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Cold Hemagglutinin Cross-Reactivity with *Mycoplasma pneumoniae*

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Convalescent sera from proven cases of infection with *Mycoplasma pneumoniae*, and rabbit antisera to *M. pneumoniae* and to human erythrocyte glycoprotein contained cold hemagglutinins which were reactive only for human erythrocytes. Only the human serum cold agglutinins were inhibited by soluble integral glycoproteins derived from human erythrocyte ghosts by treatment with chloroform-methanol. Rabbit antiserum to chloroform-methanol glycoprotein, as well as to *M. pneumoniae*, fixed complement with either *M. pneumoniae* or chloroform-methanol glycoprotein antigens. The findings support the hypothesis that the cold agglutinins elicited by *M. pneumoniae* infection represent a cross-reaction between determinants common to erythrocyte glycoprotein containing I antigen and the membrane of *M. pneumoniae*.

Cold agglutinins (CA) which appear in the serum during the course of human infection with *Mycoplasma pneumoniae* have specificity for the I antigen, the determinant(s) of which involve *N*-acetylneuraminic acid (6, 20). The I specificity is detectable in glycoproteins solubilized from erythrocyte membranes by any of several procedures (15) and in various body fluids. These soluble I-like substances display considerable heterogeneity as judged by immunochemical analysis with anti-I sera from patients with the chronic "cold agglutinin syndrome" unrelated to infection with *M. pneumoniae*. The mechanism by which CA are elicited by *M. pneumoniae* has not yet been clearly defined. We present further evidence which suggests that early (immunoglobulin M [IgM]) CA antibody evoked by *M. pneumoniae* is cross-reactive with native erythrocyte antigens which share determinants with *M. pneumoniae*.

MATERIALS AND METHODS

M. pneumoniae. The FH strain of *M. pneumoniae* was obtained through the courtesy of William Mogabgab, Tulane University School of Medicine. It had been isolated in embryonated eggs from the sputum of a patient with CA-positive pneumonia (18) and was subsequently maintained in broth and agar media as described by Chanock et al. (2). The organism was grown in bulk by the method of Somerson et al. (23) in 5-liter Povitsky bottles containing 300 ml of broth. After inoculation, bottles were incubated at 37°C until a confluent sheet of colonies had attached to the surface, usually within a period of 3 to 5 days, during which the pH of the medium dropped to ca. 6.5. The broth was decanted and discarded. The sheet of organisms adherent to the glass was washed three times with phosphate-buffered saline, pH 7.2 and scraped into phosphate-buffered saline with a rubber policeman. The organisms were washed three times more in phosphate-buffered saline by alternate centrifugation at 800 × *g* for 30 min and resuspension, and finally resuspended to the desired concentration and stored at −70°C until used. Colony counts of *M. pneumoniae* were done by the method of Kim et al. (14).

Antigens for complement fixation (CF) tests. Water-soluble glycoproteins were obtained by treatment of human erythrocyte ghosts with chloroform-methanol (CM) (9). The product of this procedure contained the major glycoprotein constituents of the membrane, including glycophorin (19). Antigen was prepared from *M. pneumoniae* by treatment of concentrated organisms with lithium diiodosalicylate (LIS)-phenol (11, 19). A 1-ml amount of suspension containing 20 mg of protein was stirred for 15 min at room temperature with 1 ml of 0.3 M LIS in 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 7.5. Two volumes of cold deionized water were added, stirred for 10 min at 4°C, and centrifuged at 80,000 × *g* for 1 h at 4°C. An equal volume of 50% phenol was added to the supernatant, the mixture was stirred 10 min at 4°C, and then centrifuged for 1 h at 4°C at 800 × *g*. The aqueous phase was removed, and the phenol phase was reextracted with 5 ml of cold water. The two aqueous phases were combined, dialyzed against deionized water in the cold for 48 h, and lyophilized.

Antisera. Antisera to *M. pneumoniae* were prepared in rabbits. In one group (1 to 3), each animal received four weekly toe pad injections of mycoplasmal suspension in complete Freund adjuvant (Difco) (500 μg of mycoplasmal protein) followed by an intramuscular injection of 0.25 ml (148 μg of protein) of suspension in adjuvant. A second group (no. 4, 8, and 19) received the same amount of mycoplasmal protein in phosphate-buffered saline without Freund adjuvant by the same routes and schedule as the first group. A third group of five rabbits (no. 20 and 22-25) each

received a total of 240 μg of mycoplasmal protein intravenously. All rabbits were bled out 1 week after the final injection. Serum was separated by centrifugation of clots at 25°C.

Antisera were prepared in rabbits to glycoprotein derived from erythrocyte ghosts treated with CM (CM glycoprotein). Soluble antigen was coupled with methylated bovine serum albumin and incorporated in complete Freund adjuvant to a final concentration of 1 mg/ml. Each animal received a total of 1.2 mg of antigen, partitioned among four weekly injections. Animals were bled out 10 days after the last injection (sera 26 and 27).

Paired sera (generously provided, along with confirmatory bacteriological and serological data, by J. A. Davis, U.S. Naval Medical Research Laboratory, Camp LeJeune, N.C.) were obtained from cases of mycoplasmal respiratory infection in which the diagnosis had been established by primary isolation of *M. pneumoniae* from the sputum and by a concomitant fourfold or greater rise in CF antibody to *M. pneumoniae*. Both acute and convalescent samples were examined for CA. From a pool of 10 sera with the highest CA titers, IgM was isolated and purified by absorption and elution with group O erythrocytes. Further purification was achieved by chromatography of eluted antibody on Sephadex G200. The column-purified IgM gave a single line of precipitation with goat antiserum to whole human serum. IgM was similarly purified from the serum of one rabbit (no. 24) showing a rise in antibody to *M. pneumoniae* as well as in Ca after intravenous immunization with *M. pneumoniae*. Anti-I from a single donor with chronic CA syndrome was obtained from Hyland Laboratories (lot no. 0344CO02AC).

Analysis of antisera. Rabbit antisera to *M. pneumoniae* were assayed by metabolic inhibition (MI) (24), microtiter CF (22), counterimmunoelectrophoresis (8), and titration of CA (3). For the latter, erythrocytes of group O from the same donor were used throughout. For assays of CA inhibition, twofold serial dilutions of substances to be tested were prepared in 0.2 ml of phosphate-buffered saline, and 0.1 ml of serum at the highest dilution giving 4+ agglutination was added. After holding the assays for 2 h in an ice bath, 0.05 ml of a 2% suspension of erythrocytes was added. After 2 more h in the cold, agglutination was read and graded 1 to 4+ macroscopically after centrifugation of the tubes for 15 s in an Adams Serofuge (Clay Adams, Inc., N.Y.). The highest dilution of inhibitor completely blocking agglutination was taken as the end point.

RESULTS

Characterization of rabbit antisera to *M. pneumoniae*. Table 1 shows the results of immunization of rabbits with *M. pneumoniae* by different routes and schedules, with or without complete Freund adjuvant. There was no significant difference, with respect to serum titers determined by MI or CF, between the animals immunized with adjuvant and those immunized without adjuvant.

The MI titers were consistently lower than the CF titers. However, all preimmunization sera

TABLE 1. *Analysis of rabbit antisera to M. pneumoniae*

Rabbit no.[a]	Route of immunization	Metabolic inhibition[b]	CF[c]		Cold agglutination human O cells[d]	CIE with *M. pneumoniae* antigen[e]
			Whole *M. pneumoniae* antigen[f]	Soluble *M. pneumoniae* antigen[g]		
1	Toepads with complete Freund adjuvant	3	512	ND	<2	+
2		4	512	ND	4	+
3		32	1,024	512	16	+
4	Toepads without adjuvant	32	256	ND	8	+
8		32	512	ND	4	+
19		2	128	ND	<2	−
20	Intravenous	<2	64	64	<2	−
22		8	128	128	16	−
23		8	128	128	128	−
24		<2	64	32	256	−
25		4	256	64	64	−
24 IgM		ND	16	8	8	ND

[a] Preimmunization sera negative by all tests.
[b] Highest dilution of serum inhibiting drop in pH of standard cultures incubated at 37°C for 3 to 5 days.
[c] Microtiter technique, overnight fixation at 4°C with 2 U of complement.
[d] Highest dilution of serum resulting in four plus agglutination of single donor group O cells at 4°C.
[e] Antigen-mycoplasmal suspension in water frozen and thawed five times. Sera absorbed with normal horse serum. +, Visible precipitate; −, no visible precipitate; CIE, counterimmunoelectrophoresis.
[f] Suspension of washed *M. pneumoniae*.
[g] LIS-extracted *M. pneumoniae* (11).
ND, Not done.

were completely negative by both tests, and, in general, those post-immunization sera with higher CF titers tended to give the highest MI titers. The overall differences between the two sets of results may perhaps be ascribed to the greater sensitivity of the CF test. Rabbits injected via the toe pads developed precipitating antibody to horse serum, a constituent of the growth medium, as well as precipitins to *M. pneumoniae*. Antibodies to horse serum were absorbed out with lyophilized horse serum (10 mg/ml), leaving undiminished the specific anti-mycoplasmal precipitins detected by counter-immunoelectrophoresis. The LIS-phenol fraction of *M. pneumoniae* fixed complement with rabbit serum no. 3 and with serum from all rabbits immunized intravenously with *M. pneumoniae*.

Characterization of human CA and rabbit antisera to erythrocyte CM glycoprotein. CA in individual human serum samples was significantly inhibited by CM glycoprotein (Table 2). The IgM purified from a pool of 10 of these CA positive sera fixed complement with *M. pneumoniae* but was not tested for CF with CM. The latter antigen, however, did not fix complement with the whole sera (no. 581 and 699) before fractionation. In response to immunization with CM glycoprotein, rabbits showed a specific increase in CF titer to homologous erythrocyte antigen and in one instance (serum 27) to *M. pneumoniae*.

DISCUSSION

The CA produced in response to natural human infection with *M. pneumoniae*, as well as the antibody found in the cold agglutinin syndrome, have specificity for the blood group I antigen present on human erythrocytes (5, 7) and contained in glycoproteins solubilized from erythrocyte membranes by any of several methods (6, 9, 20, 21). Of these methods, CM extraction of ghosts yielded the most active glycoproteins, which we have therefore examined for reactivity with human CA and the antibody elicited in rabbits by immunization with mycoplasmal antigen(s) and CM glycoprotein. The results reported here are consistent with the hypothesis that the CA which arises during *M. pneumoniae* infection and which is inhibited by erythrocyte glycoprotein (Table 2) is a cross-reactive response to mycoplasmal antigen(s), as already proposed by others (4, 17). The exact identity of the responsible antigenic determinants remains to be elucidated, however. Although glycolipids are generally acknowledged to be the major type-specific antigenic constituent of *M. pneumoniae*, at least one glycoprotein has also been identified in delipidated mycoplasmal membranes (11). Following this lead, we used the LIS-phenol extraction procedure to obtain the soluble mycoplasmal antigen which we found fixed complement with rabbit antibody to *M. pneumoniae* (Table 1).

TABLE 2. *Analysis of cross-reactive sera*

Serum	CF with:		CM glycoprotein[a] inhibiting[b] 4+ CA
	CM glycoprotein[a]	*M. pneumoniae*[c]	
Rabbit antisera to:			
CM glycoprotein[a]			
No. 26 pre-immunization	<8	32	
post-immunization	1,024	32	ND
No. 27 pre-immunization	<8	8	
post-immunization	1,024	64	ND
M. pneumoniae			
No. 24[d]	64	64	38
No. 24 IgM[e]	ND	16	ND
Human CA			
No. 581[f]	<8	64	2.5
No. 699[f]	<8	4	6
IgM (pool)[g]	ND[g]	64	Avg 8 (2.5–25)[h]

[a] See references 9 and 15.
[b] Assays on two separate preparations of CM glycoprotein gave identical results.
[c] Suspension of whole organisms.
[d] Only CA present in unfractionated serum after one intravenous injection of *M. pneumoniae*.
[e] Purified CA IgM from rabbit serum no. 24.
[f] CA positive convalescent sera.
[g] ND, Not done.
[h] CA IgM isolated from pool of 10 high-titered CA sera from cases of *M. pneumoniae* infection. Pool included convalescent sera no. 581 and 699 and four others in which CA was significantly inhibited by CM glycoprotein (range, 2.5–25 μg).

The CA evoked in rabbits by immunization with *M. pneumoniae* was reactive with human cells. Except for nonreactivity with rabbit erythrocytes, this CA may be similar to that previously reported (4) as being CA anti-I. CA anti-I could be absorbed by *M. pneumoniae* and were inhibited by alpha- and beta-galactosides. In this connection, it is of interest that polysaccharide fraction 2 of *M. pneumoniae* contains four sugars, of which galactose is present in highest molar ratio (1). Galactose is also the monosaccharide present in highest molar concentration in integral glycoprotein extracted from human membranes with warm phenol (10).

Immunization with CM glycoprotein from human erythrocytes (rabbits 26 and 27) evoked CF antibody to homologous (CM) antigen, and in one (rabbit 27) to *M. pneumoniae*. In addition, both antisera were found to contain high titers of agglutinins for erythrocytes of groups A, B, and O(H). These agglutinins were active at both 37 and 4°C and were not inhibited by highly purified and potent blood group substances from extra-erythrocytic sources (16). Part of this broadly specific agglutinin response was CA reactive with human erythrocytes but not with rabbit erythrocytes. Serum taken from rabbit 26 after the second injection of CM antigen showed a significant rise in CA titer. The CA titer was undiminished after four successive absorptions with group O cells at 37°C which reduced the titer of warm agglutinins from 256 to 2. Similar results, not otherwise reported, were obtained with the sera of three additional rabbits immunized with CM glycoprotein.

The presence, in the preimmunization serum of rabbit no. 26 (Table 2), of CF antibody for *M. pneumoniae* probably represents a cross-reactive response to bacterial antigens, either exogenous or commensal, or to related glycolipids (? galactolipids) in spinach or other vegetable matter used in feed (12, 13).

The failure of individual convalescent sera, positive in CF with *M. pneumoniae* (no. 581 and 699), to react in CF with CM erythrocyte glycoprotein remains unexplained. Nevertheless, the CA in both of these sera was inhibited by the CM erythrocyte glycoprotein (Table 2). Neither is there any clear indication as to why the antibody which is reactive with erythrocytes, either directly as CA or by inhibition of CA with soluble erythrocyte glycoprotein, should be limited to IgM. It is clear, however, that the IgM fraction also contained CF antibody for *M. pneumoniae* (Table 2). There was insufficient IgM from the serum of rabbit no. 24 to test in CF with CM glycoprotein.

The cross-reaction between *M. pneumoniae* and CM erythrocyte glycoprotein was further substantiated by the reactivity of the serum and purified IgM from rabbit no. 24 immunized with *M. pneumoniae*. Preimmunization serum contained no MI or CF antibody to *M. pneumoniae*, and, like all other normal rabbit sera tested, contained no antibody to human CM glycoprotein. The post-immunization serum from rabbit no. 24 and the IgM derived from it contained CF antibody to *M. pneumoniae*. The serum antibody to CM could, therefore, only have been produced in response to immunization with the organism. Neither the CA IgM from rabbit no. 24 nor the IgM from pooled human convalescent sera was tested for CF with CM glycoprotein. However, CA in the serum of rabbit no. 24, as well as the individual human sera represented in the pool, was inhibited by purified CM glycoprotein (Table 2). Moreover, both the rabbit and human IgM preparations have been found to be reactive with human erythrocytes when examined by immunoelectronmicroscopy (results to be reported).

The body of evidence already adduced (4, 17), together with our findings reported here, clearly supports the conclusion that CA for erythrocytes elicited in rabbits by immunization with *M. pneumoniae* and the CA which appear in humans as a result of active infection are cross-reactive responses to antigenic determinants in *M. pneumoniae*. Of the alternative explanations that have been put forward, the hypothetical effect in vivo of *M. pneumoniae* is probably excluded on quantitative considerations. *M. pneumoniae* is largely confined to the respiratory epithelium during acute infection and from that site generates the immune response, locally and systemically. Organisms gaining transient access to the blood stream would not do so in amounts sufficient to effect detectable antigenic changes on circulating blood cells, a possibility also excluded by evidence obtained in vitro (4).

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