Nature of the serum heat-labile accessory factor involved in the metabolic inhibition of Mycoplasma pneumoniae

L. H. COLEMAN, R. J. LYNN AND R. A. PATRICK

Bio-Rad Laboratories, Richmond, California and Department of Microbiology, School of Medicine, University of South Dakota, Vermillion, South Dakota 57069, U.S.A.

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Using the metabolic-inhibition (MI) test as an assay, sera from rabbits immunized with *Mycoplasma pneumoniae* were separated on the basis of a requirement for a heat-labile (56 C) accessory factor (HLAF). As in previous studies, this requirement was found in sera collected early in the immune response, but disappeared as the immunization procedure progressed. Results obtained following exposure of HLAF-requiring sera to zymosan, hydrazine, and ethylenediaminetetraacetic acid (EDTA) suggested that complement components C3, C8, and possible other components which follow C3 in the complement cascade, were required to demonstrate MI activity. Results obtained when HLAF-non-requiring sera were treated in the same manner, suggested the presence of two antibody populations. One population required component C3, and possibly C5, C6, and C9, while the other population required none of the complement components.

INTRODUCTION

In previous reports from this laboratory, immune inhibition of *Mycoplasma pneumoniae* by rabbit serum collected early in the immune response required the presence of a heat-labile accessory factor (HLAF) (Coleman and Lynn, 1972*a, b*). Other investigators have also reported this requirement and have suggested the factor involved is complement (Gale and Kenny, 1970; Fernald, Clyde and Denny, 1967*a, b*). The preliminary procedure for establishing complement-mediated immune inhibition of mycoplasma is to heat-inactivate the immune serum at 56 C for 30 min, then add a heterologous source of complement, usually normal guinea pig serum. Similarly, investigators of the specific complement components involved have treated the heterologous source with

various agents such as zymosan, hydrazine, ethylenediaminetetra-acetic acid (EDTA), aggregated gamma globulin, or bentonite (Gale and Kenny, 1970; Riggs, Sharp and Carpenter, 1967).

Several objections to this procedure exist. Firstly, evaluation of results obtained by the above procedure require the assumption that there is reciprocity between the components of the heterologous source and the immune serum. Second, assuming reciprocity, heat inactivation destroys only components C1, C2, and C8. Addition of the heterologous source adds an excess of the other components.

It is the purpose of this study to better elucidate the role of complement in metabolic inhibition of *M. pneumoniae* by both early and hyperimmune sera.

MATERIALS AND METHODS

Mycoplasma. The F. H. strain of *Mycoplasma pneumoniae* was used in this study, and grown as described previously (Coleman and Lynn, 1972*a*, *b*).

Metabolic inhibition (MI) test. The MI test was performed as described elsewhere (Coleman and Lynn, 1972*a*, *b*). Heat lability was determined by exposing sera to a temperature of 56 C for 1 hour prior to use in the MI test.

Immunization procedure. The immunization procedure was the same used in previous studies (Coleman and Lynn, 1972*a*, *b*).

Sensitized sheep erythrocytes. Sheep (lamb) red blood cells suspended in Alsever's solution were obtained from the Colorado Serum Company. The red blood cells were sensitized according to the method of Lepow et al. (1963).

Buffers. Triethanolamine buffered saline (TBS) was used at pH 7.4, ionic strength 0.15, and contained a final concentration of 1.5×10^{-4} M Ca^{++} and 5×10^{-4} M Mg^{++}. Veronal-buffered saline (VBS) was used at pH 7.4 and contained a final concentration of 0.15 M NaCl, 3.2×10^{-3} M, 5,5-diethylbarbituric acid and 2.9×10^{-3} M Na 5,5-diethylbarbiturate.

Treatment of immune sera with hydrazine hydrate. The third and fourth components of guinea pig and human complement are sensitive to the action of hydrazine (Müller-Eberhard, 1968). The technique utilized was a modification of the technique of Klein and Wellensiek (1965). A 0.1 M hydrazine hydrate solution was prepared in TBS. Increasing 0.05-ml volumes of the hydrazine solution were added to successive 0.5-ml aliquots of immune rabbit serum. Enough 0.15 M saline was then added to make a final volume of 1.0 ml. Tubes were incubated at 37 C for 60 min. At the end of the incubation period, the pH of each tube was adjusted to approximately 7.4 with 0.1 N HCl and volumes equalized with 0.15 M saline. Hemolytic activity was examined by

mixing 0.75 ml of sensitized sheep erythrocytes (in TBS), 1.75 ml phosphate buffer, pH 7.4, and 0.5 ml of the treated serum samples. Cells were incubated at 37 C for 60 min, centrifuged, and the degree of hemolysis checked by reading the supernatant optical density at 541 nm. The remainder of the treated serum samples were filter-sterilized, then assayed for metabolic inhibitory activity.

Treatment of immune sera with zymosan. Complement components C3 and possibly C5, of guinea pig and human serum are adsorbed by zymosan (Müller-Eberhard, 1968). Zymosan was prepared according to the method of Mardiney and Müller-Eberhard (1965). Increasing 0.05-ml volumes of the zymosan suspension are added to 0.5-ml aliquots of immune rabbit sera. Volumes were adjusted to 2.0 ml with 0.15 M saline. The reaction mixtures were incubated with shaking for 60 min at 37 C. The hemolytic assay was performed by reacting 0.5 ml of sensitized erythrocytes (in VBS), 0.2 ml treated serum, and 0.8 ml VBS. Following incubation at 37 C for 60 min, the reaction mixtures were centrifuged and the optical density of the supernatant measured at 541 nm. The remainder of the treated serum samples were filter-sterilized, then assayed for metabolic inhibitory activity.

Treatment of immune sera with sodium ethylenediaminetetraacetic acid (EDTA). Addition of EDTA removes the Ca^{++} required for formation of the Clqrs complex and the Mg^{++} required for formation of the $\overline{C42}$ complex (Müller-Eberhard, 1968). Three-tenths ml of immune serum was reacted with an equal volume of increasing concentrations of Na_3EDTA in VBS. The pH was adjusted to 7.4 with 5 N NaOH. The reaction mixture was incubated at room temperature for one hr. Hemolytic assays were performed by mixing 0.2 ml EDTA-treated serum, 0.5 ml sensitized erythrocytes, and 0.8 ml VBS without metals. The sensitized erythrocytes had previously been washed several times in VBS without metals to remove Ca^{++} and Mg^{++}. Incubation was at 37 C for 1 hr. Tubes were centrifuged and the optical density of the supernatant read at 541 nm. The remainder of the treated serum samples were filter-sterilized, then assayed for metabolic inhibitory activity. In order to see if Ca^{++} and Mg^{++} could be supplied to EDTA-treated sera by the MI growth media, equal volumes of treated serum and media were mixed together and reacted with sensitized erythrocytes. Following incubation at 37 C for 1 hr, cells were centrifuged, the supernatant discarded, and the remaining cells lysed with distilled water. The released hemoglobin was read at 541 nm following centrifugation.

Preimmune serum was included as a control in all of the above treatments to see if any of the reagents inhibited growth of *M. pneumoniae.*

RESULTS

The results of hydrazine treatment of HLAF-requiring and HLAF-non-requiring sera are presented in Table 1. With the HLAF-requiring sera, reduction of MI titer parallels the reduction in hemolytic activity. The HLAF-non-requiring sera, on the other hand, show a four-fold reduction in titer at 0.05 M hydrazine, but retain a significant level of MI activity even though the hemolytic activity has been destroyed.

Table 1. Effect of hydrazine treatment on MI and hemolytic activity of immune sera

Hydrazine concentration	HLAF-requiring sera[1]		HLAF-non-requiring sera[2]	
	Hemolytic O.D.[3]$_{541\ nm}$	MI titer[4]	Hemolytic O.D.$_{541\ nm}$	MI titer
0.000 M	1.680	160	2.000	320
0.005 M	0.053	80	0.030	160
0.010 M	0.050	40	0.023	160
0.015 M	0.041	<40	0.026	160
0.020 M	0.035	<40	0.032	160
0.025 M	0.037	<40	0.023	80
0.030 M	0.035	<40	0.024	80
0.035 M	0.033	<40	0.030	80
0.040 M	0 035	<40	0.029	80
0.045 M	0.035	<40	0.027	80
0.050 M	0.038	<40	0.028	80

1. Heat-labile accessory-factor-requiring sera collected 9 days after initial inoculation.
2. Heat-labile accessory-factor-non-requiring sera collected 252 days after initial inoculation.
3. Optical density.
4. Metabolic inhibition titer.

Similarly, reduction of MI activity in HLAF-requiring sera, following treatment with 0.75 mg/ml zymosan, parallels reduction in hemolytic activity (Table 2). Although there is an eight-fold reduction of MI titer in the HLAF-non-requiring sera, MI activity remains even after treatment with 1.5 mg/ml of zymosan.

EDTA treatment of HLAF requiring and non-requiring sera had no effect on MI activity although hemolytic activity was destroyed (Table 3). Hemolytic activity was not restored by exposing sera to the MI growth media.

MI and hemolytic activity could be restored to treated sera by the addition of either normal rabbit or normal guinea pig sera. Treatment of normal rabbit sera with the three reagents did not create any inhibitory activity.

Table 2. Effect of zymosan treatment on MI and hemolytic activity of immune sera

Zymosan concentration	HLAF-requiring sera[1]		HLAF-non-requiring sera[2]	
	Hemolytic O.D.[3]$_{541\,nm}$	MI titer[4]	Hemolytic O.D.$_{541\,nm}$	MI titer
0.00 mg/ml	1.680	160	2.000	320
0.25 mg/ml	0.890	160	0.054	160
0.50 mg/ml	0.465	160	0.043	160
0.75 mg/ml	0.070	<40	0.048	320
1.00 mg/ml	0.065	<40	0.044	80
1.25 mg/ml	0.049	<40	0.043	40
1.50 mg/ml	0.047	<40	0.043	40

1. Heat-labile accessory-factor-requiring sera collected 9 days after initial inoculation.
2. Heat-labile accessory-factor-non-requiring sera collected 252 days after initial inoculation,
3. Optical density.
4. Metabolic inhibition titer.

Table 3. Effect of EDTA treatment of MI and hemolytic activity of immune sera

EDTA concentration	HLAF-requiring sera[1]		HLAF-non-requiring sera[2]	
	Hemolytic O.D.[3]$_{541\,nm}$	MI titer[4]	Hemolytic O.D.$_{541\,nm}$	MI titer
Untreated	1.300	320	1.300	320
5×10^{-5} M	1.230	320	1.300	320
1×10^{-3} M	1.170	320	1.200	320
5×10^{-3} M	0.097	320	0.100	320
1×10^{-2} M	0.057	320	0.047	320

1. Heat-labile accessory-factor-requiring sera collected 9 days after initial inoculation.
2. Heat-labile accessory-factor-non-requiring sera collected 252 days after initial inoculation.
3. Optical density.
4. Metabolic inhibition titer.

DISCUSSION

Since most investigations have involved the use of a heterologous source of complement, the role of homologous complement in immune inhibition of *M. pneumoniae* was largely undefined. There are numerous reports in the literature on the danger of using complement from a heterologous source (Cushing, 1945; Muir, 1912). With rabbit antibody, immune adherence activity can actually be increased by the addition of heated guinea pig serum (Turk, 1959). On the other hand, sensitized sheep erythrocytes, exposed to heated rabbit complement, became resistant to lysis by guinea pig complement (Kempf, Gigli and Austen, 1969).

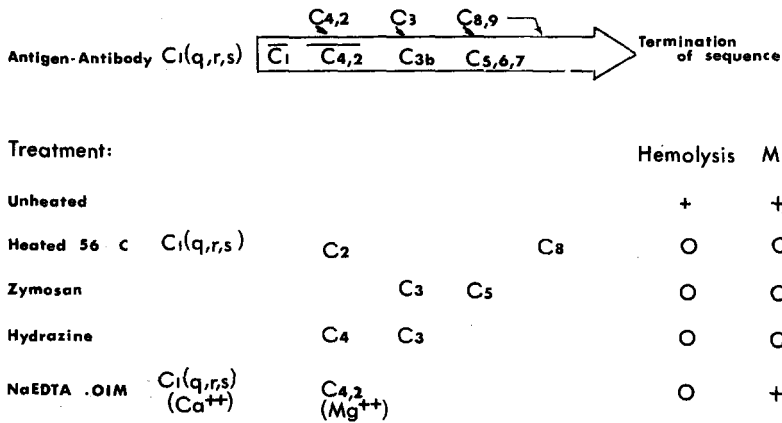Fig. 1 depicts complement components that are inactivated by the various

$\overset{C_{4,2}}{}$ $\overset{C_3}{}$ $\overset{C_{8,9}}{}$

Antigen-Antibody $C_1(q,r,s)$ | $\overline{C_1}$ $\overline{C_{4,2}}$ C_{3b} $\overline{C_{5,6,7}}$ ⟶ Termination of sequence

Treatment:					Hemolysis	MI
Unheated					+	+
Heated 56 C	$C_1(q,r,s)$	C_2		C_8	O	O
Zymosan			C_3 C_5		O	O
Hydrazine		C_4 C_3			O	O
Na₃EDTA .01M	$C_1(q,r,s)$ (Ca^{++})	$C_{4,2}$ (Mg^{++})			O	+

Fig. 1. Inactivation of complement components and its effect on hemolytic and MI activity in early immune serum.

treatments. These results have been established in guinea pig and human serum but not with rabbit serum (Müller-Eberhard, 1968). On the right hand side of the figure are the effects of the various treatments on hemolytic and MI activity of the HLAF-requiring sera. Unheated, untreated sera possess both hemolytic and MI activity. Heat inactivation at 56 C, which affects components C1, C2, and C8, destroys both hemolytic and MI activity. Treatment with zymosan, which binds components C3 and possibly C5, removes both hemolytic and MI activity. Treatment with hydrazine, which knocks out components C4 and C3, likewise destroys both hemolytic and MI activity. On the other hand, treatment with Na₃EDTA, which prevents formation of the C1qrs and $\overline{C_{4,2}}$ complexes, reduces hemolytic activity, but does not affect the MI titer. The data suggest that C3, C8 and possibly other components which follow C3 in the complement cascade are required for MI activity in HLAF requiring sera, but not components C1, C4, and C2.

The data obtained with sera which are HLAF-non-requiring indicate a heterogeneous population of antibody activities. While heat inactivation and treatment with Na₃EDTA have no demonstrable effect on MI activity, there is a partial reduction following treatment with zymosan and hydrazine. The data suggest that a population of antibodies exists which requires component C3, and possibly C5, C6, C7, and C9, while another population exists in the HLAF-non-requiring sera that requires none of the complement components.

Involvement of only certain of the nine components of complement has been established in various immune phenomena such as immune adherence and immunoconglutination (Barrett, 1970). In addition, the data support our obser-

vation that although there is a requirement for a heat-labile accessory factor in sera collected early in the immune response, no lytic activity, as measured by release of nucleic acids, is demonstrable (Coleman and Lynn, 1972a). If lytic activity analogous to immune hemolysis were present, then it would be expected that all nine components of complement would be involved. Our data indicate that this is not the case in the immune inhibition of *M. pneumoniae* by HLAF-requiring immune rabbit sera.

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