INFECTION AND IMMUNITY, July 2006, p. 4014–4020 Vol. 74, No. 7
0019-9567/06/$08.00+0 doi:10.1128/IAI.01237-05

Pneumococcal Neuraminidases A and B Both Have Essential Roles during Infection of the Respiratory Tract and Sepsis

Sonia Manco,[1] Fidelma Hernon,[1] Hasan Yesilkaya,[1] James C. Paton,[2] Peter W. Andrew,[1] and Aras Kadioglu[1]*

Department of Infection, Immunity and Inflammation, University of Leicester, Leicester LE1 9HN, United Kingdom,[1] and School of Molecular and Biomedical Science, University of Adelaide, Adelaide 5005, Australia[2]

Received 1 August 2005/Returned for modification 11 November 2005/Accepted 8 March 2006

We examined the role of the neuraminidases NanA and NanB in colonization and infection in the upper and lower respiratory tract by *Streptococcus pneumoniae*, as well as the role of these neuraminidases in the onset and development of septicemia following both intranasal and intravenous infection. We demonstrated for the first time using outbred MF1 mouse models of infection that both NanA and NanB were essential for the successful colonization and infection of the upper and lower respiratory tract, respectively, as well as pneumococcal survival in nonmucosal sites, such as the blood. Our studies have shown that in vivo a neuraminidase A mutant is cleared from the nasopharynx, trachea, and lungs within 12 h postinfection, while a neuraminidase B mutant persists but does not increase in either the nasopharynx, trachea, or lungs. We also demonstrated both neuraminidase mutants were unable to cause sepsis following intranasal infections. When administered intravenously, however, both mutants survived initially but were unable to persist in the blood beyond 48 h postinfection and were progressively cleared. The work presented here demonstrates the importance of pneumococcal neuraminidase A and for the first time neuraminidase B in the development of upper and lower respiratory tract infection and sepsis.

Streptococcus pneumoniae is a principal human pathogen responsible for respiratory tract infections, septicemia, and meningitis. The pneumococcus produces several virulence factors that are of importance in the pathogenesis of disease. Neuraminidase is believed to be one of these and is produced by all clinical isolates of *S. pneumoniae* (12, 18). The interpretation of the role of neuraminidase in pneumococcal disease is complicated by the fact that the pneumococcus produces two distinct neuraminidases, NanA and NanB (1, 4). Both neuraminidases have typical signal peptides for export; however, NanA, unlike NanB, contains a C-terminal cell surface anchorage domain (14). Both enzymes are thought to cleave sialic acid from cell surface glycans and mucin, thereby promoting the colonization of the upper respiratory tract by exposing host cell surface receptors for pneumococcal adherence (19). The two neuraminidases have different pH optima (NanA at pH 6.5 and NanB at pH 4.5), suggesting distinct roles for these enzymes in vivo (3). To date, the contributions of these two neuraminidases to pathogenesis at different in vivo sites are still unclear. Indeed, there are several conflicting publications on the precise role of NanA in pneumococcal disease, whereas the relative contribution of NanB to disease has not been reported in either a sepsis or pneumonia model.

Insofar as the role of NanA in virulence has been studied, a model of otitis media in the chinchilla has shown that NanA-deficient pneumococci are significantly less able to colonize and persist in the nasopharynx and middle ear than NanA-sufficient wild-type pneumococci (23). In addition, immunization with purified NanA has been shown to significantly reduce nasopharyngeal colonization, as well as the incidence of otitis media by pneumococci (15, 25). Furthermore, studies using in vitro adherence assays have shown that NanA-deficient pneumococci are significantly less able to adhere to chinchilla tracheal epithelium than wild-type pneumococci (24). In a previous study using BALB/c mice challenged via the intraperitoneal route with either wild-type or NanA-deficient pneumococci, no significant differences in either the median survival time or overall survival rate were observed (3), suggesting that NanA has a minimal effect upon pathogenesis in this particular infection model. Recent work in an infection model also using BALB/c mice has shown that numbers of NanA-deficient pneumococci are significantly reduced in both the nasopharynx and lungs compared to wild-type pneumococci following intranasal challenge but, interestingly, not after intraperitoneal, intratracheal, or intravenous challenge (17). Taken together, these studies suggest a host tissue/environment-specific role for NanA during pneumococcal virulence in vivo.

None of these studies, however, examined the contribution of NanB to pneumococcal virulence. In our present study, we not only investigated the role of NanA but also the role of NanB in the colonization and infection of both the upper and lower respiratory tract, as well as their role in the onset and development of septicemia following both intranasal and intravenous infection in well-established mouse models of infection.

MATERIALS AND METHODS

Bacteria. *Streptococcus pneumoniae* serotype 2 strain D39 was obtained from the National Collection of Type Cultures, London, United Kingdom (NCTC 7466). The neuraminidase A-deficient mutant (NanA$^-$) was made by insertion duplication mutagenesis. In brief, part of the *nanA* gene (HindIII nucleotide [nt] 2449 to SphI nt 3090 fragment) was cloned into pVA891, and the construct was transformed into strain D39. A single crossover mutation occurred which incor-

* Corresponding author. Mailing address: Department of Infection, Immunity and Inflammation, University of Leicester, P.O. Box 138, University Rd., Leicester LE1 9HN, United Kingdom. Phone: 44 116 2231556. Fax: 44 116 2525030. E-mail: ak13@le.ac.uk.

porated the entire plasmid, thereby disrupting the *nanA* gene and rendering the recombinant erythromycin resistant (3). Analysis of NanA$^-$ by Southern blotting confirmed that there was an insertion mutation in the *nanA* gene (3). The neuraminidase B-deficient mutant (NanB$^-$) is a deletion mutant that is an unmarked in-frame deletion of amino acids 58 to 660, constructed using overlap extension PCR, essentially as previously described (2). Bacteria were identified as pneumococci prior to experiments by Gram stain, catalase test, α-hemolysis on blood agar plates, and by optochin sensitivity. The capsular polysaccharide serotypes were confirmed by the Quellung reaction, and no difference in capsule formation between the D39 wild type or its isogenic neuraminidase A or B mutants was evident.

To standardize virulence of pneumococci, bacteria were passaged through mice as described previously (5) and subsequently recovered and stored at −70°C. When required, suspensions were thawed at room temperature and bacteria were harvested by centrifugation before resuspension in sterile phosphate-buffered saline (PBS).

Neuraminidase assay. In vivo-passaged and in vitro-grown wild-type, NanA$^-$, and NanB$^-$ pneumococci were assayed for neuraminidase activity. Bacteria were centrifuged at 13,000 rpm for 1 min, and pellets were resuspended in 250 μl 40 mM Tris (pH 7.4) to 10^7 CFU/ml. Bacterial suspensions were then sonicated on ice using a Sanyo Soniprep model 150 sonicator (amplitude, 8 μm). Cells were sonicated for 15 seconds followed by a 45-second interval; this process was repeated for a total of 8 min. The samples were then centrifuged at 13,000 rpm for 1 min, and the supernatant was kept on ice until assayed. A quantitative assay utilizing 2-*O*-(*p*-nitrophenyl)-α-D-*N*-acetylneuraminic acid (pNP-NANA; Sigma, United Kingdom) was used to assay neuraminidase activity. Aliquots of 25 μl of samples were added in triplicate to each well of a 96-well plate, and 25 μl of 0.3 mM pNP-NANA in dilution buffer at pH 6.6 was then added to the wells. The microplate was incubated statically for 2 h at 37°C before the reaction was stopped by adding to each well 100 μl ice-cold 0.5 M Na_2CO_3. The absorbance at 405 nm was determined in an MRX ELISA plate reader (Dynatech Laboratories). A reaction blank containing 25 μl of 40 mM Tris instead of the sample was included. To determine the activity of the neuraminidase, a standard curve was prepared using known concentrations of *p*-nitrophenol (Sigma).

Infection of mice. Female outbred MF1 mice were used. All mice were 8 to 10 weeks old when infected and weighed 30 to 35 g (Harlan, Bichester, United Kingdom). These mice did not have detectable levels of anti-type 2 antibodies present in their serum (data not shown). As described before (10), the mice were lightly anesthetized with 2.5% (vol/vol) fluothane (AstraZeneca, Macclesfield, United Kingdom) over oxygen (1.5 to 2 liter/min), and 50 μl PBS containing 1×10^6 CFU *S. pneumoniae* was then administered into the nostrils of the mice. The inoculum dose was confirmed by viable count after plating on blood agar plates following infection. Intravenous infections were administered as 1×10^5 CFU of *S. pneumoniae* via the dorsal tail vein. The inoculum was confirmed by plating on blood agar as above.

At prechosen time intervals following infection, groups of mice were deeply anesthetized with 5% (vol/vol) fluothane and blood was collected by cardiac puncture. Immediately afterwards, the mice were killed by cervical dislocation, and the lungs, trachea, and nasopharynx were removed separately into 10 ml of sterile PBS, weighed, and then homogenized in a Stomacher-Lab blender (Seward Medical, London, United Kingdom). Viable counts in homogenates and blood were determined by serial dilution in sterile PBS and plating onto blood agar plates (Oxoid, Basingstoke, United Kingdom) supplemented with 5% (vol/vol) horse blood. For intravenous infections, mouse tail bleeds were done 3, 6, 24, and 48 h postinfection and viable counts were performed as described above.

RNA extraction and purification. Wild-type pneumococci and the two isogenic mutants, NanA$^-$ and NanB$^-$, were grown overnight on blood agar plates (100 μg/ml erythromycin was included for the NanA$^-$ mutant). Subsequently, colonies were inoculated into brain heart infusion broth and grown overnight. The cultures were centrifuged at 3,000 rpm for 15 min, resuspended in fresh broth containing fetal calf serum, and grown until they reached an optical density at 500 nm of 0.6, at which stage all cultures contained approximately 1.8×10^8 CFU/ml. A culture volume containing 1×10^8 bacteria was added to RNAprotect (QIAGEN, Crowly, United Kingdom) for immediate stabilization of RNA, vortexed, and incubated at room temperature for 5 min. RNA was then extracted using Rneasy according to the descriptions of the manufacturer (QIAGEN, Crowley, United Kingdom). RNA was quantified at A_{260}, and its integrity was checked by ethidium bromide staining after electrophoresis through a 1% (wt/vol) agarose gel. Any contaminating DNA was removed by treatment with 2 U RNase-free DNase I (Invitrogen, Paisley, United Kingdom) for 15 min at room temperature, followed by heat inactivation for 10 min at 65°C in the presence of 2.5 mM EDTA.

Reverse transcription and quantitative reverse transcription-PCR. First-strand cDNA synthesis was performed on 250 ng DNase-treated total RNA immediately after RNA isolation using a SuperScript II RNase H reverse transcriptase kit (Invitrogen, Paisley, United Kingdom) and random hexamers according to the manufacturer's instructions. The amounts of cDNA specific for *nanA* and *nanB* mRNA were quantified by using SYBR Green PCR master mix (Applied Biosystems, Warrington, United Kingdom). The 20-μl reaction mix contained 1× SYBR Green PCR master mix, 2 μl of cDNA, 200 nM *nanA* primers (NanAF, 5′-CTACGATGAACAATAGACGTGCGC-3′; NanAR, 5′-TATCATACTGGGTCATGAAGCGTGC-3′) corresponding to nucleotides 1914 to 1938 and 2513 to 2538 in GenBank sequence X72967, respectively, or *nanB* primers (NanBF, 5′-GATTCTACTCAAGCTAAC-3′; NanBR, 5′-TCTCTAACTGTATAACGGAAATCG-3′) corresponding to nt 708 to 726 and 1136 to 1160 in GenBank sequence U43526, respectively. The amount of cDNA in samples was calculated by comparing it with the values obtained with standards constructed separately with *nanA* and *nanB* primer sets, comprising 10-fold dilutions of D39 pneumococcal genomic DNA ranging from 100 ng/μl to 1 pg/μl (corresponding to 4.5×10^7 to 4.5×10^2 copies of target). The threshold cycles (Ct; the cycle number at which the fluorescence signal crossed a fixed threshold) for quantification were defined from the baseline cycles for each gene. Each reaction was performed in triplicate.

Histology. At 12 and 24 h following infection, lungs were excised, embedded in Tissue-Tec OCT (Sakura), and frozen in liquid nitrogen with an isopentane heat buffer to prevent snap-freezing and tissue damage. Samples were stored at −70°C. A few days before sectioning, the lungs were removed to −20°C. Sections (15 μm) were taken at −18°C on a Bright cryostat and then allowed to dry at room temperature. Following acetone fixation, the sections were stained with hematoxylin and eosin and fixed with DPX mountant (BDH) for permanent storage (10). Sections from throughout the lung were taken, with at least 20 sections per lung being analyzed. Slides were analyzed independently by two observers, and lung pathology was scored blind on the following criteria: cellular infiltration around bronchioles, perivascular, and peribronchial areas, hypertrophy of bronchiole walls, and edema.

Statistical analysis. Data analysis was done by an analysis of variance followed by the Bonferroni test. Statistical significance was considered at P values of <0.05.

RESULTS

Neuraminidase activity. The neuraminidase activity of in vitro-grown and in vivo-passaged wild-type, NanA$^-$, and NanB$^-$ was assayed. There was no significant change ($P > 0.05$) in neuraminidase activity after in vivo passage compared to in vitro grown bacteria for any of the strains. The results were as follows for in vitro compared to in vivo, respectively: wild type, 43 ± 9 to 29 ± 11; NanA$^-$, 4.5 ± 1.5 to 1 ± 1; NanB$^-$, 22 ± 6 to 18.5 ± 2.5. All values are expressed as nmol pNP released/min/μg of total protein and are means ± standard errors of the means (SEM) of triplicate samples.

Transcriptional analysis of neuraminidase gene expression. Levels of *nanA* and *nanB* gene expression were quantified during in vitro growth. Both *nanA* and *nanB* genes were transcribed in vitro; however, the level of *nanA* gene transcription in mid-log phase was 10-fold higher than that of *nanB* (there was no difference in bacterial growth in vitro between the mutants and the wild-type pneumococci [data not shown]). The mean Ct value for *nanA* expression in the wild-type strain was 28.33 ± 0.26, corresponding to 114 ± 2.64 pg *nanA*-specific mRNA transcript, whereas the mean Ct value for *nanB* was 32.59 ± 0.28, corresponding to 11.3 ± 0.36 pg *nanB*-specific mRNA transcript. Analysis of both neuraminidase mutants also revealed that the mutation of neither the *nanA* nor *nanB* gene was compensated by the increased transcription of the other. The mean Ct values for *nanB* expression in the NanA$^-$ mutant or *nanA* expression in the NanB$^-$ mutant were similar to those for the wild type (31.71 ± 0.24 and 27.81 ± 0.08,

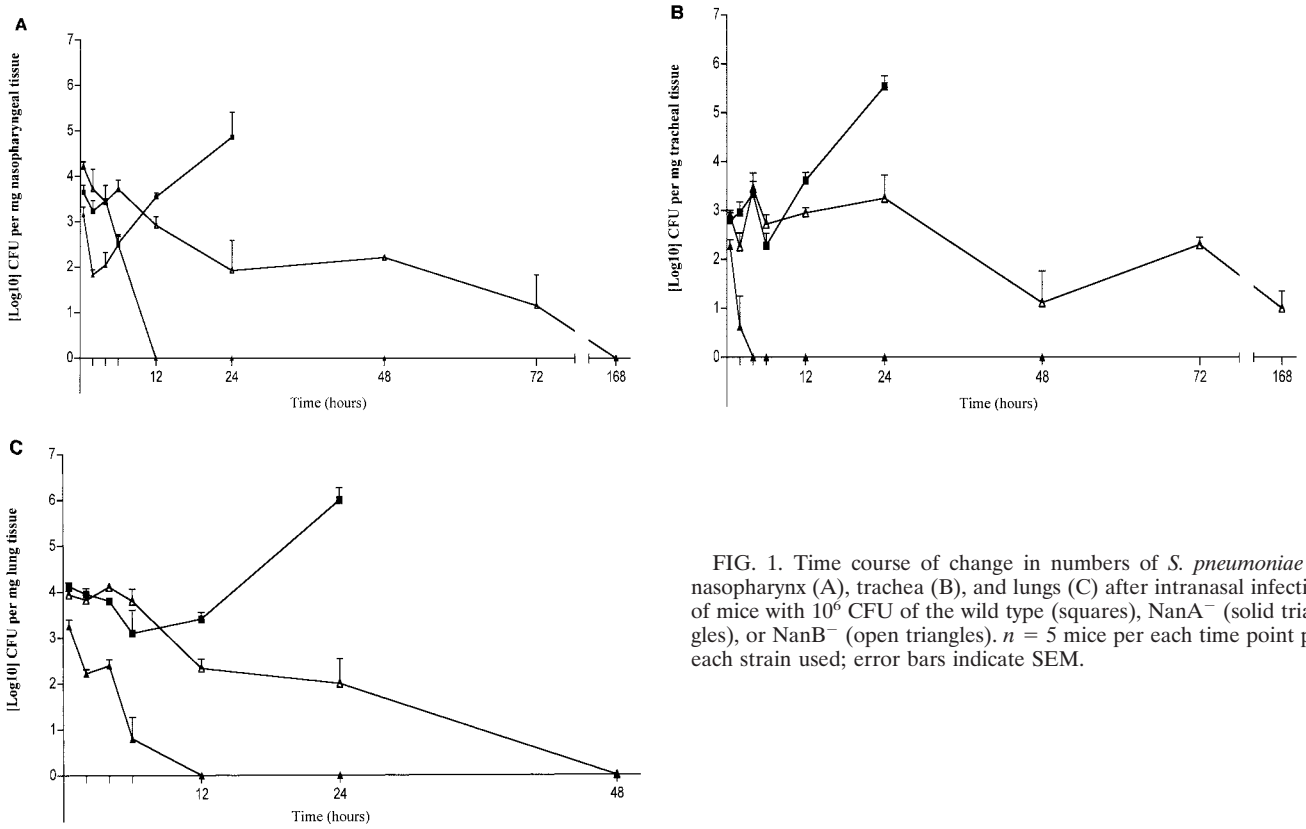

FIG. 1. Time course of change in numbers of *S. pneumoniae* in nasopharynx (A), trachea (B), and lungs (C) after intranasal infection of mice with 10^6 CFU of the wild type (squares), NanA$^-$ (solid triangles), or NanB$^-$ (open triangles). $n = 5$ mice per each time point per each strain used; error bars indicate SEM.

respectively). PCR efficiency with both *nanA* and *nanB* primer sets was also comparable. In addition, we measured the transcriptional expression of *SPR1535* (the gene downstream of *nanA*) in both the wild type and in the *nanA* isogenic mutant strain to see whether or not there was a polar effect of the insertion mutagenesis method used for the NanA$^-$ mutant. We found no significant difference in the transcriptional level of *SPR1535* in the *nanA* mutant strain compared to the parent wild-type strain (data not shown). Therefore, this rules out the possibility of a polar effect of the mutation.

Nasopharyngeal infection with wild-type and neuraminidase-deficient pneumococci. *Streptococcus pneumoniae* D39 wild type infected the nasopharynx in significantly greater numbers than NanA$^-$ at 2, 4, 6, and 12 h postinfection ($P < 0.01$) and by 6, 12, and 24 h postinfection with NanB$^-$ ($P < 0.01$ at 6 and 24 h; $P < 0.05$ at 12 h). There was no significant difference between the wild type and NanB$^-$ up to 6 h postinfection (Fig. 1A). However, NanB$^-$ was significantly better in colonizing the nasopharynx than NanA$^-$ at each time point from 2 h postinfection onwards ($P < 0.05$). NanA$^-$ was cleared rapidly within 12 h postinfection, whereas NanB$^-$ persisted in the nasopharynx for at least 72 h postinfection (Fig. 1A).

Tracheal infection with wild-type and neuraminidase-deficient pneumococci. Wild-type pneumococci infected the trachea in significantly greater numbers than both NanA$^-$ and NanB$^-$ by 24 h postinfection ($P < 0.01$) (Fig. 1B). NanB$^-$ was significantly better at colonizing the trachea than NanA$^-$ throughout the time course of infection ($P < 0.01$). NanA$^-$ was unable to successfully infect the trachea and was completely cleared by 4 h postinfection. In contrast, NanB$^-$ persisted in the trachea and was not cleared by 168 h postinfection (Fig. 1B).

Lung infection with wild-type and neuraminidase-deficient pneumococci. As was the case for the upper respiratory tract, there were significantly greater numbers of wild-type pneumococci in the lungs by 24 h postinfection than either NanA$^-$ or NanB$^-$ pneumococci ($P < 0.01$) (Fig. 1C). NanA$^-$ was completely cleared from the lungs by 12 h postinfection (Fig. 1C). Both the wild type and NanB$^-$ were present in significantly greater numbers compared to NanA$^-$ during this period ($P < 0.01$). However, NanB$^-$ was cleared from the lungs by 48 h postinfection (Fig. 1C), in contrast to its ability to colonize both the nasopharynx and trachea for longer periods. Mice infected with NanA$^-$ and NanB$^-$ survived intranasal infections, whereas mice infected with the wild type became moribund by 24 h and were sacrificed.

Blood infection with wild-type and neuraminidase-deficient pneumococci following intranasal and intravenous infections. Wild-type pneumococci caused severe bacteremia following intranasal infections. An exponential rise in wild-type pneumococcal numbers in the blood was observed during the period 24 h postinfection ($\log_{10}$ 4.2 ± 0.3 at 12 h and $\log_{10}$ 9.1 ± 0.5 at 24 h postinfection). All mice were moribund by this stage and were culled. Interestingly, however, neither NanA$^-$ nor NanB$^-$ was ever isolated from the blood at any time point following intranasal infection. To determine whether or not this was due to an inability to seed from lungs to blood or a

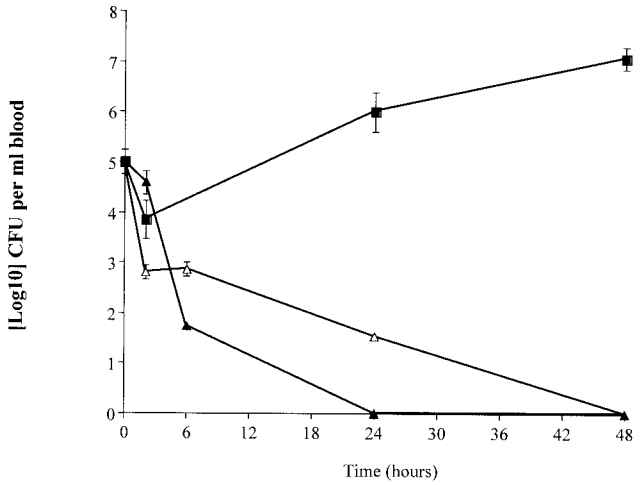

FIG. 2. Time course of change in numbers of *S. pneumoniae* in blood infected by the intravenous route. Mice were infected with 10^5 CFU of the wild type (squares), NanA$^-$ (solid triangles), or NanB$^-$ (open triangles). Tail bleeds were taken from 10 mice for each strain at 3, 6, 24, and 48 h post-intravenous infection. Error bars indicate SEM.

failure to grow in blood, both NanA$^-$ and NanB$^-$ were used in intravenous infections.

When NanA$^-$, NanB$^-$, and wild-type pneumococci were infected directly into the blood, NanA$^-$ and NanB$^-$ were unable to persist in blood beyond 24 and 48 h postinfection, respectively, and mice did not show any signs of disease (Fig. 2). On the other hand, wild-type pneumococci increased exponentially until 48 h postinfection, by which time mice became moribund and the experiment was ended.

Histopathological analysis of infected lung tissue. NanB$^-$-infected lungs exhibited lower levels of cellular infiltration and general inflammation than wild-type-infected lungs at 24 h postinfection. NanB$^-$-infected lungs exhibited mild peribronchial cellular infiltrate (Fig. 3A) and mild levels of bronchial wall hypertrophy. No interstitial alveolitis was apparent. Lung parenchyma was generally not involved in inflammation, with no clear diffusion of cellular infiltrate into these areas. Overall, NanB$^-$-infected lungs exhibited less severe inflammation than wild type at 24 h (Fig. 3B). Wild-type-infected lungs exhibited hypertrophy of inflamed bronchiole walls at 24 h (Fig. 3B) and a severe multifocal peribronchial infiltration of inflammatory cells and extensive infiltration of lung parenchyma. The bronchioles and lung alveoli appeared to be filled with exudates. Overall, at 24 h after infection, the majority of the lung surface of the mice infected with wild-type pneumococci presented consolidation of bronchiolar spaces and associated parenchyma (due to heavy infiltration of inflammatory cells and the presence of exudate) and hypertrophy of infected bronchiole cell walls.

Histopathology was also analyzed in lungs infected with either the NanA$^-$ or NanB$^-$ mutant at 12 h postinfection. For the NanB$^-$ mutant, the histopathology at 12 h was very similar to that observed at 24 h (data not shown). However, for the NanA$^-$ mutant, slight pathological features were apparent at this time point compared to both the wild type and NanB$^-$ (data not shown). In summary, in NanA$^-$-infected tissues, in contrast to the wild type and NanB$^-$, there was little general involvement or consolidation of the lung parenchyma and minimal hypertrophy of bronchiole walls (indeed, the vast majority of bronchiole walls did not appear to be inflamed). There were no major areas of cellular infiltration, either peribronchial or perivascular, and there was an absence of exudate in bronchioles and alveoli.

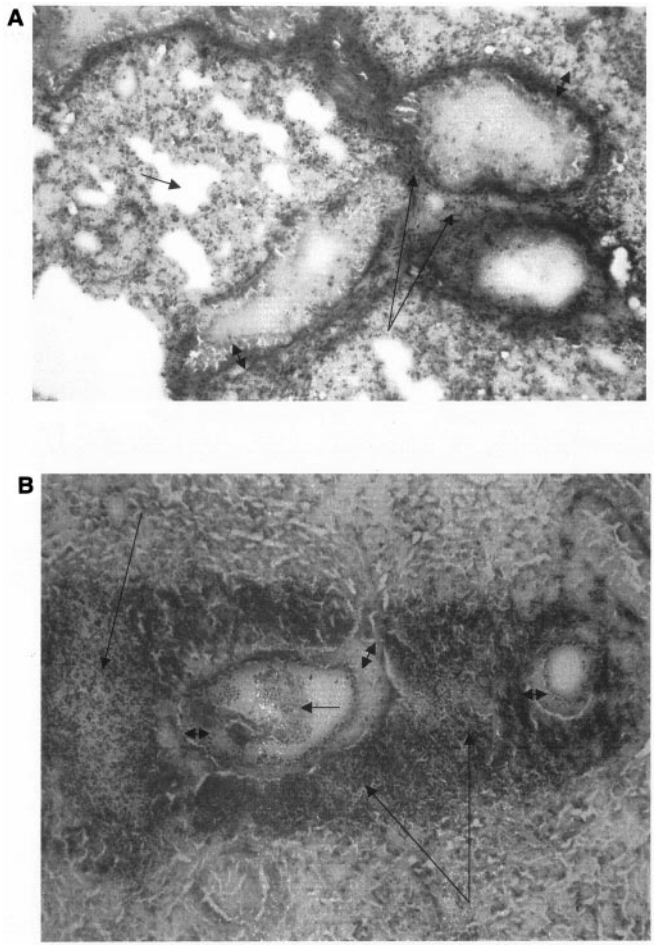

FIG. 3. Light microscopy of lung tissue from mice infected intranasally with 10^6 CFU *S. pneumoniae* NanB$^-$ at 24 h postinfection (A) or the wild type at 24 h postinfection (B). Magnification, ×250. In panel A, the long arrow indicates a mild peribronchial cellular infiltrate, the double arrow indicates mild levels of bronchial wall hypertrophy, and the short arrow shows alveolar spaces. In panel B, the double arrow indicates hypertrophy of inflamed bronchiole walls, and the long arrow indicates severe multifocal peribronchial infiltration of inflammatory cells and extensive infiltration of lung parenchyma. Exudate is represented by a short arrow.

DISCUSSION

The pneumococcus is a major human pathogen that appears to be well-adapted to colonizing the mucosal surfaces of the nasopharynx. Transmission from the nasopharynx to sterile regions of the upper and lower respiratory tract leads to infections such as sinusitis, otitis media, and pneumonia. The neuraminidase NanA has previously been shown to play an important role in the host-pneumococcal interactions in the upper

respiratory tract following intranasal infection (23, 24). However, to date no evidence has been presented to support a role for NanA beyond the confines of the respiratory tract. In addition, the contribution of another neuraminidase, NanB, to respiratory disease has not been investigated at all, although it has been reported that in a murine meningitis model the absence of NanB did not affect the course of disease or bacterial spread and multiplication compared to wild-type pneumococci (28). We have now demonstrated for the first time using outbred MF1 mice that both neuraminidases, NanA and NanB, are essential for the successful colonization of both the upper and lower respiratory tract, as well as pneumococcal survival in blood, following both intranasal and intravenous infection.

Our studies have shown that there was no significant change in neuraminidase activity after in vivo passage compared to in vitro growth for wild-type, NanA$^-$, and NanB$^-$ pneumococci. In addition, the neuraminidase activity of NanA was significantly higher than that of NanB in both in vitro and in vivo samples. This was in keeping with our mRNA data that also showed in vitro mRNA transcription levels of NanA at mid-log phase to be significantly higher than NanB. Transcript analysis also showed that inactivation of one gene was not compensated by the transcription level of the other. The transcript quantification also clearly showed that the mutations in the *nanA* and *nanB* genes had no polar effects upon each other. In addition, transcriptional expression of *SPR1535* (the gene downstream of *nanA*) in both the wild type and in the *nanA* isogenic mutant strain was not affected by the mutation in the *nanA* gene. This is in keeping with previously published work, which demonstrated that *nanA* and *nanB* are in separate regulons, consistent with these genes having distinct roles in the infection process (13). Our studies have shown that in our intranasal infection model, NanA$^-$ is cleared from the nasopharynx, trachea, and lungs within 12 h postinfection. In noteworthy contrast, NanB$^-$ persisted in both the nasopharynx and trachea for up to 168 h postinfection, and also for up to 48 h postinfection in the lungs. Furthermore, the absence of either neuraminidase resulted in survival of the infected host. Both NanA$^-$- and NanB$^-$-infected mice survived their infections, whereas all wild-type-infected mice succumbed to their infections. It is also clear from the infection data that the absence of one neuraminidase is not compensated for by the presence of the other neuraminidase, in keeping with the lack of compensatory expression of the *nanB* gene in mutants lacking the *nanA* gene and vice versa.

Intriguingly, both NanA- and NanB- are completely unable to cause sepsis following intranasal infections. There are two possible explanations for this: it may either be due to an inability of these mutants to seed from lungs to blood or to a failure of these mutants to survive in blood due to the lack of some essential process that involves neuraminidases. Our results show that in contrast to lack of sepsis following intranasal infections, when administered intravenously both NanA$^-$ and NanB$^-$ mutants persist longer in blood and are eventually cleared by 48 h postinfection. This suggests that the second scenario is more likely, namely, that neuraminidases are essential for survival in the blood in this model of infection. The reasons for this are less clear, however. Whereas proposals on the roles of neuraminidases in the colonization and infection of mucosal sites are well known, their role in nonmucosal sites, such as blood, seems less obvious. However, previous evidence has shown that the pneumococcus has the ability to bind to its surface several glycosylated protein components of the host immune response implicated in bacterial clearance. These host proteins include C-reactive protein, complement components, immunoglobulin, and lactoferrin (7–9, 20, 21, 26, 27). Although it is not clear whether or not neuraminidases interact directly with these components, recent evidence has suggested that NanA-dependent desialylation of immune components, such as human lactoferrin, secretory component, and immunoglobulin A1, does indeed occur (13). Glycosylation is known to affect the function of many proteins, and hence it is conceivable that NanA may be contributing to a protease-independent mechanism of modification of host protein components involved in bacterial clearance, thereby diminishing their protective immune functions and consequently promoting pneumococcal persistence (13).

Another interesting possibility is that neuraminidase activity may help the pneumococcus to scavenge sugars from host macromolecules as a source of carbon and possibly as capsule precursors. The lack of neuraminidase may thus inhibit the ability of the pneumococcus to successfully resist phagocytosis in the blood due to poor capsule formation, although based on colony appearance and capsule size, as judged by Quellung reaction, no obvious differences were apparent. We are, however, further investigating these possibilities.

Our study clearly demonstrates the importance of pneumococcal neuraminidases in the development of both upper and lower respiratory tract infection and sepsis in MF1 mice. Furthermore, this is the first study to report the important role of NanB in the respiratory tract and during sepsis. Our findings are in keeping with previously published in vivo work demonstrating the importance of NanA in distinct host environments, such as the upper and lower respiratory tract (17, 23–25). They contrast, however, with a study of 1-day-old rat pups, where no difference was seen in the levels or persistence of wild-type and NanA pneumococcal colonization in the nasopharynx (13). Although the route of infection was the same as ours, there are at least two important differences which may explain this discrepancy. First is the age of the animals used. Age-based differences in susceptibility to disease are well described. These are generally due to the differences in the host immune response of an adult animal to infection compared to that of a newborn; in addition, the pattern of sialylation of cell surface glycoconjugates will also differ with age. Furthermore, sialylation is known to be highly heterogenous between species (16, 22). Secondly, in our study we used a lower infectious dose (1×10^6 CFU) than King et al. (13), and the lower dose is more likely to reveal differences in virulence between different mutants. The study conducted by King et al. (13) used a high dose (1.8×10^7 CFU), which could override differences in virulence. Finally, differences in tissue-specific characteristics of the two hosts, such as pH and host tissue receptors involved in pneumococcal adherence, may also have an important role to play in the differences observed.

After a recent study of pneumococcal virulence in BALB/cJ mice (17), it was reported that NanA is not essential for survival of pneumococci when injected intraperitoneally or intravenously or when instilled directly into the lung via the intratracheal route. On the other hand, the authors also reported

that numbers of NanA-deficient pneumococci were significantly reduced in both the nasopharynx and lungs compared to wild-type pneumococci, following intranasal challenge. Hence, based on this study (17) NanA would appear to be of importance following intranasal infection only, but not following intratracheal or intraperitoneal infection. Our previous and present findings are in agreement with these data (17) in that NanA does not have a role to play in virulence following intraperitoneal infection (3) but does after intranasal infection (this study). These results clearly point to a site- and route-specific role for NanA, whereby the tissue-specific characteristics of the host site dictate the relative role of NanA. For the first time, our data also suggest the same to be true for NanB.

Our findings using MF1 mice on the important roles of NanA and NanB in blood following intravenous infection do, however, differ from those reported with NanA$^-$ using BALB/cJ mice (17). One explanation for this could be the differences in mouse strains used between the two studies. It is known that BALB/cJ mice do not develop sepsis following intranasal challenge (6). In contrast, MF1 mice develop lethal bacteremia following intranasal infection with the same dose and strain of pneumococci as those used for the BALB/cJ infections (6, 10). In addition, it was also reported in the study using BALB/cJ mice (17) that pneumolysin has no role to play in the colonization of the nasopharynx following intranasal infection. This is because these mice clear their pneumococci following infection at the doses mentioned and disease does not develop. In contrast, in MF1 mice, pneumolysin has been shown to be of vital importance in the colonization of the nasopharynx (11).

Based on previously published data and our current study, it can be concluded that pneumococcal neuraminidases are essential for both upper and lower respiratory tract infection, otitis media, and sepsis. Here we have described not only for NanA but also for NanB the important role of neuraminidase in respiratory tract and blood infections. What remains to be answered is whether or not the clearance of neuraminidase-deficient mutants in different host sites is due to their effects on local host immune response components or has more to do with particular tissue-specific characteristics of the site. Certainly, we know from our previous in vitro data that differing pH levels do affect neuraminidase activity (1). Indeed, the two neuraminidases have different pH optima (NanA at pH 6.5 and NanB at pH 4.5), suggesting distinct roles for these enzymes in vivo (3). However, this in vitro observation does not easily translate to an in vivo model, as the NanB$^-$ mutant in our model persisted significantly longer than NanA$^-$ in both the upper and lower respiratory tract following infection, thereby not clearly indicating a preference for one niche over another. However, in terms of survival and persistence, the NanB$^-$ mutant does favor the nasopharyngeal environment rather than the lower respiratory environment of the lung, perhaps suggesting a tissue/niche-specific preference. We are currently investigating these issues in different sites of the respiratory tract.

ACKNOWLEDGMENT

The Wellcome Trust supported the work at the University of Leicester.

REFERENCES

1. **Berry, A. M., R. A. Lock, and J. C. Paton.** 1996. Cloning and characterization of NanB, a second *Streptococcus pneumoniae* neuraminidase gene, and purification of the NanB enzyme from recombinant *Escherichia coli*. J. Bacteriol. **178:**4854–4860.
2. **Berry, A. M., A D. Ogunniyi, D. C. Miller, and J. C. Paton.** 1999. Comparative virulence of *Streptococcus pneumoniae* strains with insertion-duplication, point, and deletion mutations in the pneumolysin gene. Infect. Immun. **67:**981–985.
3. **Berry, A. M., and J. C. Paton.** 2000. Additive attenuation of virulence of *Streptococcus pneumoniae* by mutation of the genes encoding pneumolysin and other putative pneumococcal virulence proteins. Infect. Immun. **68:**133–140.
4. **Camara, M., G. J. Boulnois, P. W. Andrew, and T. J. Mitchell.** 1994. A neuraminidase from *Streptococcus pneumoniae* has the features of a surface protein. Infect. Immun. **62:**3688–3695.
5. **Canvin, J. R., A. P. Marvin, M. Sivakumaran, J. C. Paton, G. J. Boulnois, P. W. Andrew, and T. J. Mitchell.** 1995. The role of pneumolysin and autolysin in the pathology of pneumonia and septicemia in mice infected with a type 2 pneumococcus. J. Infect. Dis. **172:**119–123.
6. **Gingles, N. A., J. E. Alexander, A. Kadioglu, P. W. Andrew, A. Kerr, T. J. Mitchell, E. Hopes, P. Denny, S. Brown, H. B. Jones, S. Little, G. C. Booth, and W. L. McPheat.** 2001. Role of genetic resistance in invasive pneumococcal infection: identification and study of susceptibility and resistance in inbred mouse strains. Infect. Immun. **69:**426–434.
7. **Hammerschmidt, S., S. R. Talay, P. Brandtzaeg, and G. S. Chhatwal.** 1997. SpsA, a novel pneumococcal surface protein with specific binding to secretory immunoglobulin A and secretory component. Mol. Microbiol. **25:**1113–1124.
8. **Hammerschmidt, S., G. Bethe, P. H. Remane, and G. S. Chhatwal.** 1999. Identification of pneumococcal surface protein A as a lactoferrin-binding protein of *Streptococcus pneumoniae*. Infect. Immun. **67:**1683–1687.
9. **Jarva, H., T. S. Jokiranta, R. Wurzner, and S. Meri.** 2003. Complement resistance mechanisms of streptococci. Mol. Immunol. **40:**95–107.
10. **Kadioglu, A., N. Gingles, K. Grattan, T. J. Mitchell, and P. W. Andrew.** 2000. The host cellular immune response to pneumococcal lung infection in mice. Infect. Immun. **2:**492–501.
11. **Kadioglu, A., S. Taylor, F. Iannelli, G. Pozzi, T. J. Mitchell, and P. W. Andrew.** 2002. Upper and lower respiratory tract infection by *Streptococcus pneumoniae* is affected by pneumolysin deficiency and differences in capsule type. Infect. Immun. **70:**2886–2890.
12. **Kelly, R. T., S. Farmer, and D. Greiff.** 1967. Neuraminidase activities of clinical isolates of *Diplococcus pneumoniae*. J. Bacteriol. **94:**272–273.
13. **King, S. J., K. R. Hippe, J. M. Gould, D. Bae, S. Peterson, R. T. Cline, C. Fasching, E. N. Janoff, and J. N. Weiser.** 2004. Phase variable desialylation of host proteins that bind to *Streptococcus pneumoniae* in vivo and protect the airway. Mol. Microbiol. **54:**159–171.
14. **Lock, R. A., J. C. Paton, and D. Hansman.** 1988. Comparative efficacy of pneumococcal neuraminidase and pneumolysin as immunogens protective against *Streptococcus pneumoniae*. Microb. Pathog. **5:**461–467.
15. **Long, J. P., H. H. Tong, and T. F. DeMaria.** 2004. Immunization with native or recombinant *Streptococcus pneumoniae* neuraminidase affords protection in the chinchilla otitis media model. Infect. Immun. **72:**4309–4313.
16. **Mattu, T. S., R. J. Pleass, A. C. Willis, M. Kilian, M. R. Wormald, A. C. Lellouch, P. M. Rudd, J. M. Woof, and R. A. Dwek.** 1998. The glycosylation and structure of human serum IgA1, Fab, and Fc regions and the role of N-glycosylation on Fc alpha receptor interactions. J. Biol. Chem. **273:**2260–2272.
17. **Orihuela, C. J., G. Gao, K. P. Francis, J. Yu, and E. I. Tuomanen.** 2004. Tissue-specific contributions of pneumococcal virulence factors to pathogenesis. J. Infect. Dis. **190:**1661–1669.
18. **O'Toole, R. D., L. Goode, and C. Howe.** 1971. Neuraminidase activity in bacterial meningitis. J. Clin. Investig. **50:**979–985.
19. **Paton, J. C., P. W. Andrew, G. J. Boulnois, and T. J. Mitchell.** 1993. Molecular analysis of the pathogenicity of *Streptococcus pneumoniae*: the role of pneumococcal proteins. Annu. Rev. Microbiol. **47:**89–115.
20. **Poulsen, K., J. Reinholdt, and M. Kilian.** 1996. Characterization of the *Streptococcus pneumoniae* immunoglobulin A1 protease gene (*iga*) and its translation product. Infect. Immun. **64:**3957–3966.
21. **Smith, B. L., and M. K. Hostetter.** 2000. C3 as substrate for adhesion of *Streptococcus pneumoniae*. J. Infect. Dis. **182:**497–508.
22. **Spik, G., G. Strecker, B. Fournet, S. Bouquelet, J. Montreuil, L. Dorland, H. van Halbeek, and J. F. Vliegenthart.** 1982. Primary structure of the glycans from human lactotransferrin. Eur. J. Biochem. **121:**413–419.
23. **Tong, H. H., L. E. Blue, M. A. James, and T. F. DeMaria.** 2000. Evaluation of the virulence of a *Streptococcus pneumoniae* neuraminidase-deficient mutant in nasopharyngeal colonization and development of otitis media in the chinchilla model. Infect. Immun. **68:**921–924.
24. **Tong, H. H., X. Liu, Y. Chen, M. James, and T. Demaria.** 2002. Effect of neuraminidase on receptor-mediated adherence of *Streptococcus pneumoniae* to chinchilla tracheal epithelium. Acta Otolaryngol. **122:**413–419.

25. **Tong, H. H., D. Li, S. Chen, J. P. Long, and T. F. DeMaria.** 2005. Immunization with recombinant *Streptococcus pneumoniae* neuraminidase NanA protects chinchillas against nasopharyngeal colonization. Infect. Immun. **73:** 775–778.
26. **Volanakis, J. E., and M. H. Kaplan.** 1971. Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide. Proc. Soc. Exp. Biol. Med. **136:**612–614.
27. **Wani, J. H., J. V. Gilbert, A. G. Plaut, and J. N. Weiser.** 1996. Identification, cloning, and sequencing of the immunoglobulin A1 protease gene of *Streptococcus pneumoniae*. Infect. Immun. **64:**3967–3974.
28. **Wellmer, A., G. Zysk, J. Gerber, T. Kunst, M. Von Mering, S. Bunkowski, H. Eiffert, and R. Nau.** 2002 Decreased virulence of a pneumolysin-deficient strain of *Streptococcus pneumoniae* in murine meningitis. Infect. Immun. **70:**6504–6508.

Editor: F. C. Fang