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ACCELERATED PUBLICATION

O-Acetylation of sialic acid on Group B *Streptococcus* inhibits neutrophil suppression and virulence

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GBS (Group B *Streptococcus*) requires capsular Sia (sialic acid) for virulence and partially modifies this sugar by O-acetylation. In the present paper we describe serotype-specific patterns of GBS Sia O-acetylation that can be manipulated by genetic and biochemical means. *In vitro* and *in vivo* assays demonstrate that this subtle modification attenuates GBS Sia-mediated neutrophil suppression and animal virulence.

Key words: capsular polysaccharide, neutrophil, O-acetylation, sialic acid, *Streptococcus agalactiae*.

INTRODUCTION

Complex carbohydrates (glycans) on bacterial cell surfaces can sometimes elicit extraordinary phenotypes in biological systems, in particular when they closely resemble host cell-surface glycan motifs [1–3]. One prominent example of microbial carbohydrate mimicry involves the Sias (sialic acids). Sias are nine-carbon backbone acidic sugars displayed prominently on the surfaces of all vertebrate cells. In mammals, the most common Sia is Neu5Ac (*N*-acetylneuraminic acid), a cell-surface molecule with wide-ranging roles in mammalian physiology, participating in renal filtration, neuronal plasticity and suppression of innate and adaptive immune responses [4–9]. Neu5Ac is also expressed by several important human pathogens, and this molecular resemblance to the host facilitates infection by multiple mechanisms [10–14]. For example, Neu5Ac blocks cellular opsonization by the alternative pathway of complement [8,10,13,15] by interacting with a key counter-regulator of alternative complement, Factor H [8]. Neu5Ac expression also allows bacteria to engage Siglecs, a family of 14 Sia-binding immunoglobin superfamily lectins expressed on human leucocytes [1,4,16–18], many of which possess intracellular tyrosine-based inhibitory motifs thought to restrict immune activation against 'self' [4].

Genomic approaches have identified a growing number of mammalian pathogens that express Sias or related molecules, suggesting that this form of immune evasion may be more common than currently appreciated [19]. Chemical modifications of Sia structure have also been reported in a number of pathogens that engage in this form of molecular mimicry [20–25]. However, the impact of Sia structural variation on the potential for invasive bacterial infection has not been studied.

GBS (Group B *Streptococcus*) is a Gram-positive opportunistic pathogen and a model system for understanding the mechanisms and consequences of Sia molecular mimicry. A leading cause of bacterial sepsis and meningitis in newborns, GBS asymptomatically colonizes the lower gastrointestinal and vaginal mucosa in up to one-third of women sampled at a single time and two-thirds of women sampled at multiple times over a year [26,27]. Invasive GBS disease can occur following ascending infection of placental membranes or aspiration by the neonate during the birthing process [28]. A critical virulence factor of GBS is its surface CPS (capsular polysaccharide), the outermost glycan layer surrounding the bacterium. Although each serotype strain (nine in all) expresses an antigenically unique structure [29], all display terminal α2-3-linked Sia residues, which are identical with host Sia motifs and essential to GBS virulence [11]. During invasive GBS disease, Sias promote immune evasion and bacterial proliferation by suppressing the alternative complement pathway [10], impairing opsonophagocytosis, and by engaging the neutrophil receptor Siglec-9, to suppress the oxidative burst and release of granule proteases [1]. Currently available biochemical evidence suggests that CPS is the only GBS surface structure bearing Sias.

It was previously discovered that GBS partially O-acetylates terminal Sias at the 7-carbon position, a modification that spontaneously migrates to the 9-position (via the 8-position) in a pH-dependent and unidirectional manner [25]. The ultimate level of Sia O-acetylation is determined by the relative activities of a GBS Sia *O*-acetyltransferase, NeuD [30], and a Sia-specific *O*-acetylesterase. The GBS *O*-acetylesterase is linked by gene fusion to the C-terminus of a CMP-Sia synthetase essential for nucleotide activation of Sia prior to capsular assembly, together forming the dual-activity enzyme NeuA [31,32]. Using non-polar gene deletion and site-directed mutagenesis to specifically manipulate GBS Sia *O*-acetylesterase activity, we previously demonstrated that levels of surface Sia O-acetylation can be driven up or down as a single chemical variable [33]. Molecular analyses of these strains indicated that high levels of Sia O-acetylation disrupts interactions with human Siglec-9, but does not alter

Abbreviations used: cfu, colony-forming unit; CPS, capsular polysaccharide; GBS, Group B *Streptococcus*; HBSS, Hanks balanced salt solution; MOI, multiplicity of infection; Neu5Ac, *N*-acetylneuraminic acid; NICHD, National Institutes of Child Health and Development; Sia, sialic acid; THB, Todd–Hewitt Broth; UCSD, University of California, San Diego.

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deposition of complement on the GBS surface [33]. In the present study we combine biochemical analyses with cellular and animal infection models to investigate the prevalence and patterns of Sia O-acetylation in native GBS populations and, for the first time, to define the physiological significance of bacterial Sia O-acetylation during invasive infection.

EXPERIMENTAL

Bacterial strains and growth conditions

GBS strains with high (~75%) and low (<5%) levels O-acetylation were generated in the serotype III background by allelic variation of the NeuA Sia *O*-acetylesterase and were defined previously by thorough biochemical analyses [31,33]. These isogenic bacterial strains were grown in THB (Todd–Hewitt Broth; Difco, BD Diagnostics) containing 5 μg/ml erythromycin. For infection studies, bacteria were cultivated at 37 °C to mid-exponential phase and resuspended to a D_{600} of 0.4, followed by serial dilution and enumeration of cfus (colony-forming units) in each experimental inoculum. GBS isolates used in biochemical studies were from newborns who developed early-onset GBS disease (invasive strains) or newborns that were colonized but did not develop GBS disease (colonizing strains) from the NICHD (National Institutes of Child Health and Development) multi-centre study [34,35]. NICHD strains were cultivated overnight in THB without antibiotics. The strains were obtained from infants with IRB (Institutional Review Board) approval from participating institutions from the multi-centre study. GBS strains engineered to express alternate serotype Ia or III capsule polymerase genes [36] were grown in the presence of 5 μg/ml chloramphenicol.

Biochemical analysis of Sia O-acetylation

Bacterial pellets from 1 ml of culture were washed and Sias were released by mild acid hydrolysis, isolated, derivatized with 1,2-diamino-4,5-methylene dioxybenzene, and a small aliquot analysed by HPLC in parallel with Sia standards as described previously [25,31]. The percentage of Sia O-acetylation was determined by automated integration of peak areas for 7- and 9-O-acetylated Neu5Acs compared with areas for all Sias combined. In a subset of strains, 0.1 M NaOH treatment [25] was used to verify the identity of peaks corresponding to O-acetylesters; these data were very similar to results produced by the peak integration method. The rank-sum (Mann–Whitney) test was used to evaluate statistical differences in O-acetylation between serotypes.

Neutrophil isolation

Normal human volunteers donated small blood samples for the isolation of neutrophils, with informed consent under protocols approved by the UCSD (University of California, San Diego) Human Subjects Institutional Review Board. Neutrophil isolation was performed using the Polymorphprep system (Axis-Shield) and resuspended in HBSS (Hanks balanced salt solution) without Ca^{2+} or Mg^{2+}.

Neutrophil granule protease release

Bacterial strains resuspended in HBSS with Ca^{2+}/Mg^{2+} (Hyclone) were added to neutrophils at a MOI (multiplicity of infection) of 10 or 25. After incubation at 37 °C for 30 min with orbital rotation, tubes were centrifuged at 1000 ***g*** for 5 min and the supernatant collected into a 96-well microtitre plate. A 0.5 μl volume of 20 mM *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide (Sigma) dissolved in DMSO was added to each well. After incubation at room temperature (22–23 °C) for 20 min, hydrolysis of the substrate was monitored spectrofluorimetrically by the change in absorbance at 405 nm. Assays were performed in triplicate and repeated at least three times. Paired two-tailed Student's *t* tests were used for statistical evaluation.

Neutrophil oxidative burst

Neutrophils were labelled with dichlorofluorescin diacetate (Sigma) at a final concentration of 20 μM in HBSS by incubation for 20 min at 37 °C, then resuspended in 1 ml of HBSS. Approx. 10^6 neutrophils in 100 μl of HBSS were combined with bacteria (MOI 10–50) in 50 μl of HBSS with Ca^{2+}/Mg^{2+}. Cells were spun down at 1000 ***g*** for 5 min to initiate contact, then resuspended and incubated at 37 °C for up to 45 min with orbital rotation. Aliquots (50 μl) were removed at 15 min intervals and the oxidative burst was measured using a FACSCaliber flow cytometer (BD Biosciences). Neutrophils displaying positive oxidative burst were gated and the mean fluorescence intensity was calculated from this subpopulation using FlowJo software. Results shown are representative of experiments performed at least five times.

Bacterial survival in whole blood

Blood was drawn from healthy volunteers into heparinized tubes with informed consent under protocols approved by the UCSD Human Subjects Institutional Review Board. Either 10^3 or 10^4 cfus of bacteria in 100 μl of PBS were added to 300 μl of fresh whole blood and incubated at 37 °C with orbital rotation. Aliquots of 25 μl were removed and plated in serial dilutions for enumeration of surviving bacteria at various time points up to 2 h. Statistical significance was evaluated by paired two-tailed Student's *t* tests.

Mouse infection studies

All animal experiments were approved by the UCSD Committee on the Use and Care of Animals and were performed using accepted veterinary standards. Outbred 9-week-old male CD-1 mice (Charles River Laboratories) were injected intraperitoneally with approx. 4×10^7 bacteria in a total volume of 150 μl mixed 1:2 with autoclaved 10% gastric mucin (MP Biomedicals) as described previously [37]. At 13 h post-infection, blood was collected from the retro-orbital vein and bacterial titres were determined by serial dilution and plating. The statistical significance of bacterial blood titres was evaluated by a paired two-tailed Student's *t* test. Survival studies were conducted using the same procedure and animals were monitored for 10 days. Statistical comparisons of survival curves were performed using the Log-rank (Mantel–Cox) test.

RESULTS

Conserved serotype-specific patterns of GBS O-acetylation

Previous studies have shown that all tested GBS strains (24 in all, representing nine serotypes) have detectable, but highly variable, levels of Sia O-acetylation [25,31,38]. It is unclear whether patterns of GBS O-acetylation are serotype-specific or whether different levels of this modification, regardless of serotype, may be associated with different clinical outcomes. To further clarify the prevalence and patterns of O-acetylation in natural GBS populations, we performed a quantitative analysis

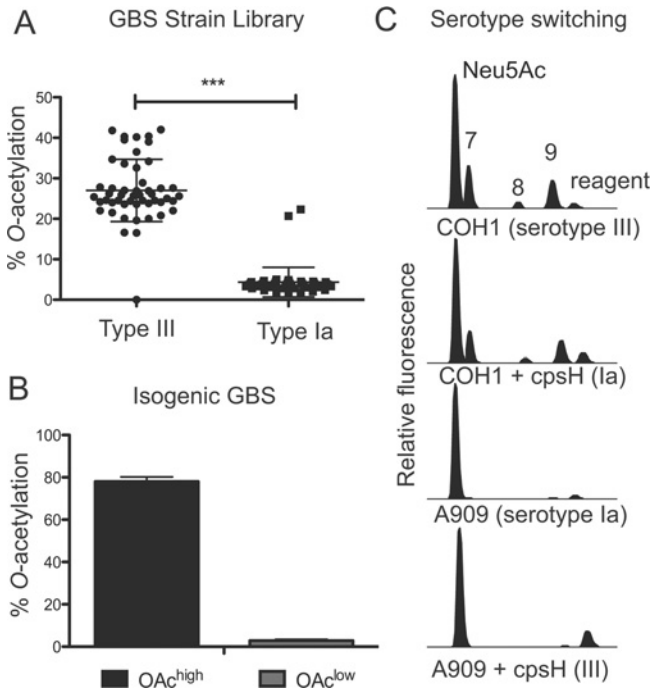

Figure 1 Natural patterns and isogenic manipulation of O-acetylation phenotypes in GBS

(**A**) Native O-acetylation levels were evaluated by HPLC following 1,2-diamino-4,5-methylene dioxybenzene derivatization as described in the Experimental section, from 99 clinical and colonizing isolates. Results were analysed using the Mann–Whitney test and indicate a significant correlation between GBS serotype and Sia O-acetylation phenotype, where type III strains have high levels of O-acetylation and type Ia strains have low levels (***$P < 0.0001$). (**B**) Experimental variation of Sia O-acetylation as a single biochemical parameter was accomplished by isogenic manipulation of the NeuA Sia *O*-acetylesterase as described previously [33]. (**C**) HPLC of fluorescently derivatized Sias from previously published strains where the capsule serotype is switched between Ia and III, by overexpression of alternate capsule polymerase genes (cpsH). COH1 is a serotype III reference strain, whereas A909 is a reference strain; both were isolated from serious neonatal infections.

Serotype	Strain	Polymorphisms		%OAc
		NeuD-88	NeuB-57	
III	**COH1**	F	A	40.5
	NICHD-1	F	A	27.1
	NICHD-2	F	A	21.8
	NICHD-3	F	A	38.5
	NICHD-4	F	A	19.6
	NICHD-5	F	A	36.6
	NICHD-6	F	A	21.4
	NICHD-7	F	A	20.7
	NICHD-8	F	A	16.4
	NICHD-9	F	A	20.1
	NICHD-10	F	A	20
	D136	C	E	3.7
	NICHD-39	ND	ND	0

Serotype	Strain	Polymorphisms		%OAc
		NeuD-88	NeuB-57	
Ia	**A909**	C	E	3.7
	NICHD-51	C	E	2.4
	NICHD-52	C	E also P176L	4.5
	NICHD-53	C	E	2.8
	NICHD-54	C	E	1.8
	NICHD-56	C	E	3.1
	NICHD-57	C	E	3.4
	NICHD-58	C	E	4.4
	NICHD-59	C	E	3.3
	NICHD-60	C	E	4.3
	NICHD-61	C	E	3.8
	NICHD-78	F	A	22.3
	NICHD-90	F	A	20.7

Figure 2 Polymorphisms in the Sia biosynthetic gene locus from strains in Figure 1(A)

Variations at amino acid positions 88 and 57 respectively of the NeuD *O*-acetyltransferase and NeuB Sia synthase sequences correlate with O-acetylation phenotypes. ND, not determined (this strain had little Neu5Ac expression).

of Sia O-acetylation in serotypes Ia and III that cause disease in the U.S.A. Structural differences in serotype Ia and III polysaccharides arise from distinct glycosyltransferases (24 % identity at the amino acid level) that are poised in the same position of the capsule operon, but which polymerize identical oligosaccharide-repeating units in a slightly different manner [36]. Nearly 100 GBS isolates were evaluated in all (44 colonizing and five invasive type Ia strains, and 43 colonizing and seven invasive type III strains).

Sia analyses were performed by acid hydrolysis and 1,2-diamino-4,5-methylene dioxybenzene derivatization, followed by reverse-phase HPLC resolution of O-acetylated and non-O-acetylated Sias as described previously [25]. The present study revealed that the level of GBS O-acetylation was not associated with clinical outcome, but rather a conserved feature of the particular serotype Ia or III capsular structure (Figure 1A). Of the 49 serotype Ia strains evaluated in the present study, 47 exhibited detectable, but very low levels, of O-acetylation (1.7–5 %). In contrast, 49 out of 50 serotype III strains had significantly higher levels of O-acetylation (16.5–41.8 %, $P < 0.0001$).

To determine whether differences in O-acetylation are due to restricted acceptor specificities of type Ia and III capsule polymerases, biochemical analyses compared type Ia and III reference strains with isogenic strains expressing the opposite CPS structure [produced by heterologous overexpression of capsule polymerase (cpsH) genes] [36]. Isogenic switching of CPS serotype (III to Ia and vice versa) did not result in a change in the overall level of O-acetylation. Strains maintained the level of O-acetylation present on the native CPS (Figure 1C). These results show that prevailing O-acetylation phenotypes are not related to the activities of CPS polymerase. The results indicate further that the architecture of the Ia or III polysaccharide does not itself constrain overall O-acetylation to a narrow range.

We previously demonstrated that polymorphism in the *O*-acetyltransferase (*neuD*) relates to differences in overall Sia O-acetylation, where phenylalanine at amino acid position 88 appears to confer higher O-acetylation than Cys[88]. In the present study we extend these findings to show that highly O-acetylated outliers among the type Ia strains display polymorphisms in Sia biosynthetic genes that are identical with type III strains (Figure 2). Consistent with other studies [34,39,40], these findings suggest that GBS strains of different serotypes engage in horizontal gene exchange. Moreover, it appears that exchange of capsule biosynthetic genes from type III to type Ia strains can result in increased overall Sia O-acetylation independent of changes in capsule serotype (see Ia outliers in Figures 1 and 2).

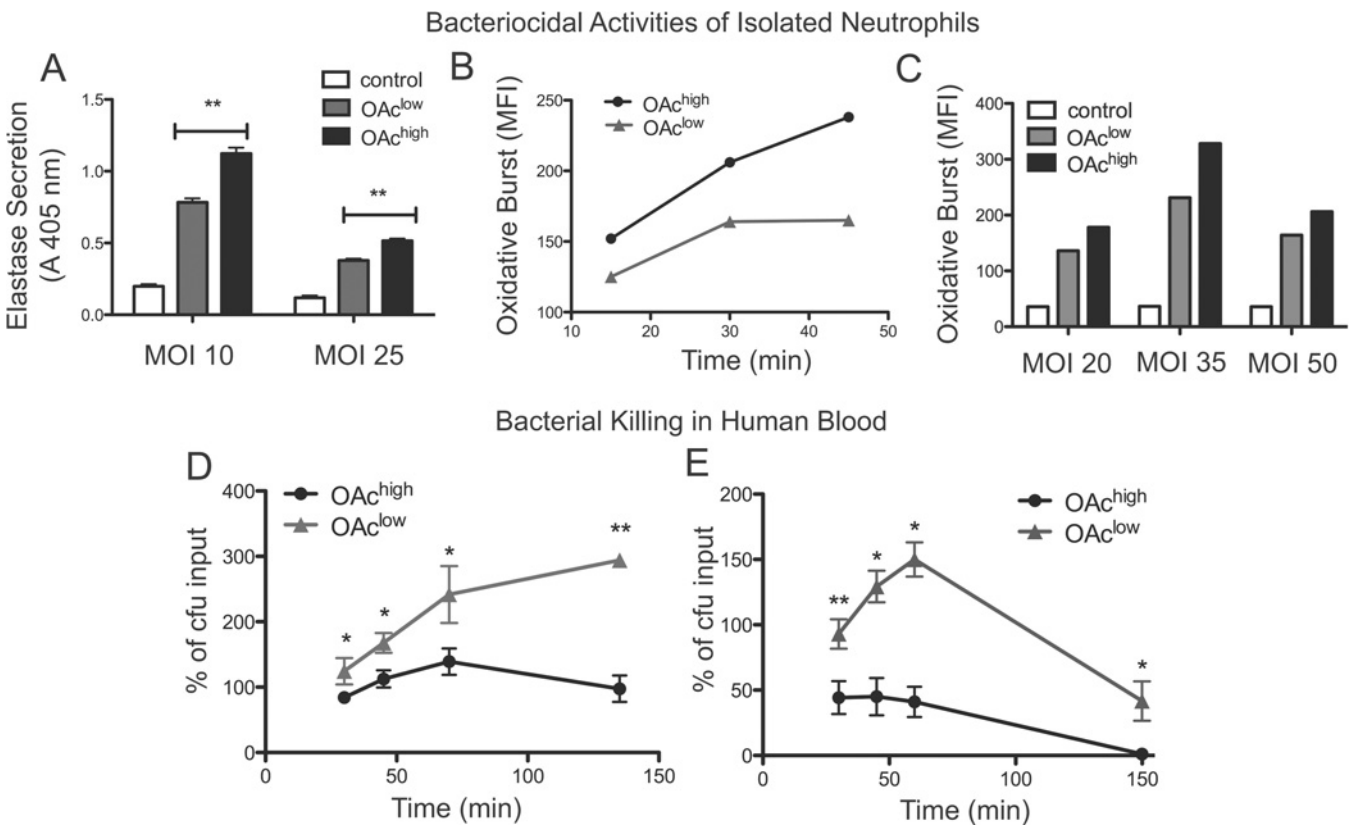

Figure 3 Sia O-acetylation stunts GBS suppression of human neutrophils and hampers bacterial killing in whole human blood

(**A**) Neutrophils were incubated with GBS at MOI 10 or 25, and granule protease activity in the supernatant was measured using the substrate MeOSuc-Ala-Ala-Pro-Val-Nmec in a fluorescence-liberation assay. The *O*Ac^{high} strain stimulated a greater secretion of elastase from isolated primary human neutrophils compared with the *O*Ac^{low} strain (**$P < 0.01$). (**B** and **C**) Oxidative burst was measured in dichlorofluorescin diacetate-labelled neutrophils by flow cytometry following incubation with GBS for 30 min at different MOIs or at a MOI 50 from 15–45 min. In all cases, the *O*Ac^{high} strain stimulates a greater oxidative burst response as compared with the *O*Ac^{low} strain. (**D**) Kinetics of bacterial proliferation (10^4 innoculum) and (**E**) bacterial killing (10^3 innoculum) in 400 μl of whole human blood which indicate greater killing of the *O*Ac^{high} strain (*$P < 0.05$; **$P < 0.005$). MFI, mean fluorescence intensity.

Sia O-acetylation reduces Sia-mediated GBS neutrophil suppression

Sias on the surface of GBS contribute to immune evasion by engaging the Sia-binding receptor Siglec-9 on the surface of neutrophils. Blocking this interaction with a monoclonal antibody allowed increased neutrophil activation and increased bacterial killing [1]. We have previously shown that O-acetylation is a natural modification of Sias that also impairs GBS interactions with purified Siglec-9 [33]. In the present study we measured characteristic responses of freshly isolated human neutrophils to isogenic GBS strains to determine whether O-acetylation impairs Sia-mediated suppression of neutrophil functions. We used previously published GBS strains [33] that vary by a single inactivating amino-acid substitution in the NeuA Sia *O*-acetylesterase, which allows variation of Sia O-acetylation as a single chemical parameter (i.e. without changing overall levels of capsule, Sias or exposed galactose residues; summarized in Figure 1B). These strains are hereafter referred to as *O*Ac^{high} and *O*Ac^{low} GBS.

Neutrophils can kill bacteria in a variety of ways. Elastase is a broad-spectrum serine protease secreted by activated neutrophils that contributes to extracellular bacterial killing. Neutrophil elastase activity assays were performed on cell supernatants following incubation of *O*Ac^{high} or *O*Ac^{low} bacteria. These experiments showed that the *O*Ac^{high} strain induced significantly higher levels of granule protease secretion than the *O*Ac^{low} strain ($P<0.01$) (Figure 3A). In contrast, intracellular killing of bacteria can occur following phagocytosis and subsequent granule fusion to create a phagolysosome in which ROS (reactive oxygen species) are produced. This process, referred to as the oxidative burst, was used as a second measure of neutrophil activation to determine whether additional Sia-dependent neutrophil evasion mechanisms are altered by structural changes in GBS capsular Sias. Freshly isolated dichlorofluorescin-diacetate-labelled human neutrophils were incubated with GBS strains for up to 45 min. Neutrophil oxidative burst was measured at 15 min intervals by flow cytometry. Assays monitored over time (Figure 3B) or with different MOIs (Figure 3C) show that the *O*Ac^{high} strain stimulates a greater oxidative burst in neutrophils than the *O*Ac^{low} strain. These results further corroborate that GBS Sia O-acetylation interrupts bacterial suppression of neutrophil bactericidal activities.

Sia O-acetylation alters the kinetics of bacterial killing in whole human blood

During systemic infection, interactions between GBS and neutrophils occur in the environment of the bloodstream, which contains many other sialylated entities, including serum proteins and additional immune and non-immune cells. To verify that GBS O-acetylation has an impact upon bacterial survival in this more complex physiological milieu, the kinetics

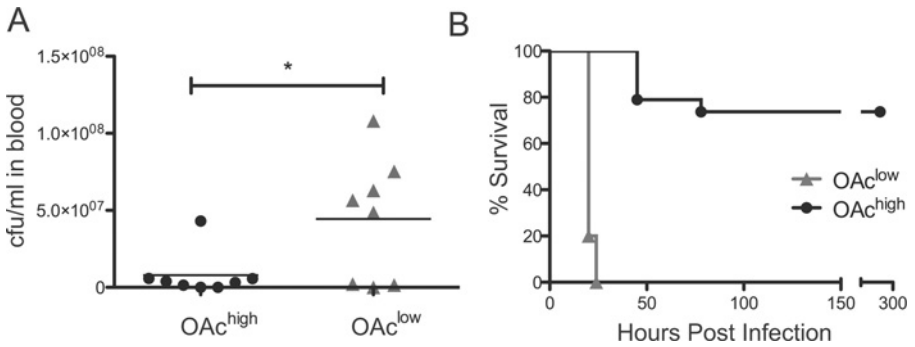

Figure 4 Sia O-acetylation attenuates strain virulence *in vivo*

CD-1 male mice (9-weeks-old) were injected intraperitoneally with 4×10^7 cfus of *O*Ac^{high} or *O*Ac^{low} GBS. (**A**) Bacterial titres from blood collected at 13 h post-infection show a higher systemic bacterial load in mice infected with the *O*Ac^{low} strain compared with the *O*Ac^{high} strain ($^*P < 0.02$). (**B**) In a separate experiment, infected mice were monitored over the course of 10 days for mortality. Consistent with all other results, the *O*Ac^{high} strain is much less virulent than the *O*Ac^{low} strain ($P < 0.0001$).

of bacterial proliferation and killing were examined in whole human blood. In experiments at higher inocula, the *O*Ac^{low} strain showed uncontrolled proliferation, whereas the *O*Ac^{high} strain was maintained near the level of inoculation (Figure 3D). At a lower initial innoculum where bacterial killing was observed, it took approximately five times longer to observe 50 % killing of the *O*Ac^{low} strain compared with the *O*Ac^{high} (Figure 3E). Taken together, these results indicate that Sia O-acetylation imparts a marked fitness cost in the milieu of whole human blood.

Sia O-acetylation attenuates GBS virulence *in vivo*

To determine whether differences in bacterial fitness *in vitro* influence the outcome of invasive infection *in vivo*, *O*Ac^{high} and *O*Ac^{low} strains were evaluated in a murine infection model. CD-1 mice were subjected to intraperitoneal injection of approx. 4×10^7 bacteria ($n = 12$ per group). At 13 h post-infection, blood drawn from mice infected with the *O*Ac^{low} strain had 5-fold higher levels of bacterial cfus than blood from mice infected with the *O*Ac^{high} strain ($P < 0.02$) (Figure 4A). Consistent with the differences observed in bacterial titres in blood, *O*Ac^{high} GBS displayed attenuated virulence in the mouse model compared with the *O*Ac^{low} GBS ($P < 0.0001$). Although all mice injected with the *O*Ac^{low} strain succumbed to infection within 24 h, the majority of mice injected with the *O*Ac^{high} strain cleared the infection (Figure 4B). Taken together, these studies indicate that a subtle chemical modification of the GBS CPS structure has a profound effect on bacterial virulence.

DISCUSSION

We add the present study to a growing body of literature demonstrating that bacterial cell-surface carbohydrates and their modifications can have profound effects on host–microbe interactions [1,2,41–43]. We show for the first time the physiological significance of bacterial capsular Sia O-acetylation (the most common structural modification of Sias) in the context of invasive GBS infection. GBS engages in an intracellular cycle of Sia O-acetylation (encoded by *neuD*) and de-O-acetylation (encoded by *neuA*) to arrive at a final level of the surface modification. We show that there are conserved serotype-specific patterns of Sia O-acetylation in GBS, which are related to a previously reported polymorphism in the NeuD *O*-acetyltransferase. Among serotype III strains, which express relatively high levels of this Sia modification, none of the 50 strains tested displayed more than 42 % Sia O-acetylation. When these natural limits were overcome by genetic methods, increased levels of Sia O-acetylation impaired the ability of GBS Sias to suppress the bactericidal functions of isolated human neutrophils. Further investigations in the more complete milieu of whole human blood or infected animals revealed even more pronounced differences between *O*Ac^{low} and *O*Ac^{high} GBS. These results show that Sia O-acetylation impairs GBS evasion of neutrophil killing mechanisms, probably mediated by reduced interaction with the neutrophil receptor Siglec-9 or its functional murine counterpart Siglec-E [1,33]. However, the profound differences in bacterial proliferation in human blood and during murine infection suggest that the effect of O-acetylation in virulence may be multifactorial.

These studies clearly demonstrate that Sia O-acetylation is not a virulence factor in the conventional sense. However, the conserved partial O-acetylation of the serotype III capsule still requires explanation. We suggest that O-acetylation may be favoured during bacterial colonization or persistence in the human vaginal or gastrointestinal tracts, environments where other bacteria express sialidases. In other contexts, sialidases are known to scavenge host Sia residues or reveal underlying structures for adhesion [44,45]. Our previous studies lend credibility to this hypothesis, showing that O-acetylation protects GBS surface Sias from degradation by several sialidases of gastrointestinal bacteria [33]. Indeed, Sia removal from the GBS surface could be an effective form of niche competition, due to increased complement deposition, loss of immune suppression through Siglec engagement, enhanced innate immune recognition of underlying galactose residues, or enhanced adaptive immune recognition of immunogenic epitopes. This potential protective role of GBS O-acetylation in the context of microbial ecology during bacterial colonization may explain why serotype III GBS have highly conserved levels of this modification, despite the apparent cost of this modification during invasive infection.

AUTHOR CONTRIBUTION

Shannon Weiman, Satoshi Uchiyama, Ajit Varki, Victor Nizet and Amanda Lewis designed the research. Shannon Weiman, Satoshi Uchiyama and Amanda Lewis collected and analysed the results. Feng-Ying Lin and Donald Chaffin contributed reagents and critical evaluation. Shannon Weiman, Ajit Varki, Victor Nizet and Amanda Lewis wrote the manuscript.

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