Group B Streptococcal Capsular Sialic Acids Interact with Siglecs (Immunoglobulin-Like Lectins) on Human Leukocytes

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Group B Streptococcus (GBS) is the leading cause of bacterial pneumonia, sepsis, and meningitis in human newborns and is increasingly recognized as a pathogen in adult populations, including diabetics, pregnant women, and the elderly. A critical factor contributing to GBS virulence is its surface capsular polysaccharide (CPS). The CPS forms the outermost layer of the bacterial surface and is typically composed of repeating subunits of four monosaccharides, i.e., glucose, galactose, N-acetylglucosamine, and N-acetylmuraminic acid, polymerized in serotype-specific configurations (10). GBS isolates are categorized as belonging to one of nine different serotypes (Ia, Ib, and II to VIII) based on antibody recognition. Although the physical structure of the CPS of each GBS serotype is unique, all GBS serotypes share 50 to 80% sequence similarity, with the highest similarity in inhibitory signaling that dampens or counteracts activating responses. In this study, we examined whether CPS Sias of various GBS serotypes can specifically engage human CD33rSiglecs present on the surfaces of leukocytes in a cell type-specific fashion suggesting distinct functional roles (11). The CD33rSiglecs share 50 to 80% sequence similarity, with the highest similarity in the V-set domain involved in Sia recognition and the two intracellular tyrosine signaling motifs. The membrane-proximal signaling domain is an immunoreceptor tyrosine-based inhibitory motif (ITIM) with the canonical sequence (I/L/V)\(\times\)Yxx(L/V), whereas the membrane-distal domain is an ITIM-like motif whose role is less well defined (29, 34). Upon phosphorylation by a Src family kinase, these motifs can recruit protein tyrosine phosphatases SHP-1, SHP-2, and SHIP. Many transmembrane proteins containing these motifs are involved in inhibitory signaling that dampens or counteracts activating signals sent by other immunoreceptors, such as those containing immunotyrosine-activating motifs.

In this study we examined whether CPS Sias of various GBS serotypes can specifically engage human CD33rSiglecs present on human leukocytes involved in control of bacterial infections. Further, we examined how O-acetyl modification of CPS Sia can modify the interaction between GBS and these leukocyte surface receptors. The implications of GBS surface expression of a human-like monosaccharide, Neu5Ac, are then discussed in the context of molecular mimicry and innate immune evasion.
MATERIALS AND METHODS

Bacteria and growth conditions. GBS wild-type (WT) strains of serotypes Ia (A909), Ib (MT09), II (DK23), III (COH1), V (NCTC10/84), and VI (NT-6) are well-characterized isolates from human neonates with invasive infections. Generation of the ΔNeuA and ΔNeuD mutants of serotype III strain COH1 was previously reported (23, 24). GBS were grown in Todd-Hewitt broth (THB), pH 7.5, or on THB agar plates. For antibiotic selection, 10 μg/ml erythromycin or 2.5 μg/ml chloramphenicol was used. Escherichia coli strains were grown in Luria-Bertani broth (LB); antibiotic selection employed 50 μg/ml chloramphenicol. For functional assays, unless otherwise noted, bacteria were grown to early exponential phase in THB, washed three times with pyrogen-free phosphate-buffered saline (PBS), resuspended in appropriate buffers, and adjusted to the desired concentrations by using a spectrophotometric method confirmed by pour plate colony counts. For enzymatic removal of CPS Sias, GBS was incubated in sterile PBS with 100 mU/ml Antrobacter ureaficiens sialidase for 1 h at 37°C and then washed three times with PBS. Trypsin-treated bacteria were incubated with 0.5% trypsin plus EDTA in PBS for 30 min at 37°C and washed five times with PBS. Prior to Chinese hamster ovary (CHO) cell binding experiments using Sia-blocking and non-Sia-blocking antibodies, GBS was preincubated for 10 min in PBS plus 5% normal goat serum and then washed twice.

GBS FITC labeling. GBS was grown overnight, diluted 1:100 in 50 ml of THB, grown to early exponential phase, pelleted, washed three times with sterile PBS (pH 7.4), and heat killed by incubation at 56°C for 40 min. GBS was then washed in 50 mM carbonate buffer (pH 8.0), resuspended in 5 ml carbonate buffer (pH 8.0) containing 1% BSA, and incubated for 45 min at 4°C. Bacteria were extensively washed in PBS to remove trace amounts of free FITC and then resuspended (20 mM HEPES, 150 mM NaCl, 1% bovine serum albumin [BSA], pH 7.4). FITC-labeled GBS cells (FITC-GBS) were then enumerated using a bacterial cytometer and a fluorescent microscope. FITC-GBS (1 × 10^6) were resuspended in PBS plus 1% BSA and analyzed by flow cytometry (BD FACSCalibur) to verify that bacterial staining was uniform throughout each sample.

Siglec-Fc binding assay. Immunol 4 enzyme-linked immunosorbent assay plates were coated with 0.5 mg/ml protein A in coating buffer (50 mM carbonate buffer, pH 9.5) overnight at 4°C. Wells were washed three times with assay buffer (20 mM HEPES, 150 mM NaCl, 1% BSA) and then blocked with assay buffer for 1 h at room temperature. Human CD33/Siglec-F chimeras diluted in assay buffer were added to individual wells at 0.5 μg/ml and allowed to adhere for at least 3 h at room temperature. Wells were washed three times with assay buffer, and 1 × 10^5 FITC-GBS suspended in assay buffer were added to each well and centrifuged. Bacteria were allowed to adhere for 10 min at 37°C. The initial fluorescence intensity was verified, wells were washed to remove unbound bacteria, and the residual fluorescence intensity (excitation, 488 nm; emission, 530 nm) was measured using a CytoFluor® fluorescent plate reader.

Soluble receptors and stable cell expression. Soluble hSiglec-Fc chimeras were produced as previously described (2). CHO-K1 cells stably expressing either full-length hSiglec-5 or hSiglec-9 were produced by transfecting CHO-KO cells either with pcDNA3.1(+)Neo-hSiglec-5 or pcDNA3.1(+)Hygro-hSiglec-9 by using Fugene-6 transfection reagent according to the manufacturer’s directions. The expression plasmid pcDNA3.1(+)Hygro-hSiglec-9 was produced by subcloning human Siglec-9 from pcDNA3.1(+)Neo into pcDNA3.1(+)Hygro. Stable transfectants were selected by growth in Ham’s F-12 medium plus 1 mg/ml G418 or 800 μg/ml hygromycin. CHO cells stably expressing hSiglec-9 were further selected using flow-activated cell sorting at the UCSD/Veteran’s Administration San Diego Flow Cytometry Core.

CHO cell adhesion assay. CHO-K1 cells with or without hSiglec expression were grown to 80%/confluence in 100-mm cell culture dishes and lifted using PBS plus 2 mM EDTA, and 2.5 × 10^5 cells were added to each well of 24 well plates and allowed to grow overnight. CHO cells were washed once with 37°C serum-free Ham’s F-12 medium and resuspended in Ham’s F-12 medium with or without 10 μg/ml antibodies for 10 min at 37°C and 5% CO2. FITC-GBS were added at a multiplicity of infection of 100:1, spun down onto the cell monolayer, and allowed to adhere for 15 min at 37°C and 5% CO2. Nonadherent bacteria were removed by repeated washing, and fluorescent images were acquired with a 5× objective lens, using a Zeiss Axiosvert 40 inverted microscope with appropriate fluorescent filters and a charge-coupled device (CCD) camera.

Isolation of human neutrophils. Venous blood was drawn from healthy volunteers under institutional review board approval, using Vacutainers containing EDTA. Neutrophils were isolated by density gradient centrifugation using PolymorphPrep solution (Axis Shield PoC AS, Oslo, Norway) according to the manufacturer’s instructions. The neutrophil layer was washed with pyrogen-free PBS without Ca2+ and Mg2+ at 4°C, contaminating erythrocytes were hypotonically lysed. Subsequently, neutrophils were washed twice and finally resuspended in Hanks’ balanced salt solution without Ca2+ and Mg2+. Viability of cells exceeded 95% as assessed by trypan blue exclusion.

Human neutrophil and GBS fluorescence microscope. FITC-GBS were added to isolated human neutrophils in RPMI 1640 without serum at a multiplicity of infection of 10:1, centrifuged to initiate contact, incubated at 37°C in 5% CO2 for 5 min, and then washed with ice cold PBS plus 1% BSA and spun down at 100 × g for 5 min at 4°C. Neutrophils were fixed using 2% paraformaldehyde in PBS for 10 min at 4°C. Human neutrophil Siglec-9 was labeled using mouse anti-hSiglec-9 antibody Fab fragments at 5 μg/ml in PBS plus 1% BSA for 30 min on ice. Anti-hSiglec-9 Fab fragments were prepared using BD Pharmingen mouse anti-hSiglec-9 clone E10-286 and the Pierce ImmunoPure IgG Fab and F(ab’), preparation kit according to the manufacturer’s instructions for producing Fab antibody fragments. Neutrophils were washed, goat anti-mouse–phycoerythrin antibody was added in PBS plus 1% BSA, and the cells were incubated at 4°C for 30 min. The cells were washed again and treated with Hoechst stain at a 1:1,000 dilution in PBS for 2 min on ice, and then cells were resuspended in 50 μl of PBS plus 1% BSA. Cells were added to glass slides and allowed to dry in the dark at 4°C, and coverslips were mounted using Gelvatol. Images were captured with a DeltaVision Restoration microscope system (Applied Precision Inc., Issaquah, WA) using a Photometrics Sony CoolSnap HQ CCD camera system attached to an inverted, wide-field fluorescence microscope (Nikon TE-200). Optical sections were acquired using a 100× (numerical aperture, 1.4) oil immersion objective in 0.2-μm steps in the z axis, using the attached Applied Precision laser-motorized stage. The fluorescent markers were excited with a standard mercury arc lamp, and fluorescence was detected using a standard DAPI (4′,6-diamidino-2-phenylindole)-FITC-rhodamine filter set.

RESULTS

Sialylated GBS bind to many different human Siglec. Well-characterized sialylated GBS strains from serotypes Ia, Ib, II, III, V, and VI (CPS structures are shown in Fig. 1) and an Sia-negative serotype III ΔNeuA allelic exchange mutant were tested for their ability to bind to hSiglec-3, -5, -6, -7, -8, -9, -10, and -11. Human Siglec-Fc chimeras were prebound to protein A-coated enzyme-linked immunosorbent assay plates in a high-avidity format, and whole-cell heat-killed FITC-GBS were added to the wells. Each of the GBS serotypes tested was capable of binding to at least one hCD33Siglec, while the Sia-negative ΔNeuA mutant did not bind to any of the hSiglec constructs tested (Fig. 2). In parallel experiments, similar interactions were observed between live (calcein-AM-labeled) GBS and hSiglec-Fc constructs (data not shown). The representative serotype Ia and Ib WT GBS strains were very effective at binding to many different hSiglecs in this high-avidity assay format. Interestingly, the serotype III strain common in

FIG. 1. All GBS serotypes described to date contain a terminal α,3-linked Sia on their CPSs. The repetitive subunits of the CPS of GBS serotypes Ia, Ib, II, III, and V were previously published as Ia/Ib (18), II (19), III (39), and V (38). The repetitive subunits of each serotype are covalently joined end to end to form the CPS. The structure of the serotype VI repetitive subunit is not known but consists of a 2:2:1 molar ratio of glucose, galactose, and N-acetylneuraminic acid, respectively (36).

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late-onset GBS disease was recognized effectively only by Siglec-9. Furthermore, in contrast to hSiglec-9, to which all GBS serotypes tested bound, not one of the GBS serotypes tested was able to bind to hSiglec-3 or hSiglec-10. Many of the hSigecls to which GBS bound are located on the cell surfaces of leukocytes involved in innate immunity to bacterial infections. Human neutrophils express Siglec-5 and -9 on their surfaces; human monocytes express Siglec-5, -7, and -9; and human macrophages express Siglec-5 and -11 (1, 14, 25).

GBS serotype III binding to hSiglec-9 is Sia specific. The significant loss of hSiglec-9 binding of the isogenic Sia-negative mutant compared to the WT serotype III parent strain (Fig. 2) suggests a Sia-dependent interaction. Independent verification of Sia dependence was achieved by treatment of WT serotype III FITC-GBS with either trypsin (to cleave bacterial cell surface proteins while leaving the bacterial CPS intact) or an exogenous sialidase (to remove all Sias attached to the surfaces of the bacteria). Consistent with our previous results using the Sia-negative /H9004 NeuA mutant, the serotype III /H9004 NeuD mutant with low Sia expression was also unable to bind to hSiglec-5, -7, and -9 (Fig. 4) or any of the other hCD33rSiglecs tested (data not shown). The restoration of CPS sialylation with greatly diminished O acetylation (complemented strain /H9004 NeuD plus pNeuD-K123A) altered the pattern of binding to hSiglec-5, -7, and -9 in comparison to that in the WT parent GBS. In the case of hSiglec-5 and -7, removal of bacterial O acetylation increased bacterial binding. However, in the case of hSiglec-9, the removal of O acetylation from CPS Sias had the opposite effect, such that binding of the bacterium was inhibited. All other hCD33rSiglecs tested showed no significant change in binding affinity between the WT highly O-acetylated serotype III GBS parent strain and its low-O-acetylated derivative /H9004 NeuD with pNeuD-K123A (data not shown).

GBS engages CHO cells expressing hSiglec-9 or hSiglec-5 in a Sia- and Siglec-specific manner. To determine if the binding

<table>
<thead>
<tr>
<th>GBS Serotype</th>
<th>la</th>
<th>lb</th>
<th>II</th>
<th>III</th>
<th>V</th>
<th>VI</th>
<th>III Sia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Siglec-3 (CD33)</td>
<td>2 ± 0.2</td>
<td>2 ± 0.1</td>
<td>4 ± 0.3</td>
<td>3 ± 0.1</td>
<td>2 ± 0.5</td>
<td>2 ± 0.1</td>
<td>3 ± 0.1</td>
</tr>
<tr>
<td>Siglec-5</td>
<td>76 ± 6</td>
<td>64 ± 8</td>
<td>9 ± 0.2</td>
<td>3 ± 0.4</td>
<td>14 ± 2</td>
<td>8 ± 0.3</td>
<td>5 ± 0.3</td>
</tr>
<tr>
<td>Siglec-6</td>
<td>54 ± 2</td>
<td>45 ± 1</td>
<td>1 ± 0.2</td>
<td>1 ± 0.1</td>
<td>13 ± 1</td>
<td>8 ± 1</td>
<td>3 ± 0.6</td>
</tr>
<tr>
<td>Siglec-7</td>
<td>73 ± 1</td>
<td>38 ± 0.9</td>
<td>5 ± 0.6</td>
<td>3 ± 0.4</td>
<td>6 ± 2</td>
<td>1 ± 0.2</td>
<td>4 ± 0.6</td>
</tr>
<tr>
<td>Siglec-8</td>
<td>48 ± 7</td>
<td>43 ± 6</td>
<td>3 ± 0.5</td>
<td>1 ± 0.1</td>
<td>17 ± 1</td>
<td>9 ± 2</td>
<td>4 ± 0.6</td>
</tr>
<tr>
<td>Siglec-9</td>
<td>63 ± 4</td>
<td>39 ± 7</td>
<td>11 ± 8</td>
<td>38 ± 2</td>
<td>10 ± 4</td>
<td>18 ± 0.3</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Siglec-10</td>
<td>2 ± 0.3</td>
<td>3 ± 2</td>
<td>3 ± 0.6</td>
<td>2 ± 0.3</td>
<td>2 ± 0.3</td>
<td>2 ± 0.3</td>
<td>2 ± 0.3</td>
</tr>
<tr>
<td>Siglec-11</td>
<td>39 ± 4</td>
<td>34 ± 4</td>
<td>1 ± 0.3</td>
<td>2 ± 0.7</td>
<td>17 ± 4</td>
<td>7 ± 0.6</td>
<td>5 ± 0.8</td>
</tr>
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</table>

**FIG. 2.** Binding of various group B Streptococcus serotypes to hCD33rSiglec. All GBS serotypes bind to at least one hCD33rSiglec, while several serotypes interact with multiple hCD33rSiglecs. FITC-GBS were added to hSiglec-Fc chimera-coated wells, and the efficiency of binding was tested. The values represent the means from at least three separate experiments ± standard deviations.

**FIG. 3.** Serotype III GBS engages hSiglec-9 through Sias present on the CPS. FITC-GBS (serotype III) were treated with trypsin in order to degrade bacterial surface proteins or with exogenous sialidase to remove all cell surface sialic acid and tested for their ability to bind hSiglec-9-Fc chimeras in comparison to serotype III /H9004 NeuA. The graphs show mean percent binding ± standard deviation for one representative experiment that was repeated three times with similar results. P values are from two-tailed t tests.

**FIG. 4.** Binding of WT serotype III GBS, its isogenic /H9004 NeuD mutant, and the /H9004 NeuD mutant complemented with pNeuD-K123A to hSiglec-5, -7, and -9. Values represent the means from three separate experiments ± standard deviations. P values are from two-tailed t tests comparing each sialylated GBS group with the nonsialylated negative control. O-Ac, O acetylation.
patterns described above were representative of GBS interactions with cell surface-expressed hCD33rSiglecs, we stably transfected CHO cells with vectors expressing hSiglec-5 and -9 and compared adherence of sialylated and nonsialylated serotype III FITC-GBS by fluorescence microscopy. A low background level of either sialylated or nonsialylated GBS adhered to the surface of untransfected CHO cells (Fig. 5A, first column). Likewise, the nonsialylated ΔNeuA mutant GBS had a relatively low level of adherence to CHO cells whether or not hSiglecs were expressed (Fig. 5A, first row). In contrast, the WT sialylated serotype Ia and III strains had greatly increased interaction with CHO cells expressing specific hSiglecs compared to the untransfected CHO cells (Fig. 5A, second and third rows). As observed using the in vitro binding assays, the serotype Ia GBS strain engaged CHO cells expressing either hSiglec-5 or -9, while the serotype III GBS strain bound only CHO cells expressing hSiglec-9. To verify that this interaction reflects an engagement of the bacterial Sia with the Siglec-9 V-set domain of hSiglec-9, we performed additional studies with Sia-blocking and Sia-nonblocking monoclonal antibodies to hSiglec-9. Binding of WT serotype III GBS was inhibited by the addition of monoclonal antibodies that block the Sia-binding pocket of hSiglec-9. In contrast, when Sia-nonblocking antibodies against hSiglec-9 were added, no inhibition of GBS binding was observed (Fig. 5B). Similar results were found in assays using the serotype Ia GBS WT strain (data not shown). Therefore, serotype III GBS interact with cell surface-expressed hSiglec-9 in a manner dependent on both bacterial Sia and the ability of hSiglec-9 to interact with Sia. The results obtained from our in vitro hSiglec-Fc binding studies closely parallel those obtained using full-length hSiglecs expressed on the surfaces of cells, indicating that our in vitro system represents a useful technique for assessing bacterial binding to hCD33rSiglecs.

Sialylated GBS interacts with hSiglec-9 present on the neutrophil surface. Neutrophils play an important role in immune defense against GBS infections (16). These specialized leukocytes also express relatively high levels of Siglec-9 and Siglec-5 on their surfaces. To test the ability of GBS to engage Siglecs on the surfaces of purified human neutrophils, polymorphonuclear leukocytes were incubated with sialylated FITC-GBS at 37°C for 5 min, followed by washing with 4°C PBS plus 1% BSA (to prevent internalization). Neutrophil-GBS interactions were visualized using fluorescence deconvolution microscopy with an hSiglec-9 specific Fab antibody fragment and a fluorescently tagged secondary antibody to label Siglec-9. Punctate staining of hSiglec-9 on the surfaces of human neutrophils was observed in the presence or absence of bacteria (Fig. 6). When sialylated GBS was allowed to interact with the neutrophil
The most promiscuous, engaging the largest number of hCD33rSiglecs with the greatest strength, whereas serotype II appears to be the least effective binder. We further demonstrate that GBS CPS Sias can engage hSiglec-9 as expressed on the surfaces of CHO cells and human neutrophils. Each serotype tested displays the same terminal α2,3 linkage of Sia; however, due to the way in which the repetitive subunit is polymerized into the CPS, types Ia and Ib will contain the highest density of Sias over a given length of CPS and serotype II will have the fewest (one Sia per two monosaccharides for Ia and Ib versus one Sia per five monosaccharides for II [Fig. 1]). Most glycan-protein interactions have low binding affinities compared to protein-protein interactions, and thus the capacity of types Ia and Ib to form dense arrays of Sias, in addition to having the proper binding epitope, may be essential for creating sufficient avidity for strong binding.

Careful analysis of the specificity of the serotype III GBS interaction with hSiglecs revealed that biochemical and genetic perturbation of bacterial sialylation or Sia O acetylation altered Siglec binding. Cell-based assays confirmed and extended the in vitro studies, showing that interactions between hSiglec-9-transfected CHO cells and GBS required both bacterial sialylation and accessibility of the hSiglec-9 Sia-binding domain for interaction with type III GBS. Furthermore, the presence of O acetylation of CPS Sias altered the binding of GBS to hSiglec-5, -7, and -9. In the context of the GBS CPS, O acetylation blocks binding to hSiglec-5 and -7 while promoting binding to hSiglec-9. Further studies are needed to determine the precise mechanism(s) by which O acetylation modifies hSiglec-GBS binding. It may be that O-acetyl modification of GBS CPS Sias directly changes the binding affinity of the V-set domains for the Sias or, alternatively, alters the three-dimensional architecture of the CPS (21), with secondary effects on the GBS-hSiglec binding interaction.

CD33rSiglecs contain an ITIM and an ITIM-like motif that can be phosphorylated and recruit Src homology phosphatases SHP-1 and SHP-2, which are thought to play a role in down-regulation of cell activation (4, 17). Numerous in vitro studies have shown the capacity of CD33rSiglecs to act in an inhibitory fashion, including negative regulation of T-cell signaling (17) and inhibition of NK cell toxicity (27). Therefore, it is possible that the GBS CPS serotypes have evolved to engage CD33rSiglecs on neutrophils and monocytes/macrophages in order to exert suppressive effects upon the innate immune response. Furthermore, hSiglec-9 can activate accelerated apoptosis in human neutrophils (35), an additional mechanism for avoidance of phagocytic killing. Conversely, it has also recently been shown that many CD33rSiglecs, including hSiglec-9, can act as endocytic receptors (7) that could theoretically aid in the internalization of bound GBS. Thus, evolutionary forces modulating hSiglec-GBS interactions could be operative from both the pathogen and host perspectives.

Sia expression by GBS and other pathogens plays an important role in resistance to host complement-mediated killing, perhaps through binding of host factor H to impair complement deposition on the bacterial surface (3, 26, 37). In addition to the data we present here supporting the interaction between GBS CPS Sias and multiple CD33rSiglecs, it was recently shown that hSiglec-5 expressed on CHO cells can bind to sialylated Neisseria meningitides (20) and that hSiglec-7 present
on dendritic cells can bind to an isolate of sialylated Campylobacter jejuni (5). Therefore, it may be that GBS and other bacteria have evolved a shared virulence mechanism of decorating their surfaces with a human-like monosaccharide, the Sia Neu5Ac, which acts as a ligand for both factor H and hCD33rSiglecs. In this fashion, the Sia-expressing bacterial pathogens could simultaneously interfere with complement and cellular components of innate immunity. In the particular case of GBS, clinical disease is seen in the neonate and other special populations, including pregnant women, the elderly, and diabetics (13, 28). Future studies could examine both the patterns of hCD33rSiglec expression and the cellular responses mediated by these receptors in these populations for correlations with increased susceptibility to GBS infection.

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