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The Affinity of a Mycobacterial Glycopeptide for Guinea-Pig Gamma-Globulin

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Summary. The affinity of a glycopeptide from culture filtrates of *Mycobacterium tuberculosis* for guinea-pig serum components, first observed as an alteration of electrophoretic mobility of γ_2-immunoglobulin in agar has been explored by a variety of techniques. It was shown that such changes in mobility were produced more readily with γ_2-globulin (Fraction I from DEAE cellulose chromatography) than with γ_1-globulin (Fraction III from DEAE cellulose chromatography). It was estimated that the treatment resulted in a rise of carbohydrate associated with γ_2-globulin from 1·3 per cent to 38–40 per cent.

INTRODUCTION

Previous investigations (White, Bernstock, Johns and Lederer, 1958; White, Jollès, Samour and Lederer, 1964) have shown that mycobacterial peptidoglycolipid fractions can reproduce the adjuvant effects of whole killed mycobacteria, including the production of increased serum antibody, when added to a water-in-oil emulsion of the antigen (ovalbumin) in mineral oil.

Guinea-pigs injected with ovalbumin in a water-in-oil emulsion, with added mycobacterial peptidoglycolipid, produced antisera with immuno-electrophoretic patterns consisting of two distinct anti-ovalbumin precipitin arcs (White, Jenkins and Wilkinson, 1963). One such arc was in the position of fast or γ_1-globulin, and the other conjoined arc was in the position of slow or γ_2-globulin. However, when ovalbumin was injected in a water-in-oil emulsion without mycobacterial peptidoglycolipid the antiserum showed a single γ_1-globulin arc only, the slower-moving component being absent.

In searching for an explanation of such adjuvant effects, it was decided to explore the possible molecular interaction between peptidoglycolipids and antibody globulin. In preliminary experiments sera from guinea-pigs injected with an oily emulsion of ovalbumin together with added peptidoglycolipid were treated with myobacterial glycopeptide; such treatment constantly resulted in a change in the serum electrophoretic pattern. The glycopeptide, available in quantity from culture filtrates of *M. tuberculosis*, was used with the assumption that it bore a structural relation to the glycopeptide component of mycobacterial peptidoglycolipid.

The subsequent investigations detailed in this paper describe the nature of the affinity between the glycopeptide and the globulin component of such antisera as revealed by different techniques.

MATERIALS AND METHODS

Preparation of the glycopeptide from culture filtrates of human type Mycobacterium tuberculosis. Culture filtrates were obtained from the Central Veterinary Laboratories, Weybridge,

and the glycopeptide (Polysaccharide I) was prepared according to the method described by Seibert (1949). Originally this product was designated Polysaccharide I but in a subsequent communication (Seibert, Soto-Figueroa and Du Four, 1955) it was established that Polysaccharide I was a glycopeptide. The fraction from the culture filtrate soluble at pH 4·6 in 70 per cent alcohol, was dialysed to remove alcohol. Remaining traces of protein were precipitated with trichloracetic acid and removed by centrifugation. The supernatant containing the glycopeptide was dialysed and evaporated to dryness.

Mycobacterial antisera used in this investigation were kindly prepared by Dr C. S. Cummins in rabbits by a primary injection of whole *Mycobacterium smegmatis* in mineral oil followed, at six weekly intervals, by two further injections of partially ruptured bacilli from a Mickle disintegrator.

Anti-ovalbumin sera were prepared in guinea-pigs using the technique described by White *et al.* (1958). Animals were bled at 21 days after a single injection of thrice crystallized ovalbumin in 0·2 ml of a water-in-mineral oil (Bayol 55, kindly provided by Esso Petroleum Co.) emulsion containing 200 μg of peptidoglycolipid (wax D) of human type *M. tuberculosis* in the oil phase. Injection was done in the left hind foot-pad.

Rabbit anti-guinea-pig globulin was produced by a globulin fraction obtained by precipitation of guinea-pig serum with an equal volume of saturated ammonium sulphate. The globulin (2 mg) was injected in water-in-oil emulsion with 1 mg of heat-killed *M. tuberculosis* (Freund's complete adjuvant) subcutaneously into rabbits in the inner aspects of both hind legs. The animals were re-injected intravenously and at two sites subcutaneously 1 month later with 2 mg globulin in 1 ml of 0·35 per cent aluminium phosphate solution and were bled 8, 10 and 12 days after this second injection.

Preparation of the serum fractions

Fast and slow guinea-pig γ-globulin fractions were separated on DEAE cellulose (Whatman DE50) as described by White *et al.* (1963). This was achieved either by concave gradient chromatography from 0·01 M PO_4 buffer, pH 7·5, to 0·3 M NaH_2PO_4 buffer as described by Fahey, McCoy and Goulian (1958) or by a stepwise elution technique. In the latter method the slow (γ_2-globulin) fraction (Fraction I) was eluted with 0·02 M PO_4 buffer, pH 7·5. Fraction III (γ_1-globulin) was then eluted with 0·1 M PO_4 buffer, pH 6·0. The remainder of the serum protein was then removed with 0·3 M NaH_2PO_4. A highly purified slow (γ_2-globulin) fraction was obtained using either technique. The fast (γ_1-globulin) fraction was not pure. It was eluted more sharply by stepwise elution (Fig. 1) but was more contaminated with other serum proteins using this method than using gradient chromatography. Protein concentration was estimated at a wavelength of 280 mμ in a Unicam spectrophotometer. Protein peaks were pooled and concentrated by dialysis against polyethylene glycol ('Carbowax 20 M', G. T. Gurr). Absolute protein values for DEAE Fraction I (γ_2-globulin) were estimated using a standard curve relating optical density at 280 mμ of weighed amounts of lyophilized γ_2-globulin to protein concentration. Immunoelectrophoretic analyses of all fractions against rabbit anti-guinea-pig globulin and against ovalbumin were performed in agar at pH 8·4 by a standard technique.

Sephadex G-200 chromatography. A slurry of 3 g Sephadex G-200 (Pharmacia, Uppsala, Sweden) in 0·15 M NaCl was prepared and allowed to stand for 48 hours. It was then poured to form a column of approximate dimensions $100 \times 1{\cdot}1$ cm. The eluant used in all experiments was 0·15 M NaCl.

Treatment of serum and serum fractions with the glycopeptide. The glycopeptide was mixed with whole serum, Fraction I or Fraction III in the proportion 50 mg/ml and incubated at 37° for 4 hours. The mixture was centrifuged at 2000 rev/min and the supernatant stored at −20°.

Chromatography of the glycopeptide preparation

Amino acids and hexosamines. The methods of hydrolysis and chromatography of the hydrolysate were as previously described (Stewart-Tull and White, 1964).

Sugars. The glycopeptide was hydrolysed in 2 N H_2SO_4 in a sealed tube at 105° for 2 hours and after cooling, was neutralized with solid $BaCO_3$. The $BaSO_4$ precipitate was removed by filtration, the filtrate was evaporated to dryness over a boiling water-bath and then re-dissolved in 0·25 ml distilled water. One-dimensional chromatography on Whatman No. 4 paper was performed using ethyl acetate + pyridine + water (160 + 40 + 20 by vol.) as the solvent (Jermyn and Isherwood, 1949). The sugars were revealed with aniline hydrogen phthalate.

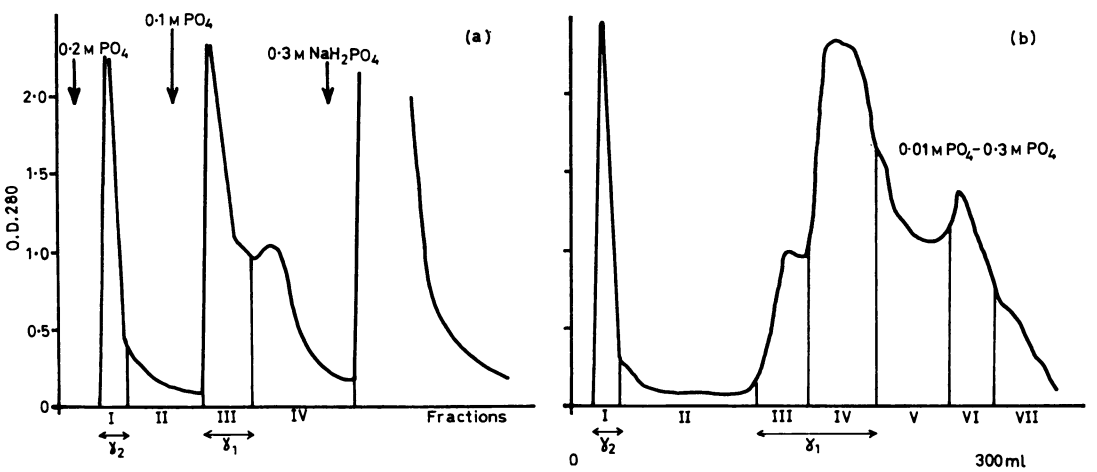

FIG. 1. Elution patterns of guinea-pig sera on DEAE cellulose. (a) Stepwise elution, and (b) gradient elution.

Carbohydrate estimation

Carbohydrate was estimated using the anthrone method. The solution to be assayed (3 ml) was placed in a chrome-cleaned test-tube, in an ice-bath. Anthrone reagent (6 ml prepared by dissolving 2 g anthrone in 1 l. of 95 per cent H_2SO_4) was added and the tube shaken to ensure complete mixing. The tube was placed in a boiling water-bath for 3 minutes and subsequently cooled. Optical density was read over the wavelength range 500–650 mμ in a Unicam spectrophotometer, in order to include the hexose peak at 625 mμ. A proportionability factor was determined from a standard curve of glucose and the amount of carbohydrate in each fraction was calculated by multiplying the optical density value at 625 mμ by this factor. The carbohydrate content of protein was expressed as a percentage by estimating the proportion of carbohydrate, determined at 625 mμ, to protein, determined at 280 mμ.

Complement fixation test

Complement-fixing antibodies against ovalbumin were estimated by a method similar to that described by White, Bass and Williams (1961), using an overnight fixation period. Two minimal haemolytic doses of complement were incubated overnight at 4° with appropriate dilutions of the test serum and antigen. Sensitized sheep red cells (2 per cent) were added next day and the test was read after incubation at 37° for 30 minutes. The antigen solution, containing 250 μg/ml ovalbumin, was titrated against a strongly positive serum before use, and in all subsequent tests the optimal dilution of antigen (1 : 4) was used. The last tube of a series of doubling serum dilutions to show complete fixation of complement was taken as endpoint.

Passive cutaneous anaphylaxis test (PCA)

The method of detecting skin sensitizing ability in guinea-pig anti-ovalbumin sera was described by White *et al.* (1963). Dilutions of serum containing 2·5 μg, 1·0 μg, 0·1 μg and 0·02 μg were injected intradermally into a guinea-pig at sites symmetrically disposed along both sides of the vertebral column. After an interval of 2 hours 45 minutes ovalbumin (2 μg antigen N) in 1 ml of 1 per cent Evans blue was injected via an ear vein. After waiting 40 minutes for the optimal development of the local lesions the animals were killed and skinned and the lesions, visible on the underside of the skin as blue areas, were measured.

RESULTS

CHROMATOGRAPHY OF GLYCOPEPTIDE (SEIBERT'S POLYSACCHARIDE I)

Chromatographic analyses of the glycopeptide fraction showed the presence of alanine, glutamic acid and α,ε-diaminopimelic acid as major components, with aspartic acid and glycine as minor components. The sugars were identified as arabinose and galactose; the hexosamines as glucosamine and muramic acid.

RELATIONSHIP BETWEEN THE GLYCOPEPTIDE AND THE HYDROSOLUBLE MOIETY OF THE PEPTIDOGLYCOLIPID (WAX D)

Preliminary attempts were made to explore the relationship between the glycopeptide prepared from the culture filtrate of *Mycobacterium tuberculosis* and the hydrosoluble moiety of the peptidoglycolipid prepared by alkaline hydrolysis. When solutions of these substances were exposed to a rabbit anti-serum against whole mycobacteria in an agar-gel double-diffusion test, a single precipitin line resulted against each antigen which joined to show a reaction of complete identity (Fig. 2).

THE EFFECT OF MYCOBACTERIAL GLYCOPEPTIDE ON THE ELECTROPHORETIC PATTERN OF WHOLE SERA FROM IMMUNIZED GUINEA-PIGS

The initial observation which prompted subsequent experiments is shown in Fig. 3. Anti-ovalbumin sera, from guinea-pigs which had received 200 μg wax D *M. tuberculosis* strain 'C' Weybridge in water-in-oil emulsion, were treated for 4 hours at 37° with the glycopeptide. Immunoelectrophoresis of these sera showed that the double, anti-ovalbumin arc produced after treatment was shorter than the arc produced by the untreated serum. In addition, when these same sera were tested against rabbit anti-guinea-pig globulin (Fig. 4) a shortening or forward movement of the γ_2-globulin arc, in treated as contrasted with untreated sera was very evident. These changes were also shown in sera from guinea-

pigs immunized with diphtheria toxoid instead of ovalbumin under otherwise identical conditions. However, sera from normal unimmunized guinea-pigs and from guinea-pigs injected with ovalbumin in incomplete Freund's adjuvant mixture also showed a slight increase in mobility of the γ_2-globulin, when tested against rabbit anti-guinea-pig globulin serum (Fig. 5); this change in γ_2-globulin mobility was less marked than that observed with sera from animals which had received the complete Freund's adjuvant mixture.

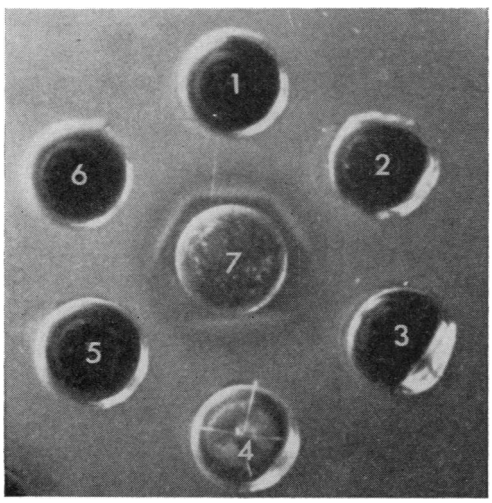

FIG. 2. Agar gel double diffusion test. Note the reaction of identity between the precipitin lines produced by the hydrosoluble moiety of wax D and the glycopeptide against rabbit anti-*M. smegmatis* serum. Basins 1 and 4 = mycobacterial glycopeptide; Basin 2 = hydrosoluble fraction of wax D of *M. tuberculosis* Canetti; Basin 6 = hydrosoluble fraction of wax D of *M. tuberculosis* H_{37} Rv.; Basins 3 and 5 = normal saline; Basin 7 = Cummins anti-*M. smegmatis* serum.

THE EFFECT OF MYCOBACTERIAL GLYCOPEPTIDE ON THE ELECTROPHORETIC BEHAVIOUR OF FRACTIONS OF GUINEA-PIG SERA FROM DEAE

Previous work has shown that the fast and slow guinea-pig precipitins may be isolated and purified by DEAE cellulose chromatography (White *et al.*, 1963). The purified fast and slow γ-globulin components were examined before and after treatment with the glycopeptide. The resulting immunoelectrophoretic patterns indicated that after treatment with the glycopeptide, Fraction I (γ_2) acquired an increase in mobility, whereas Fraction III (γ_1) showed a slight but inconsistent increase in mobility (Fig. 6).

EXPERIMENTS TO DETERMINE THE EFFECT OF GLYCOPEPTIDE ON THE BIOLOGICAL ACTIVITY OF FAST AND SLOW IMMUNOGLOBULIN COMPONENTS

Ability to produce passive cutaneous anaphylaxis in the guinea-pig is known to be associated with the fast (γ_1) fraction of guinea-pig globulin (White *et al.*, 1963; Ovary, Benacerraf and Bloch, 1963). Conversely, complement-fixing activity is found only in the slow (γ_2) fraction (Bloch, Kourilsky, Ovary and Benacerraf, 1963; Wilkinson, 1963, unpublished observation). These fractions from guinea-pig sera were tested, before and after treatment with the glycopeptide. As seen from Table 1, no change resulted in the PCA

activity of Fraction III (γ_1-globulin) or in the complement-fixing activity of Fraction I (γ_2-globulin) after treatment with the glycopeptide.

DETERMINATION OF BINDING CAPACITY OF γ-GLOBULIN FOR MYCOBACTERIAL GLYCOPEPTIDE

The possibility that the glycopeptide was binding to γ-globulin was tested by comparing carbohydrate content of Fraction I (γ_2) and Fraction III (γ_1) before and after treatment with glycopeptide. This was done by periodic acid Schiff (PAS) staining of the precipitin arcs after immunoelectrophoresis, according to the method of Stewart-Tull (1965), and by quantitative estimation of carbohydrate using the anthrone method. The polysaccharide PAS reaction of Fraction III was unchanged after treatment with glycopeptide while that of Fraction I was greatly increased (Fig. 7).

TABLE 1

COMPLEMENT FIXATION AND PASSIVE CUTANEOUS ANAPHYLAXIS TESTS

Complement fixation test			Passive cutaneous anaphylaxis test		
Guinea-pig No. 4085	Titre		Dose of anti-ovalbumin given intradermally (μg N)	Fraction III before treatment	Fraction III after treatment
	Before treatment	After treatment			
Whole serum	32	32	2·5	240	247
Fraction I	16	16	1·0	190	156
Fraction III	0	0	0·1	35	18
			0·02	0	4

Results of complement fixation tests on whole guinea-pig serum and fractions before and after treatment with glycopeptide. The titres are expressed as the reciprocal of dilution. Passive cutaneous anaphylaxis: areas of blueing (measured on under surface of skin of back in mm^2) in a guinea-pig receiving 2 μg antigen N (ovalbumin) intravenously.

For quantitative analysis of carbohydrate an aliquot of glycopeptide-treated DEAE Fraction I (γ_2-globulin) was re-chromatographed on DEAE cellulose using a phosphate buffer gradient, starting at 0·01 M PO_4. It was found that almost all of the protein was eluted from the column in one column volume, thus showing that the behaviour of slow (γ_2) globulin on DEAE cellulose was not affected by treatment with the glycopeptide. With 0·01 M PO_4 as eluant most of the free glycopeptide remained at the top of the column as a coloured band. In a control experiment 50 mg of free glycopeptide was chromatographed on DEAE cellulose at 0·01 M PO_4, and 0·52 mg (1 per cent) was eluted in the position of Fraction I γ-globulin (Table 2). Carbohydrate estimations were carried out on the treated, re-chromatographed Fraction I globulin by the anthrone method and corrections were made for the small amount of free glycopeptide known to be present in this fraction. The results in Table 2 indicate that the carbohydrate content of Fraction I (γ_2) after treatment with the glycopeptide, was increased from 1·3 per cent to 38 per cent. Immunoelectrophoresis of the treated Fraction I and treated re-chromatographed Fraction I showed that the alteration in the immunoelectrophoretic pattern was maintained after re-chromatography on a DEAE cellulose column (Fig. 8).

As preparations of glycopeptide-treated γ-globulin from DEAE cellulose contained both free and protein-bound glycopeptide an attempt was made to separate these two

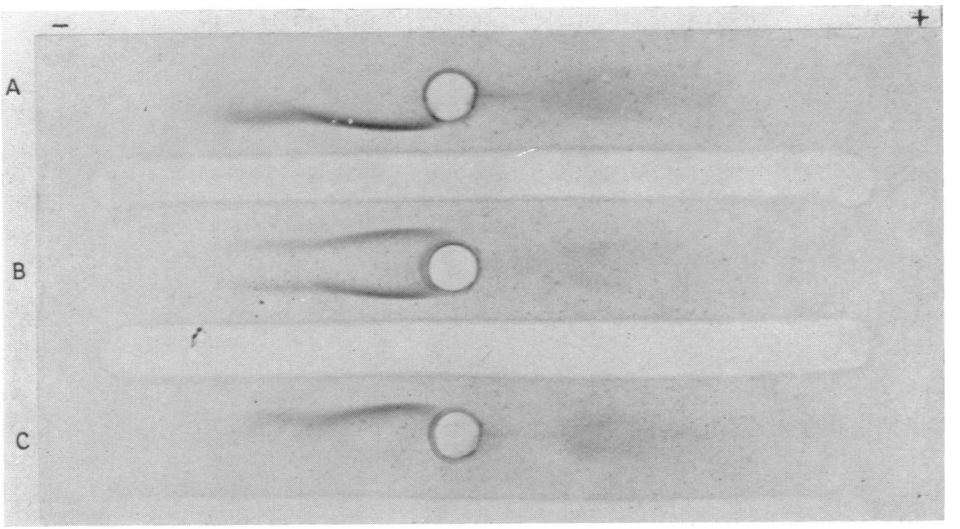

FIG. 3. Agar gel immuno-electrophoresis of the serum from a guinea-pig immunized with ovalbumin in water-in-oil emulsion containing mycobacterial peptidoglycolipid. Well A contained untreated whole guinea-pig serum No. 3954, and Wells B and C contained treated whole guinea-pig serum No. 3954. Ovalbumin (100 μg/ml) was placed in the troughs after electrophoretic separation of serum proteins. Note the shortening at the cathodal end of the double precipitin arc against ovalbumin with B and C as compared with A.

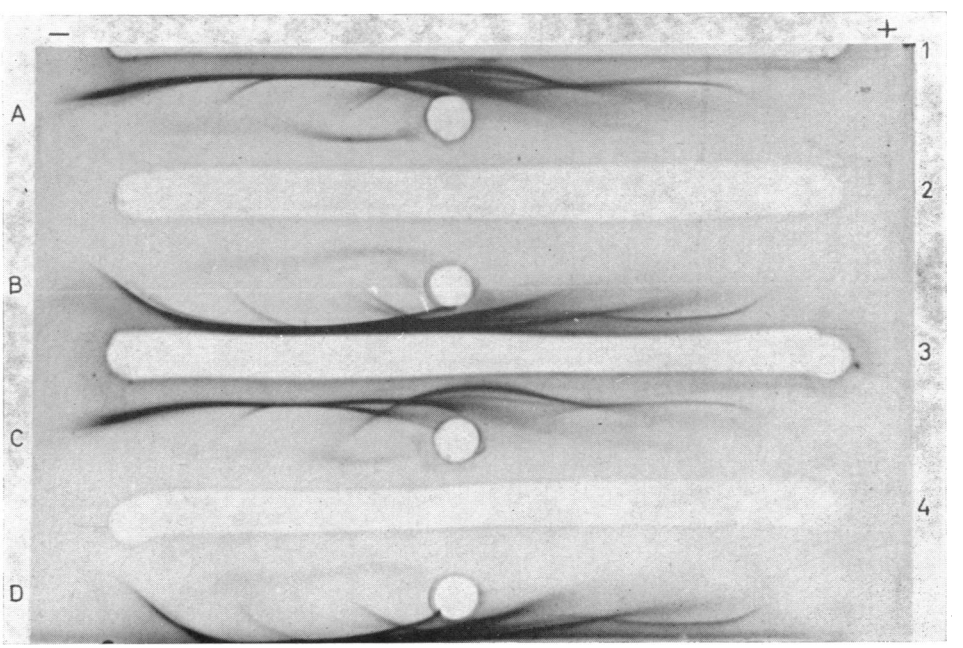

FIG. 4. Agar gel immuno-electrophoresis of guinea-pig sera before and after treatment with glycopeptide against rabbit anti-guinea-pig globulin and ovalbumin. The wells contained: A, untreated whole guinea-pig serum No. 3957; B, treated whole guinea-pig serum No. 3957; C, untreated whole guinea-pig serum No. 3954, and D, treated whole guinea-pig serum No. 3954. Troughs 1 and 3 contained rabbit anti-guinea-pig globulin and troughs 2 and 4 contained ovalbumin (100 μg/ml). Note that there is a curtailment of the cathodal end of the γ_2-globulin arc developed against rabbit anti-guinea-pig globulin, in treated as contrasted with untreated sera.

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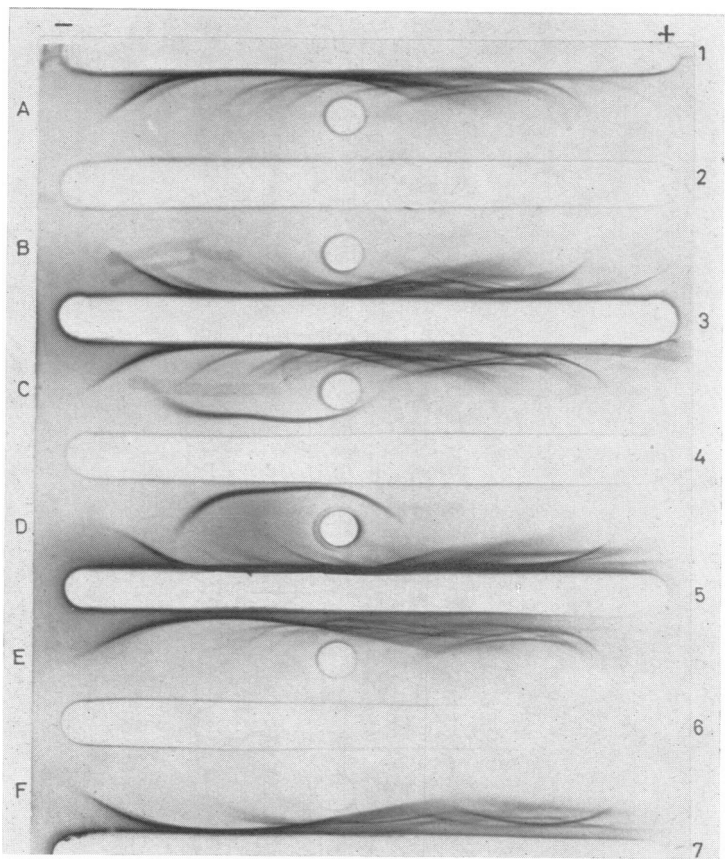

FIG. 5. Agar gel immuno-electrophoresis of sera from normal guinea-pigs and from guinea-pigs immunized against ovalbumin in Freund's complete adjuvant. The wells contained: A, normal guinea-pig serum 1; B, treated normal guinea-pig serum 1; C, untreated guinea-pig anti-ovalbumin serum; D, treated guinea-pig anti-ovalbumin serum; E, normal guinea-pig serum 2; F, treated normal guinea-pig serum 2. Troughs 1, 3, 5 and 7 contained rabbit anti-guinea-pig globulin, and troughs 2, 4 and 6 contained ovalbumin (100 μg/ml). Note that the treated immunized guinea-pig serum showed a marked shortening of the γ_2-globulin arc while in contrast sera from normal guinea-pigs showed little change after treatment with glycopeptide.

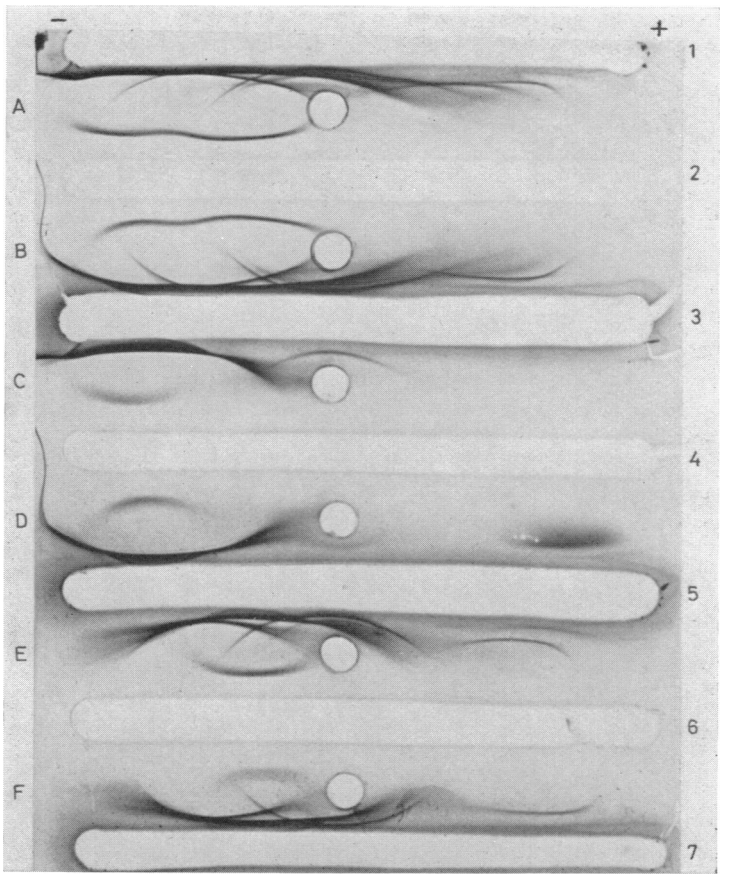

FIG. 6. Agar gel immuno-electrophoresis of treated and untreated whole serum, Fraction I and Fraction III against rabbit anti-guinea-pig globulin and ovalbumin. The wells contained: A, untreated whole guinea-pig serum No. 4073; B, treated whole guinea-pig serum No. 4073; C, untreated Fraction I of serum No. 4073; D, treated Fraction I of serum No. 4073; E, untreated Fraction III of serum No. 4073; F, treated Fraction III of serum No. 4073. Troughs 1, 3, 5 and 7 contained rabbit anti-guinea-pig globulin, and troughs 2, 4 and 6 contained ovalbumin (100 μg/ml). Note the increase in mobility (shift to the right) which is shown in the case of Fraction I after treatment.

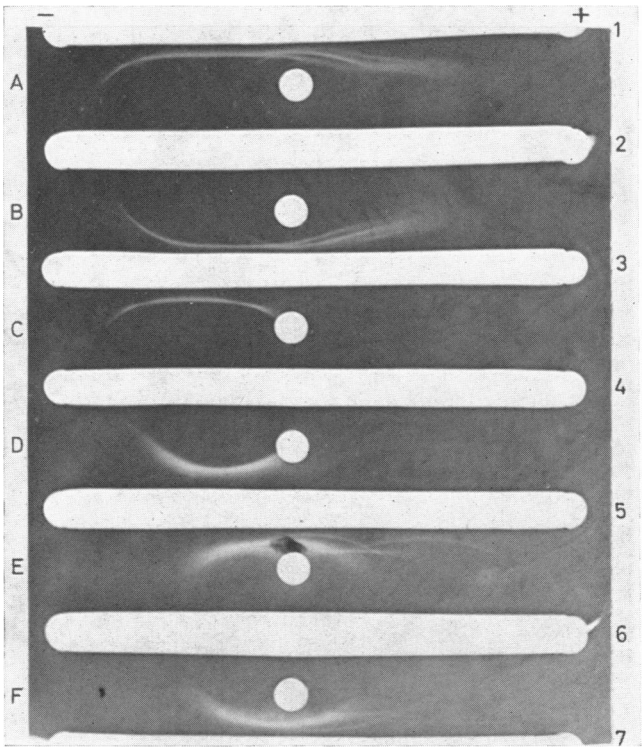

FIG. 7

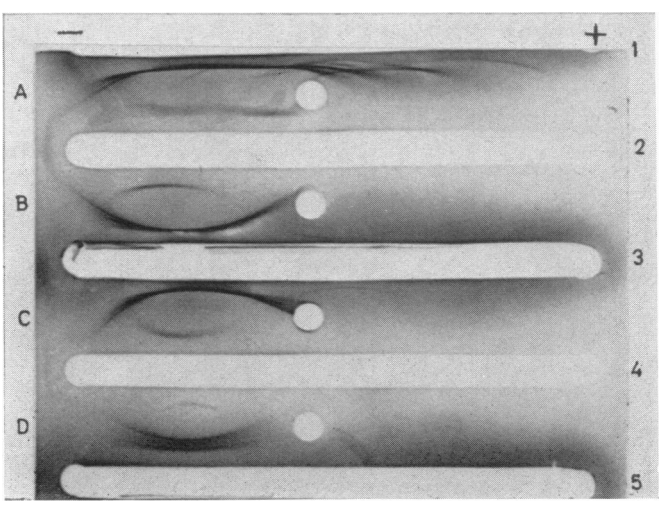

FIG. 8

fractions by gel filtration on Sephadex G-200. As the molecular weight of the glycopeptide was estimated at approximately 35,000 (Lederer, 1964) and that of γ-globulin at 150,000, clear separation of free from protein-bound glycopeptide should have been achieved on Sephadex G-200. The results of this experiment are shown in Fig. 9. The behaviour of free glycopeptide is shown in Fig. 9(a). It was eluted from Sephadex G-200 in two peaks,

TABLE 2

QUANTITATIVE ESTIMATIONS OF PROTEIN AND CARBOHYDRATE

Fraction	Protein (mg/ml serum)	Carbohydrate (mg/ml serum)	Percentage carbohydrate
Untreated DEAE Fraction I (0·01 M PO_4)	3·0	0·039	1·30
Untreated DEAE Fraction III (0·1 M PO_4)	5·0*	0·117	2·34
Treated Fraction I *ex* DEAE (0·01 M PO_4)	2·5	1·47	
Treated Fraction I *ex* DEAE, corrected for free glycopeptide†	2·5	0·95	38·0
Treated DEAE Fraction I re-chromatographed on Sephadex G-200: 7S Fraction	0·7	0·28	40·0
Treated DEAE Fraction I re-chromatographed on Sephadex G-200: 19S Fraction	0·175	0·014	7·9

* Approximate. This fraction contained a mixture of proteins.
† Free glycopeptide in Fraction I = 0·52 mg (see text).
Values of protein and carbohydrate in free glycopeptide and in chromatographic fractions of guinea-pig γ-globulin from DEAE cellulose and Sephadex G-200.

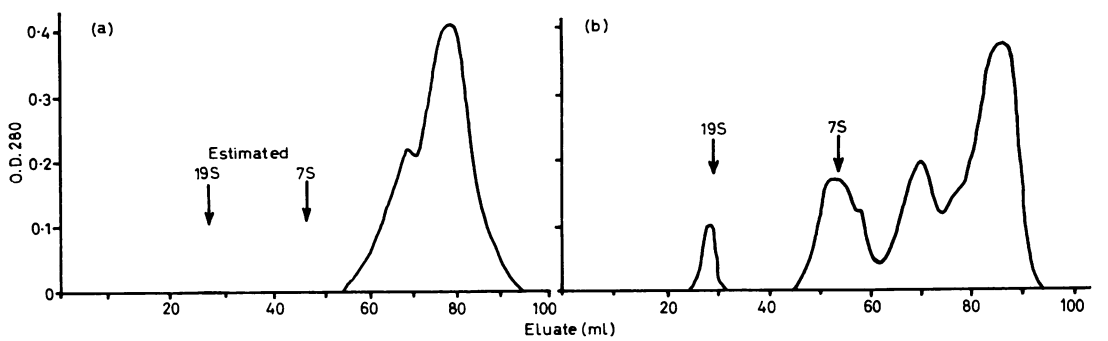

FIG. 9. Analysis on Sephadex G-200 of (a) glycopeptide alone, and (b) a mixture of glycopeptide and guinea-pig Fraction I γ-globulin. Note that the peak characteristic of free glycopeptide is eluted after the two peaks which correspond in position to 19S and 7S immunoglobulins.

FIG. 7. Agar gel immuno-electrophoresis of treated and untreated whole serum. Fraction I and Fraction III against rabbit anti-guinea-pig globulin. The resulting precipitin arcs were stained by the PAS technique. The wells contained: A, untreated whole guinea-pig serum No. 4085; B, treated whole guinea-pig serum No. 4085; C, untreated Fraction I of serum No. 4085; D, treated Fraction I of serum No. 4085; E, untreated Fraction III of serum No. 4085; F, treated Fraction III of serum No. 4085. The troughs 1, 3, 5 and 7 contained rabbit anti-guinea-pig globulin, and troughs 2, 4 and 6 were empty. Note that the narrow precipitin arc with untreated Fraction I has thickened and migrated further towards the anode after glycopeptide treatment. No such change was apparent with Fraction III similarly treated.

FIG. 8. Agar gel immuno-electrophoresis of treated and untreated serum Fraction I and of treated re-chromatographed Fraction I. The wells contained: A, untreated whole guinea-pig serum No. 5101; B, untreated Fraction I of serum No. 5101; C, treated Fraction I of serum No. 5101; D, treated re-chromatographed Fraction I of serum No. 5101. The troughs 1, 3, and 5 contained rabbit anti-guinea-pig globulin, and troughs 2 and 4 contained ovalbumin (100 μg/ml). Note that the precipitin arc against ovalbumin for Fraction I treated with glycopeptide showed increased anodal mobility. This shift was maintained in the re-chromatographed treated Fraction I.

an earlier smaller and a later larger peak. Both of these peaks were eluted later in the chromatogram than the estimated position for 7S-globulins. Fig. 9(b) shows the pattern obtained when DEAE fraction I γ-globulin treated with glycopeptide was run through G-200. A small amount of protein appeared in the estimated 19S position and next a larger peak which would correspond to 7S globulin. This was followed by the two peaks which correspond to the free glycopeptide. Protein and carbohydrate analysis of the larger and smaller molecular weight peaks showed carbohydrate contents of 7 per cent and 40 per cent respectively. The latter value was considerably in excess of the normal carbohydrate content for γ_2-globulin and indicated a binding of glycopeptide to the γ-globulin molecule. It was very close to the value previously obtained (38 per cent) when the same 7S globulin had been re-chromatographed on DEAE cellulose (Table 2).

DISCUSSION

The original observation which formed the basis of this investigation was that treatment of serum from an immunized guinea-pig with mycobacterial glycopeptide resulted in a changed appearance of the immunoelectrophoretic pattern of this serum, when developed against the homologous antigen or against rabbit anti-guinea-pig globulin. Subsequent work aimed at defining the manner in which the glycopeptide attached itself to the slow (γ_2) globulin, which seemed to be the serum fraction most obviously affected. The demonstration of an affinity between mycobacterial peptidoglycolipid and an immunoglobulin (γ_2-globulin, IgG, γG, *Bull. Wld Hlth Org.* (1964), **30**, 447) would be of possible importance in explaining the ability of mycobacterial peptidoglycolipid to increase the serum antibody levels of this immunoglobulin fraction in guinea-pigs (White *et al.*, 1963).

The results of the present work showed that glycopeptide had little effect on the immunoelectrophoretic behaviour of fast (γ_1) globulin but that it caused a shortening or forward movement of the slow (γ_2) globulin. Further experiments showed that the levels of antibody in the latter fraction as tested by complement fixation remained unaltered after treatment with glycopeptide. Similarly, after treatment this globulin fraction retained its ability to form a precipitin arc with rabbit anti-guinea-pig globulin. Thus treatment with glycopeptide did not remove the ability of this immunoglobulin to react as an antigen against its homologous antibody.

Estimation of the carbohydrate content of guinea-pig γ_2-globulin by the PAS technique and quantitatively, by the anthrone method, showed that the glycopeptide had become bound to the γ_2-globulin molecule. It therefore seemed likely that the increase in electrophoretic mobility of glycopeptide-treated γ_2-globulin was due to an increase in negative charge caused by the attachment of carbohydrate molecules.

The literature contains scattered references to a combination of bacterial polysaccharides with serum proteins. Neter, Westphal and Lüderitz (1955) and Nass, Matijevitch and Springer (1964) have described the effect of plasma proteins in inhibiting the coating of erythrocytes by bacterial lipopolysaccharide. According to the latter authors, binding of lipopolysaccharide, as revealed by such haemagglutination inhibition tests, was strongest with serum albumin, α_2-globulin and β-lipoproteins. This inhibition was increased by addition of pure β- or γ-globulins, themselves inactive at the concentrations tested. Although this communication has explored the effect of mycobacterial glycopeptide on the serum γ-globulin, the results do not imply that the glycopeptide has no effect on other serum proteins. In fact, a careful examination of the patterns produced by guinea-pig serum against rabbit anti-guinea-pig globulin (Figs. 4 and 6) shows that there is a slight

increase in electrophoretic mobility of serum proteins in the β_1 and α_2 regions as well as in the γ region, after treatment with the glycopeptide. A slight increase in mobility of γ_2-globulin from normal guinea-pigs and from those immunized with incomplete Freund's adjuvant was observed. However, the alteration in mobility of γ_2-globulin from animals immunized with Freund's complete adjuvant was consistently more marked, even though the anti-ovalbumin antibody probably constituted only a fraction of the γ_2 peak. This apparent contradiction remains unexplained.

Fraction I before treatment contained 1·3 per cent carbohydrate and after treatment this increased to 38–40 per cent carbohydrate. The molecular weight of the γ-globulin is approximately 150,000 and of the glycopeptide approximately 35,000. Therefore, a value of 38–40 per cent as reported in the present communication might be obtained if an average of three molecules of carbohydrate was bound to each γ-globulin molecule. The nature of the linkage between glycopeptide and γ-globulin remains unknown.

The evidence presented here would not suggest that the attachment of glycopeptide was limited to guinea-pig anti-ovalbumin antibody molecules alone. Further experiments, using sera from guinea-pigs immunized with diphtheria toxoid in water-in-oil emulsion with added mycobacterial peptidoglycolipid, showed the same change in electrophoretic mobility of γ_2-globulin as was observed with guinea-pig anti-ovalbumin sera. However, in experiments using sera from guinea-pigs injected with ovalbumin in water-in-oil emulsion without added peptidoglycolipid, the alteration in mobility of the γ_2-globulin when tested against rabbit anti-guinea-pig globulin serum was scarcely perceptible and was very much less marked than that obtained with sera from animals which had received Freund's complete adjuvant mixture.

The affinity of mycobacterial glycopeptide for various protein molecules may provide the basis for an explanation of the activity of mycobacteria as adjuvants. There is some evidence that the effectiveness of mycobacterial adjuvants does not extend to polysaccharide antigens (personal communication from Dr J. H. Humphrey on the basis of unpublished experiments with Type III pneumococci). Thus in this connection it would be of interest to know whether peptidoglycolipid must combine with antigen within the injection mixture in order to produce an adjuvant effect. Alternatively, peptidoglycolipid may act by combining with the newly synthesized γ_2-globulin product, in this way acting as a specific de-repressor for the ribo-nucleic acid template in the plasma cell.

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