Intranasal Immunization with Killed Unencapsulated Whole Cells Prevents Colonization and Invasive Disease by Capsulated Pneumococci

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Animal Models. (i) Mouse colonization model. C57BL/6J mice, 4 to 6 weeks old, were purchased from Jackson Laboratories (Bar Harbor, Maine) and housed four per cage. They were randomized by cage to receive vaccine or control preparations. Immunization was delivered by gently restraining the unanesthetized mouse and applying 10 μl atraumatically to the nostrils. Live pneumococcal preparations (at a concentration of 10^6 CFU/inoculation), killed Rx1AL− without or with CT, CT alone, or saline was given three times at weekly intervals. One week following the last immunization, animals in all groups received 1 mg of rifampin in 0.25 ml subcutaneously (a dose which effectively eradicates pneumococcal colonization and does not prevent subsequent colonization [data not shown]). At 1 or 7 weeks later, the mice were challenged with 10^6 CFU of S. pneumoniae type 6B applied as in the immunizations. One week after challenge, the mice were euthanized by CO₂ inhalation; the trachea was exposed and transected by careful dissection. An upper respiratory wash was done by instilling sterile, nonbacteriostatic saline retrograde through the transected trachea and collecting the first 6 drops (about 0.1 ml) from the nostrils. An animal was considered to be nasally colonized if ≥1 CFU/50 μl of wash fluid was detected on blood agar containing 2.5 μg of gentamicin/ml. In addition, quantitative cultures of these upper respiratory washes were performed; the lower limit of detection of these cultures was 1 CFU per 50 μl.

(ii) Young-rat sepsis model. Outbred virus-free Sprague-Dawley 3-week-old male rats were obtained from Charles River Laboratories (Wilmington, Mass.) and housed four per cage. They were randomized by cage to receive vaccine or control immunizations, which were administered as for the mice except that volumes of 100 μl (for the first two immunizations) and 30 μl (for the third, if applicable) were applied to the left nostril only. In pilot studies using methylene blue-containing solutions, delivery of up to 100 μl did not result in aspiration of material into the lungs (data not shown). Two weeks following the last immunization, intrathoracic challenge with S. pneumoniae type 3 was performed. The right chest was prepared with an alcohol swab, and a 0.05-ml inoculum was injected transthoracically into the right mid-lung via a 29-gauge needle on an insulin syringe. The depth of the intrathoracic injection (5 mm) was controlled by a small hemostat clipped at the base of the needle. Clinical appearance was monitored daily by an investigator blinded to immunization assignment, using a 5-point scoring system (0, well; 1, ruffled fur; 2, ruffled fur and decreased activity; 3, ruffled fur and inactivity; 4, hunched and gaunt). An animal receiving a score of ≥2 on any day of the experiment was considered to have become ill. Any animal receiving a score of 3 or above was immediately euthanized, since such a score is a reliable predictor of mortality in our experience (data not shown). Clinical appearance and mortality were assessed for 10 days after inoculation, after which time the experiment was concluded.

(iii) Passive-protection studies: infant-rat model. For passive-protection studies, we used the infant-rat sepsis model published previously [21]. Briefly, timed-pregnant Sprague-Dawley dams were allowed to deliver in our animal-housing facilities. On day 3 of life, infant rats were randomly redistributed across dams, so that each cage had approximately 12 infant rats. On day 4 of life, infant rats were randomized by cage to receive an intrathoracic injection of S. pneumoniae type 3 that had been previously incubated in various sera. The sera of main dams, so that each cage had approximately 12 infant rats. On day 4 of life, infant rats received live type 3 that had been previously incubated in various sera. The sera of main dams, so that each cage had approximately 12 infant rats. On day 4 of life, infant rats were randomized by cage to receive vaccine or control immunizations, which were administered as for the mice except that volumes of 100 μl (for the first two immunizations) and 30 μl (for the third, if applicable) were applied to the left nostril only. In pilot studies using methylene blue-containing solutions, delivery of up to 100 μl did not result in aspiration of material into the lungs (data not shown). Two weeks following the last immunization, intrathoracic challenge with S. pneumoniae type 3 was performed. The right chest was prepared with an alcohol swab, and a 0.05-ml inoculum was injected transthoracically into the right mid-lung via a 29-gauge needle on an insulin syringe. The depth of the intrathoracic injection (5 mm) was controlled by a small hemostat clipped at the base of the needle. Clinical appearance was monitored daily by an investigator blinded to immunization assignment, using a 5-point scoring system (0, well; 1, ruffled fur; 2, ruffled fur and decreased activity; 3, ruffled fur and inactivity; 4, hunched and gaunt). An animal receiving a score of ≥2 on any day of the experiment was considered to have become ill. Any animal receiving a score of 3 or above was immediately euthanized, since such a score is a reliable predictor of mortality in our experience (data not shown). Clinical appearance and mortality were assessed for 10 days after inoculation, after which time the experiment was concluded.

Mouse colonization model. The majority (85%) of mice that received intranasal saline prior to challenge with live strain type 6B were colonized by day 7 (Table 1). In contrast, mice that were immunized with killed Rx1AL− plus CT were completely protected against nasopharyngeal colonization with S. pneumoniae type 6B. In the animals challenged 2 weeks after immunization with Rx1AL− plus CT, none (0 of 29) were colonized, compared with 29 of the 34 (85%) saline controls (P < 0.0001). However, CT alone was partially protective when animals were challenged 2 weeks postimmunization (10 of 22 (45%) were colonized; P = 0.002 versus saline and P < 0.001 versus Rx1AL− plus CT), indicating that immunization with CT may provide nonspecific protection against colonization. When animals were challenged 8 weeks following the last immunization, the observed protection from CT was diminished (5 of 8 colonized) whereas the mice that received Rx1AL− plus CT were still completely protected against colonization (0 of 8 colonized; P = 0.026). The Rx1AL− vaccine without CT was not significantly protective.

The protection elicited by Rx1AL− plus CT was compared to that which can be elicited following immunization with live homologous and heterologous capsulated pneumococcal strains. All mice that were immunized with live serotype 6B (the strain used for challenge) were uniformly protected against nasopharyngeal colonization. Immunization with heterologous (type 10F or 14) live pneumococci provided partial protection, but the proportion of mice protected from carriage was significantly lower than that obtained when mice were immunized with Rx1AL− plus CT (P < 0.001) or the homologous strain (P < 0.04). When quantitative cultures were compared, animals that received Rx1AL− plus CT had significantly lower density of colonization than did mice that received saline or CT alone or were immunized with heterologous serotypes (all (P < 0.001) [data not shown]). Thus, intranasal immunization with killed Rx1AL− plus CT elicits protection against nasopharyngeal colonization equal to that provided by repeated
el (Table 3). Intrathoracic inoculation of 5 CFU of antibodies, serum of intranasally immunized rats was tested for active against both bacteremia and mortality. Preincubation in antibodies to capsule (BPIG) was, as expected, highly protective. CT alone was not protective. Preincubation in a source of serum type 3 incubated in normal rat serum was uniformly statistically significant.

CT alone; three vaccinations (experiment 2) reduced morbidity from 100 to 45% and mortality from 69 to 25% compared to sequential exposures to vaccine plus CT reduced morbidity to 32% and mortality to 28%. These differences were all statistically significant.

Protection elicited by intranasally applied killed whole cells pneumococci administered intranasally can protect against nasopharyngeal colonization and invasive disease, using two different serotypes unrelated to the capsular type from which the vaccine strain was derived. A type 6B strain was chosen because this capsular type is commonly associated with invasive disease in children and also with antimicrobial resistance. Nasopharyngeal colonization represents the initial step in the evolution of invasive pneumococcal disease. In a mouse model of nasal colonization, the vaccine was highly protective; viable pneumococci were not detected in any immunized mice. In these studies, immunization with Rx1AL- plus CT also provided significantly superior protection against nasopharyngeal colonization with serotype 6B than did repeated exposures to heterologous serotypes.

In the young-rat sepsis model, in which the initial step in pathogenesis is the development of pneumonia (21) (representing the most common form of invasive infection with pneumococcus), the vaccine was again highly protective. WU2r is a heavily capsulated type 3 strain and was chosen for this model to test whether immunization with unencapsulated cells could confer protection against invasive disease by such an organism, against which non-capule-based immune mechanisms might in theory be less effective.

Protection elicited by intranasally applied killed whole cells was reported in 1928 (9, 24) and confirmed in recent studies (16, 27). An important aspect of our studies, however, is that the immunizing strain is unencapsulated. Therefore, any protection elicited by this vaccine would be expected to be serotype independent. We have demonstrated protection against strains of two different capsular serotypes, and in future experiments we will be testing whether protection can be conferred against a broad diversity of pneumococci.

The mechanisms and antigenic specificity of the elicited protection are not known. Their elucidation will assist the practical goal of optimizing protection. It is possible that the mechanisms of protection involve both mucosal and circulating antibodies. The passive-protection experiment indicates that intranasal immunization with killed Rx1AL- plus CT elicited serum antibodies with protective potential. Whether these systemic antibodies play an important role in the observed active immunization against either colonization or sepsis or simply rep-

### Table 2. Protection of rats by intranasal immunization with killed Rx1AL- cells plus CT against intrathoracic infection with serotype 3 pneumococci

<table>
<thead>
<tr>
<th>Expt</th>
<th>Immunogen</th>
<th>No. of doses</th>
<th>Morbidity</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. ill/total no.</td>
<td>% ill</td>
<td>P vs no Rx1AL-</td>
<td>No. dead/total no.</td>
</tr>
<tr>
<td>1</td>
<td>CT</td>
<td>2</td>
<td>42/42 (100)</td>
<td>29/42 (69)</td>
</tr>
<tr>
<td></td>
<td>CT + Rx1AL-</td>
<td>2</td>
<td>18/40 (45)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>2</td>
<td>CT</td>
<td>3</td>
<td>21/24 (87)</td>
<td>20/24 (83)</td>
</tr>
<tr>
<td></td>
<td>CT + Rx1AL-</td>
<td>3</td>
<td>8/25 (32)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

*P values were calculated by the Fisher exact test between groups immunized with Rx1AL- plus CT and those immunized with CT alone.

### Table 3. Passive protection of infant rats by preincubation of serotype 3 pneumococci in sera of young rats immunized intranasally with killed Rx1AL- plus CT or in control sera

<table>
<thead>
<tr>
<th>Inoculum (CFU/intrathoracic injection)</th>
<th>Serum source</th>
<th>Bacteremia</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. bacteremic/total no. (%)</td>
<td>P vs NRS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9/10 (90)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Rats immunized with CT alone</td>
<td>10/12 (83)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Humans vaccinated with 23-valent PS vaccine</td>
<td>0/11 (0)</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>Rats immunized with Rx1AL- + CT</td>
<td>1/11 (9)</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>12/12 (100)</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>Rats immunized with CT alone</td>
<td>18/22 (82)</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Humans vaccinated with 23-valent PS vaccine</td>
<td>4/12 (33)</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>Rats immunized with Rx1AL- + CT</td>
<td>14/24 (58)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

*a Human antcapsular antibody. BPIG (hyperimmune globulin obtained from healthy adult volunteers immunized with 23-valent pneumococcal polysaccharide vaccine).
resent correlates of protection remains to be determined. CT alone, which elicited a lesser and shorter-lived active protection, notably did not elicit antibodies detectable in passive protection. It has been proposed that antibodies to several noncapsular antigens might have protective activity against many or all types of capsulated pneumococci. “Species” antigens as the cell wall polysaccharide (C substance) (7), the toxin pneumolysin (19), and various surface proteins (such as PspA, PspC, and PsaA [5, 6, 8, 18, 19, 23]) all protect mice challenged with a range of serotypes. Recently, genomic methods have been used to identify several additional proteins that may have protective potential (25). Whether these antigens would be able to elicit long-term immunity in children remains to be seen. Furthermore, since development will probably be proprietary, the affordability in low-income countries might be problematic.

Our proposed approach has the potential advantage of presenting a number of known as well as not yet known antigens, to which immunity might be synergistic (19). Additionally, the cell surface proteins are more likely to be presented in their native configuration than with purified or recombinant material. Conversely, however, not all the antigens presented by killed bacteria may contribute to protection, and some may even interfere with protection. Moreover, a particular combination of immunogenic surface antigens might prove difficult to reproduce from lot to lot in whole-cell preparations. Furthermore, from the nasopharyngeal colonization studies, it is also apparent that elicitation of protection with killed bacteria is dependent on CT. Therefore, one of our next goals is to identify a mucosal adjuvant that could be used safely and economically in humans. Although mucosal adjuvants have been tested in humans in conjunction with various vaccines (3, 12–15), it is unclear whether these would be effective with our killed pneumococcal preparation. Thus, our approach may have immunological advantages and drawbacks that must be further examined.

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