

Intranasal Immunization with Pneumococcal Polysaccharide Conjugate Vaccines with Nontoxic Mutants of *Escherichia coli* Heat-Labile Enterotoxins as Adjuvants Protects Mice against Invasive Pneumococcal Infections

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Host defenses against *Streptococcus pneumoniae* depend largely on phagocytosis following opsonization by polysaccharide-specific immunoglobulin G (IgG) antibodies and complement. Since colonization of the respiratory mucosa is the first step in pneumococcal pathogenesis, mucosal immune responses may play a significant role. In addition to inducing systemic immune responses, mucosal vaccination with an effective adjuvant has the advantage of inducing mucosal IgA antibodies. The heat-labile enterotoxin (LT) of *Escherichia coli* is a well-studied mucosal adjuvant, and adjuvant activity of nontoxic LT mutants has been demonstrated for several protein antigens. We investigated the immunogenicity of pneumococcal polysaccharide conjugate vaccines (PNC) of serotypes 1 and 3 in mice after intranasal (i.n.) immunization by using as an adjuvant the nontoxic LT mutant LT-K63 or LT-R72, which has minimal residual toxicity. Pneumococcal serotype-specific antibodies were measured in serum (IgM, IgG, and IgA) and saliva (IgA), and vaccine-induced protection was evaluated by i.n. challenge with virulent pneumococci of the homologous serotype. When administered with LT mutants, i.n. immunization with both conjugates induced systemic and mucosal immune responses, and serum IgG antibody levels were significantly higher than after subcutaneous immunization. All mice immunized i.n. with PNC-1 and LT mutants were protected against bacteremia and cleared the pneumococci from the lung 24 h after i.n. challenge; pneumococcal density correlated significantly with serum IgG antibody levels. Similarly, the survival of mice immunized i.n. with PNC-3 and LT mutants was significantly prolonged. These results demonstrate that i.n. vaccination with PNC and potent adjuvants can protect mice against invasive and lethal pneumococcal infections, indicating that mucosal vaccination with PNC may be an alternative vaccination strategy for humans.

Streptococcus pneumoniae is a major pathogen which enters the body through the respiratory mucosa and may cause serious infections such as meningitis, pneumonia, and bacteremia (36), especially in elderly people and in young children. It is also a major cause of the childhood mucosal infection otitis media (2, 14). The pneumococcus is encapsulated with polysaccharides (PS), which are the main virulence factors (39) and protect the bacteria from host defense mechanisms, particularly phagocytosis following opsonization by PS-specific antibodies and complement (25, 37).

The PS, which are poorly immunogenic in young children (20), are classified as type 2 T-cell-independent antigens. B cell activation results in immunoglobulin M (IgM) production but limited class switching, no affinity maturation, and little, if any, development of memory cells (24). By conjugation of PS to various proteins, their immunogenicity may be increased, probably by recruitment of T-cell help through linked recognition (11, 19, 27, 32).

Currently, most licensed vaccines are administered parenterally and show good efficacy in protection against various pathogens. Pneumococcal diseases can be prevented in adults by the parenteral administration of plain PS vaccines (31), and experimental pneumococcal polysaccharide-protein conjugate

vaccines (PNC) are immunogenic in infants (1, 5, 18, 33). Recently, 100% efficacy against invasive pneumococcal infections in infants was reported (3) and results from efficacy trials for acute otitis media are expected soon (10a).

Mucosal immune responses against pneumococci may be induced by mucosal vaccination, which may have additional benefits provided that it also induces sufficient systemic immune response and generates immunological memory. It is assumed that secretory IgA at mucosal surfaces inhibits the adherence and invasion of mucosal pathogens and neutralizes virulence factors (23, 35). However, mucosal vaccination has not been adequately exploited, partly due to lack of mucosal adjuvants acceptable for human use. Cholera toxin (CT) from *Vibrio cholerae* and heat-labile enterotoxin (LT) of *Escherichia coli* are strong mucosal adjuvants capable of enhancing the immune response to mucosally coadministered antigens (4, 10, 21). The toxicity of these proteins has prevented their use in humans, but recently, mutants of LT and CT with no or low toxic activity have been constructed by site-directed mutagenesis, and adjuvanticity of the LT mutants LT-K63 (nontoxic) and LT-R72 (reduced toxicity) has been demonstrated for several protein antigens (6–9, 13, 15, 26).

In this study, immune responses elicited by two serotypes of PNC after intranasal (i.n.) administration with LT-K63 and LT-R72 as adjuvants were investigated. Furthermore, vaccine-induced protection was evaluated by i.n. challenge with virulent pneumococci of the homologous serotype (30).

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TABLE 1. Pneumococcal serotype-specific antibodies in serum and saliva after immunization with PPS and PNC

Vaccine (dose [μ g])	Route	Adjuvant	Geometric mean titer (EU/ml) ^a of:			
			IgG	IgM	IgA	Salivary IgA ^b
PNC-1 (0.5)	s.c.	Saline	955 (372–2,449)	149 (93–232)	90 (59–136)	3
PNC-1 (0.5)	i.n.	Saline	27 (6–124)	52 (36–76)	56 (26–125)	5
PNC-1 (0.5)	i.n.	LT-K63	9,149 (5,074–16,495)	221 (180–271)	529 (320–874)	42
PNC-1 (0.5)	i.n.	LT-R72	8,314 (4,675–14,785)	242 (161–365)	403 (205–792)	34
PNC-1 (2.0)	s.c.	Saline	519 (158–1,706)	134 (105–169)	94 (39–224)	4
PNC-1 (2.0)	i.n.	Saline	133 (54–312)	43 (29–63)	83 (45–155)	3
PNC-1 (2.0)	i.n.	LT-K63	8,638 (5,760–12,954)	209 (149–293)	235 (129–428)	12
PNC-1 (2.0)	i.n.	LT-R72	6,227 (3,831–10,123)	345 (273–436)	210 (133–333)	15
None (control)			5 (4–7)	34 (29–41)	69 (42–113)	ND
PPS-1 ^c (2.0)	i.n.	Saline	10 (7–13)	52 (35–76)	73 (34–157)	ND
PPS-1 (2.0)	i.n.	LT-R72	25 (15–39)	50 (40–62)	96 (49–186)	ND
PNC-3 (2.0)	s.c.	Saline	339 (233–494)	109 (76–156)	34 (17–69)	12
PNC-3 (2.0)	i.n.	Saline	8 (5–11)	51 (35–75)	31 (21–44)	3
PNC-3 (2.0)	i.n.	LT-K63	3,050 (2,034–4,573)	95 (61–148)	69 (44–108)	23
PNC-3 (2.0)	i.n.	LT-R72	2,546 (1,399–4,632)	94 (70–128)	68 (45–104)	31
None (control)			4 (3–5)	35 (22–57)	31 (22–44)	5

^a The 95% confidence interval is given in parentheses.

^b Salivary samples were pooled for each group. ND, not detectable.

^c The PPS immunizations were performed in a separate experiment.

MATERIALS AND METHODS

Mice. Outbred female NMRI mice, 6 to 8 weeks old, were obtained from the Institute of Experimental Pathology at Keldur, Reykjavik, Iceland. The animals were kept in cages with free access to commercial pelleted food and water.

Vaccines and adjuvants. Experimental PNC were provided by Pasteur Mérieux Connaught, Marcy l'Etoile, France. Serotype 1 PS was conjugated to tetanus toxoid (PNC-1) and serotype 3 PS was conjugated to diphtheria toxoid (PNC-3). Pure pneumococcal polysaccharides (PPS) were purchased from the American Type Culture Collection (ATCC) (Manassas, Va.). For i.n. immunization, PNC or PPS were diluted in saline or mixed with the LT mutant LT-K63 (6, 7) or LT-R72 (15), provided by the Immunobiology Research Institute Siena, Siena, Italy. Five micrograms of LT mutant per mouse was used.

Immunization. Mice were lightly sedated by subcutaneous (s.c.) injection of Hypnorm (Janssen Pharmaceutica, Beerse, Belgium). This treatment keeps the mice conscious, which minimizes the possibility of antigen delivery into the lung during i.n. immunization. Eight to 10 mice per group were each immunized with 0.5 or 2.0 μ g of PNC or PPS. For i.n. immunization, two doses of 10- μ l vaccine solution was slowly delivered into the nares, with 30 min between each dose. For s.c. immunization, a 500- μ l vaccine solution was injected in the scapular girdle region. All groups were boosted with the same dose and route 2 weeks after primary immunization. Unimmunized mice were used as controls.

Blood and saliva sampling. The mice were bled from the retro-orbital sinus 15 days after boosting and the serum was isolated and stored at -70°C . Saliva was collected from each mouse by the insertion of absorbent sticks (Polyfintronics Inc., Rockland, Maine) into the mouth. After 5 min, the sticks were transferred to phosphate-buffered saline (PBS) containing 10.0 μ g of protease inhibitor (aprotinin; Sigma Chemical Co., St. Louis, Mo.) per ml to prevent the proteolysis of antibodies. The dissolved saliva samples for each group were pooled and stored at -70°C .

Antibody measurements. Specific antibodies (IgM, IgG, and IgA) to pneumococcal polysaccharides were determined by enzyme-linked immunosorbent assay (ELISA) designed according to the standardized ELISA protocol (3a) with a few modifications. Microtiter plates (MaxiSorp; Nunc AS, Roskilde, Denmark) were coated with 10 μ g of PS serotype 1 and PS serotype 3 (ATCC) per ml of PBS and incubated for 5 h at 37°C . For the neutralization of antibodies to cell wall PS (Statens Serum Institute, Copenhagen, Denmark), serum samples and standards were diluted 1:50 in PBS with 0.05% Tween 20 (Sigma) and incubated in 500 μ g of cell wall PS per ml for 30 min at room temperature. The neutralized sera were serially diluted and incubated in PPS-coated microtiter plates at room temperature for 2 h. For the detection of bound antibodies, horseradish peroxidase-conjugated goat anti-mouse IgG (Caltag Laboratories, Burlingame, Calif.), IgM, or IgA (Sera-Lab, Crawley Down, Sussex, United Kingdom) was diluted 1:5,000 in PBS-Tween and incubated for 2 h at room temperature. For development, 3,3',5,5'-tetramethylbenzidine peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was incubated for 10 min according to the manufacturer's instructions, and the reaction was stopped by addition of 0.18 M H_2SO_4 . Absorbance was measured at an optical density of 450 nm in an ELISA spectrophotometer (Titertek Multiscan Plus MK II; Flow Laboratories, Irvine, United Kingdom). Reference serum obtained from Pasteur Mérieux Connaught was

included on each microtiter plate for calculation of the titers expressed in ELISA units (EU) per milliliter. The titers of the reference sera (in ELISA units per milliliter) corresponded to the inverse of the serum dilution, giving an optical density of 1.0.

The assays were performed at room temperature and PBS-Tween was used for dilutions and washing. One-hundred-microliter volumes were used in all incubation steps with three washings between each step. All sera were tested in duplicate.

Pneumococci. Serotype 1 (ATCC 6301) and serotype 3 (ATCC 6303) pneumococci were maintained in tryptose broth plus 20% glycerol at -70°C . One day before challenge, stocks were plated on blood agar (Difco Laboratories, Detroit, Mich.) and incubated at 37°C in 5% CO_2 overnight. Isolated colonies were transferred to a brain heart infusion broth (Difco) with 10% horse serum, cultured at 37°C to log phase for 3.5 h, and resuspended in 0.9% sterile saline. Serial 10-fold dilutions were plated on blood agar to determine inoculum density.

Pneumococcal challenge. The challenge experiments were performed 2 days after the mice were bled. The animals were anesthetized with pentobarbitone sodium (50 mg/kg of body weight; Icelandic Pharmaceuticals, Reykjavik, Iceland) injected intraperitoneally (i.p.). They were then challenged with 50 μ l of pneumococcal suspension i.n. and were allowed to aspirate it into the lungs for 10 min (35). Blood was collected from the tail vein 24 h after challenge, plated in serial dilutions on blood agar, and cultured at 37°C in 5% CO_2 overnight. Bacteremia was determined as the number of CFU per milliliter of blood. When the mice were sacrificed, the lungs were removed and homogenized in 0.9% sterile saline, and serial dilutions were plated on blood agar that included Staph/Strep selective supplement containing nalidixic acid and colistin sulfate (Unipath Ltd., Bedford, Hampshire, United Kingdom). Pneumococcal lung infection was determined as the number of CFU/milliliter of lung homogenate. Depending on the first dilution used, the detection limit was 2.2 CFU/ml of lung homogenate and 1.6 CFU/ml of blood.

Statistical analysis. A nonparametric *t* test (a Mann-Whitney U test) was used to compare antibody titers and numbers of CFU between groups. Correlation was calculated with Pearson's correlation coefficient. The Kaplan-Meier survival test was used to compare survival rates. A *P* value of <0.05 was considered to be statistically significant.

RESULTS

Antibody responses to PNC-1. Mice were immunized i.n. with 0.5 or 2.0 μ g of PNC-1 alone or mixed with either LT-K63 or LT-R72. Administration of PNC-1 i.n. with either LT-K63 or LT-R72 elicited significantly higher antibody responses than the s.c. route for both 0.5- and 2.0- μ g doses of PNC-1 in saline ($P < 0.001$). The lower dose of PNC-1 tended to elicit a higher systemic IgG response when administered i.n. with either LT mutant, but the difference between the two doses was not significant (Table 1). In addition, for both doses of PNC-1, the

nontoxic mutant LT-K63 tended to enhance systemic IgG responses more efficiently than LT-R72, although the differences were not significant ($P = 0.337$). Administration of PNC-1 alone via the i.n. route induced a significant systemic IgG response compared to that in unimmunized control mice ($P = 0.016$ for 0.5 μg of PNC-1 and $P < 0.001$ for 2.0 μg of PNC-1), but when mixed with either LT mutant, antibody responses were significantly enhanced ($P < 0.001$).

Only those mice immunized s.c. with PNC-1 and i.n. with PNC-1 and LT mutants showed significant IgM responses, compared to unimmunized control mice ($P < 0.001$). Furthermore, significant systemic IgA responses were observed only among mice immunized i.n. with PNC-1 and LT mutants (Table 1).

Administration of PNC-1 i.n. with either LT mutant elicited the highest mucosal IgA responses (Table 1). Furthermore, salivary IgA levels were higher in pooled saliva of mice immunized i.n. with 0.5 μg than in mice given 2.0 μg of PNC-1. Mice immunized s.c. or i.n. with PNC-1 in saline rarely had detectable salivary IgA levels (Table 1).

In a separate experiment, we investigated the immunogenicity of pure serotype 1 PPS (PPS-1) when administered i.n. to two groups of mice, either with LT-R72 or in saline (Table 1). Even though a significant systemic IgG response was observed for both groups, compared to unimmunized control mice ($P = 0.005$ for PPS-1 with LT-R72 and $P = 0.010$ for PPS-1 alone), the antibody levels were significantly lower than after i.n. immunization with PNC-1 with or without LT-R72 ($P < 0.001$ for both groups). Neither mucosal nor significant systemic IgA was detectable in these immunized mice.

Antibody responses to PNC-3. Mice were immunized with 2.0 μg of PNC-3 in one of three ways: i.n., i.n. with LT mutants, or s.c. in saline. Significant serum IgG responses were observed in mice immunized i.n. with PNC-3 with either LT mutant or s.c. in saline, compared to the unimmunized group ($P < 0.001$ for all groups), but no response was observed after i.n. administration of PNC-3 in saline ($P = 0.141$; Table 1). In addition, i.n. immunization with PNC-3 mixed with either LT mutant elicited significantly higher serum IgG levels than s.c. immunization with PNC-3 in saline ($P < 0.001$).

Only those mice immunized i.n. with PNC-3 and either LT mutant had significant IgA levels in serum, compared to unimmunized control mice ($P = 0.020$ for PNC-3 mixed with LT-K63 and $P = 0.005$ for PNC-3 mixed with LT-R72).

Salivary IgA levels were slightly higher in mice immunized i.n. with PNC-3 and LT mutants than in unimmunized control mice (Table 1) but hardly detectable in mice immunized s.c. or i.n. with PNC-3 in saline.

Protection against pneumococcal infections caused by serotype 1. To evaluate vaccine-induced protection against pneumococcal bacteremia and pulmonary infection caused by serotype 1 pneumococci, immunized mice were challenged i.n. with 10^6 CFU of serotype 1 pneumococci suspended in 50 μl of saline 2 weeks after booster vaccination. Serotype 1 pneumococci were very virulent and caused severe bacteremia (mean, $\sim 10^5$ CFU/ml of blood) and lung infection (mean, $\sim 10^7$ CFU/ml of lung homogenate) in unimmunized control mice 24 h after i.n. challenge (Fig. 1).

All mice immunized i.n. with either 0.5 or 2 μg of PNC-1 with LT mutants were protected from pneumococcal bacteremia 24 h after challenge (Fig. 1A). In addition, 100% protection was observed in mice immunized s.c. with PNC-1 in saline. Of the mice immunized i.n. with PNC-1 alone, only 3 of 16 had detectable pneumococci in the blood, and all 3 had received 0.5 μg of PNC-1 (Fig. 1A). The level of bacteremia at 24 h was inversely related to serotype 1-specific IgG ($r = -0.310$; $P =$

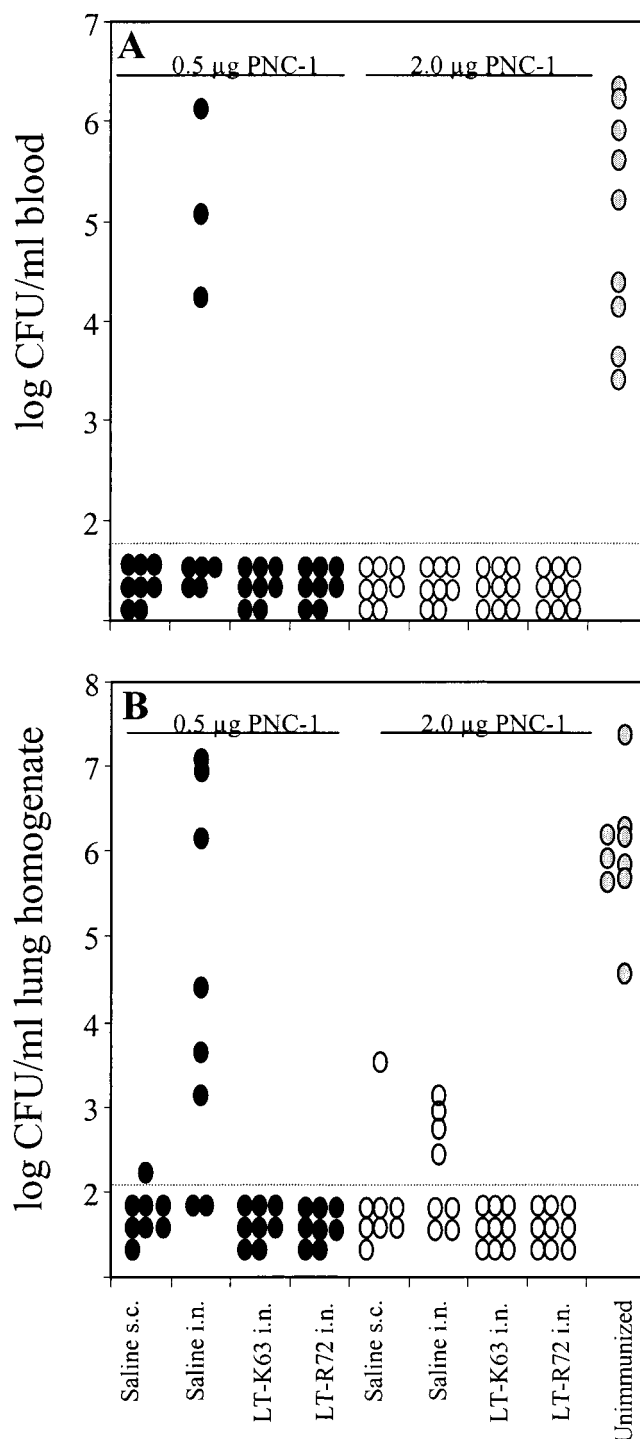


FIG. 1. Pneumococcal density (log CFU) in blood (A) and lungs (B) 24 h after i.n. challenge with serotype 1 pneumococci. Each mouse is represented by one symbol, and dotted lines indicate detection limits for CFU. The mode of immunization is indicated on the x axis (the labels apply to both panels A and B).

0.006) and IgA ($r = -0.228$; $P = 0.046$) in serum; bacteremia was not detected in mice with >20 EU of serum IgG/ml (data not shown).

Immunization i.n. with PNC-1 and LT-K63 or LT-R72 conferred 100% clearance of lung infection caused by serotype 1 pneumococci (Fig. 1B). Of the mice immunized i.n. with

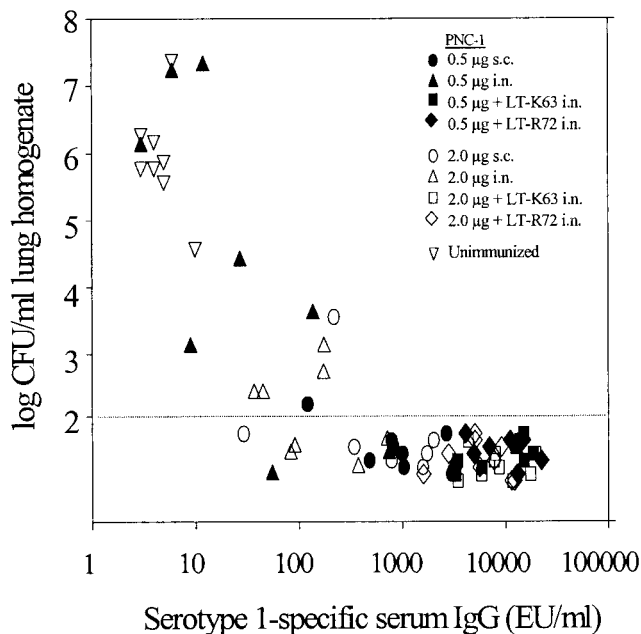


FIG. 2. Relationship between pneumococcal density in lung (as log CFU) 24 h after i.n. challenge and serotype 1-specific IgG antibody levels in serum (in ELISA units/milliliter). Each symbol represents one mouse, and the horizontal dotted line indicates the detection limit for CFU.

PNC-1 in saline, only 2 of 8 mice receiving 0.5 µg of PNC-1 and 4 of 8 receiving 2.0 µg of PNC-1 were protected from lung infection, whereas 1 of 8 in each group immunized s.c. had detectable pneumococci in the lungs. The pneumococcal density in the lungs correlated significantly with serotype 1-specific IgG (Fig. 2) and IgA antibody levels in serum ($r = -0.428, P < 0.001$ for IgG; $r = -0.285, P = 0.012$ for IgA), and no pneumococci were detectable in lungs of mice with >300 EU of serum IgG/ml.

A total of 6 of 10 mice immunized i.n. with pure PPS-1 and LT-R72 were protected against bacteremia, but only 1 of 10 mice immunized i.n. with PPS-1 alone were protected. Only 3 of 10 mice immunized i.n. with PPS-1 and LT-R72 were protected from lung infection; the lungs of all mice immunized i.n. with PPS-1 in saline were heavily infected (data not shown).

Protection against pneumococcal infections caused by serotype 3. Immunized mice were challenged i.n. with 10^4 CFU of serotype 3 pneumococci, and survival was recorded over 10 days when the experiment was terminated (Fig. 3). The challenge killed 80% of both unimmunized control mice and mice immunized i.n. with PNC-3 alone, but among mice immunized s.c. with PNC-3 in saline, survival was significantly prolonged, and 90% of these mice were protected at day 10 ($P = 0.006$). Immunization i.n. with PNC-3 mixed with LT mutants induced significantly prolonged survival, and 100% of the mice receiving LT-K63 ($P < 0.001$) and 90% of the mice receiving LT-R72 ($P = 0.006$) were still alive and looked healthy at day 10 (Fig. 3).

DISCUSSION

PNC are now in phase II and phase III clinical trials, and safety and immunogenicity has been demonstrated in infants vaccinated parenterally (1, 5, 18, 33). Antibodies elicited in infants mediate opsonophagocytosis in vitro (17, 33, 38) and protect mice against invasive infections if passively administered i.p. before i.n. challenge with virulent pneumococci (29).

Since the mucosal epithelium of the nasopharynx is the primary site of pneumococcal colonization (36), i.n. vaccination may be an alternative approach to current strategies, mainly because it may induce both mucosal and systemic immune responses. In addition, such vaccination is painless and easy to perform, which should favor these strategies for the immunization of infants and children. Most antigens are poor mucosal immunogens, partly because they lack receptor-binding properties to epithelial cells in the mucosa. Exceptions are proteins such as CT and *E. coli* LT (34, 40). These toxins are also exceptionally powerful mucosal adjuvants, inducing antibody production to mucosally coadministered unlinked antigens (4, 10, 21). Both LT and CT are ADP-ribosylating holotoxins composed of an enzymatically active A subunit and a pentameric, nontoxic B subunit which binds with high affinity to GM1 ganglioside cell surface receptors and promotes the entry of the A unit into the cell (34). Reports have suggested that the toxic A subunit is necessary for the adjuvant activity of CT and LT (22), but recently, several mutants have been constructed which have reduced or which totally lack ADP-ribosylating activity while the useful immunological properties are maintained (6-9, 13, 15, 26). The adjuvant activity of the nontoxic LT-K63, with a serine-to-lysine change at position 63, has been demonstrated for bystander antigens (6, 7), and another LT mutant, LT-R72, which contains an alanine-to-arginine substitution in position 72 in the A subunit, showed greatly reduced enzymatic activity compared to wild-type LT while adjuvant activity was maintained (15).

In this study, we demonstrated that i.n. immunization with PNC-1 and PNC-3, with LT-K63 and LT-R72 as adjuvants, elicits significant immune responses in mice. Furthermore, vaccine-induced protection against invasive infections caused by homologous pneumococcal serotypes was established. Immunization i.n. with both PNC-1 and PNC-3 with LT mutants was more efficient than immunization with PNC in saline by the s.c. route, in terms of both immunogenicity and protection. Systemic PPS-specific IgG antibodies are known to protect against

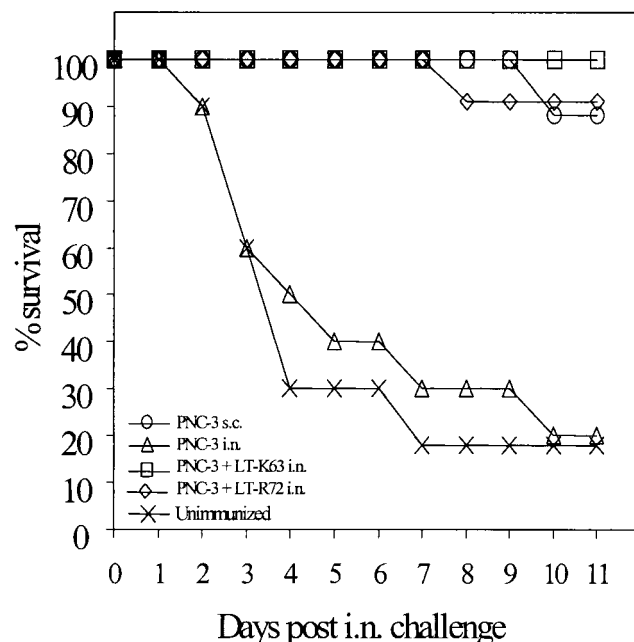


FIG. 3. Survival of mice ($n = 10$ per group) after i.n. challenge with serotype 3 pneumococci.

invasive pneumococcal infections (39), and we found that very low antibody levels were sufficient to prevent bacteremia and that clearance of serotype 1 pneumococci from the lungs was significantly related to PPS-specific serum IgG levels (Fig. 2). Immunization with PPS-1 and LT-R72 i.n. elicited a systemic IgG response, but the levels were significantly lower than the responses elicited by PNC-1 and were insufficient to clear the infection (data not shown).

Interestingly, both PNC-1 and PNC-3 tended to induce higher systemic antibody responses when mixed with the nontoxic mutant LT-K63 than with LT-R72, which appears to contradict results obtained with these two LT mutants when adjuvant activities for protein antigens were compared (15). A similar trend was observed in antibody response to PNC of other serotypes (unpublished data). Furthermore, the lower dose of PNC-1 seemed to elicit higher systemic and mucosal antibody responses than did the higher dose when administered i.n. with either LT mutant. It has already been demonstrated that PNC-1 and PNC-3 induce serum IgG antibodies in mice when administered i.n. in a glyceride-based adjuvant (16). However, the dose of PNC-1 required to elicit a 100% protective immune response was higher (16) than when the conjugates were administered with LT mutants, which suggests that the LT mutants may be more feasible adjuvants for i.n. immunization with multivalent PNCs.

Secretory IgA is considered to be an important immunological first-line defense at mucosal surfaces, and it is assumed that it inhibits adherence and invasion of mucosal pathogens and neutralizes virulence enzymes and toxins (35). In addition, it has been postulated that there is a simple transudation of serum IgG on mucosal surfaces (28). When mice were immunized i.n. with pneumococcal surface protein A (PspA) with the B subunit of CT as an adjuvant (41), significant PspA-specific levels of IgA antibodies were detected in saliva. In addition, the mice were protected against long-lasting carriage of *S. pneumoniae*, which indicates a role of mucosal IgA antibodies in the protection of mucosal surfaces. In contrast, i.n. immunization with PPS-6B conjugated to tetanus toxoid induced a marginal salivary IgA response, but significant serum IgG levels were observed (41). Furthermore, oral immunization of mice with PPS-23 conjugated to the outer membrane protein complex of *Neisseria meningitidis* in enterocoated microparticles was found to be insufficient for the induction of salivary IgA (12). However, oral immunization may induce mucosal immune responses in the presence of a mucosal adjuvant such as CT (21). In the present study, IgA antibodies were detectable in saliva only after i.n. administration of PNC with LT mutants. The salivary IgA measurements were performed on saliva samples which were pooled for each group, and thus it was not possible to evaluate the relationship between salivary IgA levels and protection against pneumococcal infections. Serum IgA may be considered a surrogate for mucosal IgA response. A weak negative correlation was found between serum IgA levels and pneumococcal density in the lungs and blood, indicating that mucosal IgA may be of importance in clearing the pneumococci. However, this may also be secondary to the correlation between IgG and IgA in serum ($r = 0.246$; $P = 0.032$).

We have demonstrated that mucosal vaccination with PNC and nontoxic LT mutants elicits mucosal as well as systemic immune responses in mice and protects against invasive pneumococcal infections. The results indicate that this vaccination strategy may be an alternative approach for preventing pneumococcal diseases and encourage its evaluation in humans.

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