

Serum Opsonic Deficiency Produced by *Streptococcus Pneumoniae* and by Capsular Polysaccharide Antigens

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The opsonic requirements for phagocytosis of *S. pneumoniae* types 6, 7, 18, and 23 were determined in normal and C₂ deficient serum, and in normal serum chelated with magnesium ethyleneglycoltetraacetic acid. All four strains were effectively opsonized via the alternative complement pathway, a finding suggesting that the capsular polysaccharides of these strains activated complement via the alternative pathway. Since bacteremic pneumococcal disease is often associated with circulating capsular polysaccharide, it was considered that this cellular component may activate complement *in vivo* and impair host defenses by producing an opsonic defect for pneumococci. To examine this hypothesis, serum was incubated with suspensions of whole *S. pneumoniae* types 6, 7, 18, or 23 or with purified capsular polysaccharide from each of these types, and residual complement activity and opsonic capacity were measured. Hemolytic C₃₋₉ complement activity and opsonic capacity for ³H-thymidine labeled *Salmonella typhimurium*, a species effectively opsonized via the alternative pathway, were reduced in serum following incubation. Polysaccharide concentrations as low as 1 μg/ml inhibited serum opsonic capacity for salmonella. Whole pneumococci and pneumococcal capsular polysaccharide also inhibited the opsonic activity of human C₂ deficient serum for salmonella, further evidence for activation of complement via the alternative pathway. Pneumococcal capsular polysaccharide markedly inhibited the opsonic capacity of normal serum for the homologous pneumococcal type. Thus, amounts of pneumococcal capsular polysaccharide, similar to those found in the serum of patients with pneumococcal disease, bring about de complementation of serum via activation of the alternative pathway and inhibit pneumococcal opsonization.

Patients with bacteremic disease due to *Streptococcus pneumoniae* frequently have circulating pneumococcal capsular polysaccharide (PCP)* with serum antigen levels as high as 50 μg/ml, detectable antigenemia for as long as 30 days and depressed serum levels of C₃ [1,2,3,4]. Since pneumococcal opsonic activity in the non-immune individual is primarily related to cleavage of C₃ and fixation of opsonically active C_{3b} to the bacterial surface [5,6], it was considered that patients with pneumococcal infection and antigenemia may have acquired a pneumococcal opsonic defect. In addition, since many pneumococcal serotypes are opsonized by activation of C₃ via the alternative pathway [5,7,8,9,10], an antigen induced opsonic defect may interfere with phagocytosis.

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*Abbreviations used in this paper: MgEGTA, magnesium ethyleneglycoltetraacetic acid; NPS, normal pooled serum; PBS, phosphate buffered saline; PCP, pneumococcal capsular polysaccharide; Pn, whole *Streptococcus pneumoniae*.

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To test this hypothesis pooled normal human serum or C₂ deficient human serum was incubated with whole *S. pneumoniae* (Pn) types 6, 7, 18, or 23 or with purified PCP from these types. Serum opsonic capacity was measured after incubation with whole bacteria or PCP by a functional assay employing neutrophil phagocytosis of methyl-³H-thymidine labeled *Salmonella typhimurium*. This species is effectively opsonized via the alternative pathway, thereby permitting evaluation of opsonic activity mediated by the alternative pathway [11]. Both whole pneumococci and PCP of the types tested caused a reduction of serum opsonic capacity and depletion of hemolytic C activity, although inhibition of opsonization varied among the serotypes. Both whole pneumococci and PCP of two representative serotypes also reduced the opsonic capacity of C₂ deficient serum, evidence for activation of the alternative pathway.

MATERIAL AND METHODS

Bacteria and PCP. *S. pneumoniae* types 18 and 23 were isolated from children with bacteremia; type 6 was obtained from the American Type Culture Collection (Rockville, MD); and type 7 was kindly provided by Dr. Robert Austrian (University of Pennsylvania, Philadelphia, PA). Serotypes were confirmed with group-specific antisera (Statens Seruminstitut, Copenhagen, Denmark), and encapsulation was maintained by monthly mouse passage. Purified PCP of types 6A, 7F, 18C, and 23F were kindly supplied by Dr. James Hill (Development and Applications Branch, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, MD). These highly purified polysaccharides were in lyophilized form without preservative and have been shown to contain less than 0.001 μ g endotoxin by both an *in vivo* rabbit assay and by the limulus lysate test; nitrogen content was less than 3% and protein content was less than 1% of their dry weight (Dr. James Hill, personal communication). They have been prepared in accordance with procedures for the manufacture of pneumococcal vaccines by the Lilly Research Laboratories, Indianapolis, Indiana. The strain of *Salmonella typhimurium* employed has been used in our laboratory for several years.

Bacterial preparation and labeling. This procedure is described in detail elsewhere [5]. Briefly, *S. pneumoniae* were grown in Mueller-Hinton broth (Difco, Detroit, MI) enriched with 3% bovine albumin, and with 1% D-glucose added during the final two hours of incubation at 37°C. A total of 0.05 mCi methyl-³H-thymidine (specific activity 6.7 Ci/mM, New England Nuclear, Boston, MA) was added to 10 ml of the growth medium. After overnight incubation at 37°C, the bacteria were harvested by centrifugation and washed three times with isotonic phosphate-buffered saline, pH 7.4 (PBS). Pneumococci were killed by heating at 65°C for 45 min, counted in a Petroff-Hauser chamber, suspended in PBS to 2.5×10^8 bacteria/ml and stored at 4°C for no longer than 7 days. For experiments employing suspensions of whole, viable *S. pneumoniae*, the bacteria were prepared by identical methods except they were not heat-killed and were used on the day of preparation.

S. typhimurium was grown in Mueller-Hinton broth enriched with 0.1 mg/ml 2'-deoxyadenosine (Sigma, St. Louis, MO) and containing 0.04 mCi methyl-³H-thymidine. The labeled bacteria were washed three times in PBS, suspended to 5×10^8 /ml and used the same day.

Leukocytes. Leukocytes were separated from heparinized blood of healthy adults by dextran sedimentation, washed and suspended in Hank's Balanced Salt Solution (Grand Island Biological Co., Grand Island, NY) with 0.1% gelatin (gel-HBSS) at a concentration of 1×10^7 neutrophils per ml.

Opsonins. Sera from three healthy adult donors were pooled, divided into 1 ml aliquots, and used throughout these experiments. Once thawed, unused serum was discarded. Type-specific pneumococcal antibody was kindly determined by Dr. Gerald Schiffman, Downstate Medical Center, Brooklyn, NY, using a sensitive radioimmunoassay method with results expressed in ng of antibody nitrogen (ngAbN) per ml [12]. The serum pool contained normal levels of antibody to types 7, 18, and 23 (214, 668, and 363 ngAbN/ml, respectively) but a lower concentration of type 6 antibody (92 ngAbN/ml). To study opsonization in the absence of the classical complement pathway, undiluted serum was chelated by the addition of 0.1 M ethyleneglycoltetraacetic acid in the presence of 0.1 M MgCl₂ (MgEGTA) to each 1 ml of serum for a final chelator concentration of 0.01 M [13]. Calcium and magnesium-free gel-HBSS was used to dilute the chelated serum. In addition, serum was obtained from a patient with inherited complete C₂ deficiency. This serum contained normal levels of all other classical and alternative complement components [14], and had a normal capacity for alternative pathway opsonization of *Staphylococcus epidermidis* and of *S. aureus*, Wood 46 [15]. All sera were divided into aliquots and stored at -70°C. Heat-inactivated serum was prepared by incubation at 56°C for 30 min.

Bacterial opsonization. Bacteria were opsonized by adding 0.4 ml of the desired opsonin to a plastic tube (12 × 75 mm, Falcon, Oxnard, CA) containing 0.1 ml of radiolabeled bacteria. After 30 min incubation at 37°C, the mixture was centrifuged at 1600 g for 15 min. The supernatant was discarded, and the bacterial pellet was resuspended in 0.5 ml gel-HBSS.

Determination of phagocytosis. The phagocytosis mixtures consisted of the opsonized bacterial suspension and 0.5 ml leukocytes suspended in gel-HBSS. The bacteria:neutrophil ratio was 5:1 for experiments with labeled pneumococci and 20:1 for studies with salmonella. Immediately following addition of leukocytes, the mixtures were aliquoted in 250 μl volumes to four polypropylene vials (Bio-vials, Beckman, Chicago, IL) which were agitated in a shaking incubator (Model 25, New Brunswick Scientific, New Brunswick, NJ) at 37°C. After 30 min incubation, 2 ml cold PBS was added to each of two vials to arrest phagocytosis. To determine neutrophil-associated bacteria, vials were centrifuged for 5 min at 160 g at 4°C, the pellets were washed twice in 2 ml cold PBS and were resuspended in 2.5 ml scintillation liquid (toluene with 20% Bio-Solve-3 in Fluoralloy, Beckman). The total bacterial population in the phagocytosis mixture was determined by adding 2 ml cold distilled water to each of the two remaining vials, centrifuging at 1600 g for 15 min and resuspending the pellet in 2.5 ml scintillation liquid. The samples were counted in a liquid beta scintillation counter (Beckman, LS-250). Chemical quenching was similar for all samples. Duplicate values were within 5% of agreement, and an average of the duplicate values was used in all calculations.

Bacterial uptake by the PMN leukocytes (% phagocytosis) at a given sampling time was calculated according to the formula:

$$\% \text{ phagocytosis} = \frac{\text{cpm in 160 g pellet}}{\text{cpm in 1600 g pellet}} \times 100.$$

The cpm in the denominator was always at least 50 times greater than background radioactivity.

Pneumococcal and salmonella opsonic requirements were defined using dilutions of normal pooled serum (NPS), MgEGTA chelated NPS, C₂ deficient serum and

NPS heated at 56°C for 30 min (Δ NPS). Bacteria were incubated with each opsonin for 30 min, and results were expressed as percent bacterial phagocytosis following an additional 30 min incubation of opsonized bacteria with leukocytes. Each value was the mean of at least four experiments, except when C₂ deficient serum was used and duplicate studies were performed. Differences were calculated by student's *t*-test for unpaired observations.

The effect of Pn and PCP on serum opsonic activity was studied by preincubating serum with Pn, PCP, and with an equal volume of saline. Residual opsonic activity, which was reflected by the percent bacterial phagocytosis, was expressed as the percentage of the saline control. Normal serum was diluted to 2.5% and C₂ deficient serum was diluted to 5% for the salmonella opsonic assay. Serum was diluted to 10% for the pneumococcal opsonic assay. Each value was the mean of at least four experiments unless otherwise indicated. The significance of opsonic inhibition was calculated using the difference in percent phagocytosis between serum preincubated with saline and Pn or PCP. Student's *t*-test for paired observations was employed. Experiments with 5 and 1 μ g/ml of PCP were performed once and were not analyzed for significance.

Hemolytic C₃₋₉ titration. The assay for hemolytic C₃₋₉ activity was carried out as previously reported [16,17]. Test samples were serially diluted in EDTA buffer. To 1.0 ml of each dilution of test sample was added 1.0 ml of EDTA buffer. EAC₁ (guinea pig) 4 (human) 2 (guinea pig) was prepared at T-max, and 1.0 ml of EAC₁₄₂ (5×10^7 /ml) was added to each tube. After 60 min incubation at 37°C, 4.5 ml of cold normal saline was added, and the tubes were centrifuged. The optical density (412 nm) of the supernatant fluid was determined and results were expressed as 50% lysis. Appropriate controls and complete blanks were run simultaneously.

RESULTS

Determination of opsonic requirements. The opsonic requirements of pneumococcal types 6, 7, 18, and 23 were investigated by incubating bacteria in dilutions of normal and MgEGTA chelated serum and comparing kinetics of neutrophil phagocytosis of the opsonized bacteria. The opsonic requirements of these serotypes are summarized in Table 1. All four types were opsonized equally well in normal and chelated serum at the 90% and 40% serum concentrations. Types 6 and 23 were also opsonized equally well in normal and chelated 20% serum. Types 7 and 18 were opsonized less effectively in concentrations of chelated serum less than 40%, although type 18 was opsonized more efficiently than the other three types in 10% and 5% chelated serum. Heat-stable factors provided negligible opsonic activity for Pn, 6, 7, and 23, and slight activity for Pn 18. Therefore, these pneumococcal strains require complement for optimal opsonization and utilize the alternative complement pathway.

S. typhimurium was effectively opsonized in normal, MgEGTA chelated and C₂ deficient serum as illustrated in Table 2. There was equal opsonic activity in 20% and 10% chelated and normal serum, while 5% and 2.5% chelated serum provided less opsonic activity. C₂ deficient serum provided less opsonic activity than either normal or chelated serum. However, at 5% and 2.5% concentrations the opsonic capacities of chelated and C₂ deficient serum were similar. Therefore, this strain of *S. typhimurium* is effectively opsonized via the alternative complement pathway.

Effect of whole pneumococci on serum hemolytic C₃₋₉ activity and opsonic capacity. Whole, viable *S. pneumoniae* were suspended to a concentration of $5 \times$

TABLE 1
Opsonic Requirements of *S. pneumoniae* types 6, 7, 18, and 23

Serotype	Opsonin	Percent phagocytosis in opsonin concentration of:					
		90%	40%	20%	10%	5%	Δ10%
6	NPS	76	72	78	39	15	2
	MgEGTA/NPS	72	59	60	3*	3*	
7	NPS	92	82	85	59	15	2
	MgEGTA/NPS	84	79	13*	6*	5*	
18	NPS	73	80	67	40	28	11
	MgEGTA/NPS	74	72	36*	22*	11	
23	NPS	78	75	77	48	24	5
	MgEGTA/NPS	76	70	63	6*	4*	

* $P < .05$

10^8 /ml in normal and C_2 deficient serum. The mixtures were incubated at 37°C for 60 min, and aliquots of serum were withdrawn at 0 and 60 min; organisms were removed by centrifugation, and the serum was frozen at -70°C for hemolytic C_{3-9} titrations. All four pneumococcal types decomplexed normal serum; hemolytic C_{3-9} activity was reduced to 28% to 53% of the time 0 titer in the single experiment illustrated by Table 3. Pn 6 was somewhat more active, and Pn 23 was least active in effecting serum hemolytic complement activity.

To demonstrate the effect whole pneumococci might have on serum opsonic capacity, pneumococci were separated by centrifugation ($1600\text{ g} \times 15\text{ min}$), and the serum was assayed for opsonic activity with salmonella. All four pneumococcal types reduced the opsonic capacity of normal serum (Fig. 1). There was reduction in both the initial rate of phagocytosis and in the maximal number of salmonella phagocytized. There was a greater reduction of serum opsonic capacity after incubation with Pn 18 than with Pn 6, 7, or 23 (Table 4). Differences between percent phagocytosis in serum preincubated with saline and with whole pneumococci were all significant at a level of $P < .05$. It was also demonstrated in a single experiment that Pn 7 and Pn 18 reduced the opsonic capacity of C_2 deficient serum for salmonella (Table 5). While Pn 18 reduced opsonic capacity to the same extent in C_2 deficient and normal serum, Pn 7 reduced opsonic capacity to a slightly greater extent in normal serum suggesting enhancement of the Pn 7 inhibition by the classical complement pathway.

No free PCP could be detected in the supernatant of the washed pneumococcal suspensions measured by counterimmunoelectrophoresis, which in our laboratory detects as little as $0.15\ \mu\text{g/ml}$ of polysaccharide using a standard assay [18]. This

TABLE 2
Opsonic Requirements of *Salmonella typhimurium*

Opsonin	Percent phagocytosis in opsonin concentration of:			
	20%	10%	5%	2.5%
NPS	69	64	61	41
MgEGTA/NPS	69	45	22†	15†
C_2 def	56*	22*	16	11

* $P < .05$

† $P < .005$

TABLE 3
Effect of Whole Pneumococci and Capsular Polysaccharide on the Hemolytic C₃₋₉ Activity of Normal Serum

Serotype	Percentage of initial serum C ₃₋₉ titer after incubation of normal serum with:			
	Whole Pn	PCP (μg/ml)		
		100	50	10
6	28%	73%	57%	67%
7	38	66	53	69
18	33	64	63	61
23	53	78	97	56

Saline incubation with serum reduced the C₃₋₉ titer by 2%.

finding suggests that the complement activating properties of whole pneumococci were not due to soluble polysaccharide in these experiments.

Effect of PCP on serum hemolytic C₃₋₉ activity and opsonic capacity. Purified PCP of each serotype was suspended in normal and C₂ deficient serum. The mixtures were incubated at 37°C for 60 min and aliquots of serum were withdrawn at 0 and 60 min and immediately frozen at -70°C for hemolytic C₃₋₉ titrations. Concentrations of polysaccharide between 10 and 100 μg/ml reduced the hemolytic C₃₋₉ titer of normal serum in the single experiment illustrated by Table 3. Serum C₃₋₉ activity was reduced to 53% to 73% of time 0 by polysaccharide of types 6, 7, and 18. These three

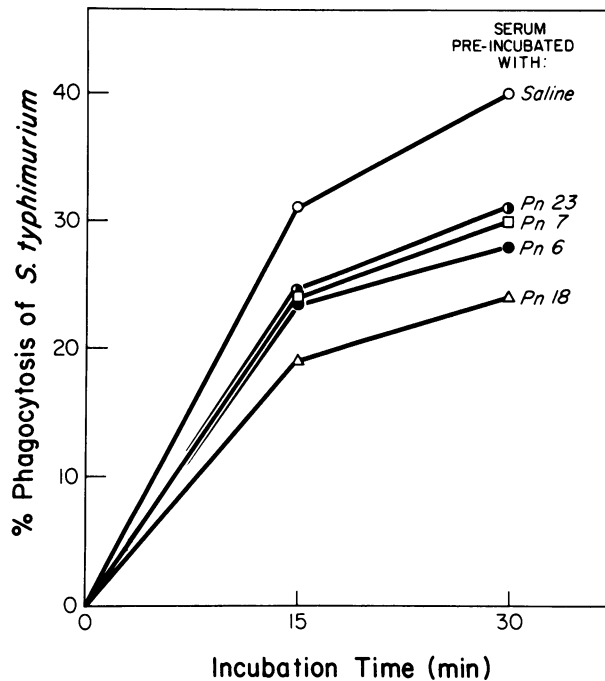


FIG. 1. Effect of whole pneumococci (Pn) on serum opsonic capacity. Serum opsonic capacity was directly related to the percent phagocytosis of salmonella using normal serum preincubated for 60 min with types 6, 7, 18, and 23 pneumococci, and serum preincubated with an equal volume of saline. Following preincubation, serum was diluted to 2.5% and used as an opsonin for salmonella. Opsonized salmonella were incubated with leukocytes, and percent phagocytosis was determined at 15 and 30 min. Each value represents the mean of four experiments. Significance of these differences are shown in Table 4.

TABLE 4
Effect of Whole Pneumococci and Capsular Polysaccharide on the
Opsonic Capacity of Normal Serum for Salmonella

Serotype	Percentage of initial serum opsonic capacity after incubation of normal serum with:					
	Whole Pn	PCP ($\mu\text{g/ml}$)				
		100	50	10	5	1
6	70%*	78%†	72%†	76%†	76%	72%
7	74#	73	45†	62†	68	85
18	59†	75†	75#	71†	76	72
23	76†	85†	87*	67†	79	74

* $P < .05$

$P < .025$

† $P < .005$

types were equally effective in decomplementing serum at high and low concentrations of PCP. Type 23 polysaccharide was most effective in the lowest concentration and had minimal activity at 50 $\mu\text{g/ml}$.

To demonstrate the effect capsular polysaccharide alone might have on serum opsonic capacity, 50 $\mu\text{g/ml}$ concentrations of each polysaccharide were incubated with serum, and the serum was assayed for opsonic capacity with salmonella. Polysaccharide of types 6, 7, and 18 partially inhibited serum opsonization while PCP 23 had little effect (Fig. 2). Lower concentrations of PCP were also tested for their effect on serum. Polysaccharide concentrations of types 6 and 18 as low as 1 $\mu\text{g/ml}$ were as active as 100 $\mu\text{g/ml}$ in reducing serum opsonic capacity (Table 4). Type 7 polysaccharide showed optimum activity at a concentration of 50 $\mu\text{g/ml}$, while type 23 polysaccharide produced the greatest inhibition when used in lower concentrations. Reduction of serum opsonic capacity was directly related to the degree of serum decomplementation produced by each PCP (Tables 3 and 4). Polysaccharide of types 7 and 18 also reduced the serum opsonic capacity of C_2 deficient serum for salmonella in a single experiment (Table 5). As with the whole organisms, type 7 polysaccharide reduced the opsonic capacity of normal serum to a slightly greater extent than of C_2 deficient serum, while PCP 18 was equally active in both sera.

To further characterize the effect of capsular polysaccharide on pneumococcal opsonic capacity, serum was preincubated with each of two polysaccharides (PCP 7 and 23) and opsonic capacity for the homologous pneumococcal type was determined. Table 6 illustrates the results of a representative experiment. Type 7

TABLE 5
Effect of Whole Pneumococci and Capsular Polysaccharide on the
Opsonic Capacity of C_2 Deficient Serum for Salmonella

Serotype	Percentage of initial serum opsonic capacity after incubation of C_2 deficient serum with:	
	Whole Pn	PCP ($\mu\text{g/ml}$) 50
7	85%	52%
18	58	73

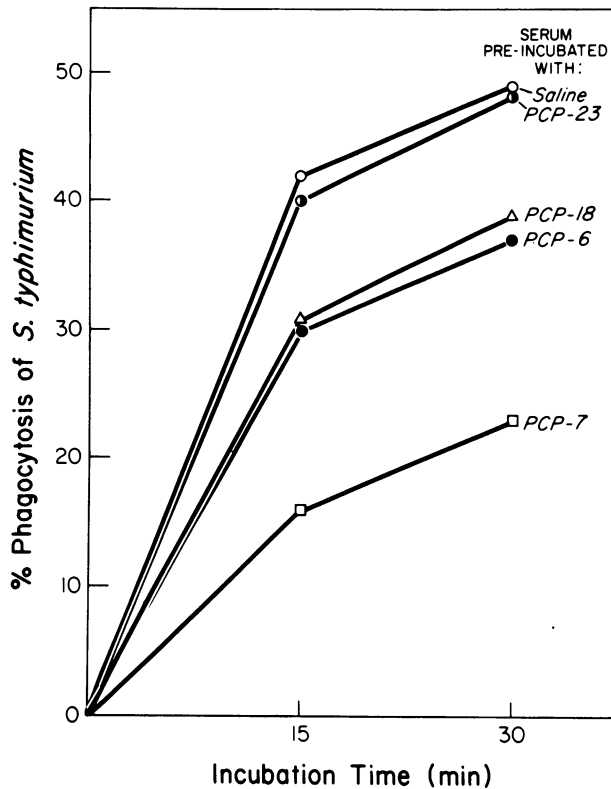


FIG. 2. Effect of pneumococcal capsular polysaccharide (PCP) on serum opsonic capacity. Serum opsonic capacity was directly related to the percent phagocytosis of salmonella using normal serum preincubated for 60 min with 50 $\mu\text{g}/\text{ml}$ polysaccharide from types 6, 7, 18, and 23 pneumococci, and serum preincubated with an equal volume of saline. Following preincubation, serum was diluted to 2.5% and used as an opsonin for salmonella. Opsonized salmonella were incubated with leukocytes, and percent phagocytosis was determined at 15 and 30 min. Each value represents the mean of four experiments. Significance of these differences are shown in Table 4.

polysaccharide at concentrations as low as 5 $\mu\text{g}/\text{ml}$ produced a striking reduction in serum opsonic capacity for type 7 pneumococci. Type 23 polysaccharide concentrations as low as 1 $\mu\text{g}/\text{ml}$ produced a marked reduction in serum opsonic capacity for type 23 pneumococci.

DISCUSSION

Streptococcus pneumoniae is a clinically significant pathogen. Although antibiotics have reduced mortality, the case-fatality rate for bacteremic pneumococcal pneumonia has remained between 20% and 30% over the past decade [19,20]. The frequency of pneumococcal disease has also not changed appreciably. Nielsen reported in 1945 that the majority (48%) of bacteria isolated from the middle ears of children with acute otitis media were *S. pneumoniae* [21]. Three decades later, Howie found a similar frequency of pneumococcal isolates (35%) from children with this condition, and also observed that first episodes of pneumococcal otitis media during infancy predispose children to recurrent acute otitis media [22,23]. *S. pneumoniae* is also the most frequent cause of occult bacteremia in febrile children and in patients with post-splenectomy sepsis [24,25].

TABLE 6
Effect of Capsular Polysaccharide on the Opsonic Capacity of Normal Serum
for the Homologous Pneumococcal Serotype

Serotype	Percentage of initial serum opsonic capacity after incubation of normal serum with:			
	PCP ($\mu\text{g/ml}$)			
	50	10	5	1
7	23%	26%	32%	72%
23	43	37	43	54

Eighty-three distinct pneumococcal serotypes have been described, but only a few types are commonly encountered in patients with pneumococcal infection. These types have remained remarkably constant over the past decade: the majority (78–84%) of isolates from patients with pneumococcal bacteremia, meningitis, and otitis media are types 1, 3, 4, 6, 7, 8, 9, 12, 14, 18, 19, and 23 [19,26,27]. Why certain serotypes predominate and how *S. pneumoniae* injures the host are questions that remain unanswered. Fine observed that pneumococcal serotypes vary in their specific opsonic requirements and described three patterns of pneumococcal opsonization [7]. Several types (7, 12, 14, and 25) decomplexed serum by activating the alternative pathway of complement in the absence of specific antibody, other types (3, 4, and 8) reduced C_3 levels by the interaction of antibody with alternative pathway components and type 1 did not activate C_3 via the alternative pathway even in the presence of specific antibody. These differences in complement fixing ability may give selective advantages to certain serotypes for host invasion and injury. Patients lacking critical pneumococcal opsonins, either because of primary deficiency or secondary to acute infection, may be unable to phagocytize and kill these microbes.

Dorff, Coonrod, and Rytel, using a rapid immunoprecipitin method, counterimmunoelectrophoresis, have detected from 0.1 to 64 $\mu\text{g/ml}$ of PCP in the serum of patients with bacteremia, meningitis, and pneumonia due to types 1, 3, 4, 6, 7, 9, 12, 15, 22, 23, and 34 [1,2,3,4,18,28]. They noted a progressive decline in the level of serum PCP after initiation of therapy but antigenemia persisted for longer than one month in 5 of 19 patients [1]. Others have speculated that PCP might be a subcellular virulence factor causing host injury. Pillemer et al. demonstrated that large amounts (3,000 μg) of types 4 and 14 PCP decomplexed human serum by removal or inactivation of properdin [29]. More recently, PCP of types 1, 4, and 25 in concentrations as low as 10 $\mu\text{g/ml}$ has been shown to reduce C_3 levels in C_4 deficient guinea pig serum, while PCP of types 2, 3, 14, and 19 did not [10]. Others have observed patients with severe pneumococcal disease due to a variety of serotypes, including types 3, 4, and 19, with low levels of factor B of the alternative pathway and normal levels of two early components (Clq , C_4) of the classical pathway [30]. Coonrod and Rylko-Bauer demonstrated that the functional activity of the alternative pathway was markedly depressed during the acute phase of pneumococcal pneumonia, especially in those patients with bacteremia [31]. Thus, we considered that patients with pneumococcal infection and antigenemia may have an acquired alternative pathway defect affecting opsonization of pneumococci.

The pneumococcal serotypes selected for this investigation were all opsonized via the alternative pathway and included both lower and higher numbered types

commonly encountered in human disease. Alternative pathway opsonization of Pn 18 was more efficient, most likely due to higher levels of type 18 pneumococcal antibody in the serum pool employed. The opsonic requirements of types 6, 18, and 23 have been more completely defined in a separate report [5]. It is important to note that our observations relate only to these single strains, and that different strains of a given serotype may vary in their ability to activate complement.

Serum incubated with suspensions of whole, viable *S. pneumoniae* and with purified PCP from each type had reduced biologic activity of C₃ measured by the hemolytic C₃₋₉ assay and by opsonic capacity for *Salmonella typhimurium*. Opsonization of salmonella was selected as a measure of serum opsonic capacity since the organism is opsonized effectively and independently of type-specific pneumococcal antibody via the alternative pathway. Suspensions of whole *S. pneumoniae* showed considerably greater activity than the homologous PCP in reducing hemolytic C₃₋₉ activity. While the quantity of PCP contained in the whole cell preparations was not measured, others have shown for types 1 and 2 *S. pneumoniae* that 5×10^8 bacteria yielded 1.5 and 2.0 μg of polysaccharide, respectively [32]. This finding suggests that PCP might not be the most active subcellular factor in reducing levels of complement. However, the same suspensions of whole types 6, 7, and 23 *S. pneumoniae* were no more active than the highest concentrations of the homologous PCP in reducing serum opsonic capacity. Whole type 18 *S. pneumoniae* were slightly more active than type 18 PCP. It is possible that PCP aggregated on the surface of this strain was more active or that there was simply more PCP in that particular whole cell preparation. We were unable to explain the different results, confirmed in repeated experiments, using the two assays. While these assays measured different endpoints along the complement cascade, the measurement of opsonic capacity would more closely reflect changes in the functional process of opsonization.

Capsular polysaccharide in concentrations as low as 1 $\mu\text{g}/\text{ml}$ significantly reduced the serum opsonic capacity. For at least two types (6 and 18), maximum opsonic inhibition was present at the lowest concentration of PCP studied, and inhibition produced by PCP 7 and 23 was maximal in the mid-range of PCP concentration used. Repeated experiments confirmed the greater activity of PCP 7 and 23 in this mid-range. Suspensions of whole *S. pneumoniae* and PCP of types 7 and 18 also reduced the opsonic capacity of C₂ deficient serum, providing additional evidence that these polysaccharides primarily activated C₃ via the alternative pathway.

The effect of PCP on serum opsonic activity for the homologous pneumococcus was greater than its effect on salmonella opsonization. The greater inhibition of pneumococcal opsonization may have been due to binding of type-specific antibody by PCP. We have shown in previous experiments that antibody enhances pneumococcal opsonization in the presence of complement [5]. Some patients with bacteremic pneumococcal disease show absent or reduced type-specific antibody production associated with high serum levels of PCP [3]. We observed that bacteremic patients with PCP antigenemia have reduced serum complement levels and reduced pneumococcal opsonic activity [33]. Testing these patient sera with the salmonella opsonic assay showed that the opsonic defect effected the alternative pathway. Opsonic activity and serum complement increased during convalescence with the disappearance of PCP antigen. These *in vivo* observations support the *in vitro* experiments with PCP antigen.

Thus, PCP antigen can activate complement and reduce serum opsonic activity. An alternative pathway opsonic defect might be expected to have its greatest impact

on host defenses during the early phase of infection, prior to the development of specific antibody required for utilization of the more efficient classical pathway for opsonization. However, PCP may not be unique in its ability to activate complement since purified pneumococcal cell wall also activates the alternative pathway [34] and inhibits neutrophil phagocytosis [35]. Pneumococcal M protein [36] and C polysaccharide [37] may also activate complement since these elements may in part determine opsonic requirements. It is possible that several or all of these subcellular elements accounted for the greater effect of whole pneumococci compared with PCP on hemolytic complement activity in our experiments. The association of increased PCP levels and increased mortality in bacteremic patients [3] may simply reflect a greater concentration of invading bacteria and, as such, a greater concentration of all the subcellular elements including PCP. To define the relative activities of these elements on inhibition of alternative pathway opsonization, studies are required to compare cell wall, cell membrane, capsular polysaccharide, and whole cell preparations from several strains and serotypes of *S. pneumoniae* in human serum.

REFERENCES

1. Coonrod JD, Drennan DP: Pneumococcal pneumonia: capsular polysaccharide antigenemia and antibody responses. *Ann Intern Med* 84:254-260, 1976
2. Coonrod JD, Leach RP: Antigenemia in fulminant pneumococemia. *Ann Intern Med* 84:561-563, 1976
3. Dee TH, Schiffman G, Sottile MI, et al: Immunologic studies in pneumococcal disease. *J Lab Clin Med* 89:1198-1207, 1977
4. Rytel MW, Dee TH, Ferstenfeld JE, et al: Possible pathogenetic role of capsular antigens in fulminant pneumococcal disease with disseminated intravascular coagulation (DIC). *Am J Med* 57:889-896, 1974
5. Giebink GS, Verhoef J, Peterson PK, et al: Opsonic requirements for phagocytosis of *S. pneumoniae* types 6, 18, 23 and 25. *Infect Immun* 18:291-297, 1977
6. Johnston RB Jr, Klemperer MR, Alper CA, et al: The enhancement of bacterial phagocytosis by serum. The role of complement components and two cofactors. *J Exp Med* 129:1275-1280, 1969
7. Fine DP: Pneumococcal type-associated variability in alternative complement pathway activation. *Infect Immun* 12:772-778, 1975
8. Jasin HE: Human heat-labile opsonins: evidence for their mediation via the alternative pathway of complement activation. *J Immunol* 109:26-31, 1972
9. Williams RC Jr, Quie PG: Opsonic activity of agammaglobulinemic human sera. *J Immunol* 106:51-55, 1971
10. Winkelstein JA, Bocchini JA, Schiffman G: The role of the capsular polysaccharide in the activation of the alternative pathway by the pneumococcus. *J Immunol* 116:367-370, 1976
11. Forsgren A, Quie PG: Influence of the alternate complement pathway on opsonization of several bacterial species. *Infect Immun* 10:402-404, 1974
12. Schiffman G, Austrian R: A radioimmunoassay for the measurement of pneumococcal capsular antigen and of antibodies thereof (abstract). *Fed Proc* 30:658, 1971
13. Fine DP, Marney SR Jr, Colley DG, et al: C₃ shunt activation in human serum chelated with EGTA. *J Immunol* 109:807-809, 1972
14. Kim Y, Friend PS, Dresner IG, et al: Inherited deficiency of the second component of complement (C₂) with membranoproliferative glomerulonephritis. *Am J Med* 62:765-771, 1977
15. Verhoef J, Peterson PK, Kim Y, et al: Opsonic requirements for staphylococcal phagocytosis: heterogeneity among strains. *Immunology* 33:191-197, 1977
16. Gewurz H, Page AR, Pickering RJ, et al: Complement activity and inflammatory neutrophil exudation in man. *Int Arch Allergy* 32:64-90, 1967
17. McLean RH, Geiger H, Burke B, et al: Recurrence of membranoproliferative glomerulonephritis following kidney transplantation. Serum complement component studies. *Am J Med* 60:60-72, 1976
18. Coonrod JD, Rytel MW: Detection of type-specific pneumococcal antigens by counterimmunoelectrophoresis. I. Methodology and immunologic properties of pneumococcal antigens. *J Lab Clin Med* 81:770-777, 1973
19. Austrian R, Gold J: Pneumococcal bacteremia with especial reference to bacteremic pneumococcal pneumonia. *Ann Intern Med* 60:759-776, 1964
20. Sullivan RJ Jr, Dowdle WR, Marine WM, et al: Adult pneumonia in a general hospital. *Arch Int Med* 129:935-942, 1972

21. Neilson JC: Studies of the aetiology of acute otitis media. Copenhagen: Munksgaard, 1945
22. Howie VM, Ploussard JH, Lester RL Jr: Otitis media: a clinical and bacteriological correlation. *Pediatrics* 45:29-35, 1970
23. Howie VM, Ploussard JH, Sloyer J: The "otitis-prone" condition. *Am J Dis Child* 129:676-678, 1975
24. Singer DB: Postsplenectomy sepsis, Perspectives in Pediatric Pathology. Edited by HS Rosenberg and RP Bolande. Chicago: Yearbook Publishers, 1973, pp 285-311
25. Teele DW, Pelton SI, Grant MHA, et al: Bacteremia in febrile children under 2 years of age: results of cultures of blood of 600 consecutive febrile children seen in a "walk-in" clinic. *J Pediatr* 87:227-230, 1975
26. Kaiser AB, Schaffner W: Prospectus: the prevention of bacteremic pneumococcal pneumonia. A conservative appraisal of vaccine intervention. *JAMA* 230:404-408, 1974
27. Sloyer JL Jr, Howie VM, Ploussard JH, et al: Immune response to acute otitis media in children. I. Serotypes isolated and serum and middle ear fluid antibody in pneumococcal otitis media. *Infect Immun* 9:1028-1032, 1974
28. Dorff GJ, Coonrod JD, Rytel MW: Detection by immunoelectrophoresis of antigen in sera of patients with pneumococcal bacteremia. *Lancet* i:578-579, 1971
29. Pillemer L, Schoenberg MD, Blum L, et al: Properdin system and immunity. *Science* 122:545-549, 1955
30. Reed WP, Davidson MS, Williams RC Jr: Complement system in pneumococcal infections. *Infect Immun* 13:1120-1125, 1976
31. Coonrod JD, Rylko-Bauer B: Complement levels in pneumococcal pneumonia. *Infect Immun* 18:14-22, 1977
32. Heidelberger M, MacLeod CM, Kaiser SJ, et al: Antibody formation in volunteers following injection of pneumococci or their type-specific polysaccharides. *J Exp Med* 83:303-310, 1946
33. Giebink GS, Dee TH, Thomas PJ, et al: Decreased opsonic activity during pneumococcal bacteremia (abstract). *Clin Research* 26:523A, 1978
34. Winkelstein JA, Tomasz A: Activation of the alternative pathway by pneumococcal cell walls. *J Immunol* 118:451-454, 1977
35. Dhingra RK, Williams RC Jr, Reed WP: Effects of pneumococcal mucopeptide and capsular polysaccharide on phagocytosis. *Infect Immun* 15:169-174, 1977
36. Austrian R, MacLeod CM: A type-specific protein from pneumococcus. *J Exp Med* 89:439-450, 1949
37. Schiffman G, Bornstein DL, Austrian R: Capsulation of pneumococcus with soluble cell wall-like polysaccharide. II. Non-identity of cell wall and soluble cell wall-like polysaccharides derived from the same and from different pneumococcal strains. *J Exp Med* 134:600-617, 1971