

## Complement Factor C3 Deposition and Serum Resistance in Isogenic Capsule and Lipooligosaccharide Sialic Acid Mutants of Serogroup B *Neisseria meningitidis*

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**Serogroup B meningococci express sialic acids on their surfaces as a modification of the lipooligosaccharide (LOS) and as capsular material consisting of  $\alpha$ 2,8-linked sialic acid homopolymers. The aim of this study was to elucidate the impact of each sialic acid component on the deposition of complement factor C3 and serum resistance. For this purpose, we used isogenic mutants deficient in capsule expression (a polysialyltransferase mutant) or sialylation of the LOS (a *galE* mutant) or both (a mutant with a deletion of the *cps* gene locus). Bactericidal assays using 40% normal human serum (NHS) demonstrated that both the capsule and LOS sialic acid are indispensable for serum resistance. By immunoblotting with monoclonal antibody MAb755 that is specific for the C3  $\alpha$ -chain, we were able to demonstrate that C3 from 40% NHS was covalently linked to the surface structures of meningococci as C3b and iC3b, irrespective of the surface sialic acid compounds. However, C3b linkage was more pronounced and occurred on a larger number of target molecules in *galE* mutants with nonsialylated LOS than in meningococci with wild-type LOS, irrespective of the capsule phenotype. C3b deposition was caused by both the classical pathway (CP) and the alternative pathway of complement activation. Use of 10% NHS revealed that at low serum concentrations, C3 deposition occurred via the CP and was detected primarily on nonsialylated-LOS *galE* mutants, irrespective of the capsular phenotype. Accordingly, immunoglobulin M (IgM) binding to meningococci from heat-inactivated NHS was demonstrated only in both encapsulated and unencapsulated *galE* mutants. In contrast, inhibition of IgA binding required both encapsulation and LOS sialylation. We conclude that serum resistance in wild-type serogroup B meningococci can only be partly explained by an alteration of the C3b linkage pattern, which seems to depend primarily on the presence of wild-type LOS, since a serum-resistant phenotype also requires capsule expression.**

The complement system is of central importance in the host defense against *Neisseria meningitidis*. Patients deficient in complement components of the membrane attack complex (MAC) or the alternative pathway (AP) frequently suffer from meningococcal disease (1, 4, 6, 10, 42). One way pathogenic *Neisseria* spp. protect themselves against the effects of the complement system is expression of sialic acids on the bacterial surface. Sialic acids have been reported to render pathogenic *Neisseria* spp. serum resistant and resistant against opsonophagocytosis (8, 15, 18, 21, 23, 24, 29, 30, 39, 46, 48, 52, 54). Meningococci of serogroups B and C are the predominant serogroups causing meningococcal disease in the Northern hemisphere (28) and express a capsule consisting of homopolymers of sialic acid (PSA) (14). Furthermore, certain lipooligosaccharide (LOS) immunotypes are sialylated exogenously in the presence of CMP-*N*-acetyl-neuraminic acid (NANA) (the activated form of sialic acid) or endogenously by CMP-NANA derived from the biosynthesis pathway of the capsular polysaccharide (36, 49). In the infant rat model, both sites of sialic acids in serogroup B meningococci appeared to be indispensable for meningococcal virulence (38, 39, 52). However, the precise impact of LOS sialylation or capsule formation in se-

rogroup B meningococci on resistance to serum and to opsonophagocytosis is still a matter of debate (18, 21–24, 30, 49).

In humans, sialic acids protect autologous cells from the complement attack by rendering surface-bound C3 accessible to the action of factor H (20). Interestingly, enzymatic removal of sialic acids from the treponemal surface increases the serum sensitivity of *Treponema pallidum* (11). Exogenous sialylation of gonococcal LOS prevents complement-dependent killing (9, 15, 23, 24, 49, 54). The same holds true for type III group B streptococci in which AP-mediated C3b deposition is inhibited by capsular terminal sialic acids (37). In contrast to investigations on eukaryotic cells (20), however, an influence of sialic acids on factor H binding has not been demonstrated in any of these bacterial models until now. One explanation for the increased serum resistance of sialylated bacteria is the reduction of binding of bactericidal antibodies to bacterial surface structures, but conflicting results concerning this mechanism have been reported in the gonococcal system (9, 41, 54).

The complement factor C3 is the site of convergence of the classical pathway (CP) and the AP of complement activation (53). Its activation results in the formation of bactericidal MACs and of C3 derivatives that interact with a variety of complement receptors (53). The deposition of C3 on bacterial surfaces has been studied in several model systems, including *Escherichia coli* (16), *Staphylococcus aureus* (16), *Listeria monocytogenes* (7), *Campylobacter fetus* (2), *Cryptococcus neoformans* (32), group B streptococci (5, 37), and *N. meningitidis*

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(21–24). Jarvis reported that the repertoire of C3 derivatives deposited on the meningococcal surface comprises iC3b and C3b (23). The investigation of several strains of serogroup C meningococci demonstrated that total C3 deposition on serogroup C meningococci did not correlate with LOS sialylation, capsule expression, and serum resistance. The capsule expression in group B meningococci did not affect iC3b deposition when a capsular strain and its acapsular variant were compared. Nevertheless, a prior study revealed that the removal of sialic acids from serogroup B meningococci by treatment with neuraminidase increased the amount of C3 deposited by the AP (21).

In the present study, therefore, we reanalyzed the impact of meningococcal LOS or capsule sialic acids on serum resistance and surface deposition of C3 in a genetically defined background by using isogenic mutants of serogroup B *N. meningitidis*.

#### MATERIALS AND METHODS

**Bacterial strains.** *N. meningitidis* B1940 (serogroup B, immunotype L3,7,9) is a clinical isolate and was obtained from U. Berger, Institute of Hygiene, Heidelberg, Germany. This strain is pilated and expresses Opa and Opc (19). Mutant strain B1940*siaD* (acapsular, sialylated LOS) is a Tn1725 knock-out mutant defective in the expression of the polysialyltransferase (18). Mutant strain B1940*galE* (capsular, LOS not sialylated) was established by replacing the *galE* and *rfb* genes with a chloramphenicol resistance cassette, resulting in the expression of a truncated LOS (18). In the mutant strain B1940*cps*, the *cps* locus comprising the genes required for synthesis of sialic acids, the polysialyltransferase gene, and the *galE* gene, was deleted (18), rendering this mutant's phenotype acapsular and negative for LOS sialylation. Bacteria were stored at  $-70^{\circ}\text{C}$  until use.

**Serum.** Normal human serum (NHS) was obtained from 10 healthy individuals with no history of bacterial meningitis, gonorrhea, or meningococcal vaccination. Blood was allowed to clot for 30 min at room temperature. Following centrifugation for 5 min at  $2,000 \times g$ , the serum was quick-frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ .

**Antibodies.** Monoclonal antibody MAb755 is directed against the  $\alpha$ -chain of C3 (31).

**Epitope mapping.** The amino acid sequence of the C-terminal 360 residues of the C3  $\alpha$ -chain (positions 1303 to 1663) was found in the Swissprot database (accession no. P01024). This section of the sequence of the C3  $\alpha$ -chain included the sequence of the complete C-terminal 40-kDa fragment of C3c (342 amino acid residues). A total of 116 overlapping pentadecapeptides with an offset of three amino acid residues were chemically synthesized as an array of N-terminally acetylated and C-terminally covalently immobilized products on cellulose sheets by using the spot synthesis technique (12) and a model ASP222 spotting robot (ABIMED Analysen-Technik, Langenfeld, Germany). Binding of MAb755 to peptide spots was assayed as described previously (13) by using a goat anti-mouse secondary antibody conjugate with alkaline phosphatase (Dianova, Hamburg, Germany). Peptides 66, 67, and 68 (comprising amino acid residues 1499 to 1519) were strongly reactive with MAb755.

**Bactericidal assay.** Meningococci grown overnight on chocolate agar were resuspended in Veronal-buffered saline (VBS) (5 mM 5,5-diethyl-barbituric acid [Serva, Heidelberg, Germany], 145 mM NaCl, 0.5 mM  $\text{MgCl}_2$ , 0.15 mM  $\text{CaCl}_2$ ) plus 0.5% bovine serum albumin (BSA) (Sigma, Deisenhofen, Germany) (VBS-BSA). The optical density at 600 nm was determined, and the bacteria were diluted appropriately. Triplicates of  $10^4$  CFU resuspended in VBS-BSA were incubated in 40% NHS at  $37^{\circ}\text{C}$  for 0, 15, 30, and 60 min. The final reaction volume was 100  $\mu\text{l}$ . The reaction was stopped by incubation on ice, and the samples were diluted immediately. Appropriate dilutions were plated on GC agar (Difco Laboratories, Detroit, Mich.) supplemented with supplement VX (Difco) and incubated overnight at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ . Bacterial counts are given as  $\log_{10}$  CFU per milliliter.

**Detection of complement factor C3b and its derivatives deposited on *N. meningitidis* strains and mutants.** Meningococci ( $2 \times 10^7$  CFU) grown overnight on chocolate agar and resuspended in VBS-BSA were added to NHS, resulting in a reaction mixture volume of 100  $\mu\text{l}$  and a final concentration of NHS of 40%. The reaction tube was immediately transferred into a waterbath at  $37^{\circ}\text{C}$  and incubated for 3, 15, and 30 min. Control experiments included NHS which was heat inactivated for 30 min at  $56^{\circ}\text{C}$  (HIS). In another set of control experiments, no serum was added. In order to block the CP of complement activation, ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA; Sigma) (final concentration, 10 mM) and 10 mM  $\text{MgCl}_2$  were added to NHS in some samples. The reaction of bacteria with complement factors was stopped by the addition of EGTA to a final concentration of 10 mM and incubation in an ice water bath for 1 min. The bacteria were pelleted by centrifugation at  $13,000 \times g$  for 5 min at  $4^{\circ}\text{C}$ . The pellet was washed three times with 500  $\mu\text{l}$  of VBS and was then

resuspended in 40  $\mu\text{l}$  of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample solution (0.3 M Tris HCl [pH 6.8], 2% SDS, 12.5% glycerol, 5%  $\beta$ -mercaptoethanol) and boiled for 5 min. Ten microliters of the lysate in sample solution was separated on an SDS-PAGE (10% polyacrylamide). For a control for the migration of the C3  $\alpha$ -chain in the gels, 1.5  $\mu\text{l}$  of NHS diluted 1:100 in VBS-BSA was used. Following electrophoresis, the proteins were transferred onto nitrocellulose filters (Bio-Rad, München, Germany) by Western blotting. The protein content of the samples was visually compared by rapid silver staining of separate SDS-PAGEs by the method of Bloom et al. (3). After unspecific binding sites were blocked with phosphate-buffered saline (PBS)–0.1% Tween 20–5% milk powder for 1 h at room temperature, C3 and its derivatives were detected by incubation with MAb755 (14  $\mu\text{g}/\text{ml}$ ) in PBS–0.1% Tween 20 overnight at  $4^{\circ}\text{C}$ . Sheep anti-mouse IgG conjugated with horseradish peroxidase (Sigma) was diluted 1:5,000 in PBS–0.1% Tween 20 and incubated for 1 h at room temperature. Antibody binding was detected by luminol-based chemiluminescence with the enhanced chemiluminescence Western blotting detection reagents (Amersham Life Sciences, Little Chalfont, United Kingdom).

**Flow cytometry analysis.** The binding of Ig to meningococci was determined by flow cytometry analysis. Meningococci ( $10^7$  CFU) were incubated with 100  $\mu\text{l}$  of HIS diluted appropriately in PBS–1% heat-inactivated fetal calf serum (FCS) (Biochrom, Berlin, Germany) for 45 min on ice. The bacteria were washed twice with 500  $\mu\text{l}$  of PBS–1% FCS and fixed with PBS–3% paraformaldehyde (Merck, Darmstadt, Germany) on ice for 20 min to reduce the infectious hazard during the flow cytometry analysis. Following two additional washes with PBS–1% FCS, the bacteria were incubated on ice for 30 min with 100  $\mu\text{l}$  of either fluorescein isothiocyanate (FITC)-conjugated anti-human IgM (Fluoline M; BioMérieux, Marcy l'Etoile, France) diluted 1:200 in PBS–1% FCS, or FITC-conjugated anti-human IgG (Fluoline G; BioMérieux) diluted 1:200, or FITC-conjugated anti-human IgA (Dianova) diluted 1:40. The bacterial pellets were washed three times and resuspended in 200- $\mu\text{l}$  portions of PBS. For a control, bacteria were incubated in PBS–1% FCS instead of HIS. The cells were analyzed by flow cytometry with a FACScan (Becton Dickinson, San Jose, Calif.) and FACScan Research Software (Becton Dickinson). Each analysis included 5,000 events. Control measurements were performed omitting HIS in the first antibody binding reaction. For one set of experiments with all strains and mutants used in this study, one positive histogram gate was defined, allowing a maximum of 2% of the events of the control experiments to appear in the positive channels.

#### RESULTS

**Bactericidal assay.** Serum resistance in pathogenic *Neisseria* spp. has been attributed to the surface expression of sialic acid. In order to dissect the roles of either LOS sialic acid or capsule sialic acid, the ability of the wild-type meningococcus strain B1940 and its isogenic capsule or LOS mutants to survive in NHS was investigated by a bactericidal assay (23). As is evident from Fig. 1, only the wild-type strain survived the action of complement in 40% NHS. The number of CFU declined from  $5.20 \log_{10}$  CFU/ml at the start of the experiment to  $3.84 \log_{10}$  CFU/ml after 1 h of serum treatment. The wild-type strain may therefore be considered partially serum resistant. In contrast, all mutants used in this assay were highly sensitive to the serum bactericidal activity, and the bacterial counts were rapidly reduced by  $2.9 \log_{10}$  CFU/ml as early as 15 min after the start of the serum incubation. It follows that both the capsule (*siaD* mutant) and the LOS sialylation (*galE* mutant) are prerequisites for resistance to the action of 40% NHS.

**Deposition of C3b and iC3b on meningococcal strains and mutants from 40% NHS.** In a variety of bacteria, alteration of complement factor C3 binding and degradation contributes to a serum-resistant phenotype. As a first approach to analyzing the mechanism responsible for serum sensitivity of either capsule or LOS mutants, we therefore investigated C3 binding to the meningococcal surface.

The complement factor C3 is the central molecule of complement activation because it is the converging point of the AP and the CP. A schematic diagram of C3 and its breakdown products is given in Fig. 2 (32, 53). Cleavage of the  $\alpha$ -chain of C3 by convertases results in the release of the anaphylatoxin C3a and the externalization of a labile thiolester bond found in the C3d region of the molecule, resulting in the formation of a covalent bond of C3b to a receptor surface (53). Inactivation of C3b to iC3b may occur by the action of factor I and H (53), and

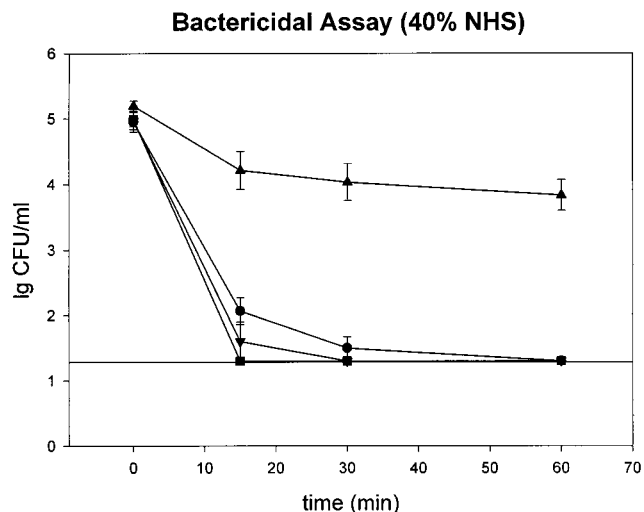


FIG. 1. Bactericidal assay. Meningococci were incubated in 40% NHS, and the number of CFU was determined at 0, 15, 30, and 60 min of incubation by plating serial dilutions. The limit of detection ( $1.3 \log_{10}$  [lg] CFU/ml) is indicated by the horizontal line. Symbols: ▲, B1940 (wild type); ●, B1940*cps*; ■, B1940*siaD*; ▼, B1940*galE*.

further proteolytic cleavage can result in the formation of C3c and C3dg.

MAB755 used in this study for the detection of C3 and its derivatives binds to the  $\alpha$ -chain of C3 (31). In this study, the epitope recognized by MAB755 was mapped between positions 1499 and 1519 of the C3 amino acid sequence (Swissprot database, accession no. P01024). This finding clearly showed that MAB755 binds to the C-terminal 40-kDa fragment of iC3b. Thus, in Western blots with reduced gels, MAB755 detects C3b deposited on meningococci as a 109-kDa fragment, most likely shifted to a higher molecular mass by covalent linkage to meningococcal surface molecules. In contrast, iC3b is represented by the 40-kDa fragment of iC3b after cleavage of the disulfate bonds under reducing conditions.

The 40-kDa iC3b fragment detected by MAB755 was deposited rapidly on all strains and mutants used in this study (Fig. 3B), indicating rapid degradation of C3b on the meningococcal surface. Nevertheless, all meningococci used were also susceptible to the deposition of intact C3b and its derivatives with high molecular masses generated by covalent ester or amide linkage to meningococcal surface structures. C3b deposition on the meningococcal surfaces correlated with the liberation of C3a to the supernatant as determined by affinity chromatography (data not shown), further demonstrating that C3 activation had occurred. This activation may be due in part to fluid-phase activation of C3 without covalent linkage of C3b, since small amounts of unshifted C3b  $\alpha$ -chain migrating slightly faster than the C3  $\alpha$ -chain (NHS control) were detected. However, the majority of C3b was present in the covalently linked form. Accordingly, the results of these experiments do not exclude the possibility that a portion of the iC3b 40-kDa fragment detected in the assay arose from fluid-phase activation. This problem might have been overcome by the use of SDS in the wash buffer (23). However, meningococci were extremely sensitive to detergents. SDS added to the wash buffer at a concentration as low as 0.03% drastically altered the protein profiles of whole-cell extracts of meningococci and extracted covalently linked C3b (data not shown). After 3 min of incubation in NHS, deposition of C3b and its derivatives on me-

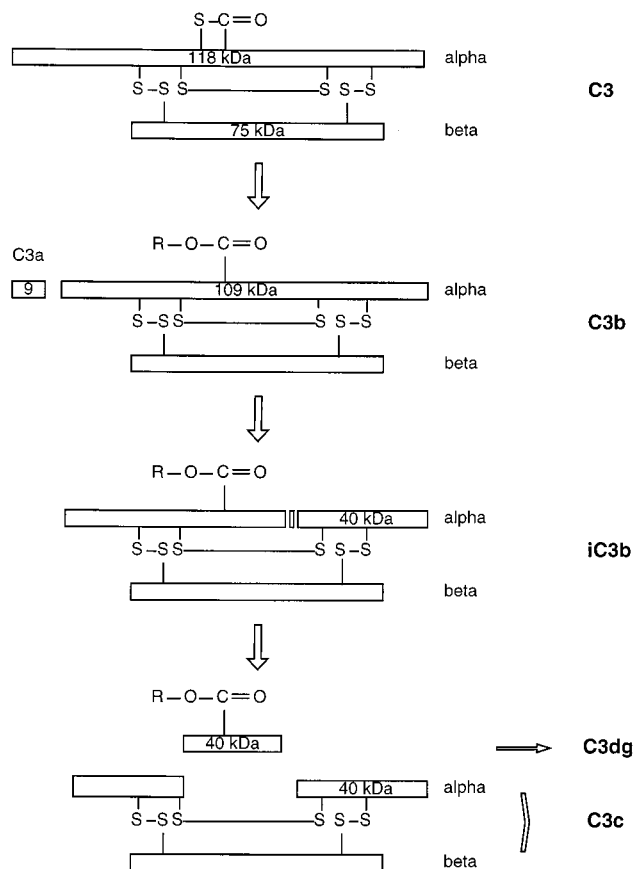


FIG. 2. Schematic illustration of the structures of the C3 molecule and its cleavage products (53) (adapted from reference 32 by permission of the publisher).

ningococci was dependent solely on the CP, since blocking the CP by EGTA-Mg<sup>2+</sup> resulted in no significant C3b binding (Fig. 3B). This early covalent linkage of C3b to meningococcal surface components resulted in equal band patterns on all strains and mutants examined, with a more pronounced binding to the *galE* mutants (*cps* and *galE* mutants). Over 30 min, however, there was only one prominent band (migrating between the 116- and 200-kDa marker proteins) detectable in the wild-type strain and its unencapsulated mutant. The meningococcal surface molecule present in this band seemed to be targeted by both the CP and the AP, because it was also detectable when the opsonization occurred in the presence of EGTA-Mg<sup>2+</sup>. In contrast, the *galE* mutants also allowed for persistent linkage of C3b to other meningococcal surface structures represented by C3b derivatives with molecular masses of >200 kDa. We therefore suggest that C3b is more accessible to cleavage when covalently linked to meningococcal surfaces with wild-type LOS than when linked to surfaces of nonsialylated-LOS *galE* mutants. This holds true especially for the meningococcal surface components represented by the double band of a molecular mass slightly higher than 200 kDa which disappeared from the wild-type LOS strains as early as 15 min after the start of the opsonization. Blocking the CP in 40% NHS by the addition of EGTA-Mg<sup>2+</sup> resulted in a delayed but equivalent targeting of meningococcal surface structures by the AP when compared to the deposition of C3b and iC3b by both pathways. We conclude from these experiments that C3b deposition from

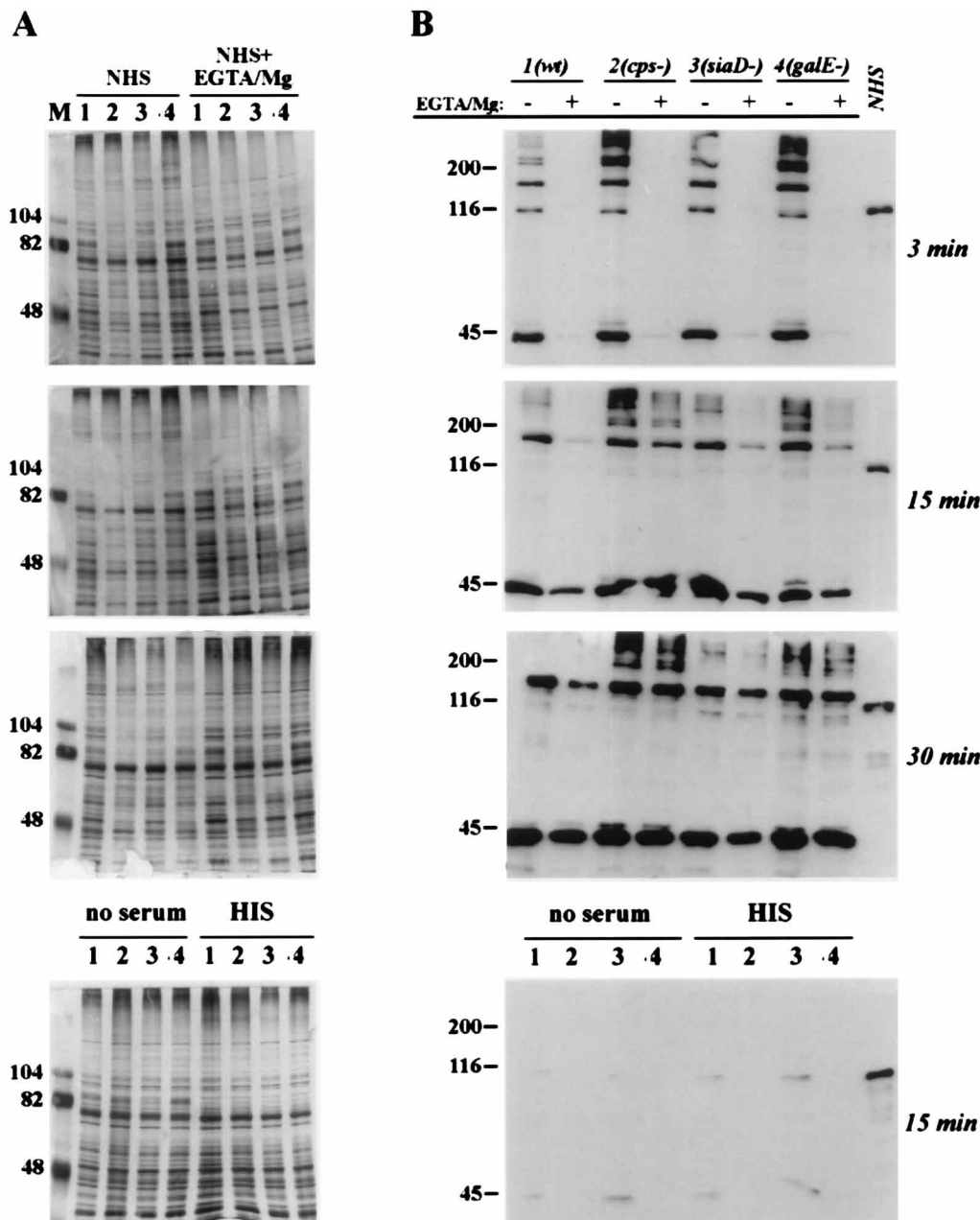


FIG. 3. C3b deposition on meningococcal strains and mutants. Meningococci were incubated in 40% NHS with or without EGTA-Mg<sup>2+</sup> for 3, 15, and 30 min. Equal sample volumes were subjected to SDS-PAGE and silver staining (A) or SDS-PAGE and immunoblotting with Mab755 that is specific for the  $\alpha$ -chain of C3 (B). Control samples included meningococci incubated without serum or with HIS. NHS was run on each immunoblotting gel to illustrate the position of the C3  $\alpha$ -chain. Lanes: 1, B1940 (wild type [wt]); 2, B1940*cps*; 3, B1940*siaD*; 4, B1940*galE*. The positions of molecular mass standards (in kilodaltons) (contained in lanes M) are indicated to the left of the gels.

40% NHS occurs by both the CP and the AP and is partially restricted by LOS sialylation but not by encapsulation.

Reduction of the serum concentration to 10% abrogated the AP activity to a large extent (data not shown). This is not surprising, since a critical concentration of NHS for AP-mediated complement activation in *L. monocytogenes* was reported to be between 10 and 20% NHS (7). With 10% NHS, only the *cps* mutant and the *galE* mutant showed deposition of covalently linked C3b, suggesting that the CP activation at low serum concentrations was abrogated by the LOS sialylation, irrespective of the capsule phenotype (data not shown).

**Binding of Igs to meningococci.** Activation of the CP usually requires the binding of the complement factor C1 to IgM or IgG bound to the activator surface (53). Because nonsialylated-LOS *galE* mutants (*galE* and *cps* mutants) differed greatly from wild-type LOS strains in C3b deposition via the CP, we used flow cytometry to analyze whether Ig binding to the meningococcal mutants was enhanced in the *galE* mutants. Heat-inactivated serum (HIS) was used as an Ig source in order to allow for Ig binding to native bacterial cells without lysis by the complement system. Binding of IgM was easily detectable in the mutant strains B1940*cps* and B1940*galE*,

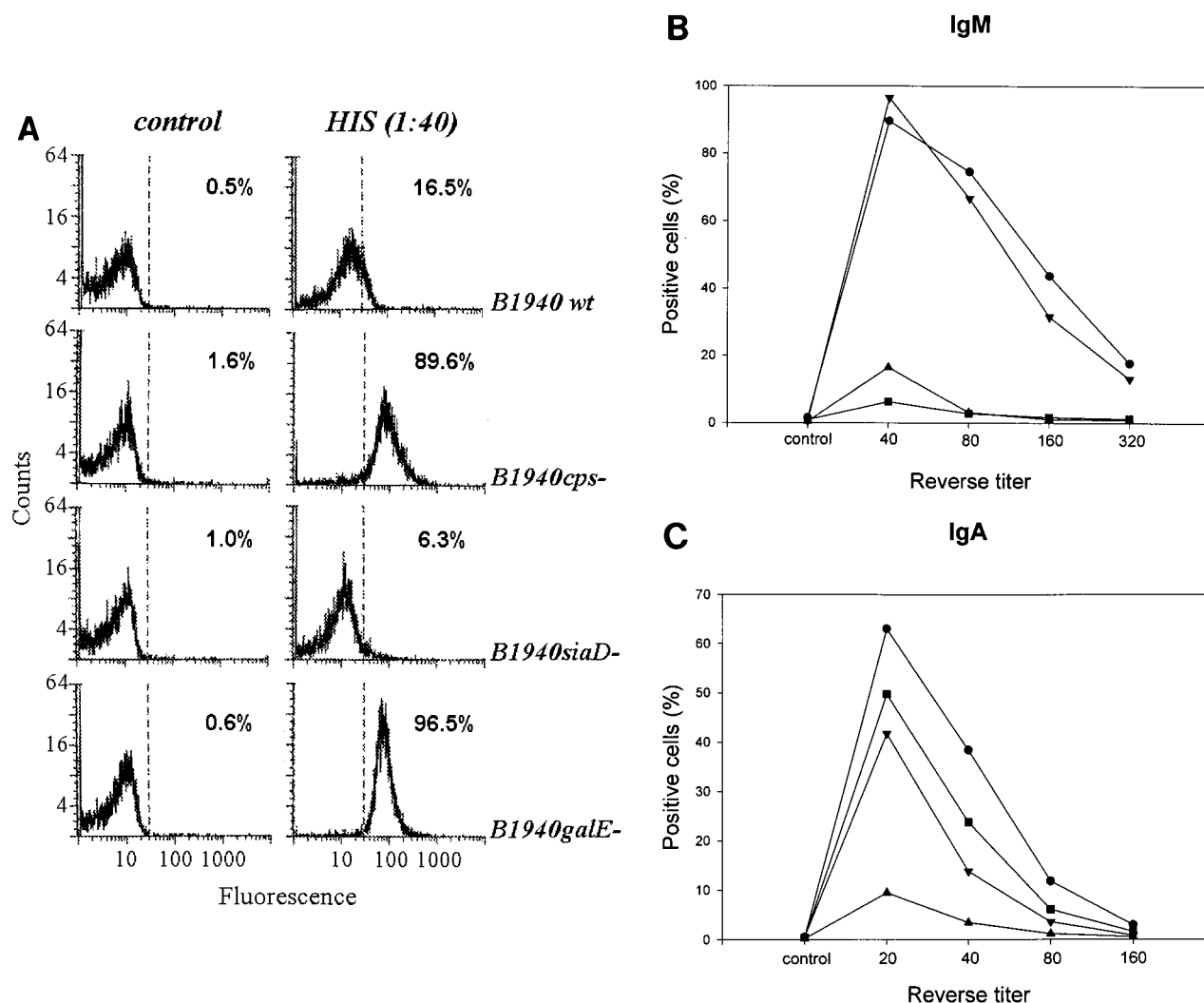


FIG. 4. Flow cytometry analysis of Ig bound from HIS to meningococci. (A) Histograms depict the distribution of fluorescence intensity after treatment of the various meningococci with 2.5% HIS or buffer alone (control), as detected by FITC-conjugated antibodies directed against human IgM. (B and C) Correlation of the numbers of IgM-positive cells and of IgA-positive cells achieved by incubation of meningococci with different serum dilutions. Symbols: ▲, *B1940* (wild type); ●, *B1940cps-*; ■, *B1940siaD-*; ▼, *B1940galE-*. Reverse titer, reciprocal of dilution.

which are deficient in LOS sialylation due to a truncated LOS, with >80% positive cells when a serum dilution of 1:40 was used (Fig. 4A). In contrast, meningococci with intact LOS sialylation bound far less IgM (<20% positive cells), irrespective of the capsular phenotype. The differences in IgM binding could even be demonstrated with serum diluted 1:160 and 1:320 (Fig. 4B). These results suggest that *galE* mutants are more accessible to IgM binding than meningococci with wild-type LOS. Enhanced IgM binding probably results in enhanced C3b deposition by the CP in these mutants, especially at low serum concentrations. In flow cytometry analysis using HIS dilutions of 1:40, 1:80, and 1:160, we were not able to demonstrate significant differences in IgG binding between the different strains and mutants used in this study (data not shown). Therefore, the impact of IgG on complement activation in meningococcal LOS and capsule mutants awaits further examination, including the differentiated analysis of the IgG subclasses.

Human serum IgA has been shown both to initiate complement-mediated killing of meningococci (22) and to have block-

ing effects on the complement lysis of meningococci (17). We therefore extended our flow cytometry analysis to cover the binding of serum IgA to the meningococcal strains and mutants. Figure 4C illustrates that the wild-type strain displayed negligible binding of IgA (approximately 10% of the cells were positive at a dilution of the HIS of 1:20), in contrast to LOS and capsule mutants, of which approximately 50% were considered positive for IgA binding. This experiment suggested that inhibition of IgA binding to the meningococcal surface required the expression of both the PSA capsule and intact sialylated LOS. Future studies should elucidate whether this inhibition is of crucial importance for the relative serum resistance of the wild-type strain in contrast to the serum-sensitive mutants.

## DISCUSSION

In this study, we have analyzed the impacts of LOS sialylation and PSA capsule expression in serogroup B meningococci on their interaction with the complement system. In bacteri-

cidal assays, the wild-type strain could be defined as relatively resistant to serum, in contrast to isogenic mutants, which were sensitive to serum, indicating that both LOS sialylation and the PSA capsule are indispensable for a serum-resistant phenotype.

In accordance with the findings of Jarvis (23), C3 was deposited from NHS on serogroup B meningococci as C3b and iC3b. C3b binding was more pronounced and occurred on a larger number of target molecules in strains and mutants with sialylated LOS. Interestingly, this effect was independent of encapsulation. Both the CP- and AP-mediated deposition of C3b were affected by LOS sialylation on the meningococcal surface. These findings suggest that (i) C3b covalently linked to surface molecules of meningococci expressing the wild-type LOS is more accessible overall to cleavage to iC3b and (ii) these meningococcal surfaces are less accessible to covalent C3 linkage. We suggest that lack of LOS sialic acid and reduction of the LOS chain length contribute to an improved presentation of surface-exposed molecules to the complement system. It is unlikely that LOS of the *galE* mutants itself serves as the major target for covalent linkage of C3, because its low molecular mass is highly unlikely to give rise to the band shifts of the C3b  $\alpha$ -chain demonstrated in Fig. 3.

Although the inhibitory effects of bacterial surface sialic acids on the activation of the alternative pathway are well established (11, 21, 37), less is known about their influence on the CP. Antibody-independent CP activation is hindered by the presence of the K1 capsule in *E. coli*, but this effect requires the presence of certain O serotypes (43, 44). Interestingly enough, the expression of terminal sialic acids in the type Ia group B streptococcal capsule mediates CP activation and C1 binding which can be eliminated by neuraminidase treatment of the organisms (34). We were able to demonstrate in this study that in serogroup B meningococci, LOS sialylation irrespective of the capsular phenotype is responsible for the inhibition of the CP. In order to give evidence about the mechanism of the enhanced CP-mediated C3b binding to *galE* mutants, Ig binding to meningococci was measured by flow cytometry. We were able to demonstrate high levels of IgM binding in *galE* mutants, in contrast to the wild-type strain and its unencapsulated mutant. Although we were not able to elucidate differences in IgG binding as well, the enhanced IgM binding could explain the increased level of CP activation in these mutants, which resulted in enhanced C3b deposition. This holds especially for the interaction of meningococci with 10% NHS, where C3b deposition is primarily mediated by the CP. The precise specificity of the IgM fraction binding to the *galE* mutants, however, remains unclear. It will therefore be interesting to determine the following in detail: (i) the meningococcal surface components targeted for C3b deposition by either the CP or the AP; (ii) the inducers of the CP or AP activation, e.g., meningococcal surface structures, antibodies, mannan-binding protein (51), or iron-binding proteins (45); and (iii) the binding specificity and Ig class of antibodies targeted at meningococcal surfaces expressing LOS species of differing length and degree of sialylation.

The *galE* mutants used in this study exhibit a truncated LOS devoid of galactose, in which the terminal three sugar molecules of the wild-type lacto-*N*-neotetraose are lacking or substituted by glucose extensions (33). *galE* mutants of the gonococcal strain MS11 exhibit increased resistance to serum compared to that of wild-type gonococci grown in the absence of CMP-NANA and bearing unsialylated LOS (47), suggesting that there are natural antibodies in NHS which are directed against the terminal portion of the gonococcal lacto-*N*-neotetraose but which do not recognize the *galE* LOS. Naturally

occurring IgM directed against subtypes of gonococcal LOS has been described as being directed against LOS terminally substituted with *N*-acetylgalactosamine (reviewed in reference 35). However, this structure does not occur in meningococci, and we were able to show in this study that B meningococcal *galE* mutants not only exhibited enhanced serum sensitivity but also bound IgM from NHS more efficiently than the wild type. Thus, a *galE* mutation in meningococci, in contrast to the one in gonococci, results in increased IgM binding to meningococcal surface molecules. Increased meningococcal serum sensitivity has also been attributed to meningococci expressing the immunotype L8 (40). The LOS of this immunotype lacks the two terminal sugars of the lacto-*N*-neotetraose. The researchers suggest that the lack of a terminal substitution with sialic acid and a reduced length of the LOS structure in this immunotype favors the insertion of MACs following the exposure to bactericidal antibodies directed against outer membrane proteins. For the *galE* mutant lacking the three terminal sugars of the lacto-*N*-neotetraose, we were able to demonstrate that mutation of the LOS also influences earlier events in the complement activation cascade, since different surface structures were targeted by C3b in *galE* mutants than in strains with wild-type LOS.

An interesting hypothesis for the serum sensitivity of *galE* mutants expressing nonsialylated LOS arises from the observation that binding of IgA specific for  $\alpha$ 1,3-galactose residues to glycosylated pili of *N. meningitidis* blocks the complement-mediated killing of the bacteria (17). *galE* mutants, in contrast to wild-type meningococci, lack terminal digalactose moieties (50), which might result in a loss of binding of blocking anti-Gal and contribute to the increased serum sensitivity of the *galE* mutants used in this study. However, the terminal glycosyl residue of meningococcal pili has been described as 1,4-linked (50), which would exclude the specific binding of anti-Gal to meningococcal pili. Future studies using purified anti-Gal antibodies should examine their impact on C3 deposition and serum resistance in the isogenic mutants used in this study.

Despite a covalent linkage of C3b to its surface, the wild-type strain was relatively resistant to the bactericidal action of 40% NHS. These findings fit in well with the results obtained by Klein et al. (30) with the same set of mutants in the whole-blood model and our recent work with the infant rat model (52), demonstrating that both LOS sialylation and the PSA capsule are indispensable for serum resistance. Interestingly, the unencapsulated mutant B1940*siad* expressing a wild-type LOS gave very similar C3b binding patterns after incubation in 10% and 40% NHS and exhibited an equally low level of IgM binding after incubation with HIS. However, this mutant differed drastically from the wild-type strain in its serum susceptibility. There are two possible explanations for this phenomenon. (i) Human serum IgA has been shown to initiate complement-mediated killing of meningococci without increasing C3 deposition (22). We were able to demonstrate in this study that the serum-sensitive unencapsulated mutant bound far more IgA than the wild-type strain, without exhibiting enhanced C3 deposition. Therefore, enhanced IgA binding by the unencapsulated mutant with wild-type LOS might be the key to its serum sensitivity without increased C3b binding. (ii) C3b is part of the C5 convertase activating the complement factor C5 in order to promote the formation of the MAC. The serum resistance of the wild-type meningococcal strain and the serum sensitivity of its unencapsulated mutant bearing wild-type LOS might be explained by differential insertion of the MAC, rather than by differential C3b binding. Early work in the gonococcal system elucidated that serum-resistant gonococci insert nonbactericidal MACs in a different molecular

configuration than serum-sensitive gonococci (25–27). We are currently investigating whether similar mechanisms also occur in meningococci. Insertion of bactericidal MACs into unencapsulated meningococci with wild-type LOS may lead to a serum-sensitive phenotype, despite levels of IgM binding and C3b deposition comparable to the levels found in the encapsulated and relatively serum-resistant wild-type meningococcus.

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