Effect of Intravenously Injected Killed Pneumococci on Leukocytes, Complement, and Phagocytosis in Rabbits

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A pneumococcal infection may be lethal in the absence of overwhelming pulmonary involvement, and death may occur even after the organisms have been killed with antibiotics. The mechanism of death is not understood but may be related to circulating pneumococcal products. For investigating the effects of nonviable pneumococci on several host defense mechanisms, rabbits were injected intravenously with 4×10^8 colony-forming units of killed sonified type 13 or type 29 pneumococci. Blood was sampled periodically for the next 24 h, and the following were measured: (i) circulating levels of leukocytes; (ii) activity of the classical and alternate complement pathways; and (iii) ability of the serum to opsonize pneumococci for ingestion and killing by polymorphonuclear leukocytes. Saline-injected control rabbits showed no change in any of the functions. Non-immune rabbits injected with either pneumococcal serotype showed progressive and profound leukopenia, no change or an increase in classical and alternate complement pathway activity, and a profound reduction in the serum-opsonizing capacity for pneumococci of the same serotype as that used in the injection. The opsonizing capacity remained normal for the other serotype. When a previously immunized animal was injected, the opsonizing capacity for the homologous organism remained intact, but leukopenia nevertheless occurred.

The mortality from severe pneumococcal infections remains disturbingly high in the first few days of treatment, even when the organisms are sensitive to antimicrobial drugs and are rapidly killed by appropriate therapy (1, 15). This early mortality in the absence of viable organisms suggests that products from nonviable bacteria or some aspect of the host response may lead to death. As early as 1932 it was shown that an injection of a type 2 soluble pneumococcal substance depressed resistance to a subsequent intravenous inoculation of the homologous organisms (17). Previous studies from this laboratory have shown that pneumococcal cell wall components added in vitro to human serum can interfere with the ability of the serum to opsonize pneumococci and promote phagocytosis by polymorphonuclear neutrophils (PMNs) (7). The present study was designed to evaluate whether killed pneumococci injected intravenously into rabbits interfere with subsequent antibacterial functions. We studied peripheral leukocyte counts, the classical and alternate complement pathways, and the in vitro opsonizing capacity of the rabbit serum after the injections.

MATERIALS AND METHODS

Bacteria. One strain each of type 13 and type 29 *Streptococcus pneumoniae* was obtained from lyophilized stock, and the serotypes were verified by the

quellung reaction. Before use the strains were grown for 16 to 18 h in 5% CO₂ at 37°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.). The organisms were then washed three times and suspended in normal saline to a concentration of 10^8 colony-forming units per ml. The suspension was sonified at a 60-W output in an ice bath for 40 min with interruptions every 3 min for cooling (Sonifier W1855 with cell disrupter; Heat Systems—Ultrasonics Inc., Plainview, N.Y.). Killing of the sonified pneumococci was verified as complete by plating the suspension on blood agar and incubating for 48 h. Thus, the material that was injected into rabbits contained the sonified debris from 10^8 colony-forming units per ml but no living organisms.

Rabbit inoculations. New Zealand white rabbits with no previous laboratory pneumococcal exposure received 0.4 ml of the sonified pneumococcal suspension (derived from 4×10^8 colony-forming units) administered intravenously into the marginal ear vein. Blood samples were withdrawn from the ear immediately before the inoculation and at the following times after the inoculation: 0.5, 1, 1.5, 2, 3, 4, and 24 h. Leukocyte and differential counts were performed on each sample, and the serum was separated and frozen at -80°C in 0.5-ml aliquots for later evaluation in batches for complement activity and pneumococcal opsonizing capacity. Control rabbits were inoculated with sterile normal saline, and blood was withdrawn and treated similarly.

Phagocytic killing test. These studies used the phagocytic test of Maaløe (12) as modified by Hirsch

and Strauss (10), with further modifications to be described. Plastic tubes with 1 ml of an incubation mixture in gel Hanks solution (1% gelatin) contained approximately 2×10^6 washed normal rabbit PMNs obtained by the sedimentation of blood in 3% swine skin gelatin in saline (Sigma Chemical Co., St. Louis, Mo.), 2×10^6 washed pneumococci from an 18-h broth culture, and 0.1 ml of the rabbit serum which was being studied for its capacity as a source of opsonins. All tests included control tubes with polymorphonuclear leukocytes omitted. After thorough mixing, 0.002 ml was removed with a calibrated platinum loop for bacterial counting. The tubes were then tightly stoppered and incubated at 37°C with end-over-end rotation at 12 rpm. Samples for bacterial counting were again removed after 30, 60, and 120 min of incubation. In the original method, the samples for bacterial counting were placed in distilled water to disrupt the polymorphonuclear leukocytes and spread the un-killed organisms. However, many strains of pneumococci are killed by distilled water, so each counting sample was diluted in 2 ml of hypotonic saline (0.63 g of NaCl per 100 ml), which was shown microscopically to disrupt the PMNs, and two samples (0.1 ml each) were pipetted into separate petri dishes and mixed with 15 ml of warm (45 to 47°C) brain heart infusion agar (Difco). Colonies were counted electronically (Biotron automated colony counter model C 111; New Brunswick Scientific Co., New Brunswick, N.J.) after incubation for 24 to 36 h at 37°C in the presence of 5% CO₂. The average counts from each pair of plates were expressed as a percentage of the initial counts, and plots of survival were made. When the same serum sample was used in five separate phagocytosis tests with the same pneumococcal strain, the mean survival after 120 min of incubation was $17 \pm 6.32\%$, with a standard error of the mean of 2.8%.

Complement activity. The overall activity of the classical and common final pathways was evaluated by measuring hemolytic complement in 50% hemolytic complement (CH₅₀) units as described for rabbit serum by Nelson and Biro (13). When the same serum sample was used in six separate determinations, the mean was 100.1 ± 14.9 CH₅₀ units, with a standard error of the mean of 3.9 CH₅₀ units. The overall functional activity of the alternate and common final pathways was measured by the ability of serum to kill immunoglobulin G-coated *Escherichia coli* O14 in the presence of 0.01 M ethylene glycol-bis(β -aminoethyl ether)-N,N-tetraacetic acid (Sigma) and 0.01 M MgCl₂ as previously described (18). In a manner analogous to that used for the assay for hemolytic complement, results were reported as the serum dilution required for the killing of 50% of 8×10^8 immunoglobulin G-coated *E. coli* O14 organisms. Units of measurement are represented by the term CB₅₀, which is used to emphasize the conceptual similarity between bactericidal (alternate pathway) and hemolytic (classical pathway) complement assays. When the same serum sample was used in four separate determinations, the mean was 38.6 ± 0.6 CB₅₀ units, with a standard error of the mean of 0.3 CB₅₀ units.

Antibody titers. Rabbit serum was evaluated for antibodies to type 13 and type 29 pneumococci by a modification of the coagglutination technique de-

scribed by Kronvall (11). This technique uses protein A on *Staphylococcus aureus* to bind immunoglobulin G by its Fc portion, leaving the Fab portion free to react with the antigen. When coated with sufficient antipneumococcal antibody, the *S. aureus* will coagglutinate with the specific pneumococci.

The Cowan I strain of *S. aureus* was grown overnight in brain heart infusion broth, washed twice in phosphate-buffered saline at pH 7.4, and suspended in 0.5% formaldehyde in phosphate-buffered saline for 3 h at room temperature. After washing 4 times in phosphate-buffered saline, the organisms were suspended to an optical density at 620 nm of 0.6. The rabbit sera were heated at 60°C for 30 min to inactivate complement and diluted 1:500, 1:1,000, 1:2,000, 1:3,000, 1:4,000, 1:6,000, 1:8,000, 1:12,000, and 1:16,000. Equal quantities of the staphylococcal suspension and the diluted serum were incubated at 37°C for 30 min.

The appropriate pneumococcal strain was grown overnight in brain heart infusion broth, washed twice in phosphate-buffered saline, suspended in phosphate-buffered saline to an optical density at 620 nm of 0.6, and then diluted 1:10 in phosphate-buffered saline. A 2- μ l amount of the pneumococcal suspension was added to 4 μ l of the antiserum-treated *S. aureus* suspension in microtiter plates, and the resulting suspension of both organisms was incubated overnight in a humid chamber at room temperature. Results were read by placing a portion of each suspension on a microscopic slide and examining microscopically with a high, dry objective for any agglutination. There was a clear distinction between the highest dilution that was positive and contained small clumps of organisms and the lowest dilution that was negative and contained single organisms or organisms in unclumped short chains. This technique is highly sensitive to immunoglobulin G antibodies, thus producing high titers even in nonimmune rabbits.

RESULTS

Leukocyte counts. Five control rabbits were inoculated intravenously with saline, and five experimental rabbits received 4×10^8 colony-forming units of sonified *S. pneumoniae* (two received type 13, and three received type 29). The five control rabbits showed no consistent change in the leukocyte count over 4 h, but the five rabbits receiving bacterial products all showed a decrease in the leukocyte count (Fig. 1). Differential counts also showed a shift, with the proportion of PMNs dropping from a normal mean of 18% before the injection to 7% at 0.5 h after the injection.

Complement. Sequential serum samples from the animals injected with the pneumococcal sonicate showed little change in the total hemolytic complement (principally a measure of classical and common final pathway activity) until two h after the injection (Fig. 2). At that time different animals showed a remarkably variable response, but the mean increased greatly and returned to normal by 24 h. The total bac-

FIG. 1. Leukocyte count of rabbits before and after the intravenous injection of pneumococcal products and saline. Vertical bars show the range of results, and the connecting lines show the mean for five rabbits in each group.

FIG. 2. Total hemolytic complement of five rabbits before and after the intravenous injection of pneumococcal products. Vertical bars show the range of results, and the connecting line shows the mean. This assay measures primarily the functional activity of the classical and common final complement pathways.

tericidal complement with the classical pathway blocked (a measure of alternate and common final pathway activity) showed little change until 3 h, at which time the animals also showed a

divergent response, and the mean level increased (Fig. 3). It should be noted that the increase in hemolytic complement occurred in rabbits different from those that showed the increase in bactericidal complement.

Opsonization. Serum was obtained from eight rabbits that received intravenous pneumococcal products (three received type 13, and five received type 29) and from five rabbits that received intravenous saline. An example of the phagocytic killing curves for type 29 pneumococci with serum obtained from a rabbit (rabbit number 1) before and at various time intervals after the injection of type 29 pneumococcal products is shown in Fig. 4. There was marked progressive impairment in the opsonizing ability of sera obtained after the injection, with the defect being most apparent in the serum sample drawn at 4 h. The defect was present at the first, or 30-min, sampling of the phagocytic incubation mixture and persisted in all postinjection sera when the mixture was sampled after 60 to 120 min of incubation. Antibody titers for sera are shown in Table 1. A titer of 1:2,000 against type 29 pneumococci was present before the injection, and this progressively decreased in the postinjection sera.

Sera drawn from the five rabbits at various

FIG. 3. Total bactericidal complement in the presence of EGTA and Mg^{2+} for the same rabbits represented by Fig. 2. Vertical bars show the range of results, and the connecting line shows the mean. This assay measures the functional activity of the alternate and common final complement pathways.

times after the injection of type 29 pneumococcal components were compared with sera from five saline-injected control rabbits for the ability to opsonize and promote the killing of type 29 pneumococci after 30 min of incubation in the phagocytic mixture (Fig. 5). Sera drawn after the pneumococcal injection progressively lost opsonizing capacity, whereas sera drawn from control rabbits up to 4 h after the saline injection maintained the ability to opsonize and kill pneumococci.

Similarly, sera from three rabbits injected with type 13 pneumococci were compared with sera from the same five control rabbits for the ability to opsonize type 13 pneumococci (Fig. 6).

FIG. 4. Phagocytic killing of type 29 pneumococci, the source of opsonin being serum drawn from a single rabbit (rabbit number 1) before and after the intravenous injection of type 29 pneumococcal products. Each postinjection serum sample is represented by a single line, with the time after the injection indicated beside the line. More surviving bacteria indicate a less effective opsonization by the serum sample.

As with type 29, there was severe impairment of the opsonizing capacity for type 13 pneumococci in those animals injected with type 13 organisms but no impairment after the saline injection.

Sera from rabbits injected with type 29 pneumococci, and those from type 13-injected rab-

FIG. 5. Survival of type 29 pneumococci after 30 min in the phagocytic killing mixture for five rabbits injected with type 29 pneumococci and five rabbits injected with saline. The number of organisms surviving in the phagocytic mixture when sera drawn at each time interval were used are compared with those surviving when preinjection sera were used. The range is shown by vertical bars, and the mean is shown by connecting lines. More surviving bacteria indicate a less effective opsonization by the serum sample.

TABLE 1. Antipneumococcal antibody titers before and after the injection of type 29 pneumococcal products into rabbit number 1

Time serum drawn after injection of	Titer after:			
	Injection no. 1 (nonimmune) for:		Injection no. 2 (immune) for:	
	Anti-type 13 pneumococci	Anti-type 29 pneumococci	Anti-type 13 pneumococci	Anti-type 29 pneumococci
0 (preinjection)	1:1,000	1:2,000	1:1,000	1:12,000
0.5	1:1,000	1:1,000	1:1,000	1:3,000
1	1:1,000	1:1,000	1:1,000	1:3,000
2	1:1,000	1:500	1:1,000	1:2,000
3	1:1,000	0	1:1,000	1:2,000
4	1:1,000	0	1:500	1:2,000

bits, were examined for the ability to opsonize type 29 pneumococci. Results showed no inhibition of phagocytosis (Fig. 7).

The rabbit injected with type 29 pneumococci (rabbit number 1) (Fig. 4) was rechallenged with type 29 pneumococci 6 months later. A similar profound leukopenia developed, but sera from this immune animal showed only minor phagocytic inhibition after the injection (Fig. 8). The antibody titer was high before the injection, and

although the titers decreased in the postinjection sera, they remained measurable (Table 1).

DISCUSSION

Defense mechanisms used against pneumococci are imperfectly understood, although it is clear that alveolar macrophages, antibody, complement, and PMNs all have important roles in killing the organisms. In systemic infection these components appear to work in concert, with the serum factors opsonizing the organisms for ingestion and killing by phagocytic cells. This study examined the effect of nonviable pneumococcal debris on aspects of the phagocytic process and has demonstrated that an intravenous injection of these components into nonimmune rabbits produces the following: (i) profound leukopenia; (ii) no change or an increase in the activity of the classical and alternate complement pathways; and (iii) type-specific inhibition of the opsonizing capacity of the postinjection serum. In a single immune animal the postinjection leukopenia still occurred, but there was only minimal inhibition of the serum opsonizing capacity for the homologous organism. Therefore, the two adverse effects of an intravenous injection of pneumococcal products shown in this study (leukopenia and decreased serum opsonizing capacity) may be independent.

The rapid occurrence of leukopenia suggests margination or sequestration of PMNs in some location, perhaps analogous to the complement-

FIG. 6. Survival of type 13 pneumococci after 30 min in the phagocytic killing mixture for three rabbits injected with type 13 pneumococci and five rabbits injected with saline. Results are presented in the same manner as in Fig. 5.

FIG. 7. Survival after 30 min in the phagocytic killing mixture of type 13 pneumococci, with serum from three rabbits injected with type 29 pneumococci as the source of opsonin, and of type 29 pneumococci, with serum from three rabbits injected with type 13 pneumococci as the source of opsonin. The range is shown by vertical bars, and the mean is shown by connecting lines.

FIG. 8. Phagocytic killing of type 29 pneumococci, with the source of opsonin being serum drawn from rabbit number 1 (Fig. 4) before and after a second intravenous injection of type 29 pneumococcal products. The prior injection had been 6 months earlier. Each postinjection serum sample is represented by a single line, with the time after the injection indicated beside the line. A comparison with the survival curves shown in Fig. 4 shows much less depletion in opsonic activity.

mediated pulmonary leukostasis that occurs after the institution of hemodialysis in humans (5) or the destruction of PMNs that may result from pneumococcal leukocidin (14). The sequestration of PMNs in the pulmonary vascular bed has been described in experimental pneumococcal sepsis (6). Current data from our laboratory (S. E. Goldblum, W. P. Reed, R. Sopher, and D. L. Palmer, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 19th, Boston, Mass., abstr. no. 1090, 1979) indicate that indeed pulmonary sequestration of PMNs regularly follows the intravenous administration of pneumococcal products. Such sequestration of PMNs at a site where localized infection is present could be interpreted as a major advantage to the host. However, in our experiments there was no focus of infection, and therefore, there was no obvious advantage to the host in having PMNs marginated or sequestered. The occurrence of leukopenia has been identified as a poor prognostic factor in human and rabbit pneumococcal infections (1, 15). It is of interest that even pneumococcal products from disrupted organisms may be involved in this process.

Previous studies from this laboratory showed non-type-specific inhibition of phagocytosis when pneumococcal mucopeptide was incubated in vitro with serum before the same serum was used as a source of opsonin in the phagocytic test and also suggested that the nonspecific inhibition occurred as a result of complement being consumed during the incubation (7). In contrast, the present in vivo studies showed that complement levels remained adequate for 24 h after the injections of killed pneumococci. Perhaps consumption of complement occurred, but if so, the components must have been rapidly replaced. Indeed, in some animals a very rapid rate of complement synthesis probably occurred and led to an increase in the activity of either the classical or alternate complement pathways.

As complement levels did not drop, it is not surprising that only type-specific inhibition of opsonization occurred, and it is also not surprising that postinjection serum from an immune animal showed less inhibition. Previous in vitro studies of the opsonic requirements for pneumococci have shown that immunoglobulin is important for initiating opsonization, although major augmentation comes from fixing complement to the bacterial surfaces (8, 9, 18, 19). The load of pneumococcal antigens during human infections is not known, but presumably it could be quite large during extensive or bacteremic infections that produce high circulating levels of pneumococcal polysaccharide (2). The load of antigen used in these studies was not sufficiently

large to decrease complement activity, and larger numbers of organisms were not used in preparing the injection mixture because preliminary trials had shown that death frequently resulted. However, decreased complement levels occur early during pneumococcal infections in humans (4, 16).

Products from whole pneumococci were injected in these studies, so it was not possible to determine which of the constituents was responsible for the observed effects. However, as the opsonic inhibition was type specific, it seems likely that the type-specific polysaccharide was responsible for that phenomenon. The striking demonstration of the in vivo depletion of serum opsonic capacity after the injection of pneumococcal products into nonimmune animals represents a dramatic example of the importance of humoral immunity in pneumococcal infections. Furthermore, it underscores the importance of prophylactic immunization for populations at risk. In contrast, the leukopenia may have been caused either by the polysaccharide or by some other constituent. For instance, pneumococcal cell wall components can activate complement (3, 7), and complement activation has been shown to lead to leukopenia under some circumstances (5).

These studies have shown that nonviable pneumococcal products given intravenously can profoundly alter host defense mechanisms. Further studies will be directed towards understanding whether these alterations can be related to the mortality still seen in treated patients with pneumococcal disease (1).

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