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Vaccines containing de-N-acetyl sialic acid elicit antibodies protective against *Neisseria meningitidis* group B and C¹

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Abstract

Murine monoclonal antibodies (mAbs) that were produced by immunization with a vaccine containing the N-propionyl derivative of *Neisseria meningitidis* group B (MenB) capsular polysaccharide (NPr MBPS) mediate protective responses against MenB but were not reactive with unmodified MBPS or chemically identical human polysialic acid (PSA). Recently, we showed that some of the mAbs were reactive with MBPS derivatives that contain de-N-acetyl sialic acid residues (Moe et al. 2005, *Infect Immun* **73**:2123–2128). In this study we evaluated the immunogenicity of de-N-acetyl sialic acid-containing derivatives of PSA (de-N-acetyl PSA) in mice. Four de-N-acetyl PSA antigens were prepared and conjugated to tetanus toxoid, including completely de-N-acetylated PSA. All of the vaccines elicited anti-de-N-acetyl PSA responses (titers $\geq 1:10,000$) but only vaccines enriched for non-reducing end de-N-acetyl residues by treatment with exoneuraminidase or complete de-N-acetylation elicited high titers against the homologous antigen. Also, non-reducing end de-N-acetyl residue-enriched vaccines elicited IgM and IgG antibodies of all subclasses that could bind to MenB. The results suggest that the zwitterionic characteristic of neuraminic acid, particularly at the non-reducing end may be important for processing and presentation mechanisms that stimulate T cells. Antibodies elicited by all four vaccines were able to activate deposition of human complement proteins and passively protect against challenge by MenB in the infant rat model of meningococcal bacteremia. Some vaccine antisera mediated bactericidal activity against a MenC strain with human complement. Thus, de-N-acetyl PSA antigens are immunogenic and elicit antibodies that can be protective against MenB and C strains.

Keywords

Neuraminic acid; polysaccharide; cancer; ganglioside

Introduction

Neisseria meningitidis is an encapsulated bacterium that causes meningitis and septicemia. Incidence peaks in infants under one year of age, and meningococcal disease remains one of the leading causes of death in childhood in developed countries (1). Even with modern antibiotics and supportive treatments, 10% to 15% of meningitis cases result in death, and 10% to 20% result in permanent neurological damage such as hearing loss or paralysis (1).

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Capsular polysaccharide-based vaccines are already available for 4 of the 5 main pathogenic meningococcal capsular groups—A, C, Y, and W135—but to date there is no broadly protective vaccine available for *N. meningitidis* group B (MenB), which causes 30% to 40% (2) of meningococcal disease in the United States. Vaccine development for MenB has been hindered by the fact that MenB capsular polysaccharide (MBPS) is composed of poly alpha 2,8 N-acetyl neuraminic acid (polysialic acid or PSA), a polymer also expressed in human tissues (3). MBPS is poorly immunogenic even when conjugated to a carrier protein possibly because of the similarity to self-antigens (4). Furthermore, antibodies against MBPS can cross-react with human PSA (5,6), which is abundant on fetal tissues including brain, heart, and kidney (3).

Using a N-propionyl MBPS (NPr MBPS)-tetanus toxoid conjugate vaccine (6,7,8), Granoff et al. produced a panel of anti-NPr MBPS monoclonal antibodies (mAbs) that were reactive with MenB but were either not cross-reactive or minimally cross-reactive with purified MBPS or human PSA antigens (6). Thus, the mAbs defined MenB-specific polysaccharide epitopes that could provide the basis for a broadly protective MenB vaccine (9–11). Although antibodies elicited by NPr MBPS are protective against MenB, the NPr derivative of MBPS is not known to occur naturally. It has been suggested that NPr MBPS mimics a conformational epitope on the MenB capsule (8) or, alternatively, that the protective Abs were elicited not by NPr MBPS but by unintended derivatives generated during the synthesis of the NPr MBPS vaccine (9). For example, incomplete re-acylation of MBPS results in contamination with polysaccharide containing de-N-acetylated residues (9).

The presence of zwitterionic de-N-acetyl residues and their possible role in eliciting protective, particularly IgG, antibodies against MenB is of considerable interest in light of recent studies by Kasper and co-workers (12–14). Uncharged or negatively charged bacterial capsular polysaccharides typically can activate B cells without T cell help and are, therefore, described as T-independent antigens. However, the antibody response is most often limited to IgM with poor induction of immunologic memory. Further, the lack of T cell help in the particular case of MBPS and the related MenC capsular polysaccharide (alpha 2,9 N-acetyl neuraminic acid), results in the production of antibodies where V_H and V_L are encoded by a restricted family of unmutated or nearly unmutated germline immunoglobulin genes whether or not the polysaccharide has been conjugated to a carrier protein (15,16). In contrast, zwitterionic polysaccharides can be processed and presented on MHC II molecules, activate T cells and are thus T-dependent antigens (12–14).

This study examines whether the presence of de-N-acetylated residues in NPr MBPS is important for eliciting antibodies that are protective against MenB bacteria without also being cross-reactive with PSA. Four anti-NPr MBPS mAbs, SEAM 2, 3, 12, and 18 described by Granoff et al. (6), were used to characterize de-N-acetylated residue-containing PSA derivatives (de-N-acetyl PSA). All four mAbs are protective against MenB strains. SEAM 2 and 3 are reactive with PSA derivatives that contain de-N-acetylated residues ((9) and *vide infra*) but not with normal human PSA antigens such as that expressed on NCAM (3). In contrast, SEAM 12 and 18 are reactive with neural cell adhesion molecule (NCAM) PSA (6). SEAM 12 is also weakly reactive with poly alpha 2,8 neuraminic acid (Table II). In the following, we describe the results of immunogenicity studies of four de-N-acetyl PSA-derivative-tetanus toxoid (TT) conjugate vaccines in CD1 mice and the functional activity of antibodies elicited by the vaccines against MenB and C strains.

Materials and Methods

Preparation of polysaccharide antigens

De-N-acetyl poly alpha 2,8 neuraminic acid (DeNAc)—Colominic acid (100 mg, Sigma-Aldrich, Saint Louis, MO) and sodium borohydride (10 mg) were suspended in water

(8.8 ml). The solution was heated to 90°C to 100°C for 2 hrs. after adding NaOH (1.8 ml of 50% solution; Fisher Scientific, Pittsburg, PA) to a final NaOH concentration of 2M. After cooling the solution to ambient temperature, 2M HCl was added to adjust the pH to 8. Precipitates were removed by centrifugation, the supernatant solution was dialyzed against 2x 4L of water (1kDa Spectrum Spectra/Por® 7 dialysis membrane; Fisher Scientific) and lyophilized.

N-Trichloroacetyl PSA (TcAc)—DeNAc (50 mg) was suspended in water (5 ml) and the pH adjusted to 8–9 with 2M NaOH. Trichloroacetyl chloride (Sigma-Aldrich) was added to the stirred solution in five 0.1 ml aliquots over a period of 1 hr. The pH was maintained between 8 and 9 by adding 2 M NaOH. The reaction mixture was dialyzed in water and lyophilized as described above.

N-propionyl PSA (NPr) and Sialidase A-treated N-propionyl PSA (NPrSia.)—NPr was prepared as described for TcAc except that propionic anhydride (Sigma-Aldrich) was used in place of the acid chloride. Exoneuraminidases are unable to degrade or degrade much slower PSA that contains Neu at the non-reducing end (T. S. Bhandari and G. R. Moe, unpublished). Therefore, a portion (20 mg) of the NPr derivative was further treated with the exoneuraminidase Sialidase A™ (Prozyme, San Leandro, CA; 0.1 U/mg of polysaccharide) to increase the fraction of molecules that terminate at the non-reducing end in Neu. The polysaccharide was incubated with Sialidase A™ in 50 mM sodium phosphate buffer, pH 7, at 37°C for two days. The reaction mixture was dialyzed in water and lyophilized as described above.

Conjugation of polysaccharide antigens to tetanus toxoid carrier protein

The polysaccharide derivatives with the exception of NPr were oxidized by combining sodium periodate (5 µmol) and 20 mg of polysaccharide (approximately 1 equivalent of periodate for every 10 residues) in 2 ml of 0.1M sodium acetate buffer, pH 6.5 and incubating for 1 hr. NPr (20 mg) was oxidized with 5 mg sodium periodate in 1 ml of 0.1M sodium acetate buffer, pH 6.5, for 30 min. Ethylene glycol (100 µl of a 10% (v/v) solution in water) was added to destroy any remaining periodate and the solution was dialyzed and lyophilized as described above.

To conjugate the polysaccharide derivative to TT, 10 mg of oxidized de-N-acetyl PSA derivative was combined with 5 mg of TT (BioVeris Corp., Gaithersburg, MD) in PBS buffer and allowed to stir at ambient temperature overnight. The following day, 5 mg of sodium cyanoborohydride (Sigma-Aldrich) was added and stirring of the mixture was continued overnight. The next day, sodium sulfate was added to 0.5M and the mixture again stirred overnight. The vaccine preparation was dialyzed against 4 L of PBS buffer, sterile filtered (0.22 µm filter), aliquoted and stored at –80°C until used.

Measurement of sialic acid concentrations

The concentration of N-acetyl neuraminic acid (NeuNAc) and neuraminic acid (Neu) in the de-N-acetyl PSA derivative preparations was determined by a modification of the Svennerholm resorcinol reaction modified as follows. The resorcinol working reagent (300 µl) was combined with the sialic acid or de-N-acetyl sialic acid sample solution (up to 50 µg of sialic acid) or standard stock solution of N-acetyl neuraminic acid or de-N-acetyl colominic acid in water (300 µl) in a polypropylene deep well (2 ml) microtiter plate (Fisher Scientific, Pittsburg, PA). The plate was sealed with a plate cover and heated in a boiling water bath for 30 minutes. After cooling to ambient temperature, isoamyl alcohol (600 µl) was added and mixed using a pipette. The phases were allowed to separate and the upper isoamyl alcohol layer was removed to a clean microtiter plate. The isoamyl alcohol extract and the lower aqueous solution (250 µl each) were transferred separately to a polystyrene microtiter plate and the absorbance at 495

nm and 580 nm was measured using a SpectraMax microtiter plate reader (Molecular Devices, Sunnyvale, CA). The amount of NeuNAc was determined by from the absorbance of the isoamyl alcohol fraction at 580 nm and the amount of Neu was determined from the absorbance of the aqueous fraction at 495 nm in comparison to a standard curve for each. The amount of Neu was corrected for the amount of de-N-acetylation that occurs during the acid hydrolysis step of the assay by measuring the amount of de-N-acetylation that occurs in the NeuNAc standard.

Western Blot

The de-N-acetyl PSA derivative-TT conjugates were tested for their ability to bind the anti-NPr mAbs SEAM 2, 3 and 18 (6) by Western blot. Each conjugate (36 µg of total sialic acid) was separated on a 4%-15% sodium dodecyl sulfate-polyacrylamide gradient gel (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose (Bio-Rad) using a Trans Blot SD Semi-dry transfer cell (Bio-Rad). The membrane was blocked for at least 1 hr in 5% (w/v) nonfat milk in PBS buffer. The mAbs SEAM 2 (20 µg/ml), SEAM 3 (0.24 µg/ml), and SEAM 18 (42 µg/ml), were incubated with the blocked membranes at ambient temperature overnight. After extensive washing with PBS buffer, bound antibodies were detected using a rabbit anti-mouse IgG polyclonal antibody conjugated to horseradish peroxidase (Zymed, South San Francisco, CA) and Western Lightning chemiluminescence reagents (PerkinElmer Life and Analytical Sciences, Waltham, MA).

Immunogenicity in mice

The immunogenicity of the de-N-acetyl PSA derivative-TT conjugates prepared above was evaluated in CD1 mice. All animal experiments were approved by the CHORI Institutional Animal Care and Use Committee. Groups of 10 female CD1 mice (6–8 wk old, Charles River Laboratories, Wilmington, MA) were immunized with de-N-acetyl PSA derivative-TT conjugate vaccine in 50% (v/v) of 0.9% saline and 50% (v/v) Freund's complete adjuvant (Pierce Biotechnology, Rockford, IL) emulsion by ip injection. Each dose contained 1–2 µg of de-N-acetyl sialic acid (i.e. Neu). Blood samples were obtained from the submandibular vein of the mice 10 days after each injection and the anti-de-N-acetyl PSA derivative titers were determined by ELISA using de-N-acetyl PSA derivative-bovine serum albumin (BSA) conjugates as solid-phase antigens as described below.

Booster doses were given at post 28 days with incomplete Freund's adjuvant (Pierce) and titers of antisera obtained 10 days post immunization were evaluated by ELISA. At 56 days post primary immunization, the groups were split in half. Five mice from each group were given unconjugated de-N-acetyl PSA derivative and the other five were given conjugated de-N-acetyl PSA derivative, both without adjuvant. Since the antibody responses of the mice that had received the unconjugated de-N-acetyl PSA derivative was minimal (titer ≤ 1:200), they were given a third dose of conjugate without adjuvant 112 days post primary immunization. The antisera from the latter group was found to be equivalent with respect to anti-DeNAc titer and functional activity to the group that was given conjugate only and were used interchangeably as three dose antisera in the assays described below.

ELISA

The anti-de-N-acetyl PSA derivative titer elicited by the vaccines was measured by ELISA using de-N-acetyl PSA derivatives conjugated to BSA prepared as described above for the de-N-acetyl PSA derivative-TT conjugates. Initially, antiserum from each mouse was tested individually, but since the titers were similar (<10-fold difference) for individuals in the group, the antisera from mice in each group were pooled and all further experiments were done with pooled antisera. ELISA plates were prepared by coating the de-N-acetyl PSA derivative-BSA conjugate (5 µg total de-N-acetyl PSA derivative) in wells of a 96-well microtiter plate

(Immunon II HB, Dynatech, Chantilly, VA) overnight at 4°C before use. Blocking the plates, adding dilutions of antisera in blocking buffer (PBS containing 1% BSA), and developing the ELISA was performed as described previously (6). The specificity of binding was tested by inhibition ELISA performed as described above, with the addition of 50 µg/ml of soluble de-N-acetyl PSA derivative as the inhibitor. Titer was defined as the dilution giving half maximal response after 1 hr of color development.

Bacterial Binding Assays—Antibody binding to MenB was measured by flow cytometry as described by Granoff et al (6). FITC-conjugated anti-mouse IgM and IgG1, IgG2a, IgG2b, and IgG3 (Bethyl Laboratories, Montgomery, TX) were used to detect bound antibodies.

Activation of Human Complement Protein Deposition

The ability of antibodies elicited by immunization with de-N-acetyl PSA derivative-conjugate vaccines to activate deposition of human complement proteins (anti-C3c) on meningococcal bacteria was determined as described by Welsch et al (17).

Serum bactericidal assay (SBA)

The ability of the antisera to mediate bacteriolysis in the presence of exogenous human complement was measured by the serum bactericidal assay using complement from a human donor that lacks antibodies to the test strain as described by Moe et al (9) except that Dulbecco's buffered saline was used in place of Gey's buffer.

Infant rat passive protection assay

Infant (4–6 d) Wistar rats were taken from the mothers and randomly divided into groups of 5 pups each. Each rat was given 100 µl of antiserum diluted 1:10 in sterile PBS containing 1% BSA ip and then returned to their mothers while the challenge bacteria were prepared. MenB strain M986 was grown to OD_{620nm}=0.6 in Mueller-Hinton broth with 0.25% glucose, washed, resuspended in PBS with 1% BSA, and diluted in the same buffer. Each rat pup was given a challenge dose of 10,400 CFU in 100 µl of buffer. The pups were randomly returned to a mother. The next day, the pups were anesthetized with isoflurane and blood was obtained by cardiac puncture using a heparanized needle. The animals were euthanized by CO₂ anoxia, and 100 µl, 10 µl, and 1 µl of the blood was plated on chocolate agar (Remel, Lenexa, KS). The plates were incubated at 37°C, 5% CO₂ overnight and then the colonies were counted using a ProtoCOL plate reader (Synoptics, Cambridge, UK).

Results

Vaccine characteristics

Four de-N-acetyl PSA derivatives as shown in Fig. 1 were prepared. The amounts of N-acyl neuraminic acid, neuraminic acid and protein are compared in Table I. The ratio of polysaccharide to protein is higher than is typically obtained in the conjugation reaction (4,6, 8), which may suggest that some of the polysaccharide was linked to other polysaccharide chains producing branched structures or that unconjugated polysaccharide aggregates with polysaccharide linked to the carrier protein.

The NPr conjugate was synthesized as a positive control (6) although it differs from the original NPr-TT conjugate vaccine in that the NPr antigen was not purified to isolate derivatives with a limited degree of polymerization range. Thus, the NPr antigen used in this study contained both shorter and longer polymers than the vaccine used to produce the prototypical SEAM 2 and 3 mAbs. In this instance, we chose not to purify the derivatives because we have observed that oligomers as small as a trimer containing as little as 20% de-N-acetyl residues forms high

molecular mass aggregates in solution (B. T. Hagen and G. R. Moe, unpublished observations). As a result purification of intermediate size fragments by size exclusion chromatography has the effect of removing derivatives that are reactive with SEAM 2 and 3.

A portion of the NPr derivative was treated with Sialidase ATM, an exoneuraminidase that progressively removes N-acetyl neuraminic acid residues from the non-reducing end (18) until the enzyme encounters a de-N-acetylated residue. The enzyme is either unable to remove de-N-acetylated residues or the rate of hydrolysis is considerably slower (T. S. Bhandari and G. R. Moe, unpublished observations). Thus, treating de-N-acetyl PSA derivatives with Sialidase ATM results in an increase in the number of chains terminating at the non-reducing end in de-N-acetylated residues. The NPrSia-TT conjugate was more reactive with SEAM 3 and less reactive with SEAM 18 (Table II, Fig. 2) than the parent NPr derivative that had not been treated with Sialidase ATM.

For reasons that are unknown, the TcAc conjugate is approximately 1000-fold more reactive with SEAM 2 (Fig. 2) than the NPr derivative as determined by ELISA (Table II). The N-trichloroacetyl group is removable under milder conditions than the N-acetyl group. Originally, the TcAc derivative was synthesized with the idea of preparing de-N-acetyl PSA derivatives with variable amounts of de-N-acetyl residues by selective removal of the protecting group under conditions that could be more easily controlled. Although the van der Waal volume of the trichloroacetyl group is similar to the N-propionyl group it was only slightly reactive with SEAM 3, SEAM 12 and SEAM 18 by ELISA (Table II) or with SEAM 3 on Western blots (Fig. 2). SEAM 12 and 18 are reactive with host PSA antigens (6,19) and were used to show the lack of autoreactive PSA epitopes in the antigen.

Lastly, the DeNAc conjugate was weakly reactive with SEAM 12 (Table II) but not reactive with SEAM 2, 3, or 18 (Table II, Fig. 2) even though SEAM 3 has been shown to recognize PSA antigens containing a small fraction (~20%) of de-N-acetylated residues (9). As with the NPrSia derivative, the DeNAc derivative is enriched for polymer chains that terminate at the non-reducing end in de-N-acetylated residues since it was almost completely de-N-acetylated. Thus, the four vaccine antigens contained a range of PSA/de-N-acetyl PSA epitopes and de-N-acetylated residue content with which to evaluate the question of whether de-N-acetyl PSA-based vaccines can be designed to elicit protective antibody responses to MenB without also eliciting antibodies that are cross-reactive with host PSA antigens.

Vaccine immunogenicity

The antibody titers for the homologous PSA derivative after each immunization were similar for individual mice and, therefore, the antisera of each group was pooled for all subsequent characterization. Antibody responses to the NPr- and TcAc-TT conjugate vaccines for the homologous antigens were very poor (Fig. 3A). In contrast, the NPrSia and DeNAc conjugate vaccines elicited high antibody titers that reached a maximum after two doses (Fig. 3A). Since the NPrSia and DeNAc PSA derivatives were enriched for non-reducing end de-N-acetyl residues, we compared the reactivity of the pooled sera with the DeNAc derivative and found that all four vaccines elicited high ($\geq 1:10,000$) anti-DeNAc antibody titers after the 3rd dose that was only slightly increased over that of the second immunization (Fig. 3B). Even though the fractional amount of de-N-acetyl residues in each vaccine varied over a wide range (from 7% to 98%) all the vaccines elicited anti-DeNAc titers of roughly the same magnitude. This suggests that the non-reducing end, zwitterionic de-N-acetyl residue component in all of the de-N-acetyl PSA antigens was immunogenic and was the immunodominant determinant of the vaccines.

The antisera were also tested by ELISA for their ability to bind to PSA. All showed minimal color development ($OD_{405nm} < 0.1$) after two hours incubation with substrate that was not

different from that of mice immunized with tetanus toxoid alone or from unimmunized mice at dilutions of 1:50 (the lowest dilution tested; data not shown). The result shows that the vaccines did not elicit PSA-reactive antibodies even though two of the vaccines (NPr, NPrSia) contained relatively large fractions of N-acyl groups. Reactivity with PSA has been associated with cross-reactivity with human PSA antigens (5,6,20). The lack of PSA cross-reacting antibodies is consistent with the fact that PSA derivatives containing de-N-acetyl residues exhibit physical and chemical properties that are distinct from PSA. Obviously, this is especially true for the DeNAc antigen, which is almost completely de-N-acetylated. For example, de-N-acetyl sialic acid antigens containing zwitterionic residues form high mass aggregates, have glycosidic bonds that are resistant to acid hydrolysis ((21), T. S. Bhandari and G. R. Moe, unpublished observations), are resistant to degradation by exoneuraminidases (T. S. Bhandari and G. R. Moe, unpublished observations) and, as shown here, are immunogenic.

The specificity of the antisera for the homologous de-N-acetyl PSA derivative and DeNAc antigens was evaluated by inhibition ELISA in the presence of 50 µg/ml of soluble DeNAc polysaccharide. However, binding actually increased slightly (10%–20%) in the presence of soluble inhibitor compared to the antisera alone. The increased binding observed may result from aggregation of soluble antigen and surface associated antigen and/or cross-linking by the antibody between surface bound and soluble antigens. As noted above, even small PSA oligosaccharides that contain a fraction of de-N-acetylated residues readily aggregate in solution to form high molecular weight complexes.

Binding to live MenB bacteria

As shown in Fig. 4A, the tetanus toxoid carrier protein and the NPr and TcAc conjugate vaccines elicited antibodies that were weakly reactive with MenB strain NMB. The NMB-reactive antibodies were primarily class IgM. In contrast, the two vaccines that were enriched for non-reducing end de-N-acetylated residues elicited IgM and IgG antibodies of all subclasses that were reactive with NMB. PSA or MBPS, even when conjugated to a carrier protein elicits mainly IgM antibodies in mice with the exception of rare IgG mAbs that have been produced in NZB mice that have a propensity to produce autoreactive antibodies (19,22).

The ability of zwitterionic PSA derivatives of normally non-immunogenic PSA to elicit IgG antibodies suggests that they may provide T cell help through the mechanism of zwitterionic polysaccharide processing and presentation on MHC II molecules described by Cobb and Kasper (12). Similarly, Gallorini et al. have recently shown that introducing a zwitterionic motif into naturally anionic bacterial polysaccharides confers the ability to activate both T cells and antigen-presenting cells such as monocytes and dendritic cells (23). Thus, some, but not all (23), zwitterionic polysaccharides are T-dependent antigens that, as shown here, can elicit IgM and IgG antibody responses.

Activation of human complement

All the antisera strongly activated complement protein (C3) deposition on MenB strain NMB (Fig. 4B), and there was little difference in the amount of complement deposited between the antisera elicited by different antigens. The uniformity in the ability of the each vaccine antiserum to activate complement is in accordance with the similarity seen in the anti-DeNAc ELISA titers (Fig. 3B) and the fact that all four vaccines elicited IgM antibodies that could bind to MenB (Fig. 4A).

Passive protection in infant rats

Several studies have shown a correlation between the ability to activate complement protein deposition on MenB bacteria and passive protection in the infant rat model of meningococcal

bacteremia (17). As shown in Fig. 5, the antisera from the mice immunized with the TT carrier protein alone did not protect the rat pups from challenge by MenB strain M986. All pups treated with the TT antisera were bacteremic and had high blood culture titers of bacteria ($>50,000$ CFU/ml). The positive control mAb SEAM 2 was completely protective (sterile blood cultures) at a dose of 6 μ g of mAb/pup. Similarly, pups treated with the 10 μ l of anti-de-N-acetyl PSA derivative-TT sera, were either completely protected (sterile blood cultures) or had reduced CFU burden compared to the pups that received the control TT antisera. Protection by vaccine antisera was significantly different than the control TT antisera ($P<0.05$) with protection by the TcAc antisera being just short of significance ($P=0.1$).

Serum bactericidal activity (SBA)

The antisera were tested for their ability to mediate SBA against MenB strain NMB. Despite showing strong activation of complement protein deposition against this strain (Fig. 4B) and passive protection against MenB strain M986 (Fig. 5), none of the vaccine antisera were able to mediate bacteriolysis of NMB in the presence of human complement (data not shown). The result is, perhaps, not unexpected based on functional characteristics exhibited by SEAM 2 and 3. SEAM 3 is not bactericidal with human complement (6) and SEAM 2 requires very high antibody concentrations (>200 μ g/ml, (24)). Yet both mAbs can activate human complement protein deposition and can passively protect in the infant rat model of MenB bacteremia at mAb concentrations on the order of 1 μ g/ml or less (see for example, Fig. 4B and Fig. 5).

Recently, we discovered that the antigens recognized by SEAM 2, 3, and 18 are expressed in all meningococcal serogroups that express sialic acid-containing capsular polysaccharides (i.e. MenB, C, Y, and W135; (24)). Furthermore, all three mAbs can mediate bactericidal activity against MenC strains with human complement (24). Therefore, we tested SBA against MenC strain 4243 with human complement. As shown in Table III, the NPr, DeNAc and TcAc antisera pools showed complement-mediated bactericidal activity against the MenC strain but the NPrSia and TT antisera had no activity in three independent assays. Thus, the differences in SBA activity of the vaccine antisera between MenB and C strains is a characteristic shared by SEAM 2 and 3, the prototypical mAbs used to develop the de-N-acetyl PSA derivatives used in this study.

Discussion

A subset of antibodies elicited by NPr MBPS-TT conjugate vaccines have the surprising characteristics of being either un-reactive or poorly reactive with purified MBPS yet are able to mediate protective responses against MenB bacteria including activation of complement protein deposition (see SEAM 2 for example, Fig. 4B), complement-dependent bactericidal activity (6,8,9), and passive protection in an infant rat model of meningococcal bacteremia (6). In a previous study, we showed that the anti-NPr MBPS mAb SEAM 3 recognizes de-N-acetyl PSA-containing derivatives (9). Since SEAM 3 binds to MenB bacteria, activates complement protein deposition, and can passively protect against a MenB bacterial challenge in an infant rat model of meningococcal bacteremia, it suggests that MenB express de-N-acetyl PSA antigens. While it is possible that mAbs such as SEAM 2 and SEAM 3 are cross-reactive with unknown non-PSA derived antigens, we consider that possibility unlikely. The specificity of both SEAM 2 and 3 have been studied extensively with a broad range of molecules including polysaccharides, polysaccharide derivatives (9), and phage display (11) and combinatorial small molecule libraries (25). Also, de-N-acetyl PSA derivatives must be immunogenic since antibodies such as SEAM 3 were elicited in response to immunization with NPr MBPS-protein conjugate vaccines that contained de-N-acetylated sialic acid residues. The purpose of the present study was to determine whether de-N-acetyl PSA derivative-TT conjugate vaccines in which the PSA derivatives were specifically reactive with mAbs SEAM 2 and 3 that are

protective against MenB bacteria but are not reactive with purified MBPS, can elicit similarly protective but not autoreactive antibodies.

The results of this study show that de-N-acetyl PSA derivatives are immunogenic. Relatively small doses (~1 µg) of de-N-acetylated residues in a de-N-acetyl PSA derivative, can elicit antibodies that bind to MenB and C bacteria, activate complement, and passively protect in an infant rat model of meningococcal bacteremia. Derivatives enriched for non-reducing end de-N-acetyl residues may be particularly efficient in eliciting IgG antibodies of all subclasses. The polyclonal antisera were not reactive with purified PSA. It is, therefore, likely that one explanation for the ability of NPr MBPS-based vaccines to elicit protective but not autoreactive antibodies is that the NPr MBPS vaccine contains some fraction of de-N-acetylated residues.

PSA that contains de-N-acetyl residues is physically and chemically different than unmodified PSA. The differences are no doubt critical in accounting for the vastly different antibody responses to the two antigens. PSA, even when conjugated to a carrier protein, is very poorly immunogenic, eliciting predominantly IgM responses where the antibodies are encoded by unmutated germline V_H and V_L genes, and fails to induce immunologic memory. In contrast, zwitterionic de-N-acetyl PSA antigens elicited high titers of IgM and IgG antibodies of all subclasses. Polysaccharides that contain zwitterionic residues can be processed differently from uncharged or negatively charged polysaccharides and presented on MHC II molecules to stimulate helper T cells (12,14,23). Therefore, it is likely that the presence zwitterionic neuraminic acid residues, particularly at the non-reducing end of de-N-acetyl PSA derivatives, is critical for eliciting the observed antibody responses.

Although bactericidal activity is widely accepted as a surrogate of protection against meningococcal disease, it may underestimate protection (26). In particular, MenB is known to be resistant to SBA and more sensitive to opsonophagocytosis than other serogroups (27), and it is possible that opsonophagocytosis is a major element of protective immunity to disease caused by MenB. Lack of SBA, therefore, may not necessarily correlate with lack of protection against MenB strains. Anti-MenB mAbs have been described that are protective but not bactericidal (17,28,29), and Toropainen et al. have reported that IgM-mediated protection against MenB as measured by the infant rat protection assay is independent of complement-mediated bacteriolysis (30).

The NPr, TcAc, and DeNAc antiserum pools were able to mediate SBA against MenC strain 4243. The result demonstrates that there is no intrinsic lack of bactericidal functionality of antibodies that are reactive with de-N-acetyl sialic acid-containing antigens expressed by meningococcal strains but that activity can be affected by the presence of structurally related PSA antigens (e.g. MBPS poly alpha 2,8 N-acetyl neuraminic acid). Similar results have been observed for the prototypic mAbs SEAM 2 and 3. Neither mAb is reactive with MCPS or MCPS containing de-N-acetyl sialic acid but both mAbs exhibit greater binding to MenC strains and are able to mediate bactericidal activity at lower antibody concentrations than MenB strains (24). Thus, it appears that both MenB and MenC strains express poly alpha 2,8 N-acetyl neuraminic acid antigens that contain non-reducing end de-N-acetyl sialic acid (neuraminic acid) that can be the target of protective antibodies elicited by de-N-acetyl PSA-containing vaccines.

Together, MenB and C strains cause 50% to >90% of meningococcal disease in developed countries (1). Although the functional activity of the de-N-acetyl PSA derivative antisera was evaluated using only a few strains in this study, a large number of genetically diverse MenB strains isolated from patients from many countries over a period of several decades and grown under a variety of conditions including human blood and plasma have been found to express antigens reactive with SEAM 2 and 3 (B. A. Flitter, J. Y. Ing, and G. R. Moe, unpublished

observations). Therefore, optimized vaccines based on de-N-acetyl PSA derivatives have the potential to provide protection against the majority of meningococcal disease occurring in developed countries.

At the present time, it is unclear whether de-N-acetyl PSA or de-N-acetyl sialic acid antigens are expressed in normal adult or fetal tissues. Hakamori and coworkers identified de-N-acetyl monosialoganglioside GM3 in A431 epithelial carcinoma and B16 melanoma cell lines (31). Subsequently, Varki and coworkers identified de-N-acetyl derivatives of the disialolylganglioside GD3 using monoclonal antibodies (32,33). By immunohistochemical (IHC) staining, they showed that de-N-acetyl GD3 was expressed at low levels in a few blood vessels, infiltrating mononuclear cells in the skin and colon, and at moderate levels in skin melanocytes (32). Using radioactive labeling experiments in melanoma cell lines, Manzi et al (34) showed that N-acetyl groups in gangliosides GD3 and GM3 turned over more rapidly than the parent molecules suggesting the existence of a sialic acid de-N-acetylase for the production of de-N-acetyl gangliosides in these cells. Recently, Popa et al isolated and determined the structure of de-N-acetyl GD3 from primary human melanoma tumor (35). The de-N-acetylated residue was shown to be at the non-reducing end of the dimer while the reducing end residue of the disialolyl moiety was N-acetylated. In unpublished work (C.P. Plested, H. Kaur and G. R. Moe), we have observed by IHC analysis that several primary human tumors including melanoma, neuroblastoma, leukemia, and tumors of the stomach, ovary and uterus express antigens recognized by SEAM 3 and polyclonal anti-de-N-acetyl PSA sera that are distinct from PSA expressed on NCAM and were not detected in the corresponding normal tissues. SEAM 3, however, does not react with de-N-acetylated GD3 derivatives (G. R. Moe and G. S. Mittendorf, unpublished observations). Understanding the biology of de-N-acetyl sialic acid antigen expression in normal and diseased tissues will be essential for establishing whether de-N-acetyl PSA-based vaccines can be safe for general use in humans to protect against meningococcal disease. Antibodies that can be produced using the de-N-acetyl PSA vaccines described here will be important in reaching that goal as well as for understanding the role of de-N-acetyl sialic acid antigens in human health and disease.

Abbreviations used in this paper

NeuNAc, N-acetyl neuraminic acid
 Neu, neuraminic acid
 PSA, polysialic acid (poly alpha 2,8 N-acetyl neuraminic acid)
 de-N-acetyl PSA, PSA containing de-N-acetyl neuraminic acid
 MBPS, *N. meningitidis* group B polysaccharide (poly alpha 2,8 N-acetyl neuraminic acid)
 DeNAc, poly alpha 2,8 neuraminic acid
 NPr, poly alpha 2,8 N-propionyl neuraminic acid
 NPrSia, NPr treated with the exonuclease Sialidase ATM
 TcAc, poly alpha 2,8 N-trichloroacetyl
 NCAM, neural cell adhesion molecule
 SBA, serum bactericidal activity
 TT, tetanus toxoid

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Vaccine

R

DeNAc*

$$\text{H}_3\text{N}^+ (98\%), \text{CH}_3\overset{\text{O}}{\parallel}\text{C} (2\%)$$

NPr

$$\text{H}_3\text{N}^+ (21\%), \text{CH}_3\text{CH}_2\overset{\text{O}}{\parallel}\text{C} (79\%)$$

NPrSia*

$$\text{H}_3\text{N}^+ (7\%), \text{CH}_3\text{CH}_2\overset{\text{O}}{\parallel}\text{C} (93\%)$$

TcAc

$$\text{H}_3\text{N}^+ (63\%), \text{Cl}_3\overset{\text{O}}{\parallel}\text{C} (37\%)$$

*Enriched at the non-reducing end for $\text{R}=\text{H}_3\text{N}^+$.

FIGURE 1.

Structure of vaccine antigens prepared from poly alpha 2,8 N-acetyl neuraminic acid. The fraction of neuraminic acid and N-acyl neuraminic acid for each antigen is indicated in parenthesis.

FIGURE 2.

Reactivity of SEAM 2, 3, and 18 with the de-N-acetyl PSA-TT vaccines by Western blot. Each of the de-N-acetyl PSA-TT vaccine antigens (10 μ g of total sialic acid) were resolved on 4–15% SDS-PAGE gels and were probed with the mAbs SEAM 2 (20 μ g/ml), SEAM 3 (0.24 μ g/ml), and SEAM 18 (42 μ g/ml) as indicated on Western blots. SEAM 18 but not SEAM 2 or 3 is reactive with PSA-NCAM (6).

FIGURE 3.

Serum antibody titer against homologous and de-N-acetylated PSA antigens by ELISA. Titer of pooled antisera against homologous de-N-acetyl PSA antigens (A) or DeNAc (B) conjugated to BSA after 1 dose (open bars), 2 doses (checkered bars), or 3 doses (filled bars).

A **B** **FIGURE 4.**

Flow cytometric analysis of (A) binding and (B) activation of complement protein deposition by vaccine antisera with MenB strain NMB. Unfilled histograms are secondary antibody binding to the bacteria in the absence of antisera. Filled histograms show fluorescence of bacteria in the presence of post 3rd dose antisera used at 1:10 dilution detected using FITC-conjugated class and subclass-specific secondary antibodies. Activation of complement deposition was measured using human complement (5%) and a FITC-conjugated anti-human C3c secondary antibody. Unfilled histograms show fluorescence of bacteria in the absence of and filled in the presence of test antisera (post 2nd dose, 1:200 dilution). The negative control TT antisera was diluted 1:20 and the concentration of the positive control mAb, SEAM 2, was 2 µg/ml.

FIGURE 5.

Passive protection against MenB challenge in infant rats. The antisera (10 μ l of post 3rd dose/pup) were tested for their ability to passively protect against bacteremia caused by challenge with MenB strain M986. The graph shows geometric mean CFU/ml for bacteria in each blood sample with error bars indicating 95% confidence intervals. The numbers above each bar indicate the number of pups infected out of the number tested. The asterisk (*) indicates $P < 0.05$ versus TT control by Student's t-Test with two-tailed distribution. SEAM 2 (6 μ g/pup) was used as a positive control.

Table 1

Comparison of N-acyl neuraminic acid (NeuAc), neuraminic acid (Neu) and protein concentration for the PSA derivative-tetanus toxoid conjugate vaccines.

Vaccine ^a	NeuNAc (mg/ml)	Neu (mg/ml)	Percent Neu	Total sialic acid (mg/ml)	Protein (mg/ml)
NPr	3.3	0.9	21	4.2	3.1
NPrSia	4.2	0.3	7	4.5	3.1
TcAc	2.2	3.7	63	5.9	3.6
DeNAc	0.2	7.8	98	8	5.1

^aNPr, N-propionyl PSA; NPrSia, Sialidase A-treated N-propionyl PSA; TcAc, N-trichloroacetyl PSA; DeNAc, de-N-acetylated PSA.

Table II

Comparing the reactivity of prototypic anti-NPr MBPS mAbs with vaccine antigen-BSA conjugates by ELISA^{a,b}.

BSA conjugate ^c	SEAM 2	SEAM 3	SEAM 12	SEAM 18
NPr	27	0.14	0.005	0.016
NPrSia	>50	0.015	0.08	0.038
TcAc	0.008	3.7	0.12	5.3
DeNAc	>50	>50	1.77	>50

^a Antigens were linked through the reducing end by reductive amination.

^b Concentration of mAb (µg/ml) giving an OD_{405nm} of 0.25 after 40 min incubation with substrate.

^c NPr, N-propionyl PSA; NPrSia, Sialidase A-treated N-propionyl PSA; TcAc, N-trichloroacetyl PSA; DeNAc, de-N-acetyled PSA.

Table III

Serum bactericidal activity of antisera pools against MenC strain 4243 with human complement.

Vaccine	1/Titer [*]		
	Expt. 1	Expt. 2	Expt. 3
TT	<8	<8	<16
NPrSia	<8	<8	<16
NPr	>256	>256	2048
DeNAc	256	96	8192
TcAc	>256	128	6144

* Serum bactericidal titers were defined as the antibody concentration resulting in a 50% decrease in colony forming units (CFU) per ml after 60 minutes incubation of bacteria in the reaction mixture, compared to the control CFU per ml at time 0. Typically, bacteria incubated with the negative control antibody and complement showed a 150 to 200% increase in CFU/ml during the 60 minutes of incubation.