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The Influence of Components of M. Tuberculosis and other Mycobacteria upon Antibody Production to Ovalbumin

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SUMMARY

The inclusion of wax D fractions of the strains Test, Canetti and H37 Rv of human type *M. tuberculosis* in water-in-oil emulsions containing ovalbumin produced an increase in serum anti-ovalbumin levels measured at three weeks following a single injection into guinea-pigs, as compared with the levels of antibody in control animals which received ovalbumin emulsions lacking these fractions.

When this activity is determined at five-fold dose differences with equal weights of whole heat-killed *M. tuberculosis* Canetti and a wax D fraction of this organism both showed the same order of activity within the levels of accuracy of the trial, and both were active at doses of 40 μg. and above.

The following fractions from Mycobacteria were found to be inactive in increasing serum anti-ovalbumin under these conditions: phosphatide of *M. tuberculosis* H37 Rv, wax C of *M. tuberculosis* Canetti, wax D of three bovine strains of *M. tuberculosis* (B.C.G., Dupré and Marmorek), wax D of *M. avium* strain No. 802, wax D of *M. phlei* and *M. smegmatis*, phthiocerol di-acetate, trehalose di-mycolate (cord factor), mycolic acid, methyl mycolate and polysaccharide from wax D of *M. tuberculosis* Canetti.

Dried bacilli from three Mycobacteria: *M. tuberculosis* B.C.G., *M. smegmatis*, *M. phlei* and delipidated *M. tuberculosis* of the bovine type Marmorek were found to be active in increasing serum anti-ovalbumin levels.

The activity of the wax D fractions of human types and the inactivity of the corresponding fractions of bovine, avian, and saprophytic types of Mycobacteria suggest that the adjuvant activity of the former is specifically due to the presence of the amino acids alanine, glutamic acid and α,ε-diaminopimelic acid they contain; these three amino acids, forming a peptide linked to the polysaccharide which is esterified with mycolic acid, seem to be necessary for the adjuvant activity observed in our experiments; hydrolysis destroys the activity of these wax D fractions.

The activity of the delipidated bacterial residues of human and bovine *M. tuberculosis*, as well as avian and saprophytic Mycobacteria, can be explained by the probable presence in these materials of a similar insoluble compound containing the three amino acids (alanine, glutamic acid and α,ε-diaminopimelic acid) linked to a polysaccharide which is esterified with mycolic acid.

Data are given of the morphological changes in guinea-pigs which constantly followed the injection of active bacilli or fractions.

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INTRODUCTION

A widely used method for the production of high titres of circulating antibody in animals was first described by Freund and McDermott (1942) and consists of the injection into animals of antigen in the aqueous phase of a water-in-oil emulsion, *M. tuberculosis* being added to the oil phase. Such adjuvant mixtures have been used successfully with a variety of antigens. They have also been widely employed for the induction of skin sensitization of the delayed type (Landsteiner and Chase, 1940), for the production of acute disseminated encephalomyelitis using emulsions containing homologous or heterologous brain tissue (Morgan, 1947; Kabat, Wolf and Bezer, 1947), and aspermatogenesis with homologous testicular tissue (Freund, Lipton and Thompson, 1953), and for the induction of immunity to certain animal parasites, e.g. influenza PR8 virus (Friedewald, 1944), poliomyelitis virus (Ward, Rader, Lipton and Freund, 1950), and *Plasmodium knowlesi* (Freund, Thomson, Sommer, Walter and Pisani, 1948).

This communication presents data concerning the role of Mycobacteria and their chemical components in potentiating the antibody production to ovalbumin. The work of Choucroun (1947) and Raffel (1948, 1950) clearly indicates that the capacity of the tubercle bacillus to produce the tuberculin type of sensitivity depends upon its content of a certain wax fraction. This is extractable from the bacillus by chloroform and is largely composed of the polysaccharide ester of mycolic acid (Raffel, 1950; Asselineau, Choucroun and Lederer, 1950). It would appear that the glycolipid or wax D is not itself an antigen in this sensitization process, but serves to promote the sensitizing ability of tuberculo-protein. The present communication is not primarily concerned with the sensitization process, although data on the development of corneal sensitivity are included.

The influence of *M. tuberculosis* (H37 Rv) in adjuvant mixtures containing ovalbumin upon the levels of serum antibody has been accurately assessed by Fischel, Kabat, Stoerk and Bezer (1952). White, Coons and Connolly (1955), also using the quantitative precipitin method, showed that the 'purified wax fraction' (Anderson, 1929) could effectively replace whole bacilli in such adjuvant mixtures, and a dose of 40 μg. was found to cause significantly increased antibody levels in the guinea-pig.

The present work carries further the characterisation of the constituents of Mycobacteria responsible for the adjuvant effect.

MATERIALS AND METHODS

ANIMALS

Guinea-pigs weighing between 390 and 550 grammes were distributed according to sex and weight in an attempt to achieve groups comparable in these respects. Each group receiving a separate chemical fraction comprised five or six animals. Diet consisted of mixed bran and oats with supplementary cabbage *ad lib.*

ANTIGEN AND INJECTION PROCEDURE

The antigen used throughout this study was thrice-crystallized egg albumin prepared by the method of Kekwick and Cannan (1936). The dose was always 5 mg. injected in 0·2 ml. of water-in-oil emulsion into the left hind footpad of the guinea-pig. This was

done in order to study the cellular reactions in the popliteal and other regional lymph nodes. To prepare a batch of antigen emulsion 25 mg. of crystalline ovalbumin was dissolved in 0·2 ml. saline, 0·2 ml. of Arlacel (Atlas Powder Company, Wilmington, Delaware, U.S.A.) added and mixed by drawing up into a 1 ml. syringe and expelling repeatedly into a test tube. A volume of 0·6 ml. of Bayol F was then added and the whole emulsified as before. When tubercle bacilli or their chemical fractions were added to the mixture they were either suspended or dissolved in the Bayol F before emulsification. Dried bacilli were suspended in oil with a Griffith type tube and pestle.

COLLECTION AND TREATMENT OF SERA

After three weeks the animals were killed with chloroform and blood collected from the incised heart into sterilized petri dishes. Serum was separated after standing 2 hrs. at 37° and overnight at 0°–4°.

In previous investigations (White *et al.*, 1955) complement was removed from the sera by the addition of the calculated quantities of a heterologous antigen-antibody system (Pneumococcus S.S.S. II and rabbit antiserum). In the present experiments, for the sake of simplicity this step was omitted, and the error due to complement content and the addition of this to the weight of precipitates was ignored, care being taken to handle all sera after collection in a uniform manner. In an initial attempt to reduce uniformly the complement content, sera after separation were allowed to remain in the refrigerator at 4° C. for six days. However, when a comparison was made between such results and those obtained with sera kept frozen at −20° C. and estimated simultaneously the differences were small, falling within the range of experimental error for the method.

ESTIMATION OF ANTIBODY NITROGEN CONTENT OF SERA

Analyses for anti-egg albumin were done in triplicate by the quantitative precipitin method modified from Kabat and Meyer (1948). Based on preliminary precipitin tests analyses were carried out on 0·2–2·0 ml. samples of serum, such as would yield approximately 50–200 μg. of nitrogen in the final precipitate. The amount of antigen for use in the estimation was calculated from a preliminary precipitin test and was such as to give maximum precipitation of antibody with a slight excess of antigen. The precipitation was allowed to proceed for three days in the refrigerator at 4°. Precipitates were washed with the minimum volume of saline in order to prevent loss due to solubility of precipitates at 0 to 4°. Tubes containing precipitates were spun in an International type PR 2 centrifuge at 1560 times gravity for 30 minutes at 0 to 4°. Supernatant fluid was removed with a Pasteur pipette. The packed precipitates were broken up after addition of a few drops of ice-cold saline and the remainder of the saline added down the sides until a total of 1·0 ml. was added. This procedure of centrifugation and replacement of supernatant with saline was repeated three times (total volume of added saline 3 ml.).

The packed precipitate was digested for 6 hr. with 0·1 ml. of 1 per cent SeO_2 and 0·1 ml. H_2SO_4 (B.D.H. 'M.A.R.'), and the ammonia nitrogen estimated by the Conway (1947) technique.

CORNEAL TESTS

After instillation of a drop of 2 per cent cocaine intra-corneal injections were made using a needle size 30 (Imperial standard wire gauge) attached to a 1 ml. tuberculin-

type 'Vim' syringe. A solution of crystalline egg albumin, 20 mg. per ml., was injected in an amount sufficient to cause a disc of opacity in the cornea 2 mm. in diameter. The eyes were examined at 24 and 48 hrs. and the extent and degree of corneal opacity, and the presence or absence of chemosis, recorded. In many cases the eye was enucleated and fixed in formol-alcohol before preparing paraffin sections. With a strong reaction, 2 or 3, most or the whole of the cornea was thickened, opaque, grey-white (resembling blotting paper). The cornea was surrounded by an injected and oedematous conjunctiva. Controls looked entirely normal at 48 hrs. except for an occasional residual trauma of the needle penetration. Histological section confirmed these findings. The strong reaction showed a cellular infiltration from limbus to limbus, within a cornea of up to three times normal thickness.

MYCOBACTERIA AND CHEMICAL FRACTIONS

Bacteria were cultivated at the Pasteur Institute, Paris, for four weeks on Sauton's medium, then filtered, washed with water on the filter and placed immediately in 5 to 10 volumes of a mixture of alcohol-ether (1 : 1). After two weeks at room temperature the first extract was filtered and extraction with alcohol-ether repeated five to six times, each extract being left in contact with the bacilli for two days. The filtered alcohol-ether extracts were combined and the three lipid fractions (acetone-soluble fat, wax A and phosphatide) separated as described by Aebi, Asselineau and Lederer (1953). After alcohol-ether extraction the bacilli were extracted several times, for two days at room temperature, with chloroform; the combined chloroform extracts were taken to dryness and waxes B, C and D separated as described by Aebi *et al.* (1953).

Before testing, the wax D fractions were dissolved in ether and centrifuged at low temperature to separate, as far as possible, bacterial debris; the wax D was then precipitated with methanol, centrifuged and dried *in vacuo*.

The polysaccharide from wax D of human type *M. tuberculosis* strain Canetti was obtained by saponification with 5 per cent alcoholic potassium hydroxide in an excess of benzene for 30 minutes. The precipitate thus formed was collected, washed with boiling benzene and alcohol, dissolved in water, neutralised and precipitated with alcohol. This preparation still contained the amino acids of the wax D linked to the polysaccharide.

The cord factor (Bloch, 1950) used in these experiments was prepared according to Noll, Bloch, Asselineau and Lederer (1956).

'Purified wax fraction' of human type *M. tuberculosis* strain H37 was kindly supplied by Dr. A. H. Coons from material prepared by Dr. Rudolf J. Anderson.

RESULTS

THE EFFECT OF VARIOUS CHEMICAL FRACTIONS OF MYCOBACTERIA ON SERUM ANTIBODY LEVELS TO OVALBUMIN INJECTED IN WATER-IN-OIL EMULSION

The data in Table 1 show that inclusion of wax D fractions of three strains of human type *M. tuberculosis*: H37 Rv, Test and Canetti, produced an increase in antibody levels three weeks after a single injection of ovalbumin in water-in-oil emulsion as compared with the levels in the group of animals which were injected with ovalbumin emulsions similar except in their lacking these fractions. Further, it is clearly shown that the

TABLE 1

EFFECT OF VARIOUS CHEMICAL FRACTIONS OF MYCOBACTERIA ON SERUM ANTIBODY LEVELS TO OVALBUMIN INJECTED AS A SINGLE DOSE IN WATER-IN-OIL EMULSION

No. in group	*Fraction and dose*	*Ref. No.*	*Anti-ovalbumin (µgN/ml. serum)*			*Corneal reaction* at 48 hours*
			Mean	*Range*	*S.D.*	
5	200 µg. phosphatide *M. tuberculosis*, human, H37 Rv	WL11	114	42–201	59	0, 0, 0, 0, 0.
5	Contemporaneous controls	—	87	39–101	61	0, 0, 0, 0, 0.
4	200 µg. 'Purified wax' (Anderson), *M. tuberculosis*, human, H37	OA1	469	345–555	86	3c, 3c, 3c, 3c.
4	Contemporaneous controls	—	155	78–226	56	0, 0, 0, 0.
5	200 µg. wax C, *M. tuberculosis*, human, Canetti	WL1	77	29–169	48	0, 0, 0, 0, 0.
5	Contemporaneous controls	—	158	100–205	43	0, 0, 0, 0, 0.
5	200 µg. wax D, *M. tuberculosis*, human, Test	WL0	660	540–762	73	Not done.
5	Contemporaneous controls	—	187	117–278	65	Not done.
5	200 µg. wax D, *M. tuberculosis*, human, Canetti	WL2	315	140–463	119	3c, 3c, 3c, 3c, 3c.
4	Contemporaneous controls	—	126	100–205	43	0, 1, 0, 1.
4	200 µg. wax D, *M. tuberculosis*, human, Test	WL6	334	125–600	171	3c, 3c, 3c, 3c.
5	Contemporaneous controls	—	91	66–128	21	0, 0, 0, 0, 0.
5	200 µg. wax D, *M. tuberculosis*, human, H37 Rv	WL28	531	356–800	154	3c, 3c, 3c, 3c, 3c.
5	Contemporaneous controls	—	127	55–245	73	0, 1, 0, 0, 0.
5	200 µg. wax D, *M. tuberculosis*, bovine, B.C.G.	WL7	91	8–195	81	0, 0, 0, 0, 1.
5	Contemporaneous controls	—	89	31–152	44	0, 0, 0, 0.
4	200 µg. wax D, *M. tuberculosis*, bovine, B.C.G.	WL21	198	42–291	100	1, 0, 0, 0.
4	200 µg. wax D, *M. tuberculosis*, bovine, Marmorek	WL18	170	44–263	84	0, 0, 0, 0.
4	Contemporaneous controls	—	136	33–313	119	0, 0, 0, 0.
5	200 µg. wax D, *M. tuberculosis*, bovine, Dupré, S.	WL26	241	61–330	100	0, 1, 0, 0, 1.
5	Contemporaneous controls	—	197	125–315	73	0, 1, 0, 0, 0.
4	200 µg. wax D, *M. avium*, 802	WL27	152	90–262	65	0, 1, 0, 0.
5	200 µg. wax D, *M. smegmatis*	WL29	64	10–125	50	0, 0, 0, 1, 0.
5	Contemporaneous controls	—	102	18–138	46	0, 0, 0, 0, 0.
5	200 µg. wax D, *M. phlei*	WL25	90	37–178	49	0, 0, 0, 0, 1.
5	Contemporaneous controls	—	99	20–176	51	1, 0, 0, 1, 0.
4	200 µg. mycolic acid from *M. tuberculosis*, human, Canetti	WL4	100	47–152	45	0, 0, 0, 0.
4	400 µg. methyl mycolate from *M. tuberculosis*, human, Canetti	WL5	47	17–88	28	0, 0, 0, 0.
4	Contemporaneous controls	—	88	57–139	31	0, 0, 0, 0.
5	200 µg. methyl mycolate from *M. tuberculosis*, human, Canetti	WL5	33	16–46	12	0, 2c, 0, 0, 0.
5	Contemporaneous controls	—	44	31–61	10	0, 0, 0, 0, 0.
5	200 µg. phthiocerol diacetate	WL3	105	62–199	48	0, 0, 0, 0, 0.
4	Contemporaneous controls	—	157	100–195	43	1, 0, 0, 1.
4	1 mg. polysaccharide from wax D of *M. tuberculosis*, human, Canetti	WL8	280	108–318	80	2, 0, 1, 0.
4	Contemporaneous controls	—	85	31–152	50	0, 0, 0, 0.

TABLE 1 (*continued*)

No. in group	*Fraction and dose*	*Ref. No.*	*Anti-ovalbumin (μgN/ml. serum)*			*Corneal reaction* at 48 hours*
			Mean	*Range*	*S.D.*	
5	200 μg. polysaccharide from wax D of *M. tuberculosis*, human, Canetti	WL8	25	3–40	13	0, 1, 0, 0, 1.
5	Contemporaneous controls	—	19	5–36	10	1, 0, 0, 0, 1.
4	200 μg. trehalose di-mycolate (cord factor) from *M. tuberculosis*, human, Brévannes	WL22	120	0–252	107	0, 0, 0, 0.
4	40 μg. trehalose di-mycolate (cord factor) from *M. tuberculosis*, human, Brévannes	WL22	88	25–160	58	0, 0, 0, 0.
4	Contemporaneous controls	—	67	20–153	53	0, 0, 1, 0.

* 1 2 3 indicate degrees of corneal response.
3 = thickening and dense opacity of whole cornea.
1 = local opacity near site of injection and/or slight general opacity of cornea.
c indicates the presence of chemosis.

inclusion of these wax D fractions in the injection mixtures resulted in high levels of corneal reactivity to ovalbumin.

Under the same experimental conditions and in the same dosage as the above (200 μg. per injection) the following failed to increase either anti-ovalbumin levels or corneal reactivity: phosphatide fraction of human type *M. tuberculosis* H37 Rv, wax C fraction of human type *M. tuberculosis* Canetti, wax D fractions of bovine type *M. tuberculosis* B.C.G. (two samples), wax D fraction of bovine type *M. tuberculosis* Marmorek, wax D fraction of bovine type *M. tuberculosis* Dupré, wax D fraction of *M. avium* strain No. 802, wax D fraction of *M. smegmatis*, wax D fraction of *M. phlei*.

The products of hydrolysis of effective wax D fractions of human type *M. tuberculosis*, i.e. mycolic acid and polysaccharide from wax D of strain Canetti, were found ineffective in increasing antibody levels over those of the control groups. Also methyl mycolate, phthiocerol di-acetate and trehalose di-mycolate (cord factor) failed to cause an increase of anti-ovalbumin levels. The corneal reactivity in all these cases was not increased.

As shown previously (White *et al.*, 1955), 200 μg. of 'purified wax fraction' (Anderson, 1929) of the human strains H37 was similarly effective in increasing ovalbumin serum antibody levels and in the production of a high level of corneal reactivity. The morphological results which accompany the injection of Purified wax fraction with ovalbumin in water-in-oil emulsion have already been described in detail (White *et al.*, 1955). The animals receiving wax D fractions in these experiments showed similar morphological changes (see also Suter and White, 1954). Briefly these may be summarized:

1. The foot injected with ovalbumin in oil emulsion containing wax D became greatly swollen and at three weeks was occupied by a granuloma consisting mainly of macrophages and fibroblasts.
2. The regional lymph nodes (the popliteal nodes and the group of nodes in the homolateral flank) became swollen and occupied by a proliferation of macrophages.
3. The remoter lymph nodes (e.g. the nodes of the contralateral flank and the lumbar abdominal nodes) as well as the nodes of the homolateral flank contained extensive proliferations of plasma cells and their precursors.

4. The lungs contained miliary granulomata of macrophages (epithelioid cells and giant cells).

All four morphological changes were constantly present in the animals which received the Purified wax fraction (Anderson) and wax D fractions of human type *M. tuberculosis*: H37 Rv, Test and Canetti. Such changes were absent from the controls. The only morphological changes in these were some swelling of soft consistency of the injected footpad and a slight infiltration of the peripheral sinuses of the regional lymph nodes with macrophages (foam cells). In those animals which received chemical fractions which failed to cause an increase in serum antibody levels to ovalbumin, i.e. the phosphatide of human type *M. tuberculosis* H37 Rv, the wax C fraction of human type *M. tuberculosis* Canetti, the wax D fractions of bovine strains of *M. tuberculosis* B.C.G. (WL7 and WL21), Marmorek and Dupré, the wax D fractions of *M. avium* No. 802, and the wax D fractions of *M. phlei* and *M. smegmatis*, the morphological changes were indistinguishable from those observed in the group of control animals.

EFFECT OF VARIOUS WHOLE AND LIPID-EXTRACTED MYCOBACTERIA ON ANTIBODY LEVELS TO OVALBUMIN INJECTED IN A SINGLE DOSE IN WATER-IN-OIL EMULSION

Table 2 shows that 200 μg. of whole heat-killed bacilli of the human type *M. tuberculosis* Canetti, 200 μg. of *M. phlei* and 200 μg. of *M. smegmatis* all produced an increase in antibody levels three weeks after a single injection of ovalbumin in water-in-oil emulsion. The animals injected with these materials showed intense reactions to the intra-corneal injection of ovalbumin when tested nineteen days later. Further, the injection of these three types of mycobacteria in oil emulsion with added ovalbumin resulted in the same gross and microscopical changes as were described above as characteristic of animals receiving wax D fractions of human type *M. tuberculosis*.

TABLE 2

EFFECT OF VARIOUS WHOLE AND FAT-EXTRACTED MYCOBACTERIA ON ANTIBODY LEVELS TO OVALBUMIN INJECTED AS A SINGLE DOSE IN WATER-IN-OIL EMULSION

No. in group	*Fraction and dose*	*Ref. No.*	*Anti-ovalbumin (μgN/ml. serum)*			*Corneal reaction at 48 hours*
			Mean	*Range*	*S.D.*	
4	200 μg. whole heat-killed *M. tuberculosis*, human, Canetti	WL9	509	238–715	171	3c, 3c, 3c, 2c.
3	500 μg. acid-delipidated *M. tuberculosis*, human, Canetti	WL12	134	22–245	91	0, 1c, 0.
4	100 μg. acid-delipidated *M. tuberculosis*, human, Canetti	WL12	165	82–375	122	0, 0, 0, 0.
7	Contemporaneous controls	—	147	31–355	99	0, 0, 0, 1, 1, 0, 0.
5	200 μg. neutral solvent-extracted *M. tuberculosis*, human, H37 Rv	WL31	395	235–456	92	3c, 3c, 3c, 3c, 3c.
5	200 μg. neutral solvent-extracted *M. tuberculosis*, bovine, Marmorek	WL30	441	274–683	143	3c, 3c, 3c, 3c, 3c.
5	Contemporaneous controls	—	62	18–138	46	0, 0, 0, 0, 0.
5	200 μg. dried whole bacilli *M. phlei*	WL32	322	206–426	71	3c, 3c, 3c, 3c, 3c.
5	200 μg. dried whole bacilli *M. smegmatis*	WL33	400	260–582	117	3c, 3c, 3c, 3c, 3c.
5	Contemporaneous controls	—	124	89–176	33	0, 0, 0, 1, 1.

On the contrary, Canetti bacilli extracted under acid conditions (WL12) failed to increase antibody levels to ovalbumin, and failed to induce a high level of corneal reactivity to ovalbumin. Human type *M. tuberculosis* H37 Rv (WL31) and bovine type *M. tuberculosis* Marmorek (WL30), which were extracted with neutral solvents, both produced increased antibody levels to ovalbumin, and both induced intense corneal reactivity to ovalbumin. Whereas *M. tuberculosis* Canetti bacilli extracted under acid conditions (WL12) failed to induce any morphological changes different from those of the controls, the neutral solvent extracted *M. tuberculosis* H37 Rv (WL31) and *M. tuberculosis* Marmorek (WL30) both caused gross and microscopical tissue changes similar to those described in the previous section as characteristic of animals receiving wax D fractions of human type *M. tuberculosis.*

A COMPARISON OF THE ACTIVITY OF VARIOUS DOSES OF HEAT-KILLED HUMAN TYPE M. TUBERCULOSIS CANETTI WITH SIMILAR DOSES OF A WAX D FRACTION OF THE SAME ORGANISM

The data of Table 3 show that inclusion of 5 mg., 200 µg. and 40 µg. doses of heat-killed human type *M. tuberculosis* Canetti organisms produced significant increases in

TABLE 3

A COMPARISON OF THE ACTIVITY OF SIMILAR WEIGHTS OF HEAT-KILLED HUMAN TYPE M. TUBERCULOSIS, CANETTI, AND A WAX D FRACTION FROM THE SAME ORGANISM

No. in group	*Fraction and dose*	*Ref. No.*	*Anti-ovalbumin (µgN/ml. serum)*			*Corneal reaction at 48 hours*
			Mean	*Range*	*S.D.*	
1	5 mg. *M. tuberculosis,* Canetti	WL9	308	—	—	3c.
1	1 mg. *M. tuberculosis,* Canetti	WL9	225	—	—	1.
4	200 µg. *M. tuberculosis,* Canetti	WL9	509	238–715	172	3c, 3c, 3c, 2c.
8	40 µg. *M. tuberculosis,* Canetti	WL9	384	196–575	137	3c, 2c, 3c, 3c, 2c, 2c, 2c, 1.
8	8 µg. *M. tuberculosis,* Canetti	WL9	130	58–204	54	1, 0, 0, 0, 2, 0, 0, 0.
4	1·6 µg. *M. tuberculosis,* Canetti	WL9	120	94–138	17	0, 0, 0, 0.
4	40 µg. wax D of *M. tuberculosis,* Canetti	WL2	493	229–623	155	3c, 3c, 3c, 1.
8	8 µg. wax D of *M. tuberculosis,* Canetti	WL2	203	55–285	81	0, 0, 0, 0, 0, 2, 1c, 0.
11	Contemporaneous controls	—	100	31–188	58	0, 0, 0, 0, 0, 0, 0, 1, 0, 1, 0.

the serum antibody levels three weeks after a single injection of ovalbumin in water-in-oil emulsion as compared with the levels in the control groups which were injected with ovalbumin emulsions similar except in their lacking mycobacteria. No significant adjuvant effect followed the use of doses of 8 and 1·6 µg. of *M. tuberculosis.* It is further shown that 40 µg. of a wax D fraction of *M. tuberculosis* Canetti is similarly effective in causing an increase in anti-ovalbumin levels, whereas 8 µg. dose may have some effect.

Corneal reactivity to ovalbumin is shown to be at a high level following 5 mg., 200 µg. and 40 µg. doses of whole *M. tuberculosis* Canetti, but to be almost constantly absent with lower doses (8 and 1·6 µg.). Corneal reactivity was found to be similarly high at the 40 µg. dose of wax D and to be almost constantly absent following a dose of 8 µg.

At the dose levels at which significantly increased antibody levels were observed

(40 μg. and above) both heat-killed *M. tuberculosis* Canetti and wax D showed similar morphological changes in the guinea-pigs, which were identical with those already described for wax D fractions of *human* type *M. tuberculosis* in a previous section above.

DISCUSSION

It has been firmly established (Choucroun, 1947; Raffel, 1948, 1950) that a wax fraction of *M. tuberculosis* along with protein of the bacillus induces in animals the state of tuberculin hypersensitivity. Raffel (1949) also showed that when an unrelated antigen, ovalbumin, is injected with the wax fraction of *M. tuberculosis* a delayed-type hypersensitivity develops to this antigen. Given in this way (ovalbumin injected at the same site as a saline emulsion of wax) delayed-type hypersensitivity developed without any demonstrable elevation of serum antibody titre. But it has been amply shown (Raffel *et al.*, 1949; Fischel *et al.*, 1952; White *et al.*, 1955) that when ovalbumin is injected in water-in-oil emulsion together with heat-killed tubercle bacilli or the 'purified wax fraction' a rise in anti-ovalbumin levels occurs. The increase in the antibody response which is due to the added tubercle bacilli or wax fraction thereof can be shown by comparison with controls injected with ovalbumin in simple water-in-oil emulsion.

Our experiments show that the following materials are active in increasing serum antibody levels to ovalbumin in the guinea-pig:

1. Whole, heat-killed and dried bacilli of human and bovine types of *M. tuberculosis*, of *M. avium* and of saprophytic strains of Mycobacteria, as already shown by Freund and McDermott (1942).
2. Wax D of three human strains of *M. tuberculosis* (Test, Canetti and H37 Rv).
3. 'Delipidated bacilli'* of human, bovine and saprophytic strains.

The following materials have been found inactive in increasing the antibody response:

1. 'Totally delipidated'† bacilli of human type *M. tuberculosis* (strain Canetti).
2. Wax D of three bovine strains (B.C.G., Marmorek and Dupré), of *M. avium*, strain No. 802, and of two saprophytic types *M. phlei* and *M. smegmatis*.
3. The phosphatide, wax C and 'cord factor' of human strains of *M. tuberculosis*.
4. Hydrolysis products of active wax D preparations (mycolic acid and polysaccharide of the human strain Canetti).

Before interpreting these results, we shall recall our main knowledge of the chemical structure of the wax D fractions.

Hydrolysis of wax D of human strains yields approximately 50 per cent mycolic acids, i.e. high molecular weight β-hydroxy-acids represented by the general formula $C_{88}H_{176}O_4$ (Asselineau and Lederer, 1953) and about 50 per cent of a nitrogenous polysaccharide of an estimated molecular weight of 6,000 to 9,000. The polysaccharide contains three sugars: arabinose, galactose and mannose; and three amino acids: alanine, L-glutamic acid and meso-α, ε-diaminopimelic acid (Asselineau, Choucroun and Lederer, 1950; Asselineau and Castel, personal communication). These three amino acids form

* 'Delipidated bacilli' have been extracted with alcohol-ether and then chloroform and still contain the 'firmly bound lipids' described by Anderson, Reeves and Stodola (1937).

† 'Totally delipidated' bacilli have been obtained by heating 'delipidated' bacilli in acid alcohol-ether, thus liberating and extracting the 'firmly bound lipids'.

a peptide which is linked to the polysaccharide (Asselineau and Lederer, 1953). The mycolic acids are esterified with the polysaccharide; wax D of human strains thus has the general structure: mycolic acid-polysaccharide-peptide (Asselineau and Castel, personal communication).

Wax D fractions of bovine, avian and saprophytic strains do not contain any amino acids and thus have the general structure: mycolic acid-polysaccharide. Some of these contain the same three sugars: arabinose, galactose and mannose.

The striking difference between the activity of the wax D of human strains and the inactivity of the wax D of bovine, avian and saprophytic strains suggests that the presence of the above-mentioned peptide, linked to the glycolipid, is essential for activity.

The inactivity of the hydrolysis products of the active wax D fractions of human strains (e.g. mycolic acid or methyl mycolate and the peptide-containing polysaccharide) indicates that activity is to be ascribed to the intact mycolic ester of the peptide-containing polysaccharide.

The inactivity of the phosphatide of the human strain H37 Rv is worth mentioning, as recent results* suggest that all our active wax D fractions are contaminated with a certain amount (up to 20 per cent) of phosphatide.

It remains to be explained why delipidated bacilli of human, bovine, avian and saprophytic strains are also active. These delipidated bacilli contain still the 'firmly bound lipids' (Anderson *et al.*, Anderson, Peck and Creighton, 1937) and moreover they all contain the above-mentioned three amino acids (alanine, glutamic acid and α,ε-diaminopimelic acid), which constitute the major nitrogenous components of the bacterial cell wall (not only of Mycobacteria but also of other bacteria) (Cummins and Harris, 1956).

The linkage of the firmly bound lipids in the bacteria delipidated with alcohol-ether and chloroform is not yet known; this linkage is destroyed by boiling with dilute acid, thus liberating the firmly bound lipids, which have a composition very similar to the wax D fractions, but are devoid of amino acids. Our results suggest that the delipidated bacteria contain the three above-mentioned amino acids of the cell wall linked to the firmly bound lipids, thus constituting a complex which is chemically very close to the wax D fractions of human strains and shows the same biological activity as the latter.

Acid hydrolysis, followed by extraction of the firmly bound lipids, destroys the active complex present in the delipidated bacterial residues.

A difficulty in deriving conclusions regarding the activity of tuberculolipid fractions is their contamination with small numbers of intact bacillary bodies (Raffel, 1948). It could possibly be maintained that the activity of a particular fraction is due to the high activity of such contaminants. In this present study small numbers (the order of 10^4 bacilli per mg. or less than 0·01 per cent) of *M. tuberculosis* were found in all the bacillary fractions. That these small numbers of contaminants could explain the adjuvant properties of the chemical fractions is unlikely from the following facts.

1. The activity of the wax D fraction of *M. tuberculosis* Canetti is at least of the same order as whole heat-killed bacilli of *M. tuberculosis* Canetti when identical weights of both are compared at five-fold dose intervals.

* Dr. Nojima (Tokyo) kindly informed us that he had isolated crystalline *meso*-inositol from wax D of B.C.G., and Asselineau and Castel (personal communication) have confirmed the presence of *meso*-inositol in our wax D preparations, but ascribe it to the presence of a certain amount of the phosphatidyl-inosito dimannoside described by Vilkas and Lederer (1956); these same wax D preparations contain also phosphorus, glycerol, lower fatty acids and mannose in amounts compatible with the presence of such a phosphatide.

2. The wax D fractions of the bovine type of *M. tuberculosis* Marmorek and of *M. smegmatis* and *M. phlei* are ineffective although they, too, are similarly contaminated with trace amounts of intact bacillary bodies, and it is shown above that intact whole *M. smegmatis* and *M. phlei*, and delipidated *M. tuberculosis* Marmorek, are active.

When whole heat-killed tubercle bacilli suspended in paraffin oil are injected subcutaneously into guinea-pigs complex and widespread histological changes occur (Rist, 1938; Saenz and Canetti, 1940). Briefly these consist of:

1. A local granuloma at the site of injection.
2. An infiltration, particularly of the regional lymph nodes, with cellular derivatives of the macrophage.
3. A widespread proliferation of plasma cells in the spleen and lymph nodes throughout the body.

The same morphological results follow the subcutaneous injection of 'purified wax fraction' (Anderson, 1929) (White *et al.*, 1955). In the animals of the present series all the above morphological features were present in those animals which received whole heat-killed *M. tuberculosis* bacilli and the wax D fractions of the human type bacilli Test, Canetti and H37 Rv and which had increased serum anti-ovalbumin values. With fractions of wax D from strains of bovine type *M. tuberculosis* (B.C.G., Dupré and Marmorek) from the *M. avium* No. 802 and from *M. phlei* and *M. smegmatis* and the other bacillary fractions which failed to cause an increased serum antibody the above histological appearances were absent, and in those respects the tissues appeared identical with those of the controls. On the basis of the results of staining frozen tissue sections with the method for revealing anti-ovalbumin (see White *et al.*, 1955), it was thought that the increased antibody production of these animals must be attributed to the widespread proliferation of plasma cell elements. This was reinforced by the evidence (Askonas and White, 1956) that the *in vitro* activity of tissue slices from such animals in incorporating ^{14}C amino acids into anti-ovalbumin correlated with their content of plasma cells. The relationship of the increased antibody levels to the extensive macrophage proliferations of these animals, if any, is unknown. No instance was met of a dissociation of the macrophage from the plasma cell response. Also no dissociation was revealed in these experiments between tissue hypersensitivity as judged by the corneal reaction and increased serum antibody levels. Thus all the compounds which caused increased serum anti-ovalbumin levels also caused a high degree of corneal reactivity to ovalbumin.

In our experience ovalbumin in oil adjuvant mixture with methyl mycolate did not give rise to corneal hypersensitivity. It has been recorded (Raffel, Lederer and Asselineau, 1954; Raffel, Asselineau and Lederer, 1955) that methyl mycolate together with the protein of the bacillus can induce the state of tuberculin hypersensitivity. However, Raffel (personal communication) has been unable in recent experiments to confirm this activity of methyl mycolate. It has been accepted (Raffel, Arnaud, Dukes and Huang, 1949) that a relatively high degree of delayed hypersensitivity is necessary for the occurrence of the corneal response.

The histological results obtained by us showed that a massive proliferation of macrophages followed injection of oily emulsions of wax D fractions of human type *M. tuberculosis* in doses as small as 40 μg. It is difficult to relate this to the many observations

which have established that certain individual lipids can act as a direct stimulus to macrophages with results closely resembling a tuberculous granuloma. It was shown by Sabin (1932) that the most active fraction in this respect was the phosphatide fraction. The wax fractions were active but to a lower degree. It has been pointed out (Rich, 1950) that very large doses were used in these experiments.

Since the work of Dr. Sabin and her colleagues, extensive investigation of the granuloma-producing properties of a range of synthetic compounds has been published (Bailey, Polgar, Tate and Wilkinson, 1955; Ungar, Coulthard and Dickinson, 1938; Ungar, 1955). We were interested in the possibility that active compounds would function as adjuvants in antibody production. Trial of two of the more active compounds (2-methyleicos-2-enylamine and 2 : 4-dimethyldocos-2-enylamine HCl in 200 μg. doses) with the same conditions as used throughout this investigation gave no increase in anti-ovalbumin levels. Neither did these compounds cause the characteristic proliferation of macrophages which, as described above, accompanies adjuvant activity. There is therefore, at present, no link between the granuloma-producing properties of these lipids following intraperitoneal injection and the widespread stimulation of macrophages seen under the conditions of the present communication.

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