Discovery and characterization of sialic acid O-acetylation in group B *Streptococcus*

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Group B *Streptococcus* (GBS) is the leading cause of human neonatal sepsis and meningitis. The GBS capsular polysaccharide is a major virulence factor and the active principle of vaccines in phase II trials. All GBS capsules have a terminal α2–3-linked sialic acid [*N*-acetylneuraminic acid (Neu5Ac)], which interferes with complement-mediated killing. We show here that some of the Neu5Ac residues of the GBS type III capsule are O-acetylated at carbon position 7, 8, or 9, a major modification evidently missed in previous studies. Data are consistent with initial O-acetylation at position 7, and subsequent migration of the *O*-acetyl ester at positions 8 and 9. O-acetylation was also present on several other GBS serotypes (Ia, Ib, II, V, and VI). Deletion of the CMP-Neu5Ac synthase gene *neu*A by precise, in-frame allelic replacement gave intracellular accumulation of O-acetylated Neu5Ac, whereas overexpression markedly decreased O-acetylation. Given the known GBS Neu5Ac biosynthesis pathway, these data indicate that O-acetylation occurs on free Neu5Ac, competing with the CMP-Neu5Ac synthase. O-acetylation often generates immunogenic epitopes on bacterial capsular polysaccharides and can modulate human alternate pathway complement activation. Thus, our discovery has important implications for GBS pathogenicity, immunogenicity, and vaccine design.

Streptococcus agalactiae | polysaccharide capsule | Neu5Ac

Group B *Streptococcus* (GBS) is the leading cause of bacterial sepsis and meningitis in human neonates (1) and is increasingly associated with invasive infections in adult populations such as pregnant women, diabetics, and the elderly (2). It is estimated that 20–30% of women of childbearing age are asymptomatic vaginal carriers of GBS (3). Newborns can be infected with GBS *in utero*, due to ascending amniotic infection, or during delivery, by aspiration of contaminated vaginal fluids. Pneumonia, sepsis, and meningitis are potential complications, reflecting an array of bacterial virulence factors that act to impede phagocytic clearance or produce host tissue injury (4). Despite antibiotic prophylaxis during delivery, it is estimated that ≈3,600 neonates develop invasive GBS infections annually in the United States (5).

Anchored to the cell wall of GBS (6) is a thick capsular polysaccharide (CPS) barrier, which is a major virulence factor in animal models of infection (7). To date, the structures of nine antigenically distinct GBS CPS serotypes have been described, containing various arrangements of galactose, glucose, GlcNAc, and the most prevalent sialic acid (Sia) of humans, *N*-acetylneuraminic acid (Neu5Ac) (8–14). Neu5Ac residues of the GBS CPS are situated on the branching terminus of each repeating unit. Studies employing GBS mutants demonstrate that Neu5Ac functions to limit the deposition of alternative complement pathway component C3b on the bacterial surface (15). This opsonization defect results in decreased phagocytosis by macrophages and neutrophils (15). Although never directly proven, GBS interference with C3b deposition is hypothesized to result from recruitment of endogenous factor H by Neu5Ac residues. Animal models of infection confirm that mutants lacking only the terminal Neu5Ac of their CPS are severely attenuated for virulence (16). Thus, the capsular Sia of GBS is a critical attribute for survival in the host. Likewise, cell surface Sia is a common component and virulence factor of many other pathogenic bacteria (17).

The risk of invasive neonatal GBS disease has been correlated with low levels of maternal antibody directed against the CPS (18). For this reason, investigators have long sought to develop a CPS vaccine that could stimulate the mother's humoral immune response and afford passive protection to the neonate by antibody passage across the placenta and through breast milk. Whereas purified GBS CPS vaccines are immunogenic in some individuals, others seem unable to generate significant antibodies against the capsule. CPS-protein conjugate vaccines for the different GBS serotypes have therefore been produced, and have yielded significantly improved antibody responses in animal studies and phase I–II human trials (19).

Here, we show for the first time, to our knowledge, that a significant portion of the native GBS capsular Sia is O-acetylated on the exocyclic polyhydroxylated side chain of Neu5Ac. Certain strains of *Escherichia coli* (20) and *Neisseria meningitidis* (21) are also known to O-acetylate Sias. However, in most instances, the O-acetylated units are internally located within a polysialic CPS. In contrast, we found O-acetylation on outer terminal Neu5Ac residues of the GBS capsule. Moreover, GBS *O*-acetyl esters appear to be added onto free Neu5Ac, rather than on to the fully assembled polysaccharide, as is the case with group C meningococci (22), and *E. coli* (23). We also emphasize that nearly three decades of studies of the GBS CPS have overlooked this modification, in part because of the use of base treatment during CPS purification. Given the potential impact of O-acetylation on host innate response, immunogenicity, and disease pathogenesis, we suggest that some current concepts and approaches regarding GBS need to be reassessed.

Materials and Methods

Bacterial Strains and Culture Conditions. GBS strains were well characterized isolates from human neonates with invasive infection. They were propagated in Todd–Hewitt broth (THB) at 37°C without shaking, unless otherwise specified. GBS stocks were streaked to isolation on THB agar plates before inoculation into broth. Antibiotic selection used 2.5–5 μg/ml chloramphenicol or 10 μg/ml erythromycin (Erm).

Sia Release and NaOH Treatment. Sias were released by using either 2 M acetic acid for 3 h at 80°C or *Arthrobacter urafaciens* sialidase (EY Laboratories) in 50 mM sodium acetate for 3 h at 37°C (24). Whereas acid gives more complete release of Sias, it also induces some migration of *O*-acetyl esters from carbon positions 7–9

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Abbreviations: GBS, group B *Streptococcus*; Sia, sialic acid; Neu5Ac, *N*-acetylneuraminic acid; DMB, 1,2-diamino-4,5-methylene dioxybenzene; CPS, capsular polysaccharide; THB, Todd–Hewitt broth; Erm, erythromycin; mQH_2O, milli-Q water; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; ManNAc, *N*-acetyl mannosamine.

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(24). Some samples were treated with 0.1 M NaOH at 37°C for 30 min to hydrolyze *O*-acetyl esters followed by neutralization with 0.1 M HCl (25).

Use of 1,2-Diamino-4,5-methylene dioxybenzene (DMB)-HPLC Analysis of Sias. Released Sias were derivatized with DMB and resolved by reverse-phase HPLC (26). Sia standards were isolated from bovine submaxillary mucin as described in ref. 24.

Electrospray MS. A Finnigan-MAT (San Jose, CA) HPLC with online mass spectrometer model LCQ-mass spectrometer system was used to verify identities of DMB-derivatized peaks observed in HPLC analysis (27). Fractions eluting from the HPLC runs were collected based on retention times of bovine submaxillary mucin Sia standards, were dried down on a shaker-evaporator, and were stored in the dark. Samples were resuspended in water for HPLC-MS analysis.

GBS Cell Wall Preparation. Serotype III GBS strain COH1 was grown overnight, pelleted, and washed with PBS. The pellet from each 40 ml of overnight culture was resuspended in 1 ml of 100 mM KH_2PO_4 (pH 6.2) with 200 units of purified mutanolysin from *Streptomyces globisporus* (Sigma). Cell wall digestion proceeded overnight at 37°C with gentle agitation. Supernatant from this digestion was dialyzed extensively by using a Spectra Por 2 12,000–14,000 molecular weight cutoff membrane, and was then lyophilized and resuspended in milli-Q water (mQH_2O). Quantitation of Sias in the pellet and supernatant indicated near-complete release of CPS under these conditions.

GBS Type III Capsule Repeating Unit Purification. The GBS cell wall preparation was digested with *Escherichia freundii* endo-β-galactosidase (V-Labs, Covington, LA), which specifically recognizes the trisaccharide-repeating unit of the GBS type III CPS backbone and cleaves the β1–4 linkage between galactose and glucose residues (28). Cell wall preparation and endo-β-galactosidase were brought to concentrations of 0.2 μM and 10 units/ml, respectively, in 50 mM sodium acetate (pH 5.5) and allowed to react for 3 days at 37°C in a toluene atmosphere. The supernatant from this reaction was applied to a Centricon 10,000 molecular weight cutoff spin column. DMB-HPLC analysis of the filtrate and retentate indicated ≈50% release of CPS fragments of a molecular weight <10,000. The filtrate was applied to a charcoal cassette (Thermo Hypersil-Keystone, Shelton, CT), washed with mQH_2O, and eluted with 60% acetonitrile plus 0.1% acetic acid. After evaporation of acetonitrile with nitrogen gas, the sample was lyophilized and resuspended in mQH_2O, then applied to a C18 cassette (Thermo Hypersil-Keystone) and washed with mQH_2O. This material was dried down on a shaker-evaporator and resuspended in mQH_2O for analysis by matrix-assisted laser desorption ionization (MALDI).

MALDI/Time-Of-Flight (TOF) MS. The m/z ratio of purified CPS repeating unit material was determined by using an Applied Biosystems DE-STR MALDI-TOF mass spectrometer. The matrix 2′,4′,6′-trihydroxyacetophenone (THAP) was prepared by dissolving THAP at 1 mg/ml in 50% acetonitrile with 1% diammonium citrate. Data were acquired in VOYAGER 5.1 in reflector/negative ion mode by using an accelerating voltage of 20,000 V, grid voltage 80%, guide wire 0.05%, and an extraction delay time of 180 ns. Data collected in this manner were reprocessed in DATA EXPLORER 4.0.0.0. LS-tetrasaccharide (Calbiochem), which has the mass expected for the unacetylated capsular repeating unit, was used as an external calibrant.

Allelic Exchange Mutagenesis and Complementation of *neu*A. The *neu*A gene, along with ≈500 bp each of upstream and downstream DNA, was amplified by PCR (using forward primer 5′-CGGAATTCCGTTACGGAGCATGATGGTTATCC-3′ and reverse primer 5′-CGGGATCCCGTCTTCTACAATAGCCAGAGCCT-3′) and T-A cloned into plasmid pCR2.1-TOPO (Invitrogen) to give pNeuA-TV. Inverse PCR of pNeuA-TV was performed by using outwardly directed primers corresponding to (*i*) the sequence immediately upstream of the *neu*A ATG start codon and (*ii*) the sequence immediately downstream of the *neu*A TAA stop codon; these primers contained 25-bp 5′ extensions corresponding to the 5′ and 3′ ends of the chloramphenicol acetyltransferase (*cat*) gene, respectively The linearized amplicon lacking the *neuA* coding region was used to cotransform *E. coli* along with a full-length *cat* gene DNA fragment. Transformants were identified where homologous recombination had generated the plasmid pNeuAΔCAT, containing an in-frame allelic replacement of *neu*A with the *cat* gene. The *cat* gene with flanking GBS DNA was subcloned into temperature-sensitive vector pHY304 bearing Erm resistance to yield the knockout vector pNeuA-KO. Wild-type GBS strain COH1 was transformed by electroporation with pNeuA-KO with selection on Erm at 30°C. Chromosomal integration events were identified by a shift to the nonpermissive temperature (37°C) under Erm plus chloramphenicol selection, followed by relaxation in the absence of antibiotics to identify double crossover mutants exhibiting chloramphenicol resistance but Erm sensitivity. Precise in-frame allelic replacement of *neu*A by *cat* in the GBS chromosome was confirmed by PCR. A plasmid for complementation analysis of the GBS COH1Δ*neu*A mutant was generated by subcloning the original *neu*A-containing PCR amplicon from pNeuA-TOPO vector into the Gram-positive expression vector pDCerm to produce pDC*neu*A.

Separation of Capsular (Bound) and Intracellular (Free) Sias. GBS were grown overnight, pelleted, and washed well with PBS. Cells were resuspended in 20 mM Tris·HCl/10 mM $MgCl_2$ (pH 7.4), boiled at 100°C for 20 min, then subjected to three rounds of freeze-thawing, and the lysate was centrifuged at 20,000 × g for 2 min. Capsular Sias were released from the insoluble cell lysate material by treatment with *Agrobacterium tumefaciens* sialidase. Intracellular and free Sias were enriched from the lysate supernatant by 70% ethanol precipitation overnight at −20°C. Supernatant from this precipitation was then passed over a Centricon 3,000 molecular weight cutoff spin column, and the low-molecular-weight fraction was analyzed by DMB-HPLC.

Results

Type III GBS Sia Is Endogenously O-Acetylated. An extensive prior literature indicates that the sole Sia of the GBS CPS is Neu5Ac. Our analysis of acid-released GBS Sias by DMB derivatization and reverse-phase HPLC unexpectedly revealed that in addition to Neu5Ac, there were peaks corresponding to retention times of DMB-derivatized standards for 7-, 8-, and 9-mono-O-acetylated Neu5Ac (Neu5,7Ac_2, Neu5,8Ac_2, and Neu5,9Ac_2; Fig. 1*A*). Because the THB medium used to grow GBS contains some O-acetylated Sias (data not shown), and some bacterial pathogens can incorporate Sias from their milieu, we next used the defined RPMI medium 1640, which lacks Sias. Although the HPLC profiles of Sias from GBS grown in the two different media varied slightly, the persistence of O-acetylated Neu5Ac peaks (Fig. 1*A*) indicates that GBS O-acetylates Sias endogenously. Because *O*-acetyl esters on Sias are susceptible to alkaline hydrolysis, we applied mild-base treatment to GBS Sias as described in *Materials and Methods*. The peaks corresponding to O-acetylated Neu5Ac did in fact disappear after NaOH treatment (Fig. 1*A*), with a corresponding increase in the Neu5Ac peak (data not shown). Tandem HPLC-electrospray MS confirmed the presence of molecules with the expected mass of DMB-derivatized O-acetylated Neu5Ac eluting at the timing expected for Neu5,7Ac_2, Neu5,8Ac_2, and Neu5,9Ac_2 (Fig. 1*B*).

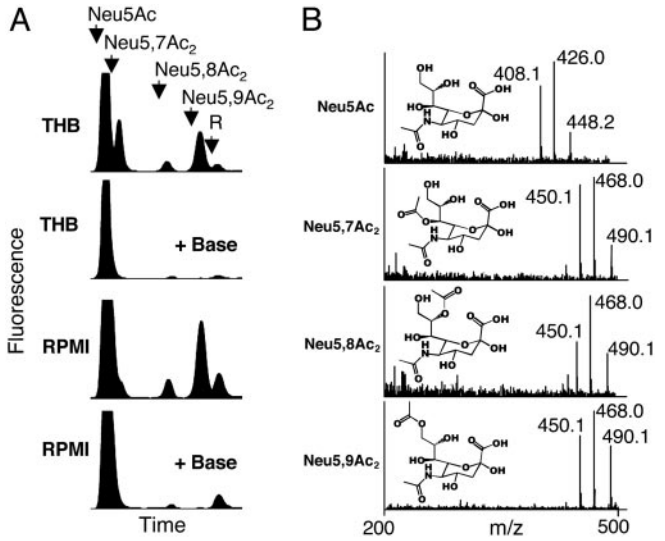

Fig. 1. GBS Sia is endogenously O-acetylated. (*A*) DMB-HPLC analysis of GBS serotype III strain COH1 grown in THB (contains Sias) or RPMI medium 1640 (chemically defined, contains no Sias). Cells were pelleted, washed, and Sias were acid-hydrolyzed and DMB-derivatized. Half of each sample was treated with 0.1 M NaOH for 30 min at 37°C before derivatization to hydrolyze *O*-acetyl esters. R, a reagent peak of unknown identity. (*B*) Tandem HPLC-electrospray MS was performed on DMB-derivatized Sia peaks, each collected separately from the analysis in *A*. The expected m/z ratios of DMB-derivatized Neu5Ac and O-acetylated Neu5Ac are 426.0 and 468.0, respectively. The other peaks likely represent the sodium adduct (+22) or dehydrated forms (−18). (*Insets*) Shown are prederivatized Sia structures based on the mass/charge ratio and the HPLC retention times in *A*.

The *Insets* in Fig. 1*B* depict underivatized Sias corresponding to the HPLC retention times and m/z ratios of DMB-derivatized molecules shown in Fig. 1 *A* and *B*, respectively. Of note, conventional methods for CPS purification include a base-treatment step to remove traces of contaminating group B carbohydrate (29), which would unavoidably hydrolyze Sia *O*-acetyl esters.

Sia O-Acetylation Is a Common Feature of GBS Strains. To determine whether GBS Sia O-acetylation is a general phenomenon or restricted to particular strains, Sias were hydrolyzed from ten GBS clinical isolates of various serotypes, followed by DMB-HPLC analysis. As shown in Fig. 2, all strains tested exhibited some level of Sia O-acetylation, ranging from ≈5% to ≈55% of total Sia. Notably, both high and low levels of O-acetylation could be seen amongst strains of the same capsule serotype (e.g.,

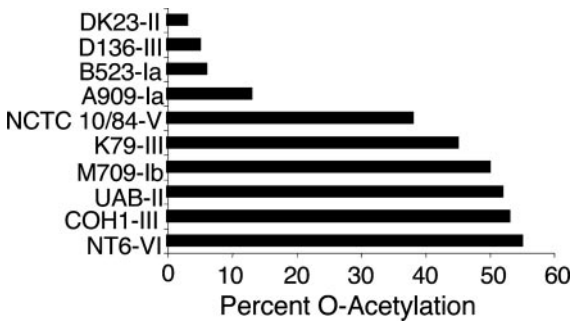

Fig. 2. GBS Sia O-acetylation is not strain-specific. Sias were acid-hydrolyzed from 10 clinical GBS isolates of various serotypes (strain-serotype) and were analyzed by DMB-HPLC. The percent of total Sias that were O-acetylated is shown.

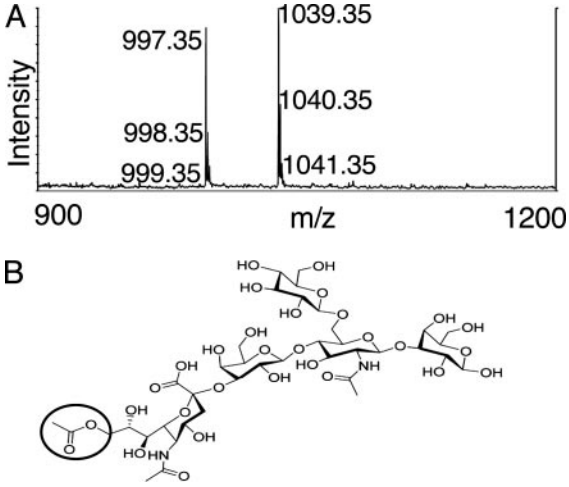

Fig. 3. GBS capsular Sia is O-acetylated. (*A*) MALDI-TOF analysis of purified type III CPS repeating unit. Cell-wall preparations from COH1 were digested and the released fragments further purified as described in *Materials and Methods*. In negative mode, the expected m/z ratios of the GBS CPS repeating units with and without O-acetylation are 997.35 and 1039.35, respectively. (*B*) Revised structure of GBS type III CPS repeat unit. Note that an *O*-acetyl ester can migrate from carbon position 7 to position 9, but is shown here at position 9 (circled). As indicated in Figs. 1 and 2, only a portion of the capsular Sia is O-acetylated.

II and III). With the exception of type Ia strain A909 that showed some variation, repeated analyses of GBS strains yielded similar relative levels of O-acetylation. The bacteria used in this analysis were grown in THB, which contains Neu5Ac and Neu5Gc, along with their O-acetylated derivatives. However, the bacteria were extensively washed before analysis, and the absence of detectable Neu5Gc and derivatives indicates effective removal of contaminating media, and lack of metabolic incorporation by the bacteria.

GBS Capsular Sia Is O-Acetylated. To confirm that the O-acetylated Neu5Ac found in the acid hydrolysate actually originated from the GBS capsule, a method was developed to purify repeating units of the CPS for analysis by MALDI-TOF. The murolytic enzyme mutanolysin was used to release a cell-wall extract of serotype III GBS as described in *Materials and Methods*. After dialysis and lyophilization, the cell-wall extract was further digested with endo-β-galactosidase, which could potentially recognize the trisaccharide-repeating unit of the GBS type III CPS backbone, cleaving the β1–4 linkage between galactose and glucose residues (8). We used the commercially available enzyme from *E. freundii*, which was previously known to hydrolyze sulfated and nonsulfated poly-*N*-acetyl-lactosaminoglycans (28), but not known to tolerate sialylated oligosaccharide side chains such as those present on the type III GBS CPS. Further purification steps were followed by MALDI-TOF analysis, which gave monoisotopic resolution, showing the expected masses of CPS repeating units with and without a single *O*-acetyl group (Fig. 3*A*). NMR was not performed on the purified repeating unit as an additional proof of its structure; however, we emphasize that there is no alternative explanation for the mass spectrometric observations presented above. A revised structure of the GBS capsular repeating unit including an *O*-acetyl group on carbon position 9 of Neu5Ac is shown in Fig. 3*B*. In reality, the *O*-acetyl ester could be at position 7, 8, or 9 (see below).

Migration of *O*-Acetyl Esters on GBS Sias. When grown in nutrient rich media, *O*-acetyl esters on GBS Sias released by acid

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hydrolysis were observed at carbon positions 7–9 (Fig. 1*A*). Notably, if hydrolysates were derivatized and analyzed within a 12-h period, much of the O-acetylation was at position 7. If the analysis was delayed over a 2- to 3-day period, more of the O-acetylation was at position 9, especially if samples were not constantly kept on ice (data not shown). *O*-acetyl esters were also observed more frequently at position 9 for RPMI medium 1640-grown cells when compared with their THB-grown counterparts (Fig. 1*A*). Also, if Sias were released directly from the bacteria by a sialidase and analyzed immediately, the *O*-acetyl esters were found predominantly at position 7 (data not shown). Migration of the GBS Sia *O*-acetyl ester from carbon positions 7–9 could also be induced by using mild ammonium hydroxide treatment conditions (data not shown; ref. 25). Based on all of the data, it appears likely that enzymatic addition of an *O*-acetyl ester occurs primarily at carbon position 7, with subsequent migration to carbon position 9, and to a lesser extent, position 8. Such O-acetyl migration on the side chain of Sias has been described in other types of biological samples (24) and is known to occur with a $t_{1/2}$ of several hours under physiological conditions (30). Migration of *O*-acetyl groups on Sias of bacterial polysaccharides was first described for types C and W-135 polysialic acid CPS of *N. meningitidis* (31).

GBS Sia O-Acetylation Occurs Before CMP Activation. The genetic basis of Sia biosynthesis in GBS has been localized to a cluster of genes at the transcriptional end of the 16-gene capsule operon (32). Similar to *E. coli* (33, 34), GBS gene products of *neuC*, *neuB*, and *neuA*, respectively, catalyze the epimerization of UDP-GlcNAc to *N*-acetyl mannosamine (ManNAc) (35), the condensation of ManNAc and phospho*enol*pyruvate to form Neu5Ac (36), and the activation of Neu5Ac with CTP, to generate CMP-Neu5Ac (37). An α2–3 sialyltransferase encoded by *cpsK* then catalyzes the transfer of activated Neu5Ac to the assembling CPS (38). To define the timing of O-acetylation in the known GBS Sia biosynthesis pathway, we created a precise allelic replacement of the GBS CMP-Sia synthase *neuA* in the type III strain COH1. A comparative analysis of capsular vs. free Sias was then performed. Briefly, free Sias were isolated in a low molecular weight fraction of ethanol-soluble cell lysate, whereas capsule-associated Sias were released by sialidase treatment of the cell lysate pellet. Sias were then analyzed by DMB-HPLC.

As expected, the COH1Δ*neu*A mutant could not CMP-activate Sias for transfer to the CPS and thus, had no Sia in its capsule (Fig. 4). However, this mutant accumulated Neu5,7Ac$_2$, Neu5,8Ac$_2$, and Neu5,9Ac$_2$ intracellularly, indicating that O-acetylation occurs before CMP activation of Neu5Ac (Fig. 4). Upon complementation of the mutant with an expression vector encoding wild-type NeuA (pDC*neu*A), capsular Sia was fully restored; however, substantially less overall O-acetylation was observed (Fig. 4). Overexpression of NeuA by transformation of wild-type COH1 with the pDC*neu*A expression vector produced a similar decrease in Sia O-acetylation (Fig. 4). Taken together, the effects of NeuA elimination and NeuA overexpression on Sia O-acetylation indicate a model wherein the GBS Sia O-acetyltransferase acts on free intracellular Neu5Ac, competing with NeuA for substrate (Fig. 5). Thus, when NeuA is absent, O-acetylated Sias accumulate in the cell. In contrast, when NeuA is overexpressed, the enzyme outcompetes the *O*-acetyltransferase for the Neu5Ac substrate, thus decreasing the overall amount of O-acetylation.

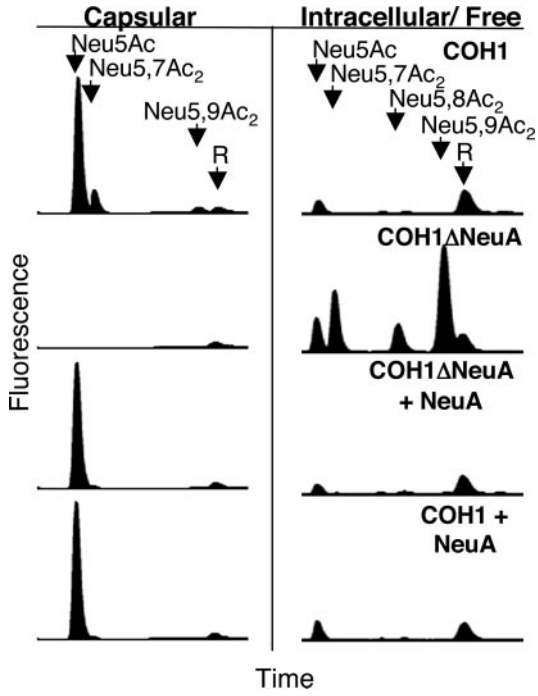

Fig. 4. GBS Sia O-acetylation occurs before CMP activation. Sias were separated based on association with either the CPS or the intracellular Sia pool, and were analyzed by DMB-HPLC. This separation was carried out for the wild-type III strain COH1, an isogenic strain (COH1Δ*neuA*) in which n*euA* (the gene encoding the GBS CMP-Sia synthase) was replaced with an antibiotic resistance gene, and both of these strains were transformed with a *neuA* expression construct pDC*neu*A (+NeuA). This experiment was performed on several occasions with similar results. It should be noted that DMB derivatization is carried out under conditions that result in a combined quantitation of Neu5Ac and CMP-Neu5Ac.

Discussion

We have used definitive structural and chemical methods to show that a significant portion of the native GBS CPS is endogenously O-acetylated. Genetic manipulation of the GBS CMP-Sia synthase *neuA* by either allelic exchange mutagenesis or overexpression provided data consistent with O-acetylation occurring at the level of free intracellular Neu5Ac rather than on the assembled polysaccharide. It appears that an unidentified GBS *O*-acetyltransferase acts on Neu5Ac to generate Neu5,7Ac$_2$. The *O*-acetyl ester can then migrate from carbon position 7 to position 9 under the conditions of our analysis and potentially *in vivo*, under physiological conditions. Based on our analysis of 10 clinical isolates, O-acetylation is common among pathogenic GBS strains, but occurs at various levels. The conservation of O-acetylation of GBS CPS Sia in all strains studied suggests it confers a selective advantage to the organism at some point in its life cycle.

In addition to the effects that O-acetylation could have on human antibody response and reactivity (see below), it is possible that this modification also alters bacterial susceptibility to certain host defense factors. For example, there is considerable evidence that Sia 9-O-acetylation can enhance activation of the alternate pathway of complement (39–41). Sia O-acetylation has also been shown to inhibit the activity of mammalian sialidases (42), which may allow GBS to preserve Sias on the CPS surface. Finally, O-acetylation could benefit GBS survival by blocking recognition of Sia by Sia-Recognizing Ig-Superfamily Lectin (Siglec) receptors on host leukocytes (43). In this regard, sialoadhesin (Siglec 1) has recently been shown to promote macrophage uptake of *N. meningitidis* by means of its sialylated lipopolysaccharide (44).

The early literature on GBS CPS purification and analysis contains a hint that Sia was O-acetylated. In the original structural description of the type III CPS, there is mention of an NMR peak and an acknowledgment that it could be

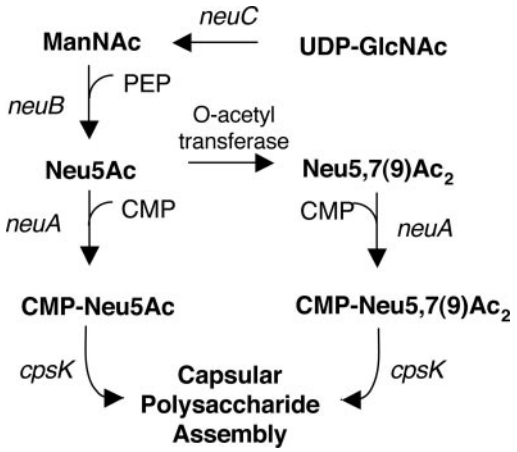

Fig. 5. A model for Sia O-acetylation in GBS. Sia biosynthesis in GBS proceeds by epimerization of UDP-GlcNAc to ManNAc, the condensation of ManNAc and phospho*enol*pyruvate (PEP) to form Neu5Ac, and the CMP activation of Neu5Ac. Gene products encoded by *neuC*, *neuB*, and *neuA* catalyze the respective steps above. An α2–3 sialyltransferase encoded by *cpsK* then catalyzes the transfer of activated Neu5Ac to the assembling CPS. Sia analysis of the NeuA mutant demonstrates that O-acetylation can occur on free Sia (Neu5Ac) through the action of an unidentified O-acetyltransferase. The observed decrease in O-acetylation upon overexpression of NeuA also supports a model in which NeuA competes with the *O*-acetyltransferase for substrate. This model suggests that the GBS CMP-Sia synthase and the sialyltransferase can act on O-acetylated Sias.

"consistent with the identification of a small amount of *O*-acetyl groups in the native type III antigen" (10). However, Sia O-acetylation was not reported as part of the CPS structure in that paper. Later publications may have been missed this modification because strains with low levels of O-acetylation were studied or because NMR instruments were less sensitive. Ultimately, studies describing purification of the CPS for vaccine preparation introduced a 1-M NaOH treatment to eliminate the contaminating group B polysaccharide (29). This treatment has the undesired effect of removing *O*-acetyl groups on Sias. Although the vast majority of the original structural characterizations did not use base treatment (except for serotypes VII and VIII), they nonetheless did not report the presence of Sia O-acetylation. Thus, the literature of the past 25 years has not addressed important issues regarding the contribution of this native GBS capsule modification to immunogenicity and disease pathogenesis.

Of obvious importance to future studies is the identification of the GBS Sia *O*-acetyltransferase. Despite discovery of O-acetylated Sias in a number of microbial and mammalian systems and biochemical characterization of several Sia *O*-acetyltransferases in biological extracts (23, 25, 45), definitive biochemical identification of a Sia *O*-acetyltransferase has yet to be achieved in any system. In this regard, a recent publication describes a putative Sia *O*-acetyltransferase in serogroup C *N. meningitidis* that is homologous to the NodL-LacA-CysE (a.k.a. the hexapeptide repeat) family of acetyltransferases (46). Although no biochemical evidence is presented, the authors provide genetic data indicating a role for this gene in Sia O-acetylation (46). GBS *neu*D is a gene homologous to the hexapeptide repeat acetyltransferase family of enzymes, which is nestled among the genes involved in GBS Sia biosynthesis. The NeuD ortholog (47) in *E. coli* K1 has been pursued but never proven responsible for Sia O-acetylation; however, as evidenced by the disappearance of Sia upon mutation of *E. coli* K1 *neu*D, the enzyme does play some role in Sia biosynthesis (47). In our preliminary studies, plasmid integrational mutagenesis of the GBS *neu*D gene likewise resulted in an asialo- phenotype that is complemented by the introduction of a *neuD* expression construct (A.L.L., V.N., and A.V., unpublished data). Assigning an O-acetylation function to this gene is thus likely to require more elaborate analyses such as site-directed mutagenesis. Analysis of functional protein sequence polymorphisms or transcriptional regulatory systems that may contribute to varied acetylation among GBS strains can be explored once the genetic and biochemical identification of the true *O*-acetyltransferase(s) is accomplished.

Three species of bacterial pathogens are now known to modify Sia by O-acetylation, but GBS displays a different O-acetylated structure and synthesizes it by a distinct mechanism. Whereas strains of *N. meningitidis* and *E. coli* have capsules made up entirely of repeating units of Sia, GBS appears unique in its display of O-acetylated Sias at branching terminal positions of a multicomponent polysaccharide capsule. Because the Sias of most meningococcal and *E. coli* capsules are linked together at either carbon position 8 or 9, (i.e., α2–8 or α2–9 linkages), O-acetylation in this context is limited to particular carbons, and is mostly internal to the polysaccharide. In contrast, the GBS capsule displays Sia in a terminal position, in an α2–3 linkage. Here, the Neu5Ac side chain is free of covalent interactions that restrain the placement and migration of an *O*-acetyl ester, similar to *N. meningitidis* W-135, which also displays an *O*-acetyl ester on a terminal Sia, which can migrate from position 7 to position 9 (31). The physiological relevance of *O*-acetyl migration is unknown, although it is tempting to speculate that such a "moving target" is more difficult for the host immune system to recognize. Another interesting aspect of GBS Sia O-acetylation is its placement within the Sia biosynthesis pathway. Whereas the *E. coli* K1 *O*-acetyltransferase is known to act at the level of the assembled polysialic acid capsule (23), GBS appears to act at the level of free intracellular Sias, adding the *O*-acetyl ester even before Sia transfer to the polysaccharide chain. This finding also stands in contrast to enzymatic studies of mammalian Sia *O*-acetyltransferases, which indicate addition of *O*-acetyl esters either to intact glycoproteins (48) or glycolipids (49), or to the activated CMP-Sia form (45). Another difference is that the mammalian *O*-acetyltransferases appear capable of adding multiple *O*-acetyl esters to a single Sia molecule, generating 7 (8)9-di-O-acetylated and 7/8/9 tri-O-acetylated Sias (25). We, however, only observed mono-O-acetylated Neu5Ac in GBS.

There are several examples where O-acetylation of bacterial polysaccharides appears to alter host immune responses, including *O*-acetyl groups on the uronic acid residues of *Staphylococcus aureus* types 5 and 8 (50); glucuronic acid, ManNAc, and glucose residues of *Streptococcus pneumoniae* 9V (51); ManNAc-phosphate residues of *N. meningitidis* serogroup A (52); *N*-acetylgalactosaminuronic acid residues of *Salmonella typhi* Vi (53); and, *N*-acetylneuraminic acid residues of *N. meningitidis* serogroup C (54–56) and *E. coli* K1 (20). In the last two instances mentioned above, the O-acetylation was present on Sia residues of polysialic acid capsules.

The unique structure of the GBS Sia epitope poses special considerations regarding its role in immunogenicity. The trisaccharide side chain of GBS serotypes Ia, III, V, and VII capsules (Neu5Acα2–3Galβ1–4GlcNAc) is identical to a common sequence terminating N- and O-glycans on the surface of all mammalian cells. Interestingly, side-chain O-acetylation of α2–3-linked Sias has never been described in any mammalian cell. Rather, endogenous side chain (7/8/9) O-acetylation in mammalian cells has so far been confined to α2–6- and 2–8-linked Sias (57). Thus, elimination of the native O-acetylation of GBS Sia by base treatment of capsule preparations potentially removes an immunogenic bacterial epitope, leaving a structure that more closely resembles host glycans.

Conceivably, the puzzling discovery of circulating antibodies in some humans that recognize O-acetylated Sia (58, 59) reflects an immunologic response to the GBS bacterium. Some investigations have suggested that conjugation of the capsule to proteins or biotin through oxidation and reductive amination of the Sia side chain may alter immunogenicity and/or recognition in ELISAs (60). This Sia side chain happens to be the very same one that we have found to be O-acetylated in the native capsule. Strain variation in GBS O-acetylation could contribute to variable human immunologic responses to the organism. The impact of this modification on the immunogenicity of GBS CPS-based vaccine formulations also merits investigation.

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