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Serological Reactions of *Mycoplasma hominis*: Differences Among Mycoplasmacidal, Metabolic Inhibition, and Growth Agglutination Tests

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Seven strains of *Mycoplasma hominis* from human genital tracts were selected to produce antisera. The complement-dependent mycoplasmacidal (MC) activity and two complement-independent activities, metabolic inhibition (MI) and agglutination during growth (GA), of these sera were compared. The GA and MI tests were equivalent in titer and generally not affected by concentration of antigen; they revealed specific cross-reacting patterns among the seven strains. There was little if any cross-reactivity with antisera to other human mycoplasmas or to *Mycoplasma arthritidis*. Control sera from unimmunized rabbits and sera against concentrated uninoculated culture media were also nonreactive. On the other hand, the sensitivity and specificity of the MC test were affected by the concentration of antigen: at about 10^2 color-changing units (CCU) of antigen, all strains were equivalently cross-reactive against the seven antisera, but at 10^2 to 10^4 CCU the individual strain specificity appeared, whereas at about 10^5 CCU little or no antibody was detectable by MC. The MC tests with the lowest concentrations of antigen gave the highest titers of antibody, but at such low concentrations of antigen there was not only loss of individual strain specificity but also some cross-reactivity against other human mycoplasmas, *M. arthritidis*, or control rabbit sera. Studies with paired human sera also indicate that the MC test with small amounts of antigen is more sensitive than GA-MI.

The most widely used serological technique in the study of human mycoplasmas has been the metabolic inhibition test (MI), but the test has not been particularly sensitive and is subject to variation (5). We have recently described a sensitive and specific mycoplasmacidal test for both T-mycoplasmas and *Mycoplasma hominis* (3, 4) and have studied the various interactions of *M. hominis* with specific antiserum, in the presence and absence of complement (J. S. Lin and E. H. Kass, manuscript in preparation). The present experiments were designed to compare differences in sensitivity and specificity of the complement-dependent mycoplasmacidal (MC) and the complement-independent MI and agglutination during growth (GA) tests. It will be shown that the MI and GA tests are similar but are in turn different from MC in sensitivity and specificity, and each has desirable characteristics for certain serological purposes.

MATERIALS AND METHODS

Strains of *M. hominis* other than the prototype strains were isolated in our laboratory from vaginal swabs of pregnant women (1). The genital prototype strain PG21 was obtained from R. H. Purcell from the National Institutes of Health and maintained in our laboratory. Six additional selected strains of *M. hominis* were purified three times by terminal dilution (4) and used to make antisera.

The procedures for preparing the organisms and the rabbit sera have been described (1). The horse serum used in the medium (1) was supplied by the Massachusetts Department of Health, and the serum from the same horse was used throughout the study. Rabbit antisera for other human mycoplasmas (*Mycoplasma orale* type I [CH-10299] and type II [CH-20247], *Mycoplasma salivarium* [PG20], *Mycoplasma fermentans* [PG18], and rabbit serum for *M. arthritidis* [PG27]) were purchased commercially (Microbiological Associates). Antiserum against strain PG21 was obtained from two different commercial sources (Microbiological Associates and Baltimore Biological Laboratories). Complement as lyophilized guinea pig serum was also purchased and reconstituted with sterile distilled water. This complement, in the dilutions used, was not inhibitory to any of the test organisms. Control sera consisted of sera from rabbits that had been immunized with concentrates of culture medium, prepared as were the antigens, or preimmunization sera from the rabbits.

Twenty-four paired human sera were obtained from pregnant women whose genital culture contained *M. hominis* (1). The first blood sample was collected at the time of delivery and the second one was obtained 4 to 6 weeks later. Sera were separated immediately after collection and stored at −20 C. Sera were heat inactivated at 56 C for 30 min prior to use.

To compare the end-point titers of the MI and the GA tests, reactions were carried out in test tubes. The reaction mixture consisted of 0.1 ml of antiserum, 0.1 ml of organism, and 0.3 ml of growth medium in plastic tubes (12 by 75 mm; Falcon Plastics). The pH changes induced by mycoplasmal growth were homogeneous and slow, so that the end points for the MI tests were reproducible on tubes if the times of operation were controlled. Tenfold serial dilutions of the mycoplasmal cultures and twofold dilutions of the antisera were used in homologous strain-antiserum combinations. In heterologous systems, $10^{2.5}$ to $10^{3.0}$ 50% color-changing units (CCU) (4) of each strain of mycoplasma were used with twofold serial dilutions of antiserum. At this concentration all seven strains induced reproducible increases in pH (from the initial level of pH 6.8 to pH 7.5 or more) after 40 h of incubation at 36 C in the growth medium. Homologous antisera were always included as controls in the heterologous systems.

In parallel, serological tests were also conducted in the microtiter system (Cooke Engineering Co.) (3). For the MC test, serial twofold dilutions of serum in 0.025-ml volumes were prepared, the diluent being 0.02 M phosphate-buffered saline at pH 6.8. To these dilutions of serum were added 0.025 ml of organisms (10^1 to 10^2 CCU except as noted), followed by 0.05 ml of guinea pig complement in 1:10 dilution. Plates were sealed and incubated at 36 C. After 1 h of incubation to permit killing, 0.1 ml of growth medium was added to each well. Finally, the plates were sealed and incubated at 36 C aerobically. The medium was observed daily for pH changes, and the reciprocal of the highest serum dilution which prevented the changes in pH after 5 days of incubation was recorded as the MC antibody titer. Invariably, the color changes remained stable for 7 additional days of incubation. For the GA or MI tests, the organisms (10^2 to 10^3 CCU in 0.025 ml, except as noted) were grown with twofold serial dilutions of antiserum (0.025 ml) and growth medium (0.15 ml) at 36 C aerobically. The wells were observed daily for pH changes of the medium and agglutinated growth at the bottom of the wells. The reciprocal of the highest antiserum dilution which showed agglutinated growth of the mycoplasmas after 5 days of incubation was recorded as the GA titer. As was observed in tubes (1), a colony-like growth appeared on the bottom of the wells after 2 days of incubation and just before changes in pH were detected, and the titers stabilized after 5 days of incubation, the colonies in the liquid then being stable for at least 7 days of incubation.

RESULTS

All the homologous and the heterologous GA and MI titers were similar, if not identical (Table 1). There were cross-reacting patterns among the seven strains as demonstrated by the reciprocal titers. The dynamics of the two reactions were also alike, e.g., the end-point titers were constant within the concentrations of antigen (10^4 to 10^2 CCU) tested. End points in the microtiter wells changed more rapidly with time than did end points in the test tube, probably reflecting the larger amount of buffer in the latter. Since it was easier to read end points for the GA test in the wells, the GA test was selected for further detailed studies.

The GA tests in the microtiter systems gave end-point titers which were not affected by the concentration of antigen present. End points were almost identical for the tube and microtiter systems (Tables 1 and 2). Serological relatedness among the seven strains was calcu-

TABLE 1. *GA and MI for seven strains of M. hominis*

Strain no.[a]	Method	GA and MI titers of antiserum						
		W_2	10	93	132	PG21	4195	183
1. (W_2)	GA	10,240	5,120	2,560	2,560	640	320	640
	MI	5,120	2,560	5,120	5,120	640	640	1,280
2. (10)	GA	10,240	40,960	20,480	10,240	2,560	5,120	2,560
	MI	10,240	20,480	20,480	10,240	2,560	5,120	5,120
3. (93)	GA	5,120	5,120	10,240	1,280	320	320	2,560
	MI	5,120	2,560	10,240	1,280	320	320	2,560
4. (132)	GA	1,280	2,560	2,560	2,560	80	160	160
	MI	2,560	5,120	2,560	5,120	320	640	320
5. (PG21)	GA	5,120	2,560	2,560	640	20,480	1,280	2,560
	MI	5,120	5,120	2,560	1,280	20,480	2,560	5,120
6. (4195)	GA	1,280	2,560	2,560	160	640	5,120	1,280
	MI	2,560	5,120	2,560	320	2,560	10,280	2,560
7. (183)	GA	320	1,280	10,280	80	160	160	5,120
	MI	320	1,280	10,280	80	160	160	5,120

[a] Strain numbers are followed by the designations generally applied to these strains in the literature.

lated by using the reciprocal GA titers (3) (Table 3). Again, relatedness was similar regardless of the system used.

MC titers. When antisera were tested against small concentrations of organisms (10^1 to 10^2 CCU), there were no differences among the homologous and the heterologous MC titers. For example, antiserum 4195 had titers of 2,048 to 8,192 against the seven strains, and antiserum W_2 had titers of 8,192 to 32,768 against the seven strains. At 10^3 to 10^4 CCU, the homologous titers remained the same, whereas some heterologous titers decreased, revealing cross-reacting patterns (Table 4). Often pH changes were seen in low dilutions of antisera, accompanied by agglutinated growth of the mycoplasmas. At concentrations of antigens of 10^5 CCU or greater, low or no MC titers were demonstrated; most of the wells showed pH changes during the 5-day observation period, accompanied by agglutinated growth of the organisms. The serum end-point titers for the MC reaction were inversely related to antigenic concentration. Figure 1 shows the relationship of serum titer to antigenic concentrations for antiserum 4195 against five strains of organism. Within the antigenic range of 10^3 to 10^6 CCU, there were straight-line relationships between antigen concentration and antibody end-point titers. Moreover, the slopes of the lines were different for each strain of mycoplasma. The figure also demonstrates the concentration of antigen, 10^3 to 10^4 CCU, that best demonstrates strain specificity.

Sensitivity of the MC and the GA tests. In rabbit antisera, the homologous MC titers were either equal to or higher than the GA titers (Tables 2 and 4). For example, antiserum 183 shared an MC titer of 8,192 and a GA titer of 2,048; antiserum 10 had a titer of 32,768 for both reactions. Differences in sensitivity were best demonstrated by studies in paired human sera (Table 5). With the serum donor's own genital isolates as antigen, the MC test detected rises in titer of fourfold or more in 9 of 18 paired sera, whereas the GA test detected such a rise in only

TABLE 2. *Antibody titers and cross-reactivity of seven strains of M. hominis as determined by the microtiter GA test*

Strain no.[a]	GA titers of antiserum						
	W_2	10	93	132	PG21	4195	183
1. (W_2)	8,192	4,096	2,048	2,048	512	256	512
2. (10)	8,192	32,768	16,384	8,192	1,024	1,024	1,024
3. (93)	2,048	4,096	8,192	512	256	128	2,048
4. (132)	1,024	2,048	1,024	2,048	64	64	128
5. (PG21)	2,048	1,024	2,048	256	16,384	1,024	1,024
6. (4195)	512	2,048	1,024	128	512	2,048	512
7. (183)	128	512	4,096	32	128	64	2,048

[a] Strain numbers are followed by the designations generally applied to the strains in the literature.

TABLE 3. *Serologic relationships of seven strains of M. hominis as determined by reciprocal GA tests of antiserum*[a]

Strain	1 (W_2)	2 (10)	3 (93)	4 (132)	5 (PG21)	6 (4195)	7 (183)
W_2	100						
10	36.0	100					
93	25.0 (36.0)[b]	50	100				
132	36.0	50	18.0 (36.0)	100			
PG21	9.0 (12.5)	4.5 (9.0)	6.3	2.2 (3.1)	100		
4195	9.0	18.0 (25.0)	9.0	4.5	12.5 (9.0)	100	
183	6.3	9.0 (12.5)	71.0	3.1	6.3	9	100

[a] All values are expressed as percentage relatedness. Calculation = $\sqrt{r_1 \times r_2} \times 100$ (5), where r_1 = heterologous titer G_2/homologous titer G_1, and r_2 = heterologous titer G_1/homologous titer G_2.

[b] Values in parentheses are those derived from the tube GA tests. Where there is a single value, the titers by tube and microfilter systems are the same.

TABLE 4. *Antibody titers and cross-reactivity of seven strains of M. hominis as determined by MC titers of antiserum*

Strain no.[a]	MC titers of antiserum[b]						
	W_2	10	93	132	PG21	4195	183
1. (W_2)	16,384	16,384	2,048	2,048	256	512	512
2. (10)	2,048	32,768	16,384	2,048	512	1,024	8,192
3. (93)	4,096	16,384	32,768	2,048	1,024	1,024	4,096
4. (132)	16,384	4,096	32,768	8,192	256	512	1,024
5. (PG21)	2,048	4,096	32,768	512	8,192	256	512
6. (4195)	8,192	4,096	32,768	512	2,048	4,096	2,048
7. (183)	4,096	32,768	32,768	1,024	512	256	8,192

[a] Strain numbers are followed by the designations generally applied to these strains in the literature.
[b] Antigen concentration was 10^3 to 10^4 CCU.

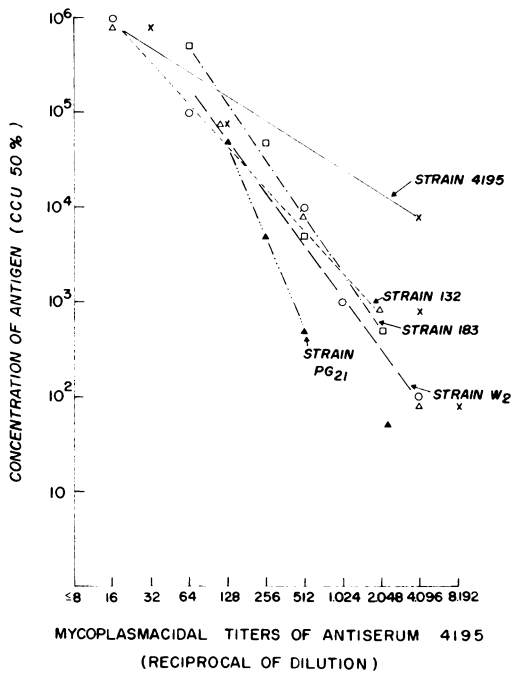

FIG. 1. *Relation of concentration of antigen to the mycoplasmacidal titers of antiserum 4195 with five different strains of M. hominis.*

one of the 18 ($P < 0.01$). Moreover, the MC titers were invariably twofold or more greater than the GA files. Among those sera that were positive for both tests, two pairs had MC titers of 256, whereas the corresponding GA titers were only 16 or 32.

Specificity of the GA and the MC tests. There were few or no detectable GA titers (≤ 16) in antisera to other human mycoplasmas, to *M. arthritidis*, and to concentrates of uninoculated culture media as well as in the sera of 23 unimmunized rabbits. The antisera to PG21 from three different sources had similar titers (Table 6). With antigenic concentrations of 10^3 to 10^4 CCU, there were also only traces or no detectable MC titers in all the nonspecific sera tested. Again antiserum PG21 from different souces had similar MC titers (Table 6) with this concentration of antigen. When smaller concentrations of antigen were used, almost all the nonspecific sera tested had some titers to all the seven strains. The MC titers in the sera from the 23 unimmunized rabbits and in antiserum PG27 ranged from 16 to 256. A few antisera to human mycoplasmas other than *M. hominis* had titers up to 1,024 when tested against 10^1 to 10^2 CCU of *M. hominis*. All of these titers were complement dependent, suggesting that the inhibitory components in these sera were probably antibody. Thus, the MC test required carefully measured concentrations of antigen to demonstrate species and strain specificity (see also Table 4).

TABLE 5. *Antibody titers to M. hominis in human sera as detected by the MC and the GA tests using serum and organism from the same ptient*

Antibody titers[a]		No. detected	
Prepartum	Postpartum	MC	GA
<8	≥16	9	1[b]
≤8	≤8	9	17[b]
>8	≥16	16	6

[a] Sera obtained from pregnant women.
[b] $\chi^2 = 6.8$ (calculated by Yates corrected chi-square); $P < 0.01$.

TABLE 6. *Antibody titers and cross-reactivity of PG21 antisera obtained from three different sources*[a]

Strain no.[b]	GA titers of antisera			MC titers of antisera[c]		
	A	B	C	A	B	C
1. (W_2)	1,024	2,048	512	512	512	256
2. (10)	512	2,048	1,024	2,048	1,024	512
3. (93)	512	1,024	256	2,048	4,096	1,024
4. (132)	128	512	64	512	1,024	256
5. (PG21)	32,768	16,384	16,384	16,384	8,192	8,192
6. (4195)	512	1,024	512	4,096	4,096	2,048
7. (183)	128	256	128	512	128	512

[a] A, Microbiological Associates; B, Baltimore Biological Laboratories; C, Channing Laboratory.
[b] Strain numbers are followed by the designations generally applied to these strains in the literature.
[c] Tested against 10^3 to 10^4 CCU of the mycoplasmas.

Reproducibility of the tests. All the GA tests for the seven reference strains were performed at least 10 different times through the course of the study. The variations for a strain-antiserum combination were twofold or less (Table 2). The MC titers with smaller concentrations of antigen were consistent, but with greater amounts of antigens, the reproducibility of the MC tests depended on the rigid control of antigenic concentrations and complement activity. Breakthrough was occasionally observed at low dilutions of antiserum, particularly with heterologous strain-antiserum combinations.

DISCUSSION

The conventional MI test for human genital mycoplasmas, which allows the organism to grow in the presence of antibody, detects a delay in metabolic activity that is induced by antiserum and generally includes complement in the test systems (5). The release of ammonia from certain metabolites, e.g., urea for T-mycoplasmas and arginine for *M. hominis*, makes it possible to detect changes in pH in the media as the mycoplasmas multiply. Because of the requirement of complement for the rapid inactivation of mycoplasmas by the antisera, and because of the presence of ammonia-sensitive components in the complement system, technical difficulty in the conventional MI tests could be attributed to the variable activity of the complement (4) under the conditions of growth.

The present data show that the complement-independent GA or MI tests and the complement-dependent MC tests are not identical in their specificity, dynamics, and sensitivity. Depending on the activity of the complement and amounts of antigen present, both the MI and the MC reactions could take place in a single reaction system, and it is difficult to determine the relative contributions from these two reactions to the end point. However, in the present studies, the complement-dependent MC reactions were carried out with definite amounts of antigens in a medium free of ammonia-yielding substrates. After the completion of the killing reaction, growth medium was added to allow the surviving organisms to multiply and the complete inhibition of pH changes was taken as the end point. In the GA or the MI, complement activity was excluded from the test systems.

The GA and MI titers for the seven strains of *M. hominis* were similar, if not identical. Therefore, these two tests can be considered to measure the same antigen-antibody system. Furthermore, serological relatedness, as calculated by the reciprocal GA titers, were very close to those calculated using the MI titers, indicating that the specificities of the two tests are similar. The similarity, and the fact that GA is detectable before MI, suggests that MI may be a reflection of agglutinated organisms in the growth phase of the mycoplasmas, thus interfering with availability of substrate or with release of product or both.

Serological heterogeneity among human *M. hominis* was previously detected by the conventional MI (2, 5), and by indirect hemagglutination tests (6). Similar heterogeneity and strain specificity could be demonstrated by appropriate use of the GA and MC tests. The MC tests were affected by many factors, such as concentration of antigen and complement activity. Subtypings of mycoplasmas by this test will probably require quantitative kinetic analysis of the reactions. On the other hand, the GA tests were simple to perform and had reproducible results. Two hundred consecutive clinical isolates of *M. hominis* could be subtyped in this laboratory by using the seven reference antisera.

All the control rabbit sera (unimmunized rabbits as well as rabbits immunized with the

concentrates of culture medium) had some MC titers to the seven strains of *M. hominis* when small amounts of antigen were used. Therefore, the significance of cross-reactivities detected in antisera to mycoplasmas other than *M. hominis* is not clear.

The cross-reactive MC activities of rabbit sera are not all directed against cell antigens that have interacted with components of the culture medium. In all preimmune sera and sera from rabbits receiving uninoculated culture media, cross-reactivity was demonstrated only with 10^1 to 10^2 CCU of antigen. Furthermore, the titers, even under such conditions, varied with different strains so that sera tested against strains 4195 and PG21 showed decreased titers after injection of the animals with uninoculated culture medium (1:32 to 1:8 or less), but no change in titer to strain 10 (1:128) and slight rises in titer from 1:32 to 1:128 to strains W_2 and 132. Strain 93 had the highest rise in titer, from 128 to 2,048 in animals receiving uninoculated medium. The basis for this strange pattern of apparent cross-reactivity requires more study.

Both the MC and GA titers in rabbit antisera and the MC titers in human sera were resistant to 2-mercaptoethanol (J. S. Lin, unpublished observations), suggesting that macroglobulin antibody was not greatly involved in these systems. The occurrence of mixed GA and MC reactions with high concentrations of antigen in the presence of complement suggests that the specificity of the antigens involved in these two tests may not be identical. Studies now in progress show that both GA and MC antibody activities were completely adsorbed or inhibited by cell membrane preparations, and that some biochemical differences were detected between antigens participating in the MC and the GA tests (J. S. Lin, unpublished results). The serological heterogeneity of human *M. hominis* is clearly established. Whether different strains play different physiological roles remains to be shown.

In brief, it appears that the complement-dependent MC test with low concentrations of organisms is more sensitive and is suitable for detecting human antibody responses, whereas the complement-independent GA test is more specific and technically less demanding and is suitable for subtyping *M. hominis*. The MI test seems to be equivalent to the GA but takes longer for the end point to appear, and breakthrough occurs.

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