF 'CLASSICAL' PROPIONIBACTERIA

| | Phagocytic index ($K$). Average of 4–5 mice | Phagocytic index ($K$). Controls Average of 4–5 mice | Ratio: $\frac{\text{Test } K}{\text{Control}(K)}$ | Ratio of spleen weights: $\frac{\text{Test}}{\text{Control}}$ |
|---|---|---|---|---|
| Anaerobic corynebacteria | | | | |
| Serological group 1 | | | | |
| *C. parvum* 0208 | 0·057 | 0·014 | 4·2 | 1·64 |
| *C. parvum* 1383 | 0·061 | 0·015 | 4·1 | 1·21 |
| *C. liquefaciens* 814 | 0·044 | 0·015 | 2·9 | 1·32 |
| *C. acnes* 737 | 0·017 | 0·014 | 1·21 | No increase |
| *C. parvum* 3085 | 0·041 | 0·015 | 2·7 | 1·9 |
| *C. parvum* 6134 | 0·036 | 0·014 | 2·6 | 2·6 |
| *C. lymphophilum* 6294 | 0·015 | 0·015 | 1·0 | 1·2 |
| Serological group II | | | | |
| *C. parvum* 10390 | 0·025 | 0·012 | 2·1 | 2·1 |
| *C. diphtheroides* 2764 | 0·025 | 0·013 | 1·9 | — |
| *C. anaerobium* 578 | 0·052 | 0·024 | 2·2 | 6·9 |
| *C. acnes* 6280 | 0·020 | 0·013 | 1·5 | 1·1 |
| *C. granulosum* 6292 | 0·015 | 0·017 | 0·9 | 1·4 |
| *C. liquefaciens* 6290 | 0·017 | 0·017 | 1·0 | 1·7 |
| Serological group III | | | | |
| *P. granulosum* 0507 | 0·044 | 0·014 | 3·1 | 1·1 |
| *C. parvum* 10387 | 0·021 | 0·014 | 1·5 | 1·2 |
| *C. parvum* A | 0·021 | 0·014 | 1·5 | 1·1 |
| *C. parvum* B | 0·019 | 0·015 | 1·3 | 1·5 |
| *C. parvum* C | 0·014 | 0·015 | 0·9 | 1·0 |
| Serological group IV | | | | |
| *P. avidum* 0575 | 0·044 | 0·024 | 1·8 | 1·4 |
| *P. avidum* 4982 | 0·055 | 0·011 | 5·0 | 3·1 |
| *P. avidum* 0589 | 0·035 | 0·015 | 2·3 | 1·7 |
| 'Classical' propionibacteria | | | | |
| *P. freudenreichii* 10470 | 0·029 | 0·024 | 1·2 | — |
| *P. arabinosum* 5958 | 0·022 | 0·024 | 0·9 | 1·0 |
| *P. jensenii* 5960 | 0·015 | 0·014 | 1·1 | 1·0 |
| *P. rubrum* 8901 | 0·014 | 0·014 | 1·0 | 1·0 |

When the results for phagocytic indices are arranged in order of decreasing magnitude for $K$ (ratio with control) the order is: *C. parvum* 1383 (group I), *P. avidum* 4982 (group IV), *C. parvum* 0208 (group I), *P. granulosum* 0507 (group III), *C. liquefaciens* 814 (group I), *C. anaerobium* 578 (group II), *C. parvum* 3085 (group II), *C. parvum* 6134 (group II), *P. avidum* 0589 (group IV) and *P. avidum* 0575 (group IV). It is therefore apparent that no single serological group shows outright superiority in causing an increase of $K$ value but

that it may be of significance that organisms derived from serological Group I include three of the five most active strains.

### EFFECT OF VARIOUS STRAINS OF ANAEROBIC CORYNEFORMS ON HYPERTROPHY OF LYMPHOID TISSUE

Table 3 includes data on the increase in spleen weight following an intraperitoneal injection of 250 μg of heat-killed coryneforms. The increase in spleen weight which resulted 10 days after injection is expressed as a ratio of the average spleen weight of a group of four to five test animals over the average of a group of contemporaneous controls. It can be seen that strains of organisms in each of the four major serological groups caused an increase in spleen weight ratio. When these results were plotted (Fig. 1) a good correl-

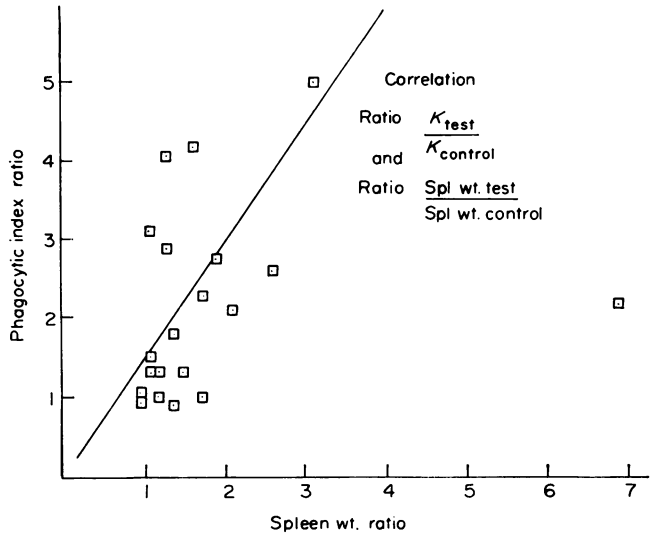


FIG. 1. Plot of the ratios of values of phagocytic index in groups of test and control animals (ordinates) against ratios of spleen weights of groups of test and control animals (abscissae).

ation emerged between the value for the ratio of spleen weights and the ratio of phagocytic index between test and control.

Weights of the livers of test and control animals were also recorded. It was apparent that animals with an increase of spleen weight also tended to show an increase of liver weight.

### EFFECT OF VARIOUS STRAINS OF ANAEROBIC CORYNEFORMS ON ANTIBODY BIOSYNTHESIS IN MICE

Table 4 includes the results of two experiments in which 250 μg of *C. parvum* 10390 in saline and 250 μg of *C. parvum* 10390 in water-in-oil emulsion were tested for their adjuvant effect upon the haemagglutinin titres against human serum albumin (HSA) in groups of mice and their contemporaneous controls. The effects on a primary response (to day 35) and on the response to a re-injection of HSA (250 μg) on (day 35) serum taken (at day 57) are shown. It is evident that both *C. parvum* in saline and *C. parvum*

in w/o emulsion failed to cause a significant increase in biosynthesis of anti-HSA in the primary responses. However, the secondary responses possibly show evidence of a slight elevation of anti-HSA (at day 57) in animals receiving *C. parvum* in saline and in w/o emulsion.

TABLE 4

HAEMAGGLUTINATION TITRES ($LOG_2$ DILUTION) AGAINST HUMAN SERUM ALBUMIN (HSA). PRIMARY RESPONSES (UP TO 35 DAYS) AND SECONDARY RESPONSES (UP TO 35 AND 22 DAYS) AFTER INTRAPERITONEAL HSA IN THE MOUSE. VALUES FOR THE STANDARD ERROR OF THE MEAN ARE GIVEN IN PARENTHESES

| | Day 0 | Day 21 | Day 35 | Day 43 | Day 57 |
|---|---|---|---|---|---|
| Group I<br>250 μg HSA + 250 μg *C. parvum* in saline | <4 | <4 | <4 | 5·8(1·2) | 6·4(1·5) |
| Group II<br>250 μg HSA in saline | <4 | <4 | <4 | <4 | 4·0(1·7) |
| Group III<br>250 μg HSA + 250 μg *C. parvum* in water-in-oil emulsion | <4 | 6·2(0·7) | 7·8(0·4) | 13·2(0·7) | 13·4(0·4) |
| Group IV<br>250 μg HSA in water-in-oil emulsion | <4 | 6·5(1·3) | 6·3(1·1) | 11·8(0·5) | 11·0(0·4) |

EFFECT OF VARIOUS STRAINS OF ANAEROBIC CORYNEFORMS ON THE IMMUNOLOGICAL RESPONSES OF GUINEA-PIGS

Guinea-pigs received 1 mg of ovalbumin (OV) antigen in water-in-oil emulsion containing varying amounts of heat-killed anaerobic coryneforms (200 μg, 1 mg and 2·5 mg) into the left footpad. Control animals were injected contemporaneously and received the same injections minus the anaerobic coryneforms. As seen from Table 5 *C. parvum* 0208 (sero-

TABLE 5

PRECIPITINS (BY RADIAL-IMMUNODIFFUSION IN ARBITRARY UNITS) AND BY COMPLEMENT FIXATION AS TITRES ($LOG_2$ DILUTION)

| Dose of organisms. | | 200 μg | | 1 mg | 2·5 mg |
|---|---|---|---|---|---|
| | | Precipitation | CFT | Precipitation | Precipitation |
| *C. parvum* 0208 | (I) | 122* | 1·6* | 182* | 124 |
| *C. parvum* 10390 | (II) | | 2 | 192 | 137 |
| *C. anaerobium* 578 | (II) | | | 220 | |
| *C. parvum* 10387 | (III) | 233 | 2 | | 80 |
| *P. granulosum* 0507 | (III) | 163 | 2·6 | | 93 |
| *P. avidum* 4982 | (IV) | | | | 85 |
| Contemporaneous Controls | | 75 | 2 | 73 | 110 |

* Average of group of three animals.

logical group I), *C. parvum* 10390 and *C. anaerobium* 578 (serological group II) showed an adjuvant effect on serum anti-HSA precipitin levels observed at 3 weeks after a single injection of antigen mixture containing 1 mg of each micro-organism. In addition, *C. parvum* 0208 (serological group I), *C. parvum* 10387 and *P. granulosum* 0507 (serological group III) and *P. avidum* (serological group IV) showed evidence of adjuvant activity in serum anti-OV levels at the 200 μg dose level. However, the several organisms tested at the 2·5 mg dose level failed to produce an adjuvant effect.

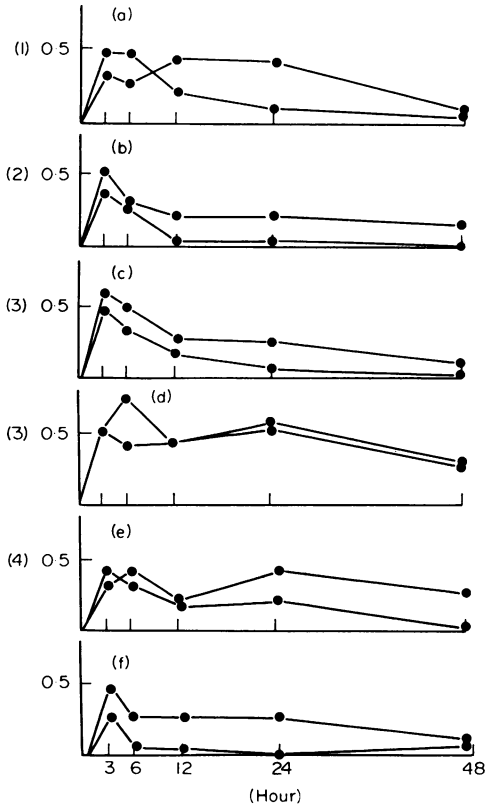


FIG. 2. Skin test responses to 50 μg ovalbumin (half double skin thickness) in individual guinea-pigs receiving various anaerobic coryneforms (1 mg) in water-in-oil emulsion, containing 1 mg ovalbumin 20 days previously. Serological group of micro-organisms indicated within parentheses. (a) *C. parvum* 0208. (b) *C. parvum* 10390. (c) *C. parvum* 10387. (d) *P. granulosum* 0507. (e) *P. avidum* 4982. (f) Control.

The delayed-type hypersensitivity responses to ovalbumin were tested in the same animals in skin and cornea 20 days after the immunizing injection. The results of skin tests to 50 μg of ovalbumin intradermally, are shown in Fig. 2; it is clear that 1 mg of the various anaerobic coryneforms, with a representative organism taken from each of the sero'ogical groups I–IV, failed to elevate the level of the delayed-type skin response. Further tests with 10 μg ovalbumin were similarly negative but are not recorded. Further skin tests were done in animals which received 1 mg and 2·5 mg of various strains of anaerobic coryneforms and these also failed to show any increase in delayed-type skin

reactivity (observable 12, 24 or 48 hours after injection). The results of an injection of ovalbumin into the cornea 21 days after the immunizing injection showed that none of the animals injected with various anaerobic coryneforms developed a positive response, i.e. the opacity of the eye injected at 24 and 48 hours with ovalbumin intracorneally was

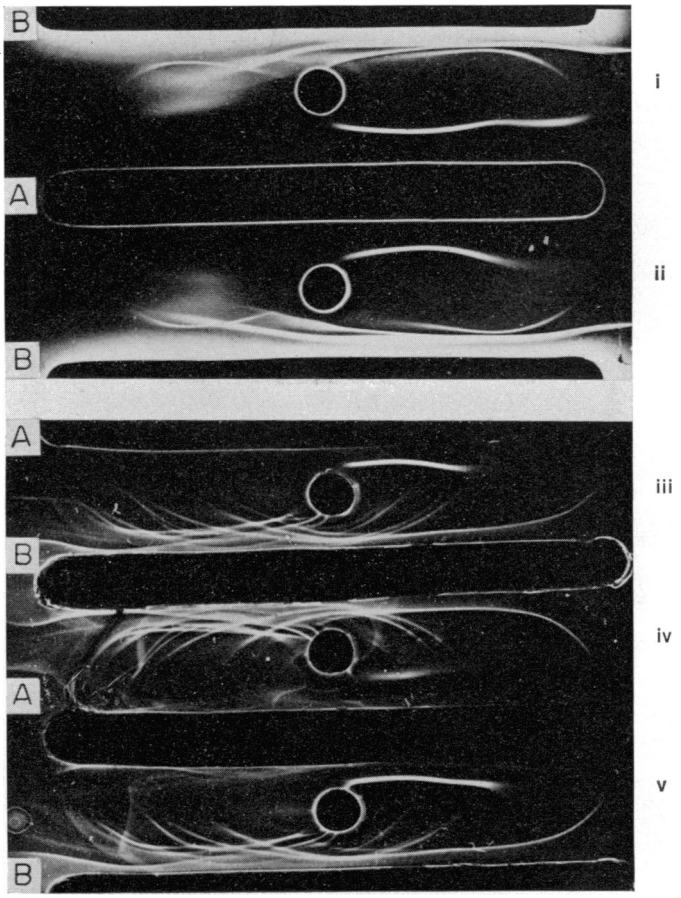


FIG. 3. Immunoelectrophoresis of serum of guinea-pigs immunized 3 weeks previously with a single injection of ovalbumin in water-in-oil emulsion (containing various anaerobic coryneforms or *M. tuberculosis*). Note $\gamma_1$ and $\gamma_2$ precipitin arcs are present in sera from animals receiving *M. tuberculosis* (Well i). Only $\gamma_1$ arcs are seen in sera from control animals (Well ii) or animals receiving anaerobic coryneforms (Wells iii, iv, v). The serological groups of anaerobic coryneforms are indicated in parentheses. (i) *M. tuberculosis*. (ii) Control. (iii) *C. parvum* 0208 (1). (iv) *C. parvum* 10390 (2). (v) *C. parvum* 10387 (3). (A) Troughs containing ovalbumin (100 $\mu$g/ml): (B) Troughs containing rabbit anti-guinea-pig $\gamma$-globulin.

limited to slight, if any, clouding of the cornea near the site of injection, which was not greater in extent than that of similar control animals which received the same immunizing injections minus anaerobic coryneforms. At the same time guinea-pigs, of the same stock and age which were injected with ovalbumin in the same immunizing injection but with 200 $\mu$g heat-killed *Mycobacterium tuberculosis* (human strain) in place of anaerobic coryneforms howed gross opacity and thickening of the cornea.

Sera of guinea-pigs subjected to the above procedure were submitted to immuno-electrophoresis in agar gel. The separated proteins after electrophoresis were developed against ovalbumin and rabbit-anti-guinea-pig globulins. The antisera from animals receiving 1 mg anaerobic coryneforms in their immunizing injections showed strong precipitin arcs of anti-ovalbumin in the $\gamma_1$ position. None of the guinea-pigs injected with anaerobic coryneforms showed a $\gamma_2$-precipitin arc in addition. This result, shown in Fig. 3, contrasts with that obtained with antisera obtained from guinea-pigs immunized with ovalbumin in water-in-oil emulsion with added 200 μg *Mycobacterium tuberculosis* (human strain) which showed two conjoined precipitin arcs of anti-ovalbumin corresponding to immunoglobulins $\gamma_1$ and $\gamma_2$.

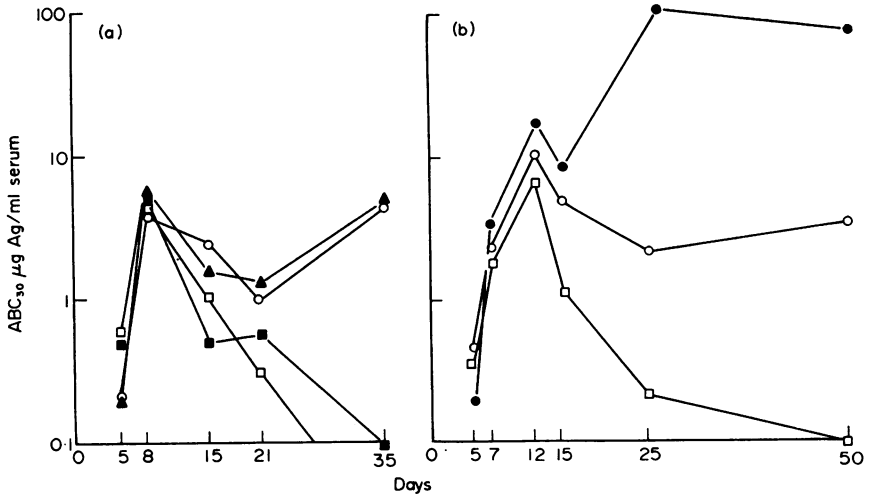


FIG. 4(a). Comparison of serum antibody responses in the chicken (average values for groups of four birds) at various times after an intramuscular injection of HSA plus *C. parvum* in water-in-oil emulsion (▲); with those from birds similarly injected with HSA in water-in-oil emulsion (controls) (○); and of HSA plus *C. parvum* 0208 in saline (■); intravenously with those of birds injected with HSA in saline (controls) (□). (b). Comparison of serum antibody responses (average values for groups of four birds) in hens injected with HSA in water-in-oil emulsion containing *M. avium* (●); with those injected with HSA in water-in-oil alone (○); with those injected with HSA in saline (□).

## HISTOLOGY OF LOCAL SITE OF INJECTION (FOOTPAD) AND OF LYMPH NODES OF GUINEA-PIGS INJECTED WITH OVALBUMIN IN WATER-IN-OIL EMULSION WITH ADDITION OF VARIOUS ANAEROBIC CORYNEFORMS

It was clear from inspection of the regional node (popliteal) that the addition of five various anaerobic coryneforms to a water-in-oil injection mixture containing ovalbumin (antigen) caused a gross expansion of the node which became rounded at 20 days after injection and considerably greater than the same nodes from control animals injected with the same injection mixture but lacking micro-organisms. Histologically the control nodes showed the accumulation of small groups of foamy macrophages in the sinuses. These often surrounded empty circular spaces which presumably represented vesicles of mineral oil (plus arlacel) which had lodged in the sinuses. These nodes showed very large and numerous germinal centres in a cortex which appeared broad in relation to the medulla and paracortex.

Popliteal nodes from guinea-pigs injected with *C. parvum* 0208 (serological group I), *C. parvum* 10390 (serological group II), *C. anaerobium* 578 (serological group II) and *P. avidum* 4982 (serological group IV) all showed varying degrees of the same histological changes. In all cases the cross section of the popliteal node became more rounded and the central part of the node was expanded owing to an increase in the extent of the para-cortex. The cortex appeared as though it were stretched and thinned over the expanded para-cortex. Germinal centres were not obviously enlarged and tended to be smaller than those of control animals. The central sinuses of the node were in places occupied by collections of foamy macrophages and occasional oil vesicles, but a constant very striking

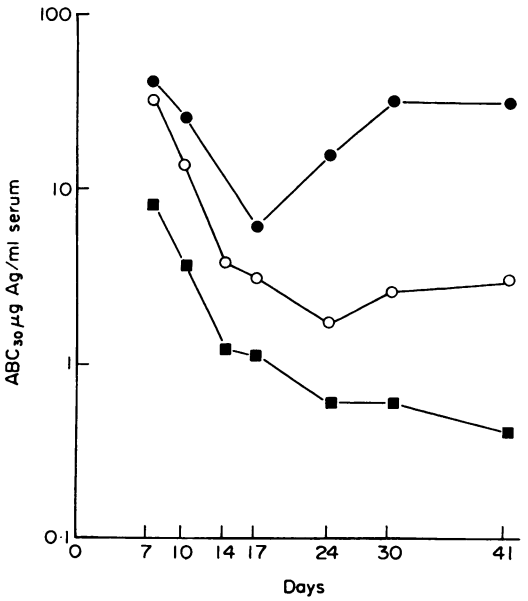


FIG. 5. Comparison of serum antibody responses (average of groups of four birds) in hens injected intravenously with HSA in water-in-oil-in-water emulsion containing *M. avium* (●); with those injected with water-in-oil-in-water emulsion alone (○); with those injected with w-o-w emulsion containing *C. anaerobium* 578 (■).

low power feature was branched elongated streaks of tightly packed lymphocytes, due to sinus-plugging with these cells (Fig. 6a). The expanded para-cortex was occupied mainly by small and medium lymphocytes. Pyroninophilic blast cells were distinctly rare. Another prominent feature of the expanded para-cortex was the prominent post-capillary venules, with hypertrophied and elongated endothelial cells (Fig. 6b).

The local site of injection in the footpad of controls showed the development of a granulomatous reaction surrounding 'empty' oil vesicles. The principal cells involved were histiocytes = macrophage with a foamy cytoplasm. In animals receiving in addition anaerobic coryneforms the local site of injection was occupied by a distinctly larger and more compact granuloma consisting mainly of dense sheets of macrophages enclosing numerous oil vesicles. These macrophages had extensive foamy cytoplasm, and were densely packed together in small pockets of cells, each having a polygonal outline. Giant cells were not seen.

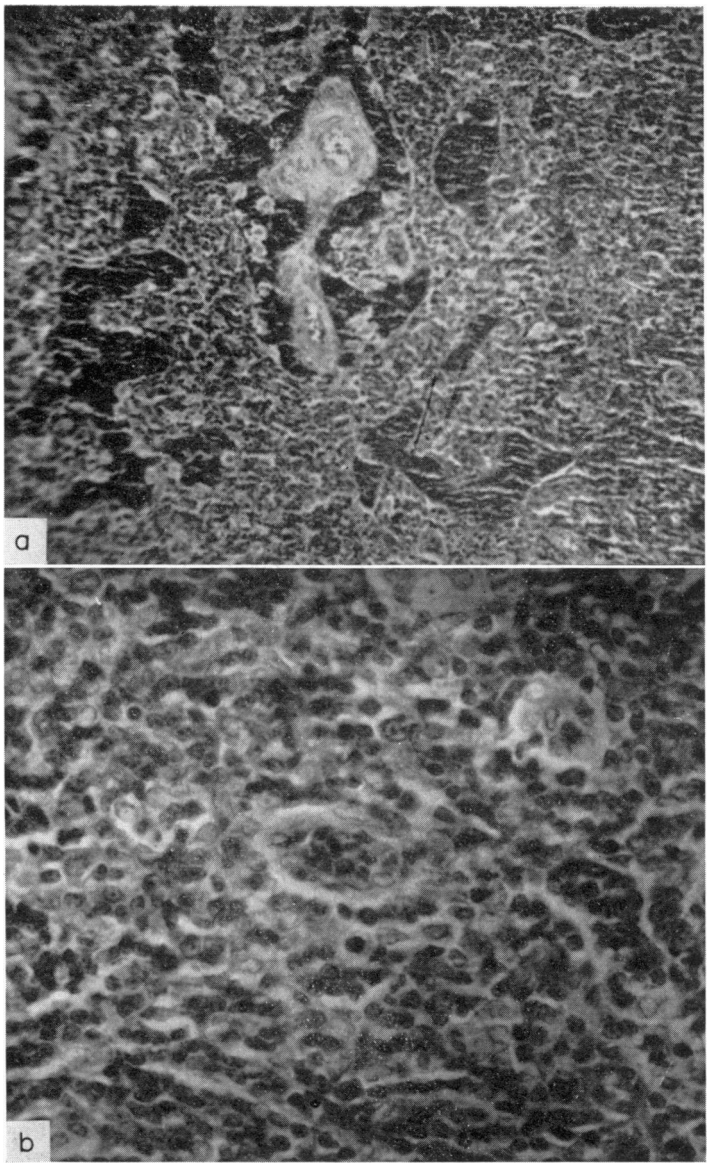


FIG. 6. (a) Photomicrograph of section of popliteal node of guinea-pig injected 3 weeks previously in the footpad with a water-in-oil emulsion containing *C. anaerobium* 578, showing prominent streaks of paracortical sinuses plugged with small lymphocytes (magnification ×10 H & E). (b) Photomicrograph of section of popliteal node of guinea-pig injected 3 weeks previously in the footpad with a water-in-oil emulsion containing *C. anaerobium* 578. Note: hypertrophic post-capillary venules with prominent elevated endothelial cell layer (magnification ×40 H & E).

EFFECT OF VARIOUS STRAINS OF ANAEROBIC CORYNEFORMS ON ANTIBODY BIOSYNTHESIS IN CHICKENS OR HENS

In view of the clear and prolonged adjuvant effect of various mycobacteria on the immunological response to HSA in the chicken (French, Stark and White, 1970) experiments were undertaken to determine whether a similar effect followed the use of anaerobic coryneforms. The results of the first experiment, in which various heat-killed anaerobic coryneforms (*C. parvum* 0208 (serological Group I), *C. parvum* 10390 (serological Group II), *C. parvum* 10387 (serological Group III), *P. granulosum* 0507 (serological Group III)) were tested for their ability to elevate serum levels of anti-HSA (Table 6) and to cause histological changes or granuloma formation at the site of injection into the muscle of the left breast, showed no evidence of an adjuvant effect upon antibody levels (estimated by a Farr test on serum samples collected at intervals up to 64 days) and little naked eye

TABLE 6

EFFECT OF VARIOUS ANAEROBIC CORYNEFORMS (500 μg) ON SERUM ANTIBODY LEVELS TO HSA (1 mg) GIVEN INTRAMUSCULARLY AS WATER-IN-OIL EMULSION IN CHICKENS AS MEASURED BY THE FARR TEST

| | Mean* ABC 30 μg Ag/ml serum (days) | | |
|---|---|---|---|
| | 8 | 29 | 64 |
| *C. parvum* 0208 (I) | 0·70 | 0·94 | 2·60 |
| *C. parvum* 10390 (II) | 1·38 | 0·21 | 0·29 |
| *C. parvum* 10387 (III) | 0·65 | 0·60 | 2·16 |
| *P. granulosum* 0507 (III) | 0·81 | 0·29 | 1·77 |

* 30 per cent binding capacity.

evidence of local granuloma formation. Sections from the breast muscle showed the histological appearances typical of the site of deposition of mineral oil on tissue, i.e. collections of foamy macrophages and fibroblast proliferation around oil vesicles. The sections from animals receiving various types of anaerobic coryneforms showed definite increase in the macrophage component of such granulomata. The macrophages were in the form of foamy histiocytes, but epithelioid cell change and giant-cell formation were not evident.

The result of the second experiment in which saline suspensions of *C. parvum* 0208 were tested for adjuvant effect after intravenous injection into chickens (3–9 weeks old) are shown in Fig. 4a, which shows no significant distinction between the average anti-HSA levels of test and control animals followed up to the thirty-fifth day of the response.

The results of the third experiment in which water-in-oil emulsions of *C. parvum* 0208 were tested for adjuvant effect after intramuscular injection into the breast of chickens (3–9 weeks old) are shown in Fig 4a which also shows no significant difference between the anti-HSA levels of test and control animals, although the values for control birds injected with water-in-oil emulsion are, as expected, higher than those for birds injected with saline suspension of antigen. As seen from Fig. 4b, the addition to a water-in-oil

emulsion of *M. avium* produces a characteristic delayed peak or elevation of anti-HSA from days 15 to 50. It can be seen that *C. parvum* 0208 has no comparable effect.

The results of the fourth experiment in which *M. avium* (1 mg) and an anaerobic coryneform: *C. anaerobium* 578 (5 mg) were compared for their adjuvant effects on anti-HSA levels after intravenous injection in water-in-oil emulsion shows (Fig. 5) that while both organisms failed to produce any marked elevation of the first peak of anti-HSA (day 7), *M. avium* caused marked elevation of anti-HSA from day 17 to day 41, but *C. anaerobium* failed to elevate the level of anti-HSA at day 41.

## DISCUSSION

An anaerobic coryneform micro-organism referred to as *C. parvum* 936B was originally used by Halpern, Prévot, Biozzi, Stiffel, Mouton, Morard, Bouthillier and Décreusefond (1964) in order to increase the clearance of carbon from the blood of mice. In subsequent papers the same and other anaerobic coryneform micro-organisms have been used for the inhibition of tumour growth (Halpern *et al.*, 1966; Smith and Woodruff, 1968; Fisher, Grace and Mannick, 1970) without clear evidence for a taxonomic identity or relationship between the organisms used. The organisms used were designated *C. parvum* 936B, a *C. parvum* organism from the National Collection of Type Cultures (possibly 10387) and a strain of *C. parvum* 'from the American Type Culture Collection'. In order to determined precisely the taxonomic status of organisms with these biological properties a wide assortment of various anaerobic coryneform organisms has been submitted to comparative tests for biological activity in this and related investigations (Wilkinson, O'Neill and Wapshaw, 1973; Cater and White, 1973).

The taxonomic position of the anaerobic coryneforms is still far from certain. We have accepted a division of anaerobic coryneforms into four groups on the basis of serology, cell wall analysis for sugars, aminoacids and aminosugars, and estimates of DNA homology. The type-strains accepted for each of these groups are *C. acnes* 0208 (serological group I) *C. acnes* 0162 (serological group II,) *C. granulosum* 0507 (serological group III), *P. avidum* 0575 (serological group IV) (Johnson and Cummins, 1972). We are grateful to Professor C. S. Cummins for confirming the serological groupings of most of the organisms used in this study. In addition, representative organisms of the so-called 'classical propionibacteria' have been included in this study (Van Niel, 1928). It is doubtful whether the term '*C. parvum*' can be justified as indicating a specific and relatively homogeneous group of anaerobic coryneform organisms, since various organisms designated originally as *C. parvum* have proved to belong to each of the three serological groups I, II and III as defined above (see also Johnson and Cummins, 1972.)

Table 1 indicates the source and designation of the twenty-five strains of anaerobic coryneforms and classical propionibacteria used. As can be seen from Table 2 these are readily divisible by biochemical tests into the groups typified by *C. acnes*, *P. granulosum* and *P. avidum*. Use of antisera prepared against cell walls of various *C. acnes* strains allows a further sharp division into two distinct groups of *C. acnes* (Johnson and Cummins, 1972). The group of organisms typified by *P. granulosum* can be further identified and segregated by means of an antiserum specific for the cell-wall antigens of the organisms of this group.

In tests of various anaerobic coryneforms for their ability to increase significantly the phagocytic index as measured in female Porton-strain mice, it is clear from Table 3 that fifteen of the twenty-one tested strains were able to produce an increase of at least 50

per cent. Micro-organisms that were effective in these tests occurred in each of the four main serological groups and many other than those bearing the designation '*C. parvum*' proved to be effective. It is, therefore, apparent that the biological property of stimulating the reticulo-endothelial system is possessed by a wide range of micro-organisms. It is of considerable interest that all of the three strains of *Propionibacterium avidum* which were tested proved to possess activity and the strain 4982 which increased the phagocytic index five-fold was clearly outstanding above all other strains in this respect. However, although no single serological group showed outright superiority in causing an increase in phagocytic index, three of the five strains which possessed highest activity belonged to serological group I.

It is also of interest that four strains of 'classical' propionibacteria (*P. freudenreichii*, *P. arabinosum*, *P. jensenii* and *P. rubrum*) all failed to produce a significant increase of phagocytic index. This appears to provide a sharp distinction between organisms such as *C. acnes* or *C. granulosum* which are isolated from cases of human disease or as commensals of the human skin and the classical propionibacteria which have their source in dairy milk products.

Many of the strains that stimulated a rise in phagocytic index also caused an increase in the weight of the liver and spleen measured at 10 days after injection. When spleen weight ratios of test and control animals were plotted against the ratios of phagocytic index between test and control animals a moderately good correlation was apparent between the magnitude of the two effects (see Fig. 1).

Tests in mice and chicken for an adjuvant action on serum levels of anti-HSA failed to show any enhancement of primary responses, although a barely significant elevation of the secondary response in the mouse was observed. It is clear that neither of the strains *C. parvum* 0208 or *C. anaerobium* 578 caused the prolonged increase in biosynthesis of anti-HSA which is typical of *M. avium* and other mycobacteria in the chicken (French, Stark and White, 1970). In the guinea-pig a clear adjuvant effect on the levels of serum antibody in a primary response to ovalbumin was found. However, no evidence could be obtained that any of several anaerobic coryneforms was able to augment cell-mediated hypersensitivity as shown by delayed-type skin tests or corneal reactions. These results contrast with those obtained by Neveu, Branellac and Biozzi (1964) who enhanced skin test reactions of the delayed-type by immunization with picrylated HSA and claimed to produce an effect equivalent to that of *Mycobacterium butyricum*. At the same time we were unable to demonstrate any augmentation of cell-mediated immunity by use of anaerobic coryneforms, it was shown that human type mycobacterium produced considerable increase in delayed-type skin responses and corneal tests in the same strain of guinea-pigs. It is also clear that whereas mycobacteria of various strains cause a considerable increase of $\gamma_2$-immunoglobulin class of anti-ovalbumin in the guinea-pig (White, Jenkins and Wilkinson, 1963) the use of anaerobic coryneforms failed to cause significant increases in this immunoglobulin form of anti-ovalbumin under the same conditions.

A study of the histological responses to a footpad injection of various anaerobic coryneforms (in water-in-oil emulsion with ovalbumin) showed that the regional (popliteal) node underwent a considerable expansion of the lymphoid cell population. Since these effects correlated with the extent of stimulation of phagocytic index it seemed probable that the histological changes within the node might relate to the various adjuvant properties of these organisms. The most significant change appeared to involve a great expansion of the lymphocyte population within the para-cortex (thymus-dependent area

or tertiary nodule). This was accompanied by extensive sinus plugging with lymphocytes (Fig. 6a) and endothelial hypertrophy of the post-capillary venules (Fig. 6b).

Such appearances correspond well with those described by Kelly, Wolstencroft, Dumonde and Balfour (1972), to follow injection of lymphocyte-activation products into the afferent lymph of guinea-pigs. In their view such histological changes could represent an activity on sinus-lining macrophages causing lymphocytes present in the sinus lumen to adhere resulting in the 'traffic jam' apparent in the lymph node sinuses. Possibly the anaerobic coryneforms could act by directly activating macrophages (see also Wilkinson, O'Neill and Wapshaw, 1973; Cater and White, 1973) or by causing the production of lymphocyte activation products. An association between these cellular events (expansion of the population of para-cortical areas, sinus plugging and hypertrophy of post-capillary venules has been described in association with the genesis of cell-mediated immunity (de Sousa and Parrott, 1969; Kelly, 1970). However, it may be significant that the guinea-pigs which received footpad injections of anaerobic coryneforms and displayed these changes to a marked degree did not develop delayed-type cell-mediated hypersensitivity (to ovalbumin) above the level of that of the control animals which lacked such histological change. This evidence therefore calls into question the rôle of these particular cellular changes in pre-conditioning the animal for cell-mediated immunity.

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