Immunization with Recombinant *Streptococcus pneumoniae* Neuraminidase NanA Protects Chinchillas against Nasopharyngeal Colonization

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**Immunization with recombinant S. pneumoniae neuraminidase NanA (rNanA) resulted in a significant reduction in pneumococcal colonization in the chinchilla model. The bacteria were eliminated from the nasopharynx 1 week earlier than that from the control cohort. Our data suggest that rNanA affords protection against pneumococcal nasopharyngeal colonization.**

*Streptococcus pneumoniae* is the most frequent cause of otitis media (OM) in children (18). It has been well documented that nasopharyngeal (NP) colonization is prerequisite for the development of OM. The nasopharynx serves as the reservoir for *S. pneumoniae*, which enters the middle ear via the eustachian tube from the nasopharynx, particularly during a viral infection. Pneumococci are frequently isolated from the nasopharynx, particularly during a viral infection. Pneumococci are isolated from the nasopharynx 1 week earlier than that from the control cohort. Our data suggest that rNanA affords protection against pneumococcal nasopharyngeal colonization.

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concentrations were 6.5 \times 10^3 CFU/ml on Columbia agar plates at 37°C with 5% CO₂, and the number of cells for mid-ear and nasal lavage samples were cultured overnight on postinoculation with 6A as previously described (28). The mid-ear and nasal lavage on days 1, 3, 7, 10, 14, 21, and 28 presenelected and randomized, were evaluated by tympanocen-

Serum antibody response determined by ELISA and Western blotting. Titters of antibody against rNanA were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (7). Each well was coated with 0.5 μg purified rNanA. Whole S. pneumoniae 6A was also used as the solid-phase antigen to determine the antibody titer to S. pneumoniae 6A as previously described (19). Western blottings were also performed, using purified rNanA (2 μg per lane) as previously described (17).

Assessment of NP colonization and the development of OM after i.n. challenge with S. pneumoniae. Fourteen days following the final immunization with rNanA, both the experimental and sham-immunized control groups were inoculated i.n. with 5 \times 10^7 CFU of S. pneumoniae 6A in a volume of 0.2 ml as previously described (17). Five chinchillas from each cohort, preselelected and randomized, were evaluated by tympanocentesis and nasal lavage on days 1, 3, 7, 10, 14, 21, and 28 postinoculation with 6A as previously described (28). The middle ear and nasal lavage samples were cultured overnight on Columbia agar plates at 37°C with 5% CO₂, and the number of CFU per milliliter was determined by a standard dilution assay and plate counting. Animals with significant tympanic membrane inflammation and middle ear fluid were considered to have developed OM as described previously (28).

Statistical analysis. Data are expressed as medians ± standard errors. Bacterial culture results below the detection limit of the viable-cell-count assay (10 CFU/ml) were ascribed values just below the detection limit (9 CFU/ml). Differences in S. pneumoniae concentrations in nasal and middle ear lavage samples between the cohorts immunized with rNanA and those treated with the buffer alone were analyzed by use of the Mann-Whitney rank sum test. Differences in the incidences of OM were analyzed by use of chi-square or Fisher-exact analysis. A P of <0.05 was set as the level of significance.

Effect of rNanA immunization on the kinetics of NP colonization. The relative ability of S. pneumoniae to colonize and persist in the nasopharynx for up to 28 days after i.n. challenge is shown in Fig. 1. There was a statistically significant reduction in the bacterial concentration in the rNanA-immunized cohort compared with that in the control group on days 1, 3, 7, and 10 postinoculation. By day 1, the median concentrations of S. pneumoniae (CFU/ml) for nasal-lavage samples were 8.0 \times 10^6 and 1.1 \times 10^7 for animals immunized with rNanA and sham-immunized controls, respectively (P = 0.016). By day 3, concentrations were 6.5 \times 10^6 and 1.7 \times 10^6 (P = 0.008); by day 7, 5.0 \times 10^6 and 3.5 \times 10^6 (P = 0.008); and by day 10, 7.6 \times 10^5 and 2.1 \times 10^5 (P = 0.008). Pneumococci were eliminated from three of the five chinchillas in the rNanA-immunized group by day 14, whereas the bacteria persisted in the naso-

Effect of rNanA immunization on the development of OM. OM developed during the first week after i.n. inoculation of S. pneumoniae (Fig. 2). Two chinchillas from each cohort developed OM on day 1; however, six chinchillas (nine ears infected) from the sham-inoculated control cohort developed OM compared with two chinchillas (two ears infected) in the rNanA-immunized cohort on days 3 and 7 after i.n. challenge (P = 0.034, based on the number of ears infected). Overall, immunization with rNanA resulted in a 50% reduction in the incidence of OM relative to that in sham-immunized controls. In the cohort receiving rNanA, only 4 of 35 chinchillas (11.4%) developed OM. In contrast, for sham-immunized animals, 8 of 35 chinchillas (22.9%) developed OM. However, there was no statistically significant difference in the incidences of OM between these two cohorts.

The median concentrations of S. pneumoniae cells for middle ear lavage samples from all animals in each cohort with positive bacterial culture were 2.4 \times 10^4 and 1.1 \times 10^5 for animals immunized with rNanA and sham-immunized controls, respectively (P = 0.045).

Analysis of chinchilla serum antibody response. The sera were analyzed for anti-rNanA by ELISA. Chinchillas immunized with rNanA exhibited a strong specific antibody response to rNanA and demonstrated a reciprocal titer of \geq 10^6 against
rNanA by 2 weeks after the final boost immunization, and the level of specific serum antibody to rNanA was maintained up to day 28 postinoculation with S. pneumoniae 6A. Serum collected from animals prior to the start of the experiment (prebleeds) as well as serum from sham-inoculated controls contained no detectable activity when tested at a 1:50 dilution. These data indicate that a higher anti-rNanA antibody titer in the immunized cohort may contribute to the lower level of pneumococcal NP colonization than that of the controls.

Antiserum generated in chinchillas immunized with rNanA recognized the 100-kDa protein band associated with neuraminidase enzymatic activity in Western blots (data not shown), and titers for antiserum against rNanA were typically in excess of 1:100,000. Serum collected from animals prior to the start of the experiment (prebleeds) as well as serum from sham-inoculated controls contained no detectable activity when tested at a 1:100 dilution.

In order to rule out the possibility that an anti-S. pneumoniae 6A antibody response induced subsequent to the i.n. challenge may be responsible for pneumococcal clearance from the nasopharynx, serum antibody against whole S. pneumoniae 6A was measured. Antibody directed against S. pneumoniae 6A was not detectable at the first week postinoculation and was detected only with low reciprocal titers (100 to 200) by days 14, 21, and 28 postinoculation in both the immunized and control cohorts. There was no correlation between this antibody titer and the level of pneumococcal colonization in both the rNanA-immunized and the sham-immunized control cohort (Fig. 3).

Two open reading frames in S. pneumoniae are known to produce proteins with neuraminidase activity. Both nanA and nanB have been cloned and sequenced (2, 6). The activity of NanA is approximately 100 times higher than that of NanB against the fluorogenic substrate (2). NanA contains an LPXTG in the C terminus and is linked covalently to the cell surface in a sortase-dependent manner (22). Moreover, nanA expression is correlated to S. pneumoniae opacity phenotypes. Compared to what occurs in opaque variants, up-regulated nanA expression and increased NanA activity were observed in S. pneumoniae transparent variants that are selected for in vivo during NP colonization (12).

We have proposed that NanA is involved in pneumococcal NP colonization in the chinchilla OM model (27). A recent study by Orihuela et al. demonstrates that NanA contributes to colonization of the nasopharynx and to the spread of pneumococci from the nasopharynx to the lungs in a mouse model (21). In contrast to these results, King et al. (12) reported that NanA does not appear to contribute to pneumococcal adherence to epithelial cells in vitro or to colonization in an infant rat model. However, they observed that the NanA-dependent desialylation of human airway glycoproteins, which bind to S. pneumoniae, may mediate bacterial clearance (12).

Despite the contradictory data regarding the role of NanA in NP colonization in different animal models, the investigation of NanA as a protective antigen and a protein-based vaccine candidate warrants further investigation (11, 23, 26). In an earlier study, native pneumococcal neuraminidase was evaluated as a protective antigen in an S. pneumoniae intraperitoneal infection model using mice (16). A comparison between neuraminidase and pneumolysin revealed that although inoculation with purified neuraminidase afforded significant protection against systemic infection and death relative to what occurred in sham-inoculated controls, it was not as effective as pneumolysin. Furthermore, immunization with neuraminidase did not prevent mortality following intraperitoneal inoculation with S. pneumoniae. Our previous studies, however, indicate that the role of neuraminidase as a protective antigen appears to be anatomically niche specific and that it induces significant protection against pneumococcal OM (17). An antecedent influenza A virus infection was used in combination with S. pneumoniae to induce OM in chinchillas during these studies. Influenza A virus plays a significant role in the increased pneumococcal NP colonization and incidence of OM, and this combined infection model is well established (28). Influenza virus neuraminidase, however, has been suggested to be an
important factor in inducing viral-bacterial synergistic effects (24) and may have masked the full effect of immunization with rNanA by removing the sialic acid substrate prior to challenge with S. pneumoniae. In the present study, we evaluated rNanA as a protective antigen against pneumococcal NP colonization and the development of OM without a concurrent influenza A virus infection in order to define the role of antineuraminidase antibody against S. pneumoniae alone. Our data show that pneumococci colonized the nasopharynx at a statistically significant lower level in the rNanA-immunized cohort than in the controls during the 28-day observation period. In addition, results from the present study indicate that specific immune responses against rNanA were associated with decreased duration of nasopharyngeal colonization. However, the bacteria were not completely eliminated until day 21 postinoculation, which may suggest the relatively subtle effects of rNanA immunization in this animal model. Pneumococcal virulence factors other than NanA contributing to NP colonization may be responsible for the bacterial persistence at the nasopharynx. Data from the present study strongly suggest that anti-rNanA antibody may enhance host clearance or inhibit pneumococcal colonization. We have shown that NanA has a significant impact on the changes of the carbohydrate moieties in the eustachian tube epithelium and may be responsible for an increased ability to colonize the nasopharynx and invade the middle ear (30). However, the mechanisms responsible for the effects of NanA need to be further explored. In conclusion, our results demonstrate the potential of NanA as a protective immunogen for pneumococcal diseases.

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REFERENCES


