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Complement-Fixing Antibody Response in Pneumococcal Pneumonia

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Previous studies of complement-fixing antibodies to pneumococcal capsular polysaccharides in humans have yielded conflicting results. We studied 65 sera from 25 patients with pneumococcal pneumonia, using both fresh sera and heat-inactivated sera with added human complement. Only 4 of the 25 patients developed detectable levels of complement-fixing anticapsular antibody. Of the 25 patients, 22 developed detectable levels of hemagglutinating anticapsular antibody, indicating that they were able to develop an immunological response during the infection. Most of the antibody detected by hemagglutination was sensitive to 2-mercaptoethanol, but some 2-mercaptoethanol-resistant antibody was also detected. In studies with rabbit antiserum, the complement fixation test was found to be as sensitive as the hemagglutination test for detection of anticapsular antibody. It is not clear why detectable levels of complement-fixing antibody do not develop more often in patients with pneumococcal pneumonia. Studies of purified anticapsular antibody would be of interest to determine whether or not these antibodies are restricted to immunoglobulin subclasses having a limited capacity to fix complement.

There is considerable variation in the ability of different species to develop complement-fixing (CF) anticapsular antibodies to pneumococci. CF antibodies develop readily in certain species (e.g., rabbits, guinea pigs, and sheep) but are usually not detectable after immunization in other species (e.g., horses, dogs, and goats) (8, 10). The few previous studies of human immune sera have yielded variable results. In early studies, Horsfall and Goodner (10) noted that guinea pig complement was not fixed during the reaction of encapsulated pneumococci with human anticapsular antibody. Similarly, Stats and Bullowa (18) found no evidence of CF anticapsular antibody in convalescent sera of eight patients with pneumococcal pneumonia. They employed a quantitative CF procedure with heat-inactivated serum and guinea pig complement. Their method for measuring CF antibody may have been somewhat insensitive, in that they employed a short time for reaction of immune complexes with complement, and they used a relatively insensitive end point (100% hemolysis) in the test. They did not evaluate possible fixation of human complement by anticapsular antibody. In contrast to the foregoing results, Heidelberger and Mayer (9) were able to detect human complement nitrogen in immune precipitates formed after addition of capsular polysaccharide to unheated serum in four of six cases, and the hemolytic complement titer decreased in two of four sera tested. In the present studies, the development of CF anticapsular antibody in 25 patients with pneumococcal pneumonia was evaluated by using heat-inactivated sera with added human complement and fresh sera without heat inactivation. CF antibodies were detected in only four of the patients.

MATERIALS AND METHODS

Patients and clinical samples. Twenty-five adults with a clinical history suggestive of bacterial pneumonia, with acute onset of fever, cough, and the production of purulent sputum, were entered in the study. All patients had roentgenographic evidence of pulmonary consolidation. Pneumococci were isolated from blood cultures in six cases, from pleural fluid in one case, and in pure culture from transtracheal aspirates in seven cases. In 11 additional cases, pneumococci were isolated as the predominant organism from expectorated sputum samples, and these patients all showed clinical improvement within 72 h after initiation of penicillin G therapy. Pneumococci were serotyped as described elsewhere (7). The serotypes of the isolates were type 3 (eight cases), type 23 (four cases), types 7, 8, and 12 (three cases each), type 9 (two cases), and types 1 and 18 (one case each).

Serum samples were divided into multiple aliquots and stored at −70°C. All sera selected for study were free of pneumococcal capsular polysaccharide, as determined by latex agglutination (for types 7 and 14) or by counterimmunoelectrophoresis (for all other

types) by procedures that have been described in detail elsewhere (5–7).

Antigens and antisera. Purified pneumococcal capsular polysaccharides were obtained from Robert S. Baker, Eli Lilly and Co., Indianapolis, Ind. Analytical data on the C-substance content of these antigens were provided by Baker or were obtained in our laboratory by immunoprecipitation analysis. Pneumococcal C-polysaccharide (C-substance) was generously provided by Henry Gewurz, Department of Immunology, Rush Medical College, Chicago, Ill. The C-substance was extracted from a Cs-capsulated mutant strain of pneumococcus (4) that was obtained originally from Gerald Schiffman, Downstate Medical Center, Brooklyn, N.Y. (3).

Rabbit antipneumococcal sera were obtained commercially from the Statens Seruminstitut, Copenhagen, Denmark.

CF tests. Serum hemolytic complement levels were measured as 50% hemolytic (CH_{50}) units by a spectrophotometric method (20) with a total reaction volume of 4.0 ml. Veronal-buffered saline (pH 7.4) containing 0.0015 M Ca^{2+}, 0.0005 M Mg^{2+}, and 0.02% gelatin was used as the diluent for sera and antigens throughout. CF antipneumococcal antibody was measured by a modification of the Stein and Van Ngu method (19). Sera were heated at 56°C for 30 min, and serial twofold dilutions were prepared beginning with a 1:8 dilution. Twofold dilutions of polysaccharide in a concentration range of 0.125 to 2.0 μg/ml were added to human sera, along with 2.5 CH_{50} units of human complement. Serum used as the source of human complement was free of anticapsular CF antibody. Rabbit antipneumococcal sera were tested with 0.0017 to 0.5 μg of polysaccharide per ml with 2.5 CH_{50} units of guinea pig complement. Titrations were carried out by mixing 1.0 ml of the heat-inactivated and diluted test sera with 1.0 ml of antigen-containing solution and 1.0 ml of complement in tubes (13 by 100 mm). The tubes were incubated for 18 h at 4°C, and residual hemolytic complement activity was measured with the sheep cell assay. Controls were included to determine any CF by serum alone or by the various concentrations of antigen alone. A control for complement deterioration was also included. The titer of CF antibody was calculated at the CH_{50} end point by graphic interpolation on a plot of the logarithm of the serum dilution versus the percentage of hemolysis (19).

The consumption of human complement in fresh serum (i.e., serum stored at −70°C) was determined by mixing the diluted test serum with an equal volume (1.0 ml) of capsular polysaccharide solution, then incubating the mixture for 18 h at 4°C. The concentrations of polysaccharide used in most cases were 1, 5, 10, and 20 μg/ml. The number of residual CH_{50} units in the reaction mixtures was determined and compared with the CH_{50} content of a control sample that contained no antigen. Sera were considered to contain CF antibody when the amount of available hemolytic complement that was fixed exceeded the probable error of the assay, i.e., ±10%, based on 95% confidence limits for replicate analyses of the CH_{50} content of normal sera. In some cases, supernatant tests for free polysaccharide were performed with a latex agglutination procedure (6).

HA antibody. An indirect hemagglutination (HA) method (1) was used to detect type-specific antipneumococcal antibody. Human group O, Rh^+ cells were employed in the test. Sensitization of the cells with pneumococcal polysaccharide was performed at 30°C. Rabbit antisera were absorbed with unsensitized human group O, Rh^+ cells for 2 h at room temperature before the test for HA antibody was performed.

Ig. Serum immunoglobulin (Ig) G and M levels were quantified by radial immunodiffusion with immunoplates from Hyland Laboratories, Costa Mesa, Calif. Fractionation of HA anticapsular antibody was performed by the method of Ammann et al. (1) by using 0.1 M 2-mercaptoethanol (2-ME) for 12 h at 4°C.

RESULTS

We studied 25 patients with pneumococcal pneumonia for the development of anticapsular antibodies. In each case, the serotype of the pneumococcal isolate from the patient was identified, and CF and HA antibodies were measured with the appropriate type-specific polysaccharides. The results are indicated in Table 1, with the data grouped according to the time of collection of samples. By using a procedure of block titration with heat-inactivated serum and 2.5

TABLE 1. *Results of tests for anticapsular antibody in 25 patients with pneumococcal pneumonia*

Phase of infection	Days after admission: mean (range)	No. studied: patients (sera)	No. of patients with CF antibody			No. of patients with HA antibody[a]
			Titration with heat-inactivated serum[a]	Polysaccharide added to fresh serum[b]	Either test	
Acute	3.6 (1–6)	11 (20)	2	—[c]	2	8
Convalescent	19.0 (7–35)	25 (34)	4	3[d]	4	22
Recovery	47.9 (36–60)	10 (11)	1	—	1	8
All		25 (65)	4	3	4	22

[a] Antibody titer ≥ 8.
[b] Fixation of >10% of CH_{50} units in fresh serum by added type-specific polysaccharide.
[c] —, Not done.
[d] Fresh serum was not available from two patients for the test.

CH_{50} units of human complement, we discovered that only 4 of 25 patients had CF anticapsular antibody titers of 8 or more. The optimal antigen concentration in this test was 0.08 to 0.17 μg of polysaccharide per ml of reaction mixture; at these concentrations, the polysaccharide antigens alone had no anticomplementary activity. The patients with positive results included one individual with bacteremic infection due to type 7 organisms and another patient with pneumonia and intrapleural empyema caused by type 3 pneumococci. The other two patients had abacteremic pneumococcal pneumonia caused by types 9 and 12. Since it has been reported that heat inactivation can decrease the immunoreactivity of antipneumococcal antibody (9), convalescent sera from 23 of the patients were studied by adding graded amounts of capsular polysaccharide to the fresh sera, then incubating the reaction mixtures at 4°C overnight. Only 3 of the 23 patients tested had CF antibody detectable by this technique (Table 1), and these patients had also had CF antibody detectable in heat-inactivated sera. The fourth patient with CF antibody in heat-inactivated serum did not have fresh serum available for testing. The pattern of complement consumption by polysaccharide and CF anticapsular antibody in fresh serum in two of the patients with positive results is shown in Fig. 1. The consumption of hemolytic complement increased progressively as larger quantities of polysaccharide were added, up to a point of maximal fixation at 5 to 10 μg of polysaccharide (2.5 to 5.0 μg of polysaccharide per ml of reaction mixture). Maximal fixation began in the region of the slight antigen excess, and a large excess of free antigen did not alter the amount of complement consumed.

Few problems with anticomplementary activity of patients' sera were encountered, and no patients were excluded from the study because of anticomplementary activity in serum samples. There were four sera that could not be tested because of anticomplementary activity at the 1:8 or 1:16 dilution. In these cases, sera that had been taken shortly before or shortly after the anticomplementary samples were available for study.

In contrast to the low incidence of detectable CF antibodies, most of the patients developed HA antibody. The majority of individuals who were sampled at each of the three phases of infection had HA antibody titers of eight or more (Table 1). The relation between titers of HA and CF antibodies for sera obtained during convalesence is illustrated in Fig. 2. There was no obvious relation between the levels of these two antibodies. Levels of HA and CF antibodies

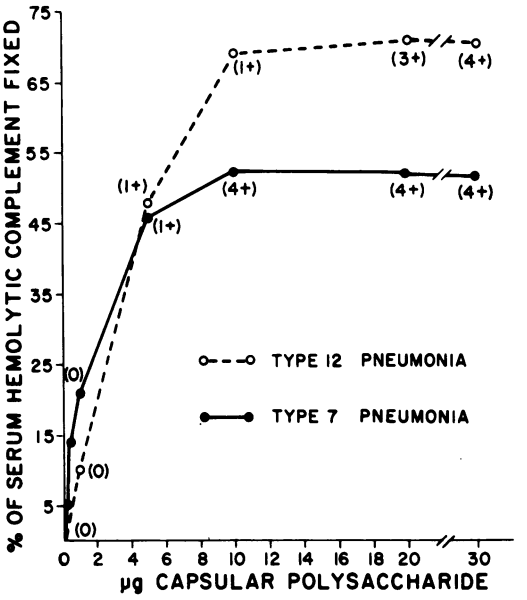

FIG. 1. *Complement consumption associated with the reaction of pneumococcal capsular polysaccharide and anticapsular antibody in convalescent sera obtained from two patients with pneumococcal pneumonia. Numbers in parentheses indicate the results of a latex agglutination test for free polysaccharide in the supernatants of the antigen-antibody reaction mixture.*

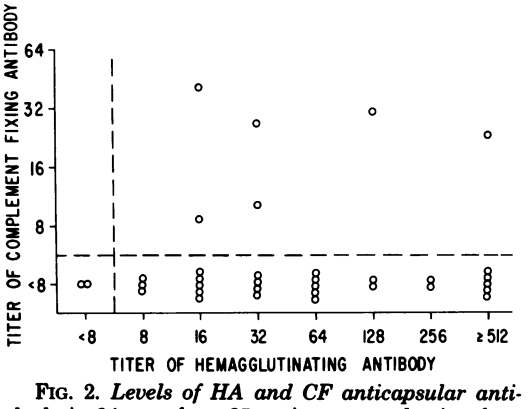

FIG. 2. *Levels of HA and CF anticapsular antibody in 34 sera from 25 patients convalescing from pneumococcal pneumonia.*

in sequential serum samples that were available in seven of the patients are given in Table 2. In these studies, the sensitivity of HA antibodies to 2-ME was also determined. Fractionation of the CF antibodies was attempted, but problems in developing a valid fractionation procedure could not be resolved satisfactorily. A major portion of the HA antibody in most of the sera

was sensitive to 2-ME, and this finding was especially true for sera obtained early in the course of the infection. In one instance (case 3), only 2-ME-resistant antibody was detected, and in three other instances (cases 1, 4, and 5) various proportions of the HA antibody were resistant to 2-ME. In two patients with CF antibodies (cases 5 and 6), the levels of CF antibody were highest in the earliest serum samples, at a time when all of the HA antibody was sensitive to 2-ME. In case 6, only 2-ME-sensitive antibody was detected.

Since relatively high titers of CF antibody were detected in several sera early in the course of infection, studies were performed to determine whether C-reactive protein, which was detectable in many of these sera, might have given CF in reaction with the trace quantities of C-substance that were present in the type-specific polysaccharides. The level of C-substance contamination varied with the capsular antigen preparation and ranged from <0.05 to 1.0% by weight. Each of the sera that were positive for anticapsular CF antibody was tested in a two-dimensional titration system with a quantity of C-substance equivalent to 1 or 2% contamination of the capsular antigens (i.e., 0.0017 or 0.0034 μg of C-substance per ml of reaction mixture). No CF was detected at these concentrations of C-substance. The antibody detected by hemag-

TABLE 2. *Levels of anticapsular antibody in sequential serum samples in seven cases of pneumococcal pneumonia*

Case	Pneumococcal serotype	Days after admission	CF antibody titer	HA antibody titer of serum:	
				Untreated	2-ME-treated
1	3	1	—[a]	<4	—
		3	<8	256	4
		7	<8	64	8
		23	<8	32	8
		43	<8	16	8
2	3	1	<8	<4	—
		3	<8	<4	—
		7	<8	8	<4
		12	<8	16	<4
		24	<8	16	<4
		59	<8	16	<4
3	3	2	<8	16	16
		6	<8	32	32
		21	<8	32	32
		55	<8	32	—
4	3	1	<8	8	<4
		5	<8	1,024	256
		14	<8	≥2,048	—
		32	<8	≥2,048	1024
5	7	2	—	8	—
		5	79.1	32	<4
		10	30.8	128	16
		16	28.4	32	16
6	12	3	—	<4	—
		6	50.1	32	<4
		15	41.9	16	<4
		25	9.1	16	<4
		39	<8	16	<4
7	12	1	<8	<4	<4
		10	<8	16	<4
		24	<8	8	<4
		43	<8	8	<4

[a] —, Not done.

glutination also appeared to be type specific in that it was effectively neutralized by an excess of the appropriate type-specific capsular polysaccharide but not by unrelated polysaccharides. Equal volumes (1.0 ml) of serum and a solution of capsular polysaccharide were mixed and incubated at 4°C for 18 h, and the HA titer of the absorbed serum was determined. Five to 10 μg of homologous capsular polysaccharide per ml of reaction mixture was required to bind the antibody in five sera with HA antibody titers ranging from 16 to 512.

Rabbit antisera were used to evaluate the relative efficacy of the HA and CF tests for detection of pneumococcal anticapsular antibodies. The titers of anticapsular antibody detected by the CF test were two- to fourfold greater than the titers measured by the HA test for four of five antisera (Table 3). HA was slightly better than CF for detecting type 8 antibody. These results suggest that the failure to detect CF antibodies in patients' sera was not the result of any technical difficulty in the CF test itself.

The failure of most patients to develop CF antibodies did not appear to be due to a lack of humoral antibody response, since most developed HA antibody. Levels of serum IgG and IgM were measured in 21 of the 25 patients, and all had values in the normal range (i.e., 0.41 to 0.260 mg/ml with a mean of 1.25 mg/ml for IgM, and 8.75 to 15.7 mg/ml with a mean of 11.1 mg/ml for IgG. Of the 25 patients, 18 had one or more serious associated diseases, including alcoholism in 12 cases, chronic obstructive airways disease in 7 cases, and diabetes mellitus in 2 cases. None of the patients, however, had neoplastic disease or clinical or laboratory evidence of cirrhosis, and none was receiving immunosuppressive drugs.

TABLE 3. *Results of HA and CF tests with rabbit anticapsular antibody*

Pneumococcal type	Antibody titer[a]	
	CF	HA
3	6,200	1,024
8	11,350	16,380
12	2,180	512
19	4,100	2,048
23	4,400	2,048

[a] Titer interpolated at the CH_{50} end point. The optimal antigen concentration was 0.002 μg/ml of reaction mixture.

DISCUSSION

Previous attempts to detect anticapsular CF antibody in patients with pneumococcal pneumonia have been limited in scope and have produced somewhat variable results (9, 10, 18). The present studies were performed with CF methods of high sensitivity. Human complement was used to allow for any possible requirement for complement of the homologous species, since this has been described as an important factor in CF by certain antibodies of avian, bovine, and human origin (2, 15, 22). In addition, because some pneumococcal antibodies may be heat labile (9), fresh serum as well as heat-inactivated serum was tested. Despite these precautions, CF anticapsular antibody was detected in only 4 of 25 patients with pneumococcal pneumonia. The positive results in these four patients appeared to be specific for anticapsular antibody. Anticomplementary activity of the sera was excluded by appropriate controls, and there were no problems with anticomplementary activity by the polysaccharide antigens. The capsular polysaccharides did contain trace amounts of C-substance, which can fix complement in combination with C-reactive protein (13), but the small amount of C-substance in our type-specific polysaccharides did not give detectable CF in control studies. In this regard, it is uncertain whether the positive results obtained by Heidleberger and Mayer (9) with human antipneumococcal serums were specific, since CF by C-substance was not excluded in their studies.

The reason for the dearth of positive results for CF anticapsular antibodies in the present studies is not clear. It seems possible that more intensive sampling during the course of the immune response might have given more positive results, but the data available in seven cases where multiple-serum samples were obtained do not support this possibility. Only 2 of 11 patients who were tested during the acute phase of the infection had positive results, and these patients also had positive results with later sera. Also, only 1 of 10 patients had positive results with late sera obtained after recovery. We cannot exclude the possibility that serum CF antibodies may have been masked by circulating soluble capsular polysaccharide, which can be present in pneumococcal pneumonia (5), but none of the sera selected for the present studies had soluble polysaccharide detectable by counterimmunoelectrophoresis or latex agglutination. Similarly, the presence of blocking antibodies in the sera cannot be excluded, but we did not observe any zone phenomena during the titration of CF anticapsular antibodies to suggest that blocking antibodies were present. It did not appear likely that the patients had an impaired immunological response, since they did not have

associated diseases that would be likely to impair their humoral antibody responses, and they had normal serum IgG and IgM levels. Moreover, a majority (22 of 25) did develop measurable levels of HA anticapsular antibodies, indicating that they were able to develop an immunological response to the capsular polysaccharides.

It was apparent that the CF test was as effective as the test for HA antibody in detecting rabbit antipneumococcal antibody, and the lack of detection of CF antibody in human sera presumably reflects differences in the qualitative or quantitative aspects of the antibody response for the different species. The human immune response in pneumococcal otitis media has been shown by indirect fluorescent-antibody methods to include IgM, IgG, and IgA antibodies (16). IgM and IgG HA antibodies have also been demonstrated by sucrose density separation and 2-ME fractionation of human sera obtained after vaccination with purified pneumococcal polysaccharides (1). The functional characteristics of purified human IgM to pneumococcal polysaccharides have not been studied, but it is known that some human IgM antibodies do not fix complement effectively (11, 14). In addition, human IgG antibody to various high-molecular-weight carbohydrates, including dextran, levan, *Haemophilus influenzae* type b polysaccharide, and teichoic acid, is predominantly IgG_2 (12, 21). Antibodies of this subclass do not fix complement as effectively as do antibodies of subclasses IgG_1 or IgG_3 (17). These observations raise a question of whether the predominant antibody response to pneumococcal capsular polysaccharides is restricted to IgM or to IgG antibodies that do not fix complement well. Such restriction has been suggested with regard to the human immune response to haemophilus and meningococcal polysaccharides (12), and it seems desirable to evaluate this possibility for pneumococcal polysaccharides as well.

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