COMPLEMENT-DEPENDENT AND COMPLEMENT-INDEPENDENT INTERACTIONS BETWEEN *MYCOPLASMA HOMINIS* AND ANTIBODIES *IN VITRO*

J.-S. LIN AND E. H. KASS

Channing Laboratory and Department of Medicine, Harvard Medical School, and Departments of Medicine and Medical Microbiology, Boston City Hospital, Boston, Mass., USA

RECENTLY, a complement-dependent, mycoplasmacidal reaction was described involving T-mycoplasmas and specific antibody (Lin and Kass, 1970). The concentration of urea in the medium was found to be critical for the demonstration of this reaction, presumably because the urease activity of the T-mycoplasmas released enough ammonia to inhibit the ammonia-sensitive components of the complement system. It was anticipated, and the present paper shows, that a similar mycoplasmacidal reaction exists for *Mycoplasma hominis* and specific antibody, and that this is dependent upon the concentration of arginine in the medium.

This mycoplasmacidal reaction was then compared with complement-independent reactions of *M. hominis* and specific antibody, the latter including agglutination, metabolic inhibition, agglutination during growth, and inhibition of multiplication. The different types of interaction between *M. hominis* and specific antibody are recorded in detail and provide a basis for future studies of the specificity and sensitivity of the various types of interaction, as well as for the development of procedures for serotyping and for the study of antibody responses to *M. hominis*.

MATERIALS AND METHODS

M. hominis, strain 4195, was isolated from the genital tract of a pregnant woman and was purified three times by a terminal dilution method. The mycoplasmas were grown in the basic medium {PPLO broth 70% (v/v), horse serum 20% (v/v), yeast extract 10% (v/v), phenol red 0.02% (w/v) and benzylpenicillin 500 U per ml, pH 7.2} supplemented with arginine 1% (w/v). The growth of the organisms was indicated by increased pH of the medium as the arginine was deaminated. Titrations of the mycoplasmas were performed in microtitration plates and the results expressed as 50% colour-change units (CCU; Lin and Kass, 1970). A single pool of an overnight culture, stored at -70°C, was used throughout this study.

To prepare antigens for immunisation, overnight cultures containing approximately 10^{8-9} CCU per ml were concentrated 100 times by centrifugation at 15,000 *g* for 30 min. at 4°C and stored at -20°C. Rabbits were immunised as previously described (Lin and Kass, 1970), each animal receiving about 10^{11} CCU of organisms. Antisera from two rabbits were pooled for use. Rabbit antisera from animals immunised against *M. hominis* (strain PG21) or *M. arthritidis* (strain PG27), and guinea-pig complement, were purchased commercially. Control antisera were obtained from rabbits immunised with concentrates of uninoculated

Received 17 Oct. 1974; accepted 13 Jan. 1975.

culture medium, prepared as were the antigens. All test sera were inactivated for 30 min. at 56°C before use.

To test the mycoplasmacidal effect of antiserum, 0.1 ml of freshly thawed organisms in 10-fold serial dilutions were mixed with 0.1 ml of test antiserum in two-fold serial dilutions, and 0.2 ml of complement in 1 in 10 dilutions in sterile polyethylene tubes (12 × 75 mm, Falcon). To test the effect of antiserum alone, 0.3 ml of the diluent (0.02M phosphate-buffered saline, pH 6.8), or PPLO broth (Difco, w/cv) or the growth medium, were added instead of 0.2 ml of complement.

Metabolic inhibition was studied twice daily by comparing the pH of the medium with a colour standard. A change of 0.5 pH units was indicated as + and of one or more pH units as ++.

RESULTS

As with T-mycoplasmas, the concentration of specific metabolite in the culture medium influences the mycoplasmacidal activity of antiserum and complement (table I). Presumably this represents the effect of ammonia, released from arginine, on the ammonia-sensitive components of complement, much as the concentration of urea influences the mycoplasmacidal activity of antisera against T-mycoplasmas (Lin and Kass, 1970).

The mycoplasmacidal activity of antiserum against *M. hominis* is shown in table I, which also shows the requirement for complement. The organisms were allowed to react for 1 h at 36°C with antiserum and complement in an arginine-free medium before complete medium was added, and further incubation up to 24 h did not alter the number of surviving mycoplasmas. However, as can be seen in table II, further decreases in the titres of organisms were observed in some tubes after incubation for 24–48 h, depending upon the concentrations of organism and antiserum in the reaction mixtures.

The decreases in titre that were observed only after incubation for 24 h were serologically specific, because the control sera had no effect comparable to that seen with specific antisera. The serum end-point titres were inversely related to the concentration of antigen, and a linear relation was found between the logarithms of concentration of antigen at end-point and the concentration of

TABLE I
Effect of arginine on complement-dependent mycoplasmacidal effect of antiserum to Mycoplasma hominis

Percentage of arginine in medium	Titre* of <i>M. hominis</i> in mixtures of			
	heated† guinea-pig serum + antiserum‡ diluted 1 in 40	unheated guinea-pig serum + antiserum‡ diluted 1 in		
		40	400	4000
0	10 ^{5.5}	10 ^{1.3}	10 ^{2.0}	10 ^{3.0}
1	10 ^{6.5}	10 ^{1.8}	10 ^{2.0}	10 ^{6.0}
4	10 ^{5.5}	10 ^{5.5}	10 ^{5.5}	10 ^{5.8}

* As 50% colour-change units. † To 56°C for 30 min.

‡ Against *M. hominis*.

TABLE II

Effect of complement on mycoplasmacidal activity of antiserum to M. hominis

Incubation time (h)	Titre* of <i>M. hominis</i>					
	Complement present			Complement absent		
	Homologous antiserum diluted 1 in 1280	No antiserum	Homologous antiserum diluted 1 in			Heterologous antiserum† diluted 1 in 10
			20	320	1280	
0	10 ^{6.0}	...	10 ^{5.0}	10 ^{3.0}	...	...
1	10 ^{1.3}	10 ^{5.5}	10 ^{5.3}	...	...	...
24	10 ^{1.0}	10 ^{5.0}	10 ^{3.3}	10 ^{2.3}	10 ^{2.5}	10 ^{2.5}
48	...	10 ^{4.0}	0	0	10 ^{1.5}	10 ^{1.8}

... = Not done. * As 50% colour-change units.

† Antiserum to *M. arthritis* or to culture medium.

TABLE III

Effect of sonication of mixtures of Mycoplasma and antiserum, without complement, on titres of M. hominis*

Suspending medium	Antiserum	Initial titre	Incubation time (days)	Titre	
				before sonic treatment†	after sonic treatment†
Diluent broth	Laboratory, homologous, 1 : 40	10 ^{5.3}	2	10 ^{3.0}	10 ^{4.3}
		10 ^{5.3}	2	10 ^{2.5}	10 ^{4.3}
Diluent broth	Commercial, homologous, 1 : 10	10 ^{5.3}	2	10 ^{2.3}	≥ 10 ^{5.5}
Diluent broth	Commercial, <i>M. arthritis</i> , 1 : 10	10 ^{5.8}	2	10 ^{4.0}	10 ^{4.3}
Complete growth broth	Laboratory, homologous, 1 : 20	10 ^{4.5}	2‡	10 ^{3.5}	10 ^{6.8}
Complete growth broth	Laboratory, homologous, 1 : 40	10 ^{4.5}	2‡	10 ^{4.5}	10 ^{7.5}

* As 50% colour-change units.

† Mycoplasmas and serum were mixed and incubated and, at indicated times, portions were treated sonically.

‡ Day when pH change of 0.5 units or more was first noted.

antiserum in producing mycoplasmal inhibition. These results suggested that agglutination was occurring in the tubes during the 2nd day of incubation. Therefore, experiments were begun to attempt to disperse the presumed aggregates by sonic treatment with a 9 kc Raytheon oscillator. Preliminary observations showed that the titre of viable *M. hominis* was diminished by treatment for 60 s at maximum intensity but was not altered measurably at 30 s. The latter duration was used in the following experiments. Mycoplasmal suspensions containing $10^{6.3}$ or $10^{5.3}$ CCU were suspended in 1 in 10 or 1 in 40 dilutions of specific antiserum and incubated at 36°C for 24–48 h. The titres decreased to approximately $10^{2.5}$ in the tubes containing specific antiserum but only to $10^{4.0}$ in the tubes containing no serum or non-specific sera. The tubes containing specific antiserum were then given a brief sonic treatment, whereupon the titres increased more than 100-fold, but the titres of the control tubes were not affected (table III).

The metabolic activity of *M. hominis* in the presence of specific antibody was examined by suspending the organisms and appropriate sera in the growth medium and incubating the mixture at 36°C . As anticipated, the degree of metabolic inhibition observed in the growth medium was directly related to the concentration of specific antiserum. In high concentrations of antiserum, such as 1 in 5 or 1 in 10 dilutions, the pH of the growth medium changed little or not at all during the observation period of 3 weeks. However, the viability of the organisms persisted. For example, $10^{6.3}$ CCU of mycoplasmas, mixed with the aforementioned dilutions of antiserum, produced no significant changes in pH of the medium, and titration after 3 weeks indicated that $10^{3.5}$ CCU were still detectable without sonic treatment (table IV). In higher dilutions of antiserum (1 in 80–1 in 2560) the mycoplasmas multiplied, as indicated by increased CCU in the tubes, but the metabolic rates as reflected by changes in pH of the medium did not rise in the same manner as in the controls; in fact, the pH change often was delayed by several days (table IV). This delay in pH change was best observed when the number of organisms in the reaction mixture was small. For example, when $10^{0.5}$ CCU of mycoplasmas were mixed with a 1 in 80 dilution of specific antiserum, there were no changes in the mycoplasmal titres or in the pH of the medium for 7 days, whereas the same inoculum of *M. hominis* without specific antiserum reached maximal titres and maximal pH changes within 3 days.

When the maximal pH change had been reached, the organisms slowly died, an effect that is independent of the presence of specific antiserum.

Changes in growth patterns of the mycoplasmas were observed in the tubes that showed decreased metabolic activity due to antiserum. Unlike the usual homogeneous and opalescent suspensions that are seen in growth broth without specific antibody, the tubes containing antiserum showed large particles that were visible to the naked eye. These particles settled to the bottom of the tubes and adhered there, leaving the supernates clear. The particles were readily visible for several hours to a day before the increase in pH of the medium was observed, and generally stabilised after incubation for 5 days. No grossly visible changes were observed after 7 additional days of incubation, once the

TABLE IV

Titres of M. hominis in relation to metabolic inhibition by homologous antiserum

Antiserum	Dilution 1 in	Titre* of <i>M. hominis</i> (and colour-change†) in the presence of the stated antiserum on day					
		0	1	2	7	11	21
Homologous	5	10 ^{6.3}	10 ^{3.8} (-)	10 ^{2.3} (-)	10 ^{2.5} (-)	10 ^{3.3} (+/-)	10 ^{3.5} (+/-)
Homologous	5	10 ^{3.3}	10 ^{1.5} (-)	0 (-)	0 (-)	10 ^{2.5} (-)	10 ^{2.0} (-)
Homologous	5	10 ^{1.3}	... (-)	... (-)	0 (-)	0 (-)	0 (-)
Homologous	80	10 ^{2.5}	... (-)	10 ^{2.5} (-)	0 (++)	...	...
Homologous	80	10 ^{0.5}	... (-)	10 ^{1.5} (-)	10 ^{5.5} (++)	...	...
Homologous	320	10 ^{2.5}	10 ^{2.5} (-)	10 ^{4.0} (+)	... (++)	...	...
Homologous	320	10 ^{0.5}	... (-)	... (-)	>10 ⁵ (++)	...	...
Homologous	640	10 ^{2.5}	10 ^{3.0} (-)	... (++)	...	...	...
Homologous	640	10 ^{0.5}	... (-)	... (-)	>10 ⁵ (++)	...	...
<i>M. arthritis</i>	10	10 ^{3.5}	≥10 ^{7.5} (++)	10 ^{1.5} (++)	0 (++)	...	...
<i>M. arthritis</i>	10	10 ^{2.5}	10 ^{7.5} (+)	10 ^{1.0} (++)	0 (++)	...	...
<i>M. arthritis</i>	10	10 ^{0.5}	10 ^{4.5} (-)	10 ^{7.8} (++)	0 (++)	...	...

... = Not done.

* As 50% colour-change units.

† Change in pH of: - = >0.5; + = 0.5-0.99; ++ = >1.0.

particles had reached maximal size. When such particles were sonically treated, as the pH of the medium started to change, the mycoplasmal titres increased more than 100-fold (table III).

In general, when metabolic inhibition by antiserum was observed, as indicated by the delay in change in pH, the delay was temporary except at the highest concentrations of antiserum, and ultimately most of the tubes in which metabolic inhibition was demonstrable developed maximal changes in pH and maximal numbers of mycoplasmas. On the other hand, the agglutinated masses tended to persist in the presence of antiserum as indicated above, although the number and sizes of the agglutinated masses varied with the concentration of mycoplasmas. Thus, at concentrations greater than 10⁴ CCU, the particles tended to be small, so that end-points were difficult to read macroscopically; but at 10³ CCU, the particles were large and clearly visible in the presence of the same amount of antiserum.

The end-points for delay in pH change and for agglutinated growth were approximately the same for two different antisera that were tested, but agglutination was invariably noted earlier than the pH change.

When the end-point titres for each of the reactions were compared, titres for specific agglutination in diluent medium, for agglutination in the growth medium, for metabolic inhibition, and for the mycoplasmacidal reaction were similar, but growth inhibition titres were much lower. Thus, for the strain used in the present studies, when tested against its homologous antiserum, growth-inhibition titres averaged 80, serum agglutination titres in the presence of diluent medium averaged 640; agglutination during growth and the

mycoplasmacidal reaction gave titres of 5120 and metabolic inhibition a titre of 10,240.

DISCUSSION

The interaction of a strain of *M. hominis* and its specific antibody has been measured in five different ways. One of these is complement dependent and results in mycoplasmal death. However, the mycoplasmacidal reaction for *M. hominis*, like the one for T-mycoplasmas, is inhibited in the presence of the specific substrate needed for growth of the organism. Consequently the mycoplasmacidal reaction for both varieties of human genital mycoplasmas is best demonstrated by mixing antibody and complement with the organism in a diluent medium free of the substrate. After a suitable incubation period, the surviving organisms are allowed to grow in the complete growth broth.

Metabolic inhibition (MI) can be demonstrated as a specific phenomenon in the absence of complement. The present observations show that MI without complement is invariably accompanied by agglutination of the multiplying organisms, and that such agglutination, herein called specific agglutination during growth (GA), can be observed as much as a day before MI is observed by pH change. That MI and GA are related phenomena is seen not only by the similarity of end-point titres, and the invariable association of the two phenomena, but also by the finding that gentle sonic treatment increases the titre of organisms in the suspension during GA and MI. GA, however, must be separated from the usual agglutination reaction in which *M. hominis* and specific antiserum will form clumps but will do so in the presence of a diluent that does not permit multiplication of the organisms. Clearly, there is a specific effect of antiserum that involves multiplying organisms, and this is to be distinguished from an effect that is independent of multiplication.

Finally, still another phenomenon, inhibition of multiplication without loss of viability, has been observed. This phenomenon, occurring in low dilutions of antiserum, was demonstrated by Bailey *et al.* (1963) and shown to be related to a water-insoluble fraction that was eluted from agglutinated *M. hominis*. At high concentrations of antiserum, the mycoplasmal cells remain viable but do not metabolise significantly.

The present observations indicate the basis for some of the mycoplasma-antibody reactions that have been studied in the past. For example, inhibition that is observed on agar plates, which requires high concentrations of antiserum and is not complement dependent (Purcell, Chanock and Taylor-Robinson, 1969), probably reflects the type of inhibition of multiplication and of metabolic activity that was observed here in liquid media in the presence of high concentrations of specific antiserum. Inhibition in liquid media by antibody in the presence of complement is widely seen in the study of mycoplasma-antibody reactions, and has been reported for strains of *M. hominis* (Bailey *et al.*, 1963), *M. pneumoniae* (Senterfit and Jensen, 1966), *M. mycoides* (Gourlay and Domermuth, 1967), and for several strains of other human mycoplasmas (Riggs and Sharp, 1970).

Although *M. hominis* was shown by Furness (1970) to grow in aggregates

during the early log phase of multiplication, the large colonies observed in the present studies do not appear in the absence of specific antibody. *M. pneumoniae* has hitherto been the only human mycoplasma that forms free-floating spherules in liquid growth media (Boatman and Kenny, 1971), but these are quite different in appearance from the agglutinated masses herein described. The present observations seem similar to those made by Gois, Cerny and Veznikova (1970), who used conventional metabolic inhibition tests and observed specific colonial growth of some species of porcine mycoplasmas in the presence of specific antibody. Agglutination during multiplication of bacteria in the presence of specific antiserum has been reported for several strains of enteric bacilli (Wynne *et al.*, 1953) and for various Gram-positive bacteria (Nicolle and Césari, 1926). Observations similar to MI have been made with mouse-leukemia cell-lines by Yang and Vas (1970), with human cell-lines by Bartholomew, Kite and Rose (1967), and with *Escherichia coli* exposed *in vitro* to specific antiserum by Portmann (1962). The significance of this general class of antigen-antibody reactions requires further study.

SUMMARY

Several *in vitro* reactions between a strain of *M. hominis* (no. 4195) and homologous antiserum have been delineated and compared. One complement-dependent and four complement-independent activities of antibody have been studied. The complement-dependent activity was mycoplasmacidal and was inhibited by the presence of arginine in the test medium. The complement-independent antibody-mediated reactions were not mycoplasmacidal and were four in number: (a) agglutination, which was manifested in buffered saline after incubation for 24–48 h at 36°C, and in which the end-points were dependent upon the concentration of antigen; (b) metabolic inhibition, in which antiserum added to liquid growth medium produced slowing of the rate at which the pH rises during growth; (c) agglutination during growth, which occurred in liquid growth medium after the addition of antiserum and coincided with, but generally preceded, metabolic inhibition; and (d) inhibition of multiplication in which high concentrations of antiserum led to inhibition of multiplication or metabolic activity, with persistence of viable mycoplasmas, under otherwise favourable conditions of growth. The end-points for each of the above methods of detecting antibody are not identical.

REFERENCES

- BAILEY, J. S., CLARK, H. W., FELTS, W. R. AND BROWN, T. M. 1963. Growth inhibitory properties of mycoplasma antibody. *J. Bact.*, **86**, 147.
BARTHOLOMEW, W. R., KITE, J. H. AND ROSE, N. R. 1967. Antigens of mammalian cell cultures. II. The nature and cellular localization of the cytotoxinogen in established cell lines. *Lab. Invest.*, **17**, 527.
BOATMAN, E. S. AND KENNY, G. E. 1971. Morphology and ultrastructure of *Mycoplasma pneumoniae* spherules. *J. Bact.*, **106**, 1005.
FURNESS, G. 1970. Effect of physical stress on the elementary bodies and colony-forming units of mycoplasmas. *J. infect. Dis.*, **122**, 64.

- GOIS, M., CERNY, M. AND VEZNIKOVA, D. 1970. Relationship between metabolism-inhibiting antibodies and colonial growth of *Mycoplasma hyorhinis* and other porcine mycoplasma in the presence of rabbit and pig sera. *Res. vet. Sci.*, **11**, 161.
- GOURLAY, R. N. AND DOMERMUTH, C. H. 1967. Growth inhibition and "neutralization" of *Mycoplasma mycoides* by immune bovine serum. I. Development of a liquid medium test. *Ann. N.Y. Acad. Sci.*, **143**, 325.
- LIN, J. S. AND KASS, E. H. 1970. Immune inactivation of T-strain mycoplasmas. *J. infect. Dis.*, **122**, 93.
- NICOLLE, M. AND CÉSARI, E. 1926. Influence des immun sérums spécifiques sur la culture des microbes pathogènes. *Annls Inst. Pasteur, Paris*, **40**, 43.
- PORTMANN, A. 1962. Action des anticorps sur la croissance d'*Escherichia coli*. *Annls Inst. Pasteur, Paris*, **103**, 141.
- PURCELL, R., CHANOCK, R. M. AND TAYLOR-ROBINSON, D. 1969. Serology of the mycoplasmas of man. In *The Mycoplasmatales and the L-phase of bacteria*, edited by L. Hayflick, New York, p. 221.
- RIGGS, S. and SHARP, J. T. 1970. Serological reactions of mycoplasmas. In *The role of mycoplasmas and L-forms of bacteria in diseases*, edited by J. T. Sharp, Springfield, Ill., p. 82.
- SENERFIT, L. B. AND JENSEN, K. E. 1966. Antimetabolic antibodies to *Mycoplasma pneumoniae* measured by tetrazolium reduction inhibition. *Proc. Soc. exp. Biol. Med.*, **122**, 786.
- WYNNE, E. S., GOTT, C. L., MEHL, D. A. AND NORMAN, J. O. 1953. Serological studies with *Vibrio metschnikovii*. *J. Immun.*, **70**, 207.
- YANG, T. J. AND VAS, S. I. 1970. Effect of antibodies on L5178Y mouse leukemia cells cultured *in vitro*. *Cancer Res.*, **30**, 1231.