Immunizations with Pneumococcal Surface Protein A and Pneumolysin Are Protective against Pneumonia in a Murine Model of Pulmonary Infection with *Streptococcus pneumoniae*

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Intranasal infection of mice with certain strains of capsular group 19 *Streptococcus pneumoniae* can result in focal pneumonia in the absence of bacteremia. Using this model of murine pneumonia, we demonstrated that immunization with recombinant forms of either pneumococcal surface protein A (PspA) or PdB (a genetically detoxified derivative of pneumolysin) elicited significant protection against focal pulmonary infection. This may be the first demonstration that a proposed vaccine antigen can protect against pneumococcal pneumonia. The best protection was obtained by immunizing mice with a mixture of PspA and PdB, indicating that the protection elicited by these antigens can complement each other. This result is in agreement with previous studies that used pneumococcal sepsis and nasal colonization models and demonstrate that the best protein vaccines for prevention of infection may be those that include more than one protection-eliciting pneumococcal protein.

Despite their successes, pneumococcal polysaccharide and polysaccharide-protein conjugate vaccines have not been ideal [1–3]. These vaccines must include multiple polysaccharide serotypes and are not protective against strains with capsular types/groups not present in the vaccines. Although evidence that these conjugates can protect against pneumococcal pneumonia per se is lacking, a recent report indicates that the licensed 7-valent conjugate vaccine does result in a 20% reduction in all-cause radiograph-positive pneumonia [4]. The complexity of polysaccharide-protein conjugate vaccines makes them expensive to produce and, at present, pre-

cludes their use in the poorest countries where most of the annual 3–5 million fatal respiratory infections in young children occur [5, 6]

A possible alternative, or complementary, approach is the use of protection-eliciting pneumococcal proteins. These antigens can be produced economically as recombinant proteins and, because they are proteins, should avoid problems of poor polysaccharide immunogenicity in infants and elderly persons. The possibility of including >1 protein in a vaccine offers the potential of targeting multiple virulence mechanisms [7, 8].

Pneumococcal surface protein A (PspA), pneumococcal surface adhesin A (PsaA), and pneumolysin are all pneumococcal protein vaccine candidates [9–11]. PsaA and PspA elicit protection against nasal carriage in mice [9, 12], and immunization with a mixture of PsaA and PspA elicits better protection against carriage than either protein alone [9]. PspA and pneumolysin each elicit protection against bacteremia and sepsis [13, 14]. In the face of very high challenge doses of a capsular type 2 strain, mixtures of PspA and pneumolysin

were clearly more protective than either protein alone [10]. In the present study, we have investigated the use of mixtures of PspA, pneumolysin, and PsaA to elicit protection against murine pneumonia.

To study protection against lung infection, we needed a model of focal pneumonia in the absence of generalized sepsis. Most strains of pneumococci (e.g. capsular types 2–6) known to cause disease in mice produce fatal sepsis in specific pathogen–free mice, whether given intravenously, intraperitoneally, or in the lung. Pneumococci of capsular types 14, 19, and 23 are common causes of invasive disease in young children, but they are relatively avirulent in mice [15] and are seldom used in mouse-infection studies. Takashima et al. [16] reported that a capsular group 19 strain could cause focal pulmonary infections in mice if given intranasally to anesthetized mice in a large volume of inoculum. We have now provided a detailed characterization of this model and have used the model to evaluate the relative ability of PspA, PsaA, and pneumolysin to elicit protection against focal pneumococcal pneumonia.

MATERIALS AND METHODS

Bacteria.   *S. pneumoniae* capsular group 19 strains L82013 and EF3030, human isolates from the Arctic Investigations Laboratory of the Centers for Disease Control and Prevention and Gothenburg, Sweden, were obtained from Alan Parkinson and Catharina Svanborg, respectively. Both strains are relatively avirulent in mice by intravenous and intraperitoneal routes [15]. Capsular group 19 strain DS2217 and type 3 strain WU2 [17, 18] were obtained from our University of Alabama at Birmingham laboratory collection. Capsular type 2 strains, D39 and its pneumolysin-lacking mutant PLN-A [19, 20], have been studied in intravenous and intraperitoneal challenge studies [10, 20, 21]. All 6 strains expressed family 1 PspA, which is present in about half of strains recovered from carriage and invasive disease [22, 23].

Mice.   Six to 8-week-old CBA/CAHN-XID/J female mice (denoted "CBA/N mice"; Jackson Laboratory) were maintained under specific pathogen–free conditions. CBA/N mice have the Btk (XID) immune-response defect [24] and fail to make natural antibodies to pneumococcal polysaccharides, including teichoic and lipoteichoic acids [25]. The absence of natural antibodies to teichoic acids makes these mice highly susceptible to pneumococcal infections [17] and much more reproducible in their susceptibility than most other strains [17, 25]. All animal experiments were done in accordance with the Institutional Animal Care and Use Committee's guidelines at the University of Alabama at Birmingham and the US Department of Health and Human Services.

Challenge with pneumococci in pneumonia, carriage, and bacteremia models.   Pneumococci were grown [26] and frozen

in aliquots for immediate use [11]. For carriage, mice were given the bacteria in a 10-μL volume in a single nostril. For lung infection, the bacteria were given in a 40-μL volume in a single nostril to mice anesthetized with methoxyflurane (Metofane; Shering-Plough), to facilitate aspiration [27]. Intravenous inoculations were given in the lateral tail vein in 0.2 mL of Ringer's injection solution (Ringer's; Abbot Laboratories), and mice were observed for 21 days for deaths.

Immunization and pneumonia challenge.   PspA for immunization was recombinant PspA/Rx1 (UAB055) [9], which comprised the first 302 of the 558 aa of PspA/Rx1, including all of the α-helical region and some of the proline-rich region [28, 29]. Rx1/PspA is of PspA family 1, which is the same family as the challenge strain EF3030 [23]. PsaA was used as a recombinant lipidated product [30]. Pneumolysin for immunization was a genetically detoxified variant of pneumolysin, PdB [31]. The molecular forms used of all 3 of these antigens have been described elsewhere [9]. The antigens were injected with aluminum hydroxide/magnesium hydroxide adjuvant (Imject Alum #77161; Pierce) with 50 μg of alum per 0.2-mL dose. Recombinant PsaA, PspA, and PdB were added to yield concentrations of 0.5, 1.0, or 20 μg/0.2-mL dose, respectively, and were mixed on a vertical wheel with a diameter of 15 cm at 6 rpm for 12 h at 4°C. Mice were immunized subcutaneously in the flank in 0.2 mL of adjuvant-protein mixture. When 2 proteins were used to immunize the same mouse, the proteins were first mixed together and then mixed with aluminum hydroxide. Control animals received Ringer's and adjuvant alone. Mice received 3 injections 14 days apart and were challenged 14 days after the last injection.

Mice were bled 7 days after challenge and were killed with an overdose of CO_2. The lungs were homogenized in 1 mL of Ringer's. The average weight of both lungs was ∼100 mg. The interior of the nose was washed with 50 μL of sterile PBS [12, 32]. The numbers of colony-forming units in blood, lung homogenate, and nasal washes were enumerated, as described elsewhere [12, 32]. In some cases, the interior of the lungs was washed with 0.5 mL of Ringer's before homogenization; the number of colony-forming units in the 2 samples were quantitated separately.

Histology.   Lungs, liver, spleen, kidney, heart, trachea, and thyroid gland were harvested from mice that were killed at 6, 8, 28, and 38 days after infection, fixed in 10% formalin, and embedded in paraffin. Sections were stained with Goodpasture's Gram stain or hematoxlyin-eosin, mounted, and examined microscopically with a 20× objective.

Phase of pneumococci.   Pneumococcal phase was determined by visual inspection while pneumococci were growing on clear agar, according to the method of Weiser [33] and Weiser et al. [34]. Phase was assigned on a relative basis by comparing the transparency/opacity of individual EF3030 colonies.

Table 1. Effects of infecting anesthetized CBA/N female mice (Jackson Laboratory) mice with *Streptococcus pneumoniae* (intranasally) in a 40-μL volume, on time to death and on infection parameters 5 days after infection.

Strain	Capsular type	Infection dose, $\log_{10}$ cfu	No. of mice	Mice alive at 5 days, %	Median time to death, h	Carriage[a]	Bacteremia[a]	Lung wash[a]	Washed lung[a]
L82013	19	6.5	5	100	>120	100	0	100	100
		7.6	3	67	>120	100	0	100	100
DS2117-94	19	6.5	3	100	>120	100	0	33	33
		6.7	5	60	>120	100	0	100	67
EF3030	19	6.8	8	100	>120	100	0	75	75
PLN-A	2	5.0	6	100	>120	100	100	100	ND
PLN-A2[b]	2	6.9	5	80	>120	100	100	100	100
		7.3	3	100	>120	100	100	33	100
D39	2	6.0	6	17	25	ND	ND	ND	ND
		4.0	5	20	72	100	100	100	ND
WU2	3	6.0	6	0	25	…[c]	…[c]	…[c]	…[c]
		4.0	5	20	72	100	100	100	ND

[a] Calculated as percentage of living mice.
[b] PLN-A2 is a subline of PLN-A with no known differences from PLN-A.
[c] No mice were still alive on day 5.

Statistical analysis. In groups in which some mice died before assay, the dead mice were assigned a number of colony-forming units in the lungs slightly larger than the largest number of colony-forming units observed in the lungs for that experiment. For carriage and bacteremia determinations, data from dead mice were omitted, because neither carriage nor bacteremia appeared to correlate with severity of lung infection with strain EF3030. Geometric mean numbers of colony-forming units/tissue were determined for groups of animals given identical treatments. The Wilcoxon 2-sample rank test

Table 2. Recovery of *Streptococcus pneumoniae* from the infected mice described in table 1.

Strain	Capsular type	Infection dose, $\log_{10}$ cfu	Days after infection	Nasal wash[a]	Blood[a]	Lung wash[a]	Washed lung[a]	P[b]
L82013	19	6.5	5	6.56 ± 0.29	<1.78	4.35 ± 0.47	3.01 ± 0.56	.029
		7.6	5	5.28 ± 0.06	<1.78	<7.59	6.71 ± 0.27	
DS2117-94	19	6.5	5	5.51 ± 0.49	<1.78	3.80[c]	2.82[c]	NS
		6.7	5	6.61 ± 0.24	<1.78	3.04 ± 0.65	2.45 ± 0.33	
EF3030	19	6.8	5	5.30 ± 0.27	<1.78	4.07 ± 0.72	3.10 ± 0.63	.010
PLN-A	2	5.0	3	4.71 ± 0.20	5.89 ± 0.34	…[d]	5.89 ± 0.12[d]	NA
PLN-A2[e]	2	6.9	5	4.65 ± 0.22	6.08 ± 0.58	3.50 ± 0.30	6.04 ± 0.46	.007
		7.3	5	4.09 ± 0.20	6.46 ± 0.70	2.40 ± 0.70	5.20 ± 0.42	.021
D39[f]	2	4.0	3	3.15[g]	>7.00[g]	…[d]	5.58[d,g]	NA
WU2[f]	3	4.0	3	3.46[g]	4.60[g]	…[d]	5.51[d,g]	NA

[a] Data are mean ± SE $\log_{10}$ cfu/mL or cfu/pair of lungs and were calculated for mice that were still alive on day 5. *P* values comparing lung wash and washed lung omit data from mice that had no detectable pneumococci in the lung and data from any dead mice. The comparison of all group 19 lung wash vs. washed lung was significant at $P = .0002$.
[b] Paired *t* test (lung wash vs. washed lung).
[c] Data provided are for the only mouse in this group with pneumococci in the lung.
[d] These lungs were not washed before homogenization.
[e] PLN-A2 is a subline of PLN-A with no known differences from PLN-A.
[f] Because all mice of this strain that were infected with 10^6 cfu either had died by day 5 (table 1) or were quite morbid when killed, no data for this cfu dose were collected.
[g] Only 1 of 4 mice infected with WU2 and 1 of 4 mice infected with D39 survived. Thus, the colony-forming unit data for mice infected with D39 and WU2 were obtained from only 1 mouse each.

(2-tailed) was used for comparisons of experimental and control groups.

RESULTS

Ability of group 19 strains to cause lung infections. Three capsular group 19 strains were inoculated separately intranasally in 40 μL of inoculum into anesthetized CBA/N mice. At 5 days after inoculation, most mice were still alive, and all surviving mice exhibited detectable nasal carriage. More than 90% of the mice had pneumococci in their lungs (table 1). Geometric mean numbers of colony-forming units in the lungs ranged from $10^{3.04}$ to $10^{4.35}$ cfu for mice inoculated intranasally with $<10^{7.0}$ cfu (table 2). The geometric mean number colony-forming units washed from the nasal tissues of the same mice ranged from $10^{5.2}$ to $10^{6.6}$ cfu. None of the 3 strains successfully invaded the blood.

To determine whether the pneumococci recovered from the lungs were free or were tissue associated, a wash of each pair of lungs was plated separately from a homogenate of the washed lungs (tables 1 and 2). For mice infected with capsular group 19 pneumococci, there were 3–10 times as many pneumococci in the wash fluid, compared to the washed lung. In contrast, for infections with capsular type 2 strain PLN-A, there were 100 times as many pneumococci associated with the washed lung as with the wash fluid. These results indicate that capsular type 2 pneumococci were more invasive in the local tissue than the group 19 pneumococci.

Virulence of EF3030 intravenous. Intravenous infection of CBA/N mice with 100–300 cfu of D39 or WU2 is known to result in bacteremia and death [13, 35, 36]. In contrast, when CBA/N mice were injected intravenously with 10^4 or 10^5 cfu of EF3030, no bacteremia was observed at 1, 4, or 7 days after inoculation, and no mice died. At an intravenous dose of 10^6 cfu of EF3030, half the infected mice died within 7 days, and virtually all exhibited bacteremia on days 1 and 4. When challenged intravenously with 10^7 cfu of EF3030, all mice died within 7 days (data not shown). In other studies, we learned

that intravenous injection of $\geq 10^6$ cfu of EF3030 produced a chronic kidney infection, which may account for the bacteremia seen at these doses of EF3030 (M. T. Coats [Department of Microbiology, University of Alabama at Birmingham] and D.E.B., unpublished data).

Intranasal challenge with type 2 and 3 strains causes bacteremia. Strains WU2, D39, and PLN-A were examined as representatives of pneumococci that readily cause bacteremia and sepsis in mice [20, 21, 35]. When anesthetized CBA/N mice were infected intranasally with $\geq 10^6$ cfu of D39 or WU2, all died within only a few days (data not shown). When infected with 10^4 cfu of D39 or WU2, 4 of the 5 mice infected with each strain died; median time of death was 3 days (table 1). In the 2 mice that were still alive at 5 days, there were significant numbers of pneumococci in the nasal wash, lungs, and blood. Only 1 of 14 mice infected with PLN-A died by day 5 (table 1). This attenuating effect of the pneumolysin-negative mutation is well known [20, 21]. The geometric mean number of colony-forming units in the nasal wash of mice given the capsular group 19 strains was 10–100-fold higher than that for mice given type 2 or 3 strains (table 2).

Because capsular type 2 and 3 challenge strains infected the blood so efficiently, we assumed that the early deaths caused by intranasal infection with these strains were probably from sepsis. To test this assumption, anesthetized mice were infected intranasally with the type 2 strains D39 and PLN-A. At 1 and 2 days after inoculation, the colony-forming units in the blood equaled or exceeded the colony-forming units in the lungs (data not shown).

Because the lungs of the infected mice weighed only 0.1 g, there would not be expected to be enough blood circulating through the lungs to have had a significant effect on the numbers of colony-forming units observed in the lungs of any of the mice in these studies.

Effects of infection dose on lung infection. When anesthetized mice were intranasally given $10^{7.75}$ cfu of EF3030, they all died within 5 days. Among the 16 mice given $10^{4.72}$–$10^{6.87}$ cfu of EF3030, pneumococci were observed in the lungs and nasal wash,

Table 3. Effect of dose of group 19 EF3030 *Streptococcus pneumoniae* on lung infection 5 days after intranasal inoculation of anesthetized mice with pneumococci in a 40-μL volume.

EF3030 dose, $\log_{10}$ cfu	No. of mice	Percentage alive	Percentage with bacteremia ($\geq \log_{10}$ 1.78 cfu/mL)	Lungs, mean $\pm$ SE, $\log_{10}$ cfu	Nasal wash, mean $\pm$ SE, $\log_{10}$ cfu
4.72	3	100	0	2.41 $\pm$ 0.43	5.52 $\pm$ 0.11
5.80	3	100	0	4.24 $\pm$ 0.78	5.90 $\pm$ 0.13
6.38	4	75	0	5.46 $\pm$ 0.33	5.67 $\pm$ 0.01
6.87	6	100	0	6.42 $\pm$ 0.26[a]	5.47 $\pm$ 0.17
7.75	3	0	All dead	All dead	All dead

[a] One mouse in this group had no pulmonary infection and was assigned $\log_{10}$ of 1.70 before averaging. The minimum colony-forming units detectable in the lung was $\log_{10}$ 1.78
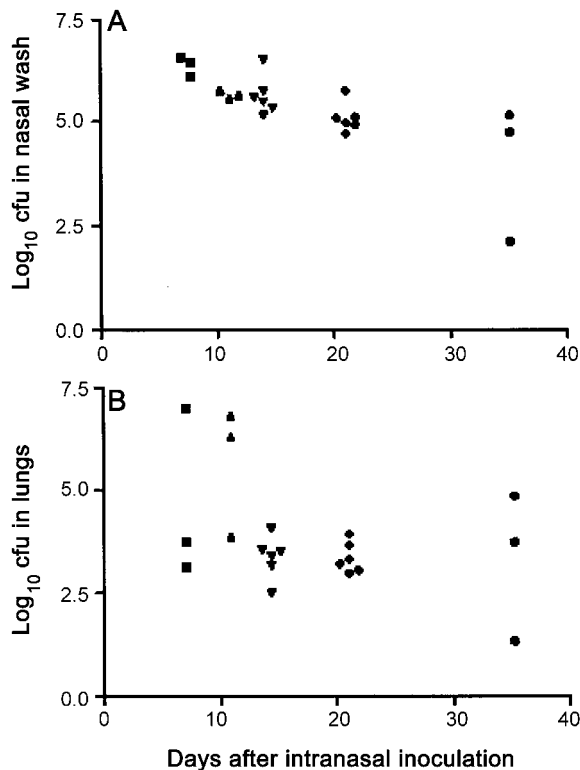
Figure 1. Duration of lung infection and nasal carriage after intranasal inoculation of anesthetized CBA/N female mice (Jackson Laboratory). Mice were inoculated with $10^{6.88}$ cfu of EF3030 in a 40-μL volume of Ringer's solution (Abbot Laboratories) and were killed on day 7, 11, 14, 21, or 35. For both the nasal wash and the lung colony-forming–unit data, statistical comparisons were made for each day that mice were killed, with the pooled data from days 7 and 11. Only day-21 and day-35 nasal wash data differed from day-7 and day-11 pooled data by these criteria. However, comparisons between days 7–11 and days 14–35 were statistically significant for both nasal wash and lung data ($P = .023$ and $P = .041$, respectively).

but not in the blood, at 5 days after challenge. Within this dose range, the numbers of colony-forming units in the lung at 5 days was proportional to the numbers of colony-forming units in the original inoculum (table 3). In contrast, the numbers of EF3030 colony-forming units in the nasal washes were independent of inoculation dose, as was observed previously [32], using a 6B strain and inoculation without anesthesia.

Kinetics of lung infection. Anesthetized mice were inoculated intranasally with $10^{6.88}$ cfu and were killed at day 7, 11, 14, 21, or 35 (figure 1). The numbers of colony-forming units in the lung and nasal wash did not change rapidly over the 35-day period. At each time point, all mice carried pneumococci in their nasopharynx, and all but 1 (on day 35) had detectable pneumococci in the lungs. The highest numbers of pneumococci in the nose were at 7 days after inoculation; the highest numbers of pneumococci in the lung were at days 7 and 11.

Phase of pneumococci in the lung and nasal cavity. Using

other strains of pneumococci, we showed that most of the colonies recovered from the blood were relatively opaque, compared with those obtained from the nasal washes [33, 34]. For EF3030, the differences in appearance of opaque and transparent colonies was quite subtle, yet still detectable. At 7–35 days after intranasal inoculation of anesthetized mice with EF3030, colonies of virtually all the pneumococci washed from the nasal cavity were transparent. At these time points, the majority (60%–90%) of colonies of pneumococci recovered from the lungs were opaque (table 4). The EF3030 inoculum was 57% opaque and 43% transparent.

Although there was much variation in the percentage of opaque colonies of pneumococci recovered from the lungs at the first few time points, there was a significant increase in the percentage of opaque colonies recovered from the lung with time (table 4). The percentages of opaque colonies from lungs harvested on days 21 and 35 was significantly higher ($P = .036$) than those recovered from the lungs harvested at days 7 and 11. Although the percentage of opaque colonies from the lung was not much higher than that of the inoculum (57%), it probably reflects a true steady-state value for the lungs. In separate studies, mice were inoculated with cultures of EF3030 exhibiting 18% and 95% opaque colonies. For mice that received each inoculum, the day 21 lungs yielded ~90% opaque colonies (data not shown).

Importance of aspiration in lung infections. In previous studies [16] and in the results described above, anesthetized mice were inoculated intranasally with a rather large volume of inoculum (40 μL). The importance of this procedure for facilitating aspiration is apparent from a comparison of the aspiration pro-

Table 4. Phase of EF3030 pneumococci recovered from the lung and nasal cavity at different times after intranasal inoculation with EF3030

| Day when killed | No. of mice | Percentage opaque[a] | | P[b] |
		Lung	Nasal wash	
7	3	63 ± 21	0	…
11	3	60 ± 12	0	…
14	6	70 ± 6	0	.39
21	6	90 ± 5	0	.065
35	3	90 ± 8	0	.18
Pooled data				
Days 7 and 11	6	62 ± 11	0	
Days 21 and 35	9	90 ± 4	0	.036

NOTE. These are the same mice depicted in figure 1 (mice infected with $10^{6.88}$ cfu of EF3030 under anesthesia). When the initial inoculum was plated, it produced 57% opaque and 43% transparent colonies. The observed percentage of opaque colonies for each tissue from mouse was based on at least 50 counted colonies.

[a] All colonies that were not scored opaque were scored transparent.
[b] P values are for day 7 vs. day 11. Statistical differences were calculated by a 2-tailed Wilcoxon 2 sample-rank test.

Table 5. Effect of inoculation protocol on lung infection with capsular group 19 strain EF3030.

Parameter	Nasal[a]	Aspiration[a]	P[b]
Log cfu inoculated	5.73	5.73	
Anesthesia	No	Yes	
Inoculum volume	10 μL	40 μL	
Log cfu/mL blood	<2.78 (NA)	<2.78 (NA)	NS
Log cfu/lungs	2.81 ± 0.42 (1.78–3.43)	6.05 ± 0.42 (4.67–7.17)	.0079
Log cfu/nose wash	5.11 ± 0.17 (4.67–5.51)	6.10 ± 0.17 (5.70–6.54)	.0159
Percentage opaque			
Lungs	95 ± 4 (77–100)	95.6 ± 2.9 (85–100)	NS
Nasal wash	20.6 ± 14.4 (2–78)	22.8 ± 5.4 (13–38)	NS

NOTE. Data are mean ± SE log (range), unless otherwise indicated. Each group contained 5 mice. NA, not applicable; NS, not significant.

[a] "Nasal" protocol was inoculation in 10 μL without anethesia. "Aspiration" protocol was inoculation in 10 μL with anesthesia.

[b] Nasal vs. lung.

tocol with a standard protocol [32] for inducing nasal carriage in adult mice (intranasal inoculation with 10 μL without anesthesia) (table 5). Although there were 10 times as many pneumococci washed from the nose of mice infected with the aspiration method, both procedures yielded solid carriage in all mice. However, when colony-forming units in the lungs were compared, there were ~2000 times as many colony-forming units in the mice inoculated with the aspiration method as with the carriage protocol. Thus, the progression of large numbers of pneumococci into the lung appears to require aspiration and is not a natural consequence of nasal colonization. This conclusion is supported by a previous study that used capsular type 6B pneumococci and the carriage protocol, in which even fewer pneumococci were observed in the lungs [32].

The larger numbers of pneumococci in the nasal passages in the aspiration-pneumonia model, compared with those in the carriage model might be explained by the movement of pneumococci from the infected lung to the nose or by the ability of the larger inoculation volume to cover more of the nasal tissue and to colonize larger fractions of niche space.

Histology of infected mice. Histological examination of the lungs removed at 6 and 8 days post inoculation revealed acute pneumonia in one or more lobes of the lungs of each mouse inoculated with EF3030 under anesthesia (figure 2B). Alveoli of the infected animals contained neutrophils, monocytes and necrotic debris. Noninfected mice had normal lungs with clear alveoli (figure 2A). A small number of diplococci, typical of *S. pneumoniae,* could be observed in gram-stained lungs of one of the 5 infected mice examined at these time points (data not shown). There was no evidence of infection in the livers or kidneys. Spleens of infected mice showed increased numbers of neutrophils in the sinuses, and extramedullary hematopoiesis also was detected.

Consistent with the decrease in numbers of colony-forming

units in the lungs at the later time points (figure 1B), day 28 and day 38 histologic examinations showed evidence of healing. Only 1 of the 3 mice examined at the day 28 time point and none at the day 38 time point showed evidence of acute pneumonia. Most of these lungs showed bronchioles filled with fibrin and macrophages consistent with focal proliferative bronchiolitis. These same lungs showed occasional perivascular lymphocytic infiltrates with focal invasion of the vessel walls. Alveoli showed foamy macrophages and some had collagen deposition. In general, less pathology was seen at day 38 than at day 28 after infection. Kidneys, livers, and spleens were unremarkable.

Although the bacteria were frequently more numerous in the nasal cavities than in the lungs (table 2), the bulk of the mucosal surface showed no pathology. However, the focal lesions that were seen included scattered lymphocytes within the mucosa, loss of cilia, and, on rare occasions, clusters of neutrophils in nasal crypts (data not shown).

Immunogenicity of PspA, PdB, and PsaA administered subcutaneously with alum in CBA/N mice. CBA/N mice were immunized with PspA, PsaA, PdB, and pair-wise combinations of the 3 antigens. PspA, PsaA, and PdB were given at doses of 1, 0.5, and 20 μg, respectively, and mice received 3 injections of the indicated antigens with alum at 2-week intervals. The differences in the doses of the 3 antigens was an attempt to compensate for anticipated differences in their immunogenicity [9]. Thirteen days after the last injection, the mice were bled for IgG antibody determinations and, on day 14, were anesthetized and challenged intranasally. Of the 3 immunogens, the highest IgG antibody responses were elicited by the lipidated PsaA, despite the fact that this antigen was given at a dose half that of PspA. The lowest antibody levels were elicited by PdB, even though it was given at a dose 20 times that of PspA (table 6). Although there appeared to be some differences in the an-
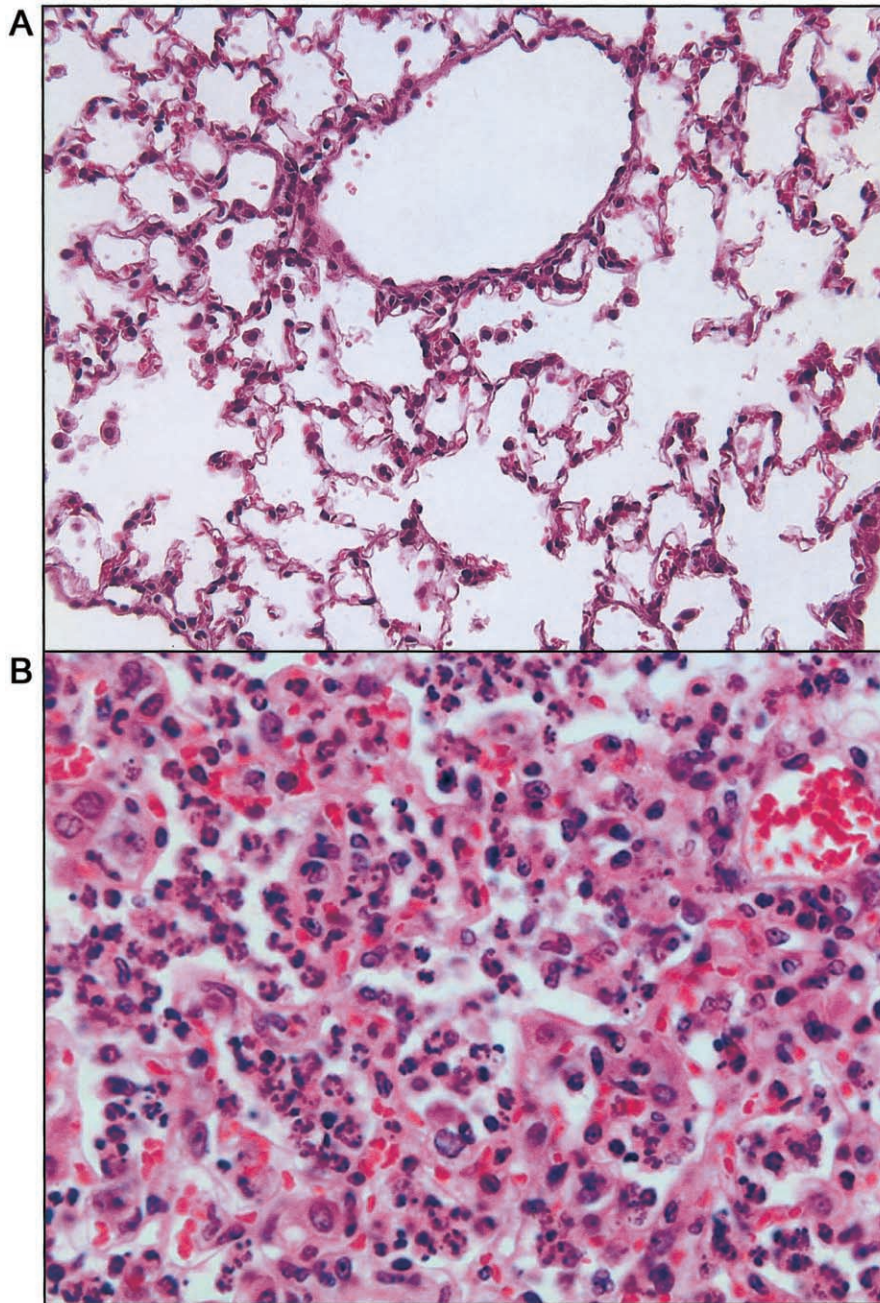
Figure 2. Sections of normal *(A)* and infected *(B)* lung stained with hemotoxylin-eosin. Anesthetized CBA/N female mice (Jackson Laboratory) were infected with $10^{6.8}$ cfu of EF3030 in 40-μL Ringer's injection solution (Abbot Laboratories) or with sterile Ringer's injection solution. Lungs were harvested and sectioned 6 days later and stained with hematoxylin-eosin. Original magnification, ×400.

tibody responses to antigens alone and in mixtures, none of these differences were statistically significant ($P > .12$, Wilcoxon 2-sample rank test).

Protection against lung infection by immunization with pneumococcal proteins. We used the highest challenge dose of EF3030 ($\sim 10^{6.8}$ cfu) that did not cause death before day 5. The protection experiments were assayed at day 7 to maximize the numbers of pneumococci in the lungs. Immunizations with PspA and PdB resulted in significant protection against pulmonary infection (figure 3). Immunization with PsaA yielded no decrease in numbers of pneumococci in the lungs of the infected mice, although it elicited 6–60 times higher antibody levels than did PspA or PdB. When the proteins were injected in pair-wise combinations, immunity to PsaA also failed to significantly enhance the protection elicited by PspA or PdB.

The most dramatic protection against lung infection was

Table 6. IgG antibody elicited by subcutaneous immunization with pneumococcal surface protein A (PspA), pneumococcal surface adhesin A (PsaA), and pneumolysin.

Immunogen	Serum IgG antibody levels, geometric mean (SE factor), μg/mL		
	PspA	PdB	PsaA
Alum	<0.001	<0.0005	<0.004
PspA	7.89 (1.72)	<0.0005	<0.004
PsaA	<0.001	<0.0005	219 (1.38)
PdB	<0.001	17.38 (1.48)	<0.004
PspA + PdB	17.0 (1.34)	9.53 (2.09)	<0.004
PspA + PsaA	32.4 (1.26)	<0.0005	347 (1.66)
PdB + PsaA	<0.001	6.25 (1.35)	407 (1.21)

NOTE. CBA/N female mice (Jackson Laboratory; 6 animals/ group) were immunized with PspA, PsaA, PdB, and binary combinations of these proteins on alum. Three subcutaneous immunizations were given at 2-week intervals. The doses of the 3 proteins per immunization were 1, 0.5, and 20 μg/mouse for PspA, PsaA, and PdB, respectively. Mice were bled 13 days after the last injection of antigen and/or alum. The SE factor is the number by which the geometric mean must be multiplied and divided to obtain the lower and upper bounds or SE).

obtained by immunization with a combination of PspA and PdB, which reduced the number of lung colony-forming units >10-fold than that achieved with PspA and >100-fold than that achieved with PdB. This observation was confirmed in a second experiment (data not shown) using groups of 10 mice immunized with alum only, PspA plus alum, PdB plus alum, and a mixture of PspA and PdB plus alum. As in the previous experiment, we observed highly significant protection, compared with that of the alum control, for immunity to PspA, PdB, or PspA plus PdB ($P < .007$, $P < .01$, and $P < .0003$, respectively). In the second experiment, the numbers of colony-forming units in the lungs of the mice immunized with the mixture of PspA and PdB was 1/245th of that in the lungs of mice injected with alum only. By pooling the results from these groups of 10 mice with the results from the groups of 6 similarly immunized mice in the first experiment (figure 3), we observed greater protection with the mixture of PspA and PdB, compared with that of PspA or PdB alone ($P = .048$ and $P = .0004$, respectively). Immunization with PspA, PdB, and/or PsaA had no effect on nasal carriage of pneumococci in mice with pneumonia (data not shown).

DISCUSSION

Our observation that subcutaneous immunization with a mixture of PspA and pneumolysin was especially protective against pneumonia in mice also was reminiscent of the fact that PspA and pneumolysin also provided optimal protection against intraperitoneal challenge [10]. These findings strengthen the conclusion that the combination of these 2 antigens may be especially useful for eliciting protection against systemic pneumococcal in-

fection. The absence of bacteremia and sepsis in the aspiration-pneumonia infection model means that the observed protection against lung infection was the result of direct protection in the lung, rather than an indirect outcome of protection against septicemia. The aspiration-pneumonia infection model exhibited an intense, but self-resolving, pneumonia. This model is especially relevant to human disease, because it mimics the situation with a large number of the human lung infections that are caused by aspiration of pneumococci, in which bacteremia is not apparent at the time of admission or treatment.

The reduced invasion of the group 19 strains versus the capsular type 2 and 3 strains was seen not only in the failure of the group 19 strains to invade the blood but also in their lower propensity to invade from the lumen of infected lungs into the pulmonary tissue. The lack of apparent invasion of the group 19 strains does not necessarily mean that they do not enter the blood, but may be a result of their inability, as was demonstrated here, to survive in the circulation.

Studies by Weiser and his colleagues [33, 34] have shown that pneumococci exhibit phase variation in colony opacity; pneumococci recovered from the nasal surface and blood tend to be transparent and opaque, respectively. As expected, EF3030 washed from the nose were virtually all transparent. Our observation that the colonies from the lung were largely opaque has not been previously reported and indicates that pneumococci treat the lung as an invasive site.

The difference in expression of virulence factors by the transparent and opaque pneumococci [33, 34] may partially explain why *S. pneumoniae* cause much less inflammation in the nose than in the lung. This difference in inflammation at these 2 sites
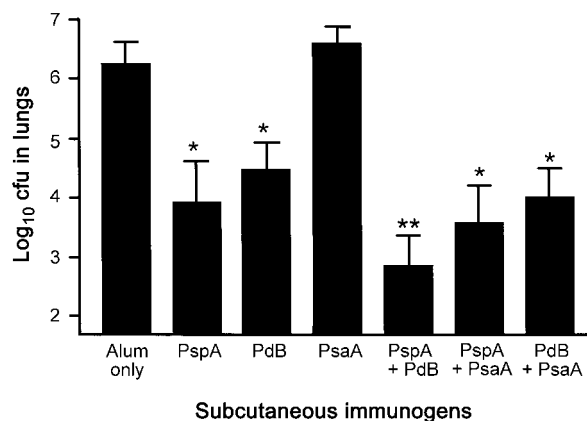

Figure 3. Immunization with pneumococcal surface protein A (PspA) and PdB (a genetically detoxified derivative of pneumolysin) can protect against pulmonary infection with *Streptococcus pneumoniae*. CBA/N female mice (Jackson Laboratory) were immunized with the indicated proteins on alum or with alum alone, challenged with $10^{6.89}$ cfu of capsular group 19 strain EF3030, and killed 7 days later to determine numbers of colony-forming units in their lungs and nasal cavity (nasal data shown). PsaA, pneumococcal surface adhesin A; *$P \leqslant .04$; **$P < .001$.

is a graphic demonstration of the degree to which the pneumococcus has evolved to asymptomatically colonize the upper airways, although the host still recognizes it as an inflammatory pathogen once it enters the lung.

The fact that PspA and pneumolysin elicited additive protection against pneumonia suggests that the antibodies to them may interfere with complementary virulence functions. PspA has been shown to interfere with activation of complement by pneumococci during their infection of mice [37, 38]. Antibody to PspA is thought to enhance surface deposition of complement by interfering with the anticomplementary activity of PspA. It has been shown that the ability of pneumolysin released from pneumococci to fix complement is an important mediator of disease [39, 40]. Antibody to pneumolysin is thought to act, in part, by interfering with pneumolysin's ability to reduce the local concentration of complement, thus making more complement available for deposition on the pneumococcal surface [41]. The antibodies to PspA and pneumolysin may act together to increase the amount of complement deposited on the bacterial surface, thus increasing opsonization, phagocytosis, and killing. The observation that elevated levels of serum antibody to these antigens can protect against pneumonia is consistent with the fact that serum (with its antibody and complement) is known to be extravasated into alveoli during inflammation [42].

Nasopharyngeal carriage is generally considered to be the primary human reservoir of pneumococci. Protection against nasal carriage is considered to be essential for herd immunity to pneumococci. Moreover, because nasopharyngeal carriage is thought to be an intermediate stage that precedes invasive disease [43], protection against carriage also should have a direct effect on the reduction of invasive disease. In previous studies, mucosal immunization with PsaA (and, to a lesser extent, PspA) was able to elicit protection against nasal carriage. The best protection against carriage was obtained with mice immunized with both PsaA and PspA [9].

Our observation here that subcutaneous immunization with pneumolysin, PspA, and/or PsaA did not protect against nasal colonization of the mice in the aspiration-pneumonia infection model is consistent with previous studies in a mouse carriage model, in which systemic immunization with PspA was not able to protect against nasal carriage, although mucosal immunization could [12]. The failure of PsaA to elicit protection against pulmonary infection in this model does not necessarily argue against its inclusion in a mucosal vaccine, in which it might elicit protection against carriage and thus prevent subsequent pneumonia.

From the results of these pneumonia studies, as well as those of the earlier studies with sepsis and carriage models [9, 10], it seems likely that mucosal immunization of humans with a mixture of PspA, PdB, and PsaA might represent an especially effective strategy. PsaA and PspA could elicit protection against

carriage and confer herd immunity. Because mucosal immunizations also can produce protective levels of serum antibody [12] the PspA and pneumolysin would be able to elicit protection against any pneumococcal pneumonia or bacteremia that occurred in individuals who still carried some *S. pneumoniae.*

If immunity to these proteins proves to be as effective in humans as the conjugate vaccines, the proteins could have some important advantages. Most importantly, the protection would be independent of capsular type. The protective responses would be T cell dependent and thus immunogenic, like the conjugate vaccine, in children. Unlike the protein carrier in the conjugate vaccine, the pneumococcal proteins will also be presented to T cells by infecting pneumococci. Thus, these pneumococcal proteins will be able to help elicit anamnestic responses among helper T cells as well as among B cells, thus possibly eliciting stronger anamnestic responses to infections. This theoretical advantage also argues for the use of these proteins as carriers for pneumococcal polysaccharides.

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