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IgtC Expression Modulates Resistance to C4b Deposition on an Invasive Nontypeable Haemophilus influenzae

Derek K. Ho,*† Sanjay Ram,‡ Kevin L. Nelson,† Paul J. Bonthuis,† and Arnold L. Smith2*†

We have previously shown that C3 binding to serum-resistant nontypeable Haemophilus influenzae (NTHi) strain R2866 is slower than C3 binding to a serum-sensitive strain. Ab-dependent classical pathway activation is required for complement-dependent killing of NTHi. To further characterize the mechanism(s) of serum resistance of R2866, we compared binding of complement component C4b to R2866 with a serum-sensitive variant, R3392. We show that C4b binding to R2866 relative to R3392 was delayed, suggesting regulation of the classical pathway of complement. Increased C4b deposition on R3392 was independent of the amount and subclass of Ab binding, suggesting that an impediment to C4b binding existed on R2866. Immunoblotting and mass spectrometry indicated that lipooligosaccharide and outer membrane proteins P2 and P5 were targets for C4b. P2 and P5 sequences and expression levels were similar in both strains. Insertional inactivation of the phase-variable lipooligosaccharide biosynthesis gene lgtC in R2866 augmented C4b deposition to levels seen with R3392 and rendered the bacteria sensitive to serum and whole blood. These results suggest a direct role of lgtC expression in the inhibition of C4b deposition and consequent serum resistance of R2866. Alteration of surface glycans of NTHi may be a critical event in determining the ability of a strain to evade host defenses and cause disseminated infection. The Journal of Immunology, 2007, 178: 1002–1012.

The Gram-negative bacterium Haemophilus influenzae is a small, nonmotile, non-spore-forming commensal of the human nasopharynx and an occasional pathogen. It is a common cause of mucosal infections such as otitis medium, sinusitis, bronchitis, and conjunctivitis. Occasionally, H. influenzae infection can result in serious invasive disease such as meningitis and epiglottitis; in these cases, encapsulated strains, most often serotype b, are usually implicated. With the widespread use of effective type b conjugate vaccines, the incidence of invasive H. influenzae infection has decreased significantly. Despite the success of this vaccine, it does not offer protection against unencapsulated (nontypeable) H. influenzae (NTHi).3 NTHi is generally considered to be an upper respiratory tract commensal in most healthy persons; most common infection results in disease restricted to respiratory mucosa. In rare instances where NTHi is implicated in invasive disease, an underlying immunological or anatomical defect is usually present in the patient. Recently, however, there have been several reports of previously healthy, apparently normal children with invasive disease due to NTHi (1–5). As mucosal NTHi disease isolates do not appear to have distinct genes involved in pathogenesis which are absent from commensal isolates (6), it is likely that these invasive isolates possess specific modifications of conserved surface components which facilitate survival in the bloodstream.

Complement is a system of over 30 plasma and cell surface proteins which play a crucial role in host defense against infection. Activation of complement results in opsonization of pathogens and immune complexes, recruitment of leukocytes, inflammation, and cell lysis (7). The three major pathways of complement include the classical pathway (CP)—which is usually initiated by Ag-Ab complexes followed by complement 1 (C1) activation, the alternative pathway—which is initiated by spontaneous hydrolysis of complement 3 (C3), and the lectin pathway—which is activated by recognition and binding to certain microbial polysaccharides (8). All three pathways converge at the C3 activation step, leading to the generation of opsonins, inflammatory peptides, and formation of the membrane attack complex. Although complement plays an indispensable role in host defense, the cytotoxic and cytolytic potential of this system presents a significant danger to host cells and tissues. To prevent excessive or inappropriate complement activation, the host has evolved elaborate regulatory mechanisms which inhibit complement at the steps of activation, amplification, and membrane attack (9). Several pathogenic microorganisms have exploited these regulatory mechanisms (10), thus facilitating complement evasion and bloodstream survival.

Several clinical and experimental observations suggest that complement plays an important role in defense against H. influenzae infection. At least one-third of the systemic infections observed in complement 2 (C2)-deficient children are due to H. influenzae (11). Patients with deficiencies in C3 or Ab production also have increased susceptibility to serious H. influenzae infections (7, 12). In animal studies, C3-depleted infant rats challenged intranasally with H. influenzae type b developed a greater incidence and magnitude of bacteremia and a higher mortality, compared with control animals (13). Similar results were observed.
when C3-depleted infant rats were challenged i.v. with *H. influenzae* serotypes a, c, or d (14). C3 depletion of infant rats also significantly increased their susceptibility to infection and bacteremia after i.p. challenge with unencapsulated *H. influenzae* (15).

A 1996 case report (2) from this laboratory was one of the first to document the normal immunological and anatomical status of a child with NTHi meningitis. The bacterial strain isolated from this child, R2866, caused bacteremia and meningitis in infant and weaning rat models of invasive *H. influenzae* infection, a phenotype not previously seen with NTHi. Furthermore, R2866 survived in defibrinated blood from normal adult humans to the same extent as a virulent, encapsulated type b strain (Elia). By ELISA analysis, we were unable to detect capsular polysaccharide production in R2866. Subsequent PCR and Southern analysis using serotype-specific primers revealed the lack of capsule biosynthetic genes (types a-I) and the bexA gene (required for capsule export), genetically corroborating the observed nontypeable phenotype (2, 16). Because bloodstream survival and serum resistance usually require evasion of Ab and complement-mediated host defenses, we used kinetic flow cytometry to characterize the deposition of various complement components on the surface of R2866 and an avirulent, serum-sensitive laboratory strain, Rd KW20. Using this approach, we have shown that C3 deposition on the surface of strain R2866 is delayed despite the binding of multiple Ab classes and C1 to a similar extent as the serum-susceptible strain Rd KW20. This phenotype is independent of alternative pathway activity, factor H recruitment, and sialylation or phosphorylcholine decoration of lipooligosaccharide (LOS) (16).

In the present work, we show that serum resistance in R2866 is facilitated by delaying C4b deposition despite efficient Ab binding. We demonstrate a critical role of LOS in these phenotypes, as inactivation of the LOS biosynthetic gene lgtC in R2866 resulted in increased C4b deposition and reduced resistance to normal human serum (NHS) and blood. These results suggest that LOS is an important mediator of complement interactions with NTHi. To our knowledge, this is the first report detailing the molecular and genetic basis of serum resistance in an invasive NTHi.

**Materials and Methods**

**Bacterial strains and growth**

Bacterial strains used in this study are listed in Table I. All *H. influenzae* strains were grown at 37°C in room air or 5% CO2 (where indicated) on chocolate agar plates supplemented with 1% isovitalex (BD Biosciences) or in Difco brain-heart infusion broth (BD Biosciences) supplemented (sBHI) with hemin (10 μg/ml) and β-NAD (10 μg/ml) and with agar for solid sBHI plates. *Escherichia coli* was grown on Luria-Bertani broth or solid agar at 37°C in room air.

**Sera and Abs**

Blood was collected from 7 to 10 healthy adult volunteers under an Institutional Review Board-approved protocol and allowed to clot. The serum was subsequently harvested, pooled, and stored at −80°C. The donors are anonymous and do not participate in *Haemophilus* research. Detection of lectin pathway activators and soluble complement regulatory proteins was performed with the following Abs: mouse monoclonal anti-human MBL, mouse monoclonal anti-human C1Inh (Antibody Shop), mouse monoclonal anti-human C4BP (Green Mountain Antibodies), mouse monoclonal anti-human t-ficolin, and anti-human H-ficolin (Cell Sciences). Sheep anti-human C4 (Biodesig) antisera is an affinity purified polyclonal Ab fraction developed against whole human C4 purified from plasma. Mouse anti-C4d (Quidel) is a mAb which recognizes an Ag expressed on the C4d domain of C4b. The anti-C4 Abs described above were used in flow cytometry and immunoblotting experiments where indicated. Alexa-fluor conjugated rabbit anti-human IgG (Molecular Probes), FITC-conjugated mouse monoclonal anti-human IgG1–4 (Sigma-Aldrich), and FITC-conjugated goat anti-human IgM Abs (Zymed Laboratories) were used in flow cytometry experiments. Secondary Alexa Fluor-conjugated donkey anti-sheep-IgG Abs and Alexa Fluor-conjugated goat anti-mouse IgG (both from Molecular Probes) were used for flow cytometry, and alkaline phosphatase-conjugated rabbit anti-sheep IgG (immunoblotting) was purchased from Molecular Probes and Pierce, respectively. Mouse monoclonal anti-*H. influenzae* P4 (a gift from Dr. B. Green, Wyeth Vaccines, Pearl River, NY), rabbit anti-*H. influenzae* P2 (a gift from Dr. T. Murphy, SUNY Buffalo, Buffalo, NY), and rabbit anti-*H. influenzae* P5 (a gift from Dr. R. Munson) Abs were used in immunoblotting. Mouse mAb 4C4 (a gift from Dr. M. Apicella, University of Iowa, Iowa City, IA) was used in whole cell ELISA.

**Flow cytometry**

*H. influenzae* were grown to mid-log phase in 5 ml of sBHI broth cultures using disposable 16 × 125-mm tubes (BD Biosciences). Bacteria were centrifuged at 10,000 × g for 3 min and resuspended in HBSS++ to a final OD600 of 0.5 (2 × 10⁶ CFU/ml). One milliliter of the bacterial suspension was centrifuged again as above and resuspended in a final volume of 0.5 ml of HBSS++. A total of 300 μl of the bacterial suspension was added to NHS (concentration specified for each experiment) or a similar volume of heat-inactivated NHS (HINHS). The final volume of the reaction mixture was maintained at 500 μl. Samples were incubated in a 37°C in a water bath. At the specified time points, 50 μl of the bacteria-serum mixture was removed, centrifuged, washed in 100 μl of HBSS++ supplemented with 1% BSA (Sigma-Aldrich), and resuspended in 50 μl of HBSS++/1% BSA. A total of 20 μl of a 1/20 dilution (or 1/50 dilution for anti-C4d) of the appropriate primary Ab (diluted in HBSS++) was added to the bacteria and incubated at room temperature for 30 min. After washing as described above, bacteria were resuspended in 50 μl of HBSS++, to which 20 μl of a 1/100 dilution of the appropriate Alexa Fluor-conjugated secondary Ab was added, followed by incubation at room temperature in the dark for 20 min. The cells were washed twice as above and resuspended in 1 ml of filtered HBSS++ containing 1% paraformaldehyde (Sigma-Aldrich). Flow cytometric analysis was performed on a Beckman Coulter EPICS XL-MCL cytometer (Beckman Coulter) using Expo32 ADC analysis software. The data was analyzed and plotted similar to previous work from this laboratory (16).
Sequenced for the genomes of *H. influenzae* strains Rd KW20 and R2866 were accessed through the Microbial Genomes pages at the website of the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/genomes/lproks.cgi).

**DNA manipulations**

All restriction enzymes were purchased from New England Biolabs. T4 DNA ligase was purchased from Promega. Biolase DNA polymerase and other PCR reagents were from Bioline. Oligonucleotide primers were purchased from Integrated DNA Technologies. Plasmid DNA was isolated using the QIAprep spin miniprep kit (Qiagen), genomic DNA from multiple colonies was isolated using the DNAeasy Tissue kit (Qiagen), while DNA from individual colonies was prepared using Chelex resin (Bio-Rad).

**Inactivation of opsX**

A PCR fragment containing opsX was amplified from Rd2866 genomic DNA using primers opsX KO F and opsX KO R (Table II). The resulting 1933-bp product was purified and ligated into pCR2.1 using the TA cloning kit (Invitrogen Life Technologies). The resulting 1.5-kb TSTE cassette containing the *opsX* ORF. Inactivation of *opsX* was achieved by ligation with an −1.5-kb TSTE *HinCl* fragment. The TSTE cassette contains an aminoglycoside phosphotransferase (*aph*) providing kanamycin resistance in E. coli or ribostamycin resistance in *H. influenzae* and is flanked by Haemophilus-specific uptake signal sequences to aid homologous recombination (18). The resulting −7.3-kb plasmid (pDH4) was linearized with XhoI and used to transform *H. influenzae* strain Rd that was rendered competent by the MIV method (19).

Dilutions of transformed cells were plated onto chocolate agar containing 30 μg/ml ribostamycin and incubated at 37°C in 5% CO2 for 24–48 h before picking colonies. Confirmation of a TSTE insertion into Rd *opsX* was performed by PCR using primers *opsX* KO F and Rd *opsX* R (Table II). Genomic DNA extracted from a PCR-confirmed, subcultured ribostamycin-resistant colony was used to transform R2866. Colonies were selected as described above and screened for TSTE cassette insertion into R2866 *opsX* by PCR using the *opsX* KO F and R primers (Table II). The resulting mutant was designated strain R3743.

**Inactivation of lgtC**

A PCR fragment containing lgtC was amplified from R2866 genomic DNA using primers lgtC KO F and lgtC KO R (Table II). The resulting 6.3-kb product was purified and ligated into pCR2.1 using the TA cloning kit (Invitrogen Life Technologies). The resulting 7.3-kb HincII fragment.

Dilutions of transformed cells were plated onto chocolate agar containing 30 μg/ml ribostamycin and incubated at 37°C in 5% CO2 for 24–48 h before picking colonies. Confirmation of a TSTE insertion into Rd *opsX* was performed by PCR using primers *opsX* KO F and Rd *opsX* R (Table II). Genomic DNA extracted from a PCR-confirmed, subcultured ribostamycin-resistant colony was used to transform R2866. Colonies were selected as described above and screened for TSTE cassette insertion into R2866 *opsX* by PCR using the *opsX* KO F and R primers (Table II). The resulting mutant was designated strain R3743.

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<td>lic1A</td>
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<tr>
<td>lic1A</td>
<td>R</td>
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<td>F</td>
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<tr>
<td>lic2A</td>
<td>R</td>
<td>CTATTTCCTATTTAGAAAAAAGC</td>
</tr>
<tr>
<td>lic3A</td>
<td>F</td>
<td>GCTAAGATACACCGGGAGTGCG</td>
</tr>
<tr>
<td>lic3A</td>
<td>R</td>
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</tr>
<tr>
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<td>ATCCGAGCAAGATTTGAAAT</td>
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<tr>
<td>lgtC</td>
<td>R</td>
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</tr>
<tr>
<td>lex2A</td>
<td>F</td>
<td>GCCGAAATTTATTTAATAC</td>
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<tr>
<td>lex2A</td>
<td>R</td>
<td>GTCTGCTATTAAGCTTTTCG</td>
</tr>
<tr>
<td>oafA</td>
<td>F</td>
<td>TTCCAGAGTTACTGTAGATCTTTTG</td>
</tr>
<tr>
<td>oafA</td>
<td>R</td>
<td>CATTTAAAAACAGCAGGAAATATAG</td>
</tr>
</tbody>
</table>

**Table II. Oligonucleotide primers**

a Primer sequences are given as 5′ to 3′.

b Restriction sites are underlined.
FIGURE 1. Delayed C4b deposition on R2866. A total of 10^6 CFU/ml H. influenzae Rd KW20 and R2866 were added to 40% NHS or HINHS and incubated at 37°C for the indicated times. Bound C4b was detected by flow cytometry using polyclonal anti-C4 Abs. The mock data point represents background Ab reactivity in the absence of NHS treatment. "Im" is the time point immediately following serum addition to bacteria. Data from four independent experiments are shown and expressed as mean fluorescence intensity (MFI) ± SD.

FIGURE 2. Restored C4b deposition on an R2866 variant (R3392) abrogates serum resistance and blood survival. A, A total of 2 × 10^5 CFU/ml Rd KW20, R2866, and R3392 were added to doubling dilutions of pooled NHS (left panel) or whole human blood from three individual donors (right panel). Following a 30-min incubation at 37°C, bacterial survival was determined by spotting samples onto sBHI plates and counting. The y-axis indicates the NHS or blood concentration required to kill 50% of the bacteria (IC_{50}). B, Flow cytometry for C4b deposition was performed as in Fig. 1 using polyclonal anti-C4 (left panel) or monoclonal anti-C4d (right panel) Abs. Data from three independent experiments are shown and expressed as MFI ± SD.

**Results**

R2866 delays C4b deposition

Our initial studies suggested that delayed C3 deposition mediates serum resistance in strain R2866 and that a functional CP is required for complement activation (16). We hypothesized that complement inhibition on R2866 occurred between the initiation of CP activation and C3 deposition. To test this possibility, we performed flow cytometry to examine the kinetics of C4b binding. As shown in Fig. 1, C4b deposition is delayed on R2866 compared with the avirulent, laboratory strain Rd KW20. The differences are most
apparent within the first 15 min of the assay. Minimal C4b binding was observed on both strains after incubation in HINHS (Fig. 1). These data suggest that complement inhibition in R2866 occurs at or before the step of C4 binding. We were unable to detect differences in IgG (total IgG and IgG subclasses) or IgM binding to both strains Rd KW20 and R2866, which suggests that differences in the amount of Ab binding are not responsible for differences in C4b deposition (16). Furthermore, we did not observe in either strain binding to lectin pathway activators (mannose binding lectin and ficolins) or recruitment of complement regulatory proteins C4b-binding protein and C1-esterase inhibitor (data not shown).

Increased C4b deposition observed on a serum-sensitive R2866 variant

In a previous study, we identified a variant of R2866 with increased sensitivity to the bactericidal activity of NHS (26) and blood (Fig. 2A). This variant, designated R3392, was derived from a single colony cultured from an infant rat infected with R2866. Using multilocus sequence typing (27), we found that both R2866 and R3392 are sequence type 99, suggesting strongly that R3392 is derived from R2866 rather than from a contaminating strain (26).

To determine whether the increased serum sensitivity observed in R3392 was also due to accelerated C4b deposition relative to R2866, we performed kinetic flow cytometry for C4b binding. As shown in Fig. 2B (left panel), R3392 bound more C4b over time compared with R2866, although at lower levels when compared with the highly serum-sensitive strain Rd KW20 (Fig. 1). Levels of C5b-9 (MAC) binding on both strains paralleled C4b binding (data not shown).

We next considered the possibility that the difference in C4b deposition between R2866 and R3392 is due to the inability of the polyclonal C4 Ab to detect the C4d fragment of C4b. Even in the
absence of detectable C4BP recruitment (C4BP is normally required for factor I activity), bound C4b could be processed by factor I into fluid-phase C4c and C4d, which remains covalently linked to the bacterial surface. As the C4d fragment (41 kDa) is considerably smaller compared with the intact C4b molecule (200 kDa), it is possible that the polyclonal Ab recognizes epitopes primarily found on the intact molecule. To address this concern, we performed kinetic flow cytometry for C4b binding using a monoclonal anti-C4d Ab. As shown in Fig. 2B (right panel), the results observed with the anti-C4d Ab parallels those observed with the polyclonal Ab. These results suggest that complement inhibition and consequent serum resistance in R2866 is not due to factor-I mediated cleavage of C4b.

Although the quantitative serum bactericidal assay (IC50) is useful for determining the correlation between complement deposition and survival in serum, it excludes the contribution of phagocytosis, which may play a critical role in vivo. It has been demonstrated that C5-knockout mice are not more susceptible to H. influenzae type b bacteremia when compared with syngenic mice with normal C5 levels (28), and that both mutant and wild-type animals can eradicate the infection at approximately equivalent rates. In contrast, mice depleted of C3 had significantly impaired clearance of both H. influenzae type B (Hib) (13) and NTHi (15). Furthermore, humans with deficiencies in C5-C9 do not exhibit increased susceptibility to H. influenzae infections. These data suggest that C3-dependent opsonophagocytosis plays an important role in host defense against H. influenzae infection. To assess the role of complement-mediated opsonophagocytosis, we performed IC50 assays using whole blood from three individual donors. As shown in Fig. 2A (right panel), strain R2866 is resistant to the bactericidal activity of whole blood compared with strain Rd KW20. Similar to the results observed in NHS, the blood IC50 of strain R3392 is decreased compared with strain R2866, approaching levels observed in Rd KW20. Collectively, these data indicate that delayed C4b deposition on R2866 mediates serum resistance as well as survival in whole blood.
Restored C4b deposition on strain R3392 is independent of Ab binding

We considered the possibility that increased C4b deposition and consequent serum sensitivity on strain R3392 could be to the result of increased Ab binding. To address this possibility, we determined IgG and IgM binding using two different serum concentrations. We performed these experiments using a 15-min incubation, a time point at which we previously observed the greatest difference in C4b deposition between strains (Fig. 2B, left panel). As shown in Fig. 3A, both R2866 and R3392 bound approximately equivalent amounts of total IgG and IgM when incubated in 40% NHS. This experiment was also performed using the serum concentration at which C4b deposition on strain R3392 was similar to that on R2866. After a 15-min incubation we observed that the amount of C4b bound to R2866 incubated with 40% NHS was similar to the amount of C4b on R3392 incubated with only 5% NHS (Fig. 3B). Despite binding similar levels of C4b, strain R2866 bound more Ab compared with R3392, commensurate with the higher serum concentration used on R2866. IgG1 was the predominant subclass that bound to both strains and R2866 bound greater amounts of IgG subclasses IgG1, IgG2, and IgG3 when compared with R3392 (Fig. 3B). Collectively, these results suggest that serum resistance in R2866 is mediated by specifically modulating C4b deposition and not because of decreased Ab binding or preference of binding of IgG subclasses that did not fix complement.

LOS and outer membrane proteins P2 and P5 are major targets for C4b

Having shown that serum resistance in R2866 was not due to a failure to bind Abs (Fig. 3) or in activating the lectin pathway (data not shown), we hypothesized that serum resistance in R2866 is mediated by a paucity or relative inaccessibility of targets for C4b. We next attempted to identify C4b targets on the two serum-sensitive strains (Rd KW20, R3392) and the serum-resistant strain (R2866) by immunoblotting for C4b (Fig. 4A). Upon activation of C4, the C4b fragment forms amide or ester bonds with its targets. The 87-kDa α’-chain of C4b contains the thioester moiety that binds covalently to the bacterial surface. The addition of 2-ME to the digestion buffer dissociates the disulfide-linked 75-kDa M and 32-kDa γ-chains from the α’-chain. As a result, bacterial targets linked to C4b will be observed migrating as a complex with the 87-kDa α’-chain. Consistent with the results in Figs. 1 and 2B, reduced C4b deposition is observed on strain R2866 compared with strains Rd KW20 and R3392 (Fig. 4A and B).

Three major C4b-target complexes at molecular masses of ~91, 130, and 160 kDa (indicated by the arrow, open arrowhead, and filled arrowhead, respectively) were observed (Fig. 4A). By subtracting the $M_i$ of the complexes, the calculated $M_i$ of the C4b targets are ~4, 43, and 73 kDa. These targets were not observed when the bacteria were incubated in heat-inactivated NHS (Fig. 4C). We hypothesized that the low $M_i$ target of ~4 kDa is LOS complexed to C4b. This hypothesis is consistent with previous observations, as we have shown that LOS is a major C4b acceptor in Neisseria meningitidis (29). We attempted to confirm the presence of LOS in the 91-kDa band using various detection techniques. Anti-LOS Abs (anti-2-keto-3-deoxyoctulosonic acid (KDO), anti-lipid A) were not successful presumably due to epitope shielding by LOS glycoside chain extensions or by the covalently linked α’-chain of C4b. We were unable to achieve high-specific activity following radio-labeling of LOS and purpald (chemical) detection (30) yielded high background when performed on polyvinylidene difluoride strips containing the putative complex. As C4b is likely bound to inner core heptoses or the corresponding glycoside chain extensions, we decided to eliminate these putative targets by inactivating the LOS biosynthetic gene opsX. opsX encodes heptosyltransferase I, the enzyme responsible for catalyzing the addition of the first heptose to KDO on the LOS inner core (31). Inactivation of this gene will yield a truncated LOS species with no extensions beyond KDO. As shown in Fig. 4D, the putative C4b-LOS complex is eliminated in strain R3743 (R2866 opsX::TSTE). The faint 87-kDa band observed in this strain likely represents a small amount of liberated C4b α’-chain. These results provide circumstantial evidence that LOS binds C4b.

To identify the other two C4b targets indicated in Fig. 4A, we separated NHS-treated bacteria on SDS-PAGE gels in duplicate. One gel was Coomassie stained while the other was transferred to PVDF. The membrane was immunoblotted for C4b and used to guide the excision of the corresponding regions on the Coomassie-stained gel for mass spectrometric analysis. The higher and lower $M_i$ complexes contained peptides whose sequences were matched to the outer membrane proteins P2 and P5 of each strain (data not shown).
from both strains indicated the loss of a hexose on R3392. Six by whole cell ELISA. MALDI-mass spectrometry analysis of LOS complexes after incubation of bacteria in the trary densitometry units are shown on

Restored C4b deposition observed on R2866 lgtC KO

Flow cytometry and immunoblotting were used to determine whether the increased susceptibility to NHS and blood

Inactivation of lgtC in R2866 increases susceptibility to NHS and blood

Specific LOS modifications mediated by phase-variable LOS biosynthetic genes have been shown to modulate serum sensitivity in NTHi (32, 33). We have previously characterized the LOS of strains R2866 and R3392 using SDS-PAGE, mAb reactivity, mass spectrometry, and the expression state of biosynthetic genes (26).

The LOS of R3392 displayed increased mobility on SDS-PAGE and reduced binding to the Gal1–4Gal epitope associated with lgtC expression in R2866. As shown in Fig. 6B, reduced 4C4 reactivity was observed in R3732 compared with R2866, while background reactivity was observed in R3735. The intermediate 4C4 reactivity observed in R3732 was expected, because the lgtC in this strain is still phase-variable, thereby allowing a minority of the bacterial population to express this gene. We also considered the possibility that the phenotypes observed in R3735 were due to a differential expression profile of other phase-variable LOS biosynthetic genes. Determination of the number of repeating units in each of these genes indicated an identical expression profile between R2866 and R3735 with the exception of a minority population of one colony of R3735 having lic1a OFF (Table III).

Table III. Analysis of tetrameric repeat regions within LOS biosynthetic genes

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<tr>
<th>Gene</th>
<th>No. of Colonies ON/No. of Colonies Screened</th>
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<td>lic1a</td>
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</tr>
<tr>
<td>lic2A</td>
<td>11/11</td>
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<td>0/11</td>
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* One colony possessed a mixed number of repeats.

* Not applicable; lgtC knockout strain.

Collectively, these data suggest that the absence of lgtC expression in R3732 is responsible for the increased serum sensitivity in this strain. To test this hypothesis, we insertionally inactivated lgtC in strain R2866, to obtain strain R3735. As shown in Fig. 5, increased serum sensitivity was observed in R3735 and was similar to levels seen with R3392. Similar results were observed using whole blood from three individual donors (Fig. 5, right panel).

A representative blot of an experiment performed three times is shown. C. Densitometry for the C4b-target complex bands was performed on the blot shown in B. Arbitrary densitometry units are shown on the y-axis. D. Absence of C4b-target complexes after incubation of bacteria in heat-inactivated NHS. Samples were prepared and processed as in B.

FIGURE 7. Restored C4b deposition on R2866 lgtC::cat. A. Flow cytometry for C4b deposition was performed as in Fig. 1. Data from three independent experiments are shown and expressed as MFI ± SD. B. Immunoblotting for C4b was performed as in Fig. 4A. A representative blot of an experiment performed three times is shown. C. Densitometry for the C4b-target complex bands was performed on the blot shown in B. Arbitrary densitometry units are shown on the y-axis. D. Absence of C4b-target complexes after incubation of bacteria in heat-inactivated NHS. Samples were prepared and processed as in B.
R3735 is also due to restored C4b deposition. As shown in Fig. 7A, we observed increased C4b deposition on R3735, similar to levels observed with R3392. Consistent with the flow cytometry data, increased C4b binding to LOS and outer membrane proteins was observed in this strain by immunoblot analysis (Fig. 7B). Densitometry values (Fig. 7C) of all three C4b-target complexes are consistent with the results observed in Fig. 7A.

Discussion
Pathogenic microorganisms which replicate in human blood must possess effective mechanisms to evade host defenses. Before the widespread use of the Hib vaccine, most cases of invasive H. influenzae disease were caused by serotype b isolates. Accordingly, it was assumed for decades that the presence of a polysaccharide capsule was a prerequisite for invasive disease. The post-Hib vaccine era has seen a shift in the epidemiology of invasive H. influenzae as NTHi is an emerging cause of bacteremia. The Active Bacterial Core Surveillance Program of the Centers for Disease Control reports that the rate of H. influenzae type b bacteremia in children less than 5 years old was 0.14 cases/100,000 population, while for NTHi it was 0.50 cases/100,000 (www.cdc.gov/nic/dd/dbmd/abs/surveirep/bht05prelim.pdf). Invasive disease caused by NTHi has usually been associated with anatomical or immunological defects in the host. Therefore, the ability of NTHi strain R2866 to cause invasive disease in an immunocompetent child was unexpected (2). In this work, we provide evidence which suggests a correlation between expression of the LOS biosynthetic gene lgtC, classical pathway regulation mediated by delayed C4b deposition, and resistance to NHS and normal human blood.

Strain R3392, a R2866 variant recovered after infant rat passage, exhibits increased C4b deposition compared with the parent strain and is sensitive to NHS (26) and normal adult blood (Fig. 2). We hypothesized that R2866 either lacked sufficient target(s) for C4b or that an alteration in the surface obscured available targets for C4b binding. By immunoblot analysis, we found that C4b bound to the same targets on R2866 and R3392 (Fig. 4A), namely outer membrane proteins P2, P5, and LOS. This suggests that the targets on R2866 possess specific modification(s) which render them less susceptible to C4b binding. LOS was the only C4b target that differed between the two strains. Due to the strong correlation between lgtC expression and resistance to NHS (26), we decided to inactivate lgtC in R2866 to confirm that it was LOS alone, and not an alteration in another molecule, that was responsible for the serum-resistant phenotype. With selective inactivation of lgtC (strain R3735), we observed restored C4b deposition on both LOS and the high M₆ outer membrane protein targets (Fig. 7B), as well as increased susceptibility to killing by NHS and blood (Fig. 5). Collectively, these data suggest that lgtC expression was responsible for decreasing the rate of C4b binding and CP regulation on R2866.

Strain R2866 is refractory to the introduction of plasmids via electroporation and no in cis-complementation system currently exists for this strain. Although we were not successful in complementing the lgtC knockout in R3735, several lines of evidence suggest that lgtC is responsible for the relevant phenotypes presented in this work. First, in the R2866 genome, there are no ORFs in the same reading frame downstream of lgtC for >8 kb, suggesting that polar effects from the lgtC mutation are unlikely. ORFs which overlap with lgtC on the opposite strand are absent. Second, we observed the same C4b targets between strains R2866, R3392, and R3735 by immunoblotting (Fig. 7B), suggesting that this mutation did not result in gross remodeling of the outer membrane. Third, the reduction in serum and blood resistance (Fig. 5) and the increase in total C4b binding (Fig. 7A) in strains R3392 and R3735 were identical. Finally, we observed identical LOS mobility and banding pattern by SDS-PAGE (Fig. 6A) and similar expression profiles of the phase-variable LOS biosynthetic genes (Table III) between strains R2866 and R3735.

We have previously described a correlation between LOS structure, C4b deposition, and serum resistance in N. meningitidis (29). In these studies, we identified LOS as an acceptor for C4b. LOS with phosphoethanolamine (PEA) at the 6-position of heptose II (6-PEA) bound significantly greater amounts of C4b via amide linkages than LOS with 3-PEA. Serum sensitivity correlated with the amount of C4b binding, with 6-PEA-bearing strains being more serum sensitive than their 3-PEA-bearing counterparts. Differences in IgG or IgM binding alone did not account for differences in C4b binding among the 3- and 6-PEA-bearing strains. The present study also does not show a correlation between the amount of IgG or IgM binding and C4b binding (Fig. 3). As we currently do not have a detailed chemical structure of the LOS of either strain R2866 or R3392, further investigation will be required to determine whether these strains use a similar mechanism to modulate C4b binding as observed with N. meningitidis.

Several LOS-mediated mechanisms of serum resistance have been described in NTHi isolated from mucosal infections. In general, the level of serum resistance observed in these strains is lower than what we describe here. Weiser and Pan demonstrated that colony variants of strain H223 which do not express choline kinase (lic1A OFF) were resistant to C-reactive protein-mediated killing. They also observed that variants which do not express the LOS epitope Galα1–4Gal (lic2A OFF) were sensitive to Ab-mediated killing (33). Hood et al. (34) have shown that mutants of NTHi strain 486 which do not express the α-2,3-sialyltransferase lic3A or the sialic acid synthetase siaB exhibit increased sensitivity to NHS. O-acetylation of the LOS inner core has also been shown to enhance serum resistance in certain strains (35).

It is not entirely clear why delayed C4b deposition on R2866, but not outright inhibition, can mediate resistance to NHS and adult blood. Incubation of R2866 in 10% NHS resulted in minimal C4b deposition throughout a 30-min assay (data not shown), as opposed to the gradual, time-dependent increase in C4b deposition observed in 40% NHS. Because C3 and C5 convertases are inherently unstable, a critical rate of C4b molecules must deposited on the bacterial surface to generate enough active convertases for effective killing. An insufficient number of convertases may be formed during the early time points on R2866, coincident with the minimal C4b deposition that is observed (Fig. 1). Consequently, as C4b deposition slowly increases on the bacterial surface, decay of previously formed convertases keeps the number of active convertases insufficient. It is also possible that the high frequency and stochastic nature of phase variation leads to input of the heterogeneous bacterial populations in our assays. In this case, it is plausible that the less resistant members of the population (such as those which have lgtC OFF), which readily bind C4b, are present in large enough numbers to give the impression of gradual increases in C4b binding over time in the entire population.

Phase variation of LOS biosynthetic genes has been established in NTHi as an effective mechanism for rapidly adapting to changing host environments (25). Accordingly, it is conceivable that lgtC expression, in the context of other LOS biosynthetic genes, allows the bacteria to adapt to a lifestyle both as a commensal and an invasive pathogen. Such a role in LOS phase variation has been suggested in the case of lic1A and phosphorylcholine (ChoP) decoration of LOS. Weiser et al. (36) have shown that during nasopharyngeal carriage in infant rats, there was a gradual selection for
variants which express ChoP (lic1A ON). However, the variants which express ChoP were more susceptible to C-reactive protein mediated killing in NHS. These results suggest that varying expression of ChoP allows the bacteria to both persist on mucosal surfaces and resist killing by NHS during invasive infection. Further investigation will be required to determine whether lgtC can play a similar role.

Currently, there is insufficient evidence to conclude that lgtC expression is a universal prerequisite for serum resistance in NTHi. The available data is limited and appears to be strain specific, which is not surprising given the highly heterogeneous nature of LOS in H. influenzae. Hood et al. (37) observed that inactivation of lgtC in type b strain RM153 resulted in fewer deaths and reduced incidence and density of bacteremia in the infant rat model of infection. These investigators also observed that lgtC is expressed in Rd KW20, which is clearly serum sensitive. Weiser and Pan (33) also observed that expression of lgtC in concert with lic2 is required for resistance to 10% NHS in NTHi strain H233. In these studies, however, the role of lgtC alone, outside the context of lic2 expression, was not specifically addressed. Finally, we have observed that inactivation of lgtC can also increase serum resistance in certain NTHi strains (data not shown), an observation in contrast to this report. Based on these data, we cannot currently predict what specific effect lgtC expression will have on LOS structure and in resistance to NHS in different strains.

An association between 4C4 expression and increased serum resistance has been observed in Rd KW20. Griffin and coworkers observed an ~2-fold increase in serum resistance (from 1C50 ~0.8% to ~1.5%) after introduction of lexA2 into strain Rd KW20, resulting in expression of the 4C4 epitope. Weiser and Pan (33) (see above) also observed a correlation between serum resistance and 4C4 expression and found that an H233 variant which does not express 4C4 can survive in IgG-depleted NHS. These workers proposed that expression of Galα1,4-Gal (4C4 epitope) allows resistance to Ab-mediated killing because this structure is a component of host cell surface glycosphingolipids, such as the PK blood group Ag. It is therefore unlikely that the host would generate Abs against this structure, consequently allowing immune evasion by H. influenzae via molecular mimicry. Although this is a plausible and attractive mechanism for serum resistance in certain NTHi strains, the data presented here (Fig. 3) suggest that specific modulation of C4b binding, as opposed to reduced Ab binding, is responsible for serum resistance in R2866. Furthermore, although direct comparisons between data acquired in different laboratories is not possible, it is worth noting that in the other studies described above, it appears that absence of 4C4 expression results in modest reductions in serum resistance, compared with the ~10-fold decrease observed in R2866.

This work integrates our knowledge of the LOS structure of R2866 and R3392 (26) with the role of complement interactions and survival in NHS and adult human blood. Collectively, the data presented here suggests that lgtC expression facilitates the generation of an LOS structure which resists C4b deposition on itself while simultaneously protecting outer membrane protein targets. Thus, modulation of LOS structure by lgtC expression in R2866 serves as an effective resistance mechanism to complement-mediated host defenses.

Disclosures
The authors have no financial conflict of interest.

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