INFECTION AND IMMUNITY, May 1983, p. 548–552
0019-9567/83/050548-05$02.00/0

Vol. 40, No. 2

Effect of Immunization with Pneumolysin on Survival Time of Mice Challenged with *Streptococcus Pneumoniae*

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Received 13 December 1982/Accepted 16 February 1983

The role of the cytolytic toxin pneumolysin in the pathogenicity of *Streptococcus pneumoniae* was investigated. Pneumolysin was purified to homogeneity and used to immunize mice. When these mice were subsequently challenged via the nasal route with virulent *S. pneumoniae*, they survived significantly longer than control mice. The mean survival times were 5.52 and 2.48 days for immunized and control mice, respectively. This work provides direct evidence for the involvement of pneumolysin in pneumococcal pathogenicity.

Pneumolysin is a sulfhydryl-activated cytolysin produced by *Streptococcus pneumoniae*. Although the toxin was first discovered nearly 80 years ago (12), the nature and extent of its role in pathogenesis remains uncertain.

Intravenous injection of pneumolysin into rabbits has been shown to cause spherocytosis and increased osmotic fragility of erythrocytes (17), effects which could also be observed during the course of a pneumococcal infection (16). In addition, it has been shown that the injection of either purified pneumolysin or virulent pneumococci into rabbit eyes results in an acute inflammatory response (6, 7), possibly because of interaction of the toxin with polymorphonuclear leukocytes (8). However, despite this evidence, some doubt has been cast on the pathogenic importance of pneumolysin, because of its intracellular location (5) and the fact that substantial amounts of the toxin can be inactivated in vitro by concentrations of cholesterol similar to those found in human serum (9).

In the present report, the importance of pneumolysin in pneumococcal pathogenicity was directly investigated by determining whether prior immunization with the purified toxin affected the survival of mice challenged with virulent *S. pneumoniae*.

MATERIALS AND METHODS

Organism and culture conditions. Cell lysates from 150 clinical isolates of *S. pneumoniae* were assayed for pneumolysin activity as described below. A type 1 strain producing large amounts of the toxin was selected for further study. This organism was grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.15 g of cysteine-hydrochloride, and 13 mg of $CaCl_2 \cdot 2H_2O$ per liter. One-liter cultures were incubated at 37°C in sealed 1-liter bottles until the end of the logarithmic phase of growth (approximately 16 h), at which time their absorbance at 600 nm (1-cm lightpath) was approximately 0.9.

Assays for pneumolysin activity. (i) Tube assay. Samples for assay were first activated for 15 min at room temperature in the presence of 30 mM 2-mercaptoethanol. Serial twofold dilutions were then prepared with 0.145 M NaCl–7 mM sodium phosphate (pH 7.2) (PBS). A 1-ml amount of each dilution was mixed with an equal volume of a 1% (vol/vol) suspension of packed human group O erythrocytes in PBS. Tubes were incubated at 37°C for 30 min and centrifuged at 3,000 × *g* for 5 min, and the absorbance of the supernatant at 541 nm was measured. The percentage of erythrocytes lysed was plotted against dilution for each sample, and the pneumolysin activity of the sample was defined as the reciprocal of the estimated dilution at which 50% of the erythrocytes would have lysed. This activity was expressed as hemolytic units (HU) per milliliter.

(ii) Microtiter plate assay. The pneumolysin activity in fractions collected from chromatography columns and preparative polyacrylamide gels was determined semiquantitatively. A 50-μl portion of each activated sample was diluted serially in PBS along one row of 12 wells in a 96-well microtiter plate. A 50-μl amount of erythrocyte suspension was added to each well, and the plates were incubated at 37°C for 30 min and then centrifuged. The highest dilution of each sample resulting in at least 50% hemolysis was then estimated visually.

Purification of pneumolysin. The method for purifying pneumolysin was adapted from that of Shumway and Klebanoff (17). Cells from 18 liters of culture were collected by centrifugation at 8,500 × *g* for 15 min at 4°C and resuspended in 250 ml of 50 mM sodium phosphate (pH 7.0). Cellular autolysis was induced by the addition of sodium deoxycholate to a concentration of 0.1% (wt/vol) and was complete after 1 h at room temperature. The crude lysate was centrifuged at 38,000 × *g* for 15 min at 4°C, and the supernatant was decanted. Solid ammonium sulfate was added slowly to the stirred supernatant until 70% saturation had been reached. This solution was left for 1 h at 4°C and

then centrifuged at 38,000 × g for 15 min. The supernatant was discarded, and the pellet, redissolved in 30 ml of 10 mM sodium phosphate (pH 7.0), was dialyzed overnight against 200 volumes of the same buffer. The dialysate was clarified by centrifugation at 38,000 × g for 15 min at 4°C, and the supernatant was applied to a column (20 by 1.6 cm) of DEAE-cellulose (Whatman DE-52) which had been preequilibrated with 10 mM sodium phosphate (pH 7.0). The column was eluted with a linear gradient of 10 to 250 mM sodium phosphate (pH 7.0) at 4°C, and fractions were assayed for hemolytic activity. Fractions with activities greater than 1,000 HU/ml were pooled and concentrated to about 10 ml in an Amicon model 52 stirred cell ultrafiltration apparatus fitted with a YM10 membrane (10,000 molecular weight retention). The concentrate was then applied to a column (100 by 2.6 cm) of Sephacryl S-200 (Pharmacia Fine Chemicals, Inc., North Ryde, New South Wales) and eluted with 50 mM sodium phosphate (pH 7.0) at 4°C. Fractions with peak activities were again pooled and concentrated by ultrafiltration, this time to a volume of about 3 ml. Glycerol (10% [vol/vol]) and bromophenol blue (0.005% [wt/vol]) were added, and the material was loaded onto a preparative 3-mm-thick discontinuous polyacrylamide gel in a Protean gel electrophoresis apparatus (Bio-Rad Laboratories, Richmond, Calif.) equipped with an electroelution attachment. The sample was subjected to electrophoresis at 100 V for 20 h at 4°C while buffer (0.375 M Tris-hydrochloride [pH 8.8]) was pumped through a channel across the bottom of the separating gel. Fractions were collected and assayed for pneumolysin as described above, and those with activities greater than 2,000 HU/ml were pooled, concentrated by ultrafiltration, and then washed by several cycles of dilution in PBS followed by reconcentration. This step removed residual acrylamide and adjusted the buffer composition.

Pneumolysin prepared in this way could be stored in 50% glycerol at −15°C for at least 3 months without significant loss of activity.

Polyacrylamide gel electrophoresis. Discontinuous polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was carried out by the method of Laemmli (11). Electrophoresis under nondenaturing conditions was carried out as described above, except that SDS was omitted from all buffers and samples were not heated before loading. Slab gels consisted of a separating gel, 15 cm wide and 11 cm long, of 10% (wt/vol) acrylamide, topped by a stacking gel, 15 cm wide and 2.5 cm long, of 3% (wt/vol) acrylamide. Analytical slab gels were 1.5 mm thick. Gels were stained with Coomassie brilliant blue R250.

Preparative slab gels were 3 mm thick and included a channel 2 mm deep formed horizontally through the separating gel approximately 3 cm from the bottom, through which elution buffer was pumped during electrophoresis. The acrylamide concentration in the separating gel below the elution channel was 20% (wt/vol).

Protein assay. Protein concentrations were measured by the method of Lowry et al. (13) and by the method of Bradford (1), with bovine serum albumin as a standard.

Immunization. Pneumolysin (about 280 μg/ml in PBS and 50% [vol/vol] glycerol) was partially inactivated by exposure to oxygen. It was then emulsified with an equal volume of Freund complete adjuvant (Commonwealth Serum Laboratories, Parkville, Victoria, Australia). Adult male outbred mice (Prince Henry strain), each weighing approximately 30 g, were injected intraperitoneally with 0.15-ml volumes containing about 20 μg of pneumolysin. At 10-day intervals, the mice were given two further injections of 20 μg of pneumolysin in PBS–50% (vol/vol) glycerol, emulsified with Freund incomplete adjuvant. Control mice were subjected to a similar course of injections of PBS-glycerol-adjuvant, but with pneumolysin omitted.

Eight days after the final injection, blood samples were collected from all mice by suborbital bleeding, and the sera were tested by the Ouchterlony gel immunodiffusion technique (14) for the presence of antibodies to pneumolysin.

The antihemolytic titers of the sera were also determined by the following method. Sera were serially diluted with PBS in microtiter plates. Each well also contained 4 HU of pneumolysin in a final volume of 50 μl. Plates were incubated at 37°C for 15 min to allow serum antibody to bind to the pneumolysin; 50 μl of a 1% erythrocyte suspension was added, and the plates were incubated at 37°C for another 30 min. For each serum, the highest dilution that inactivated the pneumolysin was determined, and the antihemolytic titer was then calculated and expressed as anti-HU per milliliter.

Counter-current immunoelectrophoresis. Counter-current immunoelectrophoresis for detection of capsular polysaccharide was carried out as described by El-Refaie and Dulake (2).

Quantitation of antibody to pneumococcal capsular polysaccharide. Antibody to pneumococcal capsular polysaccharide (serotypes 1 and 2) was measured by radioimmunoassay by the method of Schiffman et al. (15).

Challenge of mice with *S. pneumoniae*. Mice were anesthetized by intraperitoneal injection with 1.8 mg of pentobarbitone. A 50-μl amount of a 4-h serum broth culture of a virulent type 2 strain of *S. pneumoniae* (NCTC 7466) was then introduced into the nostrils of each mouse. All mice regained consciousness after 1 to 2 h.

Statistical analysis. Results of nasal challenge experiments were analyzed by the Mann-Whitney U test.

RESULTS

Purification of pneumolysin. The aim of this study was to determine whether immunization with pneumolysin afforded significant protection against pneumococcal infection in mice. Purification of pneumolysin to homogeneity represented the first step toward this goal. Samples from various stages of the purification procedure (see above) were analyzed by polyacrylamide gel electrophoresis in either the presence (Fig. 1) or absence of SDS. In both systems, the final preparation migrated as a single major protein species which accounted for greater than 97% of total protein (as determined from densitometer scans of stained gels). Comparison of the mobility of this major species with that of molecular weight marker proteins on SDS gels (Fig. 1) indicated that it had an apparent molecular

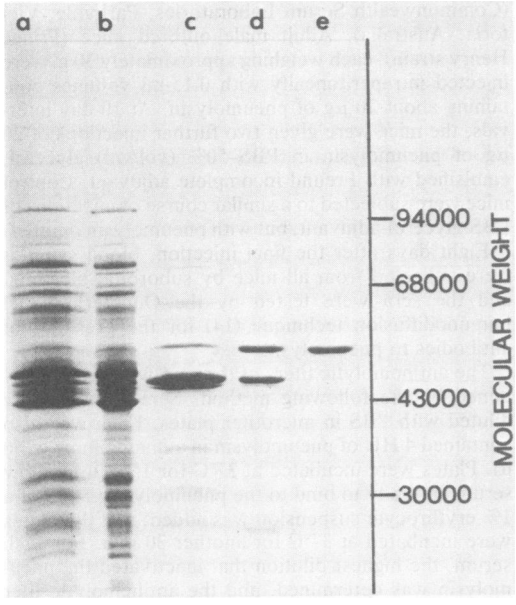

FIG. 1. SDS-polyacrylamide gel electrophoresis of samples from various stages of the pneumolysin purification procedure. Protein samples were electrophoresed as described in the text and stained with Coomassie brilliant blue. Lane a, 38,000 × *g* supernatant of crude cell lysate; lane b, post-$(NH_4)_2SO_4$ precipitation; lane c, post DEAE-cellulose chromatography; lane d, post Sephacryl S-200 chromatography; lane e final purified material. The amounts of protein loaded were approximately 110, 75, 40, 10, and 5 μg for lanes a to e, respectively. The mobility of various molecular weight marker proteins is also indicated.

weight of 52,000. No capsular polysaccharide material could be detected in the final preparation by counter-current immunoelectrophoresis. The final pneumolysin preparation had a specific activity of approximately 500,000 HU/mg of protein, which represented a net purification of about 500-fold compared with the crude cell lysate. Approximately 15% of the original activity was recovered, with a final yield of about 500 μg of highly purified pneumolysin per 18 liters of cell culture.

Antibody response of mice to immunization with pneumolysin. Pneumolysin purified as described above was used to immunize groups of mice. At the same time, control mice were injected with a placebo of PBS-glycerol-adjuvant (see above). Serum samples were collected from all mice 8 days after their last injection and analyzed by immunodiffusion. All those from mice that had been injected with pneumolysin ("immunized" mice) produced a single precipitin line when tested against either highly purified pneumolysin or a crude (post-ammonium sulfate) pneumococcal extract. No precipitin lines were visible when sera from control mice were similarly tested (Fig. 2).

All sera were tested for the presence of antibody to type 1 and type 2 pneumococcal capsular polysaccharide by radioimmunoassay. For type 1, the antibody level (mean ± standard deviation) was 566 ± 199 ng of antibody nitrogen per ml of serum for control mice, compared with 506 ± 164 ng of antibody nitrogen per ml for immunized mice. For type 2, the levels were 199 ± 40 and 164 ± 26 ng of antibody nitrogen per ml for control and immunized mice, respectively. Clearly, immunization with purified pneumolysin did not result in an increase in antibody to capsular polysaccharide of either serotype.

All sera were also assayed for antihemolytic activity. The geometric mean titer was 3,240 anti-HU/ml of serum for control mice and 121,700 anti-HU/ml for immunized mice. The low level of antihemolytic activity in control sera is probably due to inhibition by free cholesterol (9).

Challenge of mice with *S. pneumoniae*. Mice were challenged with a type 2 strain of *S. pneumoniae* (NCTC 7466) which was chosen because of its high virulence (the mouse intraperitoneal 50% lethal dose was less than 10 organisms) and because it was of a different capsular serotype from the organism used as the source of pneumolysin. Both NCTC 7466 and the source organism were shown to produce pneumolysin in similar amounts.

Groups of immunized and control mice (with 8 to 10 mice in each group) were inoculated intranasally with approximately 5×10^6 NCTC 7466 cells in a total volume of 50 μl. All mice died within 10 days, with the exception of three immunized mice which were still alive and well after 20 days. A type 2 *S. pneumoniae* could be isolated from heart blood collected from each mouse that died as a result of the challenge. Histological examination revealed evidence of pneumonic consolidation in both control and

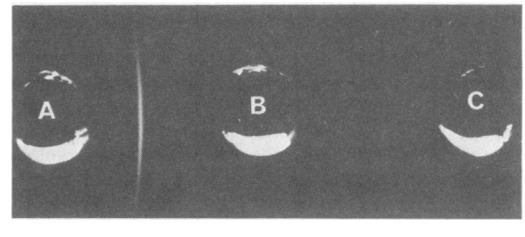

FIG. 2. Immunodiffusion analysis of sera from immunized and control mice. Well A contained 25 μl of serum from a mouse immunized with pneumolysin. Well B contained 25 μl of crude pneumococcal extract at the post-$(NH_4)_2SO_4$ precipitation stage of the pneumolysin purification procedure. Well C contained 25 μl of serum from a control mouse.

immunized mice at the time of death. The survival time of each mouse was recorded, and the results for four separate trials are shown in Fig. 3. In each trial, the mean survival time of immunized mice was greater than that of control mice. The overall mean survival time for mice immunized with pneumolysin was 5.52 days, compared with 2.48 days for control mice. For the purpose of calculating the mean survival time for immunized mice, those that did not die as a result of challenge were assigned a survival time of 10 days. When the results were analyzed by the Mann-Whitney U test, the observed differences were highly significant, with P values less than 0.005, 0.001, 0.002, and 0.001, for trials 1 to 4, respectively.

DISCUSSION

As long ago as 1946, Halbert et al. (3) immunized mice with pneumolysin preparations and showed that such treatment produced a definite, if low-grade, non-type-specific protection against pneumococcal infection. However, because of the technical limitations of the time, the pneumococcal extract used by these workers was highly impure. Specific activities quoted by Halbert et al. (3) suggest that pneumolysin constituted only about 1% of the total protein content of their vaccine. In addition, the authors pointed out that their pneumolysin preparation was contaminated with C polysaccharide. Consequently, it is impossible to draw any definite conclusions from this early study concerning the specific involvement of pneumolysin in pneumococcal pathogenicity.

Modern techniques for protein fractionation have now made it possible to prepare relatively large amounts of toxin in highly purified form. In the present work, we performed immunization experiments with high-purity antigen, and our results strongly support the hypothesis that pneumolysin does play a significant role in the pathogenicity of *S. pneumoniae*.

The homogeneity of the pneumolysin used in this study was confirmed by analytical polyacrylamide gel electrophoresis in both the presence and absence of SDS. In both systems, the final preparation migrated as a single major band comprising ≥97% of the total material. The mobility of this band on SDS gels corresponds to a molecular weight of 52,000. The molecular weight of pneumolysin has previously been estimated as 63,000 on the basis of sedimentation and gel filtration analysis of an extremely impure extract under nondenaturing conditions (10). Under such conditions, molecular weight estimates may be readily distorted by asymmetry in molecular shape and by interaction with other species. This is not the case, however, when estimates are based on electrophoretic mobility

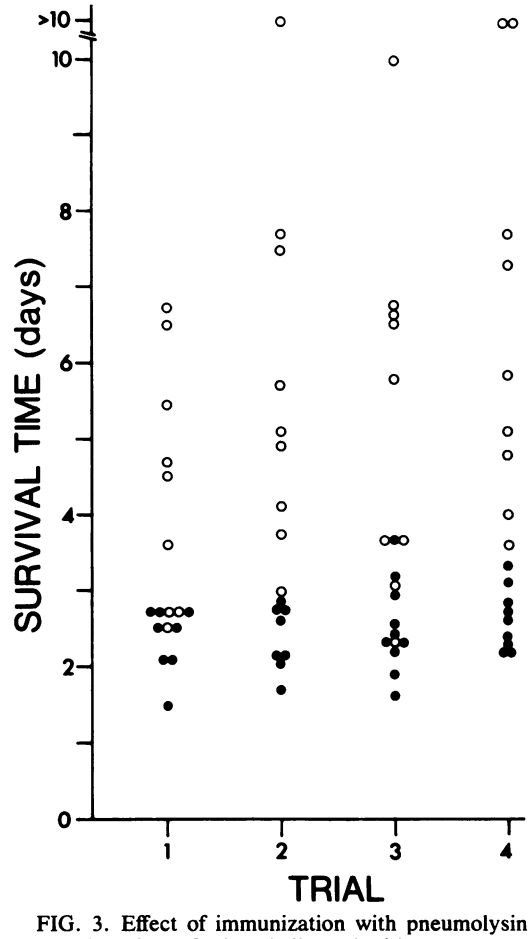

FIG. 3. Effect of immunization with pneumolysin on survival time of mice challenged with *S. pneumoniae*. Mice were challenged nasally with *S. pneumoniae* as described in the text. The survival time of immunized mice (○) and control mice (●) is indicated for each of four separate trials.

in the presence of SDS (18). The specific activity of our final pneumolysin preparations (approximately 500,000 HU/mg of protein) agrees well with that obtained by other workers (4, 17).

Immunization of mice with our pneumolysin preparation produced a strong antibody response. When sera from immunized mice were tested by immunodiffusion against either crude cell lysate or the purified toxin, only one precipitin line was observed. No precipitin lines were observed when sera from control mice were similarly tested. The antihemolytic titer of sera from immunized mice was almost 40 times higher than that of sera from control mice. These results, taken with the high degree of homogeneity of the preparation on polyacrylamide gels and the fact that immunized mice showed no increase in antibody to type 1 or type 2 capsular

polysaccharide, strongly suggest that immunization of mice with our pneumolysin preparation induced the production of significant levels of antibody to the toxin, but not to any other pneumococcal product.

The pneumococcal challenge was administered nasally to most closely mimic the natural route of infection. All mice were anesthetized before challenge to ensure deep inhalation of the organism. The dose of organism used was sufficient to be sure of killing all control mice. The overall mean survival time after challenge was only 2.48 days for control mice, but 5.52 days for immunized mice. A highly significant difference in survival time between immunized and control mice was observed in each of the four trials. It is clear that a high level of circulating antibody directed against pneumolysin correlates positively and highly significantly with increased survival time after challenge with virulent *S. pneumoniae*. Although immunization with pneumolysin did not protect mice from eventual death from pneumococcal infection, the fact that it extended their survival time is strong evidence for the involvement of the toxin in the pathogenicity of *S. pneumoniae*.

ACKNOWLEDGMENTS

We thank Tracey Dodman and Julie Magor for competent technical assistance.

This work was supported by grants from the National Health and Medical Research Council and the Adelaide Children's Hospital Research Trust.

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