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Intranasal Immunization with Pneumococcal Conjugate Vaccines with LT-K63, a Nontoxic Mutant of Heat-Labile Enterotoxin, as Adjuvant Rapidly Induces Protective Immunity against Lethal Pneumococcal Infections in Neonatal Mice

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Immunization with pneumococcal polysaccharides (PPS) conjugated to tetanus toxoid (TT) (Pnc-TT) elicits protective immunity in an adult murine pneumococcal infection model. To assess immunogenicity and protective immunity in early life, neonatal (1 week old) and infant (3 weeks old) mice were immunized intranasally (i.n.) or subcutaneously (s.c.) with Pnc-TT of serotype 1 (Pnc1-TT). Anti-PPS-1 and anti-TT immunoglobulin G (IgG) and IgM antibodies were measured in serum and saliva, and vaccine-induced protection was evaluated by i.n. challenge with serotype 1 pneumococci. Pnc1-TT was immunogenic in neonatal and infant mice when administered s.c. without adjuvant: a majority of the young mice were protected from bacteremia and a reduction of pneumococcal density in the lungs was observed, although antibody responses and protective efficacy remained lower than in adults. The addition of LT-K63, a nontoxic mutant of heat-labile enterotoxin, as adjuvant significantly enhanced PPS-1-specific IgG responses and protective efficacy following either s.c. or i.n. Pnc1-TT immunization. Mucosal immunization was particularly efficient in neonates, as a single i.n. dose of Pnc1-TT and LT-K63 induced significantly higher PPS-1-specific IgG responses than s.c. immunization and was sufficient to protect neonatal mice against pneumococcal infections, whereas two s.c. doses were required to induce complete protection. In addition, i.n. immunization with Pnc1-TT and LT-K63 induced a vigorous salivary IgA response. This suggests that mucosal immunization with pneumococcal conjugate vaccines and LT-K63 may be able to circumvent some of the limitations of neonatal antibody responses, which are required for protective immunity in early life.

Streptococcus pneumoniae (pneumococcus) is a major respiratory pathogen which enters the body through the respiratory mucosa (65) and may cause serious infections such as meningitis, pneumonia, and bacteremia, especially in young children and the elderly (4, 32). It is also the most common cause of bacterial otitis media (20). The increase in resistance to antimicrobial agents is an increasing problem worldwide (3, 10), and infants are colonized very early by pneumococcus in countries where resistant strains are prevalent (28, 35). To induce protection in early life, vaccines that rapidly induce protective immunity are required, but the immaturity of the immune system in newborns makes it difficult to induce protective immune responses by vaccination. Preclinical immunization models using various protein antigens and DNA vaccines during the neonatal period have demonstrated that induction of antigen-specific B- and T-cell responses (6, 34, 57, 58) and protection against infections (57) may be achieved. However, early life responses frequently remain delayed and weaker than those elicited in immunologically mature hosts (56).

The 23-valent pneumococcal polysaccharide (PPS) vaccine is immunogenic and protective in healthy adults (9, 52), but PPS, which are T-cell-independent type 2 antigens (36, 61), are not immunogenic in those of an early age (17). Immunization with PPS in adults induces limited class switching of activated B cells, no affinity maturation, and poor induction of memory cells. Thus, antibody responses to PPS are characterized by high levels of immunoglobulin M (IgM) and low levels of IgG that are primarily of the IgG2 subclass in humans (5, 27) and of the IgG3 subclass in mice (42). A marked improvement in the immunogenicity of polysaccharide (PS) antigens has been achieved by conjugation of PS to various protein carriers (18, 47, 55), and several PPS-protein conjugate vaccines have proven immunogenic in infants and toddlers (2, 12, 53, 59, 70; S. T. Sigurdardottir, T. Gudnason, S. Kjartansson, K. Davidsdottir, K. G. Kristinsson, G. Ingolfsdottir, M. Yaich, O. Leroy, and I. Jonsdottir, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. G-50, 2000), inducing immunologic memory (1, 41; I. Jonsdottir, G. Ingolfsdottir, E. Saeland, K. Davidsdottir, M. Yaich, O. Leroy, and S. T. Sigurdardottir, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. G-43, 2000) and reducing nasopharyngeal carriage of pneumococci (11, 40). Efficacy against both invasive disease (7) and acute otitis media (19) in infants has been demonstrated. Accordingly, PPS-protein conjugate vaccines induce protective immune responses in various adult experimental animal models (21, 22, 30, 31, 51, 66).

Recent studies have shown that mucosal delivery of antigens induces humoral and cell-mediated immune responses in both

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mucosal and systemic compartments. Mucosal immunization is especially attractive for immunization against respiratory pathogens, since the first line of defense in the upper respiratory tract is assumed to be due to IgA antibodies in mucosal secretions (62). Mucosal immunization with inactivated vaccines usually requires adjuvants. Of the many mucosal adjuvants under investigation, mutants of *Escherichia coli* heat-labile enterotoxin (LT) are among the most promising (14, 45, 46, 69). The adjuvanticity of the two mutants LT-K63 (15, 16) and LT-R72 (23) has been reported for various antigens and these molecules are ready to enter clinical trials (44). We previously demonstrated that mucosal immunization of adult mice with PPS-protein conjugate vaccines and LT mutants induces protective immunity against lethal pneumococcal infections of several serotypes (30, 31). However, little is known about mucosal immune responses in infants and neonates, and the potential advantage of LT mutants as adjuvants in immunization against pneumococcal infection in early life remained to be explored. The aim of the present study was thus to investigate the early life immunogenicity of an experimental tetanus toxoid (TT) pneumococcal conjugate vaccine of serotype 1 (Pnc1-TT) following subcutaneous (s.c.) or intranasal (i.n.) immunization and the capacity of the nontoxic LT-K63 mutant to enhance vaccine responses. Early life immunization was performed both in 3- and 1-week-old mice, which best correspond to the state of immune maturation of human infants and newborns, respectively (56). Vaccine-induced protection against lethal pneumococcal infections was assessed after i.n. challenge with a lethal dose of *S. pneumoniae* of serotype 1, which is highly virulent in mice and causes severe bacteremia and pneumonia after i.n. challenge in this mouse model (30, 31).

MATERIALS AND METHODS

Mice. Adult NMRI mice were obtained from M&B AS (Ry, Denmark). The mice were kept in microisolator cages with free access to commercial pelleted food and water and were housed under standardized conditions at the Institute of Experimental Pathology at Keldur (Reykjavik, Iceland), with regulated daylight, humidity, and temperature. Breeding cages were checked daily for new births, and the pups were kept with the mother until weaning at the age of 4 weeks.

The animal experiments were authorized by the Experimental Animal Committee of Iceland and complied with animal welfare act 15/94.

Vaccines and adjuvants. Native PPS-1 was provided by Aventis Pasteur (Marcy l'Etoile, France). An experimental lot of PPS-1 conjugated to TT (Pnc1-TT) was produced by Aventis Pasteur using a conjugation technique as previously described (63). The immunogenicity of this conjugate vaccine has previously been demonstrated in adult mice (30, 31) and in human infants 70; Sigurdardottir et al., 40th ICAAC). The mutant LT-K63 was produced and purified at Chiron SpA (Siena, Italy) as already described in detail elsewhere (23).

Immunization. Neonatal (1 week old), infant (3 weeks old), and adult (6 weeks old) mice were immunized with 0.5 μg of Pnc1-TT or 5.0 μg of PPS-1 with or without 5.0 μg of LT-K63. The LT mutant was physically mixed with the vaccine antigen 1 h prior to immunization. For i.n. immunization of infant and neonatal mice, two doses of 3.0 μl of vaccine solution were slowly delivered into the nares, with 30 min between doses. Anesthesia was avoided so as to limit aspiration into the lungs. Adult mice were immunized i.n. with two doses of 5.0 μl. For s.c. immunization of neonatal, infant, and adult mice, 50, 100, and 200 μl of vaccine solution, respectively, was injected in the scapular girdle. A second dose with the same antigen dose and route was administered 2 weeks after primary immunization, where indicated. Age-matched mice injected with sterile saline were used as controls. To assess the safety of LT-K63 as adjuvant in infant and neonatal mice, weight gain was monitored weekly and compared to mice given sterile saline.

Blood and saliva sampling. Mice were bled from the tail vein weekly for 4 weeks until 2 weeks after the second immunization for measurement of anti-PPS-1 and anti-TT antibodies in serum. Saliva was collected from each mouse by the insertion of absorbent sticks (Polyfiltronics Inc., Rockland, Maine) into the mouth. After 5 min, the sticks were transferred to phosphate-buffered saline (PBS) containing 10.0 μg of protease inhibitor (aprotinin; Sigma Chemical Co., St. Louis, Mo.) per ml to prevent proteolysis. The dissolved saliva samples for each group were pooled and stored at −70°C.

ELISA. PPS-1-specific antibodies (IgM, IgG, and IgA) were measured by enzyme-linked immunosorbent assay (ELISA) as described elsewhere (30). In brief, microtiter plates (MaxiSorp; Nunc AS, Roskilde, Denmark) were coated with 10 μg of PPS (American Type Culture Collection, Rockville, Md.) per ml of PBS and incubated for 5 h at 37°C. For neutralization of antibodies to cell wall polysaccharide (Statens Serum Institute, Copenhagen, Denmark), serum or saliva samples and standard were diluted 1:50 in PBS with 0.05% Tween 20 (Sigma) and incubated in 500 μg of cell wall polysaccharide per ml for 30 min at room temperature. The neutralized sera were serially diluted and incubated in duplicates in PPS-coated microtiter plates at room temperature for 2 h. Horseradish peroxidase-conjugated goat anti-mouse IgM, IgG, or IgA antibodies (Southern Biotechnology Associates Inc., Birmingham, Ala.) were diluted 1:5,000 in PBS-Tween and incubated for 2 h at room temperature for the detection of bound antibodies. For development of the enzyme reaction, 3,3′,5,5′-tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was incubated for 10 min according to the manufacturer's instructions, and the reaction was stopped by adding 0.18 M H_2SO_4. The absorbance was measured at 450 nm in an ELISA spectrophotometer (Titertek Multiscan Plus MK II; ICN Flow Laboratories, Irvine, United Kingdom).

For detection of TT-specific antibodies, microtiter plates (MaxiSorp) were coated with 5.0 μg of purified TT (Aventis Pasteur) per ml of 0.10 M carbonate buffer (pH 9.6) and incubated overnight at 4°C. After blocking of the coated plates with PBS containing 1% bovine serum albumin (Sigma), duplicates of samples and standard were serially diluted in PBS-Tween, added to TT-coated plates, and incubated for 2 h at room temperature. The detection of TT-specific antibodies and the development of the enzyme reaction were performed as described above.

Reference serum obtained by hyperimmunizing adult mice with the same conjugate vaccine was included on each microtiter plate. The titer of the reference serum corresponded to the inverse of the serum dilution giving an optical density of 1.0. The results of the test serum samples were calculated from the reference serum and based on a minimum of four data points and parallelism between the serum samples and the reference curve. The interassay coefficient of variation was less than 10%, and the detection limit was 1.0 ELISA units (EU)/ml. Results are expressed as mean log EU/ml ± standard deviations, and the rate of responders is defined as the number of mice with IgG titers 1 log higher than those in age-matched nonimmunized control mice. 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.

Pneumococci and mouse challenge. Serotype 1 pneumococcus (ATCC strain 6301) was maintained in tryptoset broth plus 20% glycerol at −70°C. The day before challenge, stocks were plated on blood agar (Difco Laboratories, Detroit, Mich.) and incubated at 37°C in 5% CO_2 over night. Isolated colonies were transferred to a 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 the inoculum density, which was $\approx 7 \times 10^7$ CFU/ml. The challenge experiments were performed as previously described (50). Pneumococcal bacteremia was measured as CFU per milliliter of blood, whereas pneumococcal lung infection was measured as CFU per milliliter of lung homogenate. Depending on the first dilution used, the detection limit was 2.2 CFU/ml of lung homogenate and 1.3 CFU/ml of blood.

Statistical analysis. Student's *t* test was used to compare log antibody titers and numbers of CFU (log) between groups and times. A P value of <0.05 was considered statistically significant.

RESULTS

Pnc1-TT is immunogenic in infant and neonatal mice when administered s.c. and provides protection against lethal pneumococcal infections after i.n. challenge. To assess the immune responses to systemic Pnc1-TT immunization in early murine

life, neonatal (1 week old), infant (3 weeks old), and adult mice were immunized s.c. with one or two doses of Pnc1-TT. Age-matched control mice were given sterile saline or immunized with two doses of native PPS-1 for comparison. The animals were bled 4 weeks after the first immunization (i.e., 2 weeks after the second vaccine dose, when given) for measurement of PPS-1-specific IgG and IgM antibodies. Immunization with two doses of native PPS-1 induced a significant IgG response in adults ($P = 0.002$), but not in infants ($P = 0.230$) or neonates ($P = 0.910$) (Fig. 1A). In contrast, one dose of Pnc1-TT induced a significant PPS-1-specific IgG response in both neonatal ($P = 0.004$) and infant ($P = 0.008$) mice, with three of eight neonatal and five of eight infant mice reaching PPS-1-specific IgG levels higher than 1.0 log EU/ml, previously shown to protect against bacteremia in a pneumococcal infection mouse model (30, 31). A second vaccine dose further increased IgG antibody titers in both neonatal ($P = 0.003$) and infant ($P = 0.038$) mice (Fig. 1A), with six of eight neonatal and five of seven infant mice reaching a PPS-1-specific IgG response that was higher than 1.0 log EU/ml. Antibody responses were not significantly different between mice immunized as infants or neonates after either one ($P = 0.444$) or two ($P = 0.284$) doses of Pnc1-TT (Fig. 1A). However, there was a trend towards higher anti-PPS-1 IgG titers in infant mice than in neonatal mice when data from three independent experiments were pooled (data not shown). PPS-1-specific IgG responses of neonatal and infant mice remained significantly lower than those of adult mice, even after boosting 2 weeks later ($P < 0.001$). All mice immunized with Pnc1-TT showed significantly higher PPS-1-specific IgM titers than saline-injected control mice, with similar age-related differences as observed for the IgG response (data not shown).

To assess correlation between immune responses in early life and protective capacity against lethal pneumococcal infection, mice were challenged i.n. with virulent serotype 1 pneumococci 4 weeks after the first vaccine dose, and development of pneumococcal disease was evaluated as previously described (50). All age-matched saline-injected control mice developed severe bacteremia (Fig. 1B) and had high numbers (CFU) of pneumococci in their lung homogenates (Fig. 1C). Although s.c. immunization with two doses of native PPS-1 protected 50% of adult mice from bacteremia, a majority of the young mice had detectable pneumococci in the blood (Fig. 1B). Immunization s.c. with PPS-1 did not lead to bacterial clearance from the lung of either neonatal or infant mice, whereas 30% of PPS-1-immunized adults had cleared the pneumococci from their lungs 24 h after challenge. Thus, as observed in humans, PPS-1 immunization was significantly less effective in early life than in adults.

Immunization s.c. with Pnc1-TT (either one or two doses) was sufficient to protect all adult mice from bacteremia (Fig. 1B) and lung infection (Fig. 1C), which is in agreement with previous studies using the same pneumococcal conjugate vaccine in this pneumococcal infection model (30, 31). A single dose of Pnc1-TT also protected the majority of infants and neonatal mice from bacteremia (Fig. 1B), and a significant reduction in CFU per milliliter of lung homogenates was observed in both neonates ($P < 0.001$) and infants ($P < 0.001$) compared to unimmunized control mice (Fig. 1C). After two doses of Pnc1-TT, all neonatal and five of seven infant mice

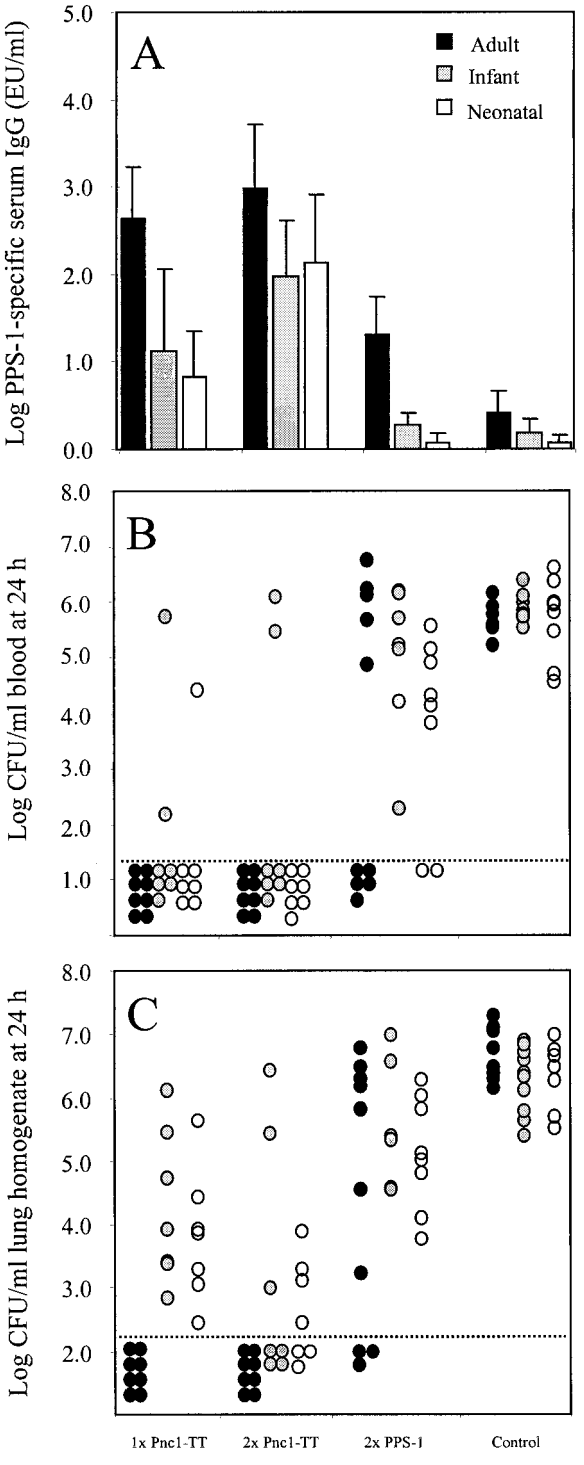

FIG. 1. Pneumococcal conjugate vaccines are immunogenic and induce protective immunity in neonatal and infant mice after s.c immunization. (A) PPS-1-specific IgG antibodies in serum 4 weeks after the first immunization with one or two doses of Pnc1-TT or PPS-1, as indicated on the *x* axis. Bars show mean antibody titers (log 10) for each group ($n = 8$) and error bars show standard deviations of the means. (B and C) For evaluation of vaccine-induced protection against lethal pneumococcal infections, mice were challenged i.n. 4 weeks after the first immunization and pneumococcal density in blood (B) and lung homogenate (C) was evaluated. One dot represents one mouse and dotted lines indicate the detection limits for CFU. The ages of the mice at administration of the first vaccine dose are indicated in the figure.

were protected from bacteremia (Fig. 1B). Furthermore, a significant reduction in CFU per milliliter of lung homogenate was observed in both neonates ($P < 0.001$) and infants ($P < 0.001$) compared to age-matched controls, as three of seven neonatal and four of seven infant mice had cleared pneumococci from their lungs 24 h after challenge (Fig. 1C).

These results show that, in contrast to PPS-1, Pnc1-TT is immunogenic and protective in both neonatal and infant mice when administered s.c. without adjuvant. However, PPS-1-specific antibody responses after s.c. immunization with Pnc1-TT in early life remain significantly lower than in adult mice, such that optimal protective efficacy requires administration of a second vaccine dose.

The nontoxic mutant LT-K63 enhances antibody responses to Pnc1-TT and protection against lethal pneumococcal infection in early murine life. As s.c. immunization with Pnc1-TT remained less effective in infant and neonatal mice than in adult mice, we investigated the possible advantage of adding an adjuvant to enhance PPS-1-specific IgG responses to Pnc1-TT and protection against lethal pneumococcal infections in this early life murine model. Neonatal, infant, and adult mice were immunized s.c. with one or two doses of Pnc1-TT and a nontoxic mutant of *E. coli* LT, LT-K63, as adjuvant. Mice were immunized s.c. with two doses of native PPS-1 and LT-K63 for comparison. To assess the safety of LT-K63 as adjuvant in infant and neonatal mice, weight gain and the number of deaths were monitored weekly and compared to age-matched mice given sterile saline. No deaths occurred, and no effect was observed on weight gain in infant and neonatal mice immunized s.c with Pnc1-TT and LT-K63 compared to unimmunized mice (data not shown), indicating that LT-K63 is safe and has a low reactogenicity profile already in early life.

Immunization s.c. with PPS-1 and LT-K63 induced significant PPS-1-specific IgG responses in adults ($P < 0.001$), but not in infants ($P = 0.347$) or neonates ($P = 0.936$) (Fig. 2A). LT-K63 did not enhance PPS-1-specific IgG responses compared to s.c. immunization with PPS-1 alone in either age group. In contrast, a significant PPS-1-specific IgG response was observed after both one and two doses of Pnc1-TT and LT-K63 for all age groups ($P < 0.001$; Fig. 2A). The IgG response after one dose of Pnc1-TT administered s.c. was comparable with or without LT-K63 in adults ($P = 0.441$) or neonates ($P = 0.343$), whereas LT-K63 enhanced the IgG antibody response in infants ($P = 0.034$). A further significant ($P < 0.001$) increase in IgG antibody titers was observed after the second dose in all age groups (Fig. 2A). Significant PPS-1-specific IgM was detected in all age groups immunized s.c. with Pnc1-TT and LT-K63, with similar age-related differences as seen for the IgG response (data not shown).

Four weeks after priming (or 2 weeks after the second immunization), mice were challenged i.n. and protection against bacteremia and lung infection was evaluated as described above. Immunization with PPS-1 and LT-K63 protected two of eight adult mice, but none of the young mice, from developing bacteremia. All neonatal and infant mice and seven of eight adults had detectable pneumococci in their lungs. One single s.c. dose of Pnc1-TT with LT-K63 protected all adult mice from bacteremia (Fig. 2B) and lung infection (Fig. 2C). In addition, all infant and seven of eight neonatal mice were protected from developing bacteremia (Fig. 2B), and a significant reduction in pneumococcal density in lungs was observed both in neonatal ($P < 0.001$) and infant ($P < 0.001$) mice, as one of eight neonatal and six of eight infant mice had completely cleared the bacteria from their lungs. Two s.c. doses of Pnc1-TT and LT-K63 successfully protected all adult, infant, and neonatal mice against both bacteremia (Fig. 2B) and lung infection (Fig. 2C).

These results demonstrate that although LT-K63 had no effect on native PPS-1 immunization, it enhanced PPS-1-specific IgG responses to a single s.c. dose of Pnc1-TT in infant mice. Most strikingly, LT-K63 enhanced PPS-1-specific IgG responses elicited by two s.c. doses of Pnc1-TT in both neonatal and infant mice, significantly improving protection against lethal pneumococcal infections in this early life pneumococcal infection model.

Intranasal immunization with a single dose of Pnc1-TT and LT-K63 is sufficient to induce protective immunity against lethal pneumococcal infections in early murine life. We previously demonstrated that i.n. immunization of adult mice with Pnc1-TT and various adjuvants provides complete protection against bacteremia and lung infection in this murine pneumococcal infection model (30, 31). To explore the potential for mucosal immunization in early murine life, neonatal and infant mice were immunized i.n., either once or twice, with Pnc1-TT with or without LT-K63 as adjuvant. Mice injected i.n. with saline were used as controls, and immunization with two doses of native PPS-1 and LT-K63 was done for comparison. To assess the safety of LT-K63 as adjuvant in infant and neonatal mice after mucosal immunization, weight gain was monitored weekly and compared to mice given sterile saline. No deleterious effect was observed on the weight gain in mice immunized i.n. with Pnc1-TT and LT-K63 compared to unimmunized mice (data not shown), and no deaths occurred, indicating the safety of LT-K63 after mucosal administration in young mice.

Intranasal immunization with native PPS-1 and LT-K63 induced a significant IgG response in adults ($P < 0.001$), but not in infants ($P = 0.251$) or neonates ($P = 0.119$) (Fig. 3A). The response was comparable to that induced by s.c. immunization with PPS-1 (with or without LT-K63) for all age groups. In contrast, one single dose of Pnc1-TT administered i.n. with LT-K63 induced a significant PPS-1-specific IgG response in all age groups tested ($P < 0.001$; Fig. 3A). Intranasal immunization of neonatal mice with Pnc1-TT and LT-K63 induced a significantly higher PPS-1-specific IgG response than s.c immunization with Pnc1-TT and LT-K63 ($P < 0.001$) (Fig. 3A and 2A). This difference was not found in infants ($P = 0.949$). A significantly enhanced response was observed after the second dose of Pnc1-TT and LT-K63 given i.n. in neonatal ($P = 0.011$), infant ($P < 0.001$), and adult ($P < 0.001$) mice. Whereas i.n. immunization with two doses of Pnc1-TT and LT-K63 elicited significantly higher PPS-1-specific IgG levels than two s.c. doses of Pnc1-TT alone in all age groups ($P < 0.001$), i.n. and s.c. immunization with two doses of Pnc1-TT and LT-K63 induced comparable PPS-1-specific IgG levels, which is seen by comparing Fig. 2A and 3A. PPS-1-specific IgG titers after i.n. immunization with either one or two doses of Pnc1-TT and LT-K63 was significantly higher in adult than in infant ($P < 0.001$) and neonatal ($P < 0.001$) mice. A significant PPS-1-specific IgM level was detected in all age groups immu-

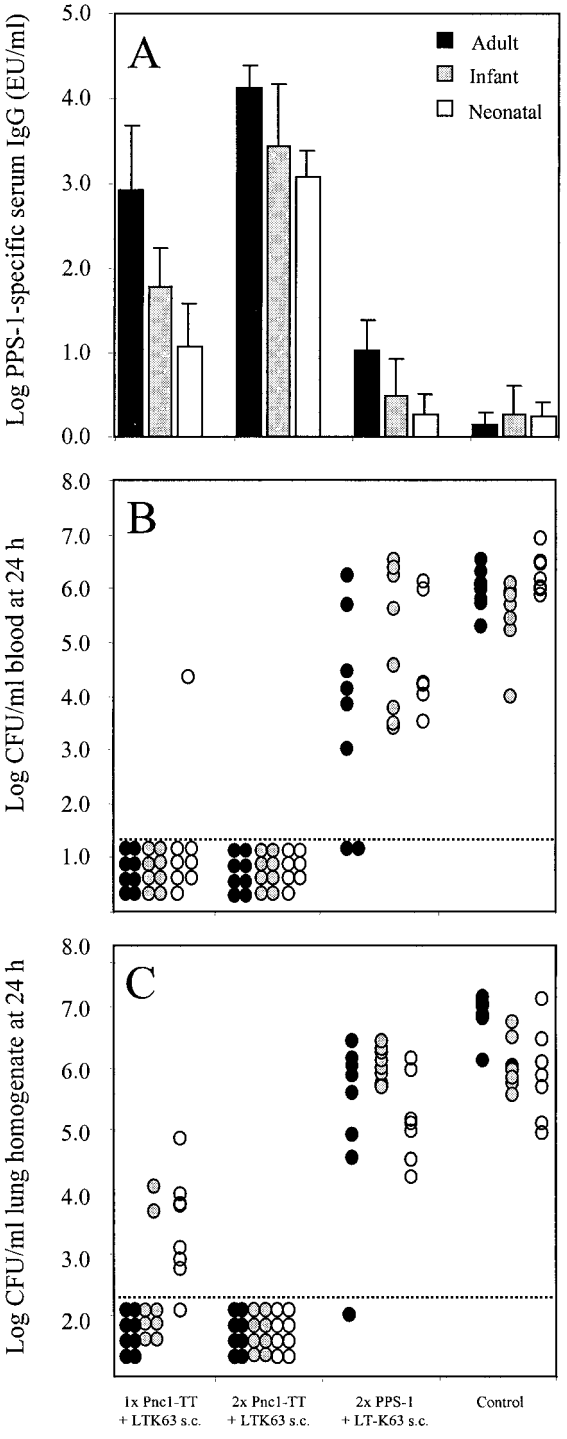

FIG. 2. LT-K63 enhances PPS-1-specific antibody responses to Pnc1-TT in neonatal and infant mice when administered parenterally and improves protection against lethal pneumococcal infections. (A) PPS-1-specific IgG antibodies in serum 4 weeks after the first s.c. immunization with one or two doses of Pnc1-TT or PPS-1 with LT-K63 as indicated on the *x* axis. Bars show mean antibody titers (log 10) for each group ($n = 8$) and error bars show standard deviations of the means. (B and C) For evaluation of vaccine-induced protection against lethal pneumococcal infections, mice were challenged i.n. 4 weeks after the first immunization and pneumococcal density in blood (B) and lung homogenate (C) was evaluated. One dot represents one mouse and dotted lines indicate the detection limits for CFU. The ages of mice at administration of the first vaccine dose are indicated in the figure.

nized i.n. with Pnc1-TT and LT-K63, with similar age-related differences as observed for the IgG response (data not shown).

To evaluate vaccine-induced protection after i.n. immunization with Pnc1-TT and LT-K63, mice were challenged i.n. as described above, and pneumococcal density in blood and lungs was evaluated 24 h after challenge. A high density of pneumococci was detected both in blood (Fig. 3B) and lung homogenates (Fig. 3C) from unimmunized control mice of all age groups. Whereas i.n. immunization with two doses of PPS-1 and LT-K63 protected 50% of adult mice from bacteremia, a majority of infant and neonatal mice immunized i.n. with PPS-1 and LT-K63 had pneumococci in their blood (Fig. 3B) and lungs (Fig. 3C). In contrast, i.n. immunization with one or two doses of Pnc1-TT and LT-K63 protected all adult, infant, and neonatal mice from developing bacteremia (Fig. 3B). Remarkably, a single i.n. dose of Pnc1-TT and LT-K63 induced complete clearance of bacteria from lungs of all adults, seven of eight infants, and five of eight neonates, whereas two doses induced complete clearance of pneumococci from lungs of all mice in all age groups (Fig. 3C).

Pnc1-TT or PPS-1 administered i.n. without adjuvant (either one or two doses) did induce significant PPS-1-specific serum IgG in adults ($P < 0.001$), but not in infants or neonates (data not shown). Immunization i.n. with Pnc1-TT alone also did not induce significant protection against lethal pneumococcal infections in neonatal or infant mice, whereas a reduction in CFU in blood ($P < 0.001$) and lungs ($P = 0.031$) was observed in adults immunized i.n. with two doses of Pnc1-TT without adjuvant (data not shown).

These results show that i.n. immunization with Pnc1-TT and LT-K63 induces a systemic PPS-1-specific IgG antibody response in neonatal and infant mice which is significantly higher than the response elicited by s.c. immunization with Pnc1-TT without adjuvant and at least as good as that elicited by s.c. injection with Pnc1-TT and LT-K63. Furthermore, i.n. immunization of neonatal and infant mice with a single dose of Pnc1-TT and LT-K63 was sufficient to induce complete protection against lethal pneumococcal disease after i.n. challenge in this early life mouse model.

i.n. immunization with Pnc1-TT and LT-K63 induces a strong salivary IgA response in neonatal and infant mice. Mucosal administration in mice of pneumococcal conjugate vaccines along with potent adjuvants has been shown to be efficient in inducing a mucosal antibody response in adult mice (30, 31). To investigate whether intranasal immunization with Pnc1-TT and LT-K63 induces a PPS-1-specific mucosal antibody response in neonatal and infant mice, saliva was sampled and PPS-1-specific IgA and IgM antibodies were measured with ELISA. As shown in Fig. 4, i.n. immunization with Pnc1-TT and LT-K63 induced a vigorous salivary IgA response in all age groups, whereas s.c. immunization with Pnc1-TT with or without LT-K63 did not. Due to volume limitation, saliva samples were pooled for each group, preventing statistical analysis. PPS-1-specific IgM antibodies were not detectable in the saliva samples (data not shown).

Intranasal immunization with Pnc1-TT and LT-K63 is optimal for rapidly enhancing PPS-1-specific antibody response in neonatal mice. As pneumococcal colonization and infection may occur very early in life, we next compared the kinetics of the IgG response to the PS and carrier moiety of pneumococ-

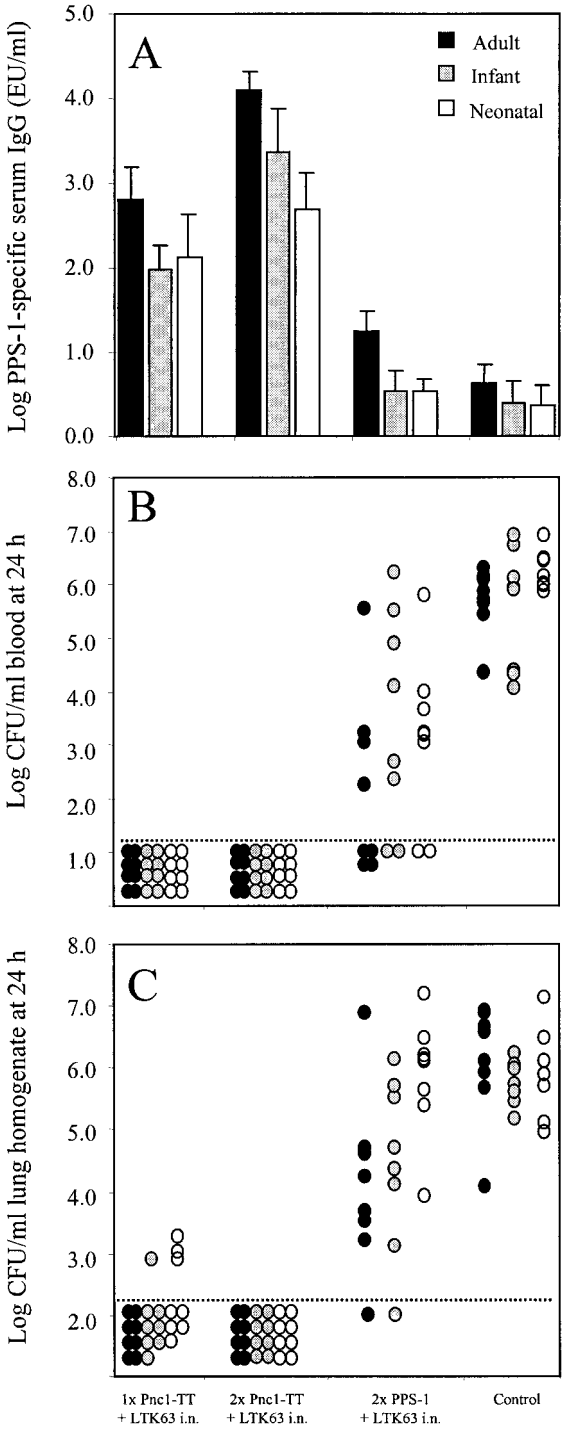

FIG. 3. i.n. immunization with one single dose of Pnc1-TT and LT-K63 is sufficient to induce protective immunity against lethal pneumococcal infections in infant and neonatal mice. (A) PPS-1-specific IgG antibodies in serum 4 weeks after the first immunization with one or two i.n. doses of Pnc1-TT or PPS-1 and LT-K63, as indicated on the *x* axis. Bars show mean antibody titers (log 10) for each group ($n = 8$) and error bars show standard deviations of the means. (B and C) For evaluation of vaccine-induced protection against lethal pneumococcal infections, mice were challenged i.n. 4 weeks after the first immunization and pneumococcal density in blood (B) and lung homogenate (C) was evaluated. One dot represents one mouse and dotted lines indicate the detection limits for CFU. The ages of mice at administration of the first vaccine dose are indicated in the figure.

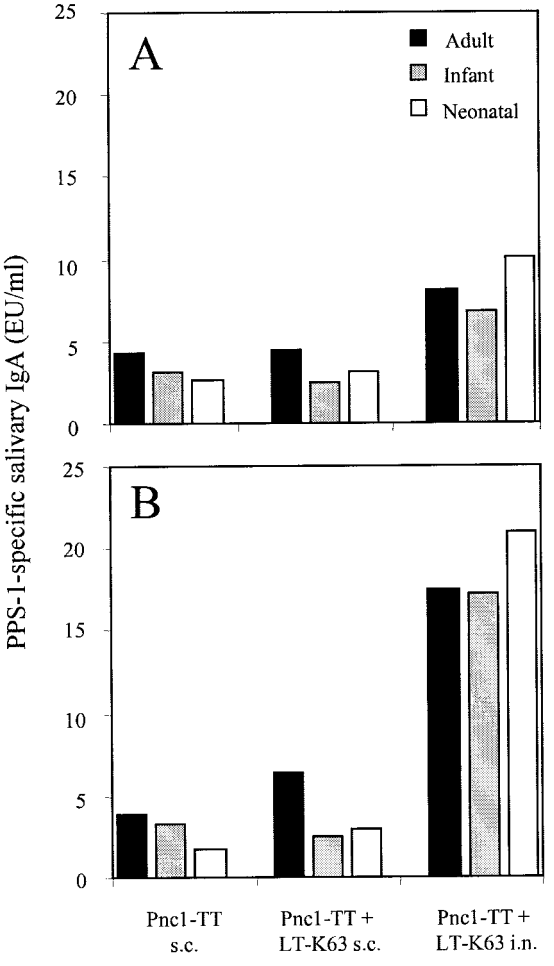

FIG. 4. i.n. immunization with Pnc1-TT and LT-K63 induces a vigorous mucosal IgA response in neonatal and infant mice. Mice were immunized once (A) or twice (B) with Pnc1-TT alone or with LT-K63 as indicated on the *x* axis. Saliva was collected 4 weeks after the first immunization and PPS-1-specific IgA antibodies were measured in saliva samples by ELISA. The ages of mice at the first immunization are indicated in the figure.

cal conjugate vaccines after either parenteral or mucosal immunization with Pnc1-TT with or without LT-K63 as adjuvant. Neonatal, infant, and adult mice were immunized either s.c. or i.n. with Pnc1-TT with or without LT-K63, and blood was sampled weekly for 4 weeks to measure TT- and PPS-1-specific IgG antibodies in the serum samples (Fig. 5). In adult mice, responses to both PPS-1 and TT after one single dose of Pnc1-TT followed similar kinetics regardless of immunization route or use of the LT-K63 adjuvant (Fig. 5A). In infant mice, both PPS-1 and TT-specific responses elicited by Pnc1-TT and LT-K63, either s.c. or i.n., were higher than those elicited by Pnc1-TT alone (Fig. 5A). The situation was different following immunization of neonatal mice, where mucosal administration of a single dose of Pnc1-TT and LT-K63 elicited optimal PPS-1 IgG responses (Fig. 5A). This superiority of mucosal immunization was not observed for responses to the TT carrier moiety, as both mucosal and s.c. immunization with Pnc1-TT and LT-K63 elicited similarly strong TT-specific IgG responses. Administration of a second vaccine dose enhanced PPS-1 re-

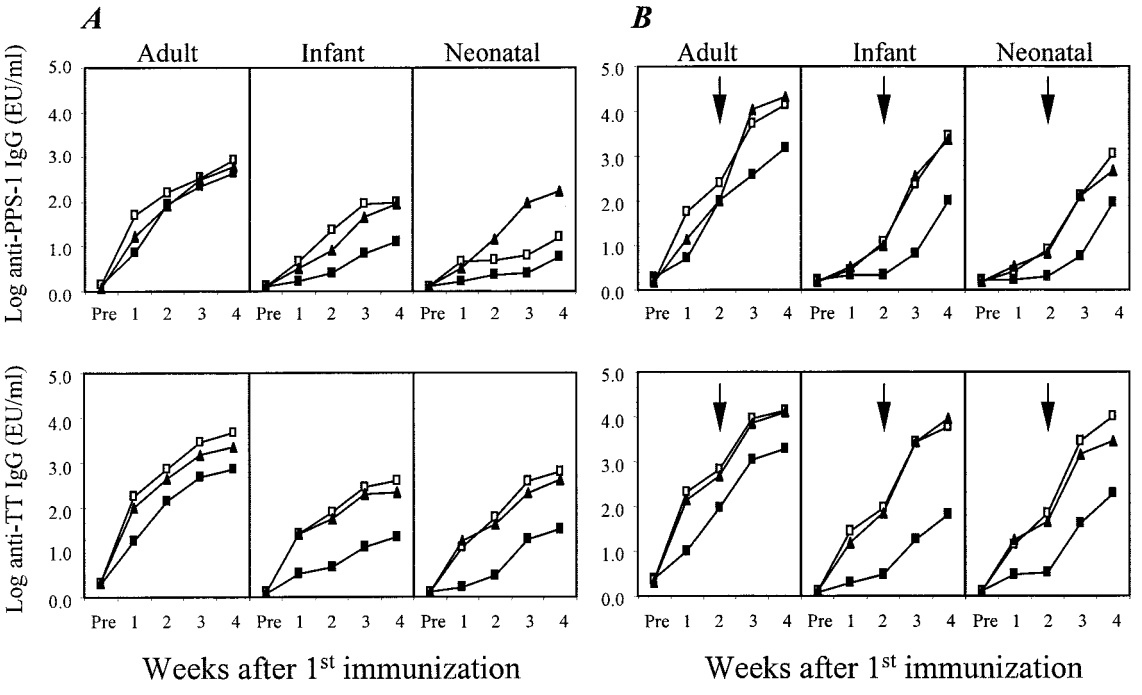

FIG. 5. i.n. immunization with Pnc1-TT and LT-K63 is optimal for rapidly enhancing antibody responses to the PS moiety of Pnc1-TT in neonatal mice. Adult, infant, and neonatal mice were immunized either once (A) or twice (B) with Pnc1-TT i.n. or s.c., with or without LT-K63. Anti-PPS-1 and anti-TT IgG antibodies were measured in serum samples taken weekly for 4 weeks after the first immunization. Modes of immunization are as follows: Pnc1-TT s.c. without adjuvant (■), Pnc1-TT s.c. with LT-K63 (□), Pnc1-TT i.n. with LT-K63 (▲). The time at secondary immunization is indicated by arrows.

sponses elicited by Pnc1-TT and LT-K63 in all age groups, regardless of the immunization route (Fig. 5B).

Thus, either i.n. or s.c. immunization with Pnc1-TT and LT-K63 elicit similar PPS-1 responses in infant mice, while mucosal immunization appears superior to s.c. immunization for the induction of primary PPS-1-specific IgG responses in neonates.

DISCUSSION

In this study, a new murine infant immunization model against *S. pneumoniae* which reproduces the main features of human infant immune responses to native capsular PPS and pneumococcal conjugate vaccines, as well as the relative protective efficacy of each vaccine, was established. Using this early life murine model, we show that protection against *S. pneumoniae* can be significantly enhanced by either parenteral or mucosal immunization with a pneumococcal conjugate vaccine and LT-K63 as adjuvant. This strategy could be of particular interest for early life immunization, as neonatal mice were only fully protected against *S. pneumoniae* following parenteral or mucosal administration of a conjugate vaccine with LT-K63. Studies on immunogenicity and protective capacity of additional serotypes of pneumococcal conjugate vaccines are presently under investigation.

IgG antibodies to the capsular PS of *S. pneumoniae* mediate opsonophagocytosis of the bacteria, which plays a major role in protecting the host from pneumococcal disease (37, 67). However, infants and young children, who are in the major risk group for pneumococcal infections, fail to respond to PPS vaccination with sufficient levels of IgG antibodies. This characteristic feature was also observed in our new early life murine model of PPS immunization: PPS-1-specific IgG responses in early murine life remained significantly weaker than in adult mice and were associated with poor protection against *S. pneumoniae* infection. In contrast, immunization with pneumococcal conjugate vaccines elicits a T-cell-dependent (TD) antibody response characterized by higher levels of PPS-specific IgG1 antibodies in both humans (68) and mice (29, 48). In the present study, we demonstrate that an experimental pneumococcal conjugate vaccine, Pnc1-TT, is immunogenic in both infant and neonatal mice after s.c. immunization. However, the early life antibody response to Pnc1-TT remained significantly lower in young than in adult mice. These observations are in agreement with results from previous studies analyzing antibody responses to various TD antigens induced by vaccination in the neonatal period, which differ from those of adults (56). Low and weak antibody responses to TD antigens early in life may be related to immaturity of antigen-presenting cells (38) and their inability to produce interleukin-12 (IL-12) (24). In addition, we have shown that the induction of antibody-secreting cells is delayed and limited in early life (43). Our recent studies suggest that this reflects limitations in germinal center induction following early life immunization with TD antigens (C.A. Siegrist et al., unpublished data). Additional factors may contribute to the low immunogenicity of PS antigens in infancy, such as low expression of complement receptor 2 on B cells (25) and immaturity of marginal zone B cells, which are considered to be crucial in the antibody response to PS antigens (26, 60). Interestingly, immunization of infants with pneu-

mococcal conjugate vaccines was recently shown to have a stronger protective efficacy against bacteremia and invasive diseases than against pneumonia (54). This feature was also observed in this early life murine immunization model, as parenteral administration of Pnc1-TT protected the majority of the young mice against bacteremia and induced a significant reduction of lung bacterial titers without, however, achieving bacterial clearance (Fig. 1B and C).

It has been indicated that mucosal immune responses, especially IgA antibodies in secretions, may contribute significantly to protection against respiratory pathogens (62). Intranasal administration of vaccines along with potent adjuvants, such as nontoxic derivatives of *E. coli* LT, is effective in inducing vaccine-specific immune responses (14, 45, 46). Numerous studies have shown that mucosal immunization with bacterial, viral, and parasitic antigens along with LT mutants induces protective immunity in various adult murine infection models (8, 31, 33, 49, 64). LT is also a potent adjuvant when given parenterally, possibly as a result of its direct and indirect effects on immunocompetent cells, causing increased expression of costimulatory molecules both on B and T cells, which subsequently leads to enhanced antigen presentation and B-cell–T-cell interactions (39, 69, 71). It is reasonable to believe that use of a fully nontoxic mutant, such as LT-K63, will have a minimum risk of adverse events (13). However, it is presently unknown whether LT-K63 will be tolerable in human infants, but clinical trials for i.n. delivery of LT-K63 are planned in order to evaluate safety in adults and eventually in the pediatric population. In the present study, LT-K63 had no deleterious effect on weight gain and did not cause any deaths in either neonatal or infant mice, thus demonstrating its low reactogenicity in early life. This is consistent with previous reports demonstrating the undetectable toxicity of LT-K63 both in vitro and in vivo (23). Parenteral immunization with Pnc1-TT and LT-K63 significantly enhanced PPS-1-specific antibody responses and protection against lethal pneumococcal infections in infant and neonatal mice compared to immunization with Pnc1-TT alone. This indicates that LT-K63 is a safe and potent adjuvant for early life parenteral immunization with this pneumococcal conjugate vaccine. Whether this strong early life adjuvanticity will be observed following immunization with other TD vaccines is presently under investigation.

In addition, mucosal immunization with Pnc1-TT and LT-K63 proved capable of inducing both strong systemic and mucosal PPS-1-specific antibody responses. Remarkably, one single i.n. dose of Pnc1-TT and LT-K63 was sufficient to protect all infant and neonatal mice from lethal pneumococcal infection, resulting in complete bacterial clearance from both blood and lungs, whereas two vaccine doses were required when Pnc1-TT and LT-K63 were given s.c. This enhanced efficacy of Pnc1-TT following mucosal rather than parenteral immunization could reflect the contribution of strong mucosal IgA responses (Fig. 4). However, enhanced systemic PPS-1-specific IgG antibody responses may also significantly contribute to rapid bacterial clearance. Interestingly, mucosal immunization of neonatal mice with one single dose of Pnc1-TT and LT-K63 induced a significantly higher PS-specific IgG response than s.c. immunization (Fig. 5), whereas TT-specific IgG responses were similar. The mechanisms responsible for this optimal induction of PPS-specific IgG responses and the effect on long-term protection following mucosal administration remain to be investigated. Although antibody responses remained slightly lower than in adults during the first 2 weeks after immunization, mucosal administration of conjugate vaccines along with LT-K63 partly circumvented the limitations of antibody responses to PS antigens that are responsible for enhanced susceptibility of neonates and infants to pneumococcal infections. This suggests that mucosal immunization in the neonatal period could be a particularly attractive approach for early life protection against pneumococcal disease.

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