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Novel Blocking Human IgG Directed against the Pentapeptide Repeat Motifs of Neisseria meningitidis Lip/H.8 and Laz Lipoproteins

Tathagat Dutta Ray, Lisa A. Lewis, Sunita Gulati, Peter A. Rice, and Sanjay Ram

Ab-initiated, complement-dependent killing contributes to host defenses against invasive meningococcal disease. Sera from non-immunized individuals vary widely in their bactericidal activity against group B meningococci. We show that IgG isolated from select individuals can block killing of group B meningococci by human sera that are otherwise bactericidal. This IgG also reduced the bactericidal efficacy of Abs directed against the group B meningococcal protein vaccine candidates factor H-binding protein currently undergoing clinical trials and Neissierial surface protein A. Immunoblots revealed that the blocking IgG was directed against a meningococcal Ag called H.8. Killing of meningococci in reactions containing bactericidal mAbs and human blocking Abs was restored when binding of blocking Ab to meningococci was inhibited using either synthetic peptides corresponding to H.8 or a nonblocking mAb against H.8. Furthermore, genetic deletion of H.8 from target organisms abrogated blocking. The Fc region of the blocking IgG was required for blocking because F(ab′)2 fragments were ineffective. Blocking required IgG glycosylation because deglycosylation with peptide:glycanase eliminated blocking. C4b deposition mediated by an anti-factor H-binding protein mAb was reduced by intact blocking IgG, but not by peptide:glycanase–treated blocking IgG, suggesting that blocking resulted from inhibition of classical pathway of complement. In conclusion, we have identified H.8 as a meningococcal target for novel blocking Abs in human serum. Such blocking Abs may reduce the efficacy of select antigroup B meningococcal protein vaccines. We also propose that outer membrane vesicle-containing meningococcal vaccines may be more efficacious if purged of subversive immunogens such as H.8. The Journal of Immunology, 2011, 186: 4881–4894.

Although Neisseria meningitidis is one of the leading causes of sepsis and bacterial meningitis worldwide (1–4), it most often resides as a commensal in the human upper respiratory tract and does not elicit symptoms. The incidence of invasive meningococcal disease (~1/100,000 in non-epidemic settings) is rare relative to the rates of colonization (population prevalence rate, ~10%) (3, 5, 6). During epidemics and in closed populations such as military recruits, rates of colonization can exceed 50% (7). Rates of invasive disease during epidemics in sub-Saharan Africa can approach 1% (8). The incidence of meningococcal disease in the United States is highest in infants. However, about a third of cases occurs in persons >30 y of age (9).

Colonization with meningococci is often an “immunizing” process (10). The studies of Goldschneider and colleagues (11) have shown that individuals who possess a serum bactericidal titer of ≥1:4 using human complement are likely to be protected against invasive disease by the test strain. Individuals who lack protective Abs are at greater risk for invasive meningococcal disease (11). Bactericidal Abs elicited against epitopes shared by heterologous strains may contribute to cross-protection.

In this study, we sought to determine the reason for the variation among individuals in serum bactericidal activity (SBA) against serogroup B meningococci. Although the lack of bactericidal Abs against the test strain intuitively is the most likely explanation, another consideration is that select bacterial Ags or epitopes may elicit an Ab response that is nonbactericidal. A subset of these nonbactericidal Abs may also interfere with killing by bactericidal Abs. Such Abs are also known as “blocking” Abs and have been identified previously in individuals recovering from meningococcal disease (12, 13). Blocking Abs against Neisseria gonorrhoeae have also been described, and in that instance, the target is reduction modifiable protein (Rmp) (14). The identification of subversive Abs in human serum that prevent killing by bactericidal Abs is important because it could represent a strategy that meningococci use to evade host immune defenses. Individuals who possess high titers of blocking Abs may be at a greater risk for development of invasive meningococcal disease. In addition, blocking Abs may undermine the effectiveness of meningococcal vaccines.

Materials and Methods

Bacterial strains

N. meningitidis strains H44/76 (B:15:P1.7,16: ST-32; invasive isolate from Norway [1976]) (15) and the serologically related strain MC58 (B:15:P1.7,16: ST-74; invasive isolate: United Kingdom [1985]) (16) have been described previously. Strain BZ198 (B:NT:PNST:ET-154) is an invasive strain isolated from The Netherlands in 1986 (17). An isogenic mutant of MC58 that lacked factor H-binding protein (fHbp) expression (MC58...
ΔfHbp) was generated using chromosomal DNA extracted from strain H44/76 ΔfHbp (18, 19). Interruption of fHbp was confirmed by PCR and loss of fHbp expression confirmed by nonreactivity with anti-fHbp mAb JAR 3 by FACS.

Human serum
Sera obtained from 19 healthy adult human volunteers without a prior history of meningococcal disease were aliquoted and stored at −80°C until used. This study was approved by the Committee for the Protection of Human Subjects in Research at the University of Massachusetts Medical School. All subjects who donated blood for this study provided written informed consent. Sera were used only once and within 1 h after thawing. Total hemolytic complement (CH50) of sera was measured using the Total Hemolytic Complement kit (The Binding Site) or the EZ Complement CH50 assay (DiaMedix Corporation) according to the manufacturer’s instructions. Heat-inactivated serum (56°C for 30 min), which destroys complement activity but leaves Ab function intact, was used as the source of Ab in some bactericidal experiments.

Anti-Neisserial Abs
mAb JAR 3 (IgG3), JAR 4 (IgG2a), and JAR 5 (IgG2b) (20) that recognize variant meningococcal fHbp that is expressed by strains H44/76 and MC58, mAb SEAM 12 (IgG2a) (21) that is specific for group B meningococcal polysaccharide and anti-Neisserial surface protein A (NspA) mAb 14C7 (22) were kindly provided by Drs. Greg Moe and Dan M. Granoff (Children’s Hospital Oakland Research Institute, Oakland, CA). Anti-porin A (anti-PorA) mAb P1.7 (ascites fluid) was purchased from the National Institute of Biological Standards and Control (Potters Bar, Hertfordshire, U.K.). mAb 2C3 (IgG1) that recognizes Lip/H.8 has been described previously (23). Bactericidal polyclonal antiserum against fHbp variant 1 (termed fHbp ID 1) and is expressed by strain H44/76 according to the classification available online at: www.neisseria.org) was raised by immunizing BALB/c mice with purified recombinant fHbp absorbed to alum (antisera was provided by Drs. Dan Granoff and Peter Beemink, Children’s Hospital Oakland Research Institute).

Serum bactericidal assays
Serum bactericidal assays were performed as described previously (24). In brief, bacteria that were harvested from an overnight culture on chocolate agar plates were repassaged onto fresh chocolate agar and allowed to grow for 6 h at 37°C in an atmosphere containing 5% CO2. Approximately 2000 CFU meningococci were incubated in serum (concentrations specified for each experiment), purified proteins, peptides, or Abs. The final reaction volumes were maintained at 150 µl. Aliquots of 25 µl of reaction mixtures were plated onto chocolate agar in duplicate at the beginning of the assay (t0) and again after incubation at 37°C for 30 min (t30). Survival was calculated as the number of viable colonies at t30 relative to t0.

Ab-depleted human serum
All manipulations were performed at 4°C. Two milliliters serum containing 10 nM EDTA to minimize complement activation was passed over a 1-ml protein G-Sepharose column (Sigma, catalog no. P3296) (25) that contained DNase and EDTA-free Protease Inhibitor Cocktail Set V (Calbiochem, catalog no. 539137) and Ni2+-affinity chromatography (Pierce) according to the manufacturer’s instructions. Purity of the protein was established by Coomassie staining after electrophoresis on a 4–12% Bis-Tris gel (Invitrogen), and the ability of the protein to react with anti-fHbp–specific mAbs JAR 1 and JAR 3 by Western blotting was confirmed.

Flow cytometry
Binding of human IgG, IgM, and C4b to N. meningitidis was determined by flow cytometry as described previously (26). In brief, 10 µl serum was added to 108 bacteria suspended in 90 µl Hank’s balanced salt solution (HBSS) containing 1 mM CaCl2 and 1 mM MgCl2 (HBSS+) for 30 min at 37°C. Bacteria were washed, and C4b, IgG, and IgM that had bound to bacteria were detected using FITC-conjugated anti-human IgG, anti-human IgM (both from Sigma), or anti-C4 (Abcam) all at a dilution of 1:100. Similarly, binding of mAbs JAR 3 and P1.7 to bacteria were detected using anti-mouse IgG FITC (Sigma) at a final dilution of 1:100. Data were collected on a BD FACS Calibur (Becton Dickinson) flow cytometer and analyzed with FlowJo software (Tree Star).

Western blotting
Western blotting was used to determine binding of human IgG to meningococcal Ags. Bacterial lysates were separated on a 4–12% Bis-Tris gel (InVitrogen Life Technologies) using 2-(N-morpholino)ethanesulfonic acid (MES) running buffer, pH 7. Proteins were transferred to PVDF membranes (Millipore) and blocked with PBS-1% dry milk for 30 min at 24°C. Membranes were then incubated with heat-inactivated normal human serum (diluted 1:100 in PBS-0.05% Tween 20) for 15 h at 4°C. Membrane-bound IgG was detected using alkaline phosphate-conjugated anti-human IgG (Sigma) at a 1:1000 dilution in PBS-0.05% Tween 20 and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NPT) purple liquid substrate (Sigma, catalog no. B3679). The subclass of the anti-Lip/H.8 human IgG was determined using polyclonal Abs against human IgG1, IgG2, IgG3, and IgG4 conjugated to alkaline phosphate (all from Sigma).

Whole-cell ELISA
Microwells were coated with strain MC58 or MC58 ΔfHbp suspended in HBSS+ at an OD660nm of 0.3 for 3 h at 37°C. Nonsporic sites were blocked with PBS/0.2% BSA for 2 h at 37°C. Serum K1 (2-fold dilutions ranging from 1:125–1000) diluted in PBS/0.05% Tween 20 either alone or in the presence of purified recombinant fHbp (50 µg/ml) were dispensed into wells for 1 h at 37°C, and bound IgG was detected using anti-human IgG conjugated to alkaline phosphatase (Sigma) diluted 1:1000 in PBS/0.05% Tween 20 and alkaline phosphate substrate buffer (Sigma).

Lip/H.8 deletion (lip) mutants and insertional inactivation of fHbp
In this study, we refer to the lipoprotein that is composed almost entirely of “AAEAP” pentapeptide repeats as Lip/H.8 and the gene encoding for this
isolated chromosomal DNA from a Laz KO gonococcal strain F62 that was used to transform N. meningitidis strain MC58, and transformants were selected on GC agar plates containing chloramphenicol (5 \(\mu\)g/ml). Loss of Laz expression was confirmed by Western blotting.

To construct a mutant of strain MC58 that lacked Laz expression, we isolated chromosomal DNA from a Laz KO gonococcal strain F62 that was constructed as follows. Base pairs 40–184 of the lac open reading frame, which includes the signal sequence, was replaced with an erythromycin resistance (erm) marker using overlap extension PCR described in this study. DNA extracted from strain F62 was used as a template, and a 329-bp fragment upstream of bp 40 and a 359-bp fragment downstream of bp 184 of the lac open reading frame were amplified using primer pairs 5'-CGGCAGATGTTGGAATTCAATGC-3' (LAZ-UP-FOR) and 5'-GTTCCAATTGATTTTTGATAACCGCCGCAATCAAGA-3' (LAZ-DOWN-REV) and 5'-TGAACAACAGTGATCTGAGCAATCCACGAGAATGC-3' (LAZ-DOWN-REV), respectively (the boldface underline sequences overlap with the erm marker). The erm marker was amplified using genomic sequence of mutant gonococcal strain F62 IgE-erm (S. Ram, unpublished results) as a template with primers 5'-GGCGGCTGATACAAAAATAGGAAACACGAAAAACAGGAC-3' (ERM-FOR) and 5'-GGTTGGATGATGTGCACTCTTGTTGATATTATCAG-3' (ERM-REV) (the boldface underlined sequences overlap with lac). The three PCR products were each gel purified and then linked by overlap extension using primers LAZ-UP-FOR and LAZ-DOWN-REV. The resulting 1527-bp product was cloned into TOPO 2.1, linearized with ScaI, and used to transform strain F62. Ern-resistant colonies were selected on GC agar containing E. coli strain MC58 (S. Ram, unpublished results) whose N-terminal 35 aa that are organized into imperfect “AAEAP” repeats and the remainder of the protein comprises an azurin-like domain.

The number of pentapeptide repeats in Lip/H.8 varies across Neisseria strains. Laz refers to the 183-aa lipoprotein (the product of the lac gene) whose N-terminal 40 aa is composed of imperfect “AAEAP” repeats and the remainder of the protein comprises an azurin-like domain. Chromosomal DNA isolated from a Lip/H.8 knockout (KO) mutant of N. gonorrhoeae strain F62 (27) was used to transform N. meningitidis strain MC58, and transformants were selected on GC agar plates containing chloramphenicol (5 \(\mu\)g/ml). Loss of Lip/H.8 expression was confirmed by Western blotting.

Similarly, the azurin domain of Laz (the N-terminal 35 aa that are organized into imperfect “AAEAP” pentapeptide repeats) was revealed by Western blotting with an antipolyhistidine mAb (Sigma, catalog No. H1029). The presence of the “Lip/H.8-like” domain of Laz (the N-terminal 35 aa that are organized into imperfect “AAEAP” pentapeptide repeats) was revealed by Western blotting with anti-Lip/H.8 mAb 2C3 (23, 28).

IgG deglycosylation

Purified NK3 IgG was deglycosylated by incubation with 500 U Peptide-N-glycanase (PNGase; New England Biolabs; catalog no. P0705S) in sodium phosphate buffer, pH 7.5, overnight at 37°C. A control reaction included purification of NK3 IgG treated with buffer alone. Deglycosylated IgG was then purified using Sepharose bound protein G beads and spin concentrated/dialyzed against TBS, pH 7, using an Amicon-15 Ultra device (30-kDa cutoff). Deglycosylation of IgG was verified by increased migration velocity of the PNGase-treated IgG relative to mock (buffer)-treated IgG on a 4–12% Bis-Tris gel that was stained with Coomassie blue.

Statistical analysis

Percentage of survival of bacteria with 95% confidence interval around the mean percentage is shown for serum bactericidal assays. Differences in percentage survival (Figs. 2B, 3, 4, 6C, 6D, 6F–H, 7C, and Table I) were assessed for significance using a two-tailed paired Student t test (p values <0.05 are considered significant).

Results

Sera from individuals vary in their ability to mediate complement-dependent killing against group B N. meningitidis

Sera obtained from 19 normal individuals were screened for their ability to kill group B N. meningitidis strain H44/76 (Fig. 1A). The final concentration of serum in each bactericidal reaction mixture was 20%. Sera that resulted in ≤50% survival were designated as “killing” (K) sera, whereas those sera in which bacteria survived >50% were called NK sera. As expected, individual sera varied widely in their ability to kill meningococci. Although <50% of bacteria survived (>50% killing) in 13 of the 19 (68%) individual sera (Fig. 1A, K1–K13), >50% of H44/76 survived (<50% killing) in the remaining 6 sera (Fig. 1A, NK1–NK6). Survival of bacteria incubated with heat-inactivated “K” sera or buffer alone at t0 usually ranged between 100 and 120% (i.e., some bacterial growth was observed).

To examine whether lack of antimeningococcal Ab explained the deficiency in bactericidal activity of nonkilling sera, we selected four of the sera that displayed the highest bactericidal activity (K1–K4) and four sera with the least bactericidal activity (NK3–NK6) to study binding of IgG and IgM to strain H44/76 by flow cytometry. There was no correlation between the amount of IgG or IgM that bound to bacteria (Fig. 1B) and the amount of killing mediated by the sera (Fig. 1A). These data suggested that differences in bactericidal activity were likely the result of quantitative differences in the Abs present in the killing and nonkilling sera. We hypothesized that Abs present in nonkilling sera were directed against nonbacterial targets on meningococci. In some instances, Abs directed against nonbacterial epitopes could have possessed blocking activity (i.e., Abs that can subvert killing by bactericidal Abs individuals) (12, 13, 29) and this possibility was addressed next.
**FIGURE 1.** Bactericidal activity of individual human sera against strain H44/76 and binding of IgG and IgM binding in select killing and nonkilling sera to H44/76. A. Hemolitically active sera obtained from 19 normal individuals were tested for complement-dependent bactericidal activity against wild-type *N. meningitidis* serogroup B strain H44/76. The final concentration of each serum in the bactericidal reaction mixture was 20%. Sera that killed ≥50% (survival ≤50%) of bacteria were designated killing (K), and those that killed <50% (survival >50%) as nonkilling (NK) sera. B. Serogroup B strain H44/76 was incubated with each of four sera that had displayed maximal bactericidal activity (K1–K4) or each of four sera with the least bactericidal activity (NK3–NK6). The amount of IgG and IgM binding was quantified by flow cytometry. Gray shaded histograms represent binding of IgG or IgM in K sera, whereas histograms depicted by the solid lines represent binding of Ig in NK sera. The x-axis represents fluorescence on a log2 scale, and the y-axis the number of events. The secondary Ab control (sec. Ab ctrl; broken lines) reaction did not contain serum.

Select nonkilling sera possess the ability to block killing by bactericidal sera

The four sera that possessed the least amount of bactericidal activity (NK3, NK4, NK5, and NK6) were screened for their ability to block killing of H44/76 by killing sera K1, K2, K3, and K4 (containing the highest bactericidal activity). In this experiment, each of the heat-inactivated (complement activity destroyed, but Ab unaffected) nonkilling sera were incubated with H44/76 (20% nonkilling serum in the final reaction mixture) followed by the addition (separately) of each of the freshly thawed killing sera K1 through K4 (10% K serum in the final reaction mixture) that contained active complement. Blocking was defined as ≥50% reduction in killing of bacteria in the presence of killing serum mixed with the heat-inactivated nonkilling serum compared with killing mediated by the same killing serum alone. Of the 16 reaction mixtures that contained a combination of active killing and heat-inactivated nonkilling serum, blocking was seen in three instances (Fig. 2A, dark gray shaded boxes). NK3 blocked killing that was mediated by K1 and K2. NK4 blocked killing only by K1. Four combinations: NK4 plus K2, NK5 plus K1, NK5 plus K2, and NK3 plus K3 yielded 25–50% blocking compared with baseline bactericidal activity in killing serum alone (Fig. 2A, light gray shaded boxes). Killing of H44/76 by sera K3 and K4 were not blocked by any of the nonkilling sera used. Thus, select nonkilling sera possessed blocking activity, and conversely, the bactericidal activity of certain killing sera could be blocked only by select nonkilling sera.

Killing of wild-type strain H44/76 by K1 (containing endogenous active complement) was blocked by heat-inactivated NK3 in a dose-responsive manner (Fig. 2B). Similar results were seen when heat-inactivated serum NK4 was added incrementally to serum K1 (data not shown). In the converse experiment, to examine whether Ab in killing serum could overcome blocking, we incubated heat-inactivated K1 (final concentration of 20%) with the organisms, followed by addition of 10% NK3 (complement active). The survival seen with heat-inactivated K1 was not statistically significant when compared with survival in NK3 alone (Fig. 2B). These data provided evidence that a heat-stable component in NK3, likely Ab, could prevent killing by K1, and that in the “competition” between killing and blocking in human serum, blocking was favored at the Ab concentrations tested.

IgG is responsible for blocking

Prior studies have shown that Ig in serum is responsible for blocking killing of Neisseriae (12–14, 29). The earlier experiments indicate that blocking is mediated by a heat-stable molecule in serum. We hypothesized that Ig in NK3 mediated blocking. IgG and IgM from NK3 were purified and each was tested for its ability to block killing of H44/76 by K1. IgG purified from NK3 (NK3 IgG) blocked killing of H44/76 by K1 in a dose-responsive manner (Fig. 3A; 2 mg/ml NK3 IgG in the reaction mixture corresponds approximately to the amount of IgG contained in 20% [v/v] serum). The converse experiment, using IgG-depleted NK3 serum, showed that depleted serum was no longer effective as a blocking agent (Fig. 3B). Only a small (∼15%) increase in survival of bacteria was seen when 0.2 mg/ml NK3 IgM (corresponding to the amount of IgM in a reaction mixture that contains ∼20% [v/v] serum) was added to K1 (10%) serum in the bactericidal reaction mixture (Fig. 3C; 5th column). These experiments indicate that IgG in NK3 was largely responsible for blocking killing by K1.
The bactericidal activity of mAbs directed against select meningococcal vaccine candidates is attenuated by blocking IgG.

Because the serogroup B meningococcal capsule is not immunogenic, several outer membrane proteins are being investigated for their vaccine potential (30). One such protein that is currently being evaluated in humans is fHbp (previously known as GNA1870 or LP2086) (18, 31–33). One classification scheme has divided fHbp into three variant families (33), and based on this classification, strains H44/76 and MC58 both express variant 1 fHbp molecules. Anti-variant 1 fHbp mAb JAR 3 is bactericidal against H44/76 (20). Two additional anti-fHbp mAbs called JAR 4 and JAR 5 are not bactericidal when used individually but show synergistic bactericidal activity when used in combination (19).

PorA is the immunodominant component of outer membrane vesicle vaccines and also evokes a bactericidal Ab response (34, 35). mAbs against PorA also form the basis for serosubtyping of meningococci, and one of these mAbs called P1.7 recognizes strain H44/76 and is bactericidal (19).

We examined blocking activity of NK3 IgG on JAR 3-mediated killing of H44/76. Bacterial survival in the presence of JAR 3 at concentrations of 0.25 and 0.5 μg/ml was greater in reaction mixtures that contained NK3 serum than in reactions containing IgG-depleted NK3 (Fig. 4A). Increasing the concentrations of JAR 3 overcame blocking by NK3 and resulted in progressively increased bacterial killing evidenced by the observation that the presence of NK3 IgG did not enhance bacterial survival at JAR 3 concentrations of 1.0 and 2.0 μg/ml compared with the corresponding reactions that lacked NK3 IgG. The shift of the survival curve to the left when IgG was depleted from NK3 provides evidence that IgG in NK3 blocked the bactericidal activity of JAR 3. Similarly, blocking IgG also decreased the bactericidal effects of a combination of JAR 4 and JAR 5 when each mAb was added to the bactericidal reaction mixture described in A. The control used heat-inactivated NK3 serum in the bactericidal reaction mixture. Incremental doses of IgM purified from NK3 failed to block killing by K1. In all experiments, the y-axis indicates percentage survival, and each column represents the mean ± SEM of three separate experiments.

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dilutions of P1.7 tested, there was no significant difference in survival in the presence or absence of NK3 IgG in the reaction. The capacity of NK3 IgG to block killing of H44/76 by antigroup B capsular mAb SEAM 12 is shown in Fig. 4C; a small, non-significant increase in survival of bacteria was seen in the presence of IgG purified from NK3 (2 mg/ml), and survival at 30 min was measured. The black bar represents survival in the presence of complement alone. Survival in the presence or absence of NK3 IgG for each concentration of SEAM 12 was not significantly different (p > 0.05). D, The bactericidal activity of murine polyclonal anti-fHbp antiserum against H44/76 is blocked by NK3 IgG. Strain H44/76 was incubated with polyclonal anti-fHbp at antisum dilutions of 1:3000 and 1:6000 either in the presence or absence of IgG purified from NK3 (2 mg/ml) and complement (40% [v/v] normal human serum depleted of IgG and IgM). Survival was measured at 30 min in a serum bactericidal assay. Survival in the presence of NK3 IgG was significantly greater than the corresponding reaction mixture that lacked NK3 IgG (p < 0.05 in each instance). In all experiments, the y-axis represents percentage survival, and each data point represents the mean (± SEM) of three separate experiments.

FIGURE 4. Blocking potential depends on the specificity of killing Ab. A, Bactericidal activity of an anti-fHbp mAb is attenuated by IgG in NK3. N. meningitidis strain H44/76 was incubated with increasing concentrations (0.25, 0.5, 1, and 2 µg/ml) of JAR 3 in a reaction mixture that contained either 20% (v/v) of blocking serum NK3 (Ab and complement intact; solid circle) or 40% (v/v) IgG-depleted NK3 (solid box). The greater concentration of IgG-depleted NK3 was used to normalize for the amount of hemolytic complement activity in the bactericidal reaction mixture (the process of IgG depletion resulted in loss of ~50% of hemolytic activity). B, IgG depletion of NK3 does not affect bactericidal activity of anti-PorA mAb P1.7. Serial dilutions (1/1000, 1/500, 1/250, and 1/125) of ascitic fluid containing murine anti-PorA mAb P1.7 were each incubated with strain H44/76 and the bactericidal assay performed as described earlier in A. C, The bactericidal activity of antigroup B capsular mAb SEAM 12 is not significantly blocked by NK3 IgG. Strain H44/76 was incubated with mAb SEAM 12 and complement (40% [v/v] normal human serum depleted of IgG and IgM), either in the absence or presence of IgG purified from NK3 (2 mg/ml), and survival at 30 min was measured. The black bar represents survival in the presence of complement alone. Survival in the presence or absence of NK3 IgG for each concentration of SEAM 12 was not significantly different (p > 0.05). D, The bactericidal activity of murine polyclonal anti-fHbp antiserum against H44/76 is blocked by NK3 IgG. Strain H44/76 was incubated with polyclonal anti-fHbp at antisum dilutions of 1:3000 and 1:6000 either in the presence or absence of IgG purified from NK3 (2 mg/ml) and complement (40% [v/v] normal human serum depleted of IgG and IgM). Survival was measured at 30 min in a serum bactericidal assay. Survival in the presence of NK3 IgG was significantly greater than the corresponding reaction mixture that lacked NK3 IgG (p < 0.05 in each instance). In all experiments, the y-axis represents percentage survival, and each data point represents the mean (± SEM) of three separate experiments.
IgG-depleted NK3 was used. Collectively, these data indicate that was significantly lower in the presence of NK3 when whole versus Sepharose. Shown in Table I, the killing efficacy of mAb 14C7 NK3 that was depleted of IgG by passage through protein G-heat-inactivated NK3 (contains blocking Ab) or heat-inactivated human serum depleted of IgG and IgM) either in the presence of strain BZ198 by anti-NspA mAb 14C7 and complement (normal that targeted an Ag other than fHbp, we compared the killing of a heterologous strain and was effective against a bactericidal Ab.

Whereas killing of H44/76 by JAR 3 was blocked by the presence of recombinant fHbp, fHbp did not decrease killing by serum K1. The identity of each serum in terms of its bactericidal activity (SBA) and blocking activity is indicated in the labels below the gel pictures. Sera that lack SBA or blocking activity are labeled as n.a. (not applicable) for that particular category.

FIGURE 5. Lip/H.8 is a meningococcal target for IgG in blocking serum. Bacterial lysates of strain MC58 and its isogenic lipoprotein Lip/H.8 KO mutant were electrophoresed on a 12% Bis-Tris gel followed by Western blotting. Parallel blots were incubated with a 1:100 dilution of NK3 serum (nonbactericidal and possessing blocking activity), NK6 (nonbactericidal and without blocking activity), K1 (bactericidal activity that could be blocked by NK3), and K3 (bactericidal activity that could not be blocked by NK3). Human IgG-reactive bands were disclosed with alkaline phosphatase-conjugated anti-human IgG. The black asterisk (on the left) indicates the Lip/H.8-reactive band. The location of PorA and lipooligosaccharide (LOS) reactive bands are indicated with arrows on the right. The identity of each serum in terms of its bactericidal activity (SBA) and blocking activity is indicated in the labels below the gel pictures. Sera that lack SBA or blocking activity are labeled as n.a. (not applicable) for that particular category.

Table I. NK3 IgG blocks killing of BZ198 by anti-NspA mAb 14C7

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Mean Survival (± SEM)</th>
</tr>
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<tbody>
<tr>
<td>C alone (20%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>139 (± 5.9)</td>
</tr>
<tr>
<td>C + 14C7 (12 µg/ml)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51 (± 5.5)</td>
</tr>
<tr>
<td>C + 14C7 (12 µg/ml) + HI-NK3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>113 (± 4.9)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C + 14C7 (12 µg/ml) + HI-NK3 minus IgG&lt;sup&gt;d&lt;/sup&gt;</td>
<td>61 (± 6.0)</td>
</tr>
<tr>
<td>C + 14C7 (24 µg/ml)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2 (± 0.5)</td>
</tr>
<tr>
<td>C + 14C7 (24 µg/ml) + HI-NK3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>88 (± 10.5)</td>
</tr>
<tr>
<td>C + 14C7 (24 µg/ml) + HI-NK3 minus IgG</td>
<td>9 (± 2.5)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Five experiments.
<sup>b</sup>Three experiments.
<sup>c</sup><i>p = 0.02 versus C + 14C7 (12 µg/ml).
<sup>d</sup>Two experiments.

C, complement; HI-NK3, heat-inactivated NK3.

Identification of lipoprotein Lip/H.8 as a target for IgG in blocking serum

To gain a better understanding of why some sera possessed blocking activity and why the bactericidal activity of only certain killing sera or mAbs could be blocked, we examined the specificity of IgG binding to meningococcal proteins by Western blots using the following representative sera: 1) blocking serum NK3, 2) nonkilling and nonblocking serum NK6, 3) killing serum K1 that could be blocked, and 4) killing serum K3 that was not blocked (Fig. 5). To determine the target(s) of blocking IgG, whole bacterial lysates of H44/76 were separated on a 4–12% Bis-Tris gel and transferred to a PVDF membrane for Western blot analysis.

Lysates of H44/76 probed with NK3 showed a prominent IgG-reactive band at ∼21 kDa (marked with a black asterisk in the middle lane [labeled “wild type”) of the NK3 blot in Fig. 5) that was not visible in the NK6 or K1 groupings and was faint in K3. This band did not correspond to the location of other known outer membrane proteins such as Opa, Opc, or Class 4 protein; the latter is the homolog of Rmp in N. gonorrhoeae, which is the target of blocking Ab in that organism (14). Parallel Coomassie blue staining failed to disclose a band at this location (data not shown), which is typical of proteins that lack (or have diminished) aromatic amino acid content. An important Neisseria outer membrane Ag that migrates at ∼21 kDa and does not stain with Coomassie blue is the lipoprotein Lip/H.8 (36–38), also called Lip.

In preliminary experiments, an anti-Lip/H.8 mAb had also shown reactivity at the ∼21-kDa band location (not shown). The identity of this band was confirmed as Lip/H.8 by loss of reactivity on deleting lip (Fig. 5, lanes marked “Lip/H.8 KO” in blots probe with NK3 and K3). Blots probed with the secondary Ab (anti-human IgG-alkaline phosphatase) alone showed no reactivity (negative control, not shown). The IgG binding pattern of blocking serum NK4 was similar to that of blocking serum NK3. Similar to data with NK3, a blot probed with NK4 also showed a Lip/H.8-reactive band (data not shown).

Interestingly, the most obvious difference between K1 and NK3 was the ∼21-kDa Lip/H.8-reactive band, which suggested that IgG directed against this Ag may have contributed to the blocking activity of NK3 (depicted by a black asterisk in Fig. 5). Serum K3, whose bactericidal activity was not blocked by NK3 (Fig. 2A), showed a strongly reactive band at ∼45 kDa that corresponds to PorA in this strain. In addition, a band at ∼5 kDa (likely lipooligosaccharide) and other undefined fainter bands in the 30- to 40-kDa range were noted. The presence of PorA Abs in K3 and the inability of NK3 to block its bactericidal activity are consistent with the data in Fig. 4B where the bactericidal activity of the anti-PorA mAb was not affected by IgG from NK3. Subsequent experiments were directed at defining the role and specificities of Lip/H.8 and a related protein called Laz that shares a region of sequence homology with Lip/H.8 as targets for blocking IgG.

Lip/H.8 and Lac are the targets of the human complement-blocking Ab against N. meningitidis

Lip/H.8 is composed entirely of 13–14 tandem repeats of pentameric sequences, and more than half of these are identical “AAEAP” sequences (38). The N-terminal ∼40 aa of another Neisserial lipoprotein called lipid-modified azurin (e.g., the Laz gene in the following strains of Neisseria: Z2491 [NC_003116.1; gene ID 908122], MC58 [NC_003112; gene ID 904058], α 14
FIGURE 6. Expression of both Lip/H.8 and Laz are required for maximal blocking. A, Alignment of amino acid sequences of Lip/H.8 and Laz of serogroup B N. meningitidis MC58. Lip/H.8 is a 98-aa protein composed almost entirely of 12–15 pentapeptide repeats (more than half are perfect “AAEAP” sequences and the remainder represent modifications [“imperfect” repeats]) depending on the strain. Laz is a 183-aa protein composed of 7 mostly imperfect AAEAP pentameric repeats that comprise the Lip/H.8-like domain at its N terminus. The pentameric repeats are highlighted as bold letters. Each pentameric repeat is marked with a bar overhead labeled with the repeat number. The C-terminal portion of Laz (amino acid residues 57–183) is homologous to bacterial copper-binding proteins called azurin. Both proteins are lipid modified at the cystine residue after cleavage of the N-terminal 17-aa signal sequence. Conserved amino acids of the Lip/H.8 domain within both proteins are shown.

B, Bacterial lysates of strain MC58 and its isogenic Lip/H.8 and Laz single KO mutants (L to R) were electrophoresed on a 12% Bis-Tris gel followed by blotting and incubation with mouse anti-Lip/H.8 mAb, 2C3. Bound 2C3 was disclosed using alkaline phosphatase-conjugated goat anti-mouse IgG. Bands specific for Lip/H.8 (Lip) and Laz are indicated.

C, Loss of either Lip/H.8 or Laz decreases blocking by NK3. Wild-type MC58 (black columns) and its Lip/H.8 (light gray columns) and Laz mutants (dark gray columns) were incubated with 10% (v/v) killing serum K1 and 20% (v/v) heat-inactivated blocking serum NK3.

D, Deleting either Lip/H.8 or Laz from
deleting Lip/H.8 or Laz diminishes blocking. We first created Lip/H.8 and Laz KO strains in the background of group B strain MC58 (Fig. 6B) and then examined the ability of heat-inactivated NK3 to block killing of the mutant strains by K1. Loss of either Lip or Laz (Fig. 6C) enabled killing by serum K1 even in the presence of heat-inactivated blocking NK3. Thus, expression of both lipoproteins was necessary to serve as targets for blocking Ab.

We next examined whether loss of Lip/H.8 or Laz expression would enhance bactericidal activity of anti-fHbp mAb JAR 3 in the presence of blocking NK3. Akin to observations with strain H44/76 (Fig. 6C), wild-type strain MC58 survived when bacteria were incubated with 2 g/ml of NK3 (complement active). A His-tagged recombinant protein containing N-terminal "imperfect" AAEAP repeats, were added either individually or in an equimolar mixture to bactericidal reaction mixtures that contained NK3 (20%) and JAR 3 (0.25 g/ml). Increasing the concentration of the two peptides in the reactions, either individually or in an equimolar mixture, resulted in increased bacterial killing (Fig. 6F). The peptide corresponding to the N terminus of Lip/H.8 restored killing slightly more than the N-terminal peptide. Control reactions included JAR 3 plus IgG-depleted NK3, with or without the Lip/H.8 peptides.

We also tested the ability of rLaz (not lipidated, but containing the N-terminal "imperfect" AAEAP repeats) to inhibit blocking using the assay described earlier. Soluble rLaz also decreased killing by JAR 3 in a dose-dependent manner (Fig. 6G). A His-tagged recombinant protein that contained only the azurin-like domain, but lacked the N-terminal AAEAP repeats, did not restore killing by JAR 3 (Fig. 6H), thereby localizing blocking specificity to the N-terminal domain of Laz that contained the pentapeptide repeats. The addition of recombinant proteins to reaction mixtures that contained killing JAR 3 and NK3 depleted of blocking IgG (used as a complement source) had no effect on bacterial killing (Fig. 6F–H, white bars). Collectively, these data provide strong evidence that the N-terminal pentapeptide motifs of Lip/H.8 and Laz contribute to bacterial survival by serving as targets for blocking IgG Abs in human serum.

Blocking requires the Fc domain of IgG

Prior studies have shown that F(ab')2 fragments of blocking human IgG directed against Rmp can block killing of N. gonorrhoeae (14, 40). Consequently, we first examined whether the F(ab')2 fragments of human anti-Lip/H.8 possessed blocking activity. F(ab')2 fragments of murine anti-Lip/H.8 mAb 2C3 inhibit blocking activity in human serum. mAb 2C3 binds to both Lip/H.8 and Laz. Unlike human IgG directed against Lip/H.8, we did not observe any blocking activity mediated by mAb 2C3, the anti-Lip/H.8 mAb (data not shown). 2C3 itself does not possess bactericidal activity against N. meningitidis because the addition of 2C3 to IgG-depleted NK3 did not decrease survival of H44/76 (data not shown).

We tested mAb 2C3 for its ability to inhibit binding of human blocking IgG that would result in restoration of killing. The ability of 2C3 to restore killing would provide additional evidence that specificity of human blocking IgG was directed against Lip/H.8 on N. meningitidis. The F(ab')2 fragment of mAb 2C3 was used to avoid confounding by its Fc region. As an example, increased killing on adding intact 2C3 to a reaction mixture containing blocking IgG and the (otherwise) bactericidal Ab could result from increased complement activation mediated by synergy between 2C3 and the bactericidal Ab. As shown in Fig. 6E, the F(ab')2 fragments of mAb 2C3 restored killing by JAR 3. In addition to providing further proof that Lip/H.8 and Laz are targets for blocking IgG in human serum, these data also suggest that blocking function is unique to human IgG because murine mAb 2C3 and human blocking IgG, although both recognizing similar or overlapping epitopes, differ in their ability to block bactericidal activity.

Killing of meningococci is restored by diverting blocking Ab from the bacterial surface with Lip/H.8 or Laz peptide fragments. We tested the ability of synthetic peptides that corresponded to the N and C termini of Lip/H.8 to inhibit or divert the binding, and therefore inhibit the function of human blocking IgG. Increasing concentrations of the two 30-mer synthetic peptides, containing the AAEAP repeats, were added either individually or in an equimolar mixture to bactericidal reaction mixtures that contained NK3 (20%) and JAR 3 (0.25 g/ml). Increasing the concentration of the two peptides in the reactions, either individually or in an equimolar mixture, resulted in increased bacterial killing (Fig. 6F).
fragments derived from NK3 IgG did not block JAR 3-mediated killing of H44/76. As expected, NK3 IgG mediated blocking in simultaneously performed control experiments (Fig. 7A). Therefore, the Fc region is necessary for the blocking function of human anti-Lip/H.8 IgG.

The N-linked oligosaccharides of the blocking serum IgG Fc region are required for blocking activity

Ig isotype (or subclass) plays an important role in dictating which of the four IgG subclasses will activate complement. The hierarchy of complement activation by human IgG subclasses is: IgG3 > IgG1 > IgG2 >> IgG4 (41). The different IgG subclasses vary in their ability to engage C1q via their respective Fc that results in complement activation. Given the new-found importance of Fc in mediating blocking (see earlier), we ascertained the subclass of human anti-Lip/H.8 IgG. Using Western blotting and subclass-specific disclosing reagents, we found that anti-Lip/H.8 IgG in NK3 and NK4 were of the IgG3 subclass (data not shown). Thus, blocking Ab did not represent a complement nonactivating subclass (such as IgG4); rather, it belonged to complement activating subclass (IgG3).

The N-linked glycan of the Fc region of IgG modulates several of its effector functions (42, 43). IgG purified from blocking NK3 serum was first deglycosylated and after that binding to H44/76 was confirmed, to determine whether Fc-linked N-glycan was important for blocking function (Fig. 7B). Deglycosylated IgG was then assessed for its ability to block JAR 3-mediated killing of H44/76. Loss of the Fc glycan resulted in loss of blocking function, restoring killing of H44/76 by JAR 3 (Fig. 7C). Collectively, these data suggest that blocking by human IgG directed against meningococcal Lip/H.8 pentapeptide repeats requires the Fc N-linked glycans.

Blocking serum IgG functions by limiting classical pathway activation and C4b deposition

Early events after binding of a complement-fixing (or bactericidal) Ab to a bacterial surface includes engagement of the C1 complex, activation of C4, and deposition of C4b on the bacterial surface. Surface-bound C4b then participates in the formation of classical pathway C3 and C5 convertases, which results in membrane attack complex insertion and bacterial killing. We asked whether human blocking IgG interfered with classical pathway activation by bactericidal Ab. Strain H44/76 was incubated with the complement source (IgG-depleted NK3) and bactericidal mAb JAR 3, either alone or in the presence of intact NK3 IgG (blocking IgG) or PNGase-treated (deglycosylated) NK3 IgG. C4b deposition on bacteria was measured by flow cytometry. The presence of intact blocking IgG in the reaction mixture significantly decreased JAR 3-mediated deposition of C4b on wild-type serogroup B strain H44/76. In contrast, C4b deposition mediated by JAR 3 remained...
unaffected when deglycosylated NK3 IgG was present in the reaction mixture (Fig. 8B). Controls for this experiment included measurement of C4b deposition on strain H44/76 in reaction mixtures that contained organisms plus: 1) the complement source alone, 2) complement plus intact blocking IgG, or 3) complement plus PNGase-treated (deglycosylated) blocking IgG. Minimal (and similar) C4b deposition was exhibited on strain H44/76 from each of the three control reactions (for simplicity, only the FACS plots depicting C4b deposition in the presence of complement alone is shown by the gray shaded histogram in Fig. 8B). The glycosylation state of NK3 IgG did not affect the amount of JAR 3 bound to bacteria (Fig. 8A). As shown earlier in Fig. 7B, similar levels of intact and deglycosylated NK3 IgG bound to H44/76.

In accordance with the observation that NK3 IgG did not block the bactericidal activity of anti-PorA mAb 1.7, NK3 IgG (whole and deglycosylated) also did not diminish C4b deposition mediated by mAb P1.7 (Fig. 8D). Akin to the earlier observations with JAR 3, NK3 IgG (whole and deglycosylated) also did not affect binding of mAb P1.7 to H44/76 (Fig. 8C).

**Discussion**

SBA is a widely accepted criterion for measurement of natural or vaccine-induced protective immunity against *N. meningitidis* (11, 44). There are substantial variations in SBA titers against meningococci among individuals. Because nasopharyngeal colonization with *N. meningitidis* is common (45), documented in the winter months in the United States and Europe and in the dry season in Africa, when epidemic potential is highest, it is likely that most individuals who have been exposed to this bacterium mount an Ab response against the colonizing strain. There is heterogeneity in the specificity of the Ab response that is elicited after colonization (35). Whether the repertoire of antimeningococcal Abs present in an individual can effect SBA against a given strain depends on several factors, including their epitope specificity, subclass, and titer.

In this study, we have shown that Ab in the sera from ~10% of individuals tested decreased serum bactericidal Ab function. Using several approaches, we provide evidence that these Abs are directed against the repeating pentapeptide “AAEAP” sequences of Lip/H.8 and the N terminus of lipid modified azurin (Laz), which possesses “imperfect” repeats. Lip/H.8 has been found in all strains of pathogenic Neisseriae that have been examined so far (the lip gene has been misannotated in published meningococcal genomes), suggesting an important role for this molecule in pathogenesis. Our data suggest that expression of this protein may serve to elicit a separate Ab response that attenuates bactericidal Ab activity, thereby providing a survival advantage to meningococci. An important role for Lip/H.8 repeat-containing proteins in the pathogenesis of invasive disease was suggested by Joseph et al. (46), who compared changes in transcriptional patterns between invasive group B meningococcal strain MC58 and a genotypically and phenotypically similar group B carriage isolate called α-522 when these bacteria were exposed to various conditions, including saliva, whole blood, or cerebrospinal fluid. When incubated in blood, MC58 upregulated *lip* and *laz* ~5- and 10-fold more than α-522 under the corresponding condition (C. Schoen, unpublished results). Differential upregulation of these genes in MC58 was not seen when bacteria were incubated with saliva or cerebrospinal fluid. These data suggest that expression of Lip/H.8 and Laz may confer an advantage to invasive strains when they encounter high levels of complement, as occurs in the bloodstream.

Azurin is involved in defense against oxidative stress and copper toxicity, and *Neisseria* mutants that lack lipid modified azurin (Laz) are hypersensitive to hydrogen peroxide and copper (47). Azurin is also expressed by other bacteria such as *Pseudomonas aeruginosa* (39). However, the presence of the N-terminal “Lip/H.8-like” motif appears to be a feature unique to pathogenic Neisseriae. The pentapeptide repeats in Laz also contribute to blocking Ab targets, and “replication” of Lip/H.8-like motifs in Laz could also serve to maximize the efficiency of blocking against Laz. Indeed, both Lip/H.8 and Laz expression are necessary to provide the maximal number of target sites for blocking Ab. It is noteworthy that a double mutant in MC58 that lacked both Lip/H.8 and Laz was more resistant to complement-dependent killing. Despite binding a similar amount of JAR 3 as the parent strain, the double mutant bound less C4 than wild-type bacteria, suggesting that loss of Lip and Laz resulted in decreased C4 activation by limiting C1 complex engagement or by blocking metastable activated C4b from accessing its targets. However, this may occur at the expense of the important functions of Laz as described earlier that might place the bacteria at a disadvantage in vivo.

Blocking Abs in normal human serum prevented killing by an anti-fHbp mAb but did not prevent killing by an anti-PorA mAb. As an example of supremacy of killing over blocking in a purely human system, serum specimen K3 contained IgG, anti-PorA, and anti-H8 (Fig. 5), and maintained killing even when additional Lip/H.8-specific human Ab (heat-inactivated NK3) was added (Fig. 2A). Abs directed against PorA, one of the most abundant proteins...
in the meningococcal outer membrane, are often present in normal sera that mediate SBA (35), and this Ag also elicits Abs mediating a significant proportion of SBA in humans vaccinated with outer membrane protein vaccines (48). fHbp is a relatively sparsely distributed outer membrane lipoprotein that is currently being investigated as a candidate for a group B meningococcal vaccine (31, 33). Our findings may have important implications for vaccine development because bactericidal activity of Abs directed against less abundant Ags may be blunted in persons with higher titers of anti-Lip/H.8 Abs. Native outer membrane vesicles that are used as vaccines (49–55) or as carriers for protein vaccine Ags contain Lip/H.8 and/or Laz, and may therefore elicit blocking Abs and attenuate bactericidal responses.

Blocking Abs directed against the two pathogenic Neisserial species vary in their target specificity. Abs against gonococcal Rmp mediate blocking against N. gonorrhoeae. Rmp is an outer membrane structural protein whose function has not been fully elucidated (56). A mAb against the class 4 protein of N. meningitidis (the Rmp counterpart in N. meningitidis) blocked the killing of meningococci by otherwise bactericidal Ab or serum in one study (57), but the same Ab showed little or no blocking activity against N. meningitidis in a separate study (58). Anti-Rmp blocking Abs activate complement and deposit C3 and C9 on the surface of N. gonorrhoeae (40). Although not formally proved to occur as a result of blocking Abs per se, serum resistance of N. gonorrhoeae is associated with larger polymers or aggregates of C5b-9 (59) or as C5b-9 complexed to distinct bacterial outer membrane constituents (60). It has been hypothesized that anti-Rmp blocking Abs divert C3 deposition from bactericidal sites on the bacteria to nonbactericidal sites (40). In contrast with the ability of anti-Rmp blocking Ab against N. gonorrhoeae to activate complement, our data show that human anti-Lip/H.8 Ab limits C4b deposition on meningococci by an otherwise bactericidal mAb. These results highlight important differences in the mechanism of blocking IgG in human serum directed against N. meningitidis and N. gonorrhoeae.

The phenomenon of blocking of bacterial killing was described as early as 1894 by Pfeiffer (61), who noted that animals given excess immune serum may be more susceptible to challenge organism. The first written account of blocking activity against Gram-negative microorganisms including Neisseriae was given by Neisser and Wechsberg in 1901 (62). Thomas and colleagues (45) showed that sera from patients convalescing from meningococcal disease were sometimes less bactericidal than acute (nonimmune) serum. They also observed that antisera from rabbits immunized with larger doses of meningococci were less bactericidal than animals immunized with a lower dose (45). Diluting the antisera obtained from heavily immunized rabbits restored bactericidal activity [a prozone effect also known as the Neisser–Wechsberg phenomenon (62)]. When these sera were heat inactivated and mixed with normal rabbit serum, killing of meningococci was abolished. Furthermore, rabbits passively immunized with blocking serum showed impaired clearance of meningococci (45). Blocking IgAl has also been identified in sera of patients after group C meningococcal disease and after serogroup C capsular vaccines (12, 13). A blocking IgG directed against the capsule of serogroup W-135 N. meningitidis was also demonstrated in the serum of a healthy C2-deficient individual after vaccination with a tetravalent polysaccharide meningococcal vaccine (63). Blocking Ab that reduces bacterial killing has been shown after Brucella abortus (64), B. melitensis (65), and Pseudomonas aeruginosa (66, 67) infections. Anti-LPS Abs have been shown to block complement-mediated killing of nontyphoidal Salmonella in convalescent sera of HIV-positive patients in Africa (68). To our knowledge, these data are the first to identify blocking anti–meningococcal Abs in otherwise healthy persons without preceding meningococcal disease or preceding meningococcal vaccination.

Classical pathway activation involves C1 complex engagement by proximate Fc regions, followed by C1 activation, C4 activation, and subsequently C4b deposition. The precise step of classical pathway activation that is blocked by human anti-Lip/H.8 IgG that results in decreased C4b deposition on bacteria is not clearly understood. An important finding in this study is that the Fc-linked oligosaccharides is required for downregulating the classical pathway and the resulting blocking activity. The Fc-linked N-glycan is necessary for a number of Ab effector functions, including complement activation and Ab-directed cell cytotoxicity (42, 43, 69–71). There is considerable heterogeneity in the structure of N-linked oligosaccharides of human IgG, and its biological effector functions are influenced by the composition of the terminal sugar residues and/or the presence of the core fucose residue (72). Kaneko et al. (73) have shown that terminal sialylation of IgG glycans inhibits inflammatory response mediated by human i.v. Ig preparations. IgG with Fc glycans that terminate in N-acetylglucosamine or high mannose residues do not activate C1 efficiently (43, 70, 74). However, terminal N-acetylglucosamine residues or IgG bearing high mannose residues may bind mannana-binding lectin, which in some instances could contribute to complement activation (74). Characterization of N-linked glycans of the Fc of human anti-Lip/H.8 blocking IgG would shed light on possible mechanisms of complement regulation, and although beyond the scope of this study, merits further investigation.

In conclusion, we have identified naturally occurring human blocking Abs that are directed against the Lip/H.8-pentapeptide repeat motifs of the Lip and Laz lipoproteins of N. meningitidis. These results suggest an important role for these peptide repeats in meningococcal pathogenesis and may explain why these repeats occur in two distinct proteins that are ubiquitously expressed in pathogenic Neisseriae. Ab-mediated, complement-dependent killing is critical for host defense against Neisseriae. We have characterized an important strategy used by this bacterium to subvert a key immune defense that may have important implications for vaccine development.

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Disclosures
All the authors have filed a patent application in the area of group B meningococcal vaccines.

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