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A Pneumolysin-Negative Mutant of *Streptococcus pneumoniae* Causes Chronic Bacteremia Rather Than Acute Sepsis in Mice

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Pneumolysin is a cytoplasmic virulence factor of *Streptococcus pneumoniae* that can interfere with phagocyte function in vitro. We have examined the effects of pneumolysin in vitro and in vivo and have found that it protects intravenously injected pneumococci against infection-induced host resistance. We employed a virulent capsular type 2 pneumococcal strain, D39, and its isogenic pneumolysin-negative mutant, PLN. Strain D39 exhibited exponential net growth in mice (doubling time, 1.4 h); 24 to 28 h after infection with 10^4 CFU, the numbers of pneumococci reached 10^9 to 10^{10} CFU/ml and the mice died. Strain PLN yielded identical net growth in mice until reaching 10^6 to 10^7 CFU/ml at 12 to 18 h postinfection. At this time, the increase in the level of PLN CFU per milliliter ceased and remained constant for several days. PLN exhibited wild-type growth kinetics in mice when coinfected simultaneously with strain D39. This observation suggests that pneumolysin exerts its effects at a distance. By 12 to 18 h postinfection with PLN, mice exhibited the following evidence of an induced inflammatory response: (i) elevated plasma interleukin-6, (ii) a halt in the net growth of PLN, and (iii) control of the net growth of pneumolysin-producing D39 pneumococci upon subsequent challenge. Our data suggest that pneumolysin plays a critical role in sepsis during the first few hours after infection by enabling pneumococci to cause acute sepsis rather than a chronic bacteremia. However, once chronic bacteremia was established, it appeared that pneumolysin was no longer able to act as a virulence factor.

Infections with *Streptococcus pneumoniae* in the respiratory tract and middle ear frequently result in shedding of pneumococci into the bloodstream, which can lead to pneumococcal sepsis. Invariably, sepsis is associated with a poor clinical outcome regardless of the site of the original infection. The pneumococcus and its products, and/or host factors (including cytokines) produced in response to pneumococcal infection, may be responsible for much of the toxicity and poor outcome associated with septicemia (20).

Pneumolysin is an intracellular protein toxin of *S. pneumoniae* that is present in virtually all clinical isolates (21) and shows minimal variation in structure (4, 26, 36). Pneumolysin is thought to be released in vivo by autolysin-dependent autolysis (5, 6, 17). Pneumolysin has been shown to be a pneumococcal virulence factor; pneumococcal strains unable to make functional pneumolysin allow increased survival times for mice challenged intranasally and a 10- to 100-fold increase in intranasal and intraperitoneal 50% lethal doses (7). Immunization with pneumolysin results in the production of antibodies to the toxin and results in an extension of life in challenge studies similar to that seen when pneumolysin is absent genetically (30, 31).

Numerous effects of pneumolysin on eukaryotic cells have been demonstrated. The toxin, like other members of the sulfhydryl-activated toxin family, damages eukaryotic membranes by binding to cholesterol present in membranes, forming oligomers, and creating transmembrane pores (19). Pneumolysin is known to interfere with the functions of cells of the immune system by apparently being released in vivo during the autolysis of pneumococci (5, 6, 17). It has been shown to inhibit human polymorphonuclear leukocyte (PMN) respiratory burst and migration (18, 29) and bactericidal activity (29). The toxin can activate the classical pathway of complement in the absence of antibodies to pneumolysin (8, 9, 32). Sublytic concentrations of purified pneumolysin have been shown to stimulate production of tumor necrosis factor alpha and interleukin-1β (IL-1β) by mononuclear phagocytes in vitro (16). Thus, pneumolysin may act as a virulence factor through a direct effect on immune cells and complement or on cytokine production.

In gram-negative infections, it is generally accepted that lipopolysaccharide stimulates tumor necrosis factor alpha secretion, which leads to the production of IL-1, IL-6, C-reactive protein, and other acute-phase proteins (1). Like gram-negative infections, gram-positive infections are associated with high levels of serum IL-6 (14). C-reactive protein was originally identified as an acute-phase protein in pneumococcal infections (2). Lethal pneumococcal septicemia in pigs is associated with a sharp rise in IL-6 levels (38). Pneumococcal cell wall preparations stimulate the production of IL-1 but not tumor necrosis factor alpha in vitro (34).

In this study, we determined the detailed kinetics of growth and clearance from the blood of CBA/N mice of a pneumolysin-deficient mutant (strain PLN) and its isogenic capsular type 2 parent (strain D39). In a previous study with time points at 1 min, 4 h, 13 h, and 30 h, we determined that the net growth of PLN appeared to be significantly slower than that of D39 in mice infected with 10^6 CFU (7). Our present data, resulting from the use of more closely spaced time points and inocula of 10^3 to 10^6 CFU, demonstrate that the growth kinetics in mice of these two strains are identical until a level of 10^6 to 10^7 CFU/ml is reached. The numbers of PLN pneumococci in the blood were then maintained at 10^6 to 10^7 CFU/ml for several days, whereas the numbers of D39 pneumococci increased exponentially from injection until the death of the mice at 24 to 28 h postinfection at 10^9 to 10^{10} CFU/ml. To examine the underlying mechanism(s) for these differences, we have obtained results indicating that an apparently protective inflammatory response is elicited when the infection with PLN reaches a level of 10^6 to 10^7 CFU/ml of blood. One aspect of

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this inflammation is a strong host immune response that can control the net growth of pneumolysin-negative as well as pneumolysin-producing pneumococci.

MATERIALS AND METHODS

Bacterial strains. We used a virulent capsular type 2 *S. pneumoniae* strain, D39 (3, 25), and its isogenic pneumolysin-deficient mutant, PLN. The pneumolysin-negative strain was created through insertion-duplication mutagenesis and contains an erythromycin resistance marker in the insert (7). Spontaneous streptomycin-resistant mutants of pneumococcal strains D39, WU2 (capsular type 3), EF3296 (capsular type 4), and BG9163 (capsular type 6B) were isolated following growth in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) or on blood agar plates containing 1 mg of streptomycin per ml. These strains were shown to be no less virulent than their antibiotic susceptible parents. S43/29R, a streptomycin-resistant mouse-virulent type M6 group A streptococcal strain, was provided by Susan Hollingshead, Department of Microbiology, University of Alabama at Birmingham.

Mice. CBA/CAHN-XID/J (CBA/N) mice, 6 to 12 weeks old, obtained from Jackson Laboratories (Bar Harbor, Maine), were used for all studies. CBA/N mice carry an X-linked immunodeficiency and are unable to produce antibodies to polysaccharides.

Intravenous infection of mice. Pneumococcal cultures were grown to the late log phase in THY. Pneumococci were diluted on the basis of the optical density at 420 nm (OD_{420}; OD of 0.2 = 5×10^7/ml) into lactated Ringer's solution, injected intravenously in a volume of 0.2 ml, and plated on blood agar to confirm the numbers of CFU per milliliter. Mice were bled retroorbitally with a 75-μl heparinized microhematocrit capillary tube (Fisher Scientific) at selected times postinfection. The number of CFU per milliliter of blood was determined by plating serial dilutions of blood obtained from individual mice on blood agar plates with or without 0.3 μg of erythromycin per ml or 1 mg of streptomycin per ml as appropriate.

In experiments in which mice were infected simultaneously with two strains of pneumococci, the strains were grown individually in THY and diluted on the basis of the OD_{420} to obtain the desired inoculum in a volume of 0.1 ml. Mice were then inoculated intravenously with 0.2 ml containing equal numbers of CFU of the two strains. The numbers of CFU of each strain per milliliter were determined as described by plating on blood agar plates with erythromycin or streptomycin.

In one experiment, mice were infected with pneumococci grown in a live mouse infected 12 h previously with strain D39. The infected mouse was bled with a heparinized capillary tube, and the blood was immediately diluted 1:10, 1:100, and 1:1000 in Ringer's solution. Groups of naive mice were injected intravenously with 0.2 ml of each diluted inoculum. Aliquots of the diluted blood were plated to calculate the numbers of CFU of *S. pneumoniae* injected.

In vitro interactions of pneumococci and PMNs. Human PMNs were isolated from 20 ml of heparinized whole blood by layering 3 parts blood over 1 part Ficoll-Paque (Pharmacia) and centrifuging to pellet erythrocytes (RBC) and leukocytes (WBC). The pellet was washed twice with phosphate-buffered saline (PBS), resuspended in 45 ml of PBS plus 5 ml of 4% dextran sulfate (Sigma) in normal saline, and incubated at 37°C for 60 min. The supernatant was removed and centrifuged to pellet PMNs and remaining RBC. Contaminating RBC were removed by hypotonic lysis. PMNs were resuspended in PBS to a concentration of approximately 3×10^6/ml. Cell viability in PBS was determined to be at least 96% by trypan blue exclusion, and the cell suspension was determined to be 90% PMNs by differential count. Pneumococci were grown in THY and diluted to 3×10^6/ml. One milliliter of the diluted bacterial culture was opsonized in 1 ml of fresh human serum for 60 min at 4°C with moderate shaking. Human serum was obtained from whole blood from a 35-year-old white female volunteer after clot retraction at room temperature for 60 min.

Opsonized cultures of pneumococci were incubated in polypropylene tubes with the suspension of PMNs in a final volume of 3 ml at 37°C with shaking. Control cultures were pneumococci without PMNs and PMNs without pneumococci. At time points of 0, 15, and 30 min, aliquots were removed and assessed for total WBC, WBC viability, and viable pneumococci. The total WBC per milliliter was determined with a hemocytometer, and the percentage of PMNs of total WBC per ml was determined by differential counts from cytocentrifuge slides. The cells were stained by the leukostat modification of Wright's stain method (Fisher Diagnostics, Orangeburg, N.Y.) and found to be 85 to 90% PMNs. The total numbers of CFU of pneumococci per milliliter were determined by plating serial dilutions on blood agar plates.

Determination of plasma levels of IL-6 and gamma interferon (IFN-γ) in infected mice. Blood was obtained at various time points from mice infected with D39, PLN, or PLN 24 h prior to infection with D39. CFU levels were determined at each time point. The blood was diluted 1:10 in RPMI medium plus 5% fetal calf serum, the cells were pelleted, and the diluted plasma was filtered through a 0.45-μm-pore-size Millex GV filter and stored at 4°C.

IL-6 and IFN-γ levels were determined by using the cytokine-sensitive cell lines 7TD1 (35) and WEHI-279 (33), respectively, by comparison of supernatant dilutions with recombinant cytokine dose-response curves as described previously (12). Samples from mice infected with the same strain at the same time point were pooled prior to performing dilutions.

Statistical analysis. All groups were compared by the two-sample rank test or Student's *t* test using two-tailed *P* values (37).

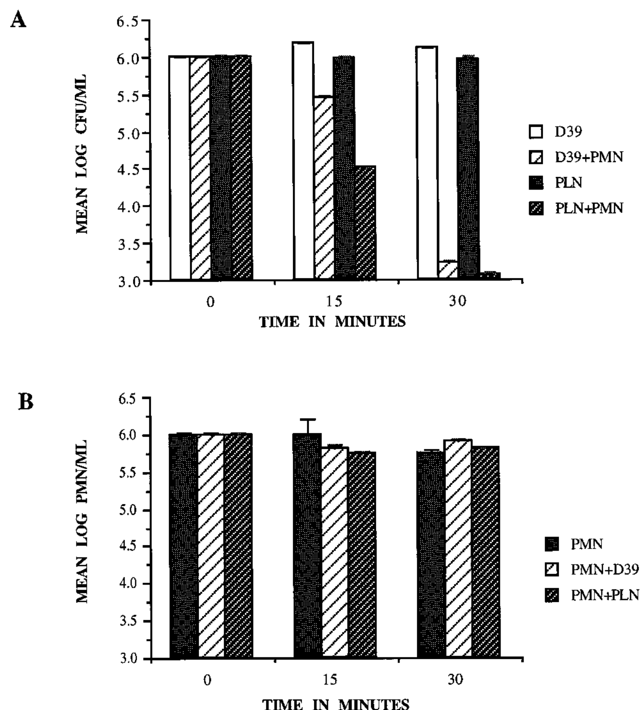

FIG. 1. In vitro interactions of pneumococci and PMNs. Pneumococci (10^6 CFU/ml) were opsonized with normal human serum and incubated with 10^6 human PMNs per ml. The numbers of pneumococcal CFU per milliliter (A) and viable PMNs per milliliter (B) were determined after 0, 15, and 30 min of incubation at 37°C. Data are shown as the geometric means (± standard errors) of triplicate CFU or PMN counts from individual samples. At each time point in panel A, the numbers of both D39 and PLN incubated with PMNs (CFU per milliliter) differ significantly from those of their respective controls lacking PMNs and at the 0 time point ($P < 0.001$) by the two-sample rank test. In panel B, no significant decreases in the numbers of PMNs were observed compared with those of control tubes lacking pneumococci.

RESULTS

Phagocytosis and killing of pneumolysin-producing pneumococci by PMNs does not reduce the viability of PMNs. Previous in vitro studies had demonstrated that purified pneumolysin can affect phagocyte function. As a partial test of the relevance of this finding, we wished to determine if pneumolysin-negative pneumococci were killed any more readily than those making pneumolysin. We also wanted to determine whether pneumolysin-producing pneumococci were able to kill PMNs.

We observed significant killing of D39 and PLN pneumococci opsonized in normal human serum by human PMNs in an in vitro assay representative of three assays performed (Fig. 1A). Pneumolysin-negative strain PLN was killed slightly faster by PMNs than the pneumolysin-positive D39 parent strain. By 15 min, the number of PLN pneumococci had decreased 1.65 logs, whereas the number of D39 pneumococci had decreased only by 0.65 log ($P < 0.001$). Within 30 min, however, the number of CFU of both D39 and PLN pneumococci per milliliter had decreased to about 1/1,000 of the starting concentration of pneumococci. No significant killing of PMNs by either strain of pneumococci was observed, strongly suggesting that in this assay the antiphagocytic effects of pneumolysin

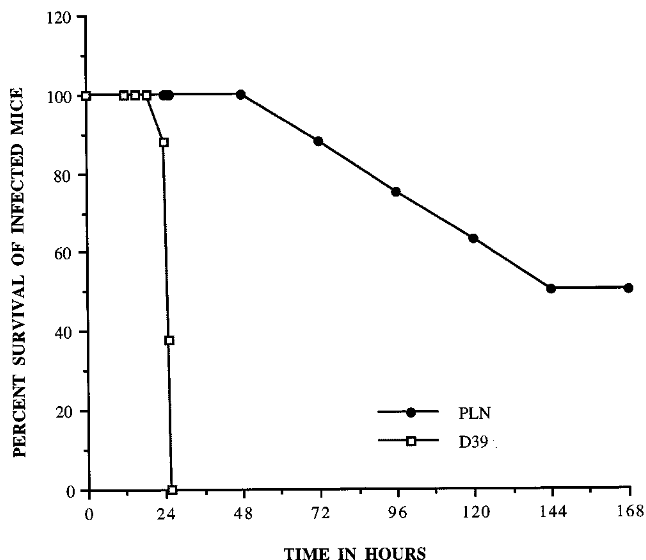

FIG. 2. Survival of mice infected intravenously with wild-type (D39) or pneumolysin-negative (PLN) isogenic pneumococci. The survival of eight mice is shown for infections with 10^5 D39 CFU or 10^5 PLN CFU. Deaths were recorded daily for 7 days as indicated, at which time all survivors were sacrificed. The difference in percentages of survival between the two groups was significant at a *P* of 0.0002 by the two-sample rank test.

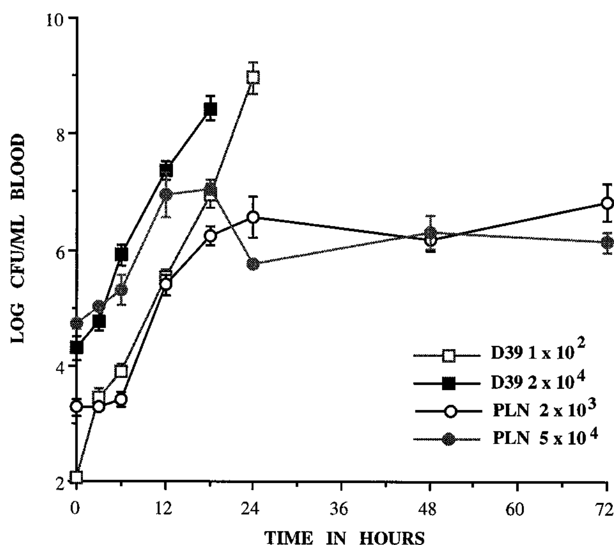

FIG. 3. Net growth of D39 and PLN in the blood of infected mice. The geometric means (± standard errors) of the log CFU per milliliter at time points of 1 min, 3 h, 6 h, 12 h, and 18 h are shown for groups of four mice infected with D39 or PLN at the indicated doses. Results for a 24-h time point are shown for all mice except those infected with 2×10^4 D39 CFU since all four of these mice died just prior to this time point. Results for a 48-h time point are shown for all mice infected with PLN; results at 72 h are shown for all mice infected with 2×10^3 PLN CFU and three of four mice infected with 5×10^4 PLN CFU since one mouse in this group died prior to this time point.

were not attributable to the destruction of phagocytosing PMNs (Fig. 1B).

Effect of pneumolysin on mouse survival postinfection. We used an in vivo mouse model to examine the effects of pneumolysin on sepsis. Previous studies have demonstrated that mice infected with the pneumolysin-negative strain PLN live several days longer than those infected with the isogenic parent strain D39 (7). This finding was confirmed under the conditions used in our current studies (Fig. 2). Strain D39 at 10^5 CFU/ml killed half of the mice within 24 h and all of the mice by 26 h postinfection. None of the mice infected with 10^5 CFU of PLN per ml died prior to 48 h postinfection, and 50% were still alive at 7 days postinfection, when the experiment was terminated. Similar results were obtained in other experiments when mice were infected with similar doses of D39 (Table 1).

Effects of pneumolysin on bacteremia kinetics. When PLN and D39 were injected intravenously at a range of doses, identical exponential growth kinetics (doubling time, 1.4 h) were observed until the numbers of CFU in the blood reached levels of 10^6 to 10^7 CFU/ml at 12 h postinfection (Fig. 3). From this point, the number of D39 CFU continued to increase exponentially until reaching 10^9 to 10^{10}/ml of blood at 24 to 28 h postinfection, at which time the mice died. No change in the net in vivo growth rate of D39 was evident over the period of infection, even in studies in which blood was collected at frequent time points just prior to the death of the mice (data not shown). Blood levels of PLN did not increase beyond 10^6 to 10^7 CFU/ml (Fig. 3), regardless of the infectious dose. The number of PLN CFU was maintained at 10^6 to 10^7 in vivo for several days to weeks (data not shown). The blood collection time points for the PLN-infected mice were not frequent enough to detect, or rule out, a possible increase in CFU immediately

TABLE 1. Effect of preinfection with PLN on D39-induced death

PLN dose ($\log_{10}$ CFU)	Time between PLN and D39 infection (h)	D39 dose ($\log_{10}$ CFU)	Survival periods for individual mice after D39 infection (h)	Median survival time (h)	*P* vs. no PLN[a]
None		2	48, 48, 48, 48	48	
None		4	21, 22, 23, 23, 23, 24, 24, 25, 25, 25, 25, 25	24	
None		5	21, 21, 21, 22, 22, 22, 22, 22, 23, 25, 25, 27, 36, 36, 36	22	
4	6	3.5	96, 120, 120, 168, 312	120	<0.02
5	6	5.9	23, 29, 30, 32	30	NS[b]
5	12	5.2	36, 96, 120, 120	108	<0.001
5	12	5.9	26, 32, 46, 72, 72, 72, 96, 144, 144, 168, 216, >336	84	<0.0001
5	24	5.6	72, 96, 144, 168, 168, 168, 168, 264, 312	168	<0.00001
5	24	5.9	96, 120, 192, >336	156	<0.00001

[a] *P* values were calculated between the PLN-plus-D39 group and the D39-only group (no PLN given) infected with the same (or next lower) number of D39 CFU. *P* value calculations were done by the two-sample rank test.
[b] NS, not significant.

prior to death. The occurrence of a plateau at 10^6 to 10^7 CFU/ml with PLN, but not with D39, was observed with PLN doses ranging from 2×10^3 to 1×10^6 CFU/ml (Fig. 3 and data not shown).

Coinfection with PLN and D39. When mice were infected simultaneously with 3×10^5 D39 CFU/ml and 3×10^5 PLN CFU/ml, the growth kinetics of both pneumococcal strains (from 4 to 24 h postinfection) and the survival times of the mice infected with both strains (Fig. 4) were identical to those of mice infected with D39 alone (Fig. 2 and 3). Even when 10^5 CFU of PLN was injected with 10^4 or 10^3 CFU of D39, strain PLN showed in vivo growth rates identical to those of strain D39. Moreover, coinfection with D39 allowed PLN to grow to greater than 10^6 to 10^7 CFU/ml. The ability of PLN to reach levels above 10^6 CFU/ml in the coinfected mice (Fig. 4) indicated that the effect of the pneumolysin produced by D39 was as advantageous to PLN growth as it was to D39 growth.

Host resistance elicited by prior infection with PLN. We reasoned that the plateau in PLN CFU per milliliter of blood (Fig. 2) that began at 12 to 18 h postinfection was due to some aspect of infection-induced antibacterial inflammation. In efforts to detect an enhanced antibacterial activity during PLN infection, we infected mice with 8×10^5 CFU of D39 per ml at 6, 12, and 24 h following infection with 3×10^5 CFU of PLN per ml. In the groups of mice preinfected with PLN for 12 or 24 h, the levels of D39 failed to increase exponentially and PLN levels held at about 10^6 to 10^7 CFU/ml. The combined levels of D39 and PLN were maintained at $\leq 10^7$ CFU/ml for at least 3 days (Fig. 5). When these results are compared with the kinetics of D39 depicted in Fig. 4, it is apparent that considerable host protection against D39 growth was induced during the first 12 h of PLN infection.

Table 1 summarizes the survival data for the PLN-preinfected mice shown in Fig. 5 as well as data from experiments using lower D39 challenge doses. As might be expected, longer preinfection times and lower challenge doses resulted in greater delays in death post-D39 infection. The protective effect of PLN preinfection was seen in mice challenged 6 h post-PLN infection with a dose of only 3×10^3 ($10^{3.5}$) D39 CFU but not when a dose of 8×10^5 ($10^{5.9}$) D39 CFU was used for challenge (Table 1; Fig. 5). With the lower D39 dose, the number of D39 CFU did not reach 10^6 until 12 h postinfection. By this time, the mice had been infected with PLN for 18 h and the number of PLN CFU was already in excess of 10^6/ml.

Plasma levels of IL-6 and IFN-γ in infected mice. One explanation for the failure of D39 to kill PLN-preinfected mice was that PLN had induced an inflammatory response. In an effort to obtain independent evidence for PLN-induced inflammation, we measured a monocyte-derived cytokine (IL-6) and a T-cell-derived cytokine (IFN-γ) in efforts to understand the roles of soluble factors and inflammation in these animals. These cytokines were measured in the plasma of mice infected with 3×10^5 D39, 1×10^5 PLN, or 1×10^5 PLN CFU 12 h prior to challenge with 1×10^6 D39 CFU. IL-6 and IFN-γ levels were determined prior to the initial infection and at 6, 12, 18, 24, and 48 h postinfection. In PLN-preinfected mice, the blood collection at the 12-h time point was done immediately prior to challenge with D39 (Fig. 6). In mice infected with 10^5 D39 CFU, 10^3 U of IL-6 per ml was detected after 6 h, and the concentration of IL-6 increased in parallel with the increase in CFU until it reached about 10^5 U/ml at 18 h postinfection. By 24 h postinfection, just prior to death of the mice, the IL-6 level had fallen to 10^3 U/ml. With mice infected with 10^5 PLN CFU only, an IL-6 response was not detected until 12 h postinfection even though these mice had an equal or greater number of PLN CFU than did the D39-infected mice. The IL-6 response in PLN-infected mice showed no net increase between 12 and 48 h postinfection. Similarly, in the PLN-preinfected mice, the levels of IL-6 remained relatively stable over the 48-h period (Fig. 6). IFN-γ was detected only in the D39-infected mice and then only at the onset of extreme sepsis, immediately prior to death.

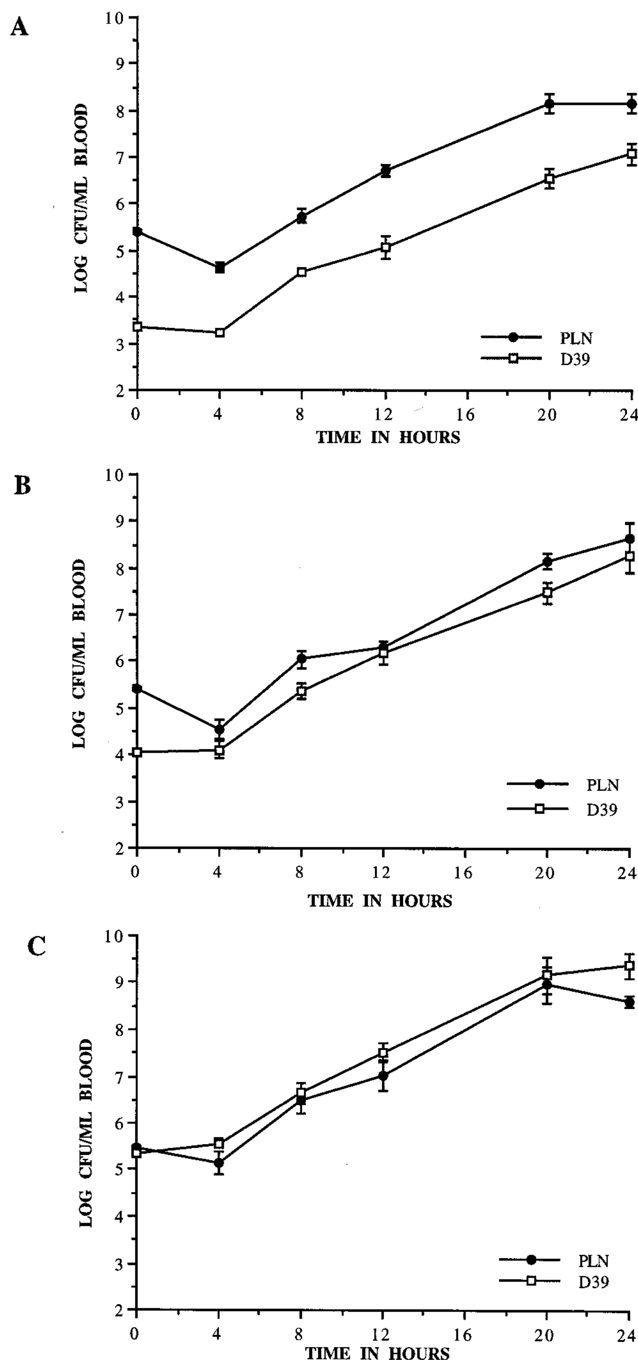

FIG. 4. Coinfection of mice with PLN plus D39. The geometric means (± standard errors) of the log CFU per milliliter of both D39 and PLN are shown for groups of four mice infected with 2×10^5 PLN and 2×10^5 D39 CFU (A), 2×10^5 PLN and 1×10^4 D39 CFU (B), and 2×10^5 PLN and 2×10^3 D39 CFU (C) for time points of 1 min and 4, 8, 12, 20, and 24 h postinfection. Mouse survival times were between 22 and 30 h (A), 30 h for two mice and between 30 and 46 h for two mice (B), and greater than 30 h but less than 46 h (C).

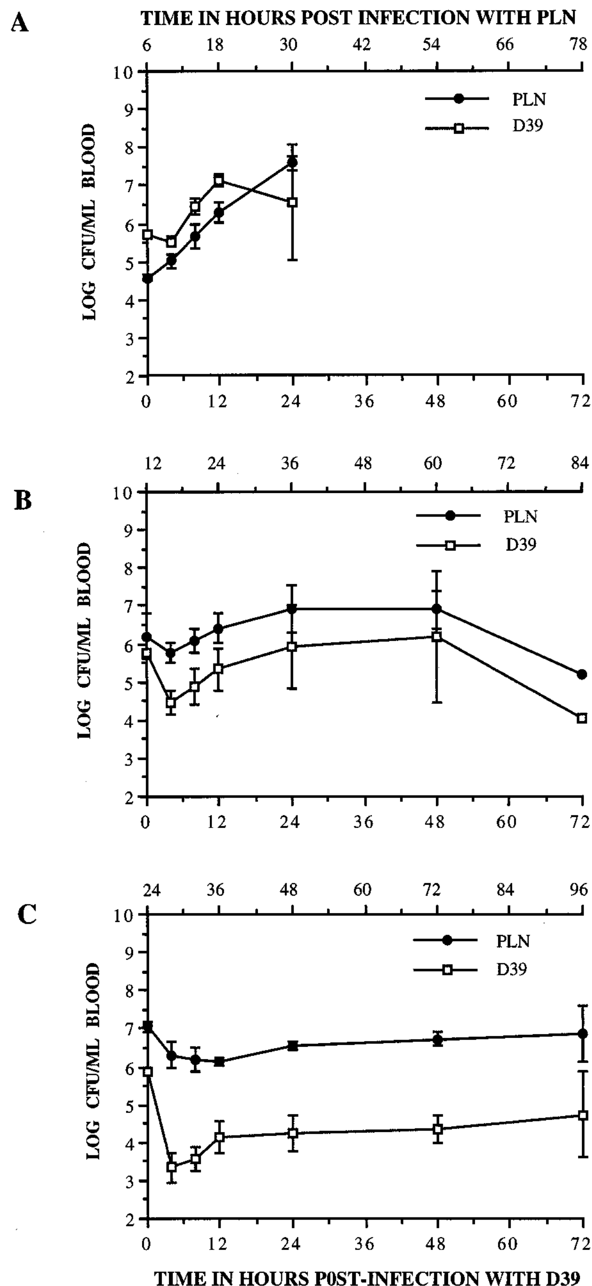

FIG. 5. Infection with PLN 6, 12, or 24 h prior to infection with D39. Groups of mice were infected with 3×10^5 PLN CFU for 6 (A), 12 (B), or 24 (C) h prior to infection with 8×10^5 D39 CFU. The 1-min bleeding was done 1 min postinfection with D39 (6, 12, or 24 h postinfection with PLN). The numbers of PLN in the mice at the time of D39 challenge were higher when preinfection times corresponding to the lengths of time of in vivo growth of PLN were longer. Median survival times post-D39 infection were 29 h for mice infected with PLN 6 h prior to infection with D39, 93 h for mice infected with PLN 12 h prior to infection with D39, and 180 h for mice infected with PLN 24 h prior to infection with D39.

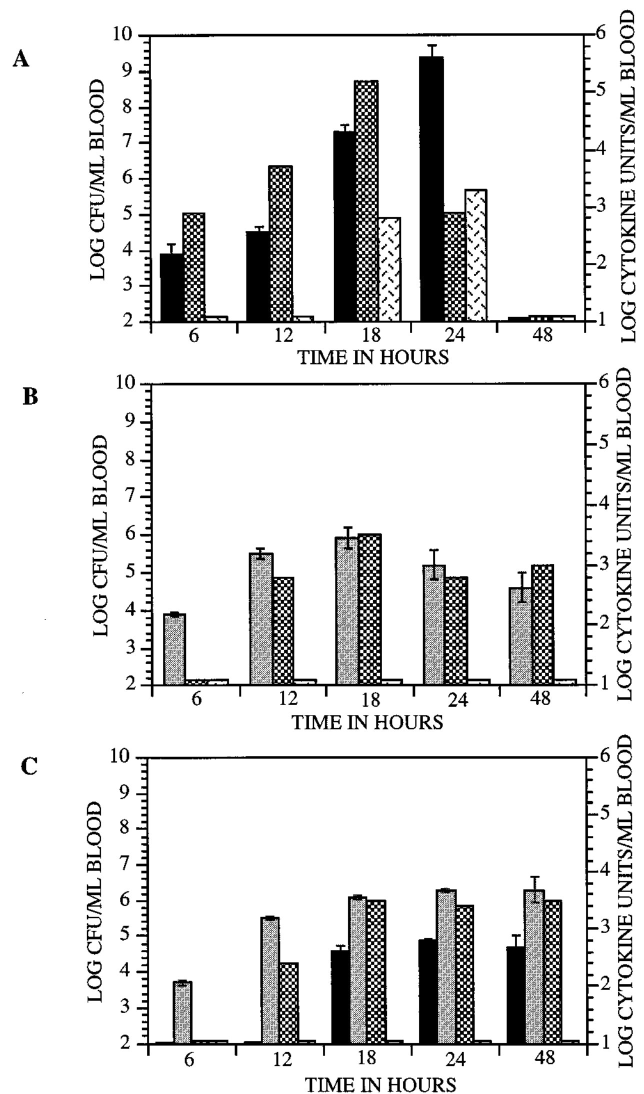

FIG. 6. Groups of four mice were infected with either 3×10^5 D39 CFU (A), 1×10^5 PLN CFU (B), or 1×10^5 PLN CFU (C) 12 h prior to infection with 1×10^6 D39 CFU. CFU levels of D39 (■) and/or PLN (▨) in the blood were determined for individual mice and are shown as the geometric means ± standard errors. Levels of IL-6 (▩) and IFN-γ (▧) were determined from blood pooled from each group of four mice. CFU determinations and cytokine assays were conducted with blood obtained prior to infection and at 6, 12, 18, 24, and 48 h postinfection. In the case of PLN-preinfected mice, time points refer to hours post-PLN infection, with the first time point postchallenge with D39 being 18 h. In the cytokine assays, coefficients of variation in replicate assays were routinely <15% and are omitted for clarity.

The ratio of IL-6 production to number of CFU was in general lower in mice infected with PLN than in mice infected with D39. This was also apparent from other experiments with slightly different challenge doses of D39 (data not shown). Since the amount of IL-6 produced is related to the level of *S. pneumoniae* CFU per milliliter, we considered the possibility that the amount of IL-6 in each pool might be more closely related to the highest number of CFU in each group of mice rather than to the geometric mean CFU of each group. To test this possibility, we calculated the ratios of IL-6 production to the largest number of CFU in the mice providing the sera for each pool. The results obtained were essentially unchanged and still indicated a greater ratio of IL-6 production/number of CFU in D39 than in PLN infections.

Lack of an effect of growth conditions on the ability of pneumococci to evade the inflammatory response induced by PLN. The studies described above demonstrate that although strain D39 can reach 10^9 to 10^{10} CFU/ml of blood and kill naive mice in a day or less, it does not reach concentrations

TABLE 2. Effect of preinfection with PLN on survival time of mice challenged with four different pathogenic streptococci

Challenge strain	Survival times postchallenge (days)		P^a
	No preinfection	Preinfected with 10^5 PLN CFU[b]	
WU2 (type 3 pneumococcus)	1, 1, 1, 1, 2, 2, 2	1, 1, 4, 4, 4, 5, 8, >21	0.04
EF3296 (type 4 pneumococcus)	2, 2, 2, 2, 2, 2, 4	2, 4, 4, 5, 6, 8, 8, 12	0.006
BG9163 (type 6B pneumococcus)	3, 4, 6, 7, >21, >21, >21	1, 2, 4, 8, 11, 17, 19	NS[c]
S43/29R (group A streptococcus)	1, 2, 2, 2, 2, 3	1, 2, 2, 2, 2, 2, 3	NS

[a] P values comparing survival times of groups of mice preinfected with 10^5 PLN CFU and groups of naive mice were calculated by the two-sample rank test.
[b] Mice were inoculated intravenously with 10^5 PLN CFU 24 h prior to intravenous inoculation with 10^5 CFU of the indicated challenge strain.
[c] NS, not significant.

above 10^7 CFU/ml in PLN-preinfected mice for at least several days. It is possible that the failure of D39 to rapidly kill the PLN-preinfected mice was due to the fact that the strain D39 in naive mice had many hours to adapt to the in vivo environment before inflammation-associated host resistance was established, whereas the D39 used to infect the PLN-preinfected mice had been grown in broth and might have been relatively less able to resist host immunity mediated by an ongoing PLN-induced host response. To test this possibility, PLN-preinfected mice were infected with D39 pneumococci either grown in THY or freshly obtained from the blood of a live mouse infected 12 h earlier. We observed that growing D39 pneumococci in the mouse did not increase their ability to evade the increased host resistance generated by PLN preinfection. The median time of death of groups of three mice infected with 10^4 D39 CFU was 27 to 29 h regardless of whether the inocula were grown in broth or in mice. When 10^4 CFU of the same broth- or mouse-grown D39 inoculum was injected into PLN-preinfected mice, the median time of death for both groups of mice was between 310 and 380 h postchallenge.

Effect of prior infection with PLN on host resistance to other pneumococcal strains and group A streptococci. If the resistance elicited by PLN was the result of nonspecific inflammation, such resistance should be able to elicit protection against pneumococci other than D39. PLN preinfection greatly extended the survival time of mice infected with type 3 or type 4 pneumococci but not of mice infected with group A streptococci (Table 2). Preinfection with PLN prior to challenge with the type 6B pneumococcal strain had no effect on time to death. Infection with type 6B pneumococci results in a chronic infection similar to that of PLN, where the median time of death was about 7 days. This stands in contrast to the much more rapid septic deaths caused by capsular types 2, 3, and 4 pneumococci (10, 11). This could be because although PLN preinfection prevents rapid sepsis, it does not affect the course of chronic *S. pneumoniae* infection.

DISCUSSION

Prior studies of the mechanism(s) by which pneumolysin affects virulence have relied on the addition of exogenous pneumolysin to in vitro systems and have shown that pneumolysin interferes with the phagocytic and antibacterial activities of phagocytes. These in vitro effects of purified pneumolysin are presumed to be important in its role in in vivo pneumococcal virulence (13, 15, 22, 27, 29, 32). For in vitro studies using purified pneumolysin to be considered relevant to the in vivo virulence effects of pneumolysin, it must be assumed that the effect of the toxin on pneumococcal sepsis is mediated by the diffusible molecule acting extracellularly on phagocytes and/or complement (9, 29, 32). This interpretation is supported, but not proven, by studies showing that antibody to pneumolysin reduces virulence as much as inactivation of the pneumolysin gene does (7, 23).

Our present data showing that pneumolysin-negative pneumococci are more susceptible than pneumolysin-producing pneumococci to in vitro killing by phagocytes further support the hypothesis that the role of pneumolysin in virulence is to decrease opsonophagocytosis. An alternative hypothesis to pneumolysin acting extracellularly on phagocytes and complement is that it kills PMNs when they destroy phagocytosed pneumococci and thus release cytoplasmic pneumolysin. This hypothesis was not supported, however, by our in vitro finding that phagocytosis and killing of pneumolysin-producing pneumococci did not affect PMN viability.

Since the release of pneumolysin from pneumococci is apparently required for the biologically important functions of pneumolysin (5, 6, 17, 22) and since it likely acts extracellularly on phagocytes, the toxin would be expected to act by causing a generalized effect on host resistance. This expectation was confirmed by the observation that when mice were coinfected with pneumolysin-producing and pneumolysin-negative pneumococci, both strains grew exponentially until the death of the mice. The generalized effect of pneumolysin on host resistance is in contrast to the virulence effects of capsule or pneumococcal surface protein A (PspA), which act at the level of the bacterium producing them; PspA produced by one strain was shown to confer no benefit on coinfecting strains lacking PspA (25).

Our studies have revealed a dynamic picture of the in vivo effects of pneumolysin. Pneumolysin did not affect the in vivo net growth of pneumococci as long as the numbers of CFU per milliliter remained less than 10^6 but was required for the numbers of CFU per milliliter of blood to increase to greater than 10^7. However, after mice have been infected with pneumolysin-negative pneumococci for at least 12 h, pneumolysin production by a subsequent challenge strain was unable to enhance the net growth of either strain.

It seemed likely that the inability of PLN pneumococci to show exponential growth beyond 10^6 or 10^7 CFU/ml of blood was due to the possibility that infections with greater than 10^6 CFU/ml induced host resistance. The observation that the number of PLN CFU in the blood remained relatively constant for several days suggests that the purported host immunity is regulated by some type of feedback control. This could be explained if higher levels of the pneumococci induced greater resistance, lower levels induced less, and the amount of immunity induced at 10^6 to 10^7 CFU/ml just balanced the in vivo growth rate of the pneumococci. Evidence for the induction of host resistance by the PLN pneumococcal infection was provided by the observation that D39 pneumococci were not able to exhibit exponential net growth in mice that were already preinfected with at least 10^6 CFU of PLN pneumococci per ml.

When mice were preinfected with 10^5 PLN CFU, host resistance was not apparent until 12 h postinfection, at which time the number of PLN in the blood reached 10^6/ml. For the establishment of host resistance, the critical factor appeared to be the level of CFU per milliliter of blood rather than the duration of the PLN infection. PLN-preinfected mice were protected against D39 challenge 6 h later if the D39 dose was sufficiently low so that the number of PLN per milliliter reached 10^6 before the D39 CFU reached 10^6/ml. It is possible that the same immunity that held PLN levels between 10^6 and 10^7 CFU also controlled the net growth of D39 in PLN-preinfected mice.

Independent evidence for the induction of inflammation in mice infected with 10^6 or more pneumococci was provided by the detection of circulating IL-6. Although the detection of IL-6 confirmed the presence of inflammation by the time levels of PLN and D39 reached 10^6 CFU/ml, it did not by itself explain why D39, but not PLN, was able to exhibit geometric net growth beyond a level of 10^7 CFU/ml. In fact, slightly higher levels of IL-6 were observed in mice infected with D39 than in those infected with PLN, even when any differences in the numbers of CFU are taken into account. The fact that circulating levels of IFN-γ were not observed until just prior to death of D39-infected mice is consistent with its being a T-cell product under transcriptional control and a purported mediator in septic shock (28). The reason for the decrease in IL-6 levels just prior to death is not clear; however, IFN-γ has been demonstrated to inhibit production by rheumatoid synovial cells of inflammatory mediators, e.g., prostaglandin and collagenase, and release of cytokines associated with inflammatory responses, e.g., IL-8 (28).

Our results indicated that pneumolysin permits the pneumococci to escape the regulatory aspects of infection-induced inflammation. It was not clear, however, why the pneumolysin produced by D39 injected 12 h after PLN infection did not enable the D39 and PLN pneumococci to exhibit exponential growth, as it did in simultaneous coinfections of both strains. One possibility was that the D39 adapted to the mouse during the first 6 to 12 h of infection and was more able to resist host immunity than when injected immediately after growth in media. This possibility was eliminated by studies in which we infected PLN-preinfected mice with D39 grown in mice rather than in media.

Two explanations could account for the ability of D39 to escape regulation by the inflammation that it elicits but be controlled by inflammation elicited by preinfection with PLN. One is that the pneumococci in an established infection are not in the same niche as those recently injected intravenously and are therefore not as susceptible to host resistance as those injected directly into the blood. Another possibility is that the continued exposure of the reticuloendothelial system of D39-infected mice to pneumolysin, even before an inflammatory response is induced, could alter the nature of the host immunity elicited. At the time that PLN-preinfected mice are challenged with D39, the inflammation is already established, prior to exposure to D39. The observation that more IL-6 units per CFU are produced in D39 than PLN infections provides at least some evidence for qualitative differences in the inflammatory responses induced by D39 and PLN pneumococci. The fact that equal or greater levels of IL-6 are elicited by D39 than by PLN must mean that although IL-6 is a measure of inflammation, it does not serve as a measure of the ability of the inflammation to control pneumococcal infection. Moreover, it is possible that the elevated IL-6 level may be detrimental to the host and may explain the rapid onset of sepsis in D39 infections.

Our results indicate that the effect(s) of pneumolysin on virulence in the mouse sepsis model is limited to the first several hours of bacteremic infection. The absence of pneumolysin during these first few hours prevents the rapid development of sepsis and can delay the death of the mice for at least several days. If, as is expected, the protective effect of antibody to pneumolysin is to block the function(s) of pneumolysin, then antibody to pneumolysin would need to be present only during the first few hours of bacteremic infection, and its presence later in the infection would exert no additional antipneumococcal effect. This interpretation would explain why antibodies to pneumolysin are readily able to delay death but frequently do not protect against death unless mice are challenged with inocula near the minimal lethal dose (24, 30). From these earlier reports and the present data, the picture of pneumococcal pathogenesis that emerges is that pneumolysin is released by autolysin-dependent autolysis in vivo and that the released pneumolysin interferes with either the function or generation of inflammation-induced immunity, thus permitting the continued exponential net growth of pneumococci.

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