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Sialic Acid O-Acetylation in Group B Streptococcus:

Impact on Pathogen-Host Interactions

A dissertation submitted in partial satisfaction of the
requirements for the degree of Doctor of Philosophy

in

Biomedical Sciences

by

Shannon Dawn Weiman

Committee in Charge:

Professor Victor Nizet, Chair
Professor Kelly Doran
Professor Joshua Fierer
Professor Richard Gallo
Professor Ajit Varki

2009

The Dissertation of Shannon Dawn Weiman is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2009

iii

DEDICATION

To the inspired minds
that have kindled my passion for and motivation to pursue biomedical science

and

To the spirit of friendship and mentorship
that has been so essential to my education and success

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LIST OF COMMON ABBREVIATIONS

GBS- Group B *Streptococcus*

CPS- capsular polysaccharide

O-Acetylation- acetylationetylation

O-Ac- O-acetylation

Sia- Sialic acid

Neu5Ac- N-*acetyl* neuraminic acid

Siglec- sialic acid recognizing immunoglobulin-like lectin

hCD33rSiglec- human CD33-related Siglec

Sn- Sialoadhesin

ITIM- immunoreceptor tyrosin-based inhibitory motif

AUS- Arthrobacter urafaciens sialidase

Phage- bacteriophage

DMB- 1,2-Diamino-4.5-methylene Dioxybenzene

WT- wild-type

A549- lung epithelial cell line

hBMEC- human brain microvascular endothelial cell line

THA- Todd-Hewitt agar

THB- Todd Hewitt Broth

PBS- phosphate buffered saline

FBS- fetal bovine serum

Cm- Chloramphenicol

Erm- Erythromycin

MOI- multiplicity of infection

CFU- colony forming units

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First and foremost, I must thank my thesis advisor Victor Nizet, who took me under his wing when I was in need of guidance. I cannot not imagine a better mentor, and am exceedingly grateful to have spent the last few years as a member of the friendly, happy and productive Nizet Lab. As a scientist, Victor is an impressive model of excellence. His scientific knowledge spanning technical laboratory skills through clinical pathology, along with his understanding of the field and its relation to society, enables him to direct important and relevant translational research. Working on projects in his lab is both exciting and motivating. His productivity is mind-boggling, and undoubtedly attributable to his work ethic and impressive ability to multi-task. His success is well deserved and he inspires greatness in those who are privileged to work with him. Even more impressive than Victor's success, is his personality and dedication to mentorship. Unfailingly supportive and optimistic, Victor effortlessly generates excitement and motivation in his pupils. His concern for personal growth, in the realms of science, career and family life fosters a community of support both inside and outside of the lab that is integral to the success of his proteges. Despite his hectic schedule and personal responsibilities, he is always accessible, willing and enthusiastic to meet on an individual basis to discuss any of these topics, providing a wealth of sound advice to those just beginning their studies as well as those establishing themselves as his colleagues. Victor also takes action on other's behalf, constantly going out of his way to network and create opportunities for his student's happiness and success. Victor is a selfless and dedicated mentor, and I am eternally grateful for his guidance in the completion of my PhD and

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****

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ABSTRACT OF THE DISSERTATION

Sialic Acid O-Acetylation in Group B Streptococcus:

Impact on Pathogen-Host Interactions

by

Shannon Dawn Weiman

Doctor of Philosophy in Biomedical Sciences
University of California, San Diego, 2008

Professor Victor Nizet, Chair

Group B Streptococcus (GBS) asymptomatically colonizes the lower gastrointestinal and urogenital tracts in 25-35% of the human population. However, in immune compromised individuals and infants, GBS is able to cause severe invasive disease and death. In newborns, GBS is the leading cause of sepsis and meningitis.

GBS is surrounded by a thick capsular polysaccharide (CPS) layer. Nine antigenically distinct serotypes have been documented, all of which display a terminal α2-3 linked sialic acid (Sia) residue. This residue is key to GBS evasion of host immune response, and as such, is a key virulence factor for the bacterium. Terminal Sias are commonly expressed on the surface of mammalian cells and in this context serve to

dampen immune response against self. This is accomplished by binding to factor H, which regulates the alternative complement pathway, as well as through interactions with Siglecs, Sia binding receptors with immunomodulatory capabilities, on the surface of immune cells. Sialic acid on the surface of GBS similarly engages these immune-suppressive mechanisms in a highly effective form of molecularly mimicry.

It has only recently been discovered that GBS biochemically modifies terminal Sia residues by adding an O-acetylationetyl group to the C-7 position of the Sia moiety. Acetylation is incomplete, ranging from 5 to 40% of total surface Sia residues, and is characteristic of different serotype strains. This level is controlled by the balance in activities of the NeuD acetyltransferase and NeuA acetylesterase. Both enzymes are dual functional, being required for surface sialylation as well as acetylation.

In this work we develop methodology using genetic techniques to manipulate O-acetylationetylation (O-acetylation) without impacting overall surface sialylation. Using isogenic strains with artificially high and low O-acetylation we proceed to analyze the impact of O-acetylation on various biological interactions and ultimate consequence for GBS virulence.

We find that O-acetylation protects GBS surface Sias from removal by various microbial sialidases, presenting a possible benefit to in the context of colonization. Looking at Sia-dependent immune interactions, we discover that GBS O-acetylation does not impact alternative complement pathway activation, with both OAc^{high} and OAc^{low} strains showing identical Sia-dependent deposition of the active opsonin C3b. O-acetylation does impair interactions with Siglec-9, thereby impairing the ability of GBS to downregulate neutrophil responses. In this regard,we document increased elastase

secretion, oxidative burst and bacterial killing by neutrophils in response to the OAc^{high} strain. In the more complex context of *ex-vivo* human whole blood, these differences are enhanced, with the OAc^{high} strain being killed more rapidly. The OAchigh strain also shows greatly attenuated virulence in a mouse IP model of infection. Thus, in the context of invasion O-acetylation appears to be a cost to bacterial survival.

Finally we document O-acetylation levels in 100 clinical and colonizing type Ia and type III GBS isolates. We find that type Ia strains consistently show very low levels of O-acetylation (5%) while type III strains display much higher O-acetylation (30%). This is correlated with the NeuD allele, possessing high or low enzymatic activity. We hypothesize that the distinct O-acetylation levels in type Ia and type III strains represent a balance between opposing forcing of selection, which differ in these different genetic backgrounds. Further studies will be necessary to elucidate these unique constraints, and their implications in disease manifestation.

Chapter I

Introduction:

Sialic Acids and the Role of O-Acetylation in Modulating

Interactions with the Innate Immune System

INTRODUCTION

Epidemiology and Clinical Manifestation of GBS Disease

Group B streptococcus, also known as *Streptococcus agalactiae*, is a Gram-positive, chain forming coccus. GBS asymptomatically colonizes the urogenital and gastrointestinal tract in 25-35% of the adult population (Campbell, 2000), causing no ill effects. Colonization represents a delicate balance in which the host is able to control and restrict bacterial proliferation, without eradicating the intruder. If the immune system is compromised, this balance is disrupted, and invasive infection can result. This is becoming increasingly common in elderly and diabetic populations (Edwards, 2005). Newborns are at greatest risk for developing GBS disease due to their exposure to bacteria *in utero* and lack of a fully developed immune system. During pregnancy, GBS can infect the fetus through ascending infection of protective placental membranes or through tears in these membranes that occur during birth. The birthing process itself leads to many infections, as the newborn aspirates infected vaginal fluids while passing through the birth canal. GBS disease is the leading cause of bacterial sepsis and meningitis in newborns, and occurs in 2 to 3 of 1,000 live births (Schuchat, 1998, Baker, 1995).

In the infant, GBS disease manifests in two possible ways. Early onset disease occurs immediately or up to 1 week after birth, and is characterized by pneumonia and septicemia. Late onset disease has a delayed manifestation, occurring up to 6 months after birth and often develops into meningitis. While it is clear that antibiotic prophylaxis at the time of labor and delivery have dramatically reduced the incidence of early onset

GBS infections, the late onset presentation has no known risk factors or prevention strategy (CDC, 2005). The determinants of early versus late onset disease remain unclear; however, a link has been made between these two disease manifestation and GBS capsular serotype (Baker, 1995, Lin, 1998, Campbell, 2000).

Capsular Polysaccharide

GBS is surrounded by a thick glycan layer termed the capsular polysaccharide (CPS). Capsules are commonly expressed on Gram-negative bacteria, as well as some Gram-positives. The capsule offers protection to the bacteria from environmental stresses, such as desiccation or viral phage infection. In the context of colonization and invasion of a host organism, the capsule offers additional protection against immune recognition and clearance mechanisms by obscuring underlying antigenic structures (Howard, 1971) as well as preventing complement deposition and phagocytic clearance (Edwards, 1982, Marques 1992, Kim, 1986). In addition capsular polysaccharides can mediate attachment to surfaces and host cells, playing roles in biofilm formation and invasion of host tissues (Jurcisek, 2005). As such, acapsular mutants are greatly attenuated and cleared rapidly.

While offering the bacterium protection, the capsule can also prove to be a liability. Some components of polysaccharide structure can be used to identify bacteria as foreign invaders. These carbohydrates, such as terminal galactose residues, are not commonly found on mammalian surfaces, and are recognized by innate immune lectins. Binding by these proteins targets the bacterial cell for destruction via the complement pathway. The capsule can also provide a target for adaptive immunity. Capsule specific

antibodies are generated against GBS and can offer protection to offspring both *in utero* and during breast feeding, demonstrating their neutralizing capabilities (Kasper, 1979).

Capsule also affects interactions with non-immune cells within the host. The capsule impairs adhesion and invasion to epithelial and endothelial cell surfaces, affecting the ability of the bacterium to traverse cellular barriers and therefore cause invasive disease. As such, the bacterium has the ability to up and downregulate capsular expression based on quorum sense and environmental factors (Gryllos, 2001).

The CPS is anchored to the bacterial outer cell wall, and is comprised of repeating oligosaccharide subunits. These subunits are widely diverse in their substituents, glycosidic linkages and overall structures, introducing the potential for differential recognition and clearance by innate and adaptive immune mechanisms. Indeed, capsular serotype is a determinant of overall strain pathogenicity in *Streptococcus pneumoniae* (Kelly, 1994) . GBS is subdivided into 9 antigenically distinct serotypes (Baker, 1995) (**Figure 1.1**), whose synthesis is encoded in a single operon that includes all essential genes for regulation, transport and synthesis of the CPS. The CpsH gene controls serotype subunit structure (Chaffin, 2000). While a causal relationship has not been established between strain serotype and pathogenicity in GBS as it has in *S. pneumoniae*, a strong correlation between strain serotype and clinical manifestation of disease has been noted. Type Ia strains are the most common colonizers and cause of early onset disease, while type III strains are over-represented in cases of late onset disease and meningitis (Campbell, 2000). Whether this difference is determined by capsular polysaccharide structure or other characteristics unique to each strain is unknown.
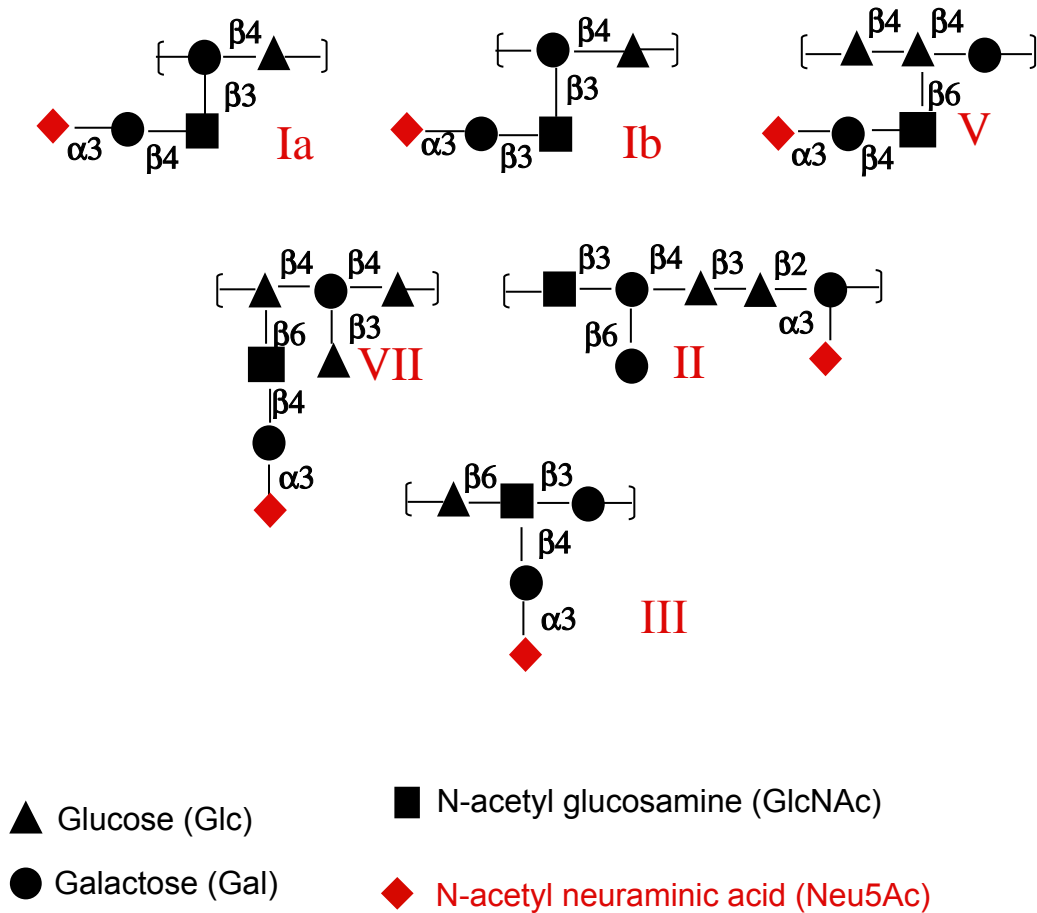
Figure 1.1 Schematic representations of the repeating oligosaccharide subunits of several GBS capsular serotypes (Wessels 1987, Jennings 1980, 1983, Kogan 1995, 1996)

Sialic Acids in Innate Immune Interactions

Common to all GBS capsular serotype structures is a terminal α2-3 linked sialic acid residue, which is of key importance to bacterial survival (Wessels, 1989). Sialic acids (Sias) of this linkage are commonly found on the surface of mammalian cells and act by a number of mechanisms to prevent immune activation against self. One mechanism is by preventing complement deposition via the alternative pathway. Alternative complement is activated when the plasma proteins C3b and factor B bind to foreign cells and act together to initiate a zymogen cascade that ultimately results in the production and deposition of proteins that recruit and activate immune cells and also opsonize foreign particles for phagocytic clearance. Sialic acids prevent pathway activation by binding factor H, which prevents complement activation by two mechanisms. Factor H binds to C3b, displacing factor B. In addition, factor H binds to and acts as a cofactor for factor I, which cleaves and inactivates C3b. Ultimately, factor H mediated displacement of factor B and depletion of active C3b prevents downstream cascade activation and recruitment of immune clearance mechanisms.

Recent evidence also shows sialic acids regulate immune activation via Siglecs, sialic acid binding receptors expressed on the surface of immune cells. The CD33 related Siglecs comprise a rapidly evolving family that are expressed on the surface of innate immune cells, such as neutrophils and macrophages. Each family member has its own unique cell-type specific expression pattern, indicative of evolution of distinct functions. In addition, recognition specificity determined by underlying Sia-glycan linkages differs between family members, implying differential cellular-recognition capabilities. Most CD33r Siglecs having intracellular inhibitory motifs (ITIMs), which transmit inhibitory

signals upon sialic acid binding, thereby suppressing immune response. However, some unique members have diverged in function, associating with activating adapter molecules, or lacking signaling capabilities altogether. These unique instances will be discussed later, but for the most part, the CD33r Siglecs inhibit immune activation upon sialic acid engagement (Avril, 2006, Crocker, Varki 2007).

Sialic acids are rarely expressed on bacterial cell surfaces. Some exceptions include human-specific pathogens such as GBS, *E. coli K1, Haemophilus influenzae, Neisseria meningitis* and *N. gonorrhoea* (Vimr, 2004). On the bacterial surface Sias act similarly to prevent immune activation in a form of molecular mimicry. This strategy is highly successful, as asialo GBS are severely attenuated and rapidly cleared from blood (Wessels, 1989). The protection conferred to GBS by surface Sias is dose dependent, as strains with higher levels are more virulent than lower expressing strains (Adderson, 2000). Immune protection of bacteria is also mediated by interactions with the factor H regulator of alternative complement and Siglecs.

Sia dependent evasion of alternative complement mediated clearance and resulting enhanced virulence has been demonstrated in a number of bacterial instances, including *H. influenzae*, a major cause of ear infections (Figueira, 2007), *Gonococci* (Gulati, 2005) and GBS (Edwards, 1982, Marquez, 1992, Maruvada, 2007). In the latter three cases, Sias have been shown to result in direct binding of factor H to the bacterial surface, thereby tempering the alternative complement pathway.

Sialylated pathogens are also able to utilize Siglec engagement to suppress immune response. GBS has been shown to interact with many members of the CD33

related Siglec family (Carlin, 2007). These interactions differ between different serotype strains, with the type Ia A909 strain strongly engaging many members of the family, and type III COH1 strain failing to engage all except for Siglec-9. The role of Siglec-9 in immune suppression by GBS has been clearly established by showing that GBS Sia engagement of Siglec-9 on human neutrophils inhibits immune activation and bacterial clearance (Carlin, Uchiyama 2009). Roles of other members of the Siglec family are less well established. While most are proposed to transmit inhibitory signals, promoting immune tolerance, one Siglec has evolved a noticeably different structure and function. Sialoadhesin, a Siglec outside of the CD33 related family, has an extended extracellular domain which positions the sialic acid binding domain far from the cell surface. In addition, this receptor lacks an intracellular inhibitory motif. Expressed on the surface of macrophage, Sialoadhesin has been identified as a phagocytic receptor for sialylated pathogens (Jones, 2003) which is upregulated upon immune activation. The divergence of this Siglec represents a counter-evolution of the mammalian immune system against molecular mimicry, and demonstrates the importance of the selective pressure imposed by these disease causing pathogens.

Differential Siglec engagement between different serotype strains provides a potential mechanism for the difference in pathogenicity and disease manifestation seen between type Ia and type III strains in the clinical setting. Underlying capsular structure may play a role in Sia-Siglec binding and therefore degree of immune suppression. It is also possible that other genetic factors associated with these strains may contribute to or even provide the predominant interaction. For example, a protein unique to the type Ia

A909 strain has been identified to interact strongly with Siglec-5, sending immune suppressive signals, in a sialic acid independent manner (Carlin, 2009).   Another contributing factor may be modification of the sialic acid moiety by O-acetylation.

O-Acetylation of Sialic Acids

Sialic acids can be biochemically modified by O-acetylation at the C-4, C-7, C-8 or C-9 positions (Varki, 1992, Klein, 1994, Varki Angata, 2002).   The degree of O-acetylation in the normal physiological state varies widely and O-acetylation signatures differ between cell types, stages of differentiation and individuals.   Acetylation level is controlled by the balance between the activities of O-acetyltransferases (OATs), which add acetyl groups to sialic acids, and O-acetylesterases (SIAEs), which remove acetyl groups. Polymorphisms in these genes that affect enzymatic activity have been shown to correlate with individual and racial differences in O-acetylation of colonic mucins (Campbell, 1994). For the most part, though, variability is attributable to transcriptional control. Differential expression and activities in different cell types results in extreme variation in characteristic O-acetylation levels. In addition, O-acetylation is temporally regulated, with developmental alterations being shown in neural, colon and hematopoetic lineages (Blum, 1987, Shen, 2004, Muchmore, 1987, Krishna, 1997, Shi 1996).

The degree of variability coupled with tight regulation of O-acetylation speaks to the importance of this modification within the mammalian system. The particular roles of Sia O-acetylation are only beginning to be elucidated, but again, are widely variable and dependent on cell type and stage of differentiation.

O-Acetylation of Host Sias Impacts Interactions with Microbes

In many cases O-acetylation has been shown to confer protection against invading microbes. In the colon, high levels of mucosal Sia O-acetylation is thought to protect the mucus layer and underlying cellular layer from degradation by microbial sialidases (Corfield, 1993, Milton, 1993). Sialidases are widespread among both colonizing and pathogenic bacteria. They are used to harvest host Sia residues for nutritional value, and expose underlying structures for adhesion and carbohydrate scavenging (Corfield, 1992, Vimr, Kalivoda, 2004). Substrate specificity can be restricted by underlying glycosydic linkage or biochemical modification such as O-acetylation, with different sialidases being more or less promiscuous than others (Powell, Varki 2001). In some cases O-acetylation provides steric hindrance to the active site of the enzyme, which recognizes the exocyclic side chain of sialic acids, thereby preventing Sia removal and allowing the host to retain this protective glycan (Sander-Wewer, 1982, Chokhawala, 2007, Varki, Diaz, 1983, 1984).

O-acetylation can also interfere with the usage of Sias as cellular receptors for invading pathogens. The abundance of Sia expression in the host makes Sias a prime candidate for targeting of microbial adhesion and invasion to host cells. Many microbes have evolved receptors to recognize Sias of a particular glycosidic linkage, allowing them to target specific hosts, and cell types within the host. This has widespread implications for both transmissibility and pathogenicity (Kumlin, 2008). For example, *Plasmodium falciparum* has evolved receptors for Sias that enable it to target erythrocytes and thereby cause malarial disease (Gilberger, 2003). O-acetylation on mouse erythrocytes has been shown to block this interaction (Reuter, 1991). O-acetylation has also been shown to

block Sia dependent binding of Influenza A and B to host cells (Higa, 1985, Rogers, 1986). By blocking interactions with pathogenic Sia-receptors, again O-acetylation is able to protect the host from damaging microbial infection.

On the other hand, O-acetylation of Sias can also prove detrimental to the host in interfacing with pathogens. In the case of Influenza C, the Sia binding receptor specifically recognizes O-acetylation as an epitope for cell specific infection (Rogers, 1986). Given their rapid evolution, it is not surprising that some microbes have evolved to take advantage of a host modification which may have originally been developed to prevent pathogenic infection. This case exemplifies the intricacies of the constant evolutionary arms race between pathogens and host immunity.

Role of O-Acetylation in the Mammalian System, Dysregulation and Impact on Host Immune Mechanisms

The role of Sia O-acetylation within the host, independent of microbial interactions, is much less clear. Its cell-type specific and temporal regulation suggests a role in development, particularly in neuronal tissues (Blum, 1987). Within the immune system, O-acetylation of Sias on the surface of T cells is thought to play a role in tissue homing and immune activation and proliferation by altering interactions between Sias and their receptors, such as Sialoadhesin, on various cell types (Krishna, 1997, Shi, 1996, Ghosh 2007). Although we do not fully understand all of the roles of Sia O-acetylation within the host, the importance of this modification is underlined by the fact that dysregulation of Sia O-acetylation is a common feature of many disease conditions. This is particularly true of cancers, in which cellular development and differentiation are

altered, thereby affecting O-acetylation programming. The direction of dysregulation varies between cancers but is consistent within a particular type, being reflective of a return to a less differentiated state. In colon cancer the normally high level of O-acetylation is strongly downregulated (Corfield, 1992, 1999, Shen, 2004). In contrast, melanomas upregulate O-acetylation from what is normally a very low level (Cheresh, 1984).

Alterations in O-acetylation impact Sia dependent interactions with both innate and adaptive immune mechanisms. In cases where O-acetylation is abnormal, the immune system is able to identify these aberrations and generate antibodies to target O-acetylation displaying cancerous cells for degradation. Sia O-acetylation in melanomas proves to be an antigenic epitope (Cheresh, 1984, Ravindranath, 1989). Sia O-acetylation can also impact interactions of Sias with innate immune mechanisms. Increased O-acetylation on MEL cells, characteristic of the undifferentiated highly proliferative state, decreases the ability of Sias to downregulate complement, resulting in increased complement deposition and complement mediated clearance (Shi, 1996). This system has also shown that O-acetylation can block Sia-Siglec interactions, altering interactions with macrophage (Shi, 1996). These examples indicate that in such cases, O-acetylation provides a mechanism by which both the adaptive and innate arms of the immune system can override Sia self-protective signals to identify and eliminate diseased self cells.

O-Acetylation of the GBS Capsular Polysaccharide

7 and 9 O-acetylation have also been documented on the surface of many sialylated pathogenic bacteria as well (Orskov, 1979). In the case of GBS, this

modification has only recently been discovered as prior studies of capsular preparations included a base treatment, which removes acetyl groups. There are many similarities in the nature of surface O-acetylation between mammalian systems and GBS. First, total surface O-acetylation is variable, ranging between 0 and 50% of total surface sialic acids (Lewis, 2007). Second, O-acetylation level is characteristic to different cell types. In GBS, glycotyping of 10 laboratory strains identified all 5 serotype Ia strains to have low O-acetylation (5%) while all 5 serotype III strains displayed higher levels (40%) (Lewis, 2007). Third, the level of O-acetylation is determined by the balance between the activities of an O-acetyl transferase, NeuD, and O-acetylesterase, NeuA. The NeuD acetyltransferase adds acetyl groups to the 7 carbon position. In acidic conditions the acetyl group remains in this position. However, at neutral or basic pH the acetyl group gradually migrates to the 9 carbon position (Varki, 1992). It is also important to note that both of these enzymes are dual functional, being required for surface sialylation as well as acetylation. Fourth, different levels of O-acetylation are associated with and caused by genetic polymorphism in the O-acetyltransferase, NeuD. High O-acetylation levels are seen in strains with the 88F allele, whereas low O-acetylation strains possess the 88C allele. The causative relationship has been established by allelic exchange mutagenesis converting the F allele to a C allele and demonstrating phenotypic conversion from high to low O-acetylation. (Lewis, 2007)

Whether differences in expression and therefore O-acetylation regulation occurs during different modes of bacterial growth, similar to developmental modulation of these enzymes in mammalian systems, is unknown. As part of the capsular polysaccharide biosynthetic operon, which is differentially regulated with different growth phases, it is

possible. However, as both genes are part of the same operon, they cannot be differentially regulated and therefore it is unlikely in the bacterial system that differential gene expression could result at different times, altering the balance of O-acetylation.

The impact of O-acetylation on interactions between Sia expressing pathogens and host remains mostly unexplored. It is clear that in the mammalian system, O-acetylation impacts interactions with both innate and adaptive immune tolerance and recognition mechanisms. Adaptive immune response is enhanced by O-acetylation in *Neisseria meningitis* with the Sia O-acetylation epitope increasing antigenicity and generation of protective antibodies in vaccine development studies (Berry, 2002). How does 7 O-acetylation on the surface of GBS impact host-microbe immune interactions? Does O-acetylation on the surface of GBS also impair Sia dependent immune evasion strategy? The body of this work further characterizes the nature of Sia O-acetylation in GBS, uses genetic tools for its controlled manipulation in isogenic strains and elucidates its impact on various biological interactions.

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Chapter II

Genetic and Biochemical Modulation of Sialic Acid O-Acetylation on Group B Streptococcus: Phenotypic and Functional Impact

ABSTRACT

Group B *Streptococcus* (GBS) is an important human pathogen and a model system for studying the roles of bacterial glycosylation in host-microbe interactions. Sialic acid (Sia), expressed prominently in the GBS capsular polysaccharide (CPS), mimics mammalian cell surface Sia, and can interact with host Sia-binding proteins to subvert immune clearance mechanisms. Our earlier work has shown that GBS partially *O*-acetylates CPS Sia residues, and employs an intracellular *O*-acetylation/de-*O*-acetylation cycle to control the final level of this surface Sia modification. Here we examine the effects of point mutations in the NeuD *O*-acetyltransferase and NeuA *O*-acetylesterase on specific glycosylation phenotypes of GBS, pinpointing an isogenic strain pair that differs dramatically in the degree of the *O*-acetyl modification (80% vs. 5%) while still expressing comparable levels of overall sialylation. Using these strains, higher levels of *O*-acetylation were found to protect GBS CPS Sia against enzymatic removal by microbial sialidases and to impede engagement of human Siglec-9, but not to significantly alter the ability of GBS to restrict complement C3b deposition on its surface. Additional experiments demonstrated that pH-induced migration of the *O*-acetyl modification from the 7- to 9-carbon positions had a substantial impact on GBS-Siglec-9 interactions, with 7-*O*-acetylation exhibiting the strongest interference. These studies show that both the degree and position of the GBS *O*-acetyl modification influences Sia-specific interactions relevant to the host-pathogen relationship. We conclude that native GBS likely expresses a phenotype of intermediate Sia *O*-acetylation to strike a balance between competing selective pressures present in the host environment.

INTRODUCTION

Group B *Streptococcus* (GBS) is a Gram-positive bacterial pathogen that colonizes the lower digestive and vaginal tract in 25%-35% of healthy women (Campbell, J.R., Hillier, S.L., et al. 2000). GBS is the leading agent of bacterial sepsis and meningitis in newborns (CDC, C.f.D.C.a.P. 2005), acquired by the fetus through invasion of placental membranes or upon aspiration of contaminated vaginal fluids during passage through the birth canal. GBS is increasingly recognized as a cause of serious infections in elderly, diabetic, and immune-compromised individuals (Edwards, M.S. and Baker, C.J. 2005, Maisey, H.C., Doran, K.S., et al. 2008).

The GBS capsular polysaccharide (CPS) is a critical virulence factor in animal models, a function largely attributable to the presence of terminal α2-3-linked sialic acids (Sias) on each of the branched repeating units of the polymer (Wessels, M.R., Rubens, C.E., et al. 1989). GBS Sia expression represents a form of molecular mimicry, wherein the bacteria decorates its own surface with a sugar moiety expressed in abundance on the surface of all mammalian cells. While there are nine antigenically- and structurally-distinct GBS capsular serotypes (Baker, C.J. and Kasper, D.L. 1985), the repeat unit of the polymer invariably contains this hallmark terminal Sia motif. GBS belonging to serotype III are disproportionately associated with late-onset bacteremia and meningitis, highlighting a particular predilection of these serotype strains to resist host innate immune clearance mechanisms. Sia expression is also an important characteristic of the human bacterial pathogens *Neisseria meningitidis*, *N. gonorrhoea*, *Haemophilus influenzae*, *Campylobacter jejuni*, and *Escherichiae coli* K1 (Vimr, E.R., Kalivoda, K.A.,

et al. 2004). *N*-acetylneuraminic acid (Neu5Ac) is the most common Sia found in nature, and the predominant Sia expressed by these microbes and their human host.

Classically, GBS capsular Sia is recognized to promote resistance to immunologic clearance by interfering with alternative complement pathway activation and accumulation of opsonic C3b on the bacterial cell surface (Edwards, M.S., Kasper, D.L., et al. 1982). In recent studies, an additional mechanism by which bacterial Sia expression can influence host-pathogen dynamics has been uncovered. The Sia-binding immunoglobin superfamily lectins (Siglecs) are expressed on leukocytes and function to regulate their activation and differentiation (Crocker, P.R., Paulson, J.C., et al. 2007). GBS of various serotypes bind human Siglecs in a Sia- and Siglec-specific fashion (Carlin, A.F., Lewis, A.L., et al. 2007), and GBS serotype III engagement of human Siglec-9 (hSiglec-9) was demonstrated to reduce neutrophil activation and bactericidal capacities (Carlin, A.F., Uchiyama, S., et al. 2009). GBS neutrophil suppression via Siglecs involves recruitment of SHP protein tyrosine phosphatases to immunoreceptor tyrosine-based inhibitory motifs (ITIM) on the receptor's intracellular domain (unpublished observations), a process known to antagonize immune signaling cascades (Avril, T., Attrill, H., et al. 2006).

A further level of complexity in understanding GBS Sia function emerged when it was discovered that GBS of all serotypes partially *O*-acetylate the Neu5Ac residues on their CPS (Lewis, A.L., Nizet, V., et al. 2004). Subsequent biochemical and mutagenesis studies identified two enzymes encoded in the GBS capsule biosynthetic operon as responsible for controlling GBS Sia *O*-acetylation levels. Addition of an *O*-acetyl group is carried out by the *O*-acetyltransferase NeuD, which also plays an essential role in GBS

Sia biosynthesis (Lewis, A.L., Hensler, M.E., et al. 2006). NeuD-catalyzed *O*-acetylation first appears on the GBS surface at the 7-carbon position of the Neu5Ac exocyclic side chain (Lewis et al. PNAS 2004), but spontaneous migration of the *O*-acetyl ester from the 7- to the 9-carbon position occurs at pH above 7.0 or below 3.0 (Kamerling, J.P., Schauer, R., et al. 1987, Lewis, A.L., Nizet, V., et al. 2004, Varki, A. and Diaz, S. 1984). Modulation of Neu5Ac *O*-acetylation levels is achieved by a Sia *O*-acetyl esterase activity present in the C-terminal domain of NeuA, itself a bifunctional enzyme that serves as the GBS CMP-Sia synthetase (Lewis, A.L., Cao, H., et al. 2007). The NeuA *O*-acetyl esterase acts intracellularly to remove a subset of the Sia modifications prior to polymerization of the CPS (Lewis, A.L., Cao, H., et al. 2007).

The *O*-acetyl modification of Sia residues in mammalian cell surface glycoconjugates is known to influence their recognition by specific lectins and their susceptibility to sialidase enzymes (Cariappa, A., Takematsu, H., et al. 2009, Chokhawala, H.A., Yu, H., et al. 2007, Powell, L.D. and Varki, A.P. 2001, Shi, W.X., Chammas, R., et al. 1996). Evaluating the potential influence of variable *O*-acetylation upon Sia-dependent molecular interactions is thus important for understanding GBS pathogen-host dynamics. However, potential pitfalls abound in the genetic manipulation of these enzymes since both the NeuD *O*-acetyltransferase and NeuA *O*-acetylesterase play roles in both Sia biosynthesis *and* regulation of the *O*-acetyl modification (Lewis, A.L., Cao, H., et al. 2007, Lewis, A.L., Hensler, M.E., et al. 2006). Here we perform thorough biochemical and phenotypic analyses of GBS strains with targeted gene deletion or active site mutation of NeuD and NeuA to successfully isolate Sia *O*-acetylation as a single chemical variable. An isogenic strain pair engineered to express

either high or low *O*-acetylation, but similar overall Sia is then utilized, to determine the effect of this modification on GBS complement activation, human Siglec-9 binding, and susceptibility to sialidases. The results suggest that GBS may have evolved a phenotype of intermediate Sia *O*-acetylation in response to competing selective pressures present in the host environment.

RESULTS

Deletion of the gene encoding either the NeuD Sia *O*-acetyltransferase or the NeuA Sia *O*-acetylesterase/CMP-Sia synthetase leads to complete loss of Sia expression in the GBS CPS (Lewis, A.L., Hensler, M.E., et al. 2006, Lewis, A.L., Nizet, V., et al. 2004). As summarized schematically in **Figure 2.1**, single amino acid substitutions to alanine in the enzyme active sites of NeuD (K123A) or NeuA (N301A) can ablate the *O*-acetyltransferase and *O*-acetylesterase activities, respectively (Lewis, A.L., Cao, H., et al. 2007, Lewis, A.L., Hensler, M.E., et al. 2006), allowing a more precise level of control of Sia modification. In addition, a naturally occurring polymorphism in NeuD (88F vs. 88C) is functionally associated through an unknown mechanism with high or low levels of acetylation, respectively (Lewis, A.L., Hensler, M.E., et al. 2006). We used these available genetic tools to generate a panel of nine strains in the serotype III GBS background (**Figure 2.1**): WT parent, WT with F88C NeuD polymorphism introduced, WT overexpressing NeuA or enzyme dead NeuA (N301A), Δ*neuD* mutant, Δ*neuD* mutant + enzyme dead NeuD (K123A), Δ*neuA* mutant, Δ*neuA* mutant + WT NeuA or enzyme dead NeuA (N301A). These strains were then analyzed for total Sia expression,
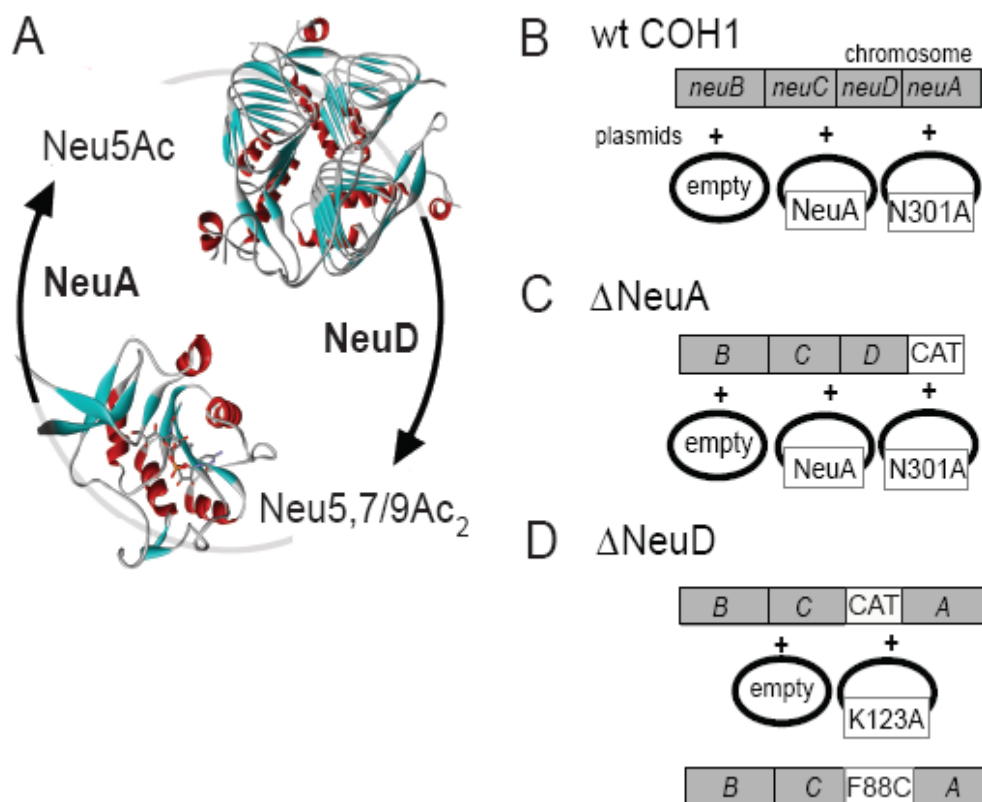
Figure 2.1 Genetic engineering of sialic acid metabolism. Schematic depictions of the intracellular cycle of Sia *O*-acetylation in GBS and the isogenic strain sets used in this study. (**A**) Homology models of NeuD, the GBS intracellular *O*-acetyltransferase and NeuA, the GBS *O*-acetylesterase, which participate in a cycle of Sia *O*-acetylation and de-*O*-acetylation resulting in the final level of surface Sia modification on GBS. Both enzymes have bifunctional roles in Sia synthesis that are not fully depicted in the schematic. Site-directed mutagenesis of transferase and esterase active sites allows specific control of the *O*-acetyl modification. Polymorphism at amino acid position 88 of NeuD can also lead to natural variations in *O*-acetylation between GBS strains (Lewis, A.L., Hensler, M.E., et al. 2006). The *O*-acetyl modification is originally located at the 7-carbon position followed by a unidirectional migration along the exocyclic side chain to the 9-carbon position where it is shown (Lewis, A.L., Nizet, V., et al. 2004). (**B-D**) Sia biosynthetic genes *neuA* and *neuD* were deleted, mutated, and/or overexpressed in the GBS parent strain COH1 as described in the *Materials and Methods*. CAT refers to the chloramphenocol acetyltransferases gene cassette used to create in-frame replacements.

Table 2.1 Summary of Isogenic Type III GBS Strains and their Phenotypes

Summary of bacterial glycosylation phenotypes as detailed in Figures 2.2-2.5. Sia and *O*-acetylation levels were determined by DMB-HPLC, exposed galactose was quantitated by flow cytometry using the lectin ECA, and CPS was evaluated by relative buoyancy as described in the *Materials and Methods*. * indicates OAc^low and ** indicates OAc^high strains used in Figures 2.6-2.8.

Strain Chromosomal	Plasmid (pDCerm)	*O*Ac	Sia	Gal	CPS
WT (COH1)	empty	++	+++	-	++
	WT *neuA**	-	++++	-	++
	N301A *neuA*	++	++++	-	++
ΔNeuA	empty	-	-	++++	+
	WT *neuA**	-	++++	-	++
	N301A *neuA***	++++	++++	-	++
ΔNeuD	empty	-	-	++++	+
	K123A *neuD*	-	++	+	++
NeuD F88C	none	-	++	+	++

degree of *O*-acetylation, level of underlying galactose exposure, hydrophobicity and Buoyancy (summarized in **Table 2.1**) -- with the aim of pinpointing a strain pair that differed only in Sia *O*-acetylation, thus allowing further examination of the functional consequences of this specific modification on the GBS surface.

GBS Buoyancy and Hydrophilicity are Influenced by Overall CPS Sia but not by Variation in Sia O-Acetylation Specific Enzyme Activities

Increased buoyancy of GBS is a strong surrogate measure for overall encapsulation (Hakansson, S., Bergholm, A.M., et al. 1988, Hakansson, S., Holm, S.E., et al. 1987). Buoyancy phenotypes were evaluated in undisturbed, non shaking overnight cultures and after slow speed centrifugation through a Percoll density gradient. While overall growth of the Sia-deficient (Δ*neuD* or Δ*neuA*) strains by colony forming unit enumeration did not differ significantly (data not shown), these strains accumulated in a flocculant pellet at the bottom of the culture tube, leaving the media transparent (**data not shown**). In contrast, the WT parent and other strains in the panel remained in suspension. Similar findings were observed in the Percoll gradient analysis, where only the Sia-deficient Δ*neuD* and Δ*neuA* strains penetrated deeper based on reduced buoyancy/increased density (**Figure 2.2A**). These findings are consistent with published observations that loss of CPS Sia due to deletion of sialyltransferase *cpsK* reduces overall GBS encapsulation (Chaffin, D.O., Mentele, L.M., et al. 2005). These data further suggest that complementation of the Δ*neuD* and Δ*neuA* strains with plasmids bearing either WT or mutated versions of the deleted genes restores GBS encapsulation to WT or near-WT levels. Bacterial CPS expression also provides an enveloping hydrophilic
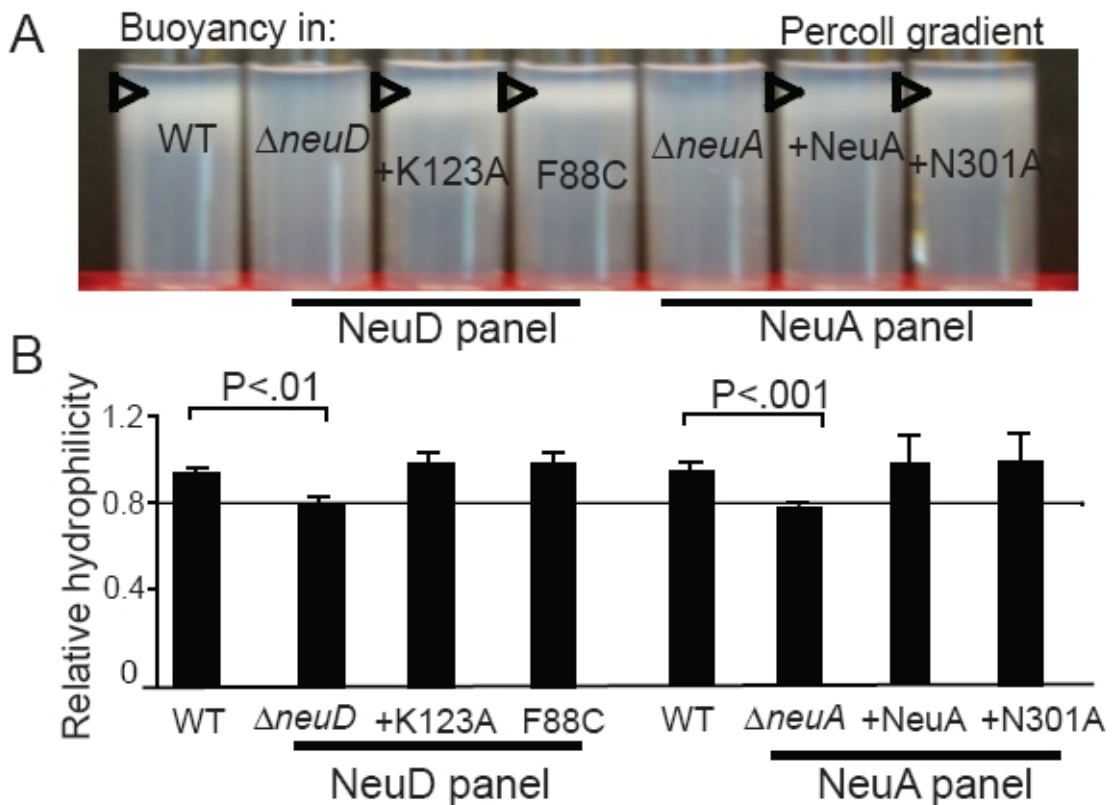
Figure 2.2 Overall buoyancy and hydrophilicity of GBS strains reveals notable differences only upon abolition of Sia synthesis. GBS strains produced by isogenic manipulation of Sia metabolic genes were evaluated for their overall buoyancy in (**A**) undisturbed cultures or (**B**) after slow-speed centrifugation through a layered Percol gradient as described in the Materials and Methods. *Strains without surface Sias were concentrated in a flocky pellet leaving a clear culture supernatant. Arrow heads emphasize a layer of buoyant bacteria that do not enter the Percol gradient. (**C**) GBS strains were evaluated for their overall hydrophilicity using a standard optical density-based assay to quantitate cells that remain in an aqueous phase when given the opportunity to adhere to a hydrophobic n-hexadecane phase.

barrier. Overall bacterial hydrophobicity was assessed using an n-hexadecane partition assay (Buchanan, J.T., Stannard, J.A., et al. 2005). Once again, only the Sia-negative strains (Δ*neuD* and Δ*neuA* mutants) were found to exhibit significantly more association with the hydrophobic phase when compared to WT (**Figure 2.2B**).

Manipulations of Sia O-Acetylation via the NeuD Acetyltransferase also Perturb Overall Sia Expression on the GBS Surface

NeuD was examined as a candidate for targeted *O*-acetyl manipulation since a naturally occurring polymorphism was correlated with high (88F, 30-50% *O*-acetylation) vs. low (88C, 5-10% O-acetylation) levels of this Sia modification in the CPS of clinical strains (Lewis, A.L., Hensler, M.E., et al. 2006). As previously published, changing the *neuD* gene sequence in GBS WT strain COH1 to encode 88C instead of 88F markedly reduced *O*-acetylation levels (Lewis, A.L., Hensler, M.E., et al. 2006). However, in the present more thorough analysis of the NeuD F88C strain (Lewis, A.L., Hensler, M.E., et al. 2006), it was apparent that decreased surface sialylation accompanies decreases in surface Sia *O*-acetylation (**Figure 2.3A**). In contrast to the Sia-negative Δ*neuD* mutant, the F88C mutant strain showed only a modest increase (compared to WT) in the extent of surface-exposed galactose, the sugar residue that immediately underlies Sia in the GBS CPS repeating units (**Figure 2.3B**). To further probe this difference, we performed an assay measuring the kinetics of Sia addition after complete enzymatic surface desialylation of the GBS WT vs. F88C mutant CPS. This assay showed that sialidase-exposed galactose residues were resialylated more quickly in the WT strain than the F88C mutant (**Figure 2.3C**). These data suggest that the F88C polymorphism of NeuD
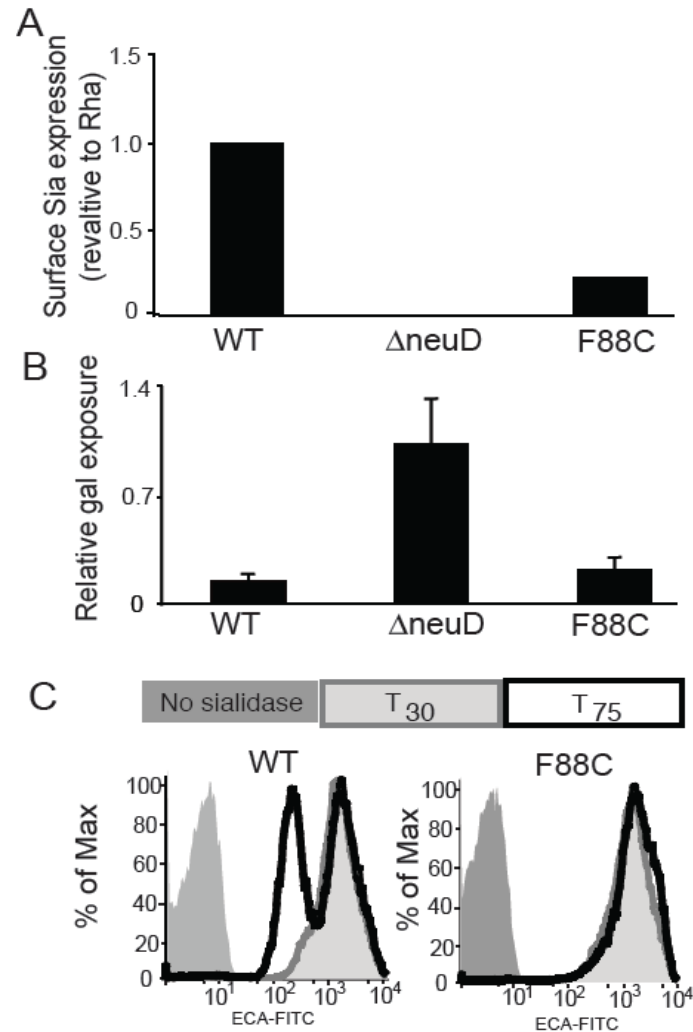
Figure 2.3 Genetic manipulation of NeuD by introduction of the natural "low-*O*-acetylation" polymorphism has unintended consequences for Sia expression. (A) Relative Sia expression was measured as described in the materials and methods in overnight cultures of WT GBS and compared to isogenic strains in which NeuD was either deleted, or replaced with the "low-*O*-acetyl" polymorphic allele of NeuD (88C, Lewis, 2006). (**B**) The extent of Galactose exposure (underlying Sia) was evaluated by flow cytometry using a FITC-conjugated ECA lectin. Data from three independent experiments were normalized based on signal from the ΔneuD strain and presented as average relative fluorescence intensity ± standard deviation. (**C**) Resialylation assays using WT and F88C GBS were carried out by desialylating GBS with with *Arthrobacter urafaciens* sialidase (AUS) and allowing the bacteria to resialylate over the designated time interval. Flow cytometry-based lectin (ECA) recognition of galactose residues, (the structure underlying GBS capsule Sias) was used to monitor the kinetics of CPS resialylation as evidenced by decreased lectin interaction.

plays a complex role in the surface glycosylation of serotype III GBS, and its impact on overall Sia content makes it an unsuitable candidate for independent manipulation of *O*-acetylation levels.

The K123A active-site mutation of NeuD was created in order to more precisely target the *O*-acetyltransferase activity of the enzyme, apart from its apparent dual role in Sia biosynthesis (Lewis, A.L., Hensler, M.E., et al. 2006). Complementation of the Δ*neuD* mutant with a plasmid expressing the mutated active site K123A NeuD enzyme markedly reduced overall *O*-acetylation (**Figure 2.4A**), but failed to restore the fully sialylated CPS of the WT strain (**Figure 2.4B**). Thus, engineering of *neuD* activity, even by active site mutation, does not provide specific variation of *O*-acetylation as an independent chemical phenotype.

GBS Sia O-Acetylation Can Be Manipulated via the NeuA Acetylesterase While Maintaining Consistent Overall Surface Sia Expression

The NeuA *O*-acetylesterase activity offered a second potential target toward a goal of manipulating GBS surface *O*-acetylation in a background of consistent total CPS sialylation. We had previously shown that a N301A mutation in the C-terminal NeuA *O*-acetylesterase active site increases GBS surface Sia *O*-acetylation by eliminating this counter-regulatory enzymatic activity (Lewis, A.L., Cao, H., et al. 2007). Here we expressed either WT NeuA or the esterase-deficient N301A NeuA on plasmids in the WT GBS parent strain COH1 or its isogenic ΔNeuA mutant. In these cases, the level of surface Sia *O*-acetylation was significantly higher in the strains expressing the esterase-deficient N301A NeuA compared to those expressing the WT NeuA enzyme (**Figure**
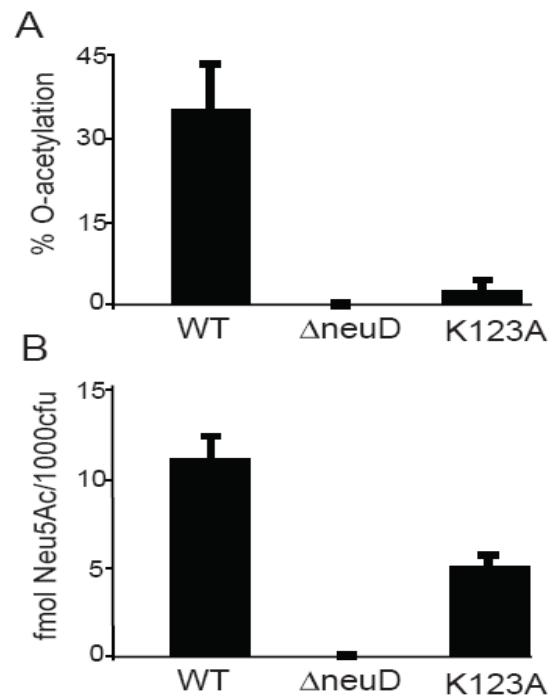
Figure 2.4 *O*-acetyltransferase activity is required for optimal surface sialylation. Isogenic strains varying in the expression or absence of the GBS *O*-acetyltransferase NeuD or the active-site mutated NeuD (K123A) were evaluated for (**A**) relative O-acetylation (as published previously) and (**B**) surface sialylation relative to colony forming units (cfu).
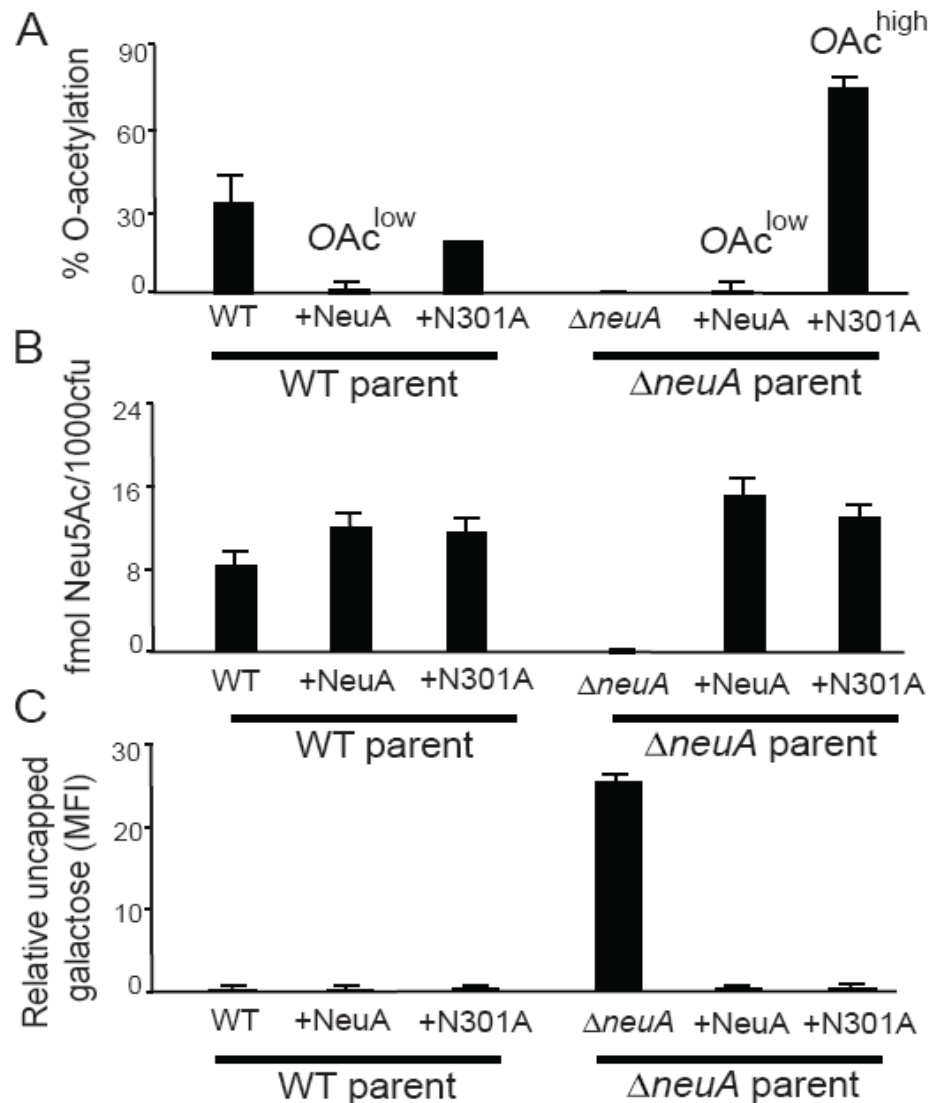
Figure 2.5 Modulation of sialic acid O-acetylesterase activity allows specific alteration of sialic acid O-acetylation without loss of sialylation. GBS strains with alterations of NeuA esterase function by chromosomal deletion, overexpression, or site-specific inactivation, as described in the *Materials and Methods* and depicted in Figure 1, were subjected to chemical analyses of (**A**) the extent of *O*-acetylation, (**B**) total surface Sia expression and (**C**) Exposure of galactose residues, which normally underlie Sias on the CPS. Data are presented as the average median fluorescence intensity of three independent experiments ± standard deviation. Strains hereafter referred to in the text as "*O*Ac^high" and "*O*Ac^low" are depicted.

2.5A), however the total amount of CPS Sia in each pair was nearly identical (**Figure 2.5B**). A slight increase in total surface Sia expression of all NeuA (WT or N301A) over-expressing strains (**Figure 2.5B**) likely reflects increased NeuA N-terminal CMP-synthetase function, which activates free intracellular Sia acid residues for incorporation into the CPS. For further analysis of the specific effect of *O*-acetylation on GBS Sia-dependent functions, we compared the very low *O*-acetylation (<5%) strains WT + NeuA or Δ*neuA* + NeuA, hereafter designated "*O*Ac^low", to the high *O*-acetylation (~80%) strain Δ*neuA* + N301A NeuA, hereafter designated "*O*Ac^high". As summarized in Table 1, these strains did not differ significantly in overall buoyancy (**Figure 2.2A**), hydrophobicity (**Figure 2.2B**), exposure of underlying galactose residues (**Figure 2.5C**), or CPS Sia content (**Figure 2.5B**).

Sia O-Acetylation Level Does Not Significantly Affect Complement Deposition on the GBS Surface

An important GBS virulence phenotype associated with CPS Sia expression is inhibition of alternative complement activation. The complement regulatory protein Factor H can recognize host and bacterial Sia, inhibit C3 convertase activity and facilitate C3b degradation (Pangburn, M.K., Rawal, N., et al. 2009), thereby reducing the amount of opsonic C3b on the cell surface. In murine erythrocytes and cancer cell lines, *O*-acetylation of Sia appears to impair human Factor H binding, resulting in increased complement-mediated lysis of these cells (Shi, W.X., Chammas, R., et al. 1996, Varki, A. and Kornfeld, S. 1980), but the effect of bacterial Sia *O*-acetylation on complement deposition has not been studied.

We incubated the isogenic OAc^{low} and OAc^{high} GBS strains in 50% serum for up to 10 minutes, washed in PBS, incubated with FITC-conjugated anti-human C3b antibody, and quantified C3b deposition by flow cytometry (**Figure 2.6A,B**). The Δ*neuA* Sia-negative strain was included in these experiments as a control. As expected from previous studies (Edwards, M.S., Kasper, D.L., et al. 1982), the Δ*neuA* strain showed ~200-fold higher C3b deposition than the OAc^{low} and OAchigh strains, even within one minute (**Figure 2.6A**). However, there was no difference in C3b deposition between the OAc^{low} and OAc^{high} strains under these conditions. Thus, in contrast to the finding with murine cells, *O*-acetylation of up to 80% of the Sia in the GBS CPS still did not reduce the ability of the bacterium to inhibit the complement deposition. A possible explanation for this difference is found in the extremely high density of Sia expression on the GBS surface compared to mammalian erythrocytes (**Figure 2.6C**). The consistent display of some non-*O*-acetylated Sia on the CPS, as ensured by the *O*-acetylesterase activity of NeuA, may preserve a sufficient reservoir on the GBS surface for Factor H binding and subsequent reduction of bacterial C3b opsonization, despite high levels of Sia *O*-acetylation.

O-Acetylation of GBS Capsular Sia Reduces Human Siglec-9 Binding

Human Siglec-9 is expressed prominently on the surfaces of neutrophils and macrophages (Angata, T. and Varki, A. 2000, Zhang, J.Q., Nicoll, G., et al. 2000) and possesses intracellular inhibitory motifs (ITIMs) that send negative signals thought to dampen immune responses upon engagement of sialylated ligands (Crocker, P.R., Paulson, J.C., et al. 2007). Recent studies have shown that GBS CPS engagement of
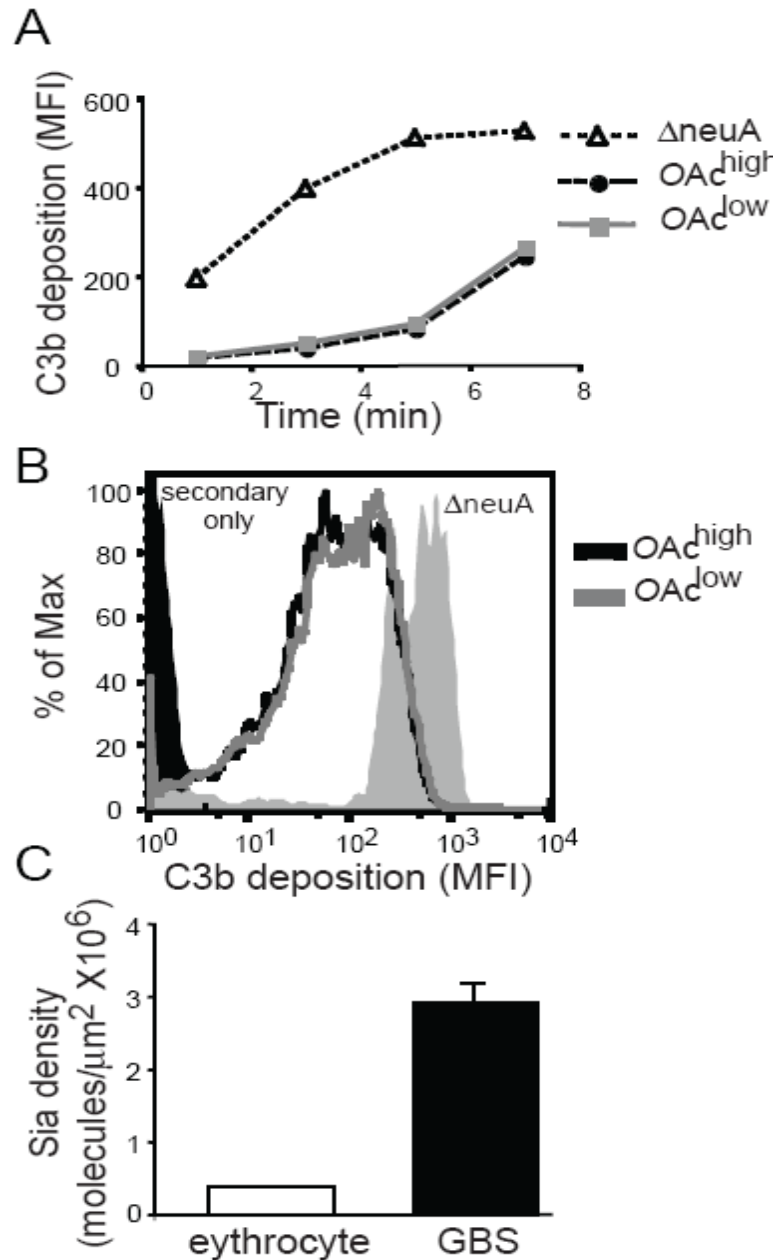
Figure 2.6   GBS O-acetylation does not impact resistance of the organism to complement opsonization. (**A**) Isogenic *O*Ac^high^ and *O*Ac^low^ GBS strains were incubated in 50% human serum and the extent of C3b deposition was analyzed by flow cytometry following staining with FITC-labeled anti-human C3b and expressed as median fluorescence intensity.
(**B**) A representative histogram of the 5-minute time point. (**C**) Sia density reveals a possible explanation for why *O*-acetylation increases susceptibility of mammalian erythrocytes, but not GBS to the effects of complement.

hSiglec-9 downregulates neutrophil responses to GBS, subverting neutrophil killing of the bacteria (Carlin, A.F., Uchiyama, S., et al. 2009). To determine whether the *defined* variable of *O*-acetylation impacts GBS interactions with hSiglec-9, we compared the isogenic *O*Ac^{high} and *O*Ac^{low} strains in a flow cytometry-based hSiglec-Fc binding assay. We found an approximately 5-fold reduction of Siglec-9 binding to the *O*Ac^{high} compared to the *O*Ac^{low} strain (**Figure 2.7A**). In this light, we can conclude that a previous measurement of decreased Siglec-9 binding following the K123A mutation of NeuD (Carlin, A.F., Lewis, A.L., et al. 2007) does not reflect the low *O*-acetylation status of the strain, but rather the decreased overall CPS Sia expression in the strain, compared to WT (**Figure 2.4A,B**)

We have previously shown that GBS *O*-acetylates Sia at the 7-carbon position (Lewis, A.L., Nizet, V., et al. 2004), and in the acidic conditions of urogenital tract colonization (pH 4-5), 7-*O*-acetylation would likely be retained at this position. However, upon entry into the lungs or bloodstream of the fetus or infant, neutral pH will likely induce non-enzymatic *O*-acetyl migration to the 9-position, a process observed for Sia O-acetyl esters in mammalian glycoconjugates (Vandamme-Feldhaus, V. and Schauer, R. 1998, Varki, A. and Diaz, S. 1984) with a calculated half life of ~5 hours (Kamerling, J.P., Schauer, R., et al. 1987). To probe the effect of 7- vs. 9-*O*-acetylation of GBS Sia on Siglec-9 binding, we took freshly grown isogenic *O*Ac^{high} and *O*Ac^{low} strains and either (a) induced complete 7- to 9-*O*-acetyl migration by incubation in pH 9 buffer at 37°C for 30min, or (b) retained 7-*O*-acetylation in another aliquot by incubation in pH 5.5 buffer. For these treatments, surface Sia was analyzed by DMB-HPLC, confirming the expected *O*-acetyl migration (or lack thereof) while retaining equivalent levels of overall Sia and

O-acetylation (**Figure 2.7B**). When these GBS were then incubated with hSiglec9-Fc chimeric protein and binding analyzed by flow cytometry, 7-*O*Ac was found to block Siglec 9 binding to a much greater extent than 9-*O*Ac (**Figure 2.7D**). This effect was specific to the migration of the *O*-acetyl ester and not related to other theoretical effects of differential pH exposure as demonstrated by the equal binding of *O*Ac^{low} strains incubated under the identical pH conditions in parallel (**Figure 2.7C**). These data are the first to demonstrate a specific influence of the position of Sia *O*-acetylation upon Siglec binding, and suggest that the impairment of GBS hSiglec-9 engagement by *O*-acetylation would be mitigated in the bloodstream by migration from the 7- to the 9-position.

O-Acetylation Blocks Removal of GBS CPS Sia by Bacterial Sialidases

When colonizing the host gastrointestinal and vaginal mucosa, GBS coexists with an abundance of other microbes, including both normal flora and transient colonizers. Many of these co-inhabiting microbes express sialidases, which remove terminal Sia residues from the mucosa of the host for use as a carbon source or to adhere to underlying structures (Corfield, T. 1992, Vimr, E.R., Kalivoda, K.A., et al. 2004). Microbial sialidases vary in their substrate specificity for different linkages and types of Sias, but most have the capacity to cleave α2-3 linked Neu5Ac (Powell, L.D. and Varki, A.P. 2001). In biochemical assays with purified enzymes, Sia 9-*O*-acetylation has been shown to interfere with substrate recognition and block the activity of sialidases (Chokhawala, H.A., Yu, H., et al. 2007, Varki, A. and Diaz, S. 1983, Varki, A. and Diaz, S. 1984); however, these observations have not been considered in the context of the intact bacterium and mucosal niche competition.
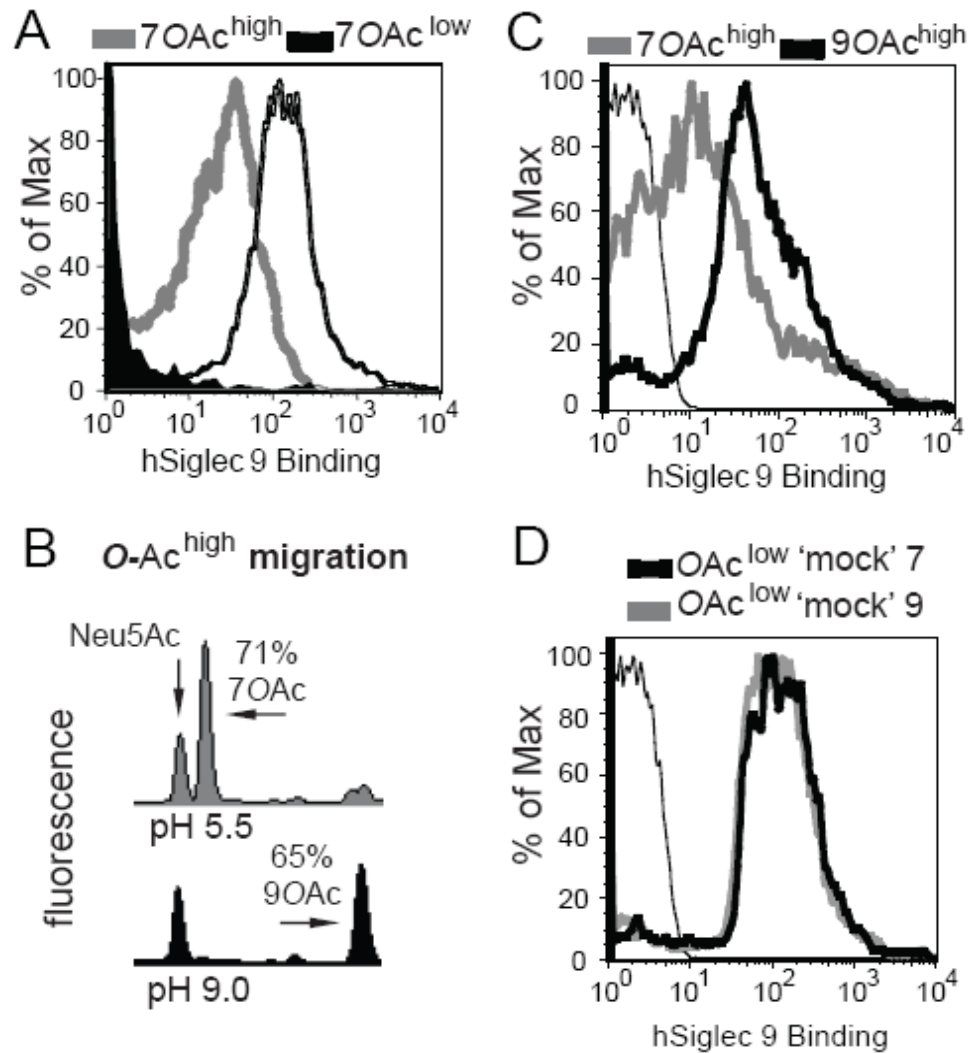
Figure 2.7 The amount and placement of CPS Sia *O*-acetylation influences GBS binding to human Siglec-9. (**A**) Isogenic *O*Ac[high] and *O*Ac[low] GBS strains were incubated with hSiglec9-Fc chimeric proteins and analyzed for binding by flow cytometry using a goat anti-human (IgG-Fc)-PE conjugated secondary antibody. (**B**) The *O*Ac[high] strain was subjected to pH-dependent *O*-acetyl migration as described in the *Materials and Methods* and analyzed by DMB-HPLC to examine the amount and position of the *O*-acetyl ester on GBS surface Sias. Percent of total Sias *O*-acetylated at each position are shown. Retention times of Neu5Ac, Neu5,7Ac$_2$ and Neu5,9Ac$_2$ were 11.8, 12.9, and 19.5 minutes respectively. (**C**) GBS displaying the *O*Ac[high] phenotype at positions 7 or 9 or (**D**) *O*Ac[low] GBS incubated under identical conditions were analyzed for interactions with hSiglec9-Fc by flow cytometry. Parallel control experiments included secondary antibody alone (**A**) and Sia-deficient ΔNeuA GBS (**C** and **D**).

We hypothesized that GBS might use *O*-acetylation to protect its critical virulence determinant from removal by microbial sialidases. The isogenic GBS OAc^{high} and OAc^{low} strains were incubated with purified sialidases from *Clostridium perfringens, Salmonella typhimurium*, and *Vibrio cholerae*, organisms that can form transient or stable associations with the human gastrointestinal tract. *Arthrobacter urafaciens* sialidase (AUS) was used as a control in each experiment since it displays relaxed substrate flexibility and releases *O*-acetylated Sias (Chokhawala, H.A., Yu, H., et al. 2007). Bacteria were incubated with a range of enzyme concentrations at 37°C for 20 min, supernatants collected and subjected directly to DMB derivatization, allowing analysis of all released Sias including modified and unmodified forms (**Figure 2.8D**), or base treated to hydrolyze *O*-acetyl esters, followed by DMB derivitization for a more accurate total Sia quantification (**Figure 2.8A-C**).

At all concentrations of each enzyme, total Sia release from the *O*Ac^{high} strain was markedly reduced in comparison to the *O*Ac^{low} strain (**Figure 2.8A-C**). Under saturating enzyme concentrations, the *S. typhimurium, C. perfringens, and V. cholerae* sialidases released approximately 20%, 50%, or 33% as much Neu5Ac from the *O*Ac^{high} strain compared to the *O*Ac^{low} strain, respectively (**Figure 2.8A-C**). In contrast, Sia release by AUS, the broad-spectrum sialidase encoded by a soil saprophyte, was unaffected by GBS *O*-acetylation (**Figure 2.8A-C**). Additional experiments evaluated the distribution of Sia types (unmodified or *O*-acetylated) that were being released from the *O*Ac^{high} strain (**Figure 2.8D**). The proportion of Sia types released by AUS (**Figure 2.8D**) mirrored the proportion of Sia types displayed by the *O*Ac^{high} strain on its surface (**Figure 2.5A**). In striking contrast, the vast majority of Sias released by *S. typhimurium, C. perfringens,*
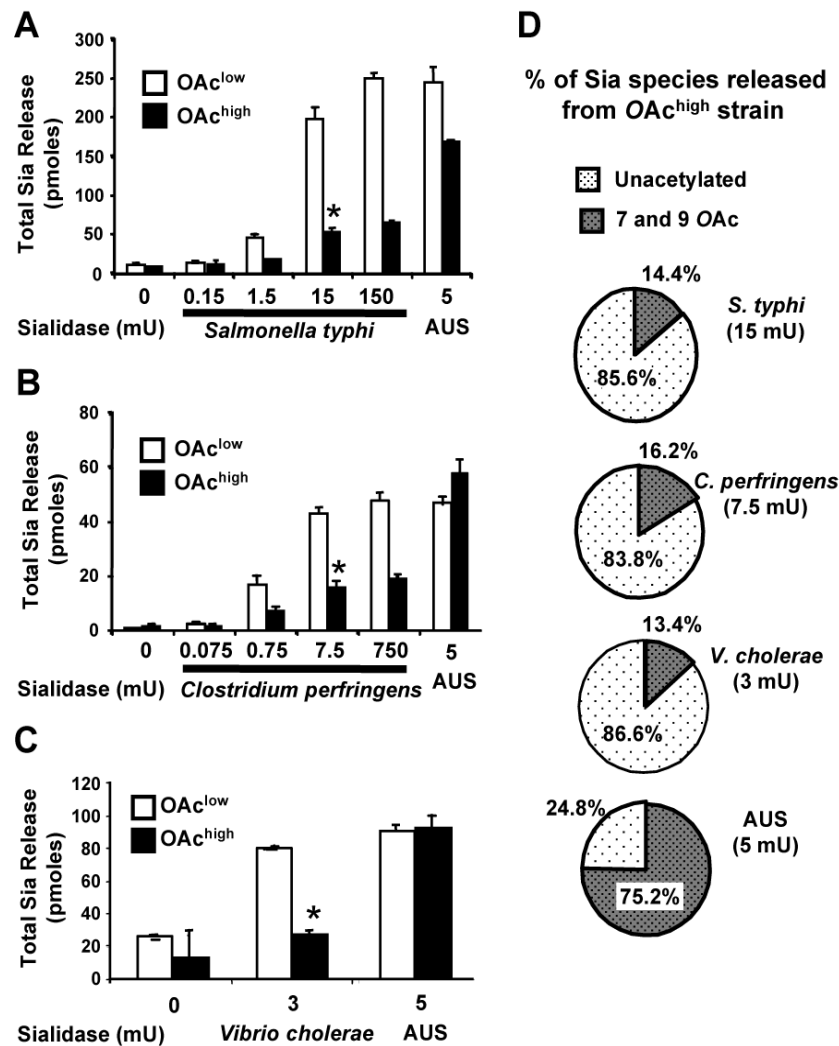
Figure 2.8 *O*-acetylation blocks GBS sialic acid removal by the sialidases of some gastrointestinal bacteria. Isogenic *O*Ac^high and *O*Ac^low GBS were treated with varying amounts of purified sialidases from the gastrointestinal pathogens (**A**) *Salmonella typhimurium* (**B**) *Chlostridium perfringens* or (**C**) *Vibrio cholerae* and the total Sia released was monitored after NaOH treatment by DMB-HPLC as described in the materials and methods. Data are presented as mean ± standard deviation from analyses performed in triplicate. *Arthrobacter urafaciens* sialidase was used as a control in all experiments as it is known to have a relaxed substrate specificity and to indiscriminately release O-acetylated Sias from GBS. (**D**) Sialidases of *S. typhimurium C. perfringens* and *V. cholerae* were incubated with GBS that express high levels of *O*-acetylation, followed by DMB-HPLC analysis without prior NaOH treatment and quantitation of *O*-acetylated and unmodified Sias. In contrast to the control *A. urafaciens* sialidase, which releases Sias in a more representative fashion, mostly unmodified Sias were released by the tested gastrointestinal pathogens.

and *V. cholerae* sialidases were unmodified (**Figure 2.8D**). The overall level of non-*O*-acetylated Sia released by each of the sialidases is comparable to that released by AUS, indicating that all non-*O*-acetylated residues available for cleavage are preferentially removed from the GBS surface under these conditions (data not shown). These data directly demonstrate that *O*-acetylation of Sias on intact GBS protects CPS Sias from cleavage by a variety of microbial sialidases. This is the first demonstration that Neu5Ac residues modified with *O*-acetyl esters at the 7-carbon position (as opposed to the 9-carbon position) are effective at blocking sialidase action.

DISCUSSION

Partial *O*-acetyl modification is a newly appreciated biochemical feature of GBS capsular Sia, which is known to be an important virulence feature of the pathogen, and the basis for molecular mimicry of the human host. The enzymatic basis of GBS Sia *O*-acetylation by the *O*-acetyltransferase NeuD and its modulation by the O-acetylesterase activity of NeuA is established. However, the required function of each enzyme in overall CPS Sia biosynthesis posed a challenge in evaluating effects of *O*-acetylation on GBS Sia-mediated phenotypes in isolation. We used over-expression of WT or esterase-deficient NeuA in GBS to generate isogenic strains that differ markedly in overall *O*-acetylation (5% vs. 80%) but express similar overall CPS Sia. These studies reveal that higher levels of *O*-acetylation of the GBS capsular Sia reduces its susceptibility to enzymatic removal by different microbial sialidases, does not

appreciably influence complement C3b accumulation on the GBS surface, but impairs the ability of GBS to engage hSiglec-9. While previous studies have examined effects of *O*-acetylation on Sia-dependent processes, ours is the first to report such influences in the context of intact bacterial cells expressing *O*-acetylation as an isolated chemical variable.

We hypothesize that a phenotype of partial Sia *O*-acetylation in GBS has evolved under competing selective pressures that benefit the organism over strategies of expressing unmodified Sia or 100% *O*-acetylated Sia. Inhibition of Sia removal by microbial or host sialidases may be one major advantage of Sia *O*-acetylation, protecting at least a proportion of this critical virulence factor from enzymatic elimination in the host environment. Increased susceptibility to the alternative complement pathway would be a theoretical disadvantage of GBS Sia *O*-acetylation (Shi, W.X., Chammas, R., et al. 1996, Varki, A. and Kornfeld, S. 1980), but these studies show that even when 80% of GBS Sia residues are *O*-acetylated, the density of GBS surface Sia expression and the preservation of a subset of non-*O*-acetylated Sia residues evidently provides a sufficient reservoir for this complement inhibitory function. Finally, *O*-acetylation reduces binding of hSiglec-9, a newly identified mechanism of GBS immune evasion. However, migration of the *O*-acetyl ester from the 7- to the 9- position under physiologic pH partially restores this binding potential.

The preservation of the NeuD/NeuA system for *O*-acetylation and de-*O*-acetylation of Sia prior to capsule assembly (**Figure 2.1**) allows GBS to generate partial Sia *O*-acetylation phenotypes. These phenotypes are likely to exhibit different optimization points based on (a) the underlying biochemical structure of each GBS serotype repeat unit that displays the terminal Sia residue and/or (b) strain variation

expression of surface proteins that influence interactions with Sia binding lectins. The approach we have outlined will facilitate future studies to understand how GBS uses *O*-acetylation to strikes a balance between sialidase protection, complement resistance and Siglec engagement to maximize its success in the host environment. *O*-acetyl modifications have also been described on Sia residues in surface expressed glycoconjugates of other bacterial pathogens (Bhattacharjee, A.K., Jennings, H.J., et al. 1976, Gamian, A., Jones, C., et al. 2000, Gamian, A. and Kenne, L. 1993, Gamian, A., Romanowska, E., et al. 1992, Orskov, F., Orskov, I., et al. 1979) and similar analyses of their influence on Sia preservation and interaction with host Sia binding lectins should prove fruitful.

MATERIALS AND METHODS

Strains and Culture Conditions

All strains used in this study were derived in an isogenic manner from the well-characterized wild-type strain COH1. NeuD and NeuA mutants were constructed as previously described (Lewis, A.L., Hensler, M.E., et al. 2006, Lewis, A.L., Nizet, V., et al. 2004) by allelic replacement with a chloramphenicol acetyltransferase cassette (Figure 1). Complementation of these mutants was achieved by transformation of the pDCerm plasmid bearing either wild-type or mutated alleles of NeuD and NeuA as previously described (Lewis, A.L., Cao, H., et al. 2007, Lewis, A.L., Hensler, M.E., et al. 2006, Lewis, A.L., Nizet, V., et al. 2004). Strains new to this study include WT and mutant

GBS bearing empty control pDCerm vectors and the WT strain transformed with the NeuA N301A vector. Although all graphs do not contain "+ empty vector" alongside the strain names, studies of strains containing overexpression constructs were always compared to WT or mutated strains containing the empty pDCerm vector and grown under identical conditions. In the case of the F88C polymorphism of NeuD, allelic replacement by homologous recombination was employed to replace the polymorphic allele into its native position on the GBS chromosome (Lewis, Hensler, 2006). GBS were grown in Todd Hewitt broth at 37°C without shaking. Strains bearing the pDCerm plasmid were grown in Todd-Hewitt broth containing 5-10µg/ml erythromycin.

Buoyancy Assays

GBS were grown overnight (without shaking). After initial inspection of buoyancy in undisturbed cultures, tubes were vortexed briefly to homogenize the bacterial suspension and 1ml of culture was pelleted, washed in PBS, and resuspended in 100ul PBS. GBS were overlaid onto a Percol gradient in glass tubes consisting of 1ml each of 60%, 50%, and 40% Percol diluted in PBS. A qualitative evaluation of buoyancy was made following 500rpm centrifugation for 10-15min. Similar results were obtained using log phase cultures.

Hydrophobicity Assays

Overall hydrophobicity of the isogenic panel of GBS strains was tested in triplicate using a variation of the method of Rosenberg (Rosenberg, M. 2006). Briefly, cells were grown to OD_{600} 0.4, washed in PBS and resuspended in 3ml of Milli-Q water

to an OD_{660} of approximately 0.1. To this, 0.6ml of n-hexadecane was added, and the suspension was vortexed 3 times for 15 seconds each. The initial and final OD_{660} values of the lower aqueous phase were collected and relative retention of bacteria within the aqueous layer was calculated by dividing the final/initial OD values. Data are represented as mean ± standard deviation and Student's t-test was used for statistical analysis.

Sialic Acid Quantitation

The quantity of sialic acid residues was measured after hydrolysis of cells or cell fractions in 2N acetic acid, or treatment with various sialidases as described in *Materials and Methods* and *Results*. Released Sias were filtration over a Centricon-10 cassette, lyophilized, and treated with 0.1N sodium hydroxide followed by neutralization as previously described (Higa, H.H., Butor, C., et al. 1989). Sialic acids were then derivatized with 1,2-diamino-4,5-methylene dioxybenzene (DMB) and quantitated by reverse phase HPLC with fluorescent detection as previously described (Lewis, A.L., Nizet, V., et al. 2004) using commercially available N-acetylneuraminic acid as a standard (Inalco). Analyses were performed in triplicate and expressed as mean ± standard deviation.

Quantitation of O-Acetylation

The levels and placement of *O*-acetyl groups on GBS Sias was determined by DMB-HPLC analysis without prior NaOH-treatment of Sias as previously described (Lewis, A.L., Cao, H., et al. 2007) using peak areas integrated by the Rainin R software package.

Fractionation of GBS for Quantitation of Surface Expressed Sia

For analysis of Sias relative to rhamnose, cell wall extracts were prepared from overnight cultures of GBS strains. 1ml of culture was washed in PBS and resuspended in 100ul Tris-HCl pH 7 containing 10 mM $MgCl_2$ and 250U mutanolysin from *Streptomyces globisporus* (Sigma). After incubation for 3 hours at 37°C, protoplasts were pelleted and the supernatant containing extracted cell wall material was split into two equal parts used for quantitation of sialic acid and rhamnose residues (of CPS or Group B carbohydrate respectively), that are both covalently attached to peptidoglycan (Deng, L., Kasper, D.L., et al. 2000).

Quantitation of Rhamnose Residues Present in the Group B Carbohydrate

Rhamnose was quantitated by the UCSD glycotechnology core resource. Briefly, cell wall extracts were hydrolyzed with 2M TFA, lyophilized, and analyzed by HPAEC-PAD using a CarboPac PA-1 column with parallel preparation and quantitation of commercially available rhamnose standard. Relative sialylation was calculated by comparing the absolute quantity of sialic acids to that of rhamnose (pmol Sia / pmol Rha) in the same volume of sample and expressed relative to the sialylation value found in the WT COH1 parent strain.

Bacterial Re-sialylation Assay

CPS Sias on GBS were removed by incubating bacteria in sterile PBS with 100mU/ml *Arthrobacter ureaficiens* sialidase (AUS) for 30 minutes at 37°C and then washed 5 times with PBS. GBS were either analyzed immediately by labeling with

ECA-FITC at 4C followed by flow cytometry (time 0).or incubated for indicated time points at 37°C prior to analysis.

Sia Quantitation Relative to Colony Forming Units

GBS were grown to mid log phase and resuspended to OD_{600} 0.4 (~10^8 cfu/ml) in PBS. 1 ml of each strain was pelleted in triplicate and resuspended in 200ul water. Bacteria were lysed using 3 rounds of freeze-thawing in a dry ice ethanol bath alternating with a 100°C heat block. Cell debris was collected by centrifugation at maximum speed on a tabletop centrifuge for 10min and any soluble intracellular materials in the supernantant, including intracellular Sias, were discarded. The pellet was washed with PBS, resuspended in 50mM sodium acetate pH 5.5 containing 7.5mU of *Arthrobacter urafaciens* sialidase (EY labs). After a 2 hour incubation at 37°C, sialic acid residues released into the supernatant were filtered, lyophilized, NaOH-treated, derivatized, and quantitated as described above. In parallel, bacteria were serially diluted and plated on Todd Hewitt Agar for evaluation of cfu, which were similar between strains. All experiments included parallel Neu5Ac standards during DMB derivatization for construction of standard curves used to estimate Sia expression. After normalization of absolute Sia levels for cfu, which did not vary considerably between samples, relative Sia levels were expressed as fmol Neu5Ac / 1000 cfu. Similar relative results were obtained when whole bacteria were subjected to 2N acetic acid hydrolysis rather than sialidase-treatment of cell-wall extracts.

ECA-Binding Assays for Estimation of Relative Uncapped Galactose

GBS were grown to mid log phase and resuspended to an OD_{600} of O.4. Approximately 10^8 cells were washed in PBS, diluted 1:5 in PBS, and 50ul of this was incubated on ice in the dark in a total volume of 100ul with the FITC-conjugated lectin from *Erythrina cristagalli* (ECA, EY labs) at a final dilution of 1:100. After a 30min incubation, cells were pelleted, washed in PBS, and resuspended for analysis using a BD Facscaliber flow cytometer.

Complement Deposition Assays

GBS were grown to mid log phase and resuspended to an OD_{600} of O.4 in PBS. Approximately 10^7 cells were resuspended in 50µl of 50% serum diluted in HEPES++ buffer (20mM Hepes, 5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 140 mM NaCl, .05% BSA, pH 7.3) and incubated at 37°C with shaking. 8µl aliquots were removed at 1, 3, 5, 7 and 10 minute intervals and diluted into 500µl ice cold PBS containing 1% BSA and 5mM EDTA. Samples were then washed with 1 ml PBS with 1% BSA and resuspended in 50 µl of 1:50 FITC conjugated goat anti-human-C3 F(ab)$_2$ antibody (Protos Immunoresearch) in PBS with 1% BSA. Samples were stained for 30 minutes on ice, washed once with 1 ml of PBS with 1% BSA, resuspended in 300 µl of PBS and analyzed using a BD Facscaliber flow cytometer.

Calculation of Sia Density

The density of Sia expression was compared between GBS and mammalian erythrocytes by calculating the number of Sia molecules expressed per cell and dividing

by the estimated surface area of each cell type. For GBS, the data collected for Figure 5 were used to calculate Sia molecules per cell and estimating GBS diameter at approximately 1μm to calculate surface area. For the erythrocyte calculations, we used estimates of Sia molecules per cell published by Powell and Hart (Powell, L.D. and Hart, G.W. 1986) for human erythrocytes and average measurements of erythrocyte diameter in normal individuals (Matsumoto, K., Nishi, K., et al. 2003) for calculation of cell surface area. Sia density was calculated as Sia molecules per cell divided by the (spherical) surface area of each cell type in μm^2.

Siglec-Fc Binding Assays

Siglec-Fc chimeric protein (0.4 μg) was pre-complexed with 0.4 μg of goat-anti human phycoerythrin conjugated secondary antibody in 50 μl of PBS for 1 hour on ice in the dark. Bacterial strains were grown to mid log phase and resuspended to OD_{600} 0.4 in PBS. $2x10^6$ bacteria in 50 μl of PBS were incubated with precomplexed mixture for 40 minutes on ice in the dark. Samples were then washed with 1 ml of PBS, resuspended in 500 μl of PBS and analyzed by flow cytometry.

O-Acetyl Migration

OAc^{high} and OAc^{low} GBS were grown to mid log phase and resuspended to an OD_{600} of O.4 in PBS. Approximately 1×10^8 bacteria were pelleted and resuspended in 1 ml 1mM sodium acetate pH 5.5 to or Tris-HCl pH 9 and incubated for 30 min at 37°C to maintain the O-acetyl ester at position 7 or induce *O*-acetyl migration to position 9

respectively. Aliquots of 100 µl were removed and treated as described above for Siglec binding (using PBS at pH 6.5), using the OAc^{low} strain as a positive control for binding and simultaneously as a negative control for the pH incubation. Additional 100 µl aliquots of pH-treated bacteria were incubated with 15 mU *Arthrobacter ureafaciens* sialidase (AUS) at 37°C for 2 hours to remove surface sialic acids, which were analyzed by DMB-HPLC to determine the extent and position of the *O*-acetyl modification as described above. Remaining cells were incubated in parallel with the Siglec incubation on ice for 40 minutes, during which time we observed little additional movement or loss of the *O*-acetyl ester.

Treatment with Sialidases of Gastrointestinal Bacteria

Bacterial strains were grown to mid log phase, washed in PBS and the 10^{-4}-10^{-6} dilutions were plated for cfu enumeration. 1×10^8 bacteria were resuspended in 20 µl of 100 mM sodium acetate buffer pH 5.5 (with 1 mM Ca for *V. cholerae*) and sialidases from *Salmonella typhimurium* (Glyko), *Chlostridium perfringens* (Sigma), *Vibrio cholerae* (Sigma), or *Arthrobacter ureafaciens* (EY Labs) were added over a 4 log range as indicated. After 37°C incubation for 20 minutes, bacterial cells were pelleted and supernatant removed for analysis by DMB-HPLC with or without prior NaOH treatment to visualize *O*-acetylated species or condense all Sias into a single peak for injections of 3-4 samples representing independent experiments in the same 50 minute elution of the reverse phase column. Peak areas were integrated and these values were corrected for original cfu, which were always very similar, to compare between OAc^{high} and OAc^{low} strains.

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ABBREVIATIONS

CPS, capsular polysaccharide; DMB, 1,2-diamino-4,5-methylene dioxybenzene; ECA; *Erythrina cristagalli* agglutinin; Gal; galactose; GBS, Group B Streptococcus; Neu5Ac, N-acetylneuraminic acid; OAc, O-acetylation; Sia, sialic acid; AUS, *Arthrobacter urafaceans* Sialidase.

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Chapter III

Sialic Acid O-Acetylation on Group B Streptococcus Impairs

Neutrophil Suppression and Attenuates Bacterial Virulence

ABSTRACT

Group B Streptococcus is the leading cause of sepsis and meningitis in newborns. Key to GBS virulence is the capsular polysaccharide, a thick glycan layer comprised of repeating oligosaccharide subunits. All GBS serotypes display terminal α2-3 linked sialic acid residues, which are identical to those presented on human host cell srufaces and prevent immune activation in a highly effective form of molecular mimicry. It was recently discovered that GBS modifies Sias by partial O-acetylation, and that this biochemical modification alters Sia-dependent molecular interactions with the host. However, the impact of O-acetylation on immune activation and bacterial pathogenicity remains unknown. Here we use previously defined isogenic serotype III strains expressing high (>75%) and low (<5%) levels of Sia O-acetylation to examine the impact of this modification in *in-vitro* and *in-vivo* models of GBS infection. GBS O-acetylation did not impact bacterial interactions with lung epithelial or brain endothelial cell monolayers *in-vitro*. However, this subtle modification significantly impaired Sia mediated human neutrophil suppression, as measured by elastase secretion and oxidative-burst, and bacterial killing by both isolated human neutrophils and in human whole blood. *In-vivo*, the OAc^{high} strain displayed attenuated virulence in mice, with significantly lower levels of bacteria in the bloodstream following intraperitoneal infection. Consistent with these data, analysis of natural levels of Sia O-acetylation among 100 clinical and colonizing GBS isolates identified limited levels of O-acetylation, and striking consistency within serotype strains. Serotype III strains

displayed intermediate levels, ever exceeded 55% of total Sias. Serotype Ia strains displayed low levels, rarely exceeded 5% O-acetylation. These data show that there is a cost of Sia O-acetylation to Sia-mediated immune evasion during invasive infection, which limits natural levels of this modification on GBS, and that the limits imposed are unique between GBS serotypes.

INTRODUCTION

Group B streptococcus is the leading cause of sepsis and meningitis in newborns GBS disease occurs when the bacterium, which asymptomatically colonizes the lower gastrointestinal and urogenital tract in 25-35% of the adult population (Campbell, 2000), crosses placental membranes during pregnancy or is aspirated by the neonate during the birthing process. In the infant, which lacks a fully developed immune system, invasive disease can result. GBS disease can manifest as early onset, which commonly presents with pneumonia and septicemia immediately after birth, or late onset, which can be delayed by 6 months and is characterized by bacteremia and meningitis. The determinants of early versus late onset disease are not understood, but multiple studies show that serotype III GBS are overrepresented among cases of late-onset disease. The recent introduction of antenatal screening and antibiotic prophylaxis has reduced the incidence of early onset GBS disease by two thirds to 0.32 cases per 1000 live births (CDC, 2005; Van Dyke, 2009). However, the incidence of late-onset GBS disease remains unchanged.

GBS is subdivided into nine antigenically distinct serotype strains defined by the oligosaccharide structure of the capsular polysaccharide (CPS), the outermost glycan layer surrounding the bacterium (Baker, 1985). While each serotype strain has an antigenically unique structure, all display a terminal α2-3 linked sialic acid (Sia) residue, shown to be essential to GBS virulence in the serotype III background (Wessels, 1989). Serotype Ia and III GBS commonly colonize the human host and result in a similar frequency of early onset disease. However, type III strains are over represented among cases of late onset GBS disease.

Sialic acids are nine carbon backbone monosaccharides that are displayed on the surface of mammalian cells but are relatively rare in bacteria. Within the host, Sias interact with various immune mechanisms to suppress innate and adaptive immune responses. Sias prevent complement pathway activation and deposition by binding to factor H, a negative regulator of alternative complement (Edwards, 1982), which allows the bacterium to evade immune clearance in animal models (Wessels, 1989). Sias also engage Siglecs, Sia-binding immunoglobin superfamily lectins, which are expressed on the surface of immune cells (Crocker, 2007), and send inhibitory signals to prevent immune activation. In the context of invasive GBS disease, Sias suppress immune activation and promote bacterial proliferation by engaging Siglec-9 on human neutrophils (Carlin, 2009).

It has recently been discovered that GBS partially O-acetylates terminal Sias (Lewis, 2004). Strains so far examined possess high (~35-50%) or low (~2-5%) levels of O-acetylation (Lewis, 2004; Panaraj, 2009) , which result from polymorphisms in the NeuD O-acetyltransferase and appear to correlate with serotype (Lewis, 2006). GBS also

encodes a specific Sia O-acetylesterase, which limits the overall level of surface O-acetylation in multiple GBS serotypes (Lewis, 2007). The GBS O-acetyl esterase is linked by gene fusion to the C-terminus of the GBS CMP-Sia synthetase (NeuA), which is necessary for nucleotide activation of sialic acid prior to capsular assembly (Vimr, 2006, Lewis, 2006). The unique and conserved glycan phenotypes between strains, along with the correlation of these strains with disease manifestation, lead us to question if O-acetylation plays a role in GBS pathogenicty by altering interactions of GBS capsular sialic acids within the host.

In order to address this question we have manipulated surface O-acetylation in an isogenic serotype III background (Weiman, 2009). An OAc^{low} strain was created by transformation of WT COH1 strain with a plasmid over-expressing the NeuA esterase, driving O-acetylation down to 5%. An OAc^{high} strain was created by transforming the ΔNeuA COH1 mutant, which lacks esterase activity as well as surface sialylation (Lewis, 2007), with a plasmid overexpressing a site-directed NeuA mutant lacking esterase activity but retaining sialylation capabilities. The resulting strain has restored surface sialylation, and displays 80% O-acetylation. Glycotyping analysis confirmed similarly matched levels of sialylation in both strains, a possible confounding variable in manipulation of the capsular biosynthetic pathway. Using these isogenic matched strains we probed various interactions with host immunity. We have previously shown that high levels of O-acetylation did not alter complement deposition on the bacterial surface of GBS, but did impair interactions with human Siglec-9 (Weiman, 2009). We now explore the physiological consequences of impaired Siglec engagement for GBS-host interactions with particular emphasis on human neutrophil activation, bacterial survival, and the

overall effects of Sia O-acetylation on pathogenicity *in-vivo.* In addition, we analyze surface O-acetylation levels in 100 clinical and colonizing type Ia and type III isolates in order to further characterize O-acetylation in native GBS populations and predict what role this biochemical modification plays *in-vivo*.

Here we show that this subtle modification of a bacterial capsular polysaccharide can have a profound effect on the kinetics of bacterial proliferation in the host

RESULTS

Sialic Acid O-Acetylation Does Not Substantially Alter GBS Adhesion or Invasion of Cellular Barriers

The ability of bacteria to cause severe invasive disease is dependent upon their ability to cross cellular barriers that are designed to prevent their penetration into host tissues. We focused on two such barriers which are of particular relevance to GBS infection. The lung epithelium provides a barrier to bacteria which may have been aspirated during birth and possibly causing pneumonia from penetrating into blood and tissue, which can result in sepsis and likely death. The blood-brain barrier, comprised of microvascular endothelial cells, prevents bacteria which have entered the blood stream from passing into the brain, where meningitis can result in brain damage and fatality.

In order to cross such barriers, bacteria must first adhere to the surface of cells. Sialic acid on the surface of GBS presents an unintended cost to bacterial adhesion by enhancing negative charge-charge repulsion between the bacterial and host cell surface.

Whether O-acetylation alters interactions between bacterial Sia and host cell surface charges is unknown. The correlations between serotype, level of O-acetylation, and prevalence of pneumonia or meningitis suggests that O-acetylation may impact adhesion and invasion to different types of host cells. Particularly, the correlation between Type III strains, incidence of meningitis, and higher levels of acetylation suggests that O-acetylation may play a role in increasing this serotype's ability to adhere to and invade blood-brain-barrier endothelial cells.

We used *in-vitro* monolayers of A549 lung epithelial cells and human Brain Microvascular Endothelial cells (hBMECs) to model cellular barriers within the host and examined the ability of Type III strains with high and low levels of O-acetylation (Weiman et. al. 2009) to adhere to and invade through these cellular barriers. Previously defined isogenic OAc^{high} and OAc^{low} strains were added to confluent monolayers, spun down to initiate contact, and incubated for 30 min at 37°C. Nonadherent bacteria were washed off, remaining cells were lysed and adherent bacteria plated in serial dilution for enumeration. Invasion studies proceeded 2 hours, followed by washing and an additional 2 hour incubation with antibiotic treatment to kill extracellular bacteria. Cells where then lysed and intracellular bacteria plated in serial dilution for enumeration. Results indicate that *O*-acetylation does not affect adhesion or invasion to A549 cells (**Figure 3.1A,B**). A slight difference in adherence to hBMECs was noted (**Figure 3.1C**), however, this difference was not statistically significant and did not translate into increased invasion (**Figure 3.1D**).
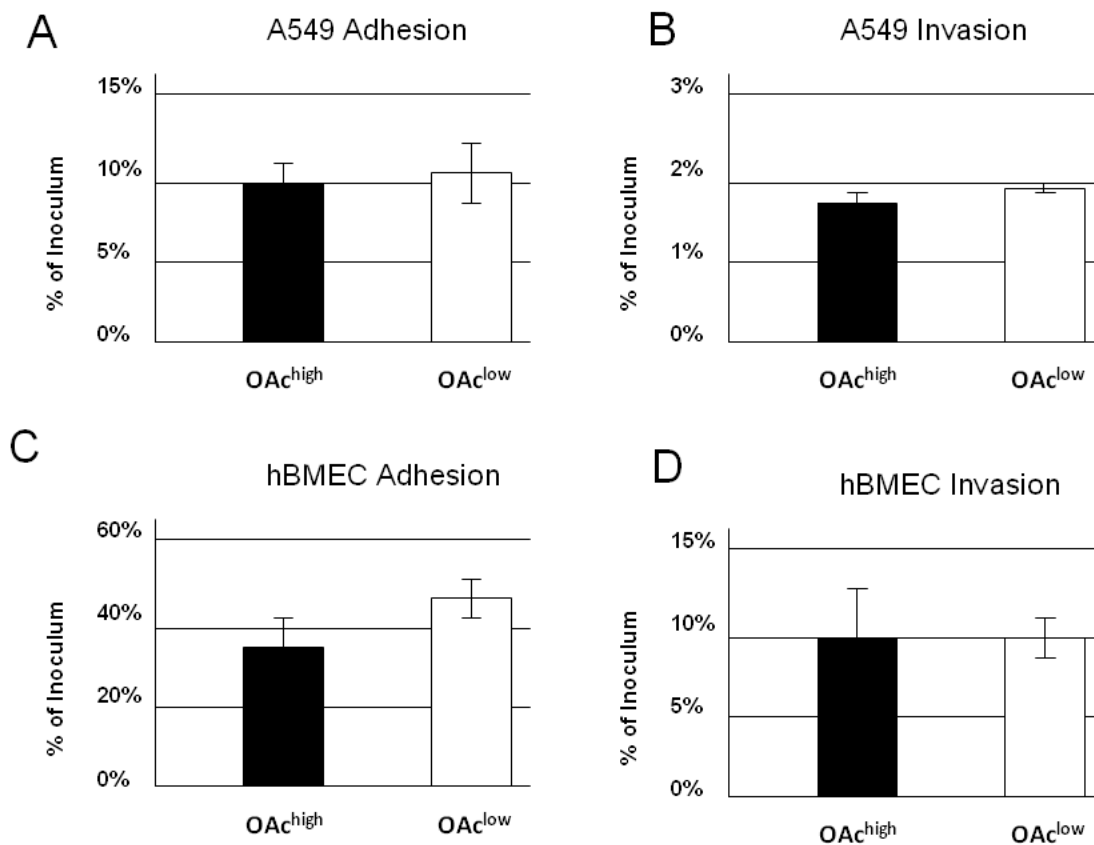
Figure 3.1 Sia O-Acetylation does not substantially alter GBS adhesion to or invasion of cellular barriers. A549 lung epithelial cell (**A**) adhesion and (**B**) invasion. hBMEC (**C**) adhesion and (**D**) invasion. Adherence was measured after 30 minute incubation, whereas invasion proceeded for 2 hours, followed by 2 hour incubation with penicillin/gentamicin to kill extracellular bacteria. All experiments were repeated at least three times. Data from representative experiment.

Sialic Acid O-Acetylation Impairs Suppression of Neutrophils

Sialic acids on the surface of GBS contribute to immune evasion strategy in part by engaging Siglec-9 on the surface of neutrophils, which send inhibitory signals. Blocking this interaction with an antibody results in increased neutrophil activation by various measures, and results in increased bacterial killing. (Carlin and Uchiyama, 2009)We have previously shown that acetylation similarly blocks Siglec-9 binding (Weiman, 2009). Whether this blockage is substantial enough to result in alterations in neutrophil activation has not been tested.

Here we use two neutrophil killing mechanisms as measures of neutrophil activation, which have been shown to be inhibited by GBS engagement of Siglec-9 (Carlin and Uchiyama, 2009). Elastase is a broad spectrum serine protease secreted by neutrophils in response to bacteria and contributes to bacterial killing. In this assay we incubated OAc^{high}, OAc^{low} and asialo strains with freshly isolated primary human neutrophils for 20 minutes. Supernatants were then analyzed for elastase activity. We found that, as expected, asialo GBS induced the greatest elastase secretion due to its inability to inhibit neutrophil activation via engagement of Siglec-9 (data not shown). OAc^{high} and OAc^{low} strains induced less elastase from neutrophils, presumably due to their surface sialylation. The OAc^{low} strain induced significantly lower levels of elastase secretion than the OAc^{high} strain (MOI 10: 69.6% p=0.00058, MOI 25: 73.1%, p=0.00030) (**Figure 3.2A**), confirming the hypothesis that GBS Sia O-Ac impairs the organism's ability to suppress neutrophil activation.

Another means for neutrophils to kill bacteria is through phagocytosis and subsequent granule fusion to create a phagolysosome in which reactive oxygen species
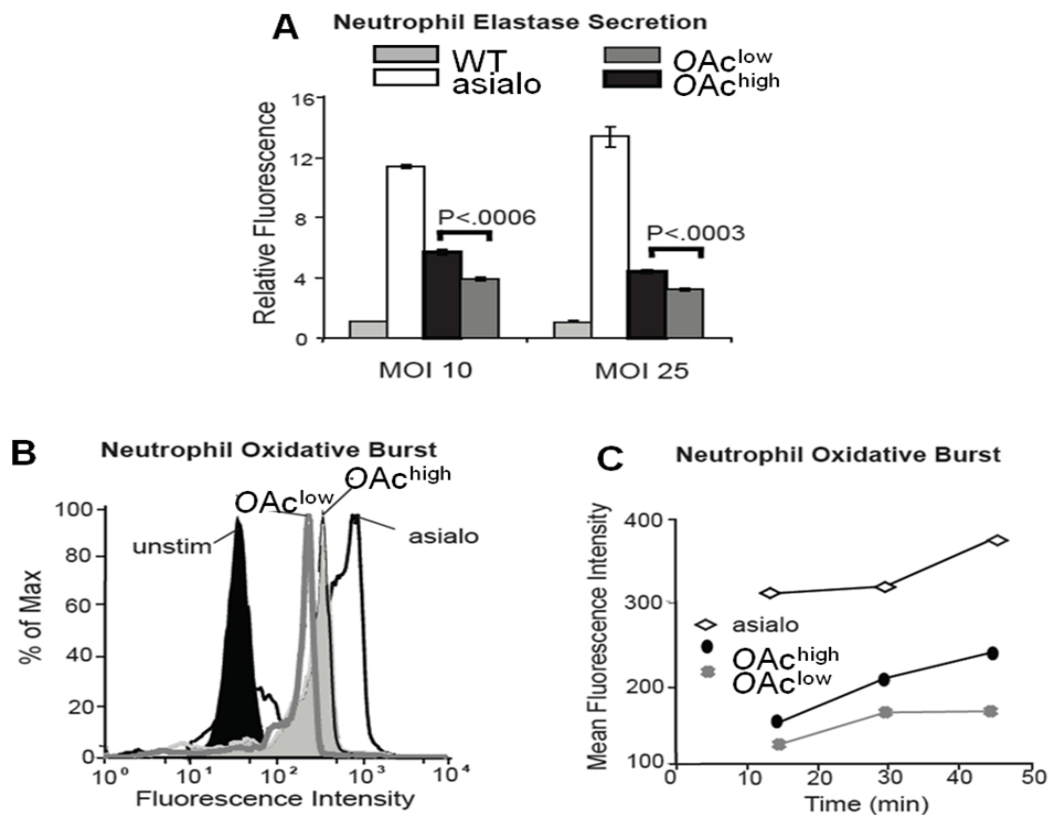
Figure 3.2 Sia O-acetylation results in increased neutrophil activation.
(**A**) OAc^{high} stimulates greater secretion of elastase from primary human neutrophils after 20 min incubation as compared to OAc^{low}. Baseline secretion by unstimulated neutrophils is defined at 1. (**B**) Individual flow cytometry tracing of oxidative burst response from a 30 minute time point at MOI 35. (**C**) OAc^{high} stimulates a greater oxidative burst response as compared to OAc^{low}. Neutrophils displaying positive oxidative burst were gated and mean fluorescence intensity calculated from this subpopulation (MOI 50). Experiments were repeated at least 3 times. Data from representative experiments.

are produced to damage and kill the ingested pathogen. This process, referred to as oxidative burst, was used as a second measure of neutrophil activation to determine if additional neutrophil killing mechanisms are affected by GBS Sia O-acetylation. Freshly isolated DCFH-DA labeled human neutrophils were incubated with OAc^{high} and OAc^{low} and asialo GBS strains for up to 45 minutes. Neutrophil oxidative burst was measured at 15 minute intervals by flow cytometry. Again we found the greatest oxidative burst was stimulated by asialo GBS. The OAc^{high} strain stimulated a greater oxidative burst than the OAc^{low} strain (OAc^{high} : OAc^{low} 1.5 fold difference) (**Figure 3.2B,C**), indicating that acetylation also impairs the ability of GBS to suppress oxidative burst.

Sialic Acid O-Acetylation Results in Increased Bacterial Killing

Next we tested whether the increase in neutrophil activation observed in response to exposure to the OAc^{high} strain in comparison to the OAc^{low} strain translated into an increase in killing of the OAc^{high} strain. Freshly isolated human neutrophils were incubated with OAc^{high} and OAc^{low} that had been pre-opsonized with naïve serum. At 30 minutes cells were serially diluted in water to lyse neutrophils and surviving bacteria were plated for enumeration. We found that the OAc^{high} strain showed more limited proliferation (190%) than the OAc^{low} strain (280%) (P<.01) (**Figure 3.3A**), confirming that O-acetylation results in failure to block immune suppression and greater bacterial killing by neutrophils.

During invasive infection, the interaction between GBS and neutrophils often occurs in the environment of the bloodstream, in the presence of serum proteins and additional immune and non-immune cells, many of which express sialic acids on their
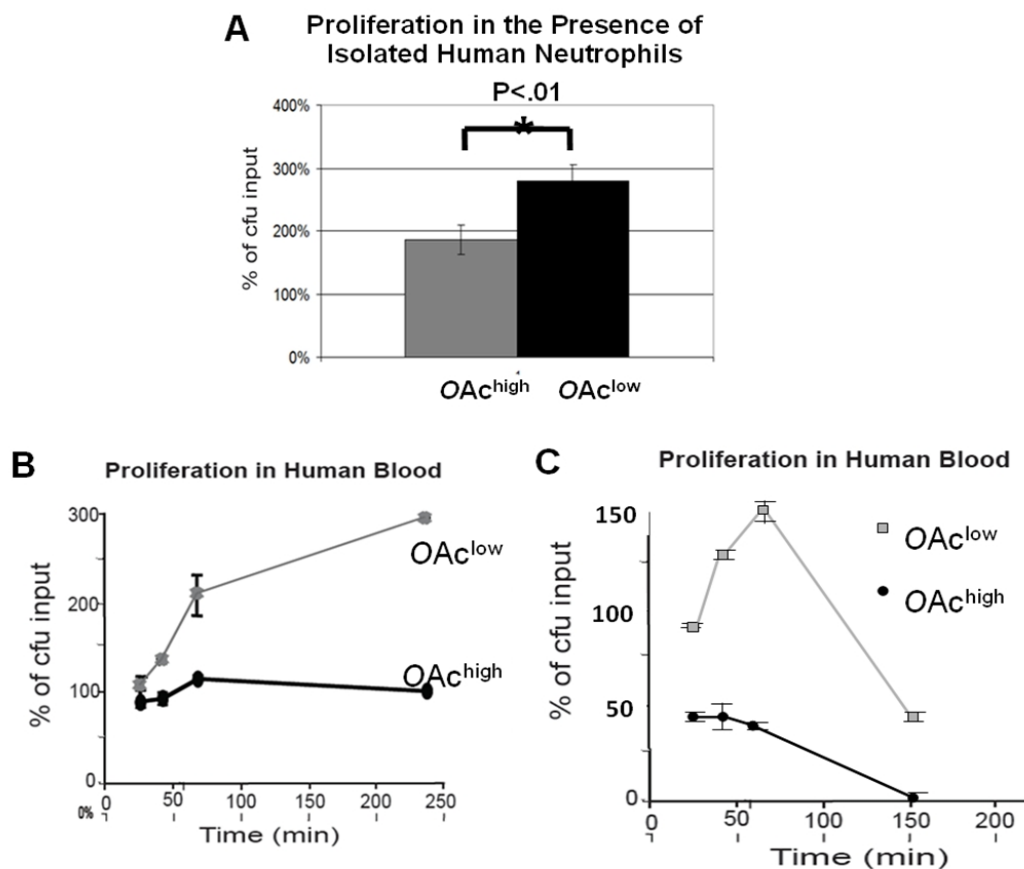
Figure 3.3 Sia O-acetylation results in increased bacterial killing *ex-vivo.*
(**A**) OAc^{low} is able to proliferate to a greater extent in the presence of primary human neutrophils as compared to OAc$^{high.}$. (**B**) OAc^{low} is able to proliferate in human whole blood while the growth of OAc^{high} is controlled. Inoculum: $1x10^4$. (**C**) At a lower inoculum, immune control of OAc^{low} is delayed, while OAc^{high} is rapidly eliminated. Inoculum: $1 x 10^3$. All experiments repeated at least 3 times. Data from representative experiments.

surface. Interactions of these additional factors with GBS and neutrophils have the potential to impact the GBS-neutrophil interaction and resulting immune response and bacterial clearance. In addition, other immune cells present within the blood may contribute to bacterial clearance, enhancing or negating overall differences in survival between strains in this context. We tested whether differences observed in survival between OAc^{high} and OAc^{low} strains in isolated neutrophil assays was also true in the more physiologic conditions present in whole blood using an *ex-vivo* approach. OAc^{high} and OAc^{low} strains were incubated in freshly drawn heparanized human whole blood. At various intervals aliquots were removed and serially diluted as described above for enumeration of surviving bacteria. Results indicate that while the OAc^{low} strain showed uncontrolled proliferation, the OAc^{high} strain was maintained at the level of inoculation (**Figure 3.3B**). At lower initial inoculum the OAc^{high} strain was completely cleared by 2.5 hours, while the OAc^{low} strain showed an initial proliferation and delayed growth control (**Figure 3.3C**). These results indicate a marked contrast in bacterial fitness in the presence of immune surveillance mechanisms in the milieu of human whole blood, with O-acetylation impairing bacterial survival.

Sia O-Acetylation Attenuates Strain Virulence *In-Vivo*

To determine whether differences in bacterial fitness *in vitro* correspond to infection outcome in a whole organism, we examined virulence of OAc^{high} and OAc^{low} strains in a murine infection model. Male 9 week old CD-1 mice were injected in the intra peritoneal cavity (IP) with approximately 4×10^7 bacteria (n=12). Blood was collected 13 hours post injection from the retro-orbital vein, serially diluted and plated for
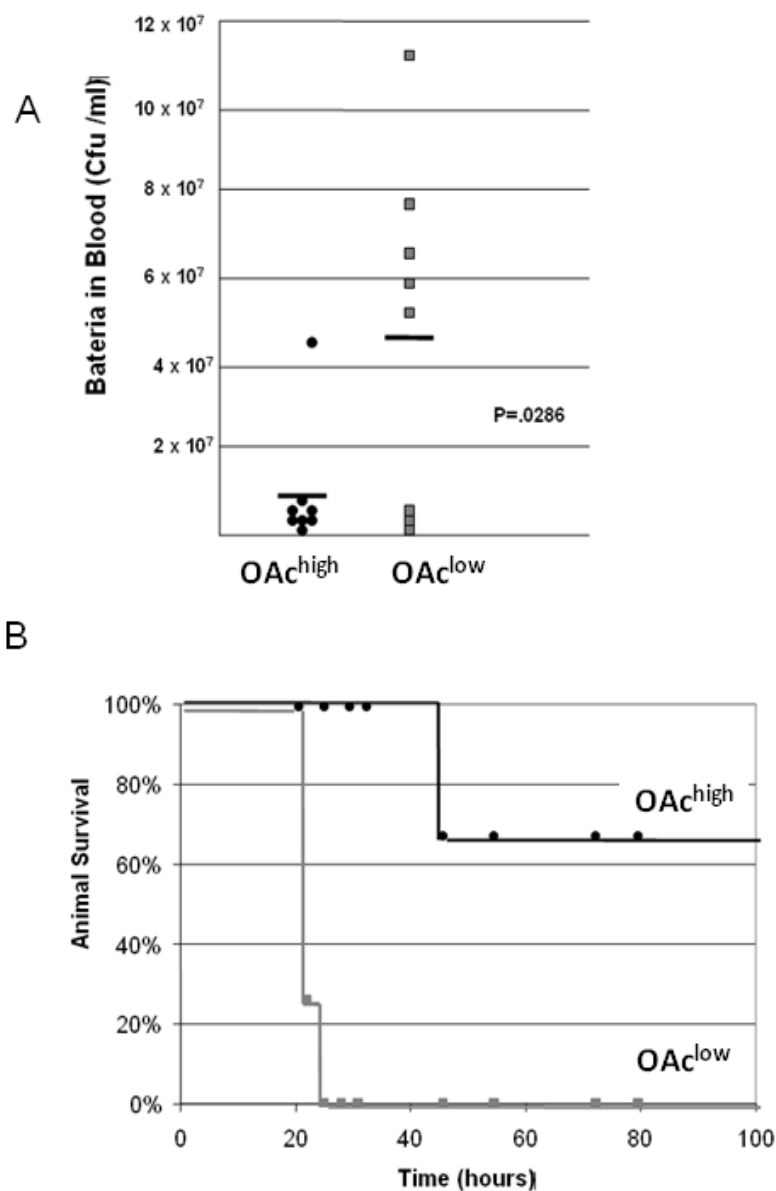
Figure 3.4 Sia O-Acetylation attenuates strain virulence *in-vivo*. 9 week old CD-1 male mice were injected intraperitonially with 4×10^7 cfu of OAc^{high} or OAc^{low} strains. (**A**) 13 hours post infection mice were bled by retro-orbital puncture. The OAc^{low} strain was found at higher levels in the blood. (**B**) In a separate experiment, mice were monitored over the course of 5 days for mortality.   Mice infected with the OAc^{low} strain exhibited 100% mortality within 24 hours, while those infected with the OAc^{high} strain exhibited only 33% mortality with delayed onset of symptoms and death.

bacterial enumeration. Mice infected with the OAc^{low} strain had 5 fold higher levels of bacteria in the blood (average 4.5×10^7 cfu/ml) than the OAc^{high} strain (average 9×10^6 cfu/ml) (p=.0286) (**Figure 3.4A**). This confirms that *in-vitro* findings are consistent in the *in-vivo* mouse model, with O-acetylation impairing bacterial survival.

The impaired proliferation of the OAc^{high} strain translates to attenuated virulence in the mouse model, with a mortality curve showing stark differences in mouse survival post IP injection with OAc^{high} versus OAc^{low} strains. All mice injected with the OAc^{low} strain succumbed to infection within 24 hours, whereas 66% of mice injected with the OAc^{high} strain survived the infection and those that succumbed did not do so until 44 hours post infection (**Figure 3.4B**). Clearly O-acetylation has a very strong impact on bacterial pathogenicity *in-vivo*.

Analysis of Sia O-Acetylation Phenotypes in a Library of Serotype Ia and III GBS

To further clarify the relationship between O-acetylation phenotype, genotype, and serotype in natural variation of GBS, we performed quantitative analyses of Sia O-acetylation levels present in one hundred clinical GBS isolates obtained from the multi center GBS study conducted by the National Institutes of Child Health and Human Development (NICHD). Both noninvasive (colonizing) and invasive strains are represented in this collection. We limited the scope of this study to serotypes Ia and III GBS; each consists of 50 randomly selected invasive and colonizing isolates (45 colonizing and 5 invasive type Ia strains, and 43 colonizing and 7 invasive type III strains). Sia analyses were performed by acid hydrolysis and DMB-derivatization, followed by reverse phase HPLC resolution of O-acetylated and non-O-acetylated Sias as
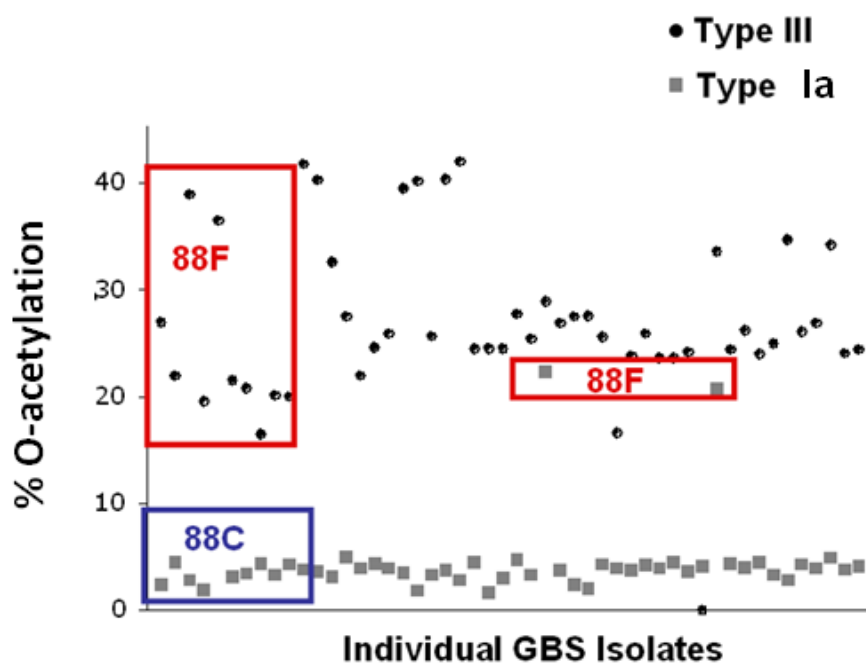
Figure 3.5 Divergent O-acetylation phenotypes between serotype Ia and III GBS. Characterization of native O-acetylation levels from 100 clinical and colonizing isolates indicates a significant correlation between GBS serotype and Sia O-acetylation phenotype, where type III strains have high-O-acetylation levels and type Ia strains have low-O-acetylation levels (P<0.0001). Polymorphism in the Sia-O-acetyltransferase, *neuD*, also correlated with O-acetylation-phenotype.

described previously (Lewis, 2004) (**Figure 3.5**). Of the 50 serotype Ia strains evaluated in this study, 48 exhibited low levels of Sia-OAc (1.7 – 5% Ave 3.6%). In contrast, 49/50 serotype III strains had high levels of Sia-OAc (16.5 - 41.8% Ave 27.5%).

In order to verify the relationship between *neuD* genotype and O-acetylation phenotype, a subset of the NICHD strain collection were chosen randomly and the coding region of *neuD*, the Sia O-acetyltransferase, was sequenced. Of the high-OAc type III strains, 10/10 encode a phenylalanine at *neuD* amino acid position 88. In contrast, 10/10 low-OAc serotype Ia strains encode a cystine at amino acid 88. As noted previously, there were no other changes in primary NeuD sequence between these strains. Interestingly, the two type Ia strains that have high levels of O-acetylation express the 88F version of NeuD, like the type III strains. These data are consistent with earlier experiments showing that chromosomal replacement of NeuD 88F for NeuD 88C converted a high-OAc type III strain to the low-OAc phenotype (Lewis, 2007). The current dataset further extends previous studies by showing that type Ia strains with NeuD 88F display the high-OAc phenotype. Given that the sequences of Sia biosynthetic genes are nearly identical between GBS strains, random mutation is unlikely to account for the 88F polymorphism and high-OAc phenotype of 2/50 type Ia strains.

DISCUSSION

Here we probe various interactions with host immunity to determine whether capsular O-acetylation plays a role in GBS pathogenicity by altering host interactions of GBS sialic acids. We show that O-acetylation presents a cost to bacterial survival in the

context of invasive infection. This may in part be mediated by impaired Siglec-9 binding on the surface of neutrophils, which results in decreased immune suppression and increased bacterial killing. The attenuated virulence of the OAc^{high} strain is demonstrated in *ex-vivo* studies using isolated primary human neutrophils. In whole blood, differences between OAc^{high} and OAc^{low} strains are enhanced. This may indicate that other yet unidentified factors in addition to neutrophil activation contribute to the observed difference in immune control of bacterial proliferation. Another possibility is that the impact of O-acetylation on neutrophil activation is amplified by interactions between other proteins and immune cells in this more complex milieu. *In-vivo* studies using an IP model of infection demonstrate even more severe attenuation of the OAc^{high} strain. There are of course many interactions between different innate immune cells and mechanisms in the whole animal that are absent in isolated *in-vitro* and *ex-vivo* systems. These include the effects of chemotaxis, chemokine mediated activation and cross-talk between cells, as well as organized immune organs such as lymph nodes and spleen which are designed to efficiently coordinate immune responses against invading pathogens. It is the coordination of the system as a whole that provides the essential power of immune response. As such, we see differences between OAc^{high} and OAc^{low} are enhanced in the intact mouse model.

It must be noted that mice to do not possess a direct homolog to Siglec-9. Instead, the role of Siglec-9 in neutrophil suppression is hypothesized to be filled by Siglec-E, a functionally equivalent paralog with the same expression pattern on neutrophils and macrophage. The increased clearance from blood and attenuated virulence of the OAc^{high} strain in the mouse model suggests that O-acetylation similarly impairs Siglec-E

engagement to Sias on the surface of GBS, resulting in impaired suppression of mouse neutrophils.

The cost of O-acetylation to bacterial survival in these studies leads us to question why clinical and colonizing isolates show any degree of O-acetylation. We have found that type Ia strains display low levels of O-acetylation. However, type III strains consistently display intermediate levels of O-acetylation, up to 40%. This suggests that O-acetylation must present some benefit to the bacteria, by some yet unidentified mechanism or in a different context.

One selective pressure favoring some degree of acetylation may be relevant in the context of colonization, which is a much more common state of existence for the bacterium. GBS colonizes the lower gastrointestinal and urogenital tracts in 25-35% of the adult population (Campbell, 2000). In this environment GBS must co-exist with other commensal and transient pathogenic microbes. Many of these microbes express sialidases, which are used to scavenge sugar residues from host cell surfaces as well as to reveal underlying sugar structures for adhesion (Corfield, 1992, Vimr, 2004). These sialidases are also active against Sias on the surface of GBS (Powell and Varki, 2001). Sia removal from the GBS surface would result in loss of immune suppression through Siglec engagement as well as reveal underlying galactose residues to immune recognition, making GBS more susceptible to immune clearance. We have previously shown that O-acetylation protects Sias on the surface of GBS from removal by a wide variety of microbial sialidases (Weiman, 2009). Thus, O-acetylation allows GBS to retain this vital sugar for immune evasion.

Another possible selective pressure favoring O-acetylation of Sias on the surface

of GBS may stem from interactions with bacteriophage (phage) in the environment. Phage often use surface carbohydrates, including epitopes of CPS structures (Stirm, 1968, Stirm, 1971, Choy, 1975, Rodriguez, 2008) and Sia residues in particular (Pelkonen, 19889), as receptors for adhesion to and penetration of host cells (Kumlin, 2008, Gilberger, 2003, Higa, 1985, Rogers, 1986, Aalto, 2001). O-acetylation of GBS CPS Sias may block adhesion of phage targeting GBS surface Sias, as we have seen with other Sia recognizing proteins, such as sialidases and Siglecs. An alternative mechanism by which O-acetylation may confer protection against phage infection, involves the CPS as a protective barrier. In some cases, bacterial CPS layers have been documented to protect against phage infection, acting as a physical barrier to prevent access to adhesive molecules on the bacterial cell wall or prevent penetration by the phage. As such, many phage have evolved enzymes to degrade CPS layers composed of various carbohydrate residues from various bacterial species (Hynes, 1995, Baker, 2002, Lindsay, 2009), including poly-sialic acids (Pelkonen, 1989). O-acetylation may inhibit CPS degrading enzymes that utilize Sias as a recognition epitope or catalytic site residue, allowing this protective barrier to maintain its integrity. Resistance to bacteriophage infection could confer a very strong selective pressure to maintain O-acetylation in GBS.

We hypothesize that O-acetylation level represents a balance between a benefit in the context of colonization and cost in the context of invasion. The optimal balance between these forces appears to be 35% in the type III background. The lower set point of type Ia strains may indicate that the selective pressures in these contexts differ between strains. Indeed there are many genetic factors unique to each strain that may impact these selective pressures. For example, type Ia strains express a β-protein which suppresses

immune response through Siglec-5 interaction (Carlin, 2009) whereas type III strains do not.

In addition, type Ia strains have been shown to interact with a wide variety of CD33r Siglecs, whereas type III strains only engage Siglec-9. (Carlin, 2004) These and other differential interactions with host immunity may influence the selective pressure imposed against O-acetylation in the context of invasion. Susceptibility to microbial sialidases and phage infection, and the role of O-acetylation during colonization may also differ for type Ia strains. Whatever the mechanisms, the selective pressures imposed against type Ia strains consistently drives O-acetylation to a much lower optimum.

Clearly much is left to explore regarding the mechanisms through which O-acetylation impacts immune response and what role O-acetylation plays in the difference between type Ia and type III strains. Here we have demonstrated a clear impact on neutrophil response and overall virulence in the type III background. Future studies will examine the impact of O-acetylation on macrophage activation and bacterial phagocytosis. Siglec transgenic and knockout mice may assess allow the contributions of these receptors to overall pathogenic phenotype. Studies using OAc^{high} and OAc^{low} in the A909 background will reveal whether the same mechanisms and selective pressures hold true in type Ia strains. By examining these relationships we hope to ultimately clarify the role of O-acetylation in pathogenesis of GBS disease.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Strains with high and low O-acetylation (COH1 background) were defined previously (Lewis et al. 2007 Weiman et. al.2009). Isogenic bacterial strains were grown in Todd-Hewitt Broth (THB; Difco, BD Diagnositics) containing 5 μg/ml of erythromycin (Erm). For infection studies, bacteria were cultivated at 37°C to mid-log phase and resuspended to OD_{600} 0.4, followed by serial dilution and enumeration of colony-forming units in experimental inocula. For biochemical studies, the GBS strains used in this study were obtained from Deng-Ying C. Lin at the NICHD and cultivated overnight in THB without antibiotics.

Cell Lines

The human brain microvascular endothelial cell line hBMEC was obtained from Kwang Sik Kim (Johns Hopkins University, Baltimore, Maryland, USA) and maintained as described previously [3, 4, 24]. A549 cells (ATCC), a human type II alveolar epithelial carcinoma cell line were maintained and passaged in RPMI 1640 tissue culture medium that contained 10% FBS. Cultures were incubated at 37° C in a humid atmosphere of 5% CO_2.

Adhesion and Invasion Assays

Eukaryotic cells were split into 24-well plates, and allowed to grow to confluence for 48 hr prior to assays. Bacteria were resuspended in PBS and added to confluent hBMEC or A549 monolayers at a multiplicity of infection (MOI) of 10. Plates were

centrifuged at 800 x g to synchronize the infection, and incubated at 37° C with 5% CO_2 for 30 minutes. Nonadherent bacteria were washed off 3 times with PBS, after which eukaryotic cells were lysed with0 .025% triton-X and cell-associated bacteria plated in serial dilution for enumeration.

Cellular invasion assays proceeded for 2 hours, after which cell associated bacteria were washed off 3 times with PBS, and 1 ml of RPMI 10% FBS containing 100 μg of gentamicin and 5 μg of penicillin G was added for two additional hours to kill extracellular adherent bacteria. After washing eukaryotic cells were lysed as described above and intracellular bacteria plated in serial dilution for enumeration. All cellular adherence and invasion assays were performed in triplicate and repeated at least three times. Student's two-tailed t-test was used to study differences and statistical significance was accepted at $p < 0.05$.

Neutrophil Isolation

Normal human volunteers donated small blood samples for the isolation of neutrophils, with informed consent obtained in accordance with the Declaration of Helsinki, under protocols approved by the University of California, San Diego Human Subjects Institutional Review Board. Isolation was performed using Polymorphprep (Axis-Shield, Oslo, Norway). Neutrophils were resuspended in HBSS without Ca/Mg.

Neutrophil Granule Protease Release

Bacterial strains were resuspended in HBSS with Ca/Mg (Hyclone) and added to neutrophils at MOI 10 or 25 as indicated and incubated at 37° C for 30 minutes with

orbital rotation. Tubes were centrifuged at 1000 g for 5 minutes and the supernatant collected into wells of a 96-well plate; 0.5 μL of MeOSuc-Ala-Ala-Pro-ValNmec dissolved in dimethyl sulfoxide at 20 mM was added to each well. After incubating at room temperature for 20 minutes, hydrolysis of the substrate was monitored spectrofluorometrically by change in absorbance at 405 nm. Assays were performed in triplicate and repeated at least three times. Student's two-tailed t-test was used to study differences and statistical significance was accepted at $p < 0.05$.

Neutrophil Oxidative Burst

Neutrophils were labeled with dichlorofluorescindiacetate at a final concentration of 20 μM in HBSS, incubated for 20 minutes at 37° C, and resuspended in 1ml HBSS. 10^6 neutrophils in 100 μl of HBSS were combined with bacteria (MOI 10-50 as indicated) in 50 μl of HBSS with Ca^{2+} / Mg^{2+} (Hyclone) for a total volume of 150μl. Cells were spun down together to initiate contact at 1000 X g for 5 minutes, resuspended, and incubated at 37°C for up to 45 minutes with orbital rotation. 50 μl aliquots were removed at 15 min intervals and oxidative burst was measured using flow cytometry. Experiments were repeated at least 5 times. Statistical significance was calculated using FlowJo software.

Bacterial Survival in the Presence of Neutrophils

Bacterial strains were resuspended in HBSS and preopsonized in 90% immune naïve serum for 20 minutes at 37° C. Bacteria were serially diluted and plated for enumeration of inoculum, and added to neutrophils at MOI 0.1 or 0.01 and incubated at

37° C with orbital rotation. 25 μl aliquots were removed at 15 minute intervals. Neutrophils were lysed in sterile H_2O and surviving bacteria plated in serial dilution for enumeration. Student's two-tailed t-test was used to study differences and statistical significance was accepted at $p < 0.05$.

Whole-Blood Bacterial Survival

Phlebotomy was performed on healthy volunteers. $5x10^4$ cfu of bacteria in 100 μl were added to 300 ml whole blood and incubated at 37° C with orbital rotation. 25 μl aliquots were removed and plated in serial dilutions for enumeration of surviving bacteria at various time points up to 2 hours. Student's two-tailed t-test was used to study differences statistical significance was accepted at $p < 0.05$.

Mouse Infection Studies

All animal experiments were approved by the Committee on the Use and Care of Animals and performed using accepted veterinary standards. Outbred 9 week old male CD-1 mice (Charles River Laboratories) were injected intraperotineally with $4x10^7$ bacteria in a total volume of 150 ul mixed 1:2 with 10% gastric mucin. (Fleming, 1982) 13 hours post infection, blood was collected via retro-orbital puncture. Bacterial counts in blood were determined by serial dilution plating. Mortality studies were conducted by the same procedure and animals monitored for survival over 5 days.

Biochemical Analysis of Sialic Acid O-Acetylation

Bacterial pellets from 1ml of culture were washed and Sias were released by mild acid hydrolysis, isolated, and derivatized with 1,2-diamino-4,5-methylene dioxybenzene (DMB), and analyzed by HPLC for percent Sia O-acetylation as previously described (Lewis, 2007).

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Chapter IV

Conclusions and Discussion

O-acetylation of sialic acids on the surface of GBS is a recent discovery. Here we provide a more thorough documentation of this phenomenon in clinical and colonizing GBS isolates, demonstrating a clear distinction between serotype Ia strains, which exhibit low levels of O-acetylation (5%), and serotype III strains, which exhibit intermediate levels of O-acetylation (30%). A serotype-specific polymorphism in the NeuA O-acetylesterase has been identified as the genetic basis for this phenomenon. However, the explanation behind the evolution of differential O-acetylation phenotypes between strains, and the implications this has in disease manifestation remains unclear. Regardless, we hypothesize that the level of surface O-acetylation reflects a balance between selective pressures for and against this biochemical modification. These selective pressures may differ in different serotypes and environmental contexts.

In order to investigate the effects of O-acetylation and its impact on GBS survival strategy, we have created isogenic OAc^{high} and OAc^{low} strains. Through genetic manipulation and subsequent glycotyping analysis we have developed a more complete understanding of the functions and roles of the the NeuD acetyltransferase and NeuA acetylesterase in GBS CPS biosynthesis.

Using the genetically engineered OAc^{high} and OAc^{low} strains we provide the first documentation of the effects of this biochemical modification on interactions between GBS and its environment. We have shown that O-acetylation can impair interactions with sialic acid recognizing proteins, including sialidases of microbial origin and Siglec immune receptors in the host. In some cases and contexts the effect of O-acetylation may benefit bacterial survival, while in others it proves costly. We have identified some of the selective pressures and mechanisms that act on and are affected by O-acetylation.

Most striking, we have shown that O-acetylation impairs GBS immune evasion as measured by, heightened neutrophil response, increased bacterial killing and overall attenuation of the OAc^{high} strain.

However, much remains to be investigated. Let us reconsider our findings, their possible implications, and the future of this research in the context of what is currently known and what remains to be discovered.

Sialidase Protection Provides a Potential Benefit in the Context of Colonization

We have shown that O-acetylation is able to protect GBS surface Sias from removal by sialidases that are expressed by a variety of co-colonizing and transient pathogenic bacteria. We presume that the removal of Sias by such sialidases would render GBS more susceptible to innate immune recognition and clearance, but this has not been directly demonstrated. It is also possible that Sias remaining on the surface after sialidase exposure are sufficient to engage immune suppressive mechanisms equally well as the fully sialylated bacterium.

Conversely, we presume that sialidase protection offered by O-acetylation conserves GBS's ability to downregulate immune activation. However, this may not be the case, as we have also demonstrated that O-acetylation impairs Siglec-9 binding and neutrophil suppression. The sialidases tested remove unacetylated residues which fully engage Siglec-9, leaving acetylated Sias on the surface. We would expect these acetylated residues to confer some degree of immune suppression, as the OAc^{high} strain binds Siglec-9 to a much greater extent than asialo GBS. However, whether the

engagement of Siglec-9 by remaining acetylated Sias is sufficient to confer immune suppression and promote bacterial survival in the context of colonization is undetermined. Thus, while we have clearly demonstrated that O-acetylation offers protection from a variety of bacterial sialidases, the impact of this protection on GBS immune evasion strategy is not definitive.

It has also not been demonstrated that O-acetylation protects GBS surface Sias from sialidases *in vivo*. It is possible that the prevalence of Sias on host cells and in thick mucins present in these environments saturates enzymatic activity, leaving GBS Sias at less of a risk for removal. If this is the case, O-acetylation would not be essential for protection. We must also keep in mind that the number of bacteria and viruses that GBS may encounter in the context of colonization is extensive, and that the diversity between these organisms is extreme. While sialidase genes are very common in both bacterial and archael genomes, the specificity of such enzymes is likely to vary widely, and whether O-acetylation offers protection will depend upon each particular case. Our studies merely indicate that O-acetylation has the ability to offer this protection in some of these cases. Whether O-acetylation ultimately protects the GBS surface *in-vivo* will depend upon the population of microbes present in the urogenital and or gastrointestinal tract at a particular time. Overall sialidase activity against GBS in these environments will depend upon the proportion of microbes producing sialidases, the level at which the sialidases are produced, their substrate specificities and whether O-acetylation blocks enzymatic activity. Recent findings indicate that each individual is host to a unique microbiome, which is in constant flux. Thus, sialidase production and profiles may differ between individuals or across time, with the potential to differentially effect GBS survival.

Similarly, GBS colonization within a particular individual also changes over time. Whether this is correlated to or influenced by sialidase production by other microbes at a particular time is an intriguing concept to consider. It would be interesting to test sialidase activity in urogenital or gastrointestinal samples in concordance with the presence of GBS, and to incubate such samples with GBS *ex-vivo* to determine if sialidase activity in the sample is able to remove GBS surface Sias, and whether this removal renders bacteria more susceptible to immune clearance. Without an appropriate colonization model we have not been able to study the impact of O-acetylation on the ability of GBS to colonize the host, but we expect that OAc^{high} strain might be better able to do so. In a 'clean mouse', without competing microbes, the OAc^{high} strain might not have this advantage.

While the intricacies and implications of sialidase protection conferred by O-acetylation have not been pinned down, it is likely that this effect presents an advantage to bacterial survival in the context of colonization, and provides a positive selective force for GBS to acetylate its surface.

O-Acetylation May Modulate Susceptibility to Bacteriophage Infection

An additional selective force favoring O-acetylation of Sias on the surface of GBS may stem from interactions with bacteriophages in the environment. Similar to viruses which target eukaryotic cells (as discussed in the introduction), bacteriophages often use surface carbohydrates, including Sias, as receptors for adhesion to and penetration of host cells (Kumlin, 2008, Gilberger, 2003, Higa, 1985, Rogers, 1986, Aalto, 2001). In many cases, epitopes recognized by bacteriophage are components of bacterial capsular

polysaccharides (Stirm, 1968, Stirm, 1971, Choy, 1975, Rodriguez, 2008). Bacteriophages targeting GBS have not been described in great detail, but may utilize epitopes containing CPS Sias. O-acetylation could potentially block this interaction, as we have seen with other Sia recognizing proteins, such as sialidases and Siglecs. By preventing bacteriophage receptors from recognizing CPS Sia epitopes on the GBS surface, O-acetylation may prevent phage adhesion, conferring GBS with resistance to infection. Alternatively, it is also possible as in the case with Influenza C (Rogers, 1986), that some phage have evolved recognition of O-acetylated CPS Sias, in which case O-acetylation would make the bacterium more susceptible.

O-acetylation may confer resistance to phage infection through a second mechanism as well. In some cases, bacterial capsular polysaccharides have been documented to confer protection against phage infection, acting as a physical barrier to prevent access to adhesive molecules on the bacterial cell wall or prevent penetration by the phage. As such, many bacteriophage have evolved enzymes to degrade CPS layers composed of various carbohydrate residues from various bacterial species (Hynes, 1995, Baker, 2002, Lindsay, 2009), including polysialic acids (Pelkonen, 1989). An enzyme which degrades the GBS CPS may utilize Sia as a recognition epitope or catalytic site residue. O-acetylation may interfere with these interactions, preventing CPS degradation and allowing this protective barrier to maintain its integrity providing resistance to phage infection.

Through these various mechanisms, modulation of susceptibility to bacteriophage infection may provide a very strong selective pressure to maintain O-acetylation in GBS.

Complement Deposition is Unaffected by O-Acetylation

We have shown that O-acetylation does not impact C3b deposition on the surface of GBS. This is counter to previous observations of mouse erythroluekemia (MEL) cells in which O-acetylation impaired factor H binding, resulting in increased complement mediated killing (Shi, 1996, Varki, Kornfeld, 1980). There are a number of possible explanations for the discrepancy between these two systems.

We have discussed the possibility that factor H binding is unaffected by O-acetylation in GBS simply due to the higher sialic acid density on the bacterial cell surface. Indeed, our calculations indicate that even in the highly acetylated strain, the density of unacetylated Sias exceeds the overall Sia density on the MEL cell surface more than 2:1. Thus, the sheer quantity of Sias may effectively recruit factor H such that acetylation does not impair binding and regulation of complement activation.

However, it is important to note that we were not able to directly measure factor H binding, as is often the case in bacterial systems. It is therefore possible that O-acetylation does alter factor H binding and alternative complement activation. However, there is an additional arm of the complement pathway that may come into play. The lectin branch is also capable of recognizing bacterial surface glycans and recruiting downstream components that activate the complement pathway, result in C3b deposition, immune activation and opsonophagocytosis (Albanyan, 2000, Aoyagi, 2005). The importance of this branch in bacterial clearance is supported by the fact that individuals with deficiencies in this pathway are highly susceptible to bacterial infection (Ruskamp, 2009, Munthe-Fog, 2009). L-ficolin and complement receptor 3 (CR3) are two such

serum lectins that recognizes sialic acids on the surface of GBS and contribute to bacterial clearance (Albanyan, 2000, Aoyagi, 2008). Multivalent binding enhances lectin affinity, and as such, the repetitive regular array of Sias on the GBS capsular polysaccharide promotes binding to a greater extent than shorter oligosaccharide chains, or the more complex surface of mammalian cells (Albanyan, 2000). As such, it is possible that the lectin branch plays a greater role in activating the complement pathway on the surface of GBS than the alternative branch, which is modulated by factor H.

Given this possibility, the discrepancy between the effect of O-acetylation on complement activation in GBS versus MEL cells may be explained by the following scenario. Since we were not able to measure factor H binding directly, it is possible that O-acetylation on the GBS cell surface does impair factor H binding as it does on MEL cells. Without a significant degree of lectin binding in the context of the host surface, these differences in factor H binding translate into differences in overall complement deposition and complement mediated clearance of MEL cells. However, significant binding of multiple lectins to GBS may mask any potential differences mediated by factor H.

It is unknown whether O-acetylation affects lectin binding and complement activation. Different lectins may or may not be affected according to their Sia recognition epitope. It is unlikely that L-ficolin binding would be affected by O-acetylation, as this lectin recognizes the Sia N-acetyl group which is not altered by O-acetyl modification of the exocyclic side chain. However, there is a possibility that O-acetyl modification could impact the ability of L-ficolin to engage in multivalent interactions with N-acetyl groups, or even that O-acetyl groups may be alternatively

recognized for binding to a lesser degree. Regardless, any impact must not be very pronounced, as we have shown that there is no difference in the downstream read out of C3b deposition. Alternatively, it is possible that O-acetylation impairs L-ficolin binding, decreasing complement activation, while also impairing factor H binding, increasing complement activation, and that these two effects balance each other out. The relative contributions of different lectins to GBS recognition and complement activation should also be considered. Whatever the explanation regarding the effects on and contributions of these two branches of the complement pathway, O-acetylation on the surface of GBS does not alter overall complement deposition. This gives GBS the freedom to acetylate its surface without impairing this essential factor of immune evasion strategy.

Impaired Siglec-9 Engagement Presents a Cost to Bacterial Survival

In contrast, we have found that O-acetylation impairs Siglec mediated immune suppression by GBS. Specifically, O-acetylation impairs binding of Siglec-9, thereby impairing neutrophil suppression, and results in increased bacterial clearance and reduced pathogenicity of artificially high O-acetylated strain.

These studies have focused on OAc^{high} and OAc^{low} strains, comparing the effects of 85% and 0% acetylation. Whether WT levels of acetylation (35%) yield intermediate phenotypes has not been tested. It is tempting to presume that the effect of O-acetylation would proceed incrementally, in a dose-dependent linear manner. However, many biological interactions are non-linear. Biochemical interactions often exhibit non-linear binding kinetics. Of particular relevance in these studies is the propensity of carbohydrate binding proteins to exhibit multivalent properties. Cooperative binding and

nucleation are important and widespread means of signal amplification in biological systems. In addition, zymogen cascades are similarly designed to rapidly amplify signals. On the cellular level, things become even more complicated, with interactions between different signaling cascades and messages being integrated into an overall cellular response. Additional complications arise from the interactions between cells. All of these mechanisms are undoubtedly involved in the complicated process of immune response. Given these multi-tiered layers of signaling interactions, and the propensity of each to exhibit non-linear dynamics, its seems unlikely that the impact of O-acetylation would exhibit a linear pattern. Instead, it is likely that intermediate O-acetylation levels do not exhibit intermediate phenotypes, but rather display "threshold" effects.

Further studies will be required to examine the impact of intermediate levels of O-acetylation in the WT type III background and why type III strains consistently display intermediate levels of O-acetylation. It is possible that there is a threshold for the effects of O-acetylation on immune evasion strategy. Below this point, increases in O-acetylation may not have a significant impact on immune suppression. However, once acetylation increases above this threshold, immune suppression is severely impaired. Glycotyping of type III clinical and colonizing isolates suggest that this threshold may exist around 40%, as all type III strains limit surface acetylation below this point.

Here we have examined the extremes of high and low acetylation in order to better understand the constraints imposed on this biochemical modification on the surface of GBS. We have outlined some of these effects, however, much is left to examine regarding the details of these complex interactions.

OTHER MECHANISMS TO CONSIDER:

Susceptibility to Neutrophil Killing Mechanisms

While we have demonstrated one mechanism by which O-acetylation alters bacterial pathogenicity, there are many other mechanisms that have yet to be investigated. It is likely that many of these contribute to differences in strain pathogenicity, as differences in neutrophil responses alone were much less striking than whole blood and *in-vivo* findings.

We have documented how GBS Sia O-acetylation impacts neutrophil elastase secretion and oxidative burst response. Differential induction of other neutrophil responses such as NET formation or cytokine and chemokine production has not been analyzed, but would be predicted to follow a similar pattern.

Bacterial susceptibility to these neutrophil killing mechanisms may also contribute to observed differences in strain survival. For example, while neutrophils use oxidative burst to kill phagocytosed bacteria, many bacteria have evolved mechanisms for resisting this killing mechanism. Indeed sialic acids have been shown to confer resistance to reactive oxygen species by acting as a hydrogen peroxide scavenger (Ogaskawara, 2007). Whether O-acetylation impacts this capability is unknown. Sias may also impact susceptibility to other killing mechanisms such as antimicrobial peptides (CRAMPs), which are secreted and concentrated in NETs to kill extracellular bacteria. The activity of these cationic pore forming toxins is affected by electrostatic interactions with negatively charged Sias in mucins (Bucki, 2008). The effect of bacterial surface Sias on CRAMP activity has not been studied, nor how O-acetylation might effect this interaction. O-acetylation modification of Sia mediated susceptibility to these and other

neutrophil killing mechanisms may contribute to, or even blunt, observed differences in bacterial survival in *ex-vivo* and *in-vivo* experiments, which has been attributed to differential neutrophil activation. Whether O-acetylation would be beneficial, neutral, or detrimental to bacterial survival in these contexts depends on the specific killing mechanism in question.

The Role of Macrophages

While our studies have focused on neutrophils, other cells of the immune system express Siglecs and contribute to bacterial clearance. Macrophages also express Siglec-9, and as such, it is likely that O-acetylation similarly impairs Siglec-9 mediated immune suppression in this cell type. This would accentuate the cost of O-acetylation to bacterial survival.

However, in this cell type there is added complexity, as macrophages also express Sialoadhesin (Sn, also termed Siglec-1), a phagocytic receptor involved in clearance of sialylated pathogens (Jones, 2003). We have shown that O-acetylation also impairs Sn binding (**Figure 4.1**), protecting GBS from phagocytosis (**Figure 4.2**). In this respect, impaired Siglec binding presents an opposing selective pressure, in which the highly sialylated bacterium is able to evade phagocytic clearance by macrophages. The final balance between the opposing effects of O-acetylation on GBS survival through Siglec-9 and Snengagement in the context of this single cell type is undetermined.

Yet, again, the story is even more complicated, as Sn is upregulated by immune activation (van den Berg, 1996). Thus, Siglec-9 mediated immune suppression effects Snexpression on the surface of macrophages. As such, a highly O-acetylated pathogen
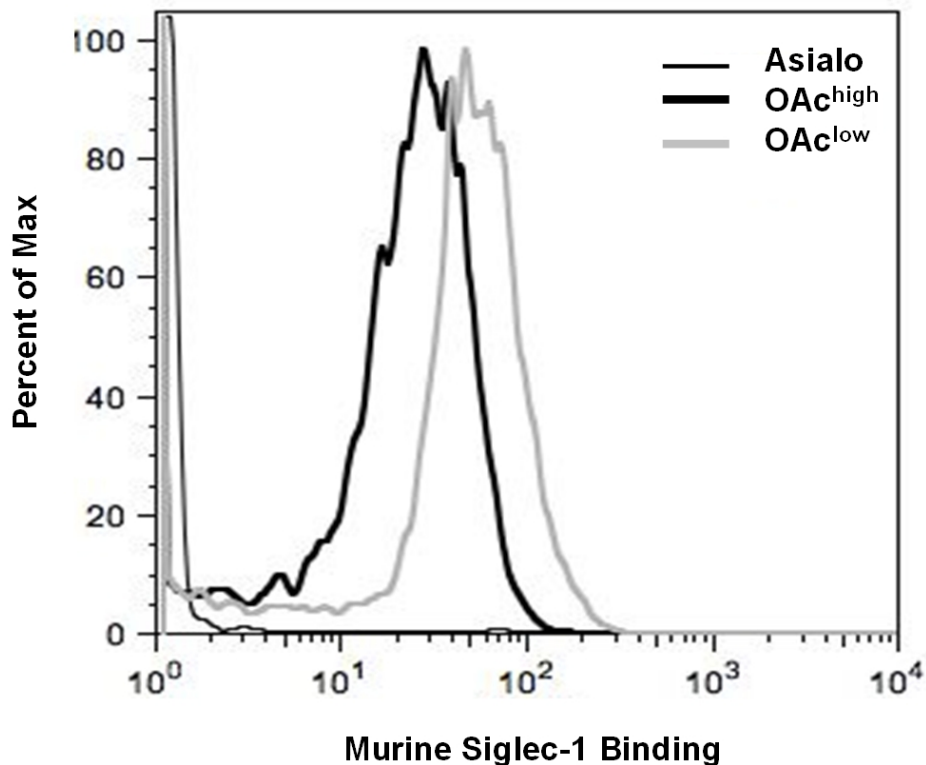
Figure 4.1   O-Ac reduces binding of murine Siglec-1 to GBS.
Isogenic OAc^high and OAc^low GBS strains were incubated with murine Siglec-1-Fc chimeric protein and analyzed for binding by flow cytometry using a goat anti-human (IgG-Fc)-PE conjugated secondary antibody. Specifically, murine Siglec-1-Fc chimeric protein (0.4 ug) was pre-complexed with goat-anti human phycoerythrin conjugated secondary antibody (0.4 ug) in 50 ul of PBS for 1 hour on ice in the dark. Bacterial strains were grown to mid log phase and resuspended to OD_{600} 0.4 in PBS. 2×10^6 bacteria in 50 ul of PBS were incubated with precomplexed mixture for 40 minutes on ice in the dark. Samples were then washed with 1 ml of PBS, resuspended in 500 ul of PBS and analyzed using a BD Facscaliber flow cytometer. Data representative of 3 independent experiments.
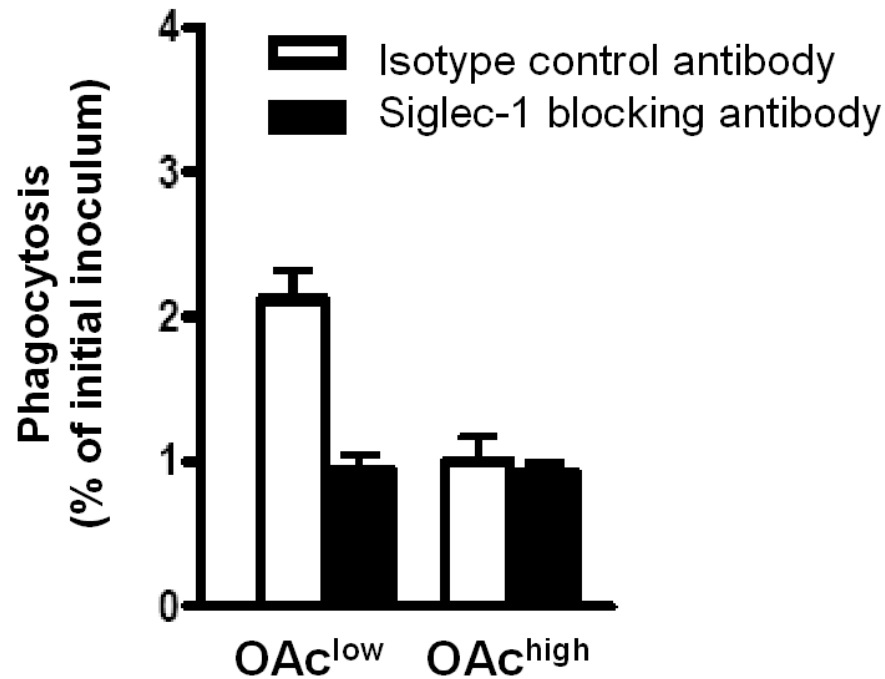
Figure 4.2 O-acetylation reduces Siglec-1 mediated phagocytosis by macrophages. Mouse bone marrow-derived macrophages (5x10^5/ml, 0.5 ml/well) were stimulated with IFN-α (500 U/ml) for 2 days to induce Siglec-1 expression. After washing with PBS twice, cells were loaded with GBS (OAc^{high} and OAc^{low} strains) at MOI=10, spun at 2000 rpm for 5 min and incubated for 1 hr at 37° C. After incubation, cells were washed with PBS three times and media containing 5 ug/ml penicillin and 100 ug/ml gentamycin was added for additional 1 hr incubation to kill extracellular bacteria. Cells were lysed in 0.025% triton X-100 and intracellular bacteria were enumerated using serial dilution plating. Siglec-1 blocking and non-blocking antibody were used as described in Jones, 2003. P= 0.0067

may be better protected from phagocytic clearance by Sn, but if initial increased activation via Siglec-9 results in increased Sn expression on the surface, this protection may be negated overall. Alternatively, while the low O-acetylated strain would be better recognized and phagocytosed by Sn, its superior ability to engage Siglec-9 and suppress immune activation may prevent surface expression of the phagocytic receptor in the first place. Thus, whether O-acetylation presents an overall cost or benefit to GBS survival against clearance by macrophages depends on the interdependence of both Siglec-9 and Sn mediated mechanisms, and opposing effects on each.

The contribution of this cell type to differential survival in whole blood and *in vivo* experiments has not been addressed. The availability of Sn knockout mice has limited these studies. In the future, use of these animals will allow the determination of Sn contribution to strain virulence.

Interactions Between Immune Cells

An additional consideration in whole blood and *in-vivo* studies is the interaction between different cell types in mounting an effective immune response. When activated, neutrophils secrete cytokines and chemokines which attract and activate other immune cells, including macrophages. Macrophages also engage in cross talk with neutrophils and other immune cells, affecting immune response. Thus O-acetylation dependent modulation of one cell type may have profound influence on overall activation of the entire immune system.

Specifically, Siglec-9 dependent suppression of neutrophils may affect macrophage activation states and therefore Sn expression. Alternatively,

macrophage activation states may affect neutrophil responses, as well as responses by other immune cells. Sn has also been shown to mediate interactions between macrophages and sialic acid expressing immune cells, modulating their activation (Wu, 2009). The effect of O-acetylation on this complicated network of interactions is unexplored, but it is in this context of the coordinated effort of the intact immune system that the weight of the costs and benefits takes its ultimate toll.

Distribution of Cell Types in Different Contexts

Within the host, immune cells are distributed in different tissues and compartments of the body in different proportions. These distributions affect potential interactions with GBS, as well as interactions between different cells of the immune system. This is of particular importance in the context of colonization versus invasive infection.

Within the gastrointestinal and urogenital tracts, patrolling macrophages are most likely to come into initial contact with colonizing GBS. Therefore the impact of O-acetylation on GBS survival would depend most upon the impact on macrophage activation and phagocytic clearance mechanisms. However, during blood stream and tissue invasion, neutrophils are more prevalent and will play a major role in bacterial clearance through their own activation, as well as their influence on macrophage activation and clearance mechanisms. The presence or absence of these different types of immune cells in different contexts, their relative distribution, potential for interaction and influence on overall immune activation all interact such that O-acetylation may have differential impact on GBS survival in different contexts.

O-Acetyl Migration

An additional variable that affects these interactions is pH, which also differs in the context of colonization versus invasive infection. Recall that GBS acetylates at the 7 carbon position of the exocyclic side chain, and that the acetyl group is retained here in acidic conditions characteristic of the urogenital tract. However, at neutral pH, characteristic of blood and tissues, this acetyl group migrates to the 9 position (Varki, 1992). We have shown that 7 O-acetylation blocks Siglec-9 binding better than the 9 O-acetylation. This suggests that in the context of colonization 7 O-acetylation would impair immune suppression to a greater extent than in the context of invasive infection. This presents an intriguing scenario, in which GBS is able to protect itself from co-colonizing bacterial sialidases during colonization in the urogenital tract where immune surveillance is minimal, but this O-acetylation is less costly to immune evasion strategy during tissue invasion where immune suppression is more critical for bacterial survival. The merits of this theory have yet to be proven. The extent of acetyl migration during invasion and whether the difference in Siglec-9 blocking translates into differences in neutrophil or macrophage activation is unknown. Also, whether the position of O-acetylation similarly impacts interactions with Sn binding and what effect this has on phagocytic clearance has not been studied. What is clear, is that the effect of O-acetylation is influenced by many interacting factors that range from biochemical to intracellular and even physiological, and that these context dependent interactions are of critical relevance.
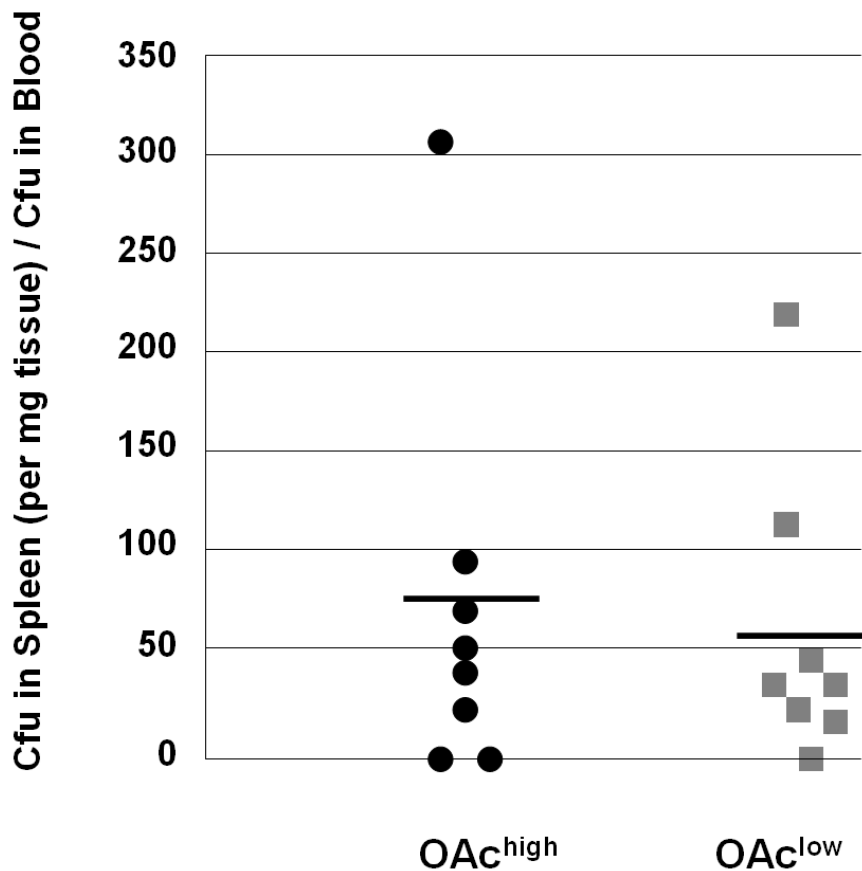
Figure 4.3   O-Ac does not impact GBS homing to spleen.
Outbred 12 week old male CD-1 mice (Charles River Laboratories) were injected intraperotineally with $4x10^7$ bacteria as described in Chapter III Materials and Methods. 13 hours post infection animals were euthanized and spleens collected aseptically. Bacterial counts in tissue homogenates were determined by serial dilution plating. Data presented as cfu/ gram of tissue, as compared to cfu found in blood (Fig. 3.3A)

Tissue Homing

O-acetylation may impact bacterial survival by actively influencing tissue localization as well. As discussed in the introduction, interactions of Sias and Siglecs is involved in tissue homing of leukocytes. This process is modified by O-acetylation. (Krishna, 1997, Shi, 1996, Ghosh 2007) Localization can subsequently influence immune response and clearance. The impact of Sia O-acetylation on tissue homing and immune clearance has been demonstrated in the context of MEL cells. O-acetylation on this cell type has been shown to impair binding to Sn on macrophages and decrease tissue homing to spleen, where Sn expression on resident macrophages is high. This leads to decrease in MEL cell clearance via reduced splenic retention. (Shi, 1996) Thus O-acetylation can impact immune clearance by influencing responses by individual cells, but also by influencing tissue localization, particularly if that tissue is an immune organ which is structured to efficiently and effectively eradicate pathogens.

Whether O-acetylation of GBS similarly effects localization of invading bacteria to spleen and immune clearance by this organ is undetermined. We have seen that O-acetylation impairs Sn binding, and therefore we might expect the OAc^{low} strain to be present at higher levels in the spleens of infected animals due to interactions with this receptor on resident splenic macrophages. If this is the case, the OAc^{low} strain might be better cleared from blood and display decreased pathogenicity, which is counter to what we have shown in our mouse model of infection. But again, the impact of splenic homing on bacterial clearance would also depend on the effect of O-acetylation on Siglec-9 mediated activation, as well as Sn mediated uptake, the balance between these two forces, and the interactions between cells expressing these receptors in the spleen.

In order to address these questions, we conducted an IP study in which mice were injected with OAc^{high} and OAc^{low} strains as described in materials and methods section of Chapter III.   Mice were sacrificed 14 hours post infection and spleens harvested, homogenized and plated for bacterial cfu.   We found no differences in splenic localization when compared to cfu in blood (**Figure 4.3**), indicating yet again that the effect of O-acetylation on GBS is distinct from that on MEL cells and studies must be conducted using the appropriate model system to accurately determine these effects. Thus, in GBS localization to spleen is not altered by O-acetylation.

Other immune organs of relevance include bone marrow and lymph nodes. We have not conducted these studies, but differential immune cell populations and Siglec expression in these tissues may affect the homing and clearance of GBS, and whether O-acetylation affects these processes.

Relative Contribution of Siglecs to Bacterial Survival *In-Vivo*

While the complexities of Sia-Siglec interactions in the context of the whole organism are difficult to unravel, one means for addressing the contribution of individual Siglecs to O-acetylation mediated differences in pathogenicity is through the use of knockout mice. There are two lines that are of particular interest in our studies.

Sialoadhesin knockout mice are currently being bred. By eliminating the impact of Sn mediated phagocytosis by macrophages, this system will allow us to address the role that this receptor plays in GBS clearance, as well as the impact of O-acetylation on this process. If Sn plays an important role in bacterial clearance *in vivo*, we would expect that these mice would be more susceptible to infection, displaying higher bacteremia and

mortality compared to WT mice, as they lack this clearance mechanism. If Sn does not play a contributing role in bacterial clearance *in-vivo*, we would not see differences in pathogenicity in this model compared to infection in WT mice.

Whether Sn differentially affects clearance of OAc^{high} and OAc^{low} GBS *in-vivo* is also undetermined. We have seen that O-acetylation impairs Sn binding, and phagocytic uptake by macrophages. As such, O-acetylation may protect bacteria from clearance, giving a survival advantage to the OAc^{high} strain. In contrast to this prediction, we have found that the OAc^{low} strain is more resistant to clearance *in vivo*, yielding higher bacteremia and mortality in a mouse IP model of infection. Thus, overall Sn is not the predominant factor influencing the difference in virulence between these two strains. However, it is possible that the impact of Sn attenuates this difference. Accordingly, infection in Sn knockout mice, in which this counterbalancing factor has been removed, might reveal an even more pronounced difference in pathogenicty between the OAc^{high} and OAc^{low} strains. Alternatively, if the impact of O-acetylation on Sn does not play in important role in bacterial clearance *in-vivo*, we would expect to see the same pattern of differential virulence of OAc^{high} and OAc^{low} strains as we see in the WT mouse model.

Analyzing the role of Siglec-9 *in-vivo* is a more complicated problem, as mice do not possess a homolog of this human Siglec. The role of Siglec-9 is predicted to be filled by Siglec-E in mice, which displays the same expression pattern on neutrophils and macrophages, and is thought to be a functionally equivalent paralog. We have shown that Siglec-E also binds to GBS. We have not determined if this interaction is Sia dependent or blocked by O-acetylation. Based on the hypothesis that GBS can mediate

immune suppression via Siglec-E, Siglec-E knockout mice will allow us to evaluate the contribution of this inhibitory Siglec on bacterial clearance *in-vivo*. If GBS engagement of Siglec-E plays a major role in immune evasion *in-vivo*, elimination of this immune suppressive receptor would render these mice more resistance to infection, with lower levels of bacteremia and increased animal survival.

Elimination of Siglec-E will also allow us to address the role that this receptor plays in mediating differences in virulence between OAc^{high} and OAc^{low} strains *in-vivo*. We have demonstrated that O-acetylation blocks Siglec-9 binding, and results in greater neutrophil activation and bacterial killing in *ex-vivo* human experiments. The impaired survival of the OAc^{high} strain *in-vivo*, is consistent with the hypothesis that Siglec-9/E plays a dominating role in contributing to differences in strain pathogenicity *in-vivo*. According to this hypothesis, we would expect that these differences would be eliminated, or muted in the Siglec-E knockout mice. Elimination of strain differences in virulence would indicate that Siglec-E plays the predominant role in contributing to these difference *in-vivo*. Muted strain differences would indicate that Siglec-E contributes, but that there are other contributing factors involved. If strain differences remain unaltered, this would indicate that strain differences are mediated by a yet unidentified mechanism and that Siglec-E is not a major contributing factor.

Using Sialoadhesin and Siglec-E knockout mice to analyze bacterial survival and strain differences in virulence *in-vivo*, will give us some insight as to the degree that each of these receptors contributes to bacterial clearance and the effect of O-acetylation on these processes within the context of the intact animal system. However, these studies

must be analyzed with the understanding that the mouse model may not directly reflect the human system, due to differences in Siglec repertoire, as well as differences in other immune mechanisms such as complement.

Developmental Changes in the Immune Landscape

It is clear that the complexities of the coordinated immune system present many variables that may differentially mediated the impact of O-acetylation in different contexts. These include variable distribution of immune cells and Siglec expression in different tissues. An added complication arises when we consider that these parameters of immune function can also change over time. Specifically, the repertoire of immune cells and their functioning capabilities is quite different in the developing neonatal immune system, compared to that of an adult. Indeed, deficiencies in neutrophil responsiveness is the main cause of neonatal susceptibility to GBS infection. It has also recently been shown that Siglec-9 expression and reactivity is decreased in neonatal neutrophils, altering overall inflammatory responsiveness and longevity of these immune effector cells. (Rashmi, 2009)

This has many implications for the impact of O-acetylation in the context of immune response. If Siglec-9 does not play as great a role in modulating immune response in neonatal neutrophils, O-acetylation may not present such a cost to bacterial survival in newborns as we have seen using adult human whole blood. Our mouse studies used young, but far from newborn, animals, and whether Siglec-E expression and reactivity exhibit similar differences over time in this model organism is unknown. Thus, it is possible that while O-acetylation proves detrimental to bacterial immune evasion

strategy in the systems we have tested, the extent of this cost may be much less in the context of neonatal invasive disease.

This could have implications for the epidemiology of serotype colonization and infection. Immune-compromised adults may be at greater risk of lowly acetylated type Ia infection, with highly acetylated type III strains being at a greater disadvantage against Siglec-9 expressing immune cells. This of course may also depend on the nature of immune-compromise. In neonates, type Ia and type III strains may have equal opportunity, due to a lack of Siglec-9 mediated immune modulation. It would be interesting to note the frequency of type Ia versus type III strains in both colonization and invasive disease in these two populations. Whether and how the immune landscape changes over the course of the first few months of life is unclear, but changes in Siglec-9 expression and neutrophil responsiveness could underly the disproportionate distribution of type III highly acetylated strains in cases of late onset GBS disease.

Here I have considered one of numerous immune parameters that may contribute to clearance of GBS, and the impact of O-acetylation on this process. Many other factors may also contribute and change as the immune system develops from birth, through infancy, childhood and adulthood. Changes in these factors may alter the response of individual immune cells to GBS, as well as modify cell-cell interactions and overall immune response, thereby affecting epidemiology of GBS disease across different age populations.

O-Acetylation in Adaptive Immunity

Addressing the role of GBS Sia O-acetylation in adaptive immunity is beyond the scope of this body of work. However, it is of critical relevance as current vaccination strategies use de-acetylated capsule as their antigenic component. Whether this impacts the efficacy of vaccination is unknown, but has many potential consequences.

First, O-acetylation might affect antibody generation by altering the immunogenicity of the CPS sialic acid residue, which forms a critical epitope for protective antibody generation against serotype III strains (Baker, 1976, Kasper, 1979). Sia O-acetylation has been shown to increase immunogenicity in both bacterial and mammalian systems. In *N. meningitis* O-acetylation increases immunogenicity of lipopolysaccharides, resulting in increased protective antibody generation against this deadly pathogen (Berry, 2002). In human melanomas, O-acetylation defines the immunogenic epitope, with de-O-acetylated Sias lacking immunogenicity altogether. (Cheresh, 1984, Ravindranath, 1989). While de-O-acetylated GBS capsule is able to invoke protective antibody generation to some extent (Pannaraj, 2009), O-acetylation of GBS could similarly increase CPS Sia dependent immunogenicity. As such, vaccination with acetylated capsule could greatly enhance vaccine efficacy.

Relative immunogenicity of acetylated versed unacetylated capsule also may affect colonization and infection in a serotype specific manner. Higher O-acetylation of type III strains may elicit a greater adaptive immune response, thereby inducing bacterial clearance. This may account for the low frequency of colonization and infection by this serotype (CDC, 2005). Meanwhile, type Ia strains displaying low levels of O-acetylation may elicit a weaker adaptive immune response, which could explain their ability to more

commonly colonize the host and cause invasive disease. (CDC, 2005, Baker, 1995, Lin, 1998, Campbell, 2000)

Of additional concern is the binding specificity of antibodies generated against O-acetylated vs. unacetylated capsule. Whether antibodies generated against one epitope are able to bind and protect against the other is again of critical importance in vaccine design and has implications in epidemiology of colonization and infection. Pannaraj et. al. (2009) have shown that human vaccinees generate antibodies against de-O-acetylated capsule that are also able to bind to acetylated GBS. However, the affinities of these antibodies have not been examined and it is possible and even likely that such antibodies would be less effective at binding to and protecting against O-acetylated GBS species than antibodies generated against O-acetylated CPS. Preliminary data shows that indeed O-acetylation blocks binding of antibodies that have been generated against unacetylated type III Sia CPS epitope. (**Figure 4.4**) As such, current vaccination strategy may offer greater protection against lowly O-acetylated type Ia strains than highly O-acetylated type III strains.

Alternatively, if O-acetylated CPS were used in vaccination, would antibodies generated against this epitope be able to bind to unacetylated capsule, protecting against lowly O-acetylated type Ia strains? It has been shown that antibodies generated against O-acetyl Sias in human melanomas are also capable of binding de-O-acetylated Sias (Cheresh, 1984, Ravindranath, 1989). But again, affinities have not been examined. Whether this true of antibodies generated against O-acetylated GBS is unknown. If such
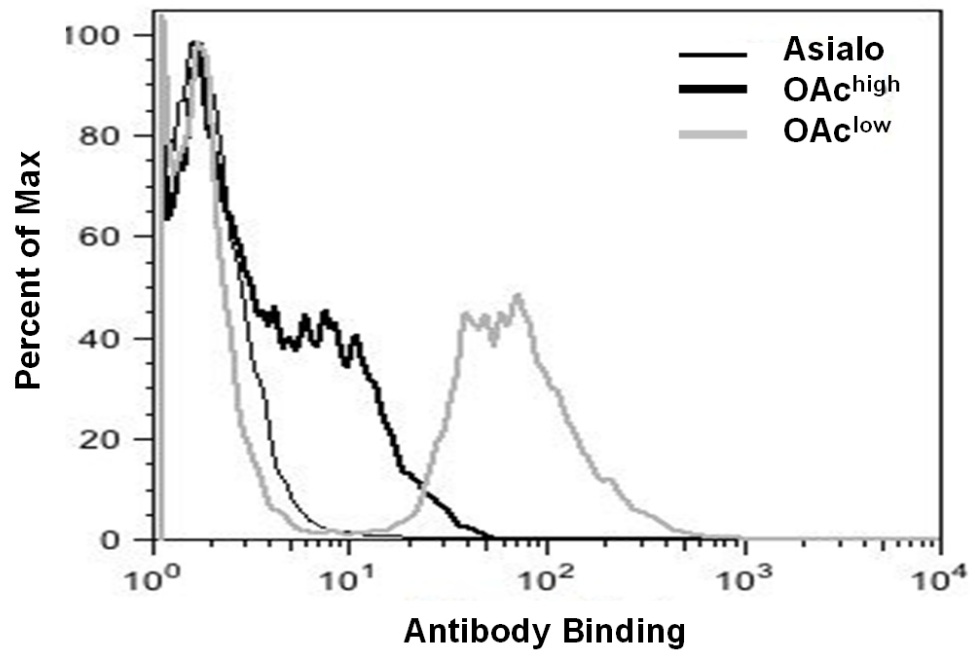
Figure 4.4 O-acetylation can block binding of one antibody directed against the type III COH1 capsular antigen.   Primary antibody directed against the COH1 capsular antigen (S9 MAB Pincus, 1988) (1:50 dilution) was preincubated with secondary Cy5-goat-anti-mouse IgM (Jackson Immuno 115-175-075, lot 71690) (1:500 dilution) for 1 hour in PBS. Isogenic OAc^{high} and OAc^{low} GBS strains were grown to mid-log phase, resuspended to OD$_{600}$ 0.4 in PBS, and diluted 1:5. 50 ul of bacteria and 50 ul of pre-conjugated antibody mixture were incubated on ice for 50 minutes on ice in the dark. Samples were then washed with 1 ml of PBS, resuspended in 500 ul of PBS and analyzed using a BD Facscaliber flow cytometer. Data representative of 3 independent experiments.

antibodies preferentially or solely bind to the acetylated epitope, vaccination with O-acetylated CPS may protect against highly O-acetylated type III strains but not lowly O-acetylated type Ia strains. As such, the optimal vaccination strategy may require antibody generations against both O-acetylated and unacetylated CPS.

The impact of O-acetylation and antibody generation in epidemiology of GBS disease is also fascinating to consider. If antibody specificities do differ between O-acetylated and unacetylated epitopes, this would imply that colonization or infection with one strain could confer adaptive immunity against strains with similar O-acetylation profiles, but not against differing strains (ie. Ia vs III and vice versa). This may be another driving force for the evolution of O-acetylation in GBS, in which O-acetyl modification can provide protection against an existing repertoire of host antibodies, in a manner reminiscent of capsular serotype evolution for immune evasion (Cieslewicz, 2005).

Another consideration is acetyl migration in the context of antibody generation and protection. Recall that GBS O-acetylates at the C-7 position of the exocyclic side chain. At the relatively acidic pH characteristic of colonization in the urogenital tract O-acetylation is retained here. However, at neutral pH characteristic of invasive infection, the acetyl group can migrates to the C-9 position. There are many complications and intricacies that come into play here. First, consider antibody generation. Whether O-acetylation at one position is more immunogenic than the other is unknown. Also, the ability of the host immune system to generate antibodies in the context of colonization vs. invasive infection could play a role in the repertoire of antibodies formed against one

epitope versus the other. Second, consider antibody specificity. Whether antibodies generated against O-acetylation at one position preferentially bind epitopes exhibiting the same position of O-acetylation is unknown. It is likely that such antibodies are not cross-reactive.

There are many implications to these possibilities. In the context of colonization, we must consider first if colonization is able to illicit an adaptive immune response. Then, are the antibodies generated in this context (7-O-Ac) protective in the context of invasion (9-O-Ac)? Looking in the opposing direction, in the context of vaccination, will antibodies generated in blood and tissue (9-O-Ac) be protective against colonization (7-O-Ac), or be more limited to protection against invasive infection?

Acetyl migration in the face of adaptive immunity presents the potential for an additional driving force supporting the evolution of acetylation in GBS. O-acetylation may act as a moving target on the GBS surface. During colonization GBS presents one epitope to the host, but during invasion the antigen is altered, enabling GBS to evade existing host adaptive immunity. Without acetylation there is no potential for epitope alteration and therefore immune evasion.

Our mouse models have addressed overall pathogenicity of high and low O-acetylated strains in the face of innate immune mechanisms, showing that O-acetylation is detrimental to GBS survival in invasive infection. However, the contribution of O-acetylation in the face of adaptive immunity remains virtually unexplored, and may provide selective pressures both for and against O-acetylation dependent on the context. The effect of O-acetylation on adaptive immune response, and vice versa, has broad

implications in vaccine design, epidemiology of serotype colonization and bacterial survival in both the context of colonization and invasive disease. While we do not currently understand these complexities, it is possible that evasion of adaptive immune mechanisms may provide a positive selective pressure to promote GBS O-acetylation.

IMPLICATIONS IN TYPE IA STRAINS

Our studies have focused on the effect of Sia O-acetylation in the type III COH1 background for a number of reasons. First, genetic manipulation has yielded strains with greater variation (0- 85% O-acetylation) in this background compared to type Ia strains (0 -35%), in which the upper limit of O-acetylation is constrained by a less active NeuD acetyltransferase. It may be possible to generate an A909 strain with similarly high levels of O-acetylation by genetic manipulation of the F88C NeuD allelic exchange A909 mutant, but these reagents have not yet been created. We expect that greater variation between OAc^{high} and OAc^{low} strains will make the effects of O-acetylation more salient in our studies. Second, the WT level of O-acetylation in the type III COH1 strain is intermediate (~35%) (Lewis, 2007), and as such, we are able to address the effects of increasing or decreasing O-acetylation compared to WT. While we have focused on the OAc^{high} and OAc^{low} strains for these studies, future experimentation will address these strains in comparison to WT COH1. In the A909 Ia background we would only be able to study the effect of increasing O-acetylation, as the WT level is very low (5%) (Lewis, 2007). Third, when tested against a panel of CD33r Siglecs, COH1 has been shown to interact only with Siglec-9, whereas A909 interacts with many members of the family

(Carlin, 2009). By studying O-acetylation in the COH1 background, we are able to isolate the effects on a single Siglec, simplifying our studies and resulting conclusions in an already highly complex system of immune interactions.

While it is tempting to presume that the results of our studies translate to the A909 strain, this cannot be assumed. Indeed, the different balance in WT levels of O-acetylation in this strain suggest that there are different selective pressures influencing and being influenced by O-acetylation in this background. We have previously hypothesized that the level of O-acetylation reflects a balance in selective pressures between benefits and costs to O-acetylation. As such, there must be additional benefit to O-acetylation in the COH1 background or additional cost in the A909 background. There are a number of selective forces and means for differential influences to consider.

Innate Immunity

First consider the role of innate immunity and Siglecs, which we have shown impose a negative selective pressure against O-acetylation. One hypothesis is that there is a greater selective pressure from the innate immune system against type Ia strains, and that any degree of O-acetylation impairs immune evasion strategy such that it is intolerable for GBS survival. There is some evidence to support this theory. First, exposure of uncapped galactose residues, which are recognized by immune lectins of the host and used to target foreign cells for destruction, is far greater on the surface of A909 than COH1. (**Figure 4.5**). To counterbalance the susceptibility to immune recognition conferred by exposed galactose residues, A909 must optimize other immune suppressive
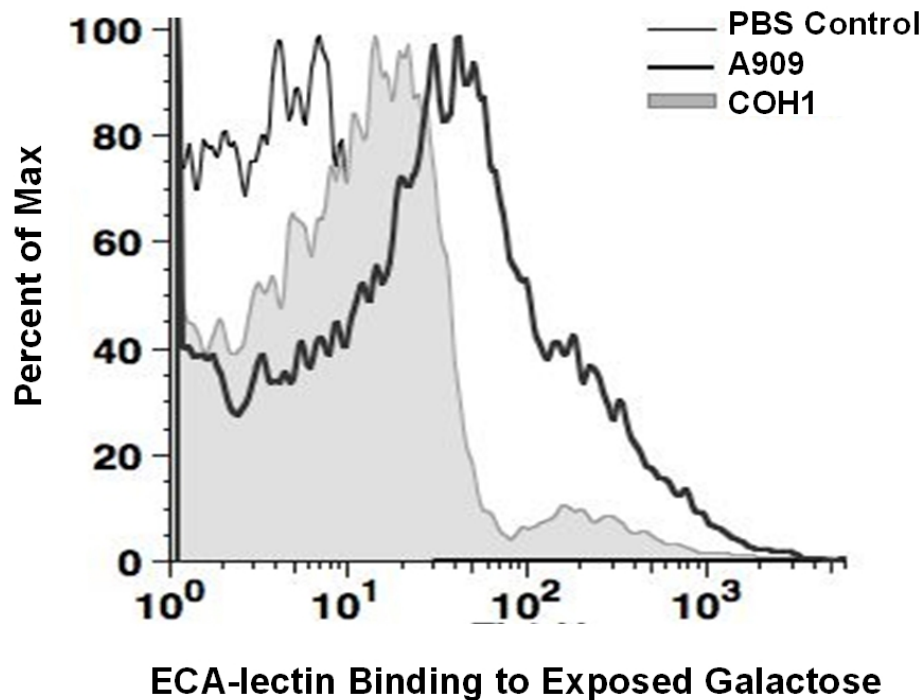
Figure 4.5 The A909 strain displays more uncapped galactose residues than COH1. The extent of galactose exposure (underlying Sia) was evaluated by flow cytometry using a FITC-conjugated ECA lectin as described in the Materials and Methods Chapter II. GBS were grown to mid log phase and resuspended to OD$_{600}$ 0.4. Approximately 10^8 cells were washed in PBS and diluted 1:5 in PBS. 50ul was incubated on ice in the dark in a total volume of 100ul with the FITC-conjugated lectin from *Erythrina cristagalli* (ECA, EY labs) at a final dilution of 1:100. After 30 minute incubation, cells were pelleted, washed in PBS, and resuspended for analysis using a BD Facscaliber flow cytometer. Data representative of 5 independent experiments.

mechanisms in order to survive in the host. Indeed, A909 strongly interacts with many members of the CD33r Siglec family (Carlin, 2009), suggests that it is optimizing immune suppression through as many Siglecs as possible.

Whether these interactions are Sia dependent or influenced by O-acetylation, however, is unproven. In fact, one such interaction has been shown to be Sia independent. Siglec-5 engagement, and resulting immune suppression, is mediated by the β-protein, which is unique to the A909 strain (Carlin, 2009). Whether interactions with other members of the family are protein or Sia dependent is unknown. As such, we addressed the Siglec-9 A909 interaction. Isogenic OAc^{high} and OAc^{low} strains were created in the A909 background using methods described in Chapter II for generation of isogenic mutants in the COH1 background. Glycotyping analysis confirmed similar surface sialylation between OAc^{high} and OAc^{low} A909 isogenic strains, and documented O-acetylation levels at 35% and 0% respectively. Siglec-9 binding experiments were performed as indicated in materials and methods section of Chapter II. Consistent with findings in the COH1 background, we found that Siglec-9 engagement by the A909 strain is Sia dependent, as the ΔNeuA asialo mutant failed to bind Siglec-9 in our flow cytometry based format (**Figure 4.6**).   In addition, O-acetylation in the A909 background, even at only 35%, blocks Siglec-9 binding (**Figure 4.6**). These findings indicate that O-acetylation has the potential to interfere with Siglec mediated immune evasion by A909 as well.

However, the effect of O-acetylation in this background is dependent upon the relative contributions of many members of the CD33r Siglec family to overall activation state of neutrophils and other immune cells. Other aspects of these interactions that must
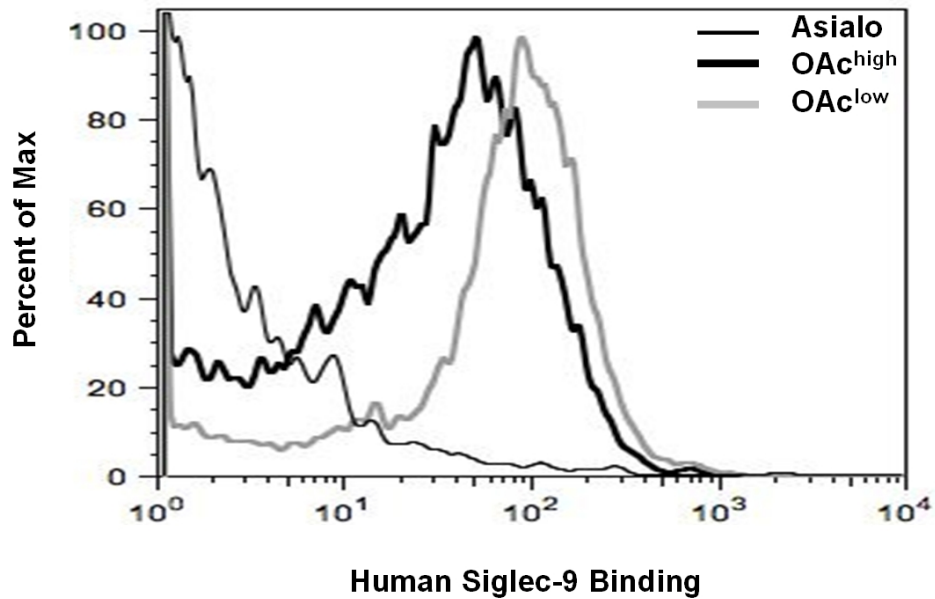
Figure 4.6   O-acetylation also reduces engagement of Siglec-9 to GBS in the A909 background. Isogenic OAc^{high} and OAc^{low} GBS A909 strains were incubated with human Siglec-9-Fc chimeric protein and analyzed for binding by flow cytometry using a goat anti-human (IgG-Fc)-PE conjugated secondary antibody. Specifically, human Siglec-9-Fc chimeric protein (0.4 ug) was pre-complexed with 0.4 ug of goat-anti human phycoerythrin conjugated secondary antibody in 50 ul of PBS for 1 hour on ice in the dark. Bacterial strains were grown to mid log phase and resuspended to OD$_{600}$ 0.4 in PBS. 2x10^6 bacteria in 50 ul of PBS were incubated with precomplexed mixture for 40 minutes on ice in the dark. Samples were then washed with 1 ml of PBS, resuspended in 500 ul of PBS and analyzed using a BD Facscaliber flow cytometer. Data representative of 3 independent experiments.

be considered are which Siglec interactions are protein dependent versus which are Sia dependent, if Sia dependent interactions are affected by O-acetylation or not, the distribution of Siglec expression on different populations of immune cells, and the relative contribution of each Siglec to the activation state of the particular cell it is being expressed on. Sia independent interactions with other innate immune mechanisms such as galactose binding lectins may also contribute to overall immune selective pressures. The balance between all of these variables ultimately determines the impact of O-acetylation in type Ia strains.

While these specifics remain unknown, we have conducted some preliminary studies on overall effects of O-acetylation to immune activation and bacterial survival. Neutrophil activation as measured by elastase secretion appears to be unaffected by O-acetylation in the A909 background (data not shown). Bacterial survival in human whole blood *ex vivo* also does not differ between strains. (data not shown) Preliminary *in vivo* experiments using IP infection model demonstrate no difference in virulence as measured by mouse mortality. (data not shown)

These studies indicate that despite impairing Siglec-9 binding, O-acetylation may not be detrimental to A909 immune evasion strategy in the context of invasive infection. This is in contrast to what we have found in COH1. However, here we compare 0% to 35% O-acetylation, whereas in the type III background we compare 0% to 85% O-acetylation. It is possible that impaired Siglec binding that results from 35% O-acetylation is not enough to affect neutrophil activation, whereas 85% is. If this is true, it might explain why the WT level of COH1 acetylation is set at 35%, as this level of O-acetylation would not impair immune evasion strategy but additional increases would

become detrimental to bacterial survival. We have the COH1 reagents to test this hypothesis, but studies have not been completed. Alternatively, it is possible that even 35% acetylation impairs COH1 survival and that 85% O-acetylation would not affect A909 survival due to the contributions of other Siglecs, or galactose binding lectins in this context.

This still leaves the question of why type Ia strains would consistently display lower O-acetylation, if partial O-acetylation does not appear to impair immune evasion. What other selective pressure prevents type Ia strains from acetylating? Or is there a selective pressure that encourages acetylation in type III strains that does not act in the type III background?

Adaptive Immunity

Adaptive immunity may play a role. There are a number of other possibilities involving antibody generation and recognition capabilities within and between strains that may influence selective forces on O-acetylation in these different backgrounds. It is possible that O-acetylation in the type Ia background is more antigenic than in the type III background, and as such adaptive immunity has created a selective pressure to lower O-acetylation in type Ia strains. I encourage the reader to refer to the previous section on adaptive immunity and to contemplate the many complex scenarios that can explain this phenomenon.

Sialidases

Next let us consider the influence of sialidases on O-acetylation levels. We have hypothesized that O-acetylation benefits COH1 in the context of colonization, by protecting from bacterial sialidases. This has not been directly demonstrated in A909 and it is possible that the underlying capsular structure in this background alters sialidase susceptibility such that O-acetylation does not have an affect. The type Ia CPS may not be as susceptible to sialidases, and therefore protection conferred by O-acetylation would be unnecessary. Alternatively, the type Ia CPS may be just as susceptible as the type III CPS, but O-acetylation may not confer protection in this background. In either case there would be no pressure to acetylate in the type Ia context, and as such type Ia strains may simply leave their surfaces unacetylated.

Bacteriophage

It is also likely that presumed selective pressure imposed by bacteriophage (phage) infection on O-acetylation, may differ between strains. Phage receptors can evolve very specific carbohydrate epitopes for recognition. If receptors or CPS degrading enzymes have evolved to recognize the CPS oligosaccharide of type Ia or type III strains uniquely, this could differentially effect the impact of O-acetylation in each of these backgrounds.

There are a number of scenarios that could support this hypothesis. First, a phage could have evolved to use the type III oligosaccharide structure for adhesion. O-acetylation may block this recognition, conferring protection against this phage, and thereby selecting for type III strains with higher O-acetylation. The inability of this

phage to adhere to the type Ia CPS would impose no selective pressure to increase O-acetylation in the the type Ia background. A second possibility is that a phage could have evolved to adhere to a different receptor that is also unique to the type III background. However, access to this receptor or penetration of the phage may be prevented by the thick CPS layer, requiring an enzyme to degrade this protective barrier. In this case, O-acetylation in the type III strain could confer protection against infection by interfering with recognition or enzymatic activity of the CPS degrading enzyme. Again this would result in a selective pressure to increase acetylation in the type III strain. No such selective pressure would be imposed on the type Ia strain as it lacks the receptor to be targeted by this phage in the first place.

Alternatively, it is possible that the selective pressure may be imposed on type Ia strains in the opposite direction. A phage may have evolved to target the type Ia strain, using O-acetylated Sia in the type Ia oligosaccharide structure in its recognition, much like the targeting mechanisms of the Influenza C virus. In this case, O-acetylation would be driven down to evade phage recognition in type Ia strains, but type III strains would not be subject to this selective pressure.

Clearly the potential for differential constraints, pressures and influences of O-acetylation between type Ia and type III strains is extensive. Future research will begin to answer these numerous questions to elucidate the guiding forces leading to differential O-acetylation in type Ia and type III strains, and what implications this has in disease manifestation.

CONCLUDING REMARKS

We have shown that in the face of innate immunity during invasive infection, GBS O-acetylation is detrimental to bacterial survival. This effect is in part mediated by Siglec-9 in the human system, with O-acetylation blocking engagement of this immune suppressive Sia receptor. As a result the OAc^{high} strain impaired neutrophil immune suppression and is unable to proliferate in the face of neutrophil killing mechanisms to the same extent as the OAc^{low} strain. While the effects of O-acetylation on neutrophil activation and killing are subtle in *in-vitro* and *ex-vivo* neutrophil isolation experiments, *ex-vivo* whole blood and *in-vivo* mouse experiments show a much greater effect. This finding is not surprising, as there are many interactions between different innate immune cells and mechanisms in the whole animal that are absent in isolated *in vitro* systems. These include the effects of chemotaxis, chemokine mediated activation and cross-talk between cells, as well as organized immune organs such as lymph nodes and spleen which are designed to efficiently coordinate immune responses against invading pathogens. It is the coordination of the system as a whole that provides the essential power of immune response. As such, we see differences between OAc^{high} and OAc^{low} strains are enhanced in the intact model.

The detrimental effect of O-acetylation on bacterial survival during invasion leads us to question why GBS would acetylate its surface Sias. There must be some contexts in which O-acetylation is beneficial to the bacterium. We have seen that Siglec-mediated selective pressure against O-acetylation can be altered by pH in different environments. The presence or absence of other factors in different contexts may also contribute to

overall O-acetylation phenotypes. We have identified some of the opposing evolutionary forces which may drive up O-acetylation.

O-acetylation impairs Sn mediated phagocytic clearance by macrophages. While the role of this receptor *in-vivo* is confounded by influences of other Siglecs and crosstalk between immune cells, the effect of O-acetylation on this receptor and its function does not appear to be a major contributing factor to overall strain virulence, as the OAc^{high} strain is less pathogenic. However, the potential benefit of O-acetylation to evading Sn mediated clearance in both the context of colonization and invasion may compound with other positive selective forces contributing to the partially O-acetylated phenotype.

Protection against other microbes in the context of colonization may provide the most powerful selective force to drive O-acetylation on the surface of GBS. We have shown that O-acetylation protects GBS surface Sias from removal by sialidases which are produced by microbes that co-exist in the same niche, thereby preserving GBS immune evasion strategy. It s also possible that O-acetylation may confer protection against bacteriophage infection, be interfering with phage adhesion to GBS CPS Sia epitopes, or by blocking CPS degrading enzymes. The importance of these influences in the context of colonization may provide strong selective pressure, as this is the most common state and intended environment for the bacterium, existing comfortably within the host without causing disease or death.

An additional contributing factor may involve O-acetylation as a means to evade adaptive immune response. O-acetylation may alter bacterial immunogenicity or the affinities of protective antibodies. In addition, the O-acetylated epitope may provide a moving target for identification by the adaptive immune system, with different serotype

strains displaying different O-acetylation phenotypes. Within the same strain, the migration of the O-acetyl epitope in the context of invasion versus colonization may present an even greater challenge to effective antibody generation, both with respect to immunogenicity as well as antibody binding affinity. Through a number of these mechanisms, O-acetylation may provide a means for GBS to evade adaptive immune response by modulating its surface.

Obviously many of the factors and mechanisms effecting O-acetylation and its impact on bacterial pathogenicity have yet to be identified and understood. As these mysteries unravel, new discoveries will give us clues about the differences between these selective pressures and mechanisms in different serotype strains. Eventually these clues will lead us towards an explanation for the very clear and distinct set points in O-acetylation between these strains and whether these differences are merely correlative or contribute in some way to disease manifestation.

There is much left to discover, but one thing is clear: The systemic effects of this subtle biochemical modification are profound. GBS must balance the detriments and benefits of O-acetylation in all contexts, colonization and invasion. The selective pressures GBS faces in these environments are numerous, include innate and adaptive immune mechanisms, as well as threats from co-colonizing microbes, both bacterial and viral. While we do not understand all the mechanisms behind the influence of O-acetylation, we cannot help but be amazed by the eloquent way in which GBS has managed to manipulate so many of these interactions through the use of a simple biochemical modification of a single sugar residue on its surface. Balancing the forces of

selection that it encounters in various contexts, GBS is able to optimize O-acetylation to achieve optimal survival. For GBS, survival within the host is exceedingly complex, and life really does hang in the balance.

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