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Anti-Idiotypic Antibody as a Potential Candidate Vaccine for *Neisseria meningitidis* Serogroup B

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Sepsis and meningitis caused by *Neisseria meningitidis* serogroup B (NMGB) are serious diseases in infants and young adults, but no effective vaccine is available. The capsular polysaccharide (PS) of NMGB has poor immunogenicity and a structural similarity to polysialic acid (PSA) on neuronal tissue that may elicit autoantibodies. Using HmenB3, a protective and nonautoreactive monoclonal antibody (MAb) to NMGB capsular PS, we produced an anti-idiotypic MAb, Naid60, which mimics the capsular PS of NMGB. We produced an anti-anti-idiotypic MAb, MoB34, by using the immunogenic site on Naid60 responsible for inducing the anti-NMGB PS antibody response. MoB34 elicited the complement-mediated killing of representative strains of serogroup B meningococci. MoB34 did not bind to CHP-134, a neuroblastoma cell line expressing α(2-8) PSA, or to mouse brain cryosections at a high concentration. Naid60-keyhole limpet hemocyanin immunization inhibited the growth of live NMGB in intraperitoneally challenged mice; in contrast, three of five control mice developed bacteremia. Thus, Naid60 has an immunogenic site that elicits antibodies with bactericidal activity against NMGB and no autoimmunity to PSA. We suggest that the immunogenic region of Naid60 is a candidate for the development of a new vaccine against NMGB.

Neisseria meningitidis is a gram-negative encapsulated bacterium and is the most common cause of bacterial meningitis. *N. meningitidis* can be divided into 13 serogroups on the basis of the structures of their capsular polysaccharides (PSs), which are chemically and immunologically distinct in each group (11, 21, 41). Moreover, the five serogroups A, B, C, Y, and W135 account for the major meningococcal disease-causing isolates in humans. The capsular PSs of *N. meningitidis* are important determinants of virulence, and the presence of serum antibody to capsular PS protects against disease. A meningococcal vaccine that uses capsular PSs from *N. meningitidis* serogroups A, C, Y, and W135 is currently licensed; however, no meningococcal vaccines are available to protect against meningococcal diseases caused by *N. meningitidis* serogroup B (NMGB). The lack of protection against NMGB in a meningococcal vaccine is a serious problem because NMGB may account for about 50% of all meningococcal meningitis infections in Europe and North America (32, 37, 40).

To produce an effective vaccine against NMGB, various approaches have been studied by targeting new bacterial proteins (9, 17, 29), after whole-genome sequencing of NMGB (45), and lipopolysaccharide (LPS) (36) in addition to outer membrane vesicle (4, 43) or N-propionylated PS (5). However, the use of bacterial proteins is problematic in a vaccine because of significant serologic heterogeneity among different strains of *N. meningitidis* serogroup B (1). With respect to the capsular PS candidate, NMGB PS is an autoantigen that may elicit autoantibodies that bind both NMGB and neuronal tissue (13, 14, 31), because NMGB PS expresses a linear α(2-8) polymer of sialic acid; thus, NMGB PS has poor immunogenicity due to immune tolerance. The structural modification of capsular PS by the substitution of *N*-propionyl for *N*-acetyl groups proved to be highly immunogenic (15) but showed the possibility of eliciting autoantibodies (2, 16, 19). In a fine-structure analysis of NMGB PS using MAbs, an immunogenic epitope was found which elicits antibodies that activate complement-mediated bacteriolysis and which has no autoantibody activity (16, 42). Thus, this epitope may be a target for molecular mimetics, such as peptide mimicry, for eliciting protective antibody without inducing autoimmunity.

Peptide mimicry approaches have been demonstrated with phage display technology for the PS of group B streptococcus type III (35), *Cryptococcus neoformans* (46, 50), and serogroups A and B of *N. meningitidis* (7, 8, 18, 42). Another approach to peptide mimicry is offered by anti-idiotypic antibody, and the feasibility of this approach has been demonstrated by an evaluation of peptide mimics of group B streptococcal infection (26) and serogroup C (48) and recently serogroup B (3) *N. meningitidis*, using a recombinant anti-idiotypic single-chain variable fragment (scFv) methodology. Previously we reported a MAb to NMGB PS, HmenB3, which has complement-mediated bactericidal activity against NMGB but no autoimmunity (42).

We now report on a monoclonal anti-idiotypic antibody to HmenB3, Naid60, which has an immunogenic and protective epitope that elicits antibodies to kill NMGB. We demonstrated that Naid60 has no cross-reactive epitope with neuronal cell

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adhesion molecules (NCAMs) by using both Naid60 immune sera and anti-anti-idiotypic MAb MoB34. Our experiments show that the anti-idiotype approach offers a means of developing a vaccine for NMGB.

MATERIALS AND METHODS

Bacterial strains and polysaccharide. The NMGB strains used were ATCC 13090 (M2092) and H44/76 (B:15:P1.7,16). Bacteria were cultured on chocolate agar plates at 37°C in a candle jar and harvested after 6 h. All bacteria were aliquoted in Hanks' balanced salt solution (HBSS) containing 20% glycerol at −70°C. Purified capsular PS of *Escherichia coli* K1 containing α(2-8)-linked polysialic acid (PSA), which is antigenically and structurally identical to the capsular PS of NMGB (22), was a gift from W. Vann (Center for Biologics Evaluation and Research, Food and Drug Administration).

Preparation of F(ab′)$_2$. HmenB3 {immunoglobulin M(κ) [IgM(κ)]} is a mouse MAb specific for NMGB PS that kills 50% of NMGB at a concentration of 1 ng/ml in the presence of rabbit complement and shows no binding to CHP-134, a human neuroblastoma cell line expressing PSA, or to mouse brain cryosections at a concentration of 25 μg/ml (42). HmenB3 was purified from the ascitic fluid of mice by Sephacryl S300 HR size-exclusion column chromatography (Amersham Pharmacia Biotech, Piscataway, New Jersey), and F(ab′)$_{2\mu}$ of HmenB3 was prepared by low-temperature pepsin proteolysis (34). Briefly, HmenB3 was dialyzed in sodium acetate buffer (0.02 M sodium acetate, 0.15 M NaCl, pH 4.0) and digested with pepsin (Sigma Chemical Co., St. Louis, MO) at an enzyme/antibody ratio of 1:1 (wt/wt) for 24 h at 4°C. The same amount of pepsin was then added to continue the reaction for another 24 h. The digestion mixture was slowly titrated back to neutrality with 2 M Tris solution. The mixture was then dialyzed against phosphate-buffered saline (PBS), and a small amount of denatured and/or aggregated material was removed by centrifugation. F(ab′)$_{2\mu}$ was purified by Sephacryl S300 HR gel filtration and confirmed by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 7.5% gels. F(ab′)$_{2\mu}$ fragments were coupled with keyhole limpet hemocyanin (KLH) or bovine serum albumin (28) for use as an immunogen for the production of anti-idiotype MAb. Briefly, the same amounts of F(ab′)$_{2\mu}$ fragments and carrier protein were mixed, an equal volume of 0.2% glutaraldehyde in PBS was added to the mixture with constant agitation, and the mixture was then incubated at room temperature (RT) for 1 h. To stop the reaction, 1 M glycine was added to a final concentration of 200 mM and incubated with stirring for 1 h. Finally the reaction mixture was dialyzed with PBS and used for immunization.

Naid60 [IgG1(κ)] is an anti-idiotype MAb to HmenB3, as described below. F(ab′)$_2$ of Naid60 was prepared by pepsin proteolysis as described above. Naid60 was digested with pepsin at an enzyme/antibody ratio of 1:20 for 8 h at 37°C, and the reaction was stopped by adding 2 M Tris solution. F(ab′)$_2$ fragments were purified using a Superdex-75 gel filtration column (Amersham Pharmacia Biotech). F(ab′)$_2$ fragments of Naid60 were coupled with carrier protein as described above.

Production of anti-idiotypic MAb (Ab2) and anti-anti-idiotypic MAb (Ab3). BALB/c mice were immunized intraperitoneally (i.p.) with 100 μg of KLH-conjugated F(ab′)$_{2\mu}$ fragments of HmenB3 emulsified in complete Freund's adjuvant (CFA) and boosted twice with incomplete Freund's adjuvant (IFA) at 2-week intervals. Finally, mice were injected intravenously with intact HmenB3 3 days before the fusion. Splenocytes were fused with P3-X63-Ag8.653 and selected with hypoxanthine-aminopterin-thymidine medium. The resulting hybridoma supernatants were screened by enzyme-linked immunosorbent assay (ELISA), as described below, using ELISA plates coated with F(ab′)$_{2\mu}$ fragments of HmenB3. The binding specificity of the anti-idiotypic MAb to HmenB3 was tested by an inhibition ELISA with NMGB as a coating antigen. An anti-idiotypic MAb was produced and named Naid60. The isotype of MAb was determined using a mouse-hybridoma subtyping kit (Roche Diagnostics GmbH, Mannheim, Germany) containing horseradish peroxidase (HRP)-conjugated goat anti-mouse isotype-specific antibodies.

To generate an anti-anti-idiotypic MAb, KLH-conjugated F(ab′)$_2$ fragment of Naid60 was used as an immunogen. The hybridoma supernatants producing Abs were screened on plates coated with killed NMGB, and a hybridoma producing an anti-anti-idiotypic MAb, named MoB34 [IgM(κ)], was produced. The bactericidal activity of MoB34 was tested, and a bactericidal inhibition assay was conducted to determine binding specificity, as described below.

ELISA and inhibition ELISA. Microtiter plates (Corning Inc., Corning, New York) were coated with 10 μg/ml of F(ab′)$_{2\mu}$ of HmenB3 for anti-idiotypic MAb screening. The plates were then washed with 0.05% Tween 20 in PBS (PBST) and blocked with 5% normal goat serum in PBST. Hybridoma culture supernatants were added to the plates and incubated for 1.5 h at 37°C. After washing, HRP-labeled goat anti-mouse IgG (Sigma) was added. After 1.5 h of incubation, *o*-phenylenediamine was added to the plates and optical densities were measured at 490 nm.

For anti-anti-idiotypic MAb screening, microtiter plates were coated with 10 μg/ml of *E. coli* K1 PS or heat-inactivated NMGB bacteria. For NMGB coating, NMGB (ATCC 13090) was killed at 65°C for 1 h and washed with PBS. One hundred microliters of PBS containing NMGB (OD at 620 nm, 0.09) was added to each well, and then the wells were dried at 37°C (18). HRP-labeled goat anti-mouse Ig was used as a secondary antibody for the screening.

Inhibition ELISA was performed to confirm whether the anti-idiotope of Naid60 has a structure similar to that of the NMGB PS epitope bound by HmenB3. On the microtiter plates coated with NMGB, a constant amount of HmenB3 was added to the wells in the presence of various concentrations of purified Naid60. After incubation for 3 h at RT with gentle agitation, the plates were washed and HRP-conjugated goat anti-mouse IgM (Sigma) was added to the wells. After washing, color was developed and OD measured.

Surface plasmon resonance (BIAcore). Analysis of Naid60 binding to HmenB3 was also carried out using the BIAcore 3000 instrument (BIAcore AB, Uppsala, Sweden). The running buffer used for sample dilution and analysis was HBS buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% Tween 20, pH 7.4). The research grade CM5 dextran sensor chip was activated with equal amounts of 0.2 M *N*-ethyl-*N*′-(3-diethylamino-propyl)-carbodiimide and 0.05 M *N*-hydroxysuccinimide. HmenB3 at a concentration of 50 μg/ml was immobilized in 10 mM sodium acetate buffer (pH 4.0) followed by 1 M ethanolamine hydrochloride (pH 8.0) to deactivate excess *N*-hydroxysuccinimide esters. This coupling resulted in 12,000 response units of immobilized antibodies per flow cell, corresponding to approximately 10 ng protein/mm^2. For the evaluation of binding, Naid60 was diluted in HBS buffer and analyzed at various concentrations. Naid60 solution was passed over the sensor chip at a flow rate of 10 μl/min. HmenB5 (18), which is another anti-NMGB PS MAb cross-reactive with NCAMs, was immobilized on the adjacent vacant flow cell under the same conditions as a control antibody. An activated and blocked flow cell without immobilized ligand was used to evaluate nonspecific binding. For all samples, response curves were also recorded on control surfaces. Results were calculated after subtraction of the control values, using the BIAevaluation 3.0 software (BIAcore AB).

Complement-mediated bactericidal assay. The bactericidal assay was performed in 96-well microtiter plates (42). Briefly, a 100-μl mixture consisting of 30 μl of *N. meningitidis* bacterial suspension (containing 2,500 CFU), 50 μl of diluted antibiotic-free Ab, and 20 μl of baby rabbit complement was incubated at 37°C for 1 h. The concentration of rabbit serum was 2% for H44/76 and 10% for ATCC 13090, depending on the susceptibility of the bacteria to the complement, and an isotype-matched irrelevant MAb was used as a negative control. After incubation, 10 μl of the reaction mixture was cultured on a chocolate agar plate to determine colony numbers. Two different strains of NMGB (ATCC 13090 and H44/76) were used to avoid killing bacteria by binding bacterial surface antigens other than capsular PS. Each assay was performed at least twice in triplicate, and means were used to calculate percent killing with the formula [(CFU without antibody − CFU of sample)/CFU without antibody] × 100. For bactericidal inhibition assay, a 100-μl mixture consisting of 30 μl of *N. meningitidis* bacterial suspension, 25 μl of diluted antibiotic-free Ab, 25 μl of various concentrations of inhibitor, and 20 μl of baby rabbit complement was incubated at 37°C for 1 h. Each assay was performed as described above.

Production of anti-idiotypic scFv antibody (Naid60-scFv). The heavy- and light-chain variable regions from the Naid60 hybridoma were cloned into pCANTAB5E vector (Amersham Pharmacia Biotech). The ligation mixture was transformed into *E. coli* TG1 cells, and the phages were rescued with helper phage M13KO7. The phage stock was prepared and phage selection was done by one round of panning on plates coated with HmenB3. Recombinant phages giving a positive ELISA signal on HmenB3-coated wells were used to produce soluble scFv antibodies. For the production of soluble scFv antibodies, the scFv gene was excised from the phage clone and inserted into the pRSET SfiI/NotI expression vector (49). Affinity-purified Naid-scFv antibodies were obtained by coexpressing the ligation mixture and pGroES/L plasmid as chaperonin (12) in *E. coli* BL21(DE3).

Flow cytometric analysis. To test whether MoB34 binds to the PSA portion of NCAMs, CHP-134 cells (24) were stained with MoB34 for flow cytometry. CHP-134 cells were mixed with MoB34 at a high concentration of 100 μg/ml and incubated for 30 min on ice. After washing, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse Ig (BD Immunocytometry Systems, San Jose, CA) for 1 h at RT. After another washing, the cells were resuspended in 400 μl of PBS containing 0.25% formaldehyde. Anti-CD56

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(PharMingen, San Diego, CA), which is specific for NCAMs, and isotype-matched irrelevant mouse MAb were used as positive and negative controls, respectively. HmenB1, which is a mouse MAb to NMGB PS and which is known to bind to CHP-134 cells (42), was also used at a concentration of 10 μg/ml as a positive control. Fluorescence was measured with a FACScaliber (Becton Dickinson, Mountain View, CA).

Histochemistry. The staining was performed with brains obtained from 1- to 2-week-old C57BL/6 mice. Five-micrometer-thick cryosections of brain were incubated with MoB34 at concentrations of 50 and 100 μg/ml for 75 min at RT, washed with PBS, and incubated with biotin-conjugated goat anti-mouse IgM antibody (Dako, Glostrup, Denmark) for 30 min. The slides were rewashed with PBS and incubated with streptavidin-peroxidase (Dako) for 30 min. After this washing, the slide was developed with a peroxidase substrate, 3-amino-9-ethylcarbazole (Dako), and counterstained with hematoxylin-eosin stain. HmenB1 was used as a positive control (42) at a concentration of 0.1 or 1 μg/ml, and isotype-matched irrelevant mouse MAb of anti-LPS Ab (Sigma) was used at a concentration of 50 and 100 μg/ml as a negative control.

Production of anti-NMGB antibody after immunization of mice with Naid60. To test the generation of anti-anti-idiotypic MAb reactive to NMGB, we injected BALB/c mice with Naid60-KLH. Female BALB/c mice 6 to 8 weeks old were i.p. injected with 100 μg of Naid60-KLH. The first injection was with CFA, and boosting was with IFA at 2-week intervals. Serum samples were collected 7 days after each injection. Serum Ab titers to NMGB were measured on NMGB-coated microtiter plates. Serum samples were 1:100 diluted with 5% normal goat serum–PBST and incubated for 1.5 h at 37°C. After washing, HRP-labeled goat anti-mouse Ig (Sigma) and *o*-phenylenediamine were used for color development.

Challenge experiments with live bacteria. BALB/c mice were subcutaneously immunized with 100 μg of Naid60-KLH with alum (Pierce, Rockford, IL) and 10 μg of CpG oligodeonucleotides and boosted 4 more times at 2-week intervals. We used alum to avoid adverse effects such as possible granulomas and abscesses cause by Freund's adjuvant. KLH-injected mice were used as a control. All mice were rendered leukopenic by three i.p. injections of a dose of 200 mg/kg cyclophosphamide on days 0, 2, and 4 to enhance their susceptibility to meningococcal infection (10, 39). Finally, a dose of 100 mg/kg cyclophosphamide was given 2 h before the bacterial challenge. Mice were then i.p. injected with 1×10^6 CFU NMGB ATCC 13090, which had been passaged three times in neutropenic mice for virulence (47). Blood specimens were obtained from the retro-orbital plexus, and 10 μl of each blood sample undiluted or diluted with HBSS at 1:10 and 1:100 was cultured on chocolate agar plates at 37°C. CFU were determined in duplicate after 18 h of incubation in a candle jar.

Nucleotide sequence accession numbers. The sequences of the V regions of Naid60 have been deposited in GenBank under accession numbers AY661813 for V_H and AY661814 for V_L.

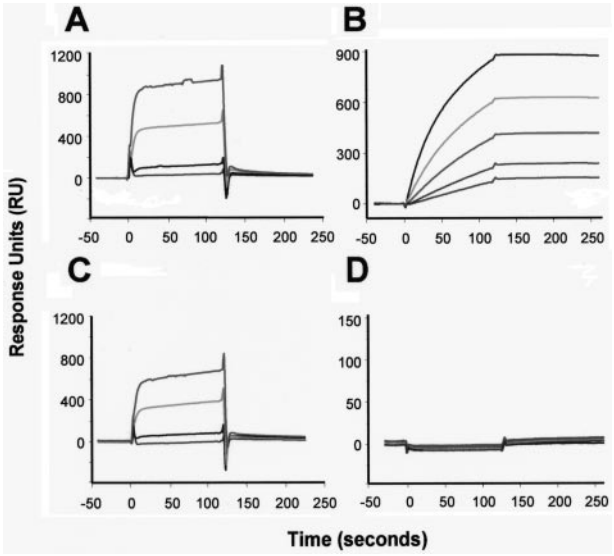

FIG. 2. Surface plasmon resonance analysis of Naid60 binding to immobilized HmenB3. The data represent five injections of Naid60 at concentrations of 50, 25, 12.5, 6.25, and 3.13 nM from top to bottom (B and D). HmenB5, which is an anti-NMGB PS MAb cross-reactive with NCAM, was used for the control antibody (D). NMGB PS was used as a positive control ligand to both HmenB3 and HmenB5, and the bindings were demonstrated at concentrations of 5, 4, 3, and 2 mg/ml of NMGB PS (A and C).

RESULTS

Characterization of the anti-idiotypic MAb of Naid60. From BALB/c mice immunized with KLH-F(ab′)$_{2\mu}$ of HmenB3 emulsified with Freund's adjuvant, we obtained one clone of a hybridoma, Naid60, that reacted with both whole Ab and F(ab′)$_{2\mu}$ of HmenB3 by ELISA (data not shown). To test whether Naid60 MAbs recognize the idiotope of HmenB3, we examined the inhibition of HmenB3 binding to wells coated with NMGB by adding various amounts of purified Naid60 as a competitor. As shown in Fig. 1, Naid60 inhibited the binding of HmenB3 to NMGB-coated microtiter plates in a dose-dependent manner, like native antigen, *E. coli* K1 PS, which is

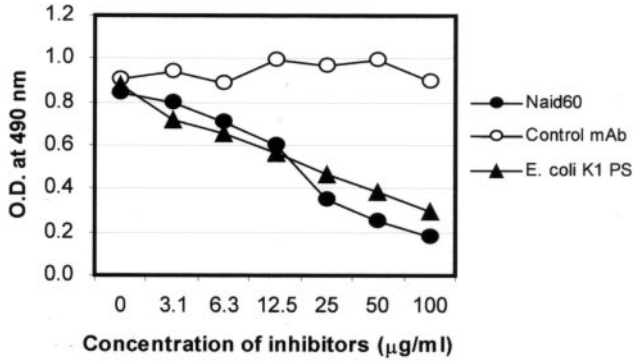

FIG. 1. Characterization of the anti-idiotype Ab of Naid60. Naid60 inhibited the binding of HmenB3 to wells coated with heat-killed NMGB in a dose-dependent manner, like *E. coli* K1 PS, which is structurally and antigenically identical to NMGB PS. Naid59, which bound to HmenB3 but did not inhibit the binding of HmenB3 to NMGB, was used as a negative control Ab.

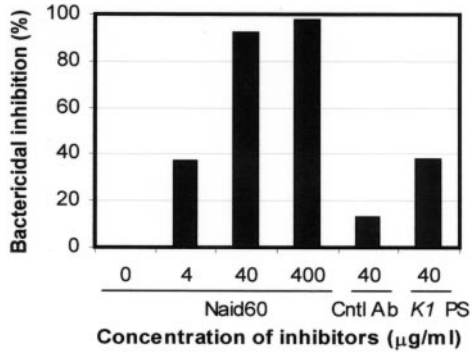

FIG. 3. Bactericidal inhibition of HmenB3 to NMGB by Naid60. NMGB bacteria were incubated with HmenB3 and various amounts of Naid60 in the presence of baby rabbit complement. Bactericidal inhibitions were 36.9%, 92.6%, and 98.0% at 4, 40, and 400 μg/ml of Naid60, respectively, and 38.3% at 40 μg/ml of *E. coli* K1 PS, a positive control inhibitor.

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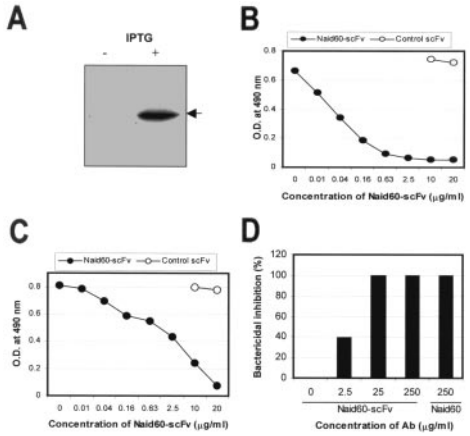

FIG. 4. Production and characterization of Naid60-scFv. (A) Recombinant scFv of Naid60 was produced as described in Materials and Methods. Affinity-purified Naid60-scFv was run on a 10% sodium dodecyl sulfate-polyacrylamide gel, and Western blotted using HmenB3 as a primary antibody after transfer to nitrocellulose, and identified as a ca. 30-kDa protein. (B and C) Naid60-scFv inhibited the binding of HmenB3 to wells coated with NMGB bacteria (B) and to wells coated with Naid60 (C) in a dose-dependent manner. (D) Naid60-scFv inhibited the complement-mediated bactericidal activity of HmenB3 to NMGB bacteria.

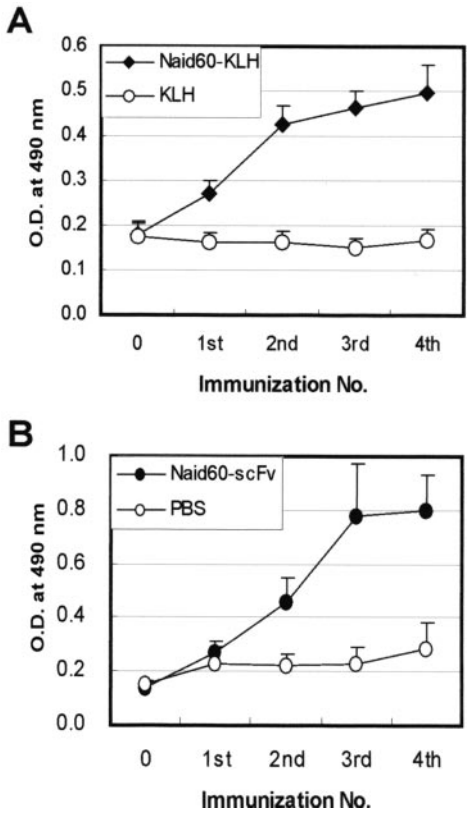

FIG. 5. Anti-NMGB Ab levels in Naid60-KLH- or Naid60-scFv-immunized mice. (A) Groups of five BALB/c mice were injected with Naid60-KLH, which was emulsified with CFA and IFA, and then boosted at 2-week intervals. After four injections, mouse sera were collected and the anti-NMGB Ab titer at a 1:100 dilution was measured using heat-killed NMGB as a coating antigen. (B) Groups of five BALB/c mice were injected with Naid60-scFv as described for panel A. Mouse sera were collected 7 days after each immunization, and the anti-NMGB Ab titer was measured at a 1:100 dilution. Error bars indicate standard deviations.

structurally and antigenically identical to NMGB PS (22). In contrast, an irrelevant isotype control antibody failed to inhibit the binding of HmenB3 to NMGB even at 100 μg/ml. We determined the V regions of Naid60.

Surface plasmon resonance analyses. We further established the ability of the BIAcore to confirm the binding specificity of Naid60 to HmenB3. As shown in Fig. 2, the sensograms represent real-time binding of Naid60 to HmenB3 at various concentrations of Naid60. Naid60 was bound to HmenB3 in a concentration-dependent manner (Fig. 2A), as for the binding of NMGB PS to HmenB3 (Fig. 2B), and the K_D was 9.2×10^{-9} M. However, Naid60 did not bind to HmenB5 (Fig. 2D), which is an isotype-matched anti-NMGB PS MAb cross-reactive with NCAMs (Fig. 2C). The binding pattern of *E. coli* K1 PS to HmenB3 was similar to those of NMGB PS (data not shown).

Naid60 inhibits the bactericidal activity of HmenB3. To determine whether Naid60 carries the mimic epitope of NMGB PS, a bactericidal inhibition assay was performed. HmenB3 has complement-mediated bactericidal activity against NMGB, and we measured the bactericidal activity of HmenB3 against NMGB ATCC 13090 as a representative in the presence of various concentrations of Naid60 as a competitor to NMGB PS and 10% rabbit complement. When Naid60 was added to concentrations of 4, 40, and 400 μg/ml, the bactericidal inhibitions of HmenB3 by Naid60 were increased to 36.9%, 92.6%, and 98.0%, respectively. Bactericidal inhibitions by *E. coli* K1 PS and irrelevant control Ab, positive and negative control inhibitors, were 38.3% and 12.8% at 40 μg/ml, respectively (Fig. 3). These results indicate that Naid60 contains the peptide mimicry site to NMGB PS and competes for binding to HmenB3.

Anti-idiotypic scFv. We developed a recombinant anti-idiotype Ab of a single-chain variable fragment of Naid60 (Naid60-scFv) for immunization to induce antibodies to NMGB. The V_H and V_L regions of Naid60 were cloned using pCANTAB5E vector, and the scFv gene was excised from the phage clone showing a positive ELISA signal to HmenB3-coated wells and then expressed in *E. coli* BL21 (data not shown). Recombinant Naid60-scFv was successfully produced with a molecular size of ~30 kDa (Fig. 4A), and Naid60-scFv dose-dependently inhibited the binding of HmenB3 to wells coated with NMGB (Fig. 4B) or with whole Naid60 Ab (Fig. 4C), as expected. Naid60-scFv also inhibited the killing of NMGB by HmenB3 in a dose-dependent manner (Fig. 4D). These results indicate that the anti-idiotypic Naid60-scFv also retains the surrogate image of the carbohydrate epitope for NMGB PS. To determine whether vaccination with Naid60 can induce anti-NMGB Ab titers and protect mice from NMGB infection, Naid60-scFv was used for immunization.

Anti-NMGB Ab production in mice. Next, we further evaluated whether the immunization of anti-idiotype Ab could elicit the production of antibodies against NMGB. First, we immunized BALB/c mice with Naid60-KLH, which was emulsified with CFA, boosted this with IFA three times at 2-week

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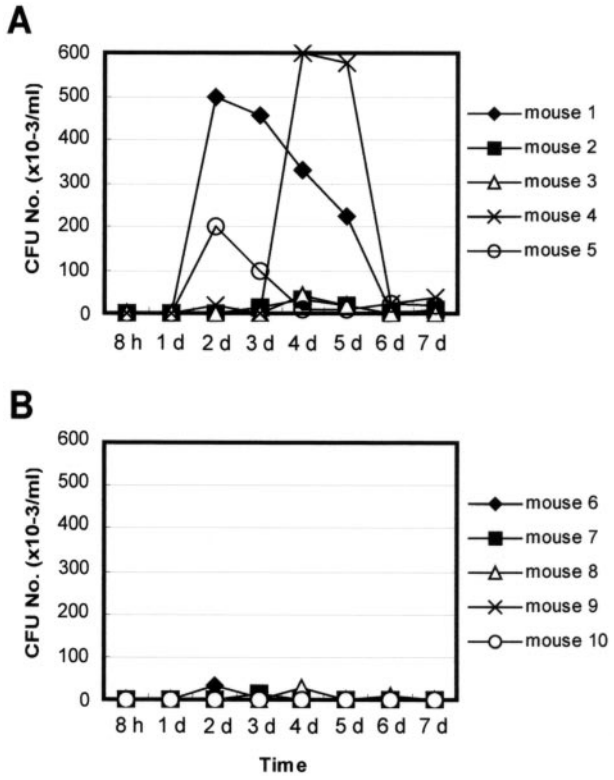

FIG. 6. Protective efficacy for Naid60-immune mice. Groups of five BALB/c mice were subcutaneously immunized with KLH (A) or 100 μg of Naid60-KLH (B) with alum and CpG ODN five times at 2-week intervals and treated with cyclophosphamide to render the mice susceptible to meningococcal infections. Subsequently the mice were challenged with 1×10^6 CFU of NMGB (ATCC 13090). To observe bacterial clearance, blood specimens were obtained from the retro-orbital plexus at the indicated times, and 10 μl of each blood sample undiluted or diluted with HBSS at 1:10 or 1:100 was cultured on chocolate agar plates at 37°C. CFU in duplicate were determined after 18 h of incubation in a candle jar.

intervals, and obtained serum samples 1 week after every injection. We then measured the anti-NMGB Ab titer according to boosting injection numbers. Anti-NMGB Ab titers were significantly elevated as the boosting injections were increased (Fig. 5). We also observed anti-NMGB Ab production after immunization with Naid60-scFv or Naid60 $F(ab')_2$-bovine serum albumin into BALB/c mice (data not shown). Anti-NMGB Ab titers were increased as injection numbers were increased and sustained a plateau level after the third injection. These data indicate that Naid60 contains an epitope that can induce an anti-NMGB antibody response in experimental mice.

Protective efficacy in Naid60-immune mice. To study the protective efficacy of Naid60, BALB/c mice were immunized five times with 100 μg of Naid60-KLH at 2-week intervals and then treated with cyclophosphamide to render them susceptible to meningococcal infections. They were subsequently challenged with 1×10^6 CFU of NMGB, and bacterial clearance was observed. In the KLH-treated control group, detectable bacteremia was noted in blood at 24 h postinfection, and rapid bacterial multiplication was observed at days 2 to 4 in three mice (no. 1, 4, and 5), with signs of weight loss and inactivity, and then bacterial multiplication declined (Fig. 6A). The other

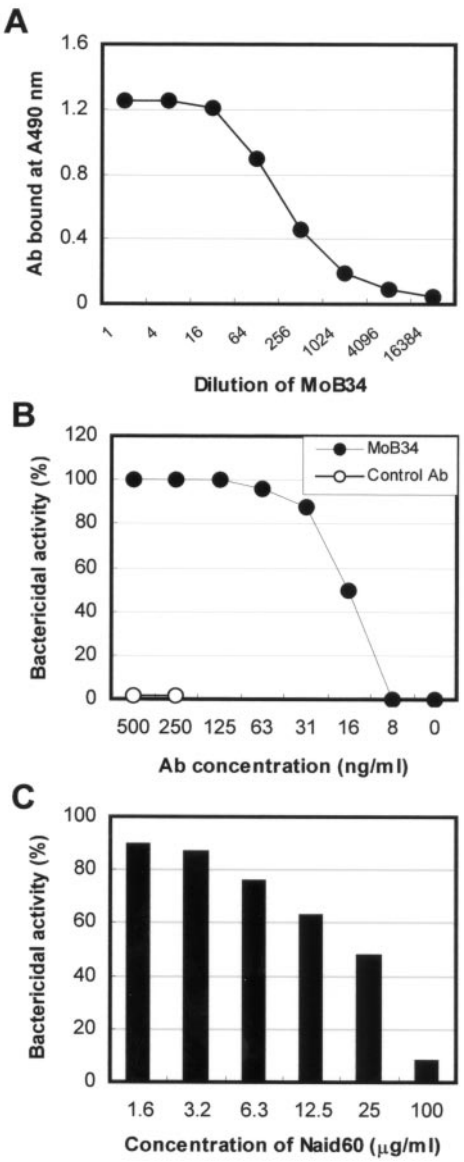

FIG. 7. Binding of MoB34 to whole bacterial cells and its bactericidal activity. (A) MoB34 purified from mouse ascites fluid bound to NMGB bacteria in dose-dependent manner. (B) The complement-mediated bactericidal activity of purified MoB34 was observed, and the 50% killing concentration was determined to be 16 ng/ml. (C) The bactericidal activity of MoB34 was reduced in the presence of Naid60.

two mice (no. 2 and 3) showed a little bacteremia on day 4 (Fig. 6A), whereas bacterial multiplication was not observed at 48 h postinfection in mice immunized with Naid60-KLH and bacteria persisted at low levels throughout the course of the experiment (Fig. 6B).

Anti-anti-idiotypic MAb. We produced a monoclonal anti-anti-idiotypic Ab to confirm the inducibility of protective Ab to NMGB by Naid60, because some antibodies were reported to bind to bacteria without promoting bactericidal activity (16, 42) and sera from mice immunized with Naid60 had poor bactericidal activity. BALB/c mice were immunized with KLH-conjugated $F(ab')_2$ of Naid60, and MAb MoB34 was produced by screening the hybridoma supernatants to both NMGB and

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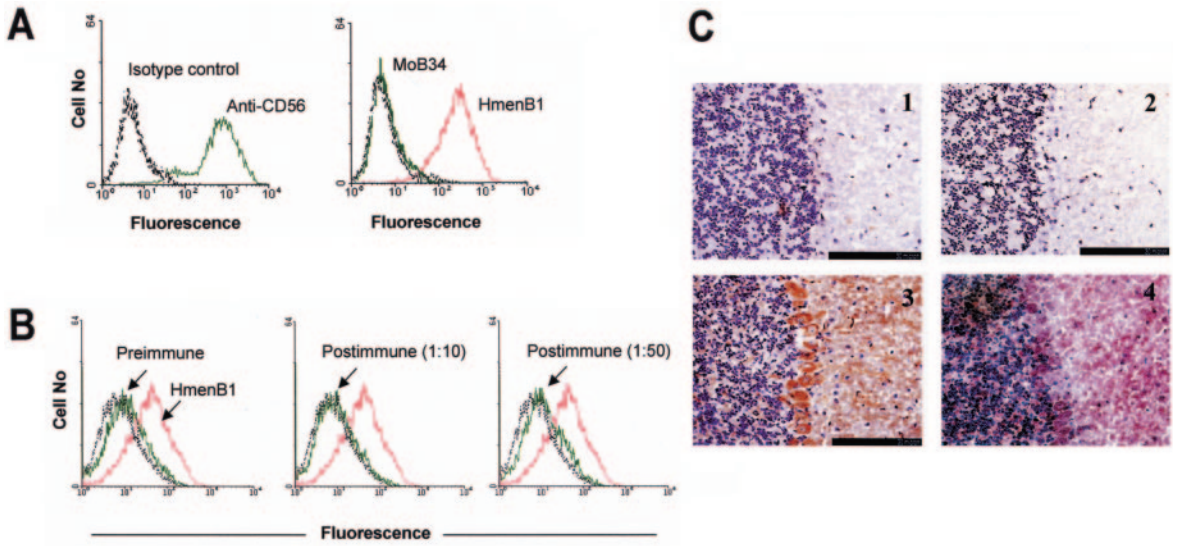

FIG. 8. Autoreactivity tests. (A) Cells were stained with anti-CD56 (green line, left) or isotype control Ab (dotted line). The culture supernatant of HmenB1 (red line) was bound to PSA on CHP-134 cells (42). One hundred micrograms per ml of MoB34 (green line, right) was used for the staining. (B) CHP-134 cells were stained with sera from mice immunized with Naid60-scFv. Five mice sera were pooled and used as a primary Ab at 1:10 (green line, center) and 1:50 (green line, right) dilutions. Preimmune sera (green line, left), the culture supernatant of HmenB1 (red line), and irrelevant Ab (dotted line) are also shown. (C) A cryosection of neonatal (less than 10-day-old) mouse brain was stained with 50 μg/ml of irrelevant Ab (anti-LPS MAb) (panel 1) and 50 μg/ml MoB34 (panel 2). HmenB1 at a concentration of 0.1 μg/ml (panel 3) and hematoxylin-eosin (panel 4) are also shown. Bars, 50 μm.

$F(ab')_2$ of Naid60. MoB34 bound to NMGB in a dose-dependent manner, showing an antigen-Ab binding pattern (Fig. 7A), and killed NMGB in the presence of rabbit complement. The concentration of MoB34 required for 50% killing of NMGB strain ATCC 13090 was approximately 16 ng/ml (Fig. 7B), and the bactericidal activity of MoB34 was inhibited by the addition of Naid60 (Fig. 7C) and purified *E. coli* K1 PS (data not shown), demonstrating that MoB34 is an anti-anti-idiotypic MAb and recognizes NMGB PS.

Autoreactivity. It is important that the Ab elicited by immunizing with Naid60 or recombinant Naid60 Ab does not bind to host cells, because NMGB PS has structural similarity to human PSA. We determined the possibility of autoreactivity of MoB34 by using a human neuroblastoma cell line, CHP-134, which expresses NCAMs decorated with a 50-mer PSA. More than 90% of the CHP-134 cells used for the study expressed a marker of NCAMs, CD56 (Fig. 8A). HmenB1, which was previously shown to bind to CHP-134 cells (42), was used for a positive control (Fig. 8B). However, MoB34 showed no binding to CHP-134 cells even at concentrations of or greater than 100 μg/ml (Fig. 8A). We tested pooled immune sera from five mice immunized with Naid60-scFv, and the mouse sera also showed no binding to CHP-134 cells at 1:10 and 1:50 dilutions (Fig. 8B). To further delineate the binding of MoB34 to brain tissue, we examined the binding of MoB34 to frozen sections of newborn mouse brain. HmenB1 clearly and densely stained Purkinje cells and neighboring cells in the cerebellum at 0.1 μg/ml (Fig. 8C, panel 3). In contrast, MoB34 did not stain the brain (Fig. 8C, panel 2), with staining comparable to the background level of irrelevant isotype control Ig (Fig. 8C, panel 1) at the same concentrations (50 or 100 μg/ml), which were >500 to 1,000-fold higher than the concentration of HmenB1. Thus, Naid60 has an epitope eliciting anti-NMGB Ab with no autoreactivity.

DISCUSSION

Our study describes an anti-idiotype antibody, Naid60, that is capable of mimicking the epitope of NMGB PS and of producing protective Ab to NMGB. Naid60 could compete the phage clones containing the peptide mimotope of NMGB PS (data not shown), which were selectively isolated using HmenB3 by biopanning a phage peptide library (33, 42). Naid60-scFv also inhibited the binding and bactericidal activity of HmenB3 to NMGB, indicating that Naid60 binds to the idiotope in the antigen-binding groove of HmenB3 and carries an epitope mimicking NMGB PS.

We produced an anti-anti-idiotypic MAb of MoB34 by using Naid60 as an immunogen to confirm that Naid60 could induce a protective Ab to NMGB and to exclude the other possibility of the involvement of a nonspecific immune reaction in the protection of Naid60-immune mice. MoB34 bound to NMGB and showed complement-mediated killing of NMGB in a dose-dependent manner; moreover, both activities were inhibited by the addition of Naid60. In our preliminary study, NMGB bacteria could not proliferate in neonatal rats when we injected live bacteria pretreated with MoB34 (data not shown). These data indicate that Naid60-immune mouse sera contained protective Ab. A knowledge of the domain of the Naid60 complementarity-determining region (CDR) responsible for inducing protective antibody is important. Considering that a synthetic peptide spanning the V_H CDR3 ($CDR3_H$) domain of anti-idiotypic Ab (48), which mimicked the meningococcal group C capsular PS, was responsible for inducing protective Ab in

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mice against a live challenge of *N. meningitidis* group C, we believe that Naid60 $CDR3_H$ is a possible candidate for the peptide mimicry of NMGB PS. Naid60 $CDR3_H$ was sequenced (data not shown) (GenBank accession no. AY661813), and it differs from any reported peptide mimotopes of NMGB PS (7, 8, 30, 33, 42). Naid60 $CDR3_H$ contained the YXY sequence motif at the central region, which has been found in peptide mimics of group B and C meningococci, and the YYYAXDY sequence motif at the C-terminal end, as for anti-idiotype G1 scFv (3). However, the N-terminal peptide sequence is different from that of anti-idiotype G1 scFv.

One novel feature of the present study is the demonstration of the lack of autoimmunity of both Naid60 immune sera and the anti-anti-idiotypic MAb, MoB34. The pooled immune sera of five mice immunized with Naid60-scFv, which contained anti-NMGB Ab, showed no binding to PSA on CHP-134 cells (background level). In addition, MoB34 did not bind to CHP-134 cells by flow cytometry or to the brain tissue of newborn mice by histochemical staining at a concentration of 100 μg/ml, which is remarkably higher than the physiologic concentration of total Ab in cerebrospinal fluid (20). These data suggest that the epitope of Naid60 which induces protective Ab is not cross-reactive with NCAMs on nerve cells.

The immunogenicity of anti-idiotype antibody is one of the main issues in regard to vaccine candidates. Our data show that anti-NMGB Ab could be identified in immune mouse sera after immunizing with Naid60-KLH, Naid60-scFv, or $F(ab')_2$ of Naid60 and that anti-NMGB Ab titers were boosted with injection number. We observed growth inhibition of NMGB bacteria in the blood when we challenged mice immunized with Naid60-KLH followed by immunosuppression with live NMGB bacteria, supporting anti-NMGB Ab production in mice. Unexpectedly, however, the sera from mice immunized with Naid60 showed no significant bactericidal activity to NMGB, in contrast to the titer of anti-NMGB Ab by ELISA, even though the immunized mice were protected from the live NMGB challenge. The reason for this is unknown, but this phenomenon could be observed in mouse immune sera of anti-idiotype G1 scFv (3) to Seam 3, which is a protective MAb raised against a chemically modified form of *N*-propionyl meningococcal B PS (16). Our study of protective efficacy in Naid60-immune mice is preliminary, and further evaluation using a more established infant rat or mouse model system is necessary.

The feasibility of using a peptide mimotope as a surrogate for the nominal antigen is important in vaccine approaches. Maitta et al. (27) reported that the peptide mimotope of *Cryptococcus neoformans* capsular PS glucuronoxylomannan can elicit an Ab response to glucuronoxylomannan in mice transgenic for the human Ig loci (XenoMouse mice), thus supporting the concept that peptide mimotope-based vaccines are likely. However, one main limitation in our study is the weak immunogenicity of anti-idiotype Ab, which was found in another study (3). To increase immunogenicity, multiepitope DNA vaccines encoding a T-cell helper epitope and a target molecule of one or more peptide epitopes in tandem have been tried, and functional antibodies have been generated in studies of a viral epitope (25), a carbohydrate mimotope of group C meningococcus (38) and the neogalactoseries Lewis Y antigen with interleukin-12 (23). Chambers and Johnston (6) showed another efficient method of genetic immunization, with constructs containing the T-cell epitope and the cartilage oligomerization matrix protein for pentameric formation of antigen (44) in the presence of genetic adjuvants of Flt3L and granulocyte-macrophage colony-stimulating factor in mice. Considering that plasmid constructs containing genomic DNA or synthetic DNA from 42 to 60 bp could efficiently induce Ab production (6), the anti-idiotope of Naid60 may be a candidate for further study.

In conclusion, a non-cross-reactive and protective epitope is located on NMGB PS, and an anti-idiotype vaccine to this epitope may be a useful NMGB vaccine.

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