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Membrane antigens of *Mycoplasma hominis*

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SUMMARY

Extraction of membranes of *Mycoplasma hominis* with *n*-butanol showed that antigenicity was associated with the non-lipid residue, which probably consisted mainly of protein, and not with the lipid itself.

Since many membrane proteins are hydrophobic, membranes were rendered soluble in various ways. Extraction with urea or phenol was the most successful, yielding extracts which were both antigenic and serologically reactive. The urea extract could not be fractionated by polyacrylamide disk electrophoresis or by column chromatography. However, serologically active components identified by gel diffusion were separated from detergent-lysed membranes by polyacrylamide disk electrophoresis. The activities of antisera against these fractions suggested that indirect-haemagglutinating or metabolic-inhibiting antibodies can be directed against several different membrane antigens. However, the antigens identified by gel diffusion probably do not represent all the components participating in indirect haemagglutination.

Treatment of membrane suspensions with heat, alkali, periodate and various enzymes showed that the four components identified by gel diffusion could be distinguished by their differing stabilities and properties. On the basis of their lability and susceptibility to proteolytic enzymes, two were identified as proteins.

INTRODUCTION

The membrane antigens of *Mycoplasma hominis* are important in indirect haemagglutination (IHA), metabolic inhibition (MI) and growth inhibition (GI) (Hollingdale & Lemcke, 1969) and as receptors for the adsorption of H-HeLa cells (Hollingdale & Manchee, 1972). The membrane is also involved in complement fixation (CF) but, in addition, antigens from the soluble cell contents (soluble fraction) participate in this reaction. Chemical extraction of whole organisms shows that serologically reactive extracts are obtained only by methods known to extract proteins. Lipids are only weakly active and aqueous phenol extracts for carbohydrates completely inactive. Since membranes are so important to the antigenicity of the cell and selective extraction of membrane antigens from whole cells by chemical methods was not possible (Hollingdale & Lemcke, 1969), membranes

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were prepared and an attempt made to separate and characterize the serologically active membrane components. Some of these results were briefly reported at the Fifth International Congress of Infectious Diseases (Lemcke & Hollingdale, 1970).

MATERIALS AND METHODS

Preparation of membrane suspensions

Membranes were prepared by ultrasonic treatment and differential centrifugation from suspensions of *Mycoplasma hominis* strain SC4, grown, harvested and washed as described by Hollingdale & Lemcke (1969). Membranes sedimented at 100,000 *g* were washed six times in dilute sodium chloride/tris buffer (0·0075 M sodium chloride, 0·0025 M tris, pH 7·4) before resuspension in the same buffer at 5–6 mg. protein/ml. and storage at −30° C. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). All preparations were tested by gel diffusion for the absence of components from the soluble fraction using antiserum to whole organisms, the membranes lysed with Triton X-100, and a soluble fraction prepared by freezing and thawing (Hollingdale & Lemcke, 1969).

Serological tests

Gel diffusion, metabolic inhibition and indirect haemagglutination tests were carried out as previously described (Hollingdale & Lemcke, 1969), except that growth medium (10%, v/v) was included in the gel diffusion agar when testing antisera to the various membrane preparations (see below), and for indirect haemagglutination the unit volume was reduced to 0·025 ml. In gel diffusion, all antigens were tested at 2 mg. protein/ml. and membranes were lysed with Triton X-100 at 5 mg./mg. membrane protein.

Preparation of 'lipid-free' membranes

The method was based on that of Rodwell, Razin, Rottem & Argaman (1967). Five volumes of membrane suspension in dilute sodium chloride/tris buffer at 5 mg. protein/ml. were extracted with two volumes of *n*-butanol in an ice-water bath for 30 min. The butanol layer was separated by centrifugation at 30,000 *g* for 20 min. The insoluble pellet and interfacial material was resuspended in the aqueous layer, re-extracted with two volumes of butanol and centrifuged as before. The pooled butanol layers and the aqueous phase were separately dialysed against buffer. The lipid was then dispersed in the buffer by sonication at 20 kc. per sec. for 2 min. (Branson S75 Sonifier, Branson Instruments Inc., Stamford, Connecticut, U.S.A.). The pellet of lipid-free membranes was washed three times and resuspended in buffer to the volume of the original membrane suspension.

For 'recombined' membranes, half the final volume of the lipid extract and the lipid-free membrane suspension were mixed and dialysed for 18 hr. at 4° C. against dilute sodium chloride/tris buffer containing 0·05 M magnesium chloride. Assuming no loss during fractionation, the lipid and lipid-free material in the recombined membranes should have been present in the same proportion as in the original membranes.

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Preparation of soluble protein extracts

The methods of Maddy (1966) and Zwaal & van Deenen (1968) for preparing soluble protein or lipoprotein extracts from mammalian erythrocyte ghosts were used with *M. hominis* membrane suspensions. Treatment with lithium chloride or lithium bromide at concentrations from 0·1 to 8 M was also tried. For urea extraction, equal volumes of membrane suspension (2 mg. protein/ml.) and 16 M urea in sodium chloride/tris buffer were held at room temperature for 30 min. with occasional shaking. The insoluble residue was deposited by centrifugation at 8000 *g* for 30 min. and the supernatant dialysed successively against 4 M and 2 M urea in buffer and buffer alone for 3, 18 and 24 hr. respectively at 4° C. Before concentration by pressure dialysis, the absence of urea was checked with urease tablets and Nessler's reagent (British Drug Houses, Poole, Dorset). Extraction with phenol was carried out by the method used for whole organisms (Hollingdale & Lemcke, 1969).

Gel filtration and polyacrylamide disk electrophoresis of urea extract

A column (1·7 × 38 cm.) of Sephadex G-50 (fine; Pharmacia AB, Uppsala, Sweden) was equilibrated with sodium chloride/tris buffer (0·015 M sodium chloride, 0·05 M tris) pH 7·4. Urea extract (2 ml. containing 4 mg. protein) was layered onto the column and eluted with the buffer used for equilibration. Fractions were collected at 3 min. intervals and monitored for absorbance at 280 nm.

The method of polyacrylamide disk electrophoresis was that of Davis (1964) except that the spacer gel was omitted and the sample containing 750 μg. of protein was layered onto the gel in sucrose (20 %, w/v).

Absorption of antiserum

Lipid-free and recombined membranes and the phenolic and urea extracts were tested for their ability to remove MI and IHA antibody from antiserum to whole organisms by absorption of 0·1 ml. of antiserum with extracts containing 1 mg. of protein (Hollingdale & Lemcke, 1969).

Separation of membrane proteins by polyacrylamide disk electrophoresis

The method was based on that of Maizel (1966). Membranes were lysed with sodium dodecyl sulphate (SDS) or Triton X-100 to give final concentrations of 2 mg. membrane protein and 20 mg. detergent/ml. Two volumes of lysate and one volume of 60 % sucrose were mixed and 0·15 ml. (containing 200 μg. protein) was layered onto each gel. Gels consisted of acrylamide (5 %, w/v) with *N*,*N*′-methylenebisacrylamide (0·13 %, w/v), and SDS (0·1 %, w/v) or Triton X-100 (0·1 %, w/v) in 0·1 M sodium phosphate, pH 7·0. Polymerization was catalysed by *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (0·05 %, v/v) and ammonium persulphate (0·075 %, w/v). Where gels were to be used for inoculation of rabbits, ammonium persulphate was removed by electrophoresis for 2 hr. at 5 mA per gel before applying the lysed membranes. Electrophoresis of detergent-lysed membranes was carried out for 2 hr. at 8 mA for each gel in 0·1 M phosphate buffer, pH 7·2, using a Shandon electrophoretic apparatus with 75 × 5 mm. tubes.

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Some gels were stained to demonstrate protein bands with naphthalene black (1%, w/v) in acetic acid (7%, v/v). To locate serologically active bands by gel diffusion, gels were sliced transversely into 3 mm. sections. With a syringe, sections were macerated into alternate wells 5 mm. in diameter and 3 mm. distant from a central 60 × 1 mm. trough. The wells containing macerated sections were filled with Triton X-100 (4%, v/v) to elute the membrane components. This was used in preference to SDS in tests with antisera, because SDS gives a non-specific precipitate with rabbit serum. The remaining wells were filled with membranes lysed with Triton X-100 and the trough with antiserum to whole organisms. Diffusion was at room temperature. Since eight gels could be run at one time and only two were required for protein staining and location of the serologically active proteins, the other gels were sliced into 3 mm. transverse sections which were numbered from the origin. Sections with the same number were pooled, immediately frozen and stored at −30° C. for future use as rabbit inocula. Altogether eight electrophoretic runs were made and the sections containing the different components identified. Before inoculating rabbits, sections from each run were retested for gel diffusion activity.

Antisera

These are listed in Table 1. Antisera to whole organisms and to purified membranes of SC4 were those prepared by Hollingdale & Lemcke (1969). For antisera to lipid-free or recombined membranes, preparations were emulsified in adjuvant (Esso Markol 52: Arlacel; 9:1 v/v) and given in two subcutaneous (s.c.) inoculations separated by an interval of 13 days. Three further intravenous (i.v.) injections of an aqueous suspension were given 13, 15 and 17 days after the second s.c. inoculation. A total of 4 mg. of protein was injected. A similar schedule was followed for antiserum to the urea extract, of which 5 mg. of protein was inoculated.

For antisera to membrane proteins separated by polyacrylamide disk electrophoresis, suitable batches of 16–19 gel sections, which contained the same precipitating component, were homogenized in saline and emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan, U.S.A.). Half of each emulsion was given subcutaneously on the inside of the right hind leg and half intramuscularly into the left flank. After 14 days, a similar emulsion was

Table 1. *Preparations of* Mycoplasma hominis *SC4 against which antisera were prepared*

Whole organisms
Membranes purified on sucrose density gradients (purified membranes)
Lipid-free membranes (butanol-extracted)
Recombined membranes (lipid-free membranes and butanol-extracted lipid)
Urea extract of membranes
Phenolic extract of membranes
Membranes lysed with sodium dodecyl sulphate
Membrane components separated by polyacrylamide disk electrophoresis:
  Antiserum A, against precipitating component 1
  Antiserum B, against precipitating component 3
  Antiserum C, against precipitating components 2*a*, 2*b* and 3
  Antiserum D, against precipitating components 2*a*, 2*b* and 3

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inoculated by the same routes in collateral positions. Rabbits were bled 7, 14, 21 and 28 days after the first inoculation. A similar schedule was followed for antisera against SDS-lysed membranes, of which the rabbits received 1·6 mg. protein, and the phenolic extract, of which 2 mg. was inoculated.

Specificity of antisera

All the antisera in Table 1 were tested by gel diffusion against the growth medium, human serum and human γ-globulin (Lister Institute, Elstree, Herts.), either alone or with Triton X-100 at the concentration used for membranes. Antisera were also tested by gel diffusion against a soluble cell fraction of *M. hominis* prepared by freezing and thawing.

Enzymes

These were obtained from the following sources: pronase (B grade), Calbiochem, Los Angeles, U.S.A.; trypsin (ex bovine pancreas, lx crystallized, 8000 BAEE units/mg.), lipase (ex hog pancreas), trypsin inhibitor (soybean) and β-glucosidase

Table 2. *Treatment of membranes of* Mycoplasma hominis *SC* 4

Agent	Final concentration	Time	Buffer	Test for enzyme activity
Heat (50°, 60°, 80°, 100° C.)	—	30 min.	A	—
Sodium meta-periodate	0·2 M	24 hr. (dark)	A	—
Sodium hydroxide	0·1 M	1 hr.	A	—
Trypsin	0·1 mg./ml.	2 hr.	B	Gelatin agar (Barer, 1946)
Pronase	0·1 mg./ml.	2 hr.	C	Gelatin agar (Barer, 1946)
Lipase	1·0 mg./ml.	16 hr.	C	Tributyrin agar (Willis, 1965)
α-Glucosidase	1·0 mg./ml.	16 hr.	C	Liberation of glucose from *p*-nitrophenyl α-D-glucoside or *p*-nitrophenyl β-D-glucoside
β-Glucosidase	1·0 mg./ml.	16 hr.	C	Liberation of glucose from *p*-nitrophenyl α-D-glucoside or *p*-nitrophenyl β-D-glucoside
α-Galactosidase	82 EU/ml.	16 hr.	C	Liberation of galactose from *p*-nitrophenyl α-D-galactoside or *o*-nitrophenyl β-D-galactoside
β-Galactosidase	3·4 EU/ml.	16 hr.	C	Liberation of galactose from *p*-nitrophenyl α-D-galactoside or *o*-nitrophenyl β-D-galactoside
Phospholipase A	0·1 mg./ml.	30 min.	D	Hydrolysis of egg yolk
Phospholipase C	1 EU/ml.	30 min.	E	Hydrolysis of egg yolk

Buffers: A 0·0075 M sodium chloride/0·0025 M tris, pH 7·4.
B 0·05 M tris, pH 7·8.
C 0·1 M phosphate, pH 7·0.
D 0·05 M tris, pH 7·2.
E 0·05 M tris/0·05 M Ca^{2+}, pH 7·2.

Sodium metaperiodate treatment at room temperature, sodium hydroxide and enzymes at 37 °C.

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(ex sweet almonds), Koch-Light Laboratories Ltd, Colnbrook, Bucks.; β-galactosidase (ex *Escherichia coli*), Boehringer Corporation, London, W.5. Phospholipase A (crystalline, ex cobra venom) and phospholipase C (*Clostridium welchii*) were kindly supplied by Dr G. M. Gray and α-galactosidase (*Trichomonas foetus*) by Professor W. M. Watkins of this Institute.

Treatment of membrane suspensions

The details are summarized in Table 2. Membranes were treated at 3–4 mg. protein/ml. In every experiment a control membrane suspension was held with buffer under the same conditions. Membranes held at room temperature for 30 min. served as the control for heated membranes. Treated and control membranes lysed with Triton X-100 were tested by gel diffusion, without removal of the agent, against antisera to whole organisms and to membrane components separated in polyacrylamide gels. In this way inactivated components could be identified.

The capacity of the treated membranes to adsorb MI and IHA antibody from antiserum to whole organisms was also determined where gel diffusion tests indicated that components had been destroyed. Antiserum previously inactivated and absorbed with washed sheep erythrocytes was mixed with membrane suspensions at a rate of 1 mg. protein to 0·1 ml. of serum and absorbed for 48 hr. at 4° C. Particulate matter was removed by centrifugation and the absorbed sera stored at −30° C. Heat-treated membranes were mixed directly with the serum, but membranes treated with sodium hydroxide or sodium periodate and the corresponding controls were first dialysed. In one experiment sodium hydroxide was neutralized with 0·1 N hydrochloric acid. Trypsin inhibitor was added to pronase or trypsin-treated membranes. In each experiment, a sample of antiserum was held with buffer as an 'unabsorbed serum' control, and was titrated at the same time as antiserum absorbed with treated or control membranes.

RESULTS

Reaction of detergent-lysed membranes in gel diffusion with whole organism antiserum

Three major precipitin bands, all apparently complex, were identified in reactions between detergent-lysed membranes of SC4 and antiserum to whole organisms. A fourth line sometimes appeared nearest to the antigen well (Hollingdale & Lemcke, 1970) but as its appearance was inconsistent, only the three major components were numbered, starting at the antiserum well. Components 1 and 3 sometimes appeared double, but, as component 2 was invariably double, the two lines were designated 2*a* and 2*b* (Pl. 1*a*). These precipitating components were distinct from those given by soluble fractions of SC4. These reactions were evidently specific to the membrane, since antiserum to whole organisms which had been raised to SC4 grown in rabbit serum medium (Hollingdale & Lemcke, 1969) did not react with any of the medium constituents or with γ-globulin (Pl. 1*a*).

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Specificity of antisera to membrane preparations

Of the antisera listed in Table 1, only those against detergent-lysed, lipid-free or recombined membranes and the serum C, which contained antibody to components 2*a*, 2*b* and 3, reacted in gel diffusion tests with growth medium, human serum and human γ-globulin, with or without Triton X-100. All three gave reactions of identity with the antisera, showing that it was the γ-globulin constituent of the human serum in the growth medium which had been adsorbed to the membrane of *M. hominis*. Detergent-lysed membranes, however, did not react with antiserum to human serum (species-precipitating serum, Burroughs Wellcome, Beckenham, Kent) in similar tests. This suggests that only a small amount of γ-globulin adsorbed to the membranes, though sufficient to elicit a response in rabbits. All antisera which had been raised to membrane fractions or extracts were therefore absorbed with an equal volume of medium for 48 hr. at 4° C. before testing for IHA antibody. Since the MI test was in medium containing human serum, any antibody to human γ-globulin would be rapidly bound and absorption with medium was therefore unnecessary. For gel diffusion tests, growth medium (10 %, v/v) included in the agar was sufficient to prevent the formation of precipitin lines between membrane antisera and human serum or γ-globulin at 2 mg. protein/ml., and should therefore have blocked any reaction with the small amounts of γ-globulin contaminating detergent-lysed membranes. Antisera to the various membrane preparations did not react with soluble fractions in gel diffusion tests. Thus, reactions between these sera and detergent-lysed membranes should have been specific to the membrane constituents.

Serological activity and antigenicity of lipid-free and recombined membranes

Both lipid-free and recombined membranes gave rise to antisera with MI and IHA titres comparable to those of antiserum against membranes purified on sucrose density gradients (Table 3). Moreover, in gel diffusion tests these antisera reacted with detergent-lysed membranes to give the same complex of precipitin lines as whole organism antisera. Both preparations also absorbed MI and IHA antibody from antiserum to whole organisms to the same degree as untreated membranes (Table 3). In gel diffusion tests with whole organism antiserum, lipid-free and recombined membranes lysed with Triton X-100 gave only one line corresponding to component 1. However, since their antigenicity and absorption capacity was comparable to that of untreated membranes, this probably reflects the failure of the detergent to lyse the butanol-treated membranes rather than a loss of antigens. The butanol-extracted lipid neither reacted in gel diffusion tests nor absorbed MI or IHA antibody from antiserum to whole organisms (Table 3). Comparison of lipids in the butanol extract with those in untreated and butanol-extracted membranes by thin layer, silica gel chromatography suggested that at least 95 % of the lipid was removed by butanol (G. M. Gray, personal communication).

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Serological activity and antigenicity of soluble protein extracts

Membrane extracts obtained by the methods of Maddy (1966) or Zwaal & van Deenen (1968) or with lithium salts contained only small amounts of protein and gave, at best, weak reactions in gel diffusion tests with antiserum to whole organisms. However, urea and phenolic extracts gave lines corresponding to components 1 and 3. When antiserum to whole organisms was absorbed with these extracts IHA titres were reduced from 2560 to $\leqslant 10$ ($\geqslant 256$-fold), although the corresponding reduction in MI titres was only 8–16-fold.

Antiserum to the urea extract contained antibody to component 3 but only very low concentrations of antibody to component 1, since it gave only a weak reaction with component 1 of detergent-lysed membranes (Pl. 1*b* and *c*) and no component 1 reaction with its homologous extract in gel diffusion. MI and IHA titres were also low (Table 4). Antiserum to the phenolic extract had MI and IHA titres comparable to those given by antiserum to purified membranes (Table 4) and gel diffusion tests showed that it contained antibody to components 1 and 3 (Plate 1*b* and *c*).

All the serologically active material in the urea extract was eluted from Sephadex G-50 in one peak immediately following the void volume and there was no separation. Similarly, no separation of proteins was obtained by polyacrylamide gel electrophoresis.

Activity of antiserum to membranes lysed with sodium dodecyl sulphate

Membranes were still antigenic after lysis with SDS. MI titres of the resulting antiserum were of the same order as those of antiserum to purified membranes (Table 4). However, IHA titres were eight-fold lower, although adjuvant was used in the preparation of this antiserum whereas serum to purified membranes was produced by i.v. inoculation of aqueous suspensions (Hollingdale & Lemcke, 1969).

Table 3. *Serological activity of* n-*butanol extracted lipid, lipid-free and recombined membranes of* Mycoplasma hominis *SC* 4

	Titre	
	Metabolic inhibition	Indirect haemagglutination*
Antiserum to:		
Purified membranes	160	5120
Lipid-free membranes	160	2560
Recombined membranes	320	5120
Antiserum to whole organisms absorbed with:		
Nil	1280	2560
Purified membranes	<10	10
Lipid	1280	1280
Lipid-free membranes	<10	<10
Recombined membranes	<10	<10

* Sera titrated against tanned erythrocytes sensitized with a sonicated suspension of SC4 at 0·125 mg. protein/ml.

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Activities of antisera to membrane fractions separated by polyacrylamide gel electrophoresis

On polyacrylamide disk electrophoresis, membranes lysed with Triton X-100 migrated poorly and gave only a few ill-resolved bands, whereas those lysed with SDS gave 12–15 protein bands. Component 1 was found in sections containing protein bands which moved fastest in the gels whereas 2 and 3 were in sections closer to the origin. By inoculating rabbits with different gel sections, sera apparently monospecific to components 1 and 3 were obtained (sera A and B) but antisera to components 2*a* and 2*b* (C and D) also contained antibody to component 3 (Pl. 1*d* and *e*). Sera with antibody to components 1 or 3 both had MI titres of 40–80 whereas antiserum with antibodies to 2*a*, 2*b* and 3 had titres at least four-fold higher. Since components 2*a* and 2*b* but not 3 were inactivated at 60° C. for 30 min. (see below), antibody to component 3 was removed from one of these antisera (C) by absorption with membranes thus heated (serum Ca, Pl. 2*c*). MI and IHA titres were then reduced four- to eight-fold, to the same level as the 'monospecific' sera to components 1 or 3 (Table 4).

Properties of membrane components

Of the precipitating components identified by gel diffusion 2*a* and 2*b* were the most heat-labile; both were eliminated after heating at 60° C. for 30 min., whereas component 1 was inactivated at 80° C. and 3 was stable at 100° C. (Table 5). The proteolytic enzymes, pronase and trypsin, destroyed component 1 (Pl. 2*a*) and part of 2*b* (Pl. 2*b* and *c*). The latter is therefore complex. All components except 3 were destroyed by periodate and all except 1 by 0·1 N sodium hydroxide. Thus, 3 was the most stable. In preliminary experiments, pancreatic lipase and α-gluco-

Table 4. *Serological activities of antisera to membrane preparations of* Mycoplasma hominis *SC* 4 (*antiserum to whole organisms included for comparison*)

Rabbit inoculum		Protein inoculated (mg.)	Precipitating antibody to component	Antiserum titre: Metabolic inhibition	Antiserum titre: Indirect haemagglutination
Whole organisms		ND	1, 2*a*, 2*b*, 3	2560	2560
Purified membranes		3·5	1, 2*, 3	160	2560
Membranes lysed SDS		1·6	1, (2*a*), 2*b*, 3	160–320	320
Membrane proteins in polyacrylamide	A	ND	1	40–80	80
	B	ND	3	80	160
	C	ND	(2*a*), 2*b*, 3	160–320	160
	Ca+	ND	(2*a*), 2*b*	40	40
	D	ND	(2*a*), 2*b*, 3	320	2560
Urea extract		5·0	(1), 3	40	20
Phenolic extract		2·0	1, 3	320	1280

ND, not determined.
SDS, sodium dodecyl sulphate.
2*, 2*a* and 2*b* fused into one line.
(1), (2*a*), weak reactions in gel diffusion tests with detergent-lysed membranes.
Ca+, antiserum C absorbed with membrane suspension heated at 60° C., 30 min.

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sidase had a similar range of activity to the proteolytic enzymes. However, both enzymes also degraded gelatin. This reaction was blocked by the addition of trypsin-inhibitor which also prevented the inactivation of the precipitating components. The lipase was still active against tributyrin in the presence of trypsin-inhibitor. Thus, both enzymes were contaminated with trypsin or a trypsin-like enzyme but neither alone had any effect on the precipitating antigens. Thin layer chromatograms of lipids extracted from membranes treated with phospholipases A or C were different from those of lipids from untreated membranes, suggesting some action on the membrane lipids. However, the precipitating components were unaffected by these enzymes. Similarly, β-glucosidase and α- and β-galactosidases were without effect.

The ability of treated membranes to absorb MI and IHA antibody decreased with the loss of precipitating components, although not strictly in parallel with the number of components destroyed (Table 5). All treatments diminished the capacity to absorb IHA antibody to a greater extent than the capacity to absorb MI antibody. In particular, heating membranes at 50° C. apparently left the precipitating antigens and the power to absorb MI antibody unaltered, but decreased IHA antibody absorption 16-fold.

DISCUSSION

The results of experiments with butanol-extracted lipid, lipid-free and recombined membranes suggest that MI and IHA antibodies are directed against membrane proteins rather than lipids or lipoproteins. This agrees with our previous results using whole organisms extracted with chloroform-methanol or aqueous acetone (Hollingdale & Lemcke, 1969). Although the chloroform-methanol and acetone extracts reacted weakly in gel diffusion and complement fixation, the butanol-extracted membrane lipid was completely inactive. This was probably

Table 5. *Serological activity of membranes of* Mycoplasma hominis *after various treatments*

Treatment	Precipitating components (gel diffusion) after treatment	Factor of decrease in antibody titre after absorption with treated membranes*	
		MI	IHA
Nil	1, 2*a*, 2*b*, 3	128–256	256–512
50°, 30 min.	1, 2*a*, 2*b*, 3	128	16–32
60°, 30 min.	1, 3	16	4–8
80°, 30 min.	3	2–4	0–2
100°, 30 min.	3	2–4	0–2
Sodium hydroxide	1	4–8	4
Periodate	3	16	2
Pronase	2*a*, (2*b*)†, 3	4	2
Trypsin	2*a*, (2*b*)†, 3	8	2

* Whole-organism antiserum with MI titre 1280–2560 and IHA titre 2560–5120 when held with buffer only.

† Part of component destroyed (Plate 2*a–c*).

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because there was less protein in the solvent phase with butanol extraction than with the more drastic chloroform-methanol or acetone extractions. Kahane & Razin (1969) also found that MI antibody was directed against membrane proteins rather than lipids of *Mycoplasma laidlawii* and *M. gallisepticum*. These species, unlike *M. hominis*, ferment glucose. In contrast, membrane proteins of another glycolytic species, *M. pneumoniae*, elicit only low titres of MI antibody in rabbits and the specific glycolipid hapten must be re-aggregated with protein to obtain an adequate MI antibody response (Razin, Prescott & Chanock, 1970). Clearly, more species of mycoplasma need to be examined to elucidate the relative importance of membrane lipids and proteins in determining antigenicity and specificity.

The membrane proteins of *M. hominis*, like those of mammalian cells, were not easily rendered soluble. However, both urea and phenol extracted some of the antigens active in gel diffusion, MI and IHA, although the precipitating components which were later found to be the most labile (2*a* and 2*b*) were not recovered. The difference between the potencies of the antisera to the urea and phenol extracts was probably due largely to the differing systems of inoculation, since both extracts appeared to contain components 1 and 3 when tested against whole-organism antiserum.

The possibility of separating components from detergent-lysed membranes was explored because of the difficulty in obtaining membrane proteins in solution and the failure to separate the proteins in the urea extract by chromatography or disk electrophoresis. Although detergents denature proteins, this seemed to occur less than with the other reagents, since detergent-lysed membranes gave more complex precipitin patterns in gel diffusion tests. Moreover, membranes lysed with SDS still elicited the production of MI, IHA and precipitating antibodies, and detergent systems have been widely used in polyacrylamide disk electrophoresis of various proteins. In polyacrylamide gels, migration and separation of the proteins of membranes lysed with SDS was better than that of membranes lysed with Triton X-100. The non-ionic character of Triton X-100 may account for this and better migration might have been achieved in a buffer system at a different pH. Because of the small amounts separated in the gels when only 200 μg. of protein was applied to each, and the problem of elution, it was not feasible to assay the separated membrane components for MI and IHA activities. However, the attempt to raise antisera to the separated components was successful with respect to two of them and it was possible to make another serum specific to components 2*a* and 2*b* by absorption. Since all these antisera had MI and IHA activity it appears that several different antigens can give rise to MI or IHA antibodies. However, the precipitating antigens may not account for all the antigens involved; others not revealed by gel diffusion may be active. For example, antigens giving rise to IgM antibody, which does not react in precipitin tests, would not be detected by this system. This would apply particularly to antigen-antibody reactions in IHA, since IHA antibodies often belong to the IgM class of immunoglobulins (Pike, 1967). Indeed, the fact that antiserum to SDS-lysed membranes had an IHA titre eight-fold lower than that of antiserum to purified membrane, although it contained the same precipitins, suggests that some non-precipitating labile antigen(s) may have been

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destroyed by the detergent. Another indication that antigen-antibody reactions other than those detected by gel diffusion occur in IHA, was the pronounced capacity of the urea and phenolic extracts for absorbing IHA antibody from whole organism antiserum. These extracts contained only components 1 and 3 and exhibited a limited capacity for absorbing MI antibody. Finally, the ability of membranes to absorb IHA antibody after heating at 50° C. was reduced even when the precipitating antigens were unaffected.

There were differences between the reactions of components 1, 2*a*, 2*b* and 3 to the various physical, chemical and enzymic treatments. Thus, 1 and 3 could be distinguished by their differing susceptibilities to heat, alkaline hydrolysis, periodate and proteolytic enzymes. The reactions of component 1, including inactivation by proteolytic enzymes, suggest that it is a protein. The resistance to the various treatments of the precipitability of component 3 militates against it being protein. However, it seems unlikely that this antigen is lipid in character, since it was neither extracted with butanol nor destroyed by pancreatic lipase or by phospholipases A or C under the conditions used. Both 2*a* and 2*b* were heat, alkali and periodate-labile. These components were also lost from membrane suspensions stored at 4° C. for 8 weeks, and even from frozen suspensions kept at −30° C. for 6–8 months. Component 2*a* differed from 2*b* in that it leached out into the suspending buffer when membrane suspensions were held at room temperature or 37° C. for several hours, whereas 2*b* remained associated with the particulate material. The lability of 2*a* and 2*b* suggest that they are proteins, although the precipitating activity of 2*a* was not destroyed by pronase or trypsin. The elimination of part of the precipitating activity of 2*b* by these enzymes shows that it is in fact a complex, part of which is protein. Thus, component 1 and part of the 2*b* complex could be identified as proteins. However, the periodate sensitivities of these components may indicate the presence of a carbohydrate determinant attached to the protein or a peptide with periodate-sensitive amino acids.

Although the determinant groups could not be precisely characterized by these experiments, their differing properties suggest that the components identified were chemically distinct from one another.

In an earlier paper we showed that the serological heterogeneity within the species *M. hominis* could be correlated with differences in the membrane antigens demonstrable by gel diffusion (Hollingdale & Lemcke, 1970). The question arises, therefore, whether strain SC4 is a typical representative of the species for antigenic analysis. However, our previous results showed that the three strains examined (including SC4), have some precipitating membrane antigens in common (Hollingdale & Lemcke, 1970).

Mycoplasma hominis is the first non-glycolytic, arginine-metabolizing mycoplasma whose membrane antigens have been examined. The membrane proteins are clearly important to the antigenicity of this organism, but more detailed biochemical work is required on this and other species of mycoplasma which do not have lipid or carbohydrate haptens like those in *M. pneumoniae* or *M. mycoides*, to characterize the determinant groups.

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EXPLANATION OF PLATES 1 AND 2

Gel diffusion reactions of membrane preparations of *Mycoplasma hominis* SC4.

PLATE 1

(*a*) Numbering of precipitating components (1, 2*a*, 2*b* and 3) in reaction between membranes lysed with Triton X-100 (*m*) and antiserum to whole organisms (*W*). Other wells contain medium, human serum and human γ-globulin all treated with Triton X-100, and Triton X-100 (2%, v/v) alone.

(*b*, *c*) Reactions between membranes lysed with Triton X-100 (*m*) and antisera to whole organisms (*W*), and to urea (*U*) and phenolic (*P*) extracts of membranes. Other wells contain medium.

(*d*, *e*) Reactions between membranes lysed with Triton X-100 (*m*) and antisera (A, B, C and D) against membrane fractions separated by polyacrylamide disk electrophoresis. Antiserum to whole organisms (*W*) for comparison. (Note components 2*b* and 3 are transposed with serum C.)

PLATE 2

(*a*–*c*) Reaction of pronase-treated membranes (*p*) with antiserum to whole organisms (*W*) and antisera (A, B, C and Ca) against membrane fractions separated by polyacrylamide disk electrophoresis. (Serum Ca is antiserum C absorbed with membranes heated at 60° C to remove antibody to component 3.) Untreated membranes (*u*) and buffer-treated membranes (*c*) as controls. All membrane preparations lysed with Triton X-100. Component 1 and part of 2*b* missing after pronase-treatment.

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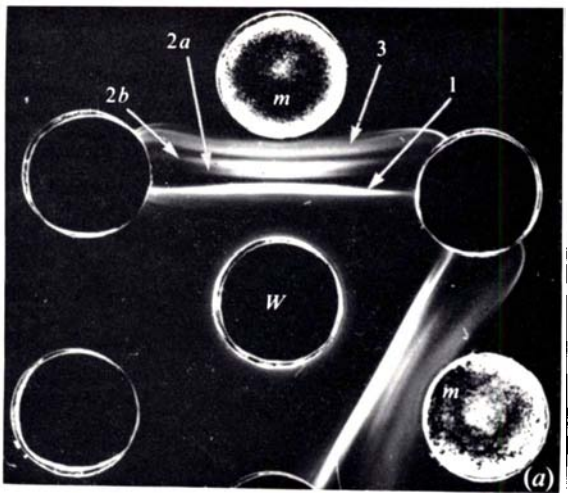

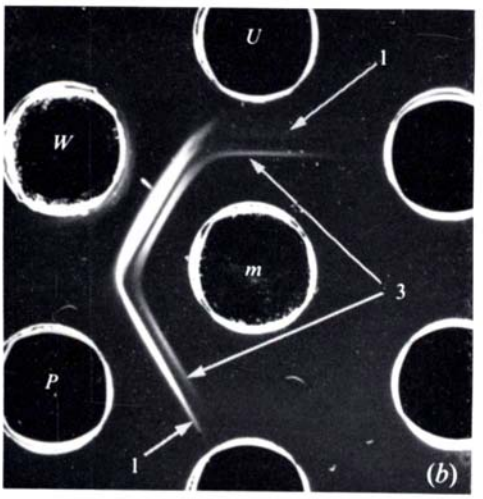

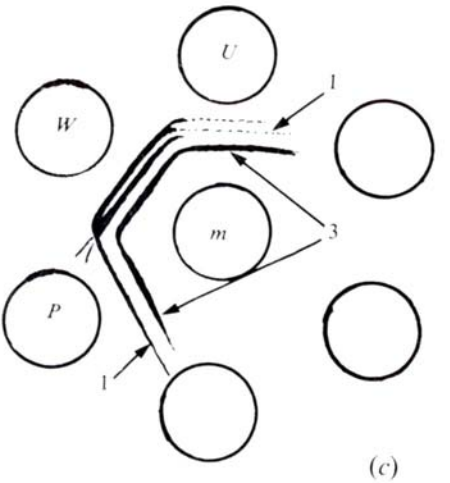

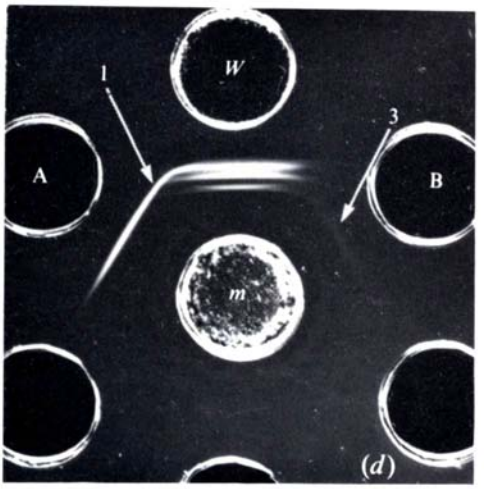

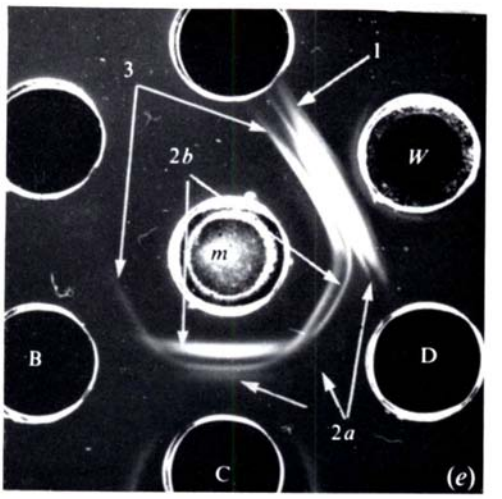

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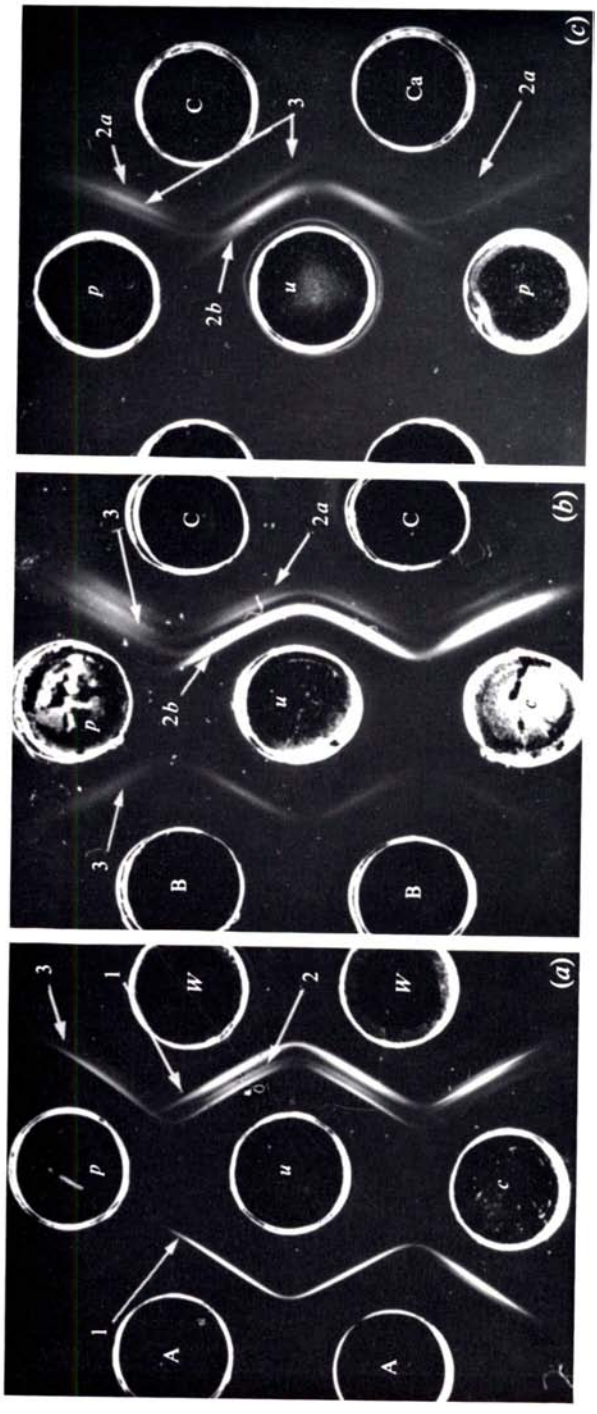

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