Sialic Acid: A Preventable Signal for Pneumococcal Biofilm Formation, Colonization, and Invasion of the Host

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The correlation between carbohydrate availability, pneumococcal biofilm formation, nasopharyngeal colonization, and invasion of the host has been investigated. Of a series of sugars, only sialic acid (i.e., *N*-acetylneuraminic acid) enhanced pneumococcal biofilm formation in vitro, at concentrations similar to those of free sialic acid in human saliva. In a murine model of pneumococcal carriage, intranasal inoculation of sialic acid significantly increased pneumococcal counts in the nasopharynx and instigated translocation of pneumococci to the lungs. Competition of both sialic acid–dependent phenotypes was found to be successful when evaluated using the neuraminidase inhibitors DANA (i.e., 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid), zanamivir, and oseltamivir. The association between levels of free sialic acid on mucosae, pneumococcal colonization, and development of invasive disease shows how a host-derived molecule can influence a colonizing microbe and also highlights a molecular mechanism that explains the epidemiologic correlation between respiratory infections due to neuraminidase-bearing viruses and bacterial pneumonia. The data provide a new paradigm for the role of a host compound in infectious diseases and point to new treatment strategies.

Recurrent colonization of the nasopharynx is a common characteristic of respiratory pathogens. Although colonization is normally asymptomatic, it is believed to be a forerunner of the invasive diseases caused by these pathogens; however, the events that bring about this key change from colonization to invasion are unknown. To address this conundrum, we present new data derived from in vitro and in vivo experiments involving *Streptococcus pneumoniae.*

S. pneumoniae is a gram-positive, anaerobic, aerotolerant bacterium responsible for a variety of acute diseases in humans, including otitis media, pneumonia, sepsis, and meningitis [1–3]. The burden of invasive pneumococcal disease is great worldwide, in both developed and developing countries. Colonization of the human nasopharynx by *S. pneumoniae* is a natural process that occurs during the first few months of life and is thought to be a prerequisite for invasive pneumococcal infection. Although most colonized individuals are asymptomatic, progression from colonization to disease has been reported to occur soon after acquisition of a new colonizing strain [3–5]. Successive episodes of colonization are common, and these episodes, along with the incidence of pneumococcal invasive disease, have a clear seasonal variation (with the peak incidence occurring in winter or early spring [4, 6]), indicating the influence of environmental factors on host-pathogen interactions.

The human nasopharynx is the principal ecological niche of many bacterial pathogens. Although this environment offers bacteria a plethora of signals, sugars present in the nasopharynx primarily have been regarded as nutrients or as foci for adhesion, although they

Received 25 August 2008; accepted 4 December 2008; electronically published 23 April 2009.

Potential conflicts of interest: none reported.

Financial support: European Union (grants LSHM-CT-2005–512099 [to M.R.O. and P.W.A.] and LSHB-CT-2005–512061 [to G.P. and M.R.O.]).

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The Journal of Infectious Diseases 2009; 199:1497–505

0022-1899/2009/19910-0013$15.00
DOI: 10.1086/598483

also may be signals that lead to enhanced virulence. In the pneumococcus, the interconnection between environmental signals and the capacity to colonize or cause disease was first indicated by genomic screening for virulence factors, which identified uptake systems (including carbohydrate uptake systems [7]) as a requirement for the development of full virulence in animal models [8–10]. In other species, sugar-utilizing systems specific for host carbohydrates have been associated with virulence, including heparin in *Staphylococcus* organisms, fucose in *Bacteroides* organisms, and sialic acid in nontypeable *Haemophilus influenzae* and *Escherichia coli* [11–15]. To our knowledge, at this time, the only example demonstrated to have a significant influence in humans is sucrose-induced colonization and cariogenicity of *Streptococcus mutans,* a model in which the dual role of the dietary sugar (as fermentable carbohydrate and as substrate for extracellular biofilm-matrix production) determines the increased virulence of the bacterium [16].

MATERIALS AND METHODS

Strains and growth conditions. The pneumococcal strains used in the present study were the serotype 2 strain D39, the serotype 4 strain TIGR4, and their respective rough derivatives DP1004 and FP23. Bacteria routinely were grown in tryptic soy broth (TSB; Becton Dickinson) or tryptic soy agar supplemented with 3% vol/vol horse blood at 37°C in an atmosphere enriched with CO_2. Sialic acid–free liquid medium was prepared with 10 g/L yeast extract (Becton Dickinson), 5 g/L $NaCl_2$, and 0.5 mol/L 3% wt/vol K_2HPO_4.

Mutant construction. Isogenic mutants were constructed by means of gene splicing by overlap extension (i.e., SOEing), as described elsewhere [17]. The primers used for deletion of the *nanA* and *nanB* locus were based on the sequences of upstream segments NanA1 (TGTAGCCGTCATTTTATTGCTAC) and NanA2 (TCCACTAGTTCTAGAGCGATTTTCTGCCTGATGTTGGTAT), downstream segments NanA3 (ATCGCTCTTGAAGGGAATGCTATTTACACCATACTTCCT) and NanA4 (CAGCTTCGCCTTGCCGTAGGT), and spectinomycin cassettes IF100 (GCTCTAGAACTAGTGGA) and IF101 (TTCCCTTCAAGAGCGAT). The size of the locus deletion was 23 kb and included open-reading frames SP1674–SP1693 in TIGR4 and open-reading frames spr1518–spr1536 in DP1004 (R6 genome). The mutant in TIGR4 was named "FP285," that in FP23 (rough TIGR4) was named "FP236," and that in DP1004 (rough D39) was named "FP240."

Microtiter biofilm methodologic approach. The methodologic approach used for the static model of *S. pneumoniae* biofilm has been described elsewhere [18]. In brief, bacteria were grown in 96-well flat-bottom polystyrene plates (Sarstedt) with inocula of 1:100. CSP1 (30 ng/mL) was used for D39 and its derivatives, and CSP2 (100 ng/mL) was used for TIGR4 and its derivatives. In all cases, microtiter plates were incubated at 37°C in an atmosphere enriched with CO_2. To detach biofilm bacteria, plates were washed 3 times, sealed, floated on a sonicator water bath (a Transonic 460/H ultrasonic bath [Elma]), and sonicated for 2 s at 35 kHz. Sugars assayed for the enhancement of biofilm formation were prepared as filter-sterilized 20% wt/vol stocks and assayed at 0.2% wt/vol. The sugars used were adonitol (Sigma 45502), arabinose (Sigma A3131), cellobiose (Sigma C7252), fructose (Fluka 47740), fucose (Sigma F8150), galactose (Carlo Erba 453125), glucose (Baker 0115), glycerol (Baker 7044), inositol (Sigma I5125), lactose (Carlo Erba 457552), maltose (Carlo Erba 459865), mannitol (Sigma M4125), mannose (Sigma M6020), melibiose (Sigma M5500), *N*-acetyl-D-galactosamine (Sigma A2795), *N*-acetyl-D-glucosamine (Sigma A8625), *N*-acetylneuraminic (sialic) acid (Sigma A9646), *N*-glycolylneuraminic acid (Sigma G9793), raffinose (Fluka 83400), rhamnose (Sigma R3875), sorbitol (Sigma S1876), stachyose (Sigma S4001), starch (Carlo Erba 417585), sucrose (Baker 0334), trehalose (Fluka P0210), xylitol (Fluka 95649), and xylose (Sigma X1500). Solutions of carbohydrates had a neutral pH in PBS, except for *N*-acetylneuraminic (sialic) acid (2% wt/vol [pH 2], 0.6% [pH 4], 0.2% [pH 6.8], and 0.06% [pH 7.0]) and *N*-glycolylneuraminic acid (2% wt/vol [pH 2], 0.6% [pH 3], 0.2% [pH 5.5], 0.06% [pH 6.0], 0.02% [pH 6.5], and 0.006 [pH 6.8]), which had acidic pH. To evaluate competition for a sialic acid–dependent effect, the neuraminidase inhibitors DANA (2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid; D9050 Sigma), zanamivir (4-guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuramic acid (Relenza; Glaxo SmithKline), and oseltamivir (i.e., oseltamivir phosphate [Tamiflu; Roche]) were used at concentrations of 0.01–300 μg/mL.

Surface protein detection. For flow cytometric analysis, bacteria were grown either in TSB to the early stationary phase or in biofilm, and $\sim 10^6$ cfu were used for the assay. Bacterial cells were washed, resuspended in PBS 1% bovine serum albumin, and incubated with rabbit anti-NanA and anti-NanB polyclonal antibodies prepared against cloned neuraminidases (data not shown). Cells were washed twice in PBS and then were resuspended in 0.5 mL of PBS and analyzed by flow cytometry (FACScan; Becton Dickinson). Data analysis was performed using CellQuest software, version 3.3 (Becton Dickinson).

Induction of gene expression by sialic acid. Pneumococci were grown in yeast extract medium. Midexponential pneumococcal cultures were aliquoted into vials containing 0.025 % wt/vol sialic acid, and samples were obtained at 5, 10, and 20 min. After being chilled on ice, samples were directly processed for RNA extraction. Experiments were performed in triplicate for TIGR and in quintuplicate for DP1004. Real-time polymerase chain reaction (PCR) analysis was performed as described elsewhere [18, 19]. Relative gene expression was analyzed using the $2^{-\Delta\Delta CT}$ method [20]. The reference gene was *gyrB,* and the reference condition was the exponential phase of growth in TSB. Statistical analysis was performed using a 2-tailed Student's *t* test, as described elsewhere [20], for quantitative PCR.

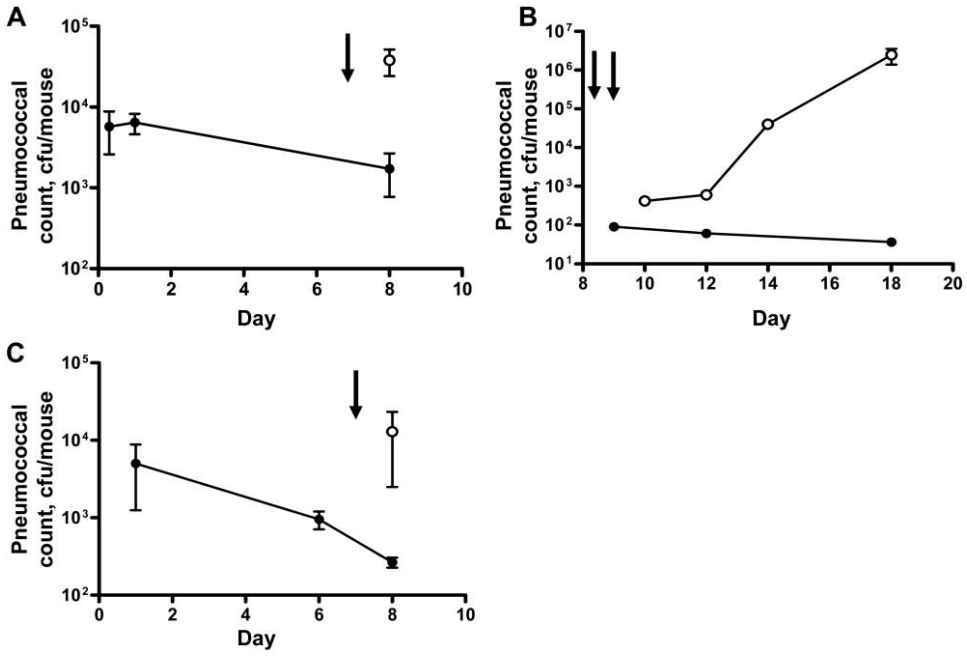

Figure 1. Influence of sialic acid on pneumococcal colonization of mice. Outbred MF1 mice were intranasally challenged with 10^4 cfu of pneumococci, and colonization was monitored by nasopharyngeal lavage *(black circles).* Pneumococcal colonization in mice intranasally inoculated with sialic acid (1 mg/mouse) *(arrow)* is denoted by white circles (with the mean count ± SD shown). *A,* Colonization with strain TIGR4 and the effect of a single intranasal dose of sialic acid on day 7 after infection. *B,* Colonization with TIGR4 and the effect of 2 intranasal doses of sialic acid on days 8 and 9 after infection and monitoring of colonization up to 18 days after infection. *C,* Colonization with the serotype 2 strain D39 and the effect of a single intranasal dose of sialic acid on day 7 after infection. The increase in the number of colonizing bacteria was statistically significant for mice given sialic acid ($P < .05$ for all panels, by 2-tailed Student's *t* test)

Colonization model. Outbred MF1 mice were purchased from Harlan Nossan and Harlan Olac. Experiments were performed at the University of Siena, Siena, Italy (for TIGR4), and at the University of Leicester, Leicester, United Kingdom (for D39), in accordance with respective national and institutional guidelines. Five different time course experiments were performed to determine (1) the influence of sialic acid on colonization by D39, (2) the influence of a 2-dose challenge with sialic acid on colonization by TIGR4, (3) the influence of a single-dose challenge with sialic acid on colonization by TIGR4 and on its spread to the lungs and blood, (4) the influence of different sugars on colonization by TIGR4, and (5) the competition in vivo of sialic acid–dependent effects by neuraminidase inhibitors.

In all experiments, the samples used for microbiological analysis were nasopharyngeal and lung lavage fluid and blood samples. For all mice, bacterial counts are expressed as the total number of colony-forming units per sample. In experiment 1, a total of 12 mice (4 mice/data point) were intranasally infected with 2×10^4 cfu of D39 in 50 μL. On day 7 after infection, each mouse in 1 additional group of 4 mice received 1 mg of sialic acid in 30 μL (40 mg/kg/day) [21, 22]; samples were obtained from these mice 24 h later (figure 1*C*). In experiment 2, a total of 21 mice (3 mice/data point) were intranasally infected with 3×10^4 cfu of TIGR4 in 30 μL. In this experiment, 2 doses of sialic acid (1 mg/mouse) were given on days 8 and 9 after infection, and colonization in nasopharyngeal lavage fluid was assayed (figure 1*B*). In experiment 3, a total of 28 mice were intranasally infected with 1×10^4 cfu of TIGR4 in 30 μL, and a single dose of sialic acid (1 mg/mouse) was given on day 7 after infection. On days 6 and 19 after infection, samples were obtained from groups of mice that had not been given sialic acid, whereas on days 8, 9, 13, 16, and 19, samples were obtained from groups of mice that had been given sialic acid. In addition to nasopharyngeal lavage fluid, lung homogenates and blood samples were also analyzed (figure 2). In experiment 4, a total of 42 mice were intranasally infected with 2×10^4 cfu of TIGR4 in 30 μL. In this experiment, samples were obtained at 6 and 24 h after infection and on day 8 after infection (figure 1*A*). Additional groups of mice were challenged with different sugars on day 7 after infection, and samples for analysis were collected on day 8. The sugars (1 mg/mouse) were given intranasally. In experiment 5, a total of 44 mice were intranasally infected with 2×10^4 cfu of TIGR4 in 30 μL. The groups of mice in experiment 5 included 10 control mice that were killed either on day 7 or day 11; ten mice that received sialic acid (1 mg/mouse) on day 7 after infection and had samples obtained on days 9 and 11; three groups of 4 mice each that were treated with either DANA, zanamivir, or oseltamivir phosphate (1 mg/mouse) on days 7 and 8; and 3 groups of 4 mice each that received a single dose of sialic acid on day 7 and subsequently were treated with either DANA, zanamivir, or oseltamivir (1

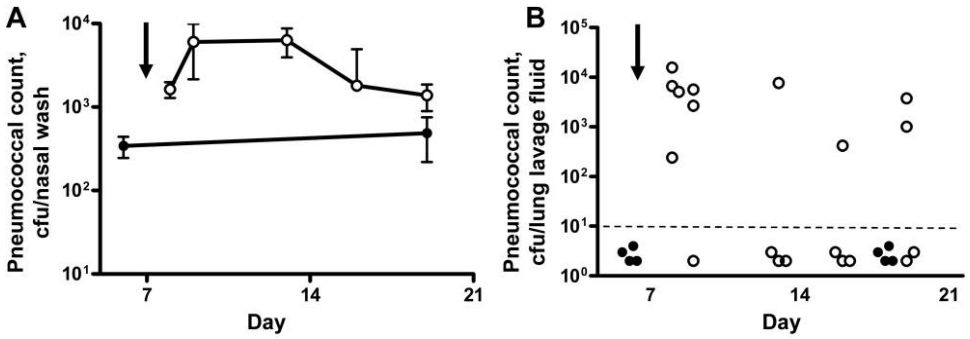

Figure 2. Influence of sialic acid on the spread of pneumococci to the lungs of colonized mice. Outbred MF1 mice were intranasally challenged with 10^4 cfu of TIGR4. Two groups of 4 mice each were colonized and received PBS intranasally as mock treatment on day 7 after infection *(filled circles),* and 5 groups of 4 mice each received sialic acid intranasally (1 mg/mouse on day 7 after infection) *(empty circles).* Bacterial counts in nasopharyngeal lavage fluid *(A)* are expressed as the mean value ± SD, whereas lung homogenate counts are expressed as individual counts (*B*; *dashed line* denotes the cutoff value). Blood samples plated in parallel had negative results at all time points. Nasopharyngeal counts were significantly greater in mice treated with sialic acid than in mice in the control group, at all time points (as determined using Student's *t* test) *(A).* Detection of pneumococci in lung samples was analyzed with Fisher's exact test; results were statistically significant at early time points *(B).*

mg/mouse) on days 9 and 10. Statistical analysis was performed using a 2-tailed Student's *t* test.

RESULTS

Pneumococcal biofilm formation is induced by sialic acid. We recently demonstrated that pneumococci grown in static biofilms in vitro exhibited the same gene expression pattern as did pneumococci from the lungs of infected mice and, hence, that this in vitro biofilm model could be used to develop hypotheses regarding events occurring during invasive pneumococcal disease [18]. In this model, adherent pneumococcal cells can be recovered after 24 h if a competence-stimulating peptide is added to the pneumococcal culture [14].

In the present study, we used this biofilm model to investigate the possible effects of carbohydrates on the pneumococcal physiologic profile during infection. Twenty-seven sugars were individually added to standard TSB medium at 0.2% wt/vol. Under these conditions, only the aminosugar sialic acid significantly increased the number of bacteria attached to the wells after 24 h of incubation (figure 3*A*); the same effect was noted after adding a competence-stimulating peptide (data not shown) [18]. Additional experiments were performed using medium based on soy or yeast extract only, because both constituents are devoid of animal products and, hence, are without sialic acid. In both media, effects on biofilm were also statistically significant at 6 h after infection and were specific to sialic acid among all sugars tested, including other monosaccharides that constitute the O-glycans of mucin (figure 3*B*). A time course experiment in yeast medium, supplemented with 0.2% wt/vol sialic acid, showed that the effect of sialic acid on the attachment of pneumococci to surfaces occurred after a few hours of incubation, corresponding to the exponential phase of growth (figure 3*C*). The sialic acid concentration found to be sufficient to induce biofilm formation was between 1 and 3 μg/mL (figure 3*D*), a concentration comparable to that of free sialic acid in human saliva [23–25]. Pneumococci carry 2–3 genes that code for sialidases/neuraminidase [26, 27]. An ~100-fold increase in the sialic acid concentration was necessary for biofilm formation by a *nanA-nanB* mutant (figure 3*D*). To exclude strain-specific factors, almost all experiments in this study were performed in parallel with the use of the serotype 2 strain D39 and the serotype 4 strain TIGR4. TIGR4 carries a third neuraminidase gene *(nanC)* in a separate chromosomal locus, but this gene was found to be irrelevant to biofilm formation (figure 3*D*) [28]. Because of the genomic organization of *nanA* and *nanB* within a single metabolic operon, the *nanAB* mutant is also deleted for cotranscribed transport systems and metabolic enzymes. In the *nanAB* mutant, recovery of the biofilm phenotype only when there is a nonphysiologic excess of sialic acid (figure 3*D*) could result from complementation of the lost high-affinity uptake by additional low-specificity transport system(s) [29]. Alternatively, a signaling event triggered by binding of sialic acid to other receptors could explain the data.

Neuraminidase production and expression. Increased expression of neuraminidase genes in pneumococcal biofilm and in animal models of infection has been reported elsewhere [18, 30, 31]. By performing cytofluorimetric analysis of pneumococci grown in liquid culture, we detected NanA antibodies in 29% of cells and noted almost no reactivity with anti-NanB serum. An increase in the detection rate to 74% for NanA and to 38% for NanB antibodies in bacteria recovered from biofilms indicates more-abundant neuraminidase production in sessile cells. The association between biofilm, neuraminidases, and sialic acid was further emphasized by the observation that sialic acid induced expression of both *nanA* and *nanB* (figure 4). The increase in expression of both *nanA* and *nanB* is steady and sig-

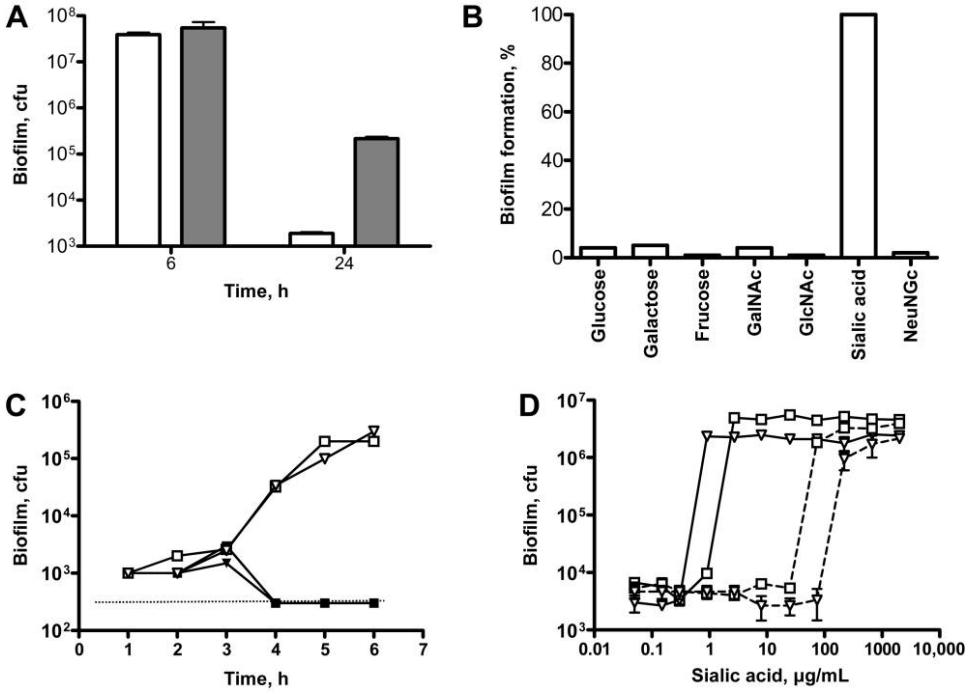

Figure 3. Biofilm formation by *Streptococcus pneumoniae* is dependent on sialic acid in the growth medium. *A,* Formation of biofilm DP1004 (a rough D39 derivative) was assayed in a static microtiter model after 6 and 24 h of incubation in unsupplemented tryptic soy broth (TSB) medium *(white bars)* and in TSB medium supplemented with 0.2% wt/vol of sialic acid *(black bars).* The effect of *N*-acetylneuraminic (sialic) acid on biofilm is significant at 24 h ($P < .001$). *B,* Biofilm formation in sialic acid–free yeast medium supplemented with 0.2% wt/vol monosaccharide after 6 h of incubation. *C,* A time course experiment performed in yeast medium and showing similar dynamics of attachment to surfaces in TIGR4 (*white triangles* and *solid line*) and DP1004 (*white squares* and *solid line*). Biofilm formation of both TIGR4 (*black triangles* and *solid line*) and DP1004 (*black squares* and *solid line,* respectively) in the absence of sialic supplementation. *D,* The effects of different sialic acid concentrations in yeast medium on biofilm formation by DP1004 (*white squares* and *solid line*), its *nanAB* mutant (*white squares* and *dashed line*), TIGR4 (*open triangles* and *solid line*), and the respective *nanAB* mutant (*white triangles* and *dashed line*) at 6 h after infection (3 replicas; the SD is shown in the graph). GalNAc, *N*-acetyl galactosamine; GlcNAc, *N*-acetyl glucosamine; NeuNGc, *N*-glycolylsialic acid.

nificant at 10 and 20 min for *nanA* in DP1004 and at 20 min for both *nanA* and *nanB* in TIGR4. This association between sialic acid and its hydrolases is typical of a substrate-inducible regulatory system [32]. In addition to the neuraminidase genes, the virulence regulator *mgrA*, which is up-regulated in biofilms [18] and is important for nasopharyngeal carriage and pneumonia [33], was induced by sialic acid (figure 4*E*). The absence of up-regulation of *nanC* (data not shown) is in accordance with the findings of a report published elsewhere [28], as well as with the observation that there is no significant variation in the capacity to form biofilm between the $nanAB^{-}/nanC^{+}$ TIGR4 mutant and the $nanAB^{-}$ D39 derivative (figure 3*D*). These data demonstrate that sialic acid functions as a regulatory signal for its own cleavage from oligosaccharides and has additional influence on more-complex phenotypes, such as biofilm and virulence.

Influence of sialic acid on carriage and invasion. To investigate the path of respiratory bacterial pathogens from colonization to invasion, we established a model of long-term nasopharyngeal carriage [3, 29, 34, 35]. Figure 1 shows that, in this model, pneumococci remained in the nasopharynx for at least 8–18 days without infection of the lower respiratory tract or bacteremia developing. However, in the present study, we made the exciting observation that intranasal administration of sialic acid after 1 week of carriage resulted in a 10- to 100-fold increase in the number of colonizing pneumococci in the nasopharynx (figure 1). The increase in the number of colonizing pneumococci was even more pronounced if 2 doses of sialic acid were administered (figure 1*B*). Experiments performed in a different laboratory with strain D39 (figure 1*C*), instead of TIGR4 (figure 1*A*), produced an identical sialic acid–dependent increase in colonization. In both cases, this sialic acid–dependent increase in nasopharyngeal colonization was accompanied by pneumococcal spread to the lungs (figure 2). It is noteworthy that no such invasion of the lower respiratory tract by bacteria can be detected in any of the colonized mice without an intranasally administered sialic acid boost. However, pneumococcal counts in the lungs of these mice were ~100 times lower than those in models of acute pneumonia [36], and blood culture results were negative. In nearly all mice without signs of acute disease, translocation of bacteria to the lung is quite reminiscent of the situation noted in humans during the seasonal increase in carriage, when very few subjects develop invasive disease. Importantly,

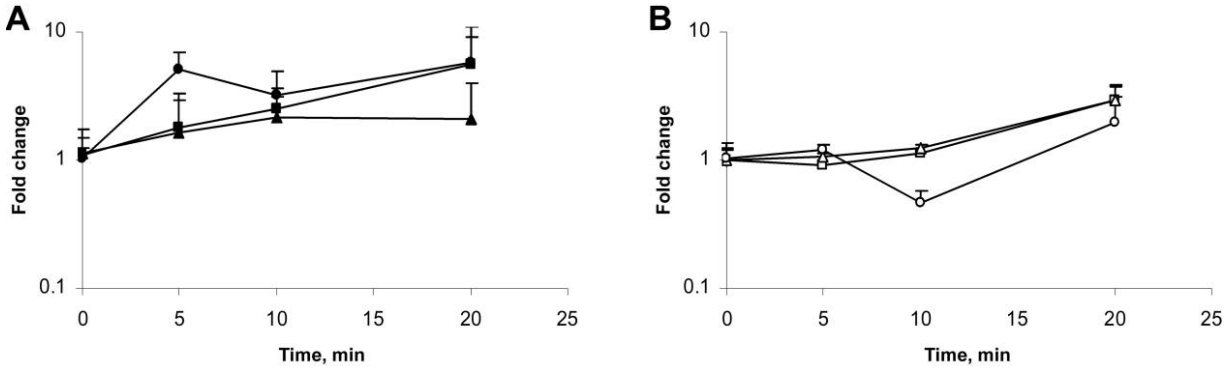

Figure 4. Changes in the expression of pneumococcal neuraminidases. Quantitative real-time polymerase chain reaction analysis was used to evaluate changes in gene expression of *nanA (squares), nanB (triangles),* and *mgrA (circles)* in response to sialic acid. Sialic acid (25 μg/mL) was added to exponentially growing pneumococci in yeast medium. A significant increase in gene expression was detected in the D39 derivative DP1004 *(A),* for *nanA* (at 10 min, a 2.5-fold increase was noted [$P < .01$]; at 20 min, a 5.6-fold increase was noted [$P < .05$]) and for *mgrA* (at 5 min, a 5.1-fold increase was noted [$P < .01$]; at 10 min, a 3.2-fold increase was noted [$P < .05$]; and at 20 min, a 5.7-fold increase was noted [$P < .05$]), as well as in the strain TIGR4 *(B),* for *nanA* (at 20 min, a 2.9-fold increase was noted [$P < .05$]) and *nanB* (at 20 min, a 3-fold increase was noted [$P < .05$]). Statistics are for $\geqslant$3 nonparallel biological replicas and were obtained using a 2-tailed Student's *t* test [20].

the booster effect on pneumococcal colonization of the nasopharynx and subsequent spread to the lower respiratory tract was specific to sialic acid; it did not occur after intranasal administration of other amino sugars (figure 5).

Competition of sialic acid–dependent phenotypes. To confirm the specificity of the 2 sialic acid–dependent phenotypes, we performed competition experiments with use of the transition state analogue of sialic acid DANA, its commercial neuraminidase inhibitor drug derivative zanamivir, and the cyclohexene-derived neuraminidase inhibitor oseltamivir [37]. Using all 3 compounds, it was possible to reduce by 1000-fold the capacity of pneumococci to form sialic acid–dependent biofilm in vitro (figure 6). In mice, intranasal delivery of DANA and of both anti-influenza drugs enabled a significant reduction in pneumococcal carriage (previously boosted by sialic acid) to levels lower than those detected in uninduced carriage (figure 7). Among mice with uninduced carriage, the reduction in pneumococcal counts was detected in all mice that were treated, although this reduction was not statistically significant (figure 7). The successful competition, both in vitro and in vivo, of the sialic acid–dependent phenotypes confirms the specificity of the observed phenomenon. Nevertheless, we want to emphasize that, although pneumococcal biofilms have been demonstrated in the host in otitis media [38], and although colonization images are suggestive of biofilms [3], the present study does not claim that a biofilm is involved in

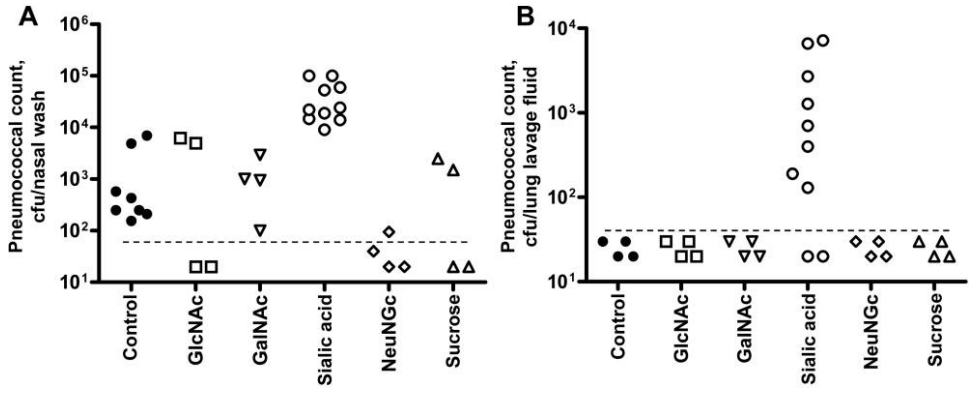

Figure 5. Modulation of pneumococcal colonization of mice by distinct sugars. Six days after intranasal infection with 10^4 cfu of TIGR4, mice received different sugars intranasally. Pneumococcal colony-forming units in the nasopharynx *(A)* and lungs *(B)* are reported for individual mice. *Dashed line,* the cutoff for detection of pneumococci. The difference in nasal carriage in mice that received sialic acid is significant with respect to the control group ($P < .01$, by 2-tailed Student's *t* test), whereas there were no significant differences ($P > .05$, by 2-tailed Student's *t* test) after administration of the other sugars. The presence of bacteria in the lungs of mice treated with sialic acid is significant ($P < .05$, by Fishers exact test). In addition to sugars used in previous experiments, *N*-glycolylsialic acid (NeuNGc), a sialic acid derivative missing in humans, was also used in the present study. GalNAc, *N*-acetyl galactosamine; GlcNAc, *N*-acetyl glucosamine.

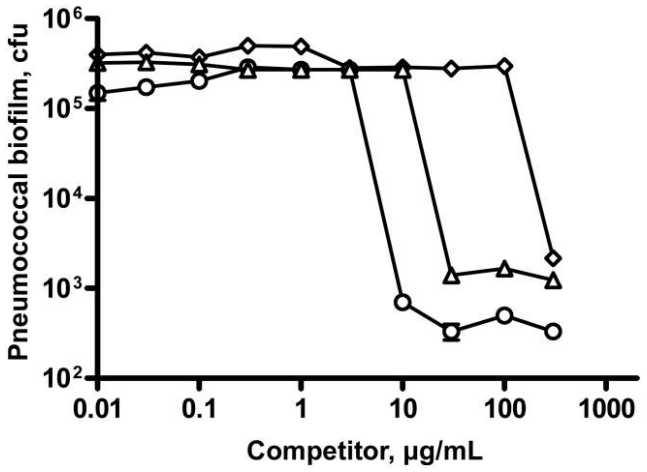

Figure 6. Competition for sialic acid–dependent biofilm formation in vitro. The effect of serial dilutions of sialic acid transition-state analogue DANA (2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid) *(triangles),* its commercial drug derivative zanamivir *(diamonds),* and the cyclohexene-derived neuraminidase inhibitor oseltamivir *(circles)* on the capacity of pneumococci to form sialic acid–dependent biofilm was evaluated in yeast medium containing 8 μg/mL free sialic acid.

pneumococcal carriage, but only that the 2 models share important analogies and that the in vitro system may serve as a suitable model for analysis of carriage phenotypes.

DISCUSSION

This study is, to our knowledge, the first to demonstrate (1) a causal association between sialic acid and pneumococcal biofilm formation in vitro and (2) a significant increase in nasopharyngeal colonization and spread to the lungs in vivo. These observations are highly relevant to our understanding of the mechanism of the virulence of this important human pathogen, and they could be more widely applicable to other bacterial pathogens that have both colonizing and invasive phenotypes. In humans, the epidemiologic profile of both carriage and invasive bacterial diseases shows clear seasonal variation (with peaks occurring in late winter and early spring) that parallels that of influenza virus, parainfluenza virus, and respiratory syncytial virus [2, 6, 39]. This correlation indicates that viral infection of the upper respiratory tract contributes to peaks in the incidence of invasive pneumococcal disease in humans [39].

Sialic acid has long been believed to play a central role in this interplay, although only because its removal was important. In humans, sialic acid is present as a terminal carbohydrate of the O-glycan chains of mucins and cell surface glycoproteins [23–25]. Binding to sialoglycoconjugates on host cells via hemagglutinin and release of viral particles by neuraminidases contributes to host range, tissue tropism, and the pathogenesis of the influenza virus and other viruses. Because of their central role in the pathogenesis of influenza A virus infection, neuraminidase inhibitors are among the best-known antiviral drugs [37]. Although it is has not been found to be directly effective against pneumococci in vivo, treatment with neuraminidase inhibitors has been shown to reduce the severity of postinfluenza pneumococcal infection [22, 40]. These observations have been explained by cleavage of sialic acids revealing new receptors for the bacteria and/or mitigating direct damage to host cells [41, 42]. The data presented in the current study suggest a new paradigm—namely, that sialic acid itself is a signaling molecule that results in fundamental alterations in the physiologic profile of bacteria, resulting in an enhanced capacity to adhere to surfaces and/or survive within the biofilm environment.

An important observation was that the sialic acid concentrations normally found in human saliva signal to the bacterium for increased production of neuraminidases, which is known to be associated with concomitant increased production of the co-transcribed transporters and metabolic enzymes [29]. The consequence of this signaling would be expected to be a local increase in the concentration of sialic acid with a positive feedback on more efficient import and metabolism. Desialylation of cell-bound and secreted glycoproteins in the nasopharynx and the

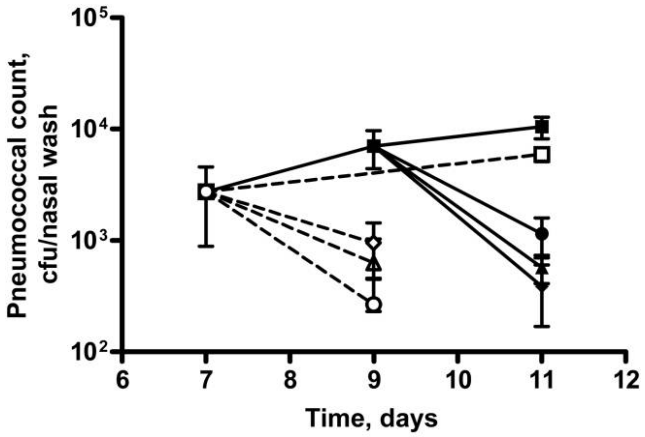

Figure 7. Competition for sialic acid–dependent colonization increases in vivo. Mice intranasally infected with pneumococci had stable nasopharyngeal colonization for 11 days (*white squares* and *dashed line*) (no significant variation was noted between days 7 and 11 after infection). After intranasal administration of sialic acid (1 mg given twice daily on days 7 and 8), pneumococcal counts increased, albeit not significantly *(black squares).* Intranasal inoculation (1 mg given twice daily on days 7 and 8 after infection) of either DANA (*white triangle* and *dashed line*), zanamivir (*white diamond* and *dashed line*), or oseltamivir (*white circle* and *dashed line*) reduced pneumococcal carriage ($P < .05$ for all 3 molecules, with respect to mice treated with sialic acid sampled at day 9; reduction was nonsignificant for mice sampled at day 7). The reduction in colonization was even more pronounced if sialic analogues (*straight lines* and *black triangle* [for DANA], *black diamond* [for zanamivir], and *black circle* [for oseltamivir]) were administered to mice (1 mg given twice daily on days 9 and 11) that had received sialic acid on day 7 and 8 ($P < .05$, for all 3 molecules with respect to mice treated with sialic acid when sampled on both day 9 and day 11). Statistical analysis was performed using a 2-tailed Student's *t* test.

lungs caused by the neuraminidases of respiratory viruses, including influenza A virus, also will increase in the concentration of free sialic acid [43, 44]. Pneumococcal neuraminidases and their role in infection have been a subject of interest for some time and have been the focus of studies describing their desialylation of host cells and involvement in adhesion in vitro and in vivo [3, 26, 27, 41, 42, 45]. Deletion of either *nanA* or *nanB* was shown to have significant effects on nasopharyngeal colonization and pneumonia [34, 46], a finding that strongly matches the proposed role of these enzymes in the novel sialic acid–inducible carriage phenotype described in the present study.

We now propose a new model for the pathogenesis of pneumococcal pneumonia in which a local, virus-induced increase in the free sialic acid concentration in the upper respiratory tract would serve as a signal for colonizing pneumococci to increase in numbers, possibly by promoting the formation of biofilm. In this context, the up-regulation of the pneumococcal neuraminidases would provide a positive feedback loop. The higher colonization density in the nasopharynx would eventually lead to passive shedding of pneumococci to the lower respiratory tract, which, worsened by virus-induced damage to bronchoalveolar cells [47], would finally initiate development of an acute pulmonary infection.

In conclusion, the data from the present study demonstrate that free sialic acid (1) is essential for biofilm formation in vitro at concentrations with physiologic relevance, (2) is able to induce the expression of neuraminidase genes and a virulence regulator, and (3) leads to an increase in nasopharyngeal pneumococcal loads and instigates the spread of pneumococci to the lower respiratory tract. These data support the hypothesis that free sialic acid is a trigger that converts a harmless colonizing pneumococcus to an invasive pathogen. This finding, in addition to adding substantial knowledge to the pathogenesis of pneumococcal disease, points to novel strategies for the prevention of invasive disease, similar to those proposed for the prevention of disease due to nontypeable *H. influenzae* [15], by treating carriage or even overt disease through interventions affecting free sialic acid on human mucosae and, thus, preventing sialic acid from acting as an environmental cue to the pneumococcus.

Acknowledgments

We thank Velia Braione, Tiziana Braccini, and Anna Cuppone for help with laboratory procedures.

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