INFECTION AND IMMUNITY, Jan. 1977, p. 7-12
Vol. 15, No. 1
Printed in U.S.A.
Copyright © 1977 American Society for Microbiology

Interactions Between *Mycoplasma pneumoniae* and the First Component of Complement

W. BREDT,[1]* B. WELLEK, H. BRUNNER, AND M. LOOS

Institut für Medizinische Mikrobiologie, Johannes-Gutenberg-Universität, D-6500 Mainz, and Institut für Medizinische Mikrobiologie, Justus-Liebig-Universität, D-6300 Giessen, Germany

Received for publication 10 August 1976

Mycoplasma pneumoniae cells were rounded and killed by fresh guinea pig serum (GPS) which did not contain detectable amounts of antibody. The first component of complement (C1) was bound by *M. pneumoniae* in considerable amounts from both GPS and purified C$\bar{1}$. The C1 bound by the cells was reacting with C4. Sequential addition of C1, C4, C2, and C-ethylenediaminetetraacetate to glass-grown *M. pneumoniae* cells resulted in rounding of a significant number of cells. *M. orale* and *M. fermentans* showed a reduced binding capacity for C1 as compared with *M. pneumoniae*. Both species were only slowly killed by fresh GPS, whereas *M. hominis* was as sensitive as *M. pneumoniae*. The results suggest an antibody-independent interaction between some components of the membrane surface of *M. pneumoniae* and C1, resulting in an activation of the complement system leading to the killing of the mycoplasma cells.

Living cells of *Mycoplasma pneumoniae* are able to activate guinea pig complement via the alternate pathway (4). However, this mode of activation does not fully explain the toxic effect (1, 5, 8, 16) of complement on some mycoplasmas. The ability of fresh guinea pig serum (GPS) to kill *M. pneumoniae* cells efficiently appears to depend on the intact classical pathway (4). This mode of activation of the complement sequence via the first component (C1) is in most instances triggered by antigen-antibody complexes. There are, however, some observations that indicate a possible activation of complement by direct interaction between a substance and the first component of complement. This has been demonstrated for lipopolysaccharide (9), some strains of gram-negative bacteria (B. Wellek, W. Opferkuch and M. Loos, unpublished observations), ribonucleic acid viruses (7), lymphocytes (7), and heart mitochondria (12).

In the present investigation the interaction of *M. pneumoniae* cells with the first component of complement was studied, and evidence was found for a possible direct activation of the complement sequence by the mycoplasma in the absence of demonstrable amounts of antibody.

MATERIALS AND METHODS

Mycoplasmas. *M. pneumoniae* strain FH was used throughout the study. Further experiments were carried out with *Mycoplasma orale* strain W1 *Mycoplasma hominis* strain 444 (both isolated in our laboratory), and *Mycoplasma fermentans* strains PG18 and K7 (kindly provided by J. G. Tully, National Institutes of Health, Bethesda, Md.). The mycoplasmas were grown in liquid medium as described previously (4). For some experiments the cells were grown in medium with agamma horse serum (Microbiological Associates Bethesda, Md.). For transfer experiments the cells were grown either on glass (*M. pneumoniae*) or in liquid medium (other species) and were harvested by scraping or centrifugation, respectively. The suspensions were checked for protein content by the method of Lowry et al. (11), and the optical density (OD) was measured at 660 nm.

Buffer solutions. Tris(hydroxymethyl)aminomethane-buffered saline (Tris buffer $^{2+}$), pH 7.2, was used as described previously (4). For some experiments Tris buffer without Ca^{2+} and Mg^{2+} was used, containing either 1 mM ethylenglycol- 2(2-aminoethyl)tetraacetic acid (EGTA) or 10 mM EGTA and 5 mM $MgCl_2$ (EGTA/Mg^{2+}).

Detection of rounding and killing activity. The rounding and killing effect of serum preparations on *M. pneumoniae* was tested as described previously (4). Rounding and killing tests with other *Mycoplasma* species were performed accordingly.

Complement. Pooled GPS was used as the "normal" complement source. It always contained some antibodies. Serum from individual guinea pigs was tested for antibodies with the mycoplasmocidal test (5). A serum was used as antibody-free serum ($GPSAb^-$) if no antibodies could be detected.

Preparation of GPS free of Ca^{2+} and Mg^{2+}. Ethylenediaminetetraacetate (EDTA) was added to GPS (final concentration, 0.01 M). Since EDTA showed toxic effects on the *M. penumoniae* cells (4), the

[1] Present address: Zentrum für Hygiene, Universität Freiburg, D-7800 Freiburg, Germany.

EDTA-ion complexes and remaining free EDTA were removed by chromatography on Sephadex G-25 (M. Dierich, personal communication). The resulting preparation (GPSCa$^-$Mg$^-$) showed no effects in a dilution of 1:5 on *M. pneumoniae* cells.

Complement reagents and assays. Buffers (veronal-buffered saline with EDTA [VBS] or sucrose [VBS-S]), preparation of cell intermediates (EA, EAC4, etc.), and molecular titration of C1 and C4 have been described by Rapp and Borsos (13). All complement titrations were done in the microliter modification (14), and the results are expressed as z values or effective molecules per milliliter. C$\bar{1}$ was purified by double zonal ultracentrifugation according to Colten et al. (6). The particular C$\bar{1}$ preparation used in the experiments yielded 1.95×10^{12} effective C$\bar{1}$ molecules/ml, and appropriate dilutions were made from this as described below. The C$\bar{1}$ preparation was free of immunoglobulin G (IgG) and IgM antibody as determined by immunoelectrophoresis (IgG and IgM) and double radioimmunoassay (for IgG). Functionally pure C2 and C4 from guinea pigs (C2 gp and C4 gp) was purchased from Cordis Corp., Miami, Fla.

C1 transfer test. The C1 transfer test was done according to Borsos and Rapp (2), with the following minor modifications. In the first (fixation) stage, the C1 source (either as a whole GPS dilution or in purified form) was incubated for 30 min at 0°C with the respective suspension of mycoplasmas, using siliconized glass tubes to prevent binding of C1 to the glass surface. The mycoplasmas were then transferred to fresh sets of glass tubes, washed four times, and suspended in the original volume. For the second (transfer) stage, 0.1-ml samples of serial dilutions of the mycoplasma suspension in VBS (μ = 0.15) were incubated with 0.1 ml of EAC4 in VBS for 15 min at 30°C. Subsequently, 0.1 ml of C2gp in sucrose buffer (μ = 0.04) was added, and incubation was continued for 10 min at 30°C. Finally, 1.0 ml of C-EDTA (GPS diluted 1/33 in VBS-EDTA) was added and further incubated for 60 min at 37°C, and the degree of hemolysis of the erythrocytes was determined spectrophotometrically at 412 nm. The number of effective C1 molecules was subsequently calculated. Controls included incubation of the respective C1 source in the absence of mycoplasmas followed by the same test procedure.

Sequential treatment of *M. pneumoniae* cells with C components. *M. pneumoniae* cells grown in cover slip chambers (3) were successively treated with C$\bar{1}$ (1:450 in VBS, 20 min, 0°C), C4 (1:20 in VBS, 20 min, 30°C), C2 (1:20 in VBS, 20 min, 30°C), and GPSCa$^-$Mg$^-$ (1:5 in Tris-EGTA, 30 min, 37°C). After the last step they were examined for signs of rounding (4). Controls were performed by omitting each one of the components in a separate preparation.

C4 inactivation. A 0.5-ml amount of mycoplasma suspension in VBS-S was preincubated for 30 min at 0°C with equal volumes of diluted serum or a dilution of purified C$\bar{1}$. Treated mycoplasma suspensions were then washed and transferred to fresh tubes and resuspended in 0.5 ml of VBS-S. A 0.5-ml portion of an appropriate dilution of partially purified guinea pig C4, and the remaining C4 activity was determined subsequently. Controls included: (i) buffer plus diluted C4gp; (ii) C1 source without mycoplasma (similar to C1 transfer test) plus diluted C4gp; (iii) mycoplasmas without C1 source to exclude inactivation of C4 by untreated cells.

RESULTS

Rounding activity. *M. pneumoniae* cells were rounded up by GPS and GPSAb$^-$ to the same extent (Table 1). Blocking of the classical pathway (by removing Ca^{2+}) by EGTA/Mg^{2+} resulted in a reduced rounding activity. *M. brale* cells showed signs of swelling and rounding only at a GPS dilution of 1:10. Higher dilutions were without effect. No rounding tests were performed with *M. hominis* or either strain of *M. fermentans*. The successive addition of purified complement components and GPSCa$^-$Mg$^-$ resulted in rounding of nearly half of the *M. pneumoniae* cells. The control cells remained intact (Table 2).

Killing activity. The killing of *M. pneumoniae* cells was only slightly lower by GPSAb$^-$ as compared to the normal GPS (Fig. 1). Destruction of some components of the alternate pathway (50°C for 20 min) resulted in a general reduction of the killing activity, probably due to the damage of some components of the classical pathway. However, both sera still showed nearly the same degree of killing after the heat treatment (GPS, 96%, and GPSAb$^-$, 84%, after 60 min). Other *Mycoplasma* species tested showed a variable sensitivity against the killing activity of normal GPS. *M. hominis* and, to a lesser extent, *M. fermentans* strain PG18 were highly sensitive. *M. hominis* was killed at

TABLE 1. *Rounding activity of normal and antibody-free GPS on M. pneumoniae*

Prepn tested	Titer of rounding activity
GPS	≧160
GPSAb$^-$	≧160
GPS in EGTA-Mg^{2+} [a]	80
GPSAb$^-$ in EGTA-Mg^{2+}	80

[a] Classical pathway blocked by removal of Ca^{2+}.

TABLE 2. *Rounding activity of purified components of complement*

M. pneumoniae cells treated with:		Cells rounded (%)
C components[a]	GPSCa$^-$Mg$^-$ [b]	
None	+	<5
C$\bar{1}$ + C4 + C2	+	30–40
C4 + C2	+	<5
C$\bar{1}$ + C2	+	<5
C$\bar{1}$ + C4	+	<5
C$\bar{1}$ + C4 + C2	−	<5

[a] Components were added successively.
[b] 1:5 dilution.

a rate comparable to that of *M. pneumoniae*. On the other hand, *M. orale* proved to be quite resistant, and the number of cells did not fall below approximately 60% after GPS treatment

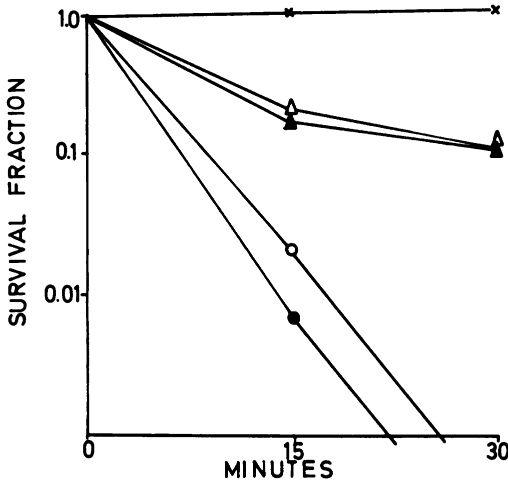

FIG. 1. *Killing of M. pneumoniae by GPS (closed symbols) and GPSAb⁻ (open symbols): ○, 1:20; △, 1:80; ×, inactivated GPS.*

(1:10, 1 h). *M. fermentans* K7 was resistant to the same degree.

Binding of C1. The results of the C1 transfer tests are summarized in Table 3. *M. pneumoniae* cells were able to bind considerable amounts of C1 from either GPS or purified C1. There was no difference between GPS and GPSAb⁻ in the number of molecules bound. C1 was bound to the same degree by *M. pneumoniae* grown in either normal horse serum or in serum with a reduced gamma globulin content. *M. hominis* reacted with C1 to the same extent as, or better than, *M. pneumoniae*. *M. orale* showed lower binding reactivity. The lowest binding values were obtained with *M. fermentans* and within this species, with strain K7. From the purified C$\bar{1}$ the lowest binding per milligram of mycoplasma protein occurred with *M. orale* and both strains of *M. fermentans*. The low binding capacities of *M. fermentans* PG18 and *M. orale* were even more obvious if the number of molecules bound from purified C$\bar{1}$ was related to the optical density of the mycoplasma suspension. With 0.06×10^{10} or 0.07×10^{10} molecules/1.0 OD unit, both species were considerably less active than *M. pneumo-*

TABLE 3. *Binding of C1 by some mycoplasma species*

Mycoplasmas tested	Protein (mg/ml)	OD (660 nm)	Source of C1[a]	Amt of C1 bound[b] (molecules × 10^{10})	Ratio C1/protein[c]	Ratio C1/OD[d]
M. pneumoniae[e]	1.57	1.7	GPS[f]	16.0	10.2	9.41
			GPSAb⁻[f]	15.0	9.6	8.8
			C$\bar{1}$uz	0.44	0.28	0.26
M. pneumoniae						
Horse serum	0.73	1.01	GPS	2.1	2.88	2.08
			C$\bar{1}$uz	0.7	0.96	0.69
Agamma serum	0.78	1.01	GPS	2.0	2.56	1.98
			C$\bar{1}$uz	0.7	0.90	0.69
M. pneumoniae	1.49	1.45	GPS	4.0	2.68	2.76
			C$\bar{1}$uz	0.6	0.40	0.41
M. orale	0.73	1.45	GPS	1.0	1.37	0.69
			C$\bar{1}$uz	0.1	0.14	0.07
M. pneumoniae	1.09	1.45	GPS	6.4	5.87	4.41
			C$\bar{1}$uz	0.65	0.60	0.45
M. fermentans PG18	0.67	1.45	GPS	0.28	0.42	0.19
			C$\bar{1}$uz	0.08	0.12	0.06
M. fermentans K7	1.87		GPS	0.11	0.06	
			C$\bar{1}$uz	0.017	0.01	
M. hominis	0.45		GPS	8.0	17.78	
			C$\bar{1}$uz	0.4	0.89	

[a] GPS was used 1:15 in VBS if not otherwise noted. (C$\bar{1}$uz) purified by ultracentrifugation was given in the amount of 1.3×10^{11} molecules/ml.
[b] Measured by transfer test.
[c] Molecules (× 10^{10}) per milligram of protein.
[d] Molecules (× 10^{10}) per 1.0 OD unit.
[e] All mycoplasmas were grown in normal inactivated horse serum, if not noted otherwise.
[f] 1:5 dilution.

niae (0.26×10^{10} to 0.69×10^{10}). Pretreatment by antiserum of *M. pneumoniae* and *M. fermentans* PG18 did not significantly increase the amount of C1 bound by the cells.

An additional experiment was performed to find out whether the C1 molecules bound by the mycoplasma surface were also activated. *M. pneumoniae* or *M. hominis* cells, which had been exposed to either GPS or $C\bar{1}$, were incubated with a dilution of purified C4gp. The results (Table 4) show that C4 was only inactivated by mycoplasmas that had been exposed previously to GPS or purified $C\bar{1}$.

DISCUSSION

The C1-binding capacity of mycoplasma cells cannot be quantitated as exactly as with erythrocytes or other large particles. The minute size of the organisms (a mycoplasma with, for example, a 0.8-μm diameter has only 1/300 of the volume of an erythrocyte) almost rules out the use of defined single-cell suspensions in experiments that require repeated washings. Therefore, aggregated cells had to be used and it was necessary to find other parameters for quantitation of the mycoplasmas. Protein content and OD were measured. Both methods can provide only approximate data about the number of particles and the size of their exposed surface area. It turned out that suspensions of different *Mycoplasma* species adjusted to equal ODs had a different protein content. The reasons for this phenomenon are possible differences in the particle size of the suspension or in the protein content of the individual cells. For comparison of the C1-binding values of different species the protein content was chosen as the more reliable parameter.

In several separate experiments C1 was bound by *M. pneumoniae* in considerable amounts. Several observations provide evidence for a direct interaction between the mycoplasma membrane and C1. (i) The globulin content of the growth medium did not influence the binding. Therefore, gamma globulins aggregated on the membrane surface seem not to play a major role in the reaction. (ii) Rounding, killing, and binding of C1 from GPS are not reduced in the absence of detectable amounts of antibodies. On the other hand, the C1-binding capacity is not significantly increased by the addition of antiserum. (iii) Purified C1 is bound in considerable amounts. (iv) Serum-C1 bound to mycoplasmas is enzymatically active; i.e., it inactivates fluid-phase C4.

These data confirm and extend those of previous reports, in which the toxic effect of fresh serum could not be absorbed by *M. pneumoniae cells* (5, 8). They suggest that binding and activation of C1 can occur in the absence of specific antibodies.

The reaction of *M. hominis* was similar to that of *M. pneumoniae*. The intense binding of C1 from GPS as compared with purified $C\bar{1}$ suggests, however, that antibodies may have been present in the GPS used in the experiments.

The two other species tested (*M. fermentans* and *M. orale*) showed a reduced binding capacity for C1 from both GPS and the purified $C\bar{1}$ preparation. This is accompanied by the low killing effect of GPS on these mycoplasmas. Only *M. fermentans* PG18 showed a higher sensitivity, which cannot fully be explained by our data. Even so, the killing rate did not reach that of *M. pneumoniae* or *M. hominis*. The

TABLE 4. *Inactivation of purified C4gp by M. pneumoniae and M. hominis preincubated with GPS or purified $C\bar{1}$*

Mycoplasma sp.	Pretreated with:[a]	Remaining activity of C4 (molecules/ml)[b]	C4 inactivation per mg of protein (molecules/ml)
M. pneumoniae[c]	GPS (1:45)	1.01×10^{8}	2.90×10^{10}
M. hominis[d]	GPS (1:25)	1.80×10^{8}	3.44×10^{10}
M. pneumoniae	$C\bar{1}$uz (0.65×10^{11})[e]	2.36×10^{8}	2.80×10^{10}
M. hominis	$C\bar{1}$uz (1.3×10^{11})[e]	0.59×10^{8}	3.45×10^{10}
M. pneumoniae	Buffer	3.75×10^{9}	
M. hominis	Buffer	3.80×10^{10}	
Buffer (*M. hominis* experiment)	Buffer	3.66×10^{10}	

[a] 30 min at 0°C.
[b] Hemolytic activity. Incubation of mycoplasma-C1 preparation with C4 was for 30 min at 30°C.
[c] 0.125 mg of protein per ml.
[d] 1.06 mg of protein per ml.
[e] $C\bar{1}$ purified by ultracentrifugation ($C\bar{1}$uz), molecules per milliliter.

tendency of *M. fermentans* to form larger clusters may have affected the results. Nevertheless, the data provide some evidence that the membrane surface of *M. fermentans* and *M. orale* does not bind and activate C1 as effectively as *M. pneumoniae* in the absence of homologous antibody.

The membrane components involved in the binding and the subsequent activation of C1 have not yet been defined. According to earlier reports on other C1-activating agents, they could be proteases (10) or lipopolysaccharide fractions (9). Experiments with membrane fractions of *M. pneumoniae* suggest an involvement of the lipid fraction in the activation of the classical pathway, whereas the alternative pathway appeared to be initiated by the protein fraction (W. Bredt and H. Brunner, unpublished observations). However, the presence of antibodies could not be totally ruled out in these experiments. It is not known whether the more resistant species do not possess the activating structures or merely have them covered by an inert substance. The difference in exposure of polysaccharide groups on the membrane surface (15) may play a role in the phenomenon. A change of surface properties by repeated subcultures comparable with the rough forms of bacteria cannot be excluded. However, the "toxic" effects of GPS were described for several *M. pneumoniae* strains, and the use of the low-passage strain Pl 1428 (5) suggests that complement sensitivity is a constant property of *M. pneumoniae*.

The direct activation of complement by membrane substances or structures, however, seems to be less efficient than the antibody-mediated reaction. The killing capacity of GPS is greatly reduced by a dilution of 1:80; however, in the presence of antiserum this dilution is sufficient for effective killing in the mycoplasmocidal test (5). This suggests that either antibodies can activate the complement sequence more efficiently or that the binding of antibodies alters the membrane structure, resulting in increased complement sensitivity of the cells. Neither possibility can be ruled out by the data presently available.

The direct interaction of *M. pneumoniae* with C1 has the same and even more detrimental biological consequences for the microorganisms as the activation of the alternate pathway (4). In the respiratory tract the organisms can probably escape from the interaction with complement under certain circumstances. However, such an interaction is unavoidable beyond the epithelial barrier, and the resulting reaction probably inhibits the possible invasion of *M. pneumoniae* in the blood stream and other tissues even if no antibodies are present. Additionally, the reaction may be involved in the pathogenesis of the disease (4).

The data obtained in this study and from the literature suggest that some microorganisms interact directly with the complement system. This reaction apparently is of some importance for both the development of early lesions in the host and the early defense against the invading microorganism.

ACKNOWLEDGMENTS

This study was supported by the Deutsche Forschungsgemeinschaft (Br 296/8, Br 346/5, SFB 107).

The skillful technical assistance of Dagmar Donarski and Christel Berger is gratefully acknowledged. We are indebted to A. Morell, Institut für Klinisch-experimentelle Tumorforschung, Bern, Switzerland, for performing the double radioimmunoassay with the purified C1 preparation.

LITERATURE CITED

1. **Barker, L. F., and J. K. Patt.** 1967. Role of complement in immune inactivation of *Mycoplasma gallisepticum*. J. Bacteriol. 94:403–408.
2. **Borsos, T., and H. J. Rapp.** 1965. Hemolysin titration based on fixation of the activated first component of complement: evidence that one molecule of hemolysin suffices to sensitize an erythrocyte. J. Immunol. 95:559–566.
3. **Bredt, W.** 1968. Growth morphology of *Mycoplasma pneumoniae* strain FH on glass surface. Proc. Soc. Exp. Biol. Med. 128:338–340.
4. **Bredt, W., and D. Bitter-Suermann.** 1975. Interactions between *Mycoplasma pneumoniae* and guinea pig complement. Infect. Immun. 11:497–504.
5. **Brunner, H., W. James, R. L. Horswood, and R. M. Chanock.** 1972. Measurement of *Mycoplasma pneumoniae* mycoplasmacidal antibody in human serum. J. Immunol. 108:1491–1498.
6. **Colten, H. R., H. E. Bond, T. Borsos, and H. J. Rapp.** 1969. Purification of the first component of complement by zonal ultracentrifugation. J. Immunol. 104:862–865.
7. **Cooper, N. R.** 1973. Activation of the complement system. Top. Mol. Immunol. 2:155–183.
8. **Gale, J. L., and G. E. Kenny.** 1970. Complement dependent killing of *Mycoplasma pneumoniae* by antibody: kinetics of the reaction. J. Immunol. 104:1175–1183.
9. **Loos, M., D. Bitter-Suermann, and M. Dierich.** 1974. Interaction of the first ($C\bar{1}$), the second (C2) and the fourth (C4) component of complement with different preparations of bacterial lipopolysaccharides and with lipid A. J. Immunol. 112:935–940.
10. **Loos, M., T. Borsos, and H. J. Rapp.** 1973. The first component of complement in serum: evidence for a hitherto unrecognized factor in C1 necessary for internal activation. J. Immunol. 110:205–212.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
12. **Pinckard, R. N., M. S. Olson, R. E. Kelley, D. H. De Heer, J. D. Palmer, R. A. Rourke, and S. Goldfein.** 1973. Antibody-independent activation of human C1 after interaction with heart subcellular membranes. J. Immunol. 110:1376–1382.

13. **Rapp, H. J., and T. Borsos.** 1970. Molecular basis of complement action. Appleton-Century Crofts, New York.
14. **Ringelmann, R., W. Opferkuch, M. Röllinghoff, and M. Loos.** 1969. Komplementmessungen mit Hilfe des Mikrolitersystems. Z. Med. Mikrobiol. Immunol. 154:329–343.
15. **Schiefer, H. G., U. Gerhardt, H. Brunner, and M. Krüpe.** 1974. Studies with lectins on the surface carbohydrate structures of mycoplasma membranes. J. Bacteriol. 120:81–88.
16. **Sethi, K. K., and M. Teschner.** 1969. A study on the role of complement in the immune inhibition of mycoplasma growth. Z. Immunitaetsforsch. 138:458–474.