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

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**A whole-cell killed unencapsulated pneumococcal vaccine given by the intranasal route with cholera toxin as an adjuvant was tested in two animal models. This vaccination was highly effective in preventing nasopharyngeal colonization with an encapsulated serotype 6B strain in mice and also conferred protection against illness and death in rats inoculated intrathoracically with a highly encapsulated serotype 3 strain. When the serotype 3 challenge strain was incubated in the sera of immunized rats, it was no longer virulent in an infant-rat sepsis model, indicating that the intranasal immunization elicited protective systemic antibodies. These studies suggest that killed whole-cell unencapsulated pneumococci given intranasally with an adjuvant may provide multiprotective protection against capsulated pneumococci.**

*Streptococcus pneumoniae* (pneumococcus) annually causes 10 million deaths worldwide, including the deaths of 1 million children in low-income countries (26). Type-specific immunity, based on the capsular polysaccharides (PS), is well established (20). The licensed 23-valent PS vaccine, however, is not efficacious in children younger than 2 years. The newly licensed heptavalent PS conjugate vaccine protects against 90% of pneumococcal invasive disease in infancy in the United States (28) but includes fewer serotypes than the PS vaccine and omits several that are prevalent worldwide (10). Other drawbacks of the conjugate vaccine include a limited effect on otitis media (2, 11), high costs, and the potential for serotype replacement, which has already been suggested in recent clinical trials (11, 17; R. Dagan, N. Givon, P. Yagupsky, N. Porat, J. Janco, I. Chang, et al., Program Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. S52, 1998).

Several investigators have identified protective antigens common to pneumococci of many or all serotypes. Several such "species" antigens in purified or vectored form have shown protection in animal models (4–6, 8, 18, 19, 23, 25), but it is uncertain whether, when, and at what cost any of these will be developed as an effective vaccine for humans, particularly in low-income countries. As an alternative presentation of species antigens, we have studied unencapsulated whole cells, which should present a number of such antigens in native configuration unoccluded by capsule. In addition, the intranasal route of immunization might elicit mucosal immunity and, with suitable adjuvant, systemic immunity as well. Finally, of importance to low-income countries, a mucosally administered whole-cell preparation has the possible advantage of low cost of produc-

tion and administration, without the need for sterile injection devices. In the present study we tested killed, unencapsulated cells applied intranasally with cholera toxin (CT) as an adjuvant (R. Malley, S. Pelton, A. Stack, R. Saladino, D. E. Briles, and P. Anderson, 2nd Int. Symp. Pneumococci Pneumococcal Dis., abstr. P25, 2000), using two animal models: nasopharyngeal colonization of mice with type 6B and lethal intrathoracic challenge of rats with type 3.

## MATERIALS AND METHODS

**Vaccine preparation.** Strain RX1 is a capsule-negative mutant derived from a pneumococcus capsular serotype 2 (22). To allow for growth to high concentrations, an autolysin (lytA)-negative mutant of Rx1 (Rx1AL<sup>-</sup>) was used to prepare the killed vaccine preparations. This strain carries an erythromycin resistance gene (1). For vaccine production, RX1AL<sup>-</sup> was grown at 37°C in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) and 0.3 µg of erythromycin/ml to about 10<sup>9</sup> cells/ml. The cells were washed and suspended in saline at 10% of the original volume. Samples were mixed 3:7 (volume/volume) with ethanol, washed and resuspended in saline, and then frozen in small aliquots. When the killed vaccine preparation was cultured on blood agar, no viable bacteria were detected (lower limit of detection, 1 CFU/0.1 ml). The final vaccine mixture also contained CT (List Biological Laboratories, Campbell, Calif.) at 1 µg of CT per dose of vaccine. Control mice or rats were immunized with 1 µg of CT in saline.

**Bacteria for animal challenge.** *S. pneumoniae* strains GA03212, SF07348, and CT80231 are of capsular serotype 6B, 10F, and 14, respectively; all clinical isolates were originally from the Active Bacterial Core Surveillance (ABCs) of the Centers for Disease Control and Prevention. Strain WU2 is a type 3 pneumococcus that has been previously described (4). WU2r was passaged intraperitoneally in rats to increase its virulence (21). These strains were stored at -70°C in either skim milk or THY with 10% glycerol. For challenge in mice, frozen suspensions of *S. pneumoniae* type 6B were thawed and diluted in saline to a concentration of 10<sup>6</sup> CFU/10 µl. For intrathoracic challenge in rats, WU2r was grown overnight on blood agar plates and then grown in THY on the morning of the experiment. In early log phase it was diluted to an estimated concentration of 8 × 10<sup>5</sup>/ml in 0.5% low-melting-point agarose to increase its virulence (21); the actual colony count was determined on blood agar. For passive-protection experiments in infant rats, frozen suspensions of WU2r were thawed, diluted in sera, and incubated at 4°C for 90 min. Following incubation, the bacterial preparations were further diluted in 0.5% low-melting-point agarose to the desired concentration.

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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 mice and applying 10  $\mu$ l atraumatically to the nostrils. Live pneumococcal preparations (at a concentration of 10<sup>6</sup> CFU/inoculation), killed Rx1AL<sup>-</sup> 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<sup>6</sup> CFU of *S. pneumoniae* type 6B applied as in the immunizations. At 1 week after challenge, the mice were euthanized by CO<sub>2</sub> 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  $\geq 1$  CFU/50  $\mu$ l of wash fluid was detected on blood agar containing 2.5  $\mu$ g 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  $\mu$ l.

**(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  $\mu$ l (for the first two immunizations) and 30  $\mu$ l (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  $\mu$ l 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  $\geq 2$  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 as 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 interest were pools from the young rats immunized (in experiment 2 of Table 2) three times with Rx1AL<sup>-</sup> plus CT or with CT alone. Additional controls were normal rat serum (NRS) and a positive control consisting of bacterial polysaccharide immune globulin (BPIG; hyperimmune serum obtained from healthy adult volunteers immunized with 23-valent pneumococcal PS vaccine [gift of the Massachusetts Biological Laboratories, Jamaica Plain, Mass.]). Low and high inocula (corresponding to a final challenge of ca 5 and 50 CFU, respectively) were added to each serum or BPIG sample. Following incubation for 90 min at 4°C, bacteria were diluted to the desired concentration in 0.5% low-gelling-point agarose, as described above. Bacterial challenge via the intrathoracic route was performed as described above, except that the volume of the injection was 25  $\mu$ l. Blood cultures of specimens from all infant rats were performed 24 h after challenge. For this purpose, the tail vein of each infant rat was swabbed with 70% ethanol and punctured aseptically with a sterile lancet. Approximately 10  $\mu$ l of blood was plated on a blood agar plate containing 2.5 mg of gentamicin per liter (to suppress growth of the normal skin flora) and incubated overnight at 37°C with 5% CO<sub>2</sub>. Bacteremia was defined as the presence of  $\geq 1$  CFU of *S. pneumoniae*. The lower limit of detection of bacteremia was 100 CFU/ml. The end point of these experiments was mortality by day 7 after challenge.

**Statistical Analysis.** Fisher's exact test was used to compare the frequency of nasopharyngeal colonization following challenge in mice immunized with various preparations. To compare the density of colonization in mice, log-transformed counts of CFU of pneumococci were compared using Student's *t* test. For these purposes, the density of colonization in noncolonized animals was assigned a value equal to half the lower limit of detection, or 0.5 CFU per 50  $\mu$ l. Fisher's test

TABLE 1. Effect of intranasal immunization on the nasopharyngeal colonization of mice by serotype 6B pneumococci

Immunogen	Results when challenged 2 wk post-immunizations	
	No. colonized/total no. (% colonized)	<i>P</i> vs control <sup>a</sup>
Without adjuvant		
Control (saline alone)	39/44 (85)	— <sup>b</sup>
Live serotype 6B	0/10 (0)	<0.0001
Live serotype 14	5/12 (42)	0.0017
Live serotype 10	5/12 (42)	0.0017
Killed Rx1AL <sup>-</sup>	8/11 (72)	0.33
With adjuvant (CT)		
Control (CT alone)	10/22 (45)	— <sup>b</sup>
Killed Rx1AL <sup>-</sup> + CT	0/29 (0)	<0.0001

<sup>a</sup> *P* values were calculated by the Fisher exact test.

<sup>b</sup> *P* = 0.002 for comparison between groups that received CT versus saline alone.

was used to compare the mortality rates following intrathoracic challenge in the rats that were immunized with Rx1AL<sup>-</sup> plus CT versus CT alone as well as in the passive-protection experiments.

## RESULTS

**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<sup>-</sup> plus CT were completely protected against nasopharyngeal colonization with *S. pneumoniae* type 6B. In the animals challenged 2 weeks after immunization with Rx1AL<sup>-</sup> 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%] colonized; *P* = 0.002 versus saline and *P* < 0.001 versus Rx1AL<sup>-</sup> 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<sup>-</sup> plus CT were still completely protected against colonization (0 of 8 colonized; *P* = 0.026). The Rx1AL<sup>-</sup> vaccine without CT was not significantly protective.

The protection elicited by Rx1AL<sup>-</sup> 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<sup>-</sup> plus CT (*P* < 0.001) or the homologous strain (*P* < 0.04). When quantitative cultures were compared, animals that received Rx1AL<sup>-</sup> 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<sup>-</sup> plus CT elicits protection against nasopharyngeal colonization equal to that provide by repeated

TABLE 2. Protection of rats by intranasal immunization with killed Rx1AL<sup>-</sup> cells plus CT against intrathoracic infection with serotype 3 pneumococci

Expt	Immunogen	No. of doses	Morbidity		Mortality	
			No. ill/ total no. (% ill)	<i>P</i> vs no Rx1AL <sup>-a</sup>	No. dead/ total no. (% dead)	<i>P</i> vs no Rx1AL <sup>-a</sup>
1	CT	2	42/42 (100)		29/42 (69)	
	CT + Rx1AL <sup>-</sup>	2	18/40 (45)	<0.0001	18/40 (45)	0.043
2	CT	3	21/24 (87)		20/24 (83)	
	CT + Rx1AL <sup>-</sup>	3	8/25 (32)	0.0001	7/25 (28)	0.0001

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

exposure to the challenge strain and greater than that provided by heterologous capsulated strains.

**Young-rat sepsis model.** In the young-rat sepsis model of direct intrapulmonary challenge, essentially all control animals sickened and most died within 1 week after the challenge with capsular type 3 *S. pneumoniae* (Table 2). In experiment 1, two sequential exposures to vaccine plus CT reduced morbidity from 100 to 45% and mortality from 69 to 25% compared to CT alone; three vaccinations (experiment 2) reduced morbidity to 32% and mortality to 28%. These differences were all statistically significant.

**Passive protection.** To examine a possible role of serum antibodies, serum of intranasally immunized rats was tested for protection of infant rats in the intrapulmonary challenge model (Table 3). Intrathoracic inoculation of 5 CFU of *S. pneumoniae* type 3 incubated in normal rat serum was uniformly fatal in infant rats. Preincubation in the serum of rats given CT alone was not protective. Preincubation in a source of serum antibodies to capsule (BPIG) was, as expected, highly protective against both bacteremia and mortality. Preincubation in serum of rats given Rx1AL<sup>-</sup> plus CT was also highly protective against both bacteremia and mortality (*P* < 0.0001 for both bacteremia and mortality compared to preincubation with serum from normal rats or rats that received CT alone).

When the challenge inoculum was increased 10-fold, serum from Rx1AL<sup>-</sup>/CT-immunized animals provided lower but still highly significant protection against death compared to serum from normal rats (*P* = 0.006) or rats that received CT alone (*P* < 0.001).

## DISCUSSION

We have demonstrated that unencapsulated killed whole-cell 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 serotype 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<sup>-</sup> 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-capsule-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<sup>-</sup> 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 3. Passive protection of infant rats by preincubation of serotype 3 pneumococci in sera of young rats immunized intranasally with killed Rx1AL<sup>-</sup> plus CT or in control sera

Inoculum (CFU/intrathoracic injection)	Serum source	Bacteremia		Mortality	
		No. bacteremic/total no. (%)	<i>P</i> vs NRS	No. dead/total no. (%)	<i>P</i> vs NRS
5	NRS	9/10 (90)		11/11 (100)	
	Rats immunized with CT alone	10/12 (83)	1.0	11/11 (100)	1.0
	Humans vaccinated with 23-valent PS vaccine <sup>a</sup>	0/11 (0)	<0.0001	0/11 (0)	<0.0001
	Rats immunized with Rx1AL <sup>-</sup> + CT	1/11 (9)	0.0003	1/11 (9)	<0.0001
50-75	NRS	12/12 (100)		12/12 (100)	
	Rats immunized with CT alone	18/22 (82)	0.27	22/22 (100)	1.0
	Humans vaccinated with 23-valent PS vaccine <sup>a</sup>	4/12 (33)	0.001	4/12 (33)	0.001
	Rats immunized with Rx1AL <sup>-</sup> + CT	14/24 (58)	0.015	13/24 (54)	0.006

<sup>a</sup> Human anticapsular 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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