USE OF INSERTIONAL INACTIVATION TO FACILITATE
STUDIES OF BIOLOGICAL PROPERTIES OF
PNEUMOCOCCAL SURFACE PROTEIN A (PspA)

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Infants and very young children are poor responders to polysaccharide anti-
gen (1–4) and fail to make protective antibody responses to a number of
polysaccharide-containing vaccines including the 23-valent pneumococcal vaccine
(2, 5) currently used to immunize adults (6). One way to attempt immunization
of children against encapsulated bacteria is to modify the capsular polysaccharides
so that they will be antigenic in children. Another is to identify bacterial proteins
that can elicit sufficiently protective responses. The studies described in this
paper are based on our recent demonstration that two mAbs reactive with a
pneumococcal surface protein A (PspA) present on the nonencapsulated pneu-
mococcal strain R36A, and on some encapsulated strains (7), can protect mice
from fatal pneumococcal infection with certain type 3 and type 2 strains (8).
Whether or not such pneumococcal proteins can elicit protective responses in
animals or humans, and whether or not these proteins play a role in pneumococcal
virulence, has not previously been established.

The ability of pneumococcal surface components, in addition to the capsule,
to elicit protective immune responses could account for the early observations
that the most protective horse antipneumococcal sera were made against highly
virulent pneumococci (9) and that the presence of anticapsular antibody in the
sera did not necessarily mean that they would be highly protective (10). Although
there were a number of early reports (11–15) of protective antibodies directed
against antigens other than the capsular polysaccharide, the subject of antigenic
pneumococcal proteins ceased to be a major topic of investigation after Avery
and Goebel demonstrated (16) in 1933 that the protective properties of an
immune serum from a horse could be completely removed by absorption with
isolated type 1 polysaccharide. Protective antibodies to protein components of
the pneumococcus may have been difficult to detect, in part because antibodies

This work was supported by U.S. Public Health Service grants AI-21548, CA-16673, and CA-13148.
D. E. Briles is the recipient of Research Development award AI-00498 from the National Institutes
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1 Abbreviations used in this paper: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CTM, complete
transformation medium; CWE, cell wall extract; PBST, PBS/Tween 20; PspA, pneumococcal surface
protein A.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/87/02/0381/14 $1.00
Volume 165 February 1987 381–394
**Table I**

**Bacterial Strains**

| Strain          | Relevant genotype/phenotype | Reference |
|-----------------|----------------------------|-----------|
| *S. pneumoniae* |                            |           |
| R36A*           | PspA*                      | 8, 19     |
| Rx1*            | *hex, PspA*                | 20        |
| DP1617          | *ery-2, str-1, fus, sul-d, nov-1, sig* | 21        |
| WG44.1          | *ery-2, PspA*              | This paper|
| WG44.2          | *ery-2, PspA*              | This paper|
| WG44.3          | *ery-2, PspA*              | This paper|
| D39             | PspA*                      | 19        |
| JY53            | *ery-2, PspA*              | This paper|
| JY54            | *ery-2, PspA*              | This paper|
| WU2             | PspA*                      | 22        |

**Escherichia coli**

| DH1             | F*, *endA1, hisdR17, (r17*, m1*), supE44, thi-1, Lac, recA1, gyrA96, relA | 23        |

* R36A is a nonencapsulated derivative of the virulent type 2 strain D39.
* Rx1 is a highly transformable derivative of R36A.

to the cell wall and capsular polysaccharides may predominate in the immune response to live and killed whole pneumococci (8) and because anticapsular antibodies are probably more protective than antibodies to pneumococcal proteins.

Injection of intact cells of the nonencapsulated strain R36A can elicit protection against certain encapsulated *Streptococcus pneumoniae* in *xid* mice (17). It seemed likely that this protection was due to antiprotein antibodies because the immunogen used did not contain capsular polysaccharide and because *xid* mice fail to make antibodies to polysaccharides including the teichoic acids of pneumococcal cell walls (18).

To facilitate subsequent investigation of the functional and structural properties of PspA, including its ability to elicit protective immune responses, we have constructed mutants of *S. pneumoniae* strain Rx1, a variant of R36A (19), which fail to express this cell surface protein. From one of these mutants we have cloned a 550 bp DNA fragment that appears to be part of the *pspA* structural gene. In this report we describe the generation of this cloned fragment and studies in which the plasmid containing the cloned fragment was used to produce congenic PspA* pneumococcal strains that were used to evaluate the biological effects of PspA.

### Materials and Methods

**Bacterial Strains.** The strains used in this study are listed in Table I.

**Growth and Maintenance of Strains.** All strains were maintained as 10% glycerol frozen stocks at −80°C. Pneumococcal cultures were grown according to procedures routinely used in our lab (24) and were harvested by centrifugation (4,000 g, 10 min). Their concentrations were confirmed by plating on blood agar. *Escherichia coli* cultures were grown in LB medium (25) unless otherwise specified.

**Mice.** CBA/N mice were obtained from Dominion Laboratories, Dublin, VA. (CBA/N
× DBA/2)F1 mice were raised by crossing CBA/N females with DBA/2 males obtained from The Jackson Laboratory, Bar Harbor, ME.

**Bacterial Transformation.** Transformations of pneumococcal strain Rx1 and mutants derived from it were carried out by established procedures (26). Cells were grown to high competence in the complete transformation medium (CTM) of Smith et al. (27). Alternatively, Todd-Hewitt broth containing 0.5% yeast extract was substituted as the basal medium then supplemented after autoclaving to 1 mM CaCl2 and 2 mg/ml of BSA. Similar transformation frequencies of Rx1 were observed with both media. Competent pneumococci were produced by growing a culture of recipient pneumococci to a density of 3 × 10⁸ cells/ml in basal medium, diluting 100-fold into competence medium, and removing a sample of cells at 10-min intervals over a period of about 200 min. Aliquots of the cells removed at each time point were stored frozen in 10% glycerol at −80°C. To determine the time point at which competent cells were obtained, cells from each time point were used for transformation as described below. From then on, transformation studies were done with cells grown under the same conditions to the optimum time point, made 10% in glycerol, and stored frozen at −80°C.

For transformation, cultures were thawed at 37°C and added to 0.1 volume of DNA (usually at a concentration of 1 µg/ml) at the same temperature. The cells were then incubated for 1 h to allow phenotypic expression. Dilutions were then made and plated on blood agar containing the appropriate antibiotic. Virulent, encapsulated pneumococci were transformed by using competent, heat-killed broth cultures of Rx1 to induce competence (28).

**E. coli** strain DH1 at ~10⁷ CFU/ml was transformed with <10 µl containing ~0.2 µg of plasmid DNA by the method of Hanahan (23).

**Insertion Inactivation Mutagenesis.** Extracted pneumococcal chromosomal DNA was digested with Hind III, Bam H1, or Sau 3A according to the manufacturer's (Boehringer Mannheim Biochemicals, Indianapolis, IN) instructions and ligated to the appropriate digest of pVA891 (29) that can replicate autonomously in *E. coli* but not streptococci. The product of this ligation reaction was used to transform strain Rx1 at about 20 ng DNA/ml of culture (30). A portion of each culture was plated to score the number of Em' transformants. To select for Em' transformants, the rest of the culture was diluted 100-fold into growth medium containing 2 µg/ml erythromycin and was grown to 5 × 10⁸ bacteria per ml. The cultures were supplemented to 10% glycerol and stored frozen at −80°C. Individual colonies were tested for inactivation of genes coding for surface antigens detected by our mAbs by the colony immunoblot procedure described below.

Three isolates that did not express PspA were designated WG44.1, WG44.2, and WG44.3. The remainder of the isolates screened still produced PspA. Five of these, designated WG44.5, WG44.6, WG44.7, WG44.9, and WG44.12, were used as donors for the introduction by chromosomal transformation of pVA891 into non-PspA portions of the D39 chromosome. D39 isolates obtained all were Em' and still produced PspA. These isolates have been identified as LC10.1, LC10.2, LC10.3, LC10.5, and LC10.8.

**Colony Blot Procedure for the Detection of PspA⁻ mutants of Rx1.** Pneumococci were plated onto blood agar plates containing 2 µg/ml of erythromycin at about 100 CFU/plate and were incubated overnight at 37°C in a candle jar. Individual colonies were blotted onto a sterile nitrocellulose membrane by placing the membrane over the colonies on the surface of the agar and then gently lifting it off. The membrane was incubated for 1 h at room temperature with 1% BSA in PBS and transferred to a 10% solution of mAb tissue culture supernatant in 0.05% Tween 20 in PBS (PBST) for 2 h at room temperature on a rocking platform. The membrane was washed three times for 5 min each with PBST, and then incubated for 2 h at room temperature with isotype-specific affinity-purified goat anti-mouse alkaline phosphatase–conjugated antibody (0.2 µg/ml) diluted in PBST. As a control, all new lots of goat immune sera were tested for reactivity with pneumococcal antigens. The membrane was again washed with PBST, and the alkaline phosphatase conjugates were visualized by placing the membrane in 0.5 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma Chemical Co., St. Louis, MO) in 1 M Tris, pH 8.8, on
A PNEUMOCOCCAL IMMUNOGEN AND VIRULENCE FACTOR

The development of blue spots (i.e., colonies) was stopped by rinsing the membrane several times with deionized water.

Immunostaining of Electroblotted SDS Gels (Immunoblotting). Electrophoresed PspA was visualized by standard immunoblotting techniques. Crude lysates were prepared by detergent-augmented autolysis as described previously (8). We used a modification of the method described by Lacks and Neuberger (33) for autoplast formation to produce a cell wall extract (CWE) of pneumococcal strains (7). Pneumococci were grown to ~1 x 10⁷ cells/ml in 500 ml of Todd-Hewitt broth supplemented with 0.5% yeast extract. Cells were harvested by centrifugation, washed once with PBS, and resuspended in 3 ml of PBS containing 20% sucrose and 0.05 M MgCl₂. After 24 h at room temperature, the suspension was centrifuged (4000 g, 10 min, 4°C) to remove the protoplasts. The supernatant was dialyzed against three changes of PBS at 4°C and centrifuged at 10,000 g for 10 min to remove insoluble material. The amount of protein in the CWE was determined by a microassay (Bio-Rad Laboratories, Richmond, CA). The CWE was aliquoted and stored at −20°C until use.

SDS-PAGE was carried out on unlabeled CWE using 20 μl of cell material (1 μg/μl total proteins) added to 20 μl of SDS-PAGE sample buffer. This was placed in a boiling water bath for 5 min before loading in the well of a 5–12% gradient gel (34). After electrophoresis, the proteins were transferred to nitrocellulose (Millipore, Bedford, MA) using a Trans-Blot apparatus (Bio-Rad Laboratories) with the transfer buffer described by Burnette (35). After transfer of the proteins, the membrane was processed as described in the colony blot assay.

Preparation of ³²P-labeled DNA probe. Plasmid DNA containing the pneumococcal insert, pKSD300, was purified by the method of Birnboim and Doly (36). The purified plasmid DNA was digested with Sau 3A to generate a unique pneumococcal fragment, which was separated from the vector DNA on a 0.7% agarose gel. The pneumococcal fragment was isolated by the method of Dretzen et al. (37). The purified DNA fragment was ³²P-labeled by nick translation synthesis (38) using a commercially available kit (Amersham Corp., Arlington Heights, IL).

Southern Blot Analysis. High molecular weight pneumococcal genomic DNA was prepared by resuspending 10⁹ bacteria in 0.5 ml of lysis buffer (0.1% sodium deoxycholate, 0.01% SDS, and 0.15 M sodium citrate) for 60 min at 65°C. Proteinase K was added to 100 μg/ml, and the suspension was incubated overnight at 57°C. It was then extracted twice with phenol, once with CHCl₃/isoamyl alcohol (24:1), and dialyzed overnight at 4°C against Tris/EDTA (10 mM Tris, pH 8.0, and 1 mM EDTA). RNase was added to 100 μg/ml, and the suspension was incubated for 2 h at 37°C followed by a second 2-h incubation in the presence of 10 μg/ml of proteinase K. The DNA was extracted twice with phenol and once with CHCl₃/isoamyl alcohol, and was dialyzed extensively against Tris/EDTA at 4°C.

For Southern analysis, the pneumococcal genomic DNA was digested with various enzymes, the fragments were separated by agarose gel electrophoresis, and were transferred to a nitrocellulose membrane. Hybridization was at 42°C in 50% formamide in sealed plastic bags. Membranes were washed as stringently as possible, and then autoradiographed on Kodak XA film using intensifying screens (25).

Mouse Immunization Studies. We immunized xid mice with two weekly intravenous injections of 2 x 10⁹ heat-killed Rxl (PspA+) or WG44.1 (PspA⁻). After a 6-d rest, the mice were injected with 25 or 100 CFU of WU2, type 3, intravenously in 0.2 ml of Ringer's lactate (the LD₅₀ for WU2 in xid mice is 1–2 CFU). Mice were observed and deaths were recorded for a 10-d period after inoculation.

Blood Clearance Assays. The rate of removal of pneumococci from the blood was determined using previously described procedures (24). Mice were challenged intravenously with mixed cultures containing equal numbers of PspA⁺ (D39) and PspA⁻ (JY53
or JY54) pneumococci. The total number of pneumococci injected was $\sim 10^5$. Blood samples were collected from the retroorbital plexus at the indicated times in calibrated heparinized capillary pipettes. The samples were serially diluted in Ringer's lactate and plated on blood agar to determine the total number of viable pneumococci in the blood or on blood agar containing 5 μg/ml of erythromycin to determine the number of mutants.

### Results

**Isolation of Insertionally Inactivated PspA mutants.** We used the colony blot procedure to screen for PspA mutants of Rx1 that had occurred as a result of insertion mutagenesis after transformation with ligation mixtures of the plasmid pVA891 and random restriction fragments of Rx1 chromosomal DNA. Only one of three chromosomal libraries yielded mutants that failed to react with anti-PspA antibody XI164. This was the library constructed using Sau 3A endonuclease. We identified three mutants from this library and designated them WG44.1, WG44.2, and WG44.3. The frequency of mutation observed was $\sim 1$ per 380 Em$^r$ colonies.

PspA was not detected by immunoblot analysis of lysates of the three mutants (Fig. 1), indicating that the PspA$^-$ phenotype was not due merely to a failure to export the protein to the surface.

We used the colony blot assay to test the ability of our anti-PspA mAbs XI64, XI69, XI126, and HPR36A to bind to the mutants. Although our previous data had suggested that our four anti-PspA mAbs reacted with different epitopes (8), it appeared that they all detected the same molecule (7). This interpretation was consistent with our colony blot results since all four antibodies reacted with the wild-type Rx1 pneumococci, but none of them reacted with any of the mutants.

**Cloning of a Pneumococcal Fragment from the Inactivated Gene.** By extracting DNA from the mutants, and using this DNA to transform *E. coli*, we were able to isolate a single clone that carried a fragment of about 550 bp. When this chimeric plasmid, derived from pVA891 and designated pKSD300, was used to transform wild-type Rx1, 32 of 32 of the resulting transformants failed to produce PspA. This indicated that we had cloned a fragment that carried homology with a gene necessary for the production of PspA.

**Southern Blot Analysis.** We examined the ability of our cloned fragment to bind restriction enzyme fragments of chromosomal DNA isolated from PspA-
producing wild-type cells. $^{32}$P-labeled probe isolated from pKSD300 hybridized to unique fragments of Rx1 chromosomal DNA that had been digested with Bam HI, Hind III, or Eco RI and electrophoresed in agarose (Fig. 2).

**Colony Hybridization Analysis.** To further confirm the specificity of our DNA probe, we did colony hybridization experiments in which the $^{32}$P-labeled probe from pKSD300 was tested for its ability to bind the DNA from 102 pneumococcal isolates representing 21 serotypes. These isolates were also tested for their ability to react with mAbs Xi64 and Xi126 in a colony blot assay. In agreement with our previous study (7), the reactivity of the mAbs with the isolates was relatively independent of their capsular serotypes (data not shown). The reactivity of the probe with the isolates was also relatively independent of their capsular serotypes (Table II, footnote).

When the reactivity of the anti-PspA antibodies with the isolates was compared with the reactivity of the probe with the isolates, it was observed that although there was not a one to one correlation in the ability to react with the antibody and the DNA probe, 18 of the 25 isolates that produced PspA as detected by either Xi64 or Xi126, did react with the probe and only 3 of 77 isolates that failed to react with either antibody reacted with the probe (Table II). These results indicated that our probe reacted preferentially with pneumococcal strains that produce a PspA that is immunochemically similar to that of strain Rx1. We also tested five group A and five group B streptococcal isolates in these assays. None of these bacteria reacted with either of our antibodies or the DNA probe.

**Transformation and Growth Rate of PspA Mutants.** We examined the effect of
TABLE II
Pattern of Reactivity of Anti-PspA Antibody and PspA Probe with Pneumococcal Isolates

| Number of isolates | Reactivity with: | 550 bp pspA probe |
|--------------------|-----------------|------------------|
|                    | Xi64            | Xi126            |                   |
| 8                  | +               | +                | +                 |
| 7                  | +               | -                | +                 |
| 3                  | -               | +                | +                 |
| 3                  | -               | -                | +                 |
| 1                  | +               | +                | -                 |
| 1                  | +               | -                | -                 |
| 5                  | –               | +                | –                 |
| 74                 | –               | –                | –                 |

* The reactivity of the probe with strains of the 21 capsular serotypes was as follows: type 1, 0/3 (isolates reactive with the probe per isolates of serotype tested); type 2, 3/4; type 3, 4/11; type 4, 0/10; type 5, 1/1; type 6, 3/14; type 7, 1/7; type 8, 0/2; type 9, 2/8; type 10, 0/4; type 11, 0/1; type 12, 1/1; type 14, 2/7; type 16, 0/3; type 18, 0/5; type 19, 1/8; type 21, 0/2; type 23, 1/6; type 27, 0/1; type 31, 1/3; and type 33, 1/1.

TABLE III
Transformation of S. pneumoniae with a Chromosomal Str* Marker

| Recipient strain | Chromosomal pVA891 expression | PspA expression | Transformation frequency* |
|------------------|-------------------------------|-----------------|--------------------------|
| Rx1              | –                             | +               | 1/100                    |
| WG44.1           | +                             | –               | 1/970                    |
| WG44.2           | +                             | –               | 1/940                    |
| WG44.3           | +                             | –               | 1/890                    |
| WG44.7           | +                             | +               | 1/230                    |
| WG44.9           | +                             | +               | 1/230                    |
| WG44.13          | +                             | +               | 1/210                    |

* The frequency of transformants per viable cells. These data represent averages obtained from four separate experiments, each of which provided results indicating that PspA* pneumococci transform about 4–10 times more efficiently than PspA* pneumococci. In each of the four experiments the PspA* and PspA* cell lines used were grown to identify cells in the stage of growth showing maximal transformation efficiency. The data given are based on values obtained using cells from each strain that were harvested on the time point of highest transformation efficiency of that strain in that particular experiment.
PspA+ vs. PspA− strains. This finding suggests that the presence of PspA on the bacterial surface may have some effect on the frequency of transformation.

Using one of the mutants, we also observed that a lack of PspA had no effect on the rate of growth of these bacteria in a complex medium (Fig. 3).

**Immune Response of xid Mice to PspA.** To evaluate the protective potential of PspA, we immunized xid mice with two weekly intravenous injections of Rx1 (PspA+) or WG44.1 (PspA−), rested them, and infected them with WU2, type 3, pneumococci. We were able to avoid complicating factors caused by antibodies to the PC determinant of the teichoic acids and to the capsule in part by using xid mice that cannot produce antibodies to most polysaccharides including both of these antigens (18). Potential problems with anticapsular antibodies were doubly eliminated since the immunizing strains were nonencapsulated derivatives of a type 2 strain. The survival of the mice is depicted in Fig. 4. These results
McDANIEL ET AL.

TABLE IV
Mortality of Mice Infected with PspA+ and PspA− S. pneumoniae

| Mice                  | Intravenous challenge | Number of mice | Median hours to death | Alive at 20 d | PspA+ vs. PspA−*4 |
|-----------------------|-----------------------|----------------|-----------------------|---------------|--------------------|
| (CBA/N × DBA/2)F1     | 300 PspA+             | 10             | 41 (37–46)           | 10            | <0.03              |
| males (xid)           | 500 PspA−             | 17             | 54 (48–60)           | 12.5          | <0.03              |
| (CBA/N × DBA/2)F1     | 106 PspA+             | 8              | 58 (46–78)           | 25            | <0.06              |
| females (non-xid)     | 106 PspA−             | 16             | 133 (111–162)        | 56            | <0.06              |

PspA+ is D39. Data from experiments with both JY53 and JY54 were pooled to obtain the PspA+ data. There were no statistically significant differences between the data obtained with JY53 and JY54.

* Deaths were recorded at intervals of every 1–3 h during the experiment. Mice alive at 10 d were arbitrarily assigned a time of death of 240 h for the calculations of the reciprocal mean and p value.
† The median days to death was calculated as the reciprocal mean and expressed with the range of standard error.
‡ p values; calculated by the two sample rank test from the time of death of the individual mice in each group.

indicate that the presence of PspA on pneumococci can elicit protective immunity against pneumococcal infection.

Relative Pathogenesis of D39 and D39 Mutants Lacking PspA. To analyze its effect on virulence, the PspA− mutation was introduced into the type 2 strain D39 by transformation with pKSD300. Seven of seven isolates tested were PspA−, as determined by the colony blot assay, and two of these, JY53 and JY54, were chosen for the virulence studies. Although the PspA− mutants, JY53 and JY54, could still cause fatal infection of both xid and non-xid mice, the median time to death of mice infected with them was significantly later than that of mice infected with D39 (Table IV). The reduction in the virulence resulting from the loss of PspA was more apparent in blood-clearance studies. The number of mutant bacteria remaining in the blood 1 h after infection was reduced ~10-fold when compared with the parent D39 strain in a mixed infection (Fig. 5). After this first hour, no further decreases occurred and the ratios of PspA− to PspA+ isolates remained essentially unchanged.

Control strains were prepared for the blood-clearance studies that carried the pVA891 plasmid in their chromosome but still expressed PspA. These were constructed by transforming D39 with chromosomal DNA from individual pneumococci obtained from the Rx1 library of random pVA891 inserts. Independently transformed D39 colonies, detected by screening for erythromycin resistance, were used as sources of DNA to backcross the chromosomally inserted pVA891s once more onto the D39 background. Using these strains, it was possible to show that the rapid early blood clearance of JY53 and JY54 was because of their lack of PspA and not because of some effect of simply carrying pVA891 in their chromosomes. Five D39 strains carrying pVA891 but still expressing PspA showed exactly the same blood-clearance patterns as D39 (Table V).

Discussion

We have isolated mutants of S. pneumoniae that fail to produce PspA and have cloned a 550 bp fragment of a gene required for its production into the plasmid
A PNEUMOCOCCAL IMMUNOGEN AND VIRULENCE FACTOR

FIGURE 5. Comparison of blood clearance of PspA⁺ (D39) vs. PspA⁻ (JY53 or JY54) pneumococci. Both xid ([CBA/N × BALB/c]F, males, left panels) and normal ([CBA/N × BALB/c]F, females, right panels) mice were infected intravenously with mixed cultures containing approximately equal numbers of D39 and JY53 (panels A, B, and E) or D39 and JY54 (panels C, D, and F). The number of bacteria was determined by bleeding at 1 min (time 0), 1 h, and 4 h and plating for total CFU (i.e., D39 plus mutant) and for Ery⁺ CFU (mutant only). A–D show the numbers of D39 and the numbers of mutant recovered from a single mouse in a typical experiment. E and F indicate the ratios of JY53 and JY54, respectively, to D39. These ratios were obtained from the averages of six and five mice, respectively.

TABLE V
The Effect of Random pVA891 Insertions on the Virulence of D39

| Strain     | Source of pVA891 | PspA | CFU/ml of blood* |
|------------|------------------|------|-------------------|
|            |                  |      | 1 min             | 1 h               |
| D39        | —                | +    | 1.8 × 10⁵         | 1.8 × 10⁵         |
| JY33       | pSD300           | —    | 4.4 × 10⁵         | 5.1 × 10⁵         |
| LC10.1†    | WG44.5           | +    | 1.4 × 10⁶         | 6.0 × 10⁵         |
| LC10.2     | WG44.6           | +    | 8.8 × 10⁵         | 2.8 × 10⁵         |
| LC10.3     | WG44.7           | +    | 1.5 × 10⁶         | 7.5 × 10⁵         |
| LC10.5     | WG44.9           | +    | 2.2 × 10⁵         | 2.7 × 10⁵         |
| LC10.8     | WG44.12          | +    | 3.8 × 10⁵         | 1.0 × 10⁵         |

* Mice were injected intravenously with ~10⁶ pneumococci in 0.1 ml and bled at 1 min and 1 h after inoculation. Data represent averages obtained from two mice. In all cases the duplicate values were within 2.7-fold of each other, except for the 1 h data for JY53, which was 2 × 10⁵ and 8.5 × 10⁵ CFU per ml, and the 1 h data for LC10.3, which was 1.1 × 10⁵ and 4.0 × 10⁵ CFU/ml.

† LC10.1, LC10.2, LC10.3, LC10.5, and LC10.8 were made by transforming D39 with Rx1 derivatives WG44.5, WG44.6, WG44.7, WG44.9, and WG44.12 that carry pVA891 as random inserts in their DNA. Transformants were selected as Em⁺ colonies.
When the recombinant plasmid, pKSD300 is introduced by transformation into Rx1 or into encapsulated pneumococcal strains, it insertionally inactivates the *pspA* gene and totally blocks the production of PspA.

When we examined the pattern of reactivity of a 32P-DNA probe made from the 550 bp fragment with a panel of 102 pneumococcal strains, we saw that the probe reacted with most, but not all of the isolates that reacted with the anti-PspA antibodies. The simplest interpretation of this data is that the probe detects a portion of the *pspA* structural gene that is different from those portions coding for the antigenic determinants detected by our antibodies. The possibility that our cloned gene is a fragment of a regulatory gene required for PspA production seems unlikely since some of the isolates made detectable PspA but did not react with the probe.

If the probe were reacting with a regulatory gene, the existence of those strains could have only occurred if those particular strains did not need the regulatory gene, or if both the regulatory gene and the structural gene were polymorphic. If the latter was the case, it seems unlikely that the PspA protein variant detected by the antibodies and the regulatory gene variant detected by the probe would cosegregate in such a high frequency of randomly chosen pneumococcal isolates. Likewise, if the regulatory gene were not needed in all pneumococcal strains that express PspA, it is surprising that the ability to make PspA in the absence of this gene would not show a more random association with the production of the PspA variant detected by our antibodies.

It seems likely that many of the 74 strains that failed to react with either the antibodies or the probe may make PspA, but that their PspA molecules may differ enough from the PspA of Rx1 that it is not detected by our probe or our antibodies. This possibility is presently under investigation.

Our past data have indicated that passive administration of two of our four anti-PspA hybridoma antibodies protected mice from infection with certain strains of pneumococci (8). This finding suggested that immunization with PspA might be able to elicit protection against pneumococcal infection. In the work described here we were able to test this supposition by comparing the degree of protection against the type 3 strain WU2 that was elicited by immunizing xid mice with normal PspA + Rx1 pneumococci, and with Rx1 pneumococci that fail to produce PspA. Our finding indicated that the presence of PspA in Rx1 pneumococci was required for protection, and that of all the surface proteins on Rx1, PspA was apparently more important than any others at eliciting protection against strain WU2.

The function of the PspA protein is unknown. Its absence does not affect either the growth rate of pneumococci in medium or block their ability to be
transformed. It does, however, appear to have a detectable effect on the rate of
clearance of D39 pneumococci from the blood during the first few hours after
intravenous injection. As such, it may be one of the factors that contributes to
their virulence.

Summary

PspA is a cell surface protein of Streptococcus pneumoniae that is present on a
number of clinical isolates as well as the nonencapsulated laboratory strain Rx1.
In a previous report (8) we have shown that mAbs directed against PspA can
protect mice from at least some of the pneumococcal strains bearing this protein.
In our present report we have produced insertional inactivation mutants that
lack PspA and have used these mutants to demonstrate that PspA can play a role
in pneumococcal virulence and that anti-PspA immunity can lead to protection
against pneumococcal infection.

PspA- mutants were obtained using derivatives of plasmid pVA891 carrying
chromosomal fragments from Rx1. From one of the mutants, we cloned a 550
bp fragment of the pneumococcal gene into pVA891 and transferred this
chimeric plasmid, designated pKSD300, into Escherichia coli. After transforma-
tion of pKSD300 into Rx1, PspA production is not detected. In colony hybridi-
zation experiments, the 550 bp fragment hybridizes specifically to pneumococcal
isolates in a pattern consistent with the hypothesis that the fragment is a portion
of the psbA structural gene that is different from the portions coding for the
antigenic determinants detected by mAbs Xi64 or Xi126. When X-linked immu-
nodeficient (xid) CBA/N mice were immunized with wild-type Rx1, they were
resistant to challenge with type 3 strain WU2. However, when these mice were
immunized with a PspA- mutant of Rx1, they failed to survive the subsequent
challenge, indicating that immunity to PspA can contribute to the resistance to
pneumococcal infection. Using pKSD300 we insertionally inactivated psbA in
D39, a virulent strain of S. pneumoniae. When injected intravenously there was a
10-fold greater reduction of the mutant pneumococci in the blood, as compared
to the wild-type D39.

We would like to acknowledge Nora Liu and Colynn Forman for their excellent technical
assistance, Maxine Aycock for preparation of the illustrations, and Ann Brookshire for
preparation of the manuscript. We also acknowledge the advice and encouragement of
Drs. Peter Burrows, Olga McDaniel, William Benjamin, Douglas Waltman, and Roy
Curtiss.

Received for publication 3 September 1986 and in revised form 20 October 1986.

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