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Effect of Defined Point Mutations in the Pneumolysin Gene on the Virulence of *Streptococcus pneumoniae*

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The thiol-activated toxin pneumolysin is a known pneumococcal virulence factor, with both cytotoxic (hemolytic) and complement activation properties. Copies of the pneumolysin gene carrying defined point mutations affecting either or both of these properties were introduced into the chromosome of *Streptococcus pneumoniae* D39 by insertion-duplication mutagenesis. The virulences of these otherwise isogenic strains were then compared. There was no significant difference in either the median survival time or overall survival rate between mice challenged with D39 derivatives producing the wild-type toxin and those expressing a pneumolysin gene with an Asp-385→Asn mutation, which abolishes the complement activation property. However, mice challenged with strains carrying either His-367→Arg or Trp-433→Phe plus Cys-428→Gly mutations, which reduce hemolytic activity to approximately 0.02 and 0.0001% of the wild-type level, respectively, had significantly greater median survival times and overall survival rates than mice challenged with D39 derivatives expressing a wild-type pneumolysin gene. No additional reduction in virulence was observed when mice were challenged with a D39 derivative carrying Trp-433→Phe, Cys-428→Gly, and Asp-385→Asn, rather than Trp-433→Phe and Cys-428→Gly, mutations in the pneumolysin gene. Thus, it appears that in the intraperitoneal challenge model, the contribution of pneumolysin to virulence is largely attributable to its hemolytic (cytotoxic) properties rather than to its capacity to activate complement. Interestingly, however, the amount of pneumolysin required for full virulence may be very small, as D39 derivatives carrying the Trp-433→Phe mutation (which reduces hemolytic activity to 0.1% of the wild-type level) had intermediate virulence.

Streptococcus pneumoniae (the pneumococcus) is a human pathogen of major importance, causing life-threatening, invasive diseases such as pneumonia, bacteremia, and meningitis. Although the pneumococcal polysaccharide capsule is generally considered to be essential for virulence (2), there is a substantial body of evidence that protein factors such as the toxin pneumolysin play an important role in pathogenesis (20).

Pneumolysin is a member of a family of toxins produced by a number of gram-positive genera, which are referred to as thiol-activated cytolysins (31). These toxins appear to share a common mode of action, which involves an initial interaction with the target cell membrane (possibly via cholesterol) followed by oligomerization of toxin molecules to form transmembrane pores, resulting in cell lysis (6). Studies using purified pneumolysin have demonstrated that in addition to potent hemolytic activity, this toxin has a variety of direct cytotoxic effects on human cells and tissues in vitro (12, 19, 22, 27, 28, 33). Free cholesterol is a potent inhibitor of the cytotoxic properties of pneumolysin (13). However, pneumolysin is also capable of direct activation of the classical complement pathway in the absence of specific antibody, with concomitant reduction in serum opsonic activity (25), a property mediated by its ability to directly bind the Fc region of human immunoglobulin G (17). The fact that cholesterol did not inhibit this capacity suggested that a separate pneumolysin domain was responsible and that the toxin was therefore bifunctional (25).

Structure-function studies involving oligonucleotide-directed mutagenesis of the cloned toxin gene (7, 17, 29) have demonstrated that the cytotoxic and complement activation properties of pneumolysin are indeed determined by different regions of the molecule. An 11-amino-acid domain (Glu-Cys-Thr-Gly-Leu-Ala-Trp-Glu-Trp-Trp-Arg) (residues 427 to 437 in the pneumolysin sequence) is conserved among other members of the thiol-activated cytolysin family, and mutations within this region, particularly Cys-428→Gly and Trp-433→Phe, reduce hemolytic (cytotoxic) activity by as much as 10^3-fold (29). A His-367→Arg mutation, which blocks oligomerization of toxin in the host cell membrane, also results in a 5×10^3-fold reduction in hemolytic activity (7). An indication of the regions of pneumolysin which may be involved in complement activation came from the observation that pneumolysin has limited homology with human C-reactive protein (17). Site-directed mutagenesis of amino acids within one of the regions of pneumolysin homologous to C-reactive protein, particularly Asp-385→Asn, reduced the ability of the toxin to bind immunoglobulin G and abolished its ability to activate the complement pathway (17). The cytolytic activity of these mutants was not affected.

We have previously demonstrated that disruption of the pneumolysin gene in the *S. pneumoniae* chromosome by insertion-duplication mutagenesis significantly reduces the virulence of type 2 and type 3 pneumococci. However, full virulence could be reconstituted by back-transformation of the pneumolysin-negative pneumococcus with a cloned DNA fragment carrying an intact copy of the pneumolysin gene (4, 5). These studies confirmed the importance of pneumolysin as a pneumococcal virulence factor but did not determine the relative in vivo contributions of the cytotoxic and complement activation properties of the toxin. This question has been addressed in the present study by introduction of copies of the pneumolysin gene containing one or more defined point mu-

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tations into the *S. pneumoniae* chromosome and comparing the virulences of pneumococci which differ by single amino acid residues.

MATERIALS AND METHODS

Bacterial strains and cloning vectors. The *S. pneumoniae* strains used were the virulent type 2 strain D39 (3) (obtained from the National Collection of Type Cultures, London, England; strain number NCTC7466) and its nonencapsulated, highly transformable derivative Rx1 (30). These organisms were routinely grown in Todd-Hewitt broth containing 0.5% yeast extract or on blood agar. *Escherichia coli* K-12 strain DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was grown in Luria-Bertani medium (16) with or without 1.5% Bacto Agar (Difco Laboratories, Detroit, Mich.). Plasmid pVA891 has been described previously (15). Where appropriate, chloramphenicol was added to growth media at a concentration of 25 μg/ml.

Purification of pneumolysin derivatives. Pneumolysin derivatives were purified from *E. coli* JM109 carrying pJCP202 or derivatives thereof as previously described (24). Cytotoxicity was determined by hemolysis assay using human erythrocytes as previously described (23).

Transformation. Transformation of *E. coli* with plasmid DNA was carried out with $CaCl_2$-treated cells as described by Brown et al. (8). *S. pneumoniae* strains were transformed with chromosomal or plasmid DNA as described previously (5, 35), and transformants were selected on blood agar containing 0.2 μg of erythromycin per ml.

DNA manipulations. *S. pneumoniae* chromosomal DNA used in Southern hybridization experiments was extracted and purified as previously described (21). Plasmid DNA was isolated from *E. coli* by the alkaline lysis method (18). Analysis of recombinant plasmids was carried out by digestion of DNA with one or more restriction enzymes under the conditions recommended by the supplier. Restricted DNA was electrophoresed in 0.8 to 1.5% agarose gels with a Tris-borate-EDTA buffer system as described by Maniatis et al. (16).

Southern hybridization analysis. Chromosomal DNA (1 to 2.5 μg) was digested with appropriate restriction enzymes, and the digests were electrophoresed on agarose gels in Tris-borate-EDTA buffer. DNA was then transferred to a positively charged nylon membrane (Hybond N^+; Amersham, England) as described by Southern (32) and hybridized to probe DNA labelled with digoxigenin (DIG) by the method of Feinberg and Vogelstein (10). Filters were washed and then developed by using anti-DIG-alkaline phosphatase conjugate (Boehringer, Mannheim, Germany) and 4-nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate substrate according to the manufacturer's instructions. DIG-labelled lambda DNA, restricted with *Hin*dIII, was used as a DNA molecular size marker.

SDS-PAGE and Western blot (immunoblot) analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (14), and proteins were electrophoretically transferred from gels onto nitrocellulose filters as described by Towbin et al. (34). Filters were probed with mouse antipneumolysin serum or control serum (used at a dilution of 1:1,000) followed by goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Bio-Rad Laboratories, Richmond, Calif.). Enzyme-labelled bands were visualized with a 4-chloro-1-naphthol–H_2O_2 substrate system.

PCR and DNA sequencing. Two oligonucleotide primers, 5′-ATTAGGAGG TAGCATATGGCAAATAAA-3′ and 5′-GTCATTTTCTACCTTATCCTC-3′, were used to amplify a 1,428-bp fragment containing the complete pneumolysin coding region from the *S. pneumoniae* chromosome. The PCR product was then purified, and the C-terminal-encoding portion was directly sequenced by using an oligonucleotide primer (5′-ACAACTTCTTTTTTACGTGAC-3′) which anneals to the region 84 to 105 nucleotides upstream of the His-367 codon. Sequencing was carried out with dye-labelled terminators on an Applied Biosystems model 373A automated DNA sequencer.

Virulence studies. *S. pneumoniae* strains from fresh overnight blood-erythromycin plates were inoculated into serum broth (meat extract broth plus 10% horse serum) and incubated at 37°C for 4 h. Production of type 2 capsule was confirmed by quellung reaction, using antisera obtained from Statens Seruminstitut, Copenhagen, Denmark. Cultures were then diluted to a density of 2×10^6 CFU/ml, and 0.1-ml volumes were injected intraperitoneally into groups of 16 BALB/c mice. Survival time was recorded, and differences in median survival time between groups were analyzed by the Mann-Whitney *U* test (two-tailed). Differences in the overall survival rate between groups were analyzed by the χ^2 test (two-tailed).

RESULTS AND DISCUSSION

Construction of pneumococci carrying defined pneumolysin point mutations. The purpose of this study was to construct pneumococci carrying pneumolysin genes with defined mutations in regions affecting hemolytic activity and/or complement activation. Introduction of various single point mutations into the cloned pneumolysin gene by oligonucleotide-directed mutagenesis has been described previously (7, 17, 29); the double and triple mutations were constructed as part of this study. The Asp-385→Asn mutation abolishes complement activation without affecting hemolytic activity (17). The specific cytotoxicities of purified recombinant pneumolysin carrying the Trp-433→Phe, His-367→Arg, Trp-433→Phe Cys-428→Gly, and Trp-433→Phe Cys-428→Gly Asp-385→Asn mutations were 0.1, 0.02, 0.0001, and 0.0001% of that of the wild-type toxin, respectively.

Derivatives of *S. pneumoniae* D39 carrying pneumolysin point mutations were constructed by insertion-duplication mutagenesis using the vector pVA891 (15). This is a deletion derivative of the *Escherichia-Streptococcus* shuttle plasmid pVA838 which has lost the capacity to replicate autonomously in streptococci but retains a streptococcal gene encoding erythromycin resistance. The first stage of the mutagenesis procedure involved subcloning a 700-bp *Hin*dIII fragment from pJCP202 (or derivatives thereof) (24) encoding the C-terminal portion (codons 265 to 471) of the various pneumolysin derivatives into pVA891 (Fig. 1). When this construct is used to transform *S. pneumoniae*, recombination between the cloned C-terminal fragment and the homologous region of the chromosomal pneumolysin gene incorporates the recombinant pVA891 derivative into the chromosome. This also replaces the C-terminal portion of the native toxin gene with sequences derived from the donor construct, with the native C terminus separated from the reconstituted pneumolysin gene by the vector sequences (Fig. 1). Of course, recombination could occur at any point within the cloned C-terminal portion, and only specific point mutations downstream from the crossover point would be incorporated into the intact copy of the pneumolysin gene. Thus, it is necessary to determine the DNA sequence of the appropriate region of the intact pneumolysin gene to determine whether specific mutations have been incorporated.

In a previous study (5), we found that the efficiency of direct transformation of the encapsulated type 2 strain D39 to erythromycin resistance, using recombinant pVA891 derivatives, was very low, even in the presence of competence factor derived from the highly transformable D39 derivative Rx1. Therefore, we adopted a two-step approach to introduce the pneumolysin mutants into D39. First, we transformed the nonencapsulated strain Rx1 with pVA891 derivatives carrying each of the C-terminal pneumolysin mutations listed above or with a pVA891 derivative carrying the C-terminal portion of the wild-type pneumolysin gene and then isolated erythromycin-resistant transformants. Chromosomal DNA from one of the transformants from each reaction was subjected to Southern hybridization analysis to confirm interruption of the pneumolysin gene with the pVA891 sequences. The complete copy of the pneumolysin gene was also PCR amplified, and the C-terminal portion of the 1,428-bp amplicon was sequenced as described in the Materials and Methods. This confirmed that each of the point mutations had been incorporated into the complete copy of the pneumolysin gene in the chromosome in the respective pneumococcal strain.

The various mutated pneumolysin loci were then introduced into the chromosome of the virulent encapsulated strain D39 by transformation with chromosomal DNA extracted from the respective Rx1 derivative. Two independent transformation experiments were carried out for each strain to minimize the possibility of cotransformation of spurious *S. pneumoniae* sequences along with the mutated pneumolysin and erythromycin resistance loci. Each experiment yielded several erythromycin-resistant D39 transformants, all of which produced a type 2 capsule (as determined by quellung reaction), and one from each of these was selected for further study.

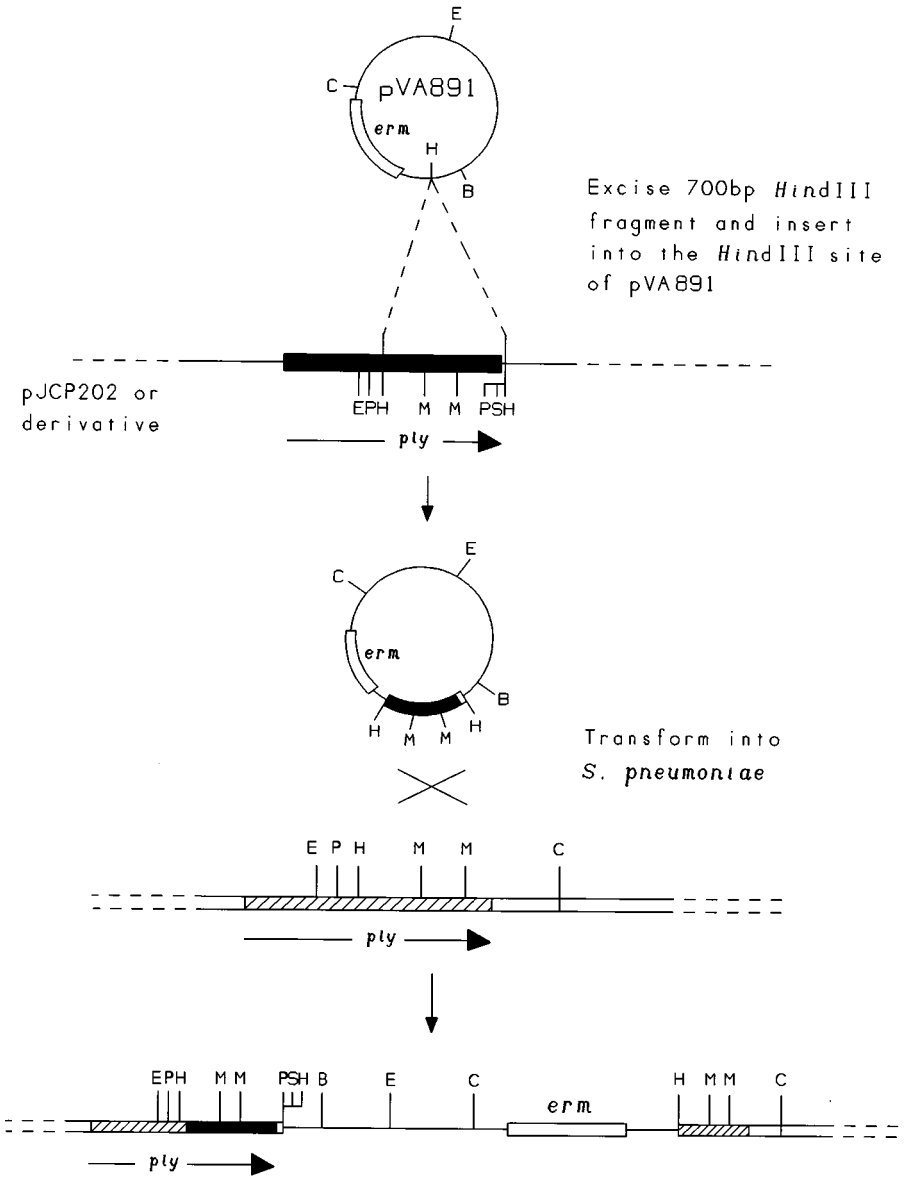

FIG. 1. Scheme for introduction of point mutations into the C-terminal portion of the chromosomal pneumolysin gene. The solid region represents the mutated pneumolysin gene encoded by derivatives of pJCP202. The hatched region represents the chromosomal copy of the pneumolysin gene. Restriction sites are abbreviated as follows: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; M, *Mst*II; P, *Pst*I; S, *Sph*I.

To confirm insertion of the pVA891 derivative into the pneumolysin locus (thereby duplicating the pneumolysin C terminus), chromosomal DNA was extracted from each strain, restricted with *Hin*dIII, and subjected to Southern hybridization analysis using the 204-bp *Mst*II fragment within the C-terminal portion of the pneumolysin gene as a probe (Fig. 2). D39 digests contained only one probe-reactive 5.6-kb fragment, whereas digests of all of the erythromycin-resistant derivatives of D39 contained 5.6- and 0.7-kb *Hin*dIII fragments which hybridized to the probe (Fig. 2). This is consistent with incorporation of the pVA891 derivatives into the pneumolysin locus, as shown in Fig. 1. To confirm that an intact pneumolysin gene had been reconstituted and was expressed in each transformant, crude cell lysates of the various strains were separated by SDS-PAGE and subjected to Western blot analysis using mouse antipneumolysin serum as described in the Materials and Methods (Fig. 3). All strains produced a 53-kDa species which reacted with the antiserum. The slightly weaker labelling in some of the tracks was due to lower total protein content of the loaded extract. In addition, the intact pneumolysin gene was PCR amplified from each D39 derivative, and the C-terminal portion was sequenced as described above. This confirmed that the intact copy of the pneumolysin gene in each transformant contained the expected point mutations (result not shown).

Comparative virulence of D39 derivatives. To determine the impact of the various pneumolysin point mutations on virulence, groups of 16 BALB/c mice were inoculated intraperitoneally with 2×10^5 CFU of the various D39 derivatives. Within each group, the two D39 derivatives from the independent transformation reactions were administered to eight mice each, and the results were pooled. The survival times for the

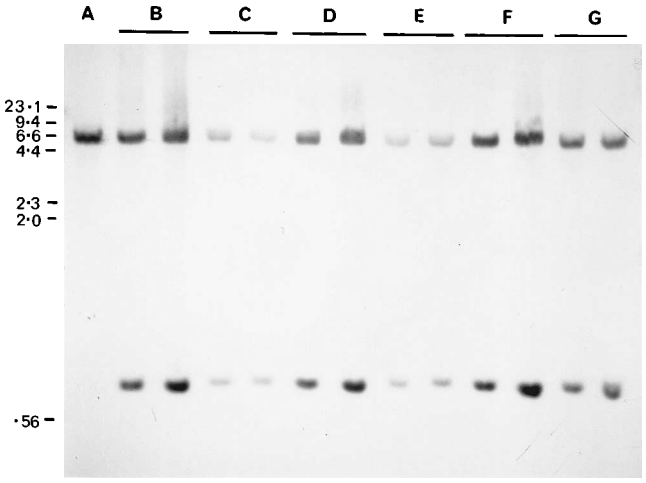

FIG. 2. Southern hybridization analysis. Chromosomal DNA from the various D39 derivatives was digested with *Hin*dIII, electrophoresed, blotted, and probed with a DIG-labelled 204-bp *Mst*II fragment from the C-terminal portion of the pneumolysin gene. Lanes: A, D39; B, two independent D39/Pn$^+$ transformants; C, two D39/His-367→Arg transformants; D, two D39/Asp-385→Asn transformants; E, two D39/Trp-433→Phe transformants; F, two D39/Cys-428→Gly Trp-433→Phe transformants; G, two D39/Asp-385→Asn Cys-428→Gly Trp-433→Phe transformants. The mobility of DNA size markers (in kilobases) is also indicated.

various mice are shown in Fig. 4, and the statistical analysis of the data is presented in Table 1. There was no significant difference in the virulence of D39 and D39/Pn$^+$ derivatives, in which pVA891 insertion reconstituted a wild-type pneumolysin gene; the respective survival rates were 2 of 16 and 0 of 16, and the median survival times were identical (1.7 days). This result indicates that the mutagenesis procedure used did not result in polar effects which impact on virulence. There was no significant difference in the median survival time (1.7 and 2.0 days, respectively) or overall survival rate (0 of 16 for both groups) between mice receiving D39/Pn$^+$ and those challenged with D39/Asp-385→Asn, which indicates that in this animal model,

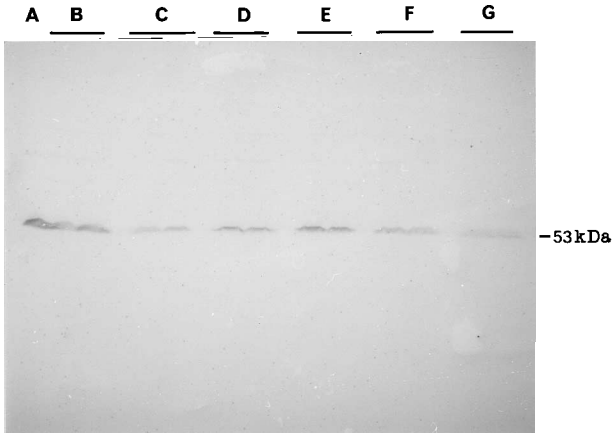

FIG. 3. Western blot analysis. Lysates of fresh overnight cultures of the various D39 derivatives were separated by SDS-PAGE, blotted, and probed with antipneumolysin serum. Lanes: A, D39; B, two independent D39/Pn$^+$ transformants; C, two D39/His-367→Arg transformants; D, two D39/Asp-385→Asn transformants; E, two D39/Trp-433→Phe transformants; F, two D39/Cys-428→Gly Trp-433→Phe transformants; G, two D39/Asp-385→Asn Cys-428→Gly Trp-433→Phe transformants. The mobility of purified pneumolysin (53 kDa) is indicated.

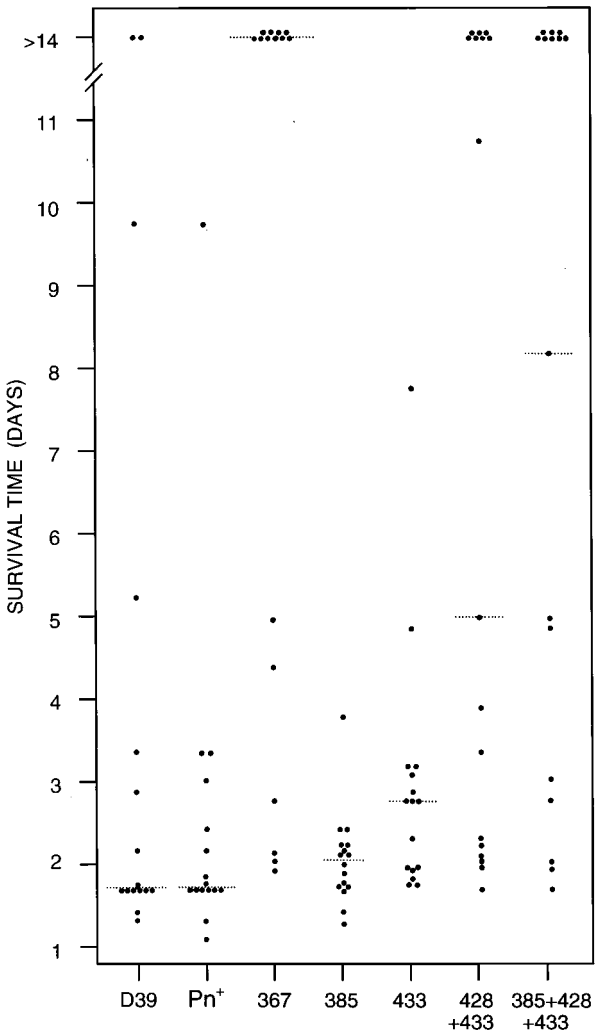

FIG. 4. Survival time of mice challenged with D39 derivatives. Groups of 16 mice were injected intraperitoneally with 2×10^5 CFU of *S. pneumoniae* D39, D39 derivatives expressing a wild-type pneumolysin gene (Pn$^+$), or D39 derivatives expressing pneumolysin genes with the indicated point mutations. The broken lines indicate the median survival time for each group.

complement activation by pneumolysin does not contribute significantly to virulence. Mutations affecting hemolytic activity, however, had a significant impact. Mice challenged with either D39/His-367→Arg or D39/Trp-433→Phe Cys-428→Gly had significantly greater median survival times (>14 and 5.0 days, respectively) and overall survival rates (10 of 16 and 7 of 17, respectively) than those receiving D39/Pn$^+$ (median survival time of 1.7 days, with no survivors). The His-367→Arg mutation reduces the specific hemolytic activity of pneumolysin to approximately 0.02% of the wild-type level, while the double mutation Trp-433→Phe Cys-428→Gly retains only 0.0001% of the wild-type hemolytic activity. Neither of these mutations, however, affects complement activation activity. Moreover, no additional reduction in virulence was observed when mice were challenged with D39/Trp-433→Phe Cys-428→Gly Asp-385→Asn (median survival time, 8.2 days; survival rate, 8 of 16) rather than D39/Trp-433→Phe Cys-428→Gly. However, we have recently found that triple-mutant pneumolysin purified from recombinant *E. coli*, unlike other purified pneumolysin derivatives, does not react with a mono-

TABLE 1. Statistical analysis of survival data[a]

Strain	*P* value[a]				
	D39/Pn$^+$	D39/His-367→Arg	D39/Asp-385→Asn	D39/Trp-433→Phe	D39/Trp-433→Phe Cys-428→Gly
D39	NS (NS)				
D39/His-367→Arg	<0.002 (<0.001)				
D39/Asp-385→Asn	NS (NS)	<0.002 (<0.001)			
D39/Trp-433→Phe	<0.05 (NS)	<0.002 (<0.001)	<0.05 (NS)		
D39/Trp-433→Phe Cys-428→Gly	<0.002 (<0.02)	NS (NS)	<0.002 (<0.02)	<0.02 (<0.02)	
D39/Trp-433→Phe Cys-428→Gly Asp-385→Asn	<0.002 (<0.01)	NS (NS)	<0.002 (<0.01)	<0.02 (<0.01)	NS (NS)

[a] Data represent Mann-Whitney *U*-test *P* values (two-tailed) for the differences in survival time between the groups of mice challenged with the indicated D39 derivatives. Figures in parentheses are χ^2 *P* values (two-tailed) for differences in overall survival rate between groups. NS, not significant.

clonal antibody which recognizes an epitope near the C terminus of the toxin (16a). Thus, it is possible that the combination of the three point mutations renders the resultant protein susceptible to proteolytic cleavage in recombinant *E. coli*, which is not apparent from SDS-PAGE analysis. Whether this also occurs in *S. pneumoniae* is not known. However, regardless of this caveat, the results of the present study demonstrate unequivocally that in the intraperitoneal challenge model, the contribution of pneumolysin to virulence is largely attributable to its hemolytic (cytotoxic) properties rather than to its capacity to activate complement.

Interestingly, the amount of pneumolysin cytolytic activity required for full virulence of pneumococci may be very small, as D39 derivatives carrying the Trp-433→Phe mutation (which reduces hemolytic activity to 0.1% of the wild-type level) had intermediate virulence. The median survival time of mice challenged with this strain was 2.7 days, which was significantly greater than for mice challenged with D39/Pn$^+$ (median survival time of 1.7 days). Both the median survival time and overall survival rate of mice challenged with D39/Trp-433→Phe were significantly less than for those challenged with either D39/His-367→Arg or D39/Trp-433→Phe Cys-428→Gly (Table 1). This result implies that very low levels of pneumolysin production may be sufficient for near-maximum virulence of a given strain of *S. pneumoniae*. This would be consistent with our observation that there does not appear to be a correlation between pneumolysin production and virulence of clinical isolates and that immunization with pneumolysin provides a significant degree of protection against strains of *S. pneumoniae* producing both low and high levels of toxin (1).

Conclusions. This study is the first to compare the virulence of isogenic pneumococci which differ only by defined amino acid substitutions in the toxin pneumolysin. The results confirm the involvement of pneumolysin in the pathogenicity of *S. pneumoniae* and indicate that in the intraperitoneal challenge model, the cytotoxic property of the toxin is more important than its capacity to activate complement. It should be emphasized, however, that in other models, this may not necessarily be the case. Indeed, there is evidence that the capacity of pneumolysin to activate complement plays a role in the course of infection during pneumococcal pneumonia and in the pathological changes seen. Feldman et al. (11) found that when purified pneumolysin was put into the lungs of rats, the histological changes seen had all of the salient features of pneumococcal pneumonia. The extent of these changes was diminished when toxins with either the Trp-433→Phe (reduced cytolytic activity) or Asp-385→Asn (complement activation deficiency) mutation was used. When pneumolysin-negative D39 derivatives (5) were instilled into mice, either endotracheally or intranasally, they had a reduced capacity to multiply in the lungs and the onset of bacteremia was delayed (9, 26). Rubins et al. (26) reported that this was due to the absence of both the cytolytic and complement-activating activities of pneumolysin. Coadministration of recombinant pneumolysin resulted in the pneumolysin-negative pneumococci having a pattern of multiplication in the lung comparable to that of the parental D39 strain. However, pneumolysin affected the course of infection in two ways. It influenced the extent of multiplication in the lung and the time that pneumococci persisted. Cytolytic activity determined the extent of multiplication in the lung, but it was the ability of pneumolysin to activate complement which determined its effect on persistence of pneumococci within the lungs. Pneumolysin-negative D39 coinstilled with the Asp-385→Asn version of pneumolysin did not persist to the same extent as the parent strain.

It appears, therefore, that the ability of pneumolysin to activate complement may be of little consequence in a systemic infection, but it is important locally. To confirm our view that both complement-activating and cytolytic activities of pneumolysin are important in pathogenesis of pneumonia, the behavior of the strains constructed in the present study is being examined in mice infected by both endotracheal and intranasal routes.

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