Opsonic Requirements for Phagocytosis of *Streptococcus pneumoniae* types VI, XVIII, XXIII, and XXV

G. SCOTT GIEBINK,† JAN VERHOEF,‡ PHILLIP K. PETERSON,§ AND PAUL G. QUIE†

Departments of Pediatrics† and Medicine,‡ University of Minnesota School of Medicine, Minneapolis, Minnesota 55455

Received for publication 24 January 1977

An assay system employing radiolabeled, heat-killed *Streptococcus pneumoniae* and human polymorphonuclear leukocytes was utilized to study serum pneumococcal opsonic requirements. Comparing the kinetics of phagocytosis in normal serum, heat-inactivated serum, immunoglobulin G (IgG)-deficient serum, C2-deficient serum, and magnesium dichloride ethyleneglycol-tetraacetic acid (MgEGTA)-chelated serum allowed definition of the opsonic requirements for four pneumococcal serotypes: VI, XVIII, XXIII, and XXV. All four serotypes were efficiently opsonized in 10% normal serum. Only type XVIII was opsonized in heat-inactivated serum. All four were also opsonized in IgG-deficient serum but not as efficiently as in normal serum. Opsonization via the alternative pathway was diminished for all four serotypes in 10% MgEGTA-chelated and C2-deficient serum. Furthermore, by varying the concentration of MgEGTA-chelated serum, it was found that type XXV was least efficiently opsonized via the alternative pathway. The quantitative nature of this assay system will permit measurement of bacterial and host factors that may contribute to host susceptibility to pneumococcal infection.

Optimal phagocytosis and subsequent intracellular killing of most bacteria, including *Streptococcus pneumoniae*, require the participation of opsonins, including serum complement and immunoglobulin (Ig). Employing rabbit serum, Johnston et al. showed that the opsonic activity of hyperimmune serum was amplified by complement, whereas opsonization in nonimmune serum was primarily mediated by complement (12). Human antibodies against pneumococcal capsular polysaccharide apparently do not fix complement via the classical complement pathway (10, 21). More recently, experiments have shown that an opsonically active fragment (C3b) can be generated by complement activation via the alternative pathway in the absence of specific antibody (11, 25).

Employing an assay that measured complement consumption via alternative pathway activation, Fine described three distinct patterns of pneumococcal opsonization: (i) C3 activation via the alternative pathway in the absence of antibody (types VII, XII, XIV, and XXV), (ii) C3 activation via the alternative pathway only in the presence of specific antibody (types III, IV, and VIII), and (iii) failure to activate C3 via the alternative pathway even in the presence of specific antibody (type I) (6). He suggested a relationship between virulence and the pattern of opsonization for each serotype. However, seven of the eight types tested are invasive and are frequently recovered from patients with bacteremic infections (1, 13, 15). It would be of interest to compare the opsonic requirements of pneumococcal types that rarely cause bacteremic disease with the commonly recovered types. Fine (6) and Winkelstein et al. (27) were unable to show a difference between noncapsulated and capsulated variants of single serotypes in their degree of complement consumption. Since others have demonstrated the type specificity of the M protein antigen of pneumococcus (2) and the heterogeneity of both capsular and C polysaccharides among pneumococcal strains (19), it is possible that these mucoprotein moieties also determine pneumococcal opsonic requirements.

In this report, pneumococcal opsonic requirements in human serum were directly studied by a sensitive, functional assay. Polymorphonuclear (PMN) leukocyte uptake of heat-killed [3H]thymidine-labeled pneumococci, opsonized in human serum, was measured. The opsonic capacities of normal, heat-inactivated, and IgG-deficient sera were studied. Alternative complement pathway opsonization was assessed in serum chelated with magnesium dichloride ethyleneglycol-tetraacetic acid (MgEGTA) and in human C2-deficient serum. Serum chelated with MgEGTA has been shown by others to support

† Present address: Microbiology Laboratory, Catharijnesingel 59, Utrecht, The Netherlands.
alternative but not classical complement pathway function (7). Normal serum was compared with these selectively deficient sera to determine the contribution of different heat-stable and heat-labile serum factors to the phagocytosis of *S. pneumoniae*. The opsonic requirements of type VI, XVIII, XXIII, and XXV pneumococci were studied, representing both lower- and higher-numbered serotypes frequently isolated from children with pneumococcal otitis media (20).

**MATERIALS AND METHODS**

Pneumococci. *S. pneumoniae* types XVIII, XXIII, and XXV were isolated from children with pneumococcal bacteremia; type VI was obtained from the American Type Culture Collection (Rockville, Md.). Serotypes were confirmed with type-specific antisera (Statens Seruminstitut, Copenhagen, Denmark). Virulent, encapsulated organisms were maintained by mouse passage every 2 to 4 weeks. Several colonies of bacteria were inoculated from sheep blood agar plates into 10 ml of Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) enriched with 3% bovine serum albumin and containing 0.20 mCi of [methyl-3H]thy midine (specific activity, 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.). After 14 to 16 h of incubation at 37°C, 1 ml of sterile 10% d-glucose and an additional 0.20 mCi of [3H]thy midine were added, and the cultures were incubated for an additional 2 h. The bacteria were washed three times with sterile isotonic phosphate-buffered saline (pH 7.4), counted in a Petroff-Hauser chamber, and suspended in phosphate-buffered saline to the desired concentration. The concentration of viable organisms was determined by pour plate colony counts. The bacteria were heat-killed in a 65°C water bath for 45 min, washed once in phosphate-buffered saline, and stored at 4°C for no longer than 14 days.

**Leukocytes.** Blood was drawn from healthy adult donors into a heparinized syringe (10 U of heparin per ml). One-third volume of 6% dextran (Cutter Laboratories, Berkeley, Calif.) was added, and the mixture was allowed to settle at room temperature for 1 h. The leukocyte-rich plasma was removed and centrifuged at 150 × g for 5 min. The leukocyte pellet was washed twice in heparinized saline (10 U of heparin per 10 ml of saline). The concentration of PMN leukocytes was determined by quantitative and differential cell counts and adjusted to 107 PMN leukocytes per ml in Hanks balanced salt solution (Grand Island Biological Co., Grand Island, N.Y.) with 0.1% gelatin (gel-HBSS).

**Opsonins.** Sera from three healthy adult donors were pooled and divided into portions. This serum contained normal levels of specific antibody for *S. pneumoniae* types XVIII and XXIII (688 and 363 ng of antibody nitrogen per ml, respectively) and somewhat lower levels of type VI antibody (92 ng of antibody nitrogen per ml). Antibody determinations and normal values were kindly provided by G. Schifferman, Downstate Medical Center, Brooklyn, N.Y. To study opsonization in the absence of the classical complement pathway, undiluted serum was chelated with Mg-EGTA, in a final concentration of 10 mM, as previously described (7). 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. (Serum was kindly provided by Y. Kim, University of Minnesota.) This serum contains normal levels of all other classical and alternative complement components (12a), and has a normal capacity for alternative pathway opsonization of *Staphylococcus epidermidis* and *S. aureus* Wood 46 (24a) and *Salmonella typhimurium* (unpublished data). To study opsonization in the absence of Ig, serum was obtained from an infant with congenital rubella syndrome (M.E.) in which IgG, IgA, and IgE were nondetectable by Mancini radial immunodiffusion and by Ouchterlony methods; this serum contained 30 ng of IgM per ml and had normal opsonic activity for two strains of *S. aureus*, Cowan I and 502A (24a). All sera were divided into portions and stored at −70°C. Heat-inactivated serum was prepared by incubation at 56°C for 30 min.

**Bacterial opsonization.** To eliminate the inhibitory effect of cation depletion on phagocytosis during the study of MgEGTA-chelated serum (23), and to remove factors in heat-inactivated serum that might adversely affect phagocytosis (9), bacteria were first incubated with each opsonic source; they were then centrifuged, the supernatant was discarded, and leukocytes were added. Specifically, 0.4 ml of the desired opsonin was added to a plastic tube (12 by 75 mm; Falcon Plastics, Oxnard, Calif.) that contained 0.1 ml of radiolabeled, heat-killed pneumococci at 2.5 × 109/ml. After 30 min of incubation at 37°C, the mixture was centrifuged at 1,600 × g for 15 min. The supernatant was discarded, and the bacterial pellet was suspended in 0.4 ml of gel-HBSS. The kinetics of opsonization were evaluated by incubating pneumococci with opsonin for 5, 15, and 30 min before phagocytes were added. In one series of experiments opsonization of live and of heat-killed pneumococci were compared.

**Phagocytosis mixture.** The phagocytosis mixtures consisted of 0.5 ml of the opsonized bacteria (suspended in gel-HBSS) and 0.5 ml of leukocytes. The bacteria/PMN leukocyte ratio was approximately 5:1. Tubes were tumbled at 10 rpm on a rotating rack (Fisher Roto-Rack, Fisher Scientific Co., Chicago, Ill.) at 37°C.

**Determination of phagocytosis.** To determine the leukocyte-associated bacterial population, duplicate 100-μl samples were taken with an Eppendorf pipette after 1, 15, and 30 min of incubation and were placed in 2 ml of cold phosphate-buffered saline in polypropylene vials (Bio-Vials, Beckman, Chicago, Ill.). The vials were centrifuged for 5 min at 160 × g at 4°C. After the supernatants were discarded, the pellets were washed in 1 ml of cold phosphate-buffered saline. The final pellet was suspended in 2.5 ml of scintillation liquid (toluene with 20% Bio-Solve-3 in Fluorollo; Beckman Instruments, Inc., Fullerton, Calif.). To determine the total bacterial population, representing phagocytized and non-leukocyte-associated bacteria, duplicate 100-μl samples were taken at the end of the assay period, placed in 2 ml of phosphate-buffered saline, and centrifuged at 1,600 × g for 15 min. The
pellets were suspended in 2.6 ml of scintillation liquid. The samples were counted in a liquid beta scintillation counter (Beckman, LS-250). Chemical quenching was similar for all samples. An average of the duplicate values was used in all calculations. The large majority of duplicate values were within 20% agreement.

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

\[
\text{% phagocytosis} = \frac{\text{cpm in } 160 \times g \text{ pellet}}{\text{cpm in } 1,600 \times g \text{ pellet}} \times 100
\]

The counts per minute in the denominator was always at least 50 times greater than background radioactivity.

RESULTS

Opsonization of type VI, XVIII, XXIII, and XXV pneumococci. Figure 1 illustrates the kinetics of phagocytosis of \textit{S. pneumoniae} types VI, XVIII, XXIII, and XXV when opsonized in 10% normal, heat-inactivated, IgG-deficient, C2-deficient, and MgEGTA-chelated sera. Approximately 40% of type VI pneumococci opsonized in normal serum were phagocytized after 30 min of incubation, whereas IgG-deficient serum provided only sufficient opsonic activity to allow 20% phagocytosis after 30 min (Fig. 1a). Heat-inactivated serum, C2-deficient serum, and MgEGTA-chelated serum provided negligible opsonin for this serotype, as evidenced by the low leukocyte-associated radioactivity.

Figure 1b illustrates the phagocytosis of type XVIII pneumococci. There was moderate opsonic activity for this serotype in both C2-deficient and MgEGTA-chelated sera. However,
both of these sera were less efficient opsonins than was normal serum. IgG-deficient and heat-inactivated sera provided less opsonic activity, although heat-inactivated serum provided more opsonic activity for type XVIII than for type VI pneumococci.

Type XXIII pneumococci were more efficiently opsonized in 10% normal serum than the other serotypes; 70% were phagocytized after 30 min of incubation (Fig. 1c). No appreciable phagocytosis was observed after opsonization in either heat-inactivated serum or C2-deficient serum. There was slight opsonic activity in IgG-deficient serum and in chelated serum, but phagocytosis was only 20% of that observed when this serotype was opsonized in normal serum.

There was efficient phagocytosis of type XXV pneumococci opsonized in 10% normal serum, whereas IgG-deficient serum provided approximately one-half the opsonic activity of normal serum (Fig. 1d). For this serotype, little opsonic activity was demonstrated in C2-deficient and MgEGTA-chelated sera.

**Effect of serum concentration on opsonization.** Normal serum and MgEGTA-chelated serum were used as opsonins for each of the four pneumococcal serotypes. Samples obtained from the phagocytosis mixtures after 30 min of incubation reflected maximal phagocytosis. Pneumococcal opsonization increased as serum concentration was raised (Fig. 2). Twenty percent normal serum provided as much opsonic activity as did 90% serum for each type. Concentrations of normal serum less than 20% gave more striking reductions in the opsonization of types VI, XVIII, and XXIII than of type XXV. In contrast, type XXV was poorly opsonized in chelated serum at concentrations less than 40%, whereas higher concentrations of chelated and normal serum provided nearly equal opsonizations of this type. Types VI and XXIII were equally well opsonized in chelated and normal serum at concentrations of 20% or more, whereas opsonization of these two types was negligible in concentrations of chelated serum less than 20%. Type XVIII was opsonized less efficiently

![Figure 2](http://iai.asm.org/)

**Fig. 2.** Phagocytosis of type VI, XVIII, XXIII, and XXV S. pneumoniae after opsonization in serial dilutions of normal serum and MgEGTA-chelated serum. Bacteria were opsonized for 30 min before addition of PMN leukocytes, and samples were obtained after a 30-min incubation of the phagocytosis mixtures.
than types VI and XXIII in 20% chelated serum, but was opsonized more efficiently than type XXV at this serum concentration \((P < 0.01, \text{Student's} \ t \text{ test for unpaired observations})\). In addition, type XVIII was opsonized significantly more efficiently than the other three types in 10% chelated serum \((P < 0.005)\).

**Kinetics of alternative pathway opsonization.** Experiments were performed to compare the time required for opsonization of pneumococci in normal and MgEGTA-chelated serum. Type XXIII pneumococci were incubated for 5 and 15 min in 20% normal and 20% MgEGTA-chelated serum, and the kinetics of phagocytosis were compared (Fig. 3). After incubation of pneumococci with opsonin, the opsonin was removed by centrifugation before leukocytes were added, thereby preventing any influence of the chelator might have on phagocytosis. Opsonization in normal serum was complete within 5 min since there was no difference between the rates of phagocytosis of bacteria opsonized for 5 or 15 min. Opsonization in chelated serum, however, was incomplete after 5 min of incubation; bacteria opsonized for the shorter period showed less than those opsonized for 15 min.

**Phagocytosis of live, heat-killed, and stored pneumococci.** Live and heat-killed pneumococci were opsonized in 20% normal pooled serum, and rates of phagocytosis were compared. The kinetics of phagocytosis of live and heat-killed organisms were similar during the first 30 min of incubation for all four serotypes. Heat-killed pneumococci that had been stored for up to 2 weeks at 4°C retained their type-specific Quellung reaction and the original bacterial concentration. The kinetics of phagocytosis of heat-killed bacteria remained the same during the storage period. In addition, there was no detectable free capsular polysaccharide in the supernatant of heat-killed pneumococcal suspensions when measured by counterimunoelectrophoresis, which in our laboratory detects as little as 0.15 µg of polysaccharide per ml by a standard method (4).

**DISCUSSION**

A frequently employed method for determination of pneumococcal opsonic activity has been the phagocytic index, which is an enumeration of the number of neutrophils ingesting bacteria during incubation with an opsonic source (12, 28). Due to the low degree of sensitivity inherent in this technique, several more sensitive but indirect assays have been developed for measuring serum pneumococcal opsonic activity. Fine used residual hemolytic complement activity as an index of opsonic activity (6). Although this method is sensitive, it is dependent on the measurement of residual activity of multiple serum factors and does not necessarily reflect the functional aspect of opsonization. Forsgren and Quie used a functional assay for describing serum opsonic activity that depended on intracellular killing as a measure of phagocytosis (8). This method is indirect, in that some bacterial strains may be killed at a slow rate even though they are opsonized and phagocytized efficiently. Others have used an assay system that measures the reduction of nitroblue tetrazolium dye by phagocytic cells as an index of phagocytosis (12). Although this is a functional assay, the end point depends on intracellular metabolic activity of the phagocytes, which may reflect more than pneumococcal opsonization and ingestion.

Recently, methods have been described for measurement of bacterial phagocytosis, utilizing radiolabeled bacteria in functional assay systems (5, 12, 14, 16, 18, 24). These methods, and the modifications used for the investigation reported here, allow quantitation of phagocytized radiolabeled bacteria and provide a precise estimation of opsonization and phagocytosis. Phagocytosis in this context has been defined as comprising
both bacterial attachment to the phagocyte membrane and internalization (17).

Our assay system employed [3H]thymidine-labeled, heat-killed pneumococci and human granulocytes. Phagocytes were maintained in suspension in this assay, in contrast to glass-adherent phagocytes used in a previous report (12). The availability of IgG-deficient serum and C2-deficient serum allowed comparison of the kinetics of phagocytosis in several opsonic sources and permitted the study of opsonic requirements of four pneumococcal serotypes.

All four serotypes were efficiently opsonized in 10% normal serum. Unlike the other serotypes, type XVIII was opsonized in heat-inactivated serum, suggesting that heat-stable serum factors alone were opsonic for this serotype. It is possible that the higher level of type XVIII antibody found in the serum pool employed for these experiments provided these additional heat-stable factors and that type-specific pneumococcal antibody may enhance opsonic activity against the autologous serotype.

All four pneumococcal serotypes were opsonized effectively via the alternative pathway in 90% MgEGTA-chelated serum. Others have also observed efficient alternative pathway opsonization of S. pneumoniae (type XXV) in undiluted human serum (6) and in C4-deficient guinea pig serum (18). This report illustrates that testing dilutions of chelated and normal sera for opsonic activity more precisely describes differences among serotypes in the efficiency of alternative pathway opsonization. Ten percent chelated and C2-deficient sera provided sufficient alternative pathway opsonic activity only for type XVIII. Although pneumococcal antibody concentration was not determined in the C2-deficient patient, it is possible that type XVIII antibody mediated the enhanced opsonization of this type via the alternative pathway in both sera. Although only type XVIII was opsonized in 10% chelated serum, serum concentration had to be raised to 40% to obtain maximal opsonization of this type via the alternative pathway. In contrast, types VI and XXIII were maximally opsonized via the alternative pathway in 20% chelated serum. Type XXV was most difficult to opsonize via the alternative pathway, since 40% chelated serum was required to obtain maximal alternative-pathway opsonization.

These observations give evidence that pneumococcal types vary in their capacity to activate the alternative pathway. In addition, we observed that opsonization of pneumococci is slower when classical pathway opsonization is blocked by MgEGTA chelation. Root et al. observed a similar lag in alternative pathway opsonization with C4-deficient guinea pig serum (18). Thus, to evaluate the contribution of the alternative pathway to serum opsonic activity, bacteria must be incubated for sufficient time to allow maximal opsonization in test sera, and it may be necessary to use several dilutions of serum.

Serum deficient in IgG provided opsonic activity for all four pneumococcal serotypes tested, suggesting that complement alone acted as an effective opsonin. We did not, however, exclude any contribution to complement-mediated opsonization made by the small amount of IgM present in this serum.

The results of this study confirm a previous observation of variable opsonic requirements among pneumococcal serotypes (6). Phagocytosis of radiolabeled opsonized pneumococci in selectively deficient sera demonstrated differences in the relative degree of IgG-independent and alternative-pathway opsonization among four pneumococcal serotypes. There may also be strain differences within particular serotypes. Stephens et al. have shown variability among pneumococcal strains for reactivity with the Fc region of IgG (22). In addition, Bjorksten et al. have observed strain differences for alternative-pathway opsonization among Escherichia coli (3).

The heterogeneity among pneumococcal serotypes for alternative-pathway opsonization may in part explain the predominance of certain types in human infection and may be of importance in determining host response. Others have reported that whole pneumococcal cells and pneumococcal capsular polysaccharide antigen from some types depress C3 levels in vitro by activating the alternative pathway (27). Winkelstein et al. have shown that reduction in serum C3, produced in C5-deficient mice after treatment with cobra venom factor, significantly reduced serum pneumococcal opsonic activity and increased susceptibility to pneumococcal disease (29). Pre-opsonization of pneumococci with heat-labile serum factors before inoculation of treated animals prevented the increased susceptibility to infection. Thus, a defect in pneumococcal opsonization may occur during overwhelming pneumococcal infection, with subsequent depletion of C3. This defect may be more striking for those serotypes requiring greater concentrations of alternative-pathway factors for opsonization. The specific pneumococcal opsonic requirements may also explain the predisposition of certain patients, having selective deficiencies of specific antibodies, nonspecific IgM or IgG, complement components, or other serum cofactors, to pneumococcal infection (6, 22, 26). The precise quantitation provided by these
methods will allow measurement of bacterial and host factors that may contribute to host susceptibility to pneumococcal infection.

ACKNOWLEDGMENTS

We thank Karen Petty for her technical skills and Kathy Hoogenakker and Patti Lorenz for typing the manuscript. This research was supported by Public Health Service contract NO1-AI-52533 and grant AI-08821 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED


