An Antigenic Analysis for Membranes of *Mycoplasma hominis* by Cross-absorption

By JUEY-SHIN L. LIN

*Channing Laboratory and Department of Medicine, Harvard Medical School and
Peter Bent Brigham Hospital, Division of the Affiliated Hospitals Center Inc.,
Boston, Massachusetts 02115, U.S.A.*

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Antigenic components at the outer surface membranes of seven serotypes of *Mycoplasma hominis* were analysed by the mycoplasmacidal reaction and the agglutination during growth reaction. Antibody absorbing capacities of the mycoplasma cells were compared with the absorbing capacities of membranes. It was shown that serologically active membrane antigens were mainly heat-labile proteins. No major antigens common to all seven serotypes were detected and each strain had its own specific antigens at the cell surface. Results of analysis indicate that there is a complex antigenic structure exposed in *M. hominis* and that 7 to 14 cross-reacting antigens may be present at the outer surface in the different serotypes examined. Additional cross-reacting antigens, presumably inner membrane in origin and not exposed at the cell surface, were also demonstrable.

INTRODUCTION

Serological heterogeneity within strains of *Mycoplasma hominis* had been demonstrated repeatedly (Purcell *et al.*, 1969). Recently, *M. hominis* was shown to consist of at least seven serotypes by the mycoplasmacidal (MC), the agglutination during growth (GA) and the growth inhibition (GI) tests (Lin & Kass, 1974; Lin *et al.*, 1975), and more than 200 strains of *M. hominis* were serotyped using the GA test and antisera from the seven serotypes (Lin *et al.*, 1976). It is of interest that all these tests showing strain specificities require live cells for the serological reactions to be evident. Since mycoplasmas do not have cell walls (Razin, 1973), the serological heterogeneity suggests a complex antigenic structure of the surface membrane. Therefore, an analysis was undertaken of the MC and GA antigens at the outer surface of *M. hominis*. This paper presents the results of such an analysis and shows that there are at least 7 to 14 different antigens at the outer surface of the different serotypes of *M. hominis*.

METHODS

Reference strains and antisera. The origins of the seven reference strains of *M. hominis* were described previously (Lin *et al.*, 1975). They are designated as serotypes 1 to 7. Mycoplasmas were grown in a basic medium containing 70% (v/v) PPLO broth, 20% (v/v) horse serum, 10% (v/v) yeast extract, 0.002% (w/v) phenol red and 500 units penicillin ml⁻¹, pH 7.2, supplemented with 1% (w/v) arginine (Braun *et al.*, 1970). Antisera against each strain were prepared in rabbits (Lin & Kass, 1975).

Cell and membrane preparations. Overnight cultures which contained 10⁸ to 10¹⁰ 50% colour changing units of organisms ml⁻¹ (c.c.u. ml⁻¹) (Lin & Kass, 1970) were centrifuged at 15000 g for 30 min. The cells were washed twice with and suspended in phosphate-buffered saline (PBS; 0.02 M-phosphate, pH 6.8) and were used directly to absorb sera. The final cell preparations contained 10¹¹ to 10¹² c.c.u. mycoplasmas ml⁻¹ and 8 to 12 mg protein ml⁻¹, determined by Lowry's method using crystalline bovine serum albumin as standard. The remaining cells were stored at -70 °C for the preparation of membrane fractions.

Membrane fractions were isolated essentially according to the procedure of Hollingdale & Lemcke (1969).

Cell preparations were diluted with an equal volume of PBS and treated for 3 min with a 9KC Ratheon sonic oscillator at maximum intensity. The sonicated cells were centrifuged at 15000 *g* for 30 min and the pellets, presumably unbroken cells, were resuspended in the same volume of PBS and sonicated again. A total of three sonications were performed in this manner and the combined supernatants were centrifuged at 40000 *g* for 30 min. The pellets were washed twice with and resuspended in PBS and stored at -70°C . The membrane fractions derived from 10^{11} to 10^{13} c.c.u. mycoplasmas ml^{-1} usually contained 0.5 to 1.0 mg protein ml^{-1} . The final preparations contained few or no viable cells (< 10 c.c.u.).

Measurement of serological activity. Serological tests were conducted using the microtitre system previously described (Lin & Kass, 1974). Both MC and GA tests were performed with 10^1 to 10^9 c.c.u. antigens. All the sera were heat-inactivated at 56°C for 30 min prior to the tests. Fresh guinea pig sera which had a haemolytic activity of ≥ 500 units (Kabat & Mayer, 1961) were included as complement sources in the MC tests. Twofold serial dilutions of serum were tested and the reciprocal of the highest dilution of serum in which serological activities were demonstrated was used to express antibody titre. Antibody titres to the serotype used for immunization are referred to as 'homologous' and those to other serotypes as 'heterologous'.

Treatment of cell preparations. The cells were treated in various ways in order to characterize the biochemical properties of the antigenic components: (i) a preparation was incubated with freshly prepared sodium periodate (final concn 5 mM) at room temperature for 1 h (Morgan & Watkins, 1951) and then washed once with PBS or dialysed against PBS at 4°C overnight; (ii) a preparation was incubated with trypsin [Difco, 1:250, final concn 0.2% (w/v), previously filtered through a $4\ \mu\text{m}$ Millipore filter] at 37°C for 30 min; (iii) a preparation was freeze-thawed 10 times, alternating between dry ice in ethanol and a 37°C water bath; (iv) a preparation was incubated at 56°C for 30 min.

Serum absorption. In order to absorb antibodies, 0.1 ml of antisera were incubated with 0.3 ml of the whole cell or membrane preparations at 37°C for 1 h and then centrifuged for 30 min at 9000 *g* to remove cells and at 40000 *g* to remove membranes. The supernatants were incubated further with 0.4 ml of the same preparation at 4°C overnight in a roller drum and re-centrifuged. In the final supernatants, the antiserum had been diluted to 1:8. Twofold serial dilutions of the preparations were tested with the homologous antiserum. The lowest concentrations of the cell and membrane preparations which reduced the homologous titre to ≤ 64 were considered as containing 1 unit of antigen. Preparations containing 1 or 2 units of antigen were used to absorb concurrently the homologous antiserum, and the six heterologous antisera, respectively. Seven preparations which contained pooled membranes of six serotypes were prepared so that the final concentration of antigens for each serotype was equivalent to 1 to 2 units. The pooled preparations were used to absorb the homologous antiserum of the serotype which had not been included in the preparation. A fourfold or more decrease in titre after the absorption with heterologous cells was considered to be significant. Cell and membrane preparations having 1 to 2 units of antigen usually contained 1 to 2 and 0.5 to 1.0 mg protein ml^{-1} , respectively.

Analysis of antigenic components. It was assumed that antigens corresponding to antibodies which were absorbed by and interacted with whole cells were present at the cell surface. Thus, when a given cell preparation had significantly absorbed antibodies, the preparation shared an antigen with the serotype that was used as the test antigen in the serological test. This assumption was tested by reciprocal absorption. If the reciprocal absorption gave results similar to that expected from the initial studies, then the antigen was considered a major one. If the absorption was not reciprocal, then the antigen was considered as a minor one, i.e. present in too small an amount to absorb a significant amount of antibody but capable of inducing and reacting with antibodies. Major antigens were represented by capital letters A, B, C, D, E, F, G for serotypes 1, 2, 3, 4, 5, 6 and 7, respectively and minor antigens were represented by small letters a, b, c, d, e, f, g. Thus, if cross-reacting activities of BC, CA and AB were demonstrated in sera of serotypes 1, 2 and 3, respectively, then an antigen labelled ABC would be present and shared by serotypes 1, 2 and 3. Antigen A does not cross-react with antigens AB or ABC.

RESULTS

Biochemical properties of membrane antigens

Preparations of cell antigens were subjected to various treatments in order to achieve a basic characterization of their nature. Representative results of these studies with serotype 6 and the homologous antiserum are shown in Table 1. Most, if not all, of the absorbing capacities of the whole cells were destroyed by heating at 56°C for 30 min and by exposure to trypsin. Treatment with periodate partially inactivated the capacities of the antigens to absorb GA antibodies. Repeated freeze-thawing did not alter the serological capacity of the cell preparations. The results indicated that the major antigens are heat-labile proteins,

Table 1. *Sensitivity of surface antigens of M. hominis (serotype 6) to various treatments*

Serological test	Titre before absorption	Titre after absorption with whole cells* that had been exposed to:					
		No treatment	37 °C	56 °C	Freeze-thawing	Periodate	Trypsin
Mycoplasma-cidal (MC)	2048	≤ 16	≤ 16	4096	32	≤ 16	2048
Agglutination during growth (GA)	2048	≤ 32	≤ 32	2048	≤ 32	256	1024

* Whole cell preparations contained 1 to 2 mg protein ml⁻¹ or 1 unit of antigenic activity as defined in Methods.

Table 2. *Differential antibody-absorbing capacities between cells and membrane antigens of M. hominis demonstrated in heterologous antisera*

Serotype of absorbing antigen	Serotype of antiserum absorbed	Homologous MC titre of antiserum			
		Before absorption		After absorption with:	
				Cells*	Membranes*
2	6	4096		2048	128
2	7	16384		8192	1024
3	5	8192		16384	2048
3	6	4096		4096	512
6	5	16384		16384	2048
7	1	16384		16384	512

* Each preparation contained 1 to 2 units of antigenic activity against the homologous antiserum as defined in Methods.

Table 3. *Serotype-specific antibody* demonstrated in seven antisera of M. hominis after absorption of each serum with pooled membranes of the six heterologous serotypes*

Serological test	Antiserum absorbed with:	Homologous MC titre of antiserum to serotype:						
		1	2	3	4	5	6	7
MC	PBS	8192	8192	16384	8192	8192	4096	8192
	Membranes	256	1024	4096	1024	1024	256	512
GA	PBS	2048	16384	4096	2048	8192	2048	1024
	Membranes	1024	4096	512	512	4096	2048	256

* Very low titres (≤ 64) to the other six heterologous serotypes.

that polysaccharides may be involved in the GA reaction and that lipids are not significantly involved as antigens.

Absence of common antigens

When the six heterologous cell preparations were used to absorb each of the seven sera, respectively, there was no significant alteration of the homologous titre of the sera, indicating that there are no major antigens at the cell surface that are common to the seven serotypes of *M. hominis*. When membrane preparations were used in the same manner, those obtained from serotypes 1, 4 and 5 also did not alter the homologous titres of the six antisera, whereas those from serotypes 2, 3, 6 and 7 significantly lowered the homologous MC titres of some sera (Table 2). Lowering of antibody titres by the latter membrane fractions was not caused by non-specific inactivation of antibodies, since a large proportion of antisera were not affected. Presumably therefore, some cross-reacting antigens are present in the membrane preparations of serotypes 2, 3, 6 and 7 but these are not expressed at the outer surface of the cells.

Table 4. *Absorption of antibodies from antiserum to M. hominis serotype 1 by heterologous cells*

Serotype of antigens used for test	Serotype of cells used for absorption						
	None	2	3	4	5	6	7
MC titre after absorption							
1	8192	8192	8192	4096	8192	4096	8192
2	8192	≤ 64	4096	512	4096	2048	1024
3	8192	512	≤ 32	1024	8192	512	1024
4	8192	1024	4096	≤ 32	4096	4096	1024
5	8192	2048	1024	1024	≤ 32	4096	8192
6	8192	1024	2048	2048	4096	≤ 32	512
7	8192	2048	8192	2048	8192	4096	≤ 32
GA titre after absorption							
1	8192	1024	8192	1024	2048	1024	2048
2	8192	≤ 64	1024	512	4096	4096	2048
3	2098	≤ 32	≤ 32	64	1024	1024	256
4	1024	128	128	≤ 32	1024	1024	1024
5	2048	256	2048	512	≤ 32	1024	512
6	512	64	256	256	512	≤ 64	256
7	128	≤ 32	≤ 32	≤ 32	64	≤ 32	≤ 32

Table 5. *Absorption of antibodies from antiserum to M. hominis serotype 1 by heterologous membranes*

Serotype of antigens used for test	Serotype of membranes used for absorption						
	None	2	3	4	5	6	7
MC titre after absorption							
1	8192	4096	8192	4096	8192	8192	512
2	8192	≤ 64	8192	256	2048	512	2048
3	8192	512	≤ 64	2048	1024	256	256
4	8192	512	2048	≤ 64	512	256	2048
5	8192	1024	256	≤ 64	≤ 64	512	4096
6	8192	256	2048	1024	2048	≤ 64	512
7	8192	≤ 64	256	2048	4096	1024	≤ 64
GA titre after absorption							
1	8192	2048	4096	1024	2048	1024	4096
2	8192	≤ 64	2048	≤ 64	4096	8192	2048
3	2048	128	≤ 64	128	1024	256	512
4	1024	128	256	≤ 64	1024	1024	512
5	2048	512	1024	≤ 64	≤ 64	512	1024
6	512	≤ 64	512	256	512	≤ 64	256
7	128	≤ 64	≤ 64	≤ 64	≤ 64	≤ 64	≤ 64

Serotype-specific antigens

The presence of type-specific antigens was demonstrated by absorbing each antiserum with the preparation which contained pooled membranes from six heterologous serotypes (Table 3). Each serum retained significant homologous antibody titres after the absorption but only low titres (≤ 64) to the other six heterologous serotypes could be demonstrated. Relatively more MC than GA antibodies were removed from sera 1, 5, 6 and 7, results similar to those shown in Table 2.

Table 6. *Antigens detected in membranes of M. hominis by reciprocal absorption tests*

Serotype	Outer surface		Inner surface†		Total
1	ABC, ABG*, ACD, ACE, ACF, ADG, AFG	ab, ac, ad, AE, af, AG	BD, CG, EG	A	17
2	ABC*, ABG*, BCF, BCG*, BFG	AB, BC, BD, be, BF, bg	AF, DE, DF, EF, GE	B, C, G	19
3	ABC, ACD, ACE*, ACF, CBF, CBG, CDF, CFG*	AC, bc, CD, ce, CF, CG	AG, DG, EF	C, E, F	20
4	ACD, ADG*, CDF, DFG	AD, bd, CD, de, DF, dg	AE, AF	D	13
5	ACE*	ab, be, ce, de, ef, eg	AB, AD, AG, BD, BG, CG	E	14
6	ACF*, AFG*, BCF*, BFG, CDF*, FDG*	af, bf, CF, DF, ef, GF	AB, AD, AE, BE	F, E	18
7	ABG, AGD, AFG, BFG, CFG, DFG	AG, BG, cg, DG, eg, FG	BE	G, A	15

* Also exposed at the inner surface.

† Not exposed at the outer surface.

Antigenic components of membranes

Titres after absorption of antibodies from antiserum to serotype 1 by heterologous cells are presented in Table 4 as a representative of the seven sera. The minimum number of cross-reacting antigens at the outer surface of the seven serotypes of *M. hominis*, estimated from the results of reciprocal absorptions, was 7 to 14 (Table 6). There were 15 antigens shared by three serotypes and 30 antigens shared by two serotypes. Various heterologous antibody activities, which were not absorbed or detected by the homologous cells, were present in all seven sera, indicating that some membrane antigens were not exposed at the cell surface. Antibodies to such antigens, if exposed at the inner surface, would be selectively absorbed by membranes but not by whole cells. Therefore, results of reciprocal absorptions with membranes (Table 5) were compared with those with cells (Table 4) and the antibodies which were absorbed preferentially by membranes were derived. Inner membrane antigens, which absorbed a significant amount of antibody from heterologous sera, invariably induced antibodies in the homologous serum. As summarized in Table 6, 3 to 8 such antigens were detected. In total, 13 to 20 membrane antigens were detected in each serotype of *M. hominis*.

DISCUSSION

Surface antigens of *M. hominis* active in the MC and GA reactions were inactivated by incubation at 56 °C for 30 min and by treatment with trypsin. Thus, serologically active antigens contain predominantly heat-labile proteins. Although periodate treatment lowered the antibody-absorbing capacity of cell preparations for the GA reaction, it is not certain whether carbohydrates participated in the GA reaction; some amino acids may react with periodate (Kabat & Mayer, 1961). Repeated freeze-thawing failed to lower any of the antigenic activities of the preparation, suggesting that lipoproteins were not involved in the reactions. Past studies suggest that little or no glycolipid and glycoprotein occur in membranes of *M. hominis* (Rottem & Razin, 1973; Schiefer *et al.*, 1974). Results presented here were similar to those of Hollingdale & Lemcke (1972) who treated membrane preparations of a strain of *M. hominis* in various ways and tested their antigenicity by the metabolism inhibition, indirect haemagglutination and gel diffusion tests. They showed that antigenicity

was associated with non-lipid residues and probably consisted mainly of proteins. Antigenic components with various sensitivities to heat, alkali and periodates were also demonstrated.

Both the mycoplasmacidal reaction, i.e. killing of *M. hominis* by antibodies in the presence of complement, and the agglutination during growth of *M. hominis* in broth cultures in the presence of antibody (Lin & Kass, 1974) require live cells to manifest the serological reactions. Therefore, antigens participating in these reactions are exposed at the outer surface of *M. hominis*. In the present experiments, live unfrozen cells were used to absorb antisera, indicating that the antigens which absorbed antibody from appropriate antisera are also located at the outer surface of the mycoplasmas. It is unlikely that much destruction of cells occurred during the process of antibody absorption. It has been shown that antibody to *M. hominis* agglutinated and inhibited the growth of mycoplasmas but only in the presence of complement were mycoplasmas killed by antibody (Lin & Kaas, 1975). Although the membrane fractions used in the present experiments were not further characterized, it was presumed that cytoplasmic components were not involved (Razin, 1973). The results presented here are aimed not at identifying specific antigens or the number of antigens present in membranes of *M. hominis*, but merely to illustrate the antigenic heterogeneity of the species. The present methods of analysis are based on some assumptions which may not be quantitative. For example, 1 to 2 units of antigen activity may not be sufficient to remove minor antibody activities. It was also assumed that immunogenicity and absorbing capacity of antigens were equivalent. Moreover, the inner membrane components detected by using sonicated membrane fragments (Kahane & Razin, 1969; Pollack *et al.*, 1965) may not be optimal.

Serological heterogeneity within other species of mycoplasmas has been demonstrated in *M. pulmonis* (Forshaw & Fallon, 1972), *M. hyorhinis* (Gois *et al.*, 1974) and *Ureaplasma urealyticum* (Purcell *et al.*, 1969; Black, 1970; Lin *et al.*, 1972). The cross-reacting patterns demonstrated within each mycoplasmal species are as complex as in *M. hominis*. The high sensitivity and specificity of the MC and GA reactions made it possible to examine antigenic components of *M. hominis* in detail. Results presented here have shown that a total of 13 to 20 antigens are demonstrable by serological techniques in each serotype of *M. hominis*. Hollingdale & Lemcke (1970) observed 12 to 15 protein bands and Amar *et al.* (1974) reported 18 different polypeptide bands in some strains of *M. hominis* using electrophoresis of detergent-lysed membrane preparations.

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