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Evaluation of Recombinant Transferrin-Binding Protein B Variants from *Neisseria meningitidis* for Their Ability To Induce Cross-Reactive and Bactericidal Antibodies against a Genetically Diverse Collection of Serogroup B Strains

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Transferrin-binding protein B (TbpB) is a surface-exposed protein, variable among strains of *Neisseria meningitidis*, that has been considered as a vaccine candidate. To define a TbpB molecule that would give rise to broadly cross-reactive antibodies with TbpB of many strains, specific antisera were produced against three recombinant TbpB variants from strain M982: one corresponding to the full-length TbpB; one in which stretches of amino acids located in the central part of the molecule, described as hypervariable, have been deleted; and one corresponding to the N-terminal half of the molecule, described as the human transferrin binding domain. The reactivity of these antisera against 58 serogroup B strains with a 2.1-kb *tbpB* gene representing different genotypes, serotypes, and subtypes and different geographic origins was tested on intact meningococcal cells. In parallel, the bactericidal activity of the antisera was evaluated against 15 of the 58 strains studied. Of the 58 strains, 56 (98%) reacted with the antiserum specific for the N-terminal half of TbpB M982; this antiserum was bactericidal against 9 of 15 strains (60%). On the other hand, 43 of 58 strains reacted with the antiserum raised to full-length TbpB while 12 of 15 (80%) were killed with this antiserum. The antiserum specific to TbpB deleted of its central domain gave intermediate results, with 53 of 58 strains (91.3%) recognized and 10 of 15 (66.6%) killed. These results indicate that the N-terminal half of TbpB was sufficient to induce cross-reactive antibodies reacting with the protein on meningococcal cells but that the presence of the C-terminal half of the protein is necessary for the induction of cross-bactericidal antibodies.

Meningococcal disease caused by serogroup B meningococci remains an important health problem in many parts of the world. Because group B polysaccharide is poorly immunogenic in humans (22, 53), several vaccine approaches based on noncapsular surface antigens are being pursued in different laboratories to provide an efficacious protection against this disease. Among these approaches are outer membrane vesicle vaccines, lipopolysaccharides, and class 1 protein-based vaccines (30, 36, 37, 46, 54, 55). So far, however, the protection conferred by two doses of outer membrane vesicle vaccines in Norwegian teenagers over a 29-month period was only 57% (41). These results, along with others (7, 14, 33, 34, 36), are both encouraging, demonstrating that disease incidence can be reduced by vaccination, and disappointing, because higher efficacy had been anticipated with such preparations. These results leave room for improved vaccines, notably for cross-reactive vaccines which would confer broad protection against meningococcal strains of different serotypes and serosubtypes; many studies have focused on other meningococcal surface antigens, notably iron-regulated proteins (6). *Neisseria meningitidis* grown in iron-restricted media express novel proteins which include Fbp (32), a 70-kDa protein (FrpB) (15), lactoferrin-binding proteins (Lbps) (43), hemoglobin-binding proteins (HmbR and Hpu) (27, 48, 49), and transferrin-binding proteins (Tbps) (44).

Transferrin-binding proteins are composed of two subunits, TbpA (formerly Tbp1), which is described as a transmembrane protein, and TbpB (formerly Tbp2), a lipoprotein, presumably anchored to the outer membrane through its lipid tail. The polypeptide part of TbpB is predicted to be largely external to the outer membrane (5, 11, 12, 25, 26). TbpA and TbpB, with apparent molecular masses of 98 and 66 to 85 kDa, respectively (16, 20), have several attributes of good vaccine candidates: (i) they are surface-exposed molecules (3); (ii) they are expressed in vivo during infection (2, 4, 19); (iii) they elicit protective and bactericidal antibodies in laboratory animals (13, 28); and (iv) as of today, no one has reported the occurrence of natural mutants lacking Tbps: our laboratory has tested some 200 strains and all expressed these proteins (unpublished results). The results of different studies showed that recombinant or native TbpB from different bacterial pathogens confer protection or induce protective antibodies against the homologous strain. The protective role of TbpB has been shown with native TbpB from *N. meningitidis* (28), recombinant TbpB from *Actinobacillus pleuropneumoniae* (42), and recombinant TbpB from *Haemophilus influenzae* (29). The variability observed for TbpB has raised some questions about its capacity to be used as a broadly cross-reactive antigen (20, 47). Previous studies have shown that while TbpB varied among strains, antigenic and genomic features of TbpB and *tbpB* genes allowed the meningococcal strains to be classified into two major families: B16B6-like strains and M982-like strains, expressing a TbpB in

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the range of 67 and 85 kDa, respectively (40). Antisera raised to B16B6 Tbps are bactericidal for B16B6-like strains (13), while the cross-reactivity of antisera raised to M982 TbpB had not been assessed.

In the present study, with the aim of developing a well-defined subunit vaccine and circumventing the low yields of Tbps isolated from *N. meningitidis*, recombinant TbpB from strain M982 was produced in *Escherichia coli* and purified, and specific antisera were raised in rabbits. The reactivity of these antisera on whole meningococcal cells was assessed in a dot blot assay on a total of 58 serogroup B isolates. In addition, the bactericidal activity of the antisera was evaluated on 15 of these strains and compared to that of an antiserum raised to the TbpA-TbpB complex isolated from *N. meningitidis.*

MATERIALS AND METHODS

Bacterial strains and culture conditions. The 63 isolates examined included 39 of the 50 serogroup B meningococci described by Seiler et al. (45) and the reference strains B16B6, M982, M978, S3032, and 6940 (25, 31). To broaden the geographical representativeness of the strains analyzed, this collection was supplemented with a set of 19 strains from Australia (54250), Brazil (SP111/88 and SP160/88), China (2713 and 3839), Israel (M692, M870, M912, and M918), Japan (106, 126, and 130), Russia (344 and 568), South Africa (313, 315, 349, and 351), and the United States (13763). The isolates were characterized by their combination of alleles at 14 enzyme loci as described previously (8) and serotyped and subtyped with monoclonal antibodies (38).

For DNA extraction, *N. meningitidis* strains were grown on Mueller-Hinton agar plates (Difco). To detect Tbps and for dot-blot assays with antisera, strains were grown for 5 h at 37°C in flasks containing 50 ml of Mueller-Hinton broth (Difco) supplemented with 30 μM EDDA (ethylene diamine di-*ortho*-hydroxyphenyl acetic acid [Sigma]). For bactericidal assays, the same iron-chelated medium was used but the time of incubation was 4 h. For adsorption of sera, *E. coli* was grown in Luria-Bertani medium (Difco) supplemented with 100 μg of ampicillin per ml when necessary.

PCR amplification of *tbpB*. DNA was extracted from each strain by a rapid method with guanidium isothiocyanate (35). PCRs to amplify *tbpB* genes were performed on genomic DNA with the two primers (5′-TGCTATGGTGCTGC CTGTG-3′ and 5′-TGCCGTCGAAGCCTTATTC-3′) described previously (31). The products of each PCR were analyzed on 1% agarose gel, and the size of the *tbpB* gene was determined with the molecular weight marker λ *Hin*dIII (New England BioLabs).

Construction of *E. coli* vectors expressing *tbpB*. The arabinose-inducible expression vector (pARA13) was used for *tbpB* expression in *E. coli* as described previously (26, 51). The *tbpB* sequence encoding the full-length TbpB (TbpB-100%) of strain M982 was cloned into the pTG5768 vector as described previously. The *tbpB* nucleotide sequence encoding the minimal human transferrin (hTf)-binding domain (amino acids [aa] 1 to 350), referred to as the TbpB-53% domain by Von der Haar et al. (51), was cloned in the pTG5782 vector. The *tbpB* sequence encoding the TbpB protein with part of its hypervariable central domain deleted (TbpB-80%) was cloned in the pTG5783 vector; four deletions were made by site-specific mutagenesis (23) and correspond to nucleotides encoding aa 354 to 379, aa 415 to 444, aa 465 to 481, and aa 500 to 520 (according to the numbering of the amino acid sequence of the TbpB M982 protein published previously [25]). All three constructs were kindly provided by M. Legrain (TRANSGENE, Strasbourg, France

Expression and purification of recombinant proteins. Recombinant *E. coli* B cells harboring the different plasmids were grown in a 10-liter fermentor with TGM16 medium as described previously (26), and a fraction was kindly provided by D. Speck (TRANSGENE). Expression of the various forms of TbpB was induced by the addition of L-arabinose (0.2%, wt/vol) to the medium when the culture reached an absorbance of about 60 at 600 nm. After 1 h of induction, bacterial cells were harvested and stored at −20°C. A control culture of *E. coli* harboring only the cloning vector without the *tbpB* gene cassette was produced under identical conditions. Membrane fractions containing each form of recombinant TbpB (rTbpB) were prepared as described previously for the Tbp complex (13). Different forms of rTbpB were purified by preparative gel electrophoresis. Membrane fractions were solubilized in Laemmli electrophoresis buffer. A 20-mg portion of total protein was loaded on slab gels (14 by 16 by 0.5 cm), and electrophoresis was conducted as described by Laemmli (24). After electrophoresis, a strip of gel corresponding to the size of recombinant TbpB (88 kDa for rTbpB-100%, 67 kDa for rTbpB-80%, and 43 kDa for rTbpB-53%) was cut out and minced. Three strips (88, 67, and 43 kDa) were cut out from the gel run with the *E. coli* control crude extract. Proteins were extracted mechanically from the minced gel in buffer containing 6 M urea, precipitated from the urea extract, and dialyzed against phosphate buffer containing 0.05% Sarkosyl (*N*-lauroylsarcosine [Sigma]).

Purification of meningococcal Tbp complex. The Tbp complex was purified from membrane fraction of *N. meningitidis* M982 as described previously (13). Briefly, crude total membranes were obtained by passing the bacterial suspension in a cell-lysing apparatus operated under high pressure (Rannie model 8.30H) followed by differential centrifugations. The membrane fraction was solubilized in 50 mM Tris-HCl (pH 8.0) containing 1 M NaCl, 10 mM EDTA, and 0.5% Sarkosyl (buffer A) and incubated with a Sepharose-4B resin (Pharmacia) onto which hTf (Sigma) had been immobilized. After incubation, the resin was packed into a column, washed with 3 column volumes of buffer A and then with buffer A containing 0.75 M guanidine-HCl. The proteins were eluted with 50 mM Tris-HCl (pH 8.0) containing 1 M NaCl, 10 mM EDTA, 0.05% Sarkosyl, and 2 M guanidine-HCl. Fractions containing the transferrin-binding proteins were pooled, dialyzed, and concentrated. The protein solution was stored at −70°C in 50 mM phosphate buffer (pH 8.0) containing 0.05% Sarkosyl.

Preparation of membrane fraction and Western blotting. Bacterial pellets were resuspended in 125 mM Tris-HCl (pH 8.0), sonicated, solubilized in a 1% solution of Triton X-100, and centrifuged for 15 min at 8,000 × *g*. The supernatant was centrifuged for 30 min at 10,000 × *g*, and the pellet was resuspended in 125 mM Tris-HCl (pH 8.0). A 50-μg portion of protein in Laemmli sample buffer was loaded onto 10% polyacrylamide gels (24), electrophoresed, and electrotransferred to nitrocellulose membranes (50). The membranes were incubated with either hTf conjugated to peroxidase (100 μg/ml) (Jackson Immunoresearch Laboratories) or specific antisera followed by incubation with the conjugate (goat anti-rabbit immunoglobulin G [IgG] conjugated to peroxidase [Zymed]). The reaction was developed by incubating the membrane in a substrate solution containing 4-chloro-1-naphthol and H_2O_2 as previously described (40).

Preparation and treatment of rabbit antisera. New Zealand White rabbits were immunized with three injections of 50 μg of recombinant TbpB, the corresponding *E. coli* extract as a control, or a meningococcal TbpA-TbpB complex purified from strain M982 as described previously (13). Briefly, the proteins were emulsified in Freund's adjuvant and injected into the rabbits on days 0, 21, and 42 by both the subcutaneous and intramuscular routes. On day 57, the animals were sacrificed and blood was collected. To ensure their specificity, the antisera were adsorbed on meningococci grown in the presence of iron as described previously (13). For dot-blot assays, antisera were further adsorbed three times on *E. coli* B. The ratio of serum to cells was 5 ml to 1 × 10^{10} CFU. For bactericidal assays, IgGs were affinity purified on protein G-Sepharose 4FF columns (Pharmacia) (21).

Peptide synthesis and enzyme-linked immunosorbent assay. Seven-mer peptides overlapping by 4 aa spanning the entire sequence of TbpB M982 (229 overlapping peptides) were synthesized as previously described (39), with the epitope-scanning kit (Cambridge Research Biochemicals, ICI). The pin-based enzyme-linked immunosorbent assay was carried out as described previously (39). Briefly, the blocks of pins were immersed in microtiter plates containing 200 μl of blocking buffer (bovine serum albumin, 10 g · liter^{-1}; ovalbumin, 10 g · liter^{-1}; sodium azide, 0.05%; and Tween 20, 0.1% in phosphate-buffered saline per well. After 1 h at 25°C, the pins were incubated with rabbit antisera diluted 1:150 in blocking buffer and further incubated overnight at 4°C. The pins were washed and incubated with specific alkaline phosphatase conjugate diluted 1:2,500 in blocking buffer (Dako A/S). After incubation and washes, substrate was added and the reactivity of the antisera against each peptide was measured at 405 nm after a 1-h incubation. For repetitive use of the pins, the bound Igs were removed as recommended by the manufacturer's.

Dot blot assay. The dot blot assay was performed by the method of Schryvers and Morris (44) with slight modifications. Briefly, bacterial suspensions (2 × 10^9 CFU/ml) grown under iron starvation were twofold serially diluted, and 50 μl of each dilution was spotted onto nitrocellulose filters (pore size, 0.45 μm; Schleicher & Schuell) under vacuum. The filters were incubated either with hTf-peroxidase (Jackson Immunoresearch Laboratories) or with adsorbed rabbit antisera and then with a goat anti-rabbit IgG-peroxidase conjugate (Zymed). The reaction was detected with a colorimetric substrate (4-chloro-1-naphthol) or a chemoluminescent substrate for peroxidase (enhanced chemoluminescence [ECL]; Amersham). The reactions were quantified by using a densitometer (Sebia preference). The titers are expressed as the last dilution giving a detectable signal (optical density at 530 nm > 0.1).

Bactericidal assay. The bactericidal activity of purified rabbit IgG was tested as described previously (13) with slight modifications. Briefly, 50-μl volumes of serial twofold dilutions of IgG solutions were added to 96-well microplates (Nunc) and incubated with 25 μl of an iron-starved meningococcal suspension adjusted to 2 × 10^4 CFU/ml and 25 μl of baby-rabbit complement. After a 1-h incubation at 37°C, 20 μl of the mixture in each well was plated onto Mueller-Hinton agar plates. The plates were incubated overnight at 37°C under 10% CO_2. The bactericidal titer of each serum was expressed as the last dilution of serum at which 50% or greater killing was observed compared to the complement control. For strains that were directly killed by the complement without addition of purified IgG, the complement was first adsorbed on formaldehyde-fixed bacteria (10^{10} CFU; 3 ml of complement) before being used for the bactericidal assay. All bactericidal assays were performed at least twice (three times for most strains, and six times or more for some strains).

RESULTS

Characteristics of strains analyzed. The 63 *N. meningitidis* strains of serogroup B analyzed were assigned to 59 different electrophoretic types, 10 serotypes, and 8 subtypes. The strains were genetically diverse, and all major clone complexes of serogroup B strains causing systemic disease (9, 10, 52) were represented: ET-5 complex (seven strains), ET-37 complex (four strains), cluster A4 (eight strains), and lineage III (two strains) (Table 1).

The ability to express Tbps when cultured under iron starvation conditions was measured by a dot blot assay on whole cells (Table 1). All these strains expressed Tbps with an average titer of 128, with the level of expression ranging from 16 (one strain, NG6/88) to 512 (strains 106, NGPB24, 84250, 344, 349, 126, M982, and 313). To evaluate the reproducibility of determining Tbp expression, the titer was measured four times for 10 strains. The expression was quite stable, with ±1 dilution accuracy. The level of expression varied among genetically related strains: for example, the expression titer ranged from 64 to 512 among the seven strains belonging to the ET-5 complex.

The *tbpB* gene size of all these strains was determined after amplification by PCR. A PCR product was obtained for all the strains, and the *tbpB* gene size was either 1.8 kb (5 strains) or 2.1 kb for (58 strains) (Table 1); the two distinct gene sizes were in agreement with the identification of two major families of strains based on *tbpB* gene size (31, 39). Four of the five strains with a 1.8-kb *tbpB* gene belonged to the ET-37 complex (strains B16B6, NGP139, 2713, and 344). The only exception was strain 106, isolated in Japan.

Characterization of recombinant TbpB and antisera. In an attempt to produce tailored TbpB molecules that were able to induce broadly cross-reactive antisera, three variant forms of TbpB from strain M982 were produced in *E. coli*: one referred to as rTbpB-100% (full-length TbpB molecule, aa 1 to 691), one referred to as rTbpB-80% (aa 1 to 691 with aa 351 to 379, 415 to 444, 465 to 481 and 500 to 520 deleted), and the third referred to as rTbpB-53% (aa 1 to 350) corresponding to the minimal hTf-binding domain as defined by Vonder Haar et al. (51). The three recombinant TbpB molecules were purified to homogeneity by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). All three proteins were able to bind hTf on Western blots (Fig. 1A). The position of TbpB of *N. meningitidis* M982 was visualized on a Western blot with hTf (Fig. 1A, lane 4). The purified proteins were used to immunize rabbits, and the immune sera obtained were assessed for their specificity on outer membrane proteins of M982 grown under iron starvation; all the sera specifically recognized TbpB of strain M982 and no other outer membrane protein (Fig. 1B).

To illustrate the epitope specificity of the antisera, their reactivity was analyzed by the Pepscan technique with heptapeptides synthesized on pins. As shown in Fig. 2, antisera raised against the full-length TbpB (anti-rTbpB 100%) recognized almost all the linear epitopes across the whole protein (Fig. 2B). This reactivity was specific, since antisera raised against proteins from a mock purification (anti-*E. coli* control) showed almost no reactivity against the same peptides (Fig. 2A). Antisera against the deleted TbpB (anti-rTbpB-80%) showed similar reactivity against the heptapeptides throughout the protein (Fig. 2C), but the reactivity was weaker than that observed for rTbpB-100%. When protein was truncated at the C terminus (rTbpB-53%), no antibodies were generated against peptides localized at the C-terminal half of the molecule as expected. However, instead of having strong reactivity

TABLE 1. Characteristics of the *N. meningitidis* group B strains analyzed

Strain[a]	Clonal group[b]	Serogroup type, subtype	Tbp expression[c]	*tbpB* gene size (kb)[d]
106		B:NT:P1.5	512	1.8
NGH36		B:8:P1.2	128	2.1
EG329	ET-5 complex	B:15:P1.6	128	2.1
BZ83*	ET-5 complex	B:15:−	128	2.1
NGBP37*	ET-5 complex	B:15:P1.7,16	64	2.1
8680*	ET-5 complex	B:15:P1.3	128	2.1
NGP355*	ET-5 complex	B:15:P1.15	256	2.1
NGPB24	ET-5 complex	B:21:P1.7,16	512	2.1
84250	ET-5 complex	B:15:P1.16	512	2.1
528		B:ND	128	2.1
EG328*		B:NT:−	64	2.1
M692		B:4:P1.15	256	1.8
BZ133		B:NT:−	128	2.1
1000*		B:ND	256	2.1
NGH15		B:8:P1.15	256	2.1
315		B:4:P1.15	64	2.1
NGH38*		B:NT:P1.3	128	2.1
EG011		B:NT:P1.3	64	2.1
B16B6*	ET-37 complex	B:2a:P1.2	128	1.8
NGP139	ET-37 complex	B:2a:P1.2	32	1.8
2713	ET-37 complex	B:NT:P1.2	64	1.8
344	ET-37 complex	B:ND	512	1.8
DK353		B:ND	128	2.1
BZ232		B:NT:P1.2	128	2.1
568		B:ND	64	2.1
M918		B:15:−	128	2.1
M912		B:4:−	128	2.1
349		B:NT:−	512	2.1
M870		B:NT:−	128	2.1
NGF26		B:NT:P1.16	128	2.1
NG6/88		B:NT:P1.1	16	2.1
130		B:NT:P1.14	128	2.1
DK24		B:ND	128	2.1
SWZ107*		B:4:−	128	2.1
NGE31		B:NT:−	256	2.1
3839		B:NT:P1.15	256	2.1
SP111/88		B:8:−	256	2.1
351*		B:NT:P1.7	256	2.1
NGE28		B:4:−	256	2.1
NGG40*		B:1:−	128	2.1
NG4/88		B:4:−	128	2.1
NGH41*	Cluster A4	B:NT:−	256	2.1
BZ163	Cluster A4	B:2b:P1.16	128	2.1
13763	Cluster A4	B:ND	32	2.1
BZ162	Cluster A4	B:2b:P1.2	128	2.1
BZ157*	Cluster A4	B:2b:P1.2	64	2.1
3906	Cluster A4	B:4:−	256	2.1
NGE30	Cluster A4	B:4:P1.16	64	2.1
BZ159	Cluster A4	B:2b:P1.2	64	2.1
NG3/88		B:8:P1.1	256	2.1
BZ147		B:15:−	256	2.1
BZ47		B:NT:−	64	2.1
126		B:NT:−	512	2.1
EG327		B:NT:−	64	2.1
BZ138	Lineage III	B:15:−	128	2.1
BZ198	Lineage III	B:15:−	256	2.1
M982*		B:9:P1.9	512	2.1
297-0		B:4:P1.15	128	2.1
313		B:NT:−	512	2.1
6940*		B:19:P1.6	64	2.1
M978*		B:8:P1.1	64	2.1
S3032		B:12:P1.12	64	2.1
SP160/88		B:8:−	256	2.1

[a] The strains are ordered according to their genetic similarities based on the analysis of 14 gene loci encoding metabolic enzymes. The strains used in the bactericidal assay are indicated by an asterisk.

[b] Strains belonging to the four major epidemiological clonal complex (ET-5, ET-37, cluster A4, and lineage III) are indicated.

[c] Transferrin-binding protein expression was determined by serially twofold diluting a bacterial suspension (initial concentration, 2×10^9 CFU/ml). Then 50 μl of each dilution was spotted onto nitrocellulose followed by an incubation with hTf conjugated to peroxidase (1 μg/ml). The titer of Tbp expression was defined as the inverse of the last dilution of bacteria able to bind transferrin.

[d] The size of *tbpB* gene was determined after PCR amplification.

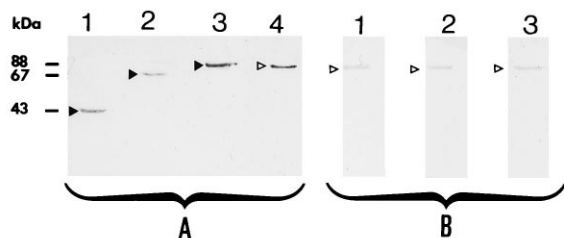

FIG. 1. Western blot characterization of rTbpB M982 proteins and rTbpB-specific antisera. (A) Outer membrane proteins from *E. coli* cells expressing rTbpB-53% (43 kDa) (lane 1), rTbpB-80% (67 kDa) (lane 2), and rTbpB-100% (88 kDa) (lane 3) and outer membrane proteins from iron-starved *N. meningitidis* M982 (lane 4) were separated on homogeneous 10% polyacrylamide gel, transferred onto nitrocellulose, and then incubated with hTf conjugated to peroxidase at 100 μg/ml. (B) Outer membrane proteins from iron-starved *N. meningitidis* M982 were also incubated with polyclonal rabbit antiserum (dilution, 1/500) raised either to rTbpB-100% (lane 1), rTbpB-80% (lane 2), or rTbpB-53% (lane 3). Nitrocellulose strips from panel B were incubated with a goat-anti rabbit IgG-horseradish peroxidase conjugate (1/1,000; Zymed). Solid and open triangles indicate the positions of rTbpB and TbpB, respectively, from strain M982.

against peptides across the N-terminal half of the protein, only distinct linear epitopes were identified (Fig. 2D). Since the Pepscan technique measures only linear epitopes, the amount and the importance of conformational epitopes present in the sera would have to be measured by other biological tests.

Reactivity of anti-rTbpB antisera with the different meningococcal strains. The cross-reactivity of sera raised against rTbpB-100%, rTbpB-80%, or rTbpB-53% was analyzed against the 58 strains whose TbpB is encoded by a 2.1-kb gene. To compare the reactivity of each antiserum, the level of expression of the hTf receptor was determined for each strain. Typical results with four strains are given in Fig. 3. The reactivity of the antiserum raised against the negative control (Fig. 3, lanes 2) was low for all the strains tested. Moreover, although all the strains expressed the hTf receptor (lanes 1), some of them, like 8680 and NGH41, did not react with serum raised against rTbpB-100% (lanes 3). In contrast, strain NGH41 reacted with the sera raised against rTbpB-80% and rTbpB-53% (lanes 4 and 5). To quantify the cross-reactivity of each antiserum against the different strains, it was necessary to normalize the titers obtained with sera by the hTf-binding titer of each strain. This normalized value (titer of antiserum/hTf-binding titer) allowed the comparison of strains with different hTf-binding levels. The results obtained for all the 58 strains show that 15 strains of the 58 tested did not react with the anti-rTbpB-100% serum (normalized value, 0) while 5 did not react with the anti-rTbpB-80% serum and only 1 failed to react with the anti-rTbpB-53% serum. These results indicate that the cross-reactivity of an antiserum raised against a M982-rTbpB protein can be increased when parts of the molecule are deleted. The only strain that was not recognized by the anti-rTbpB-53% was strain 8680 from Chile (which belongs to the ET-5 complex) (lanes 5). The absence of reactivity was observed in three independent experiments, and, furthermore, this strain was not recognized by either anti-rTbpB-100% or anti-rTbpB-80%. Six other strains from the ET-5 complex isolated in Europe and Australia were tested in this study (strains

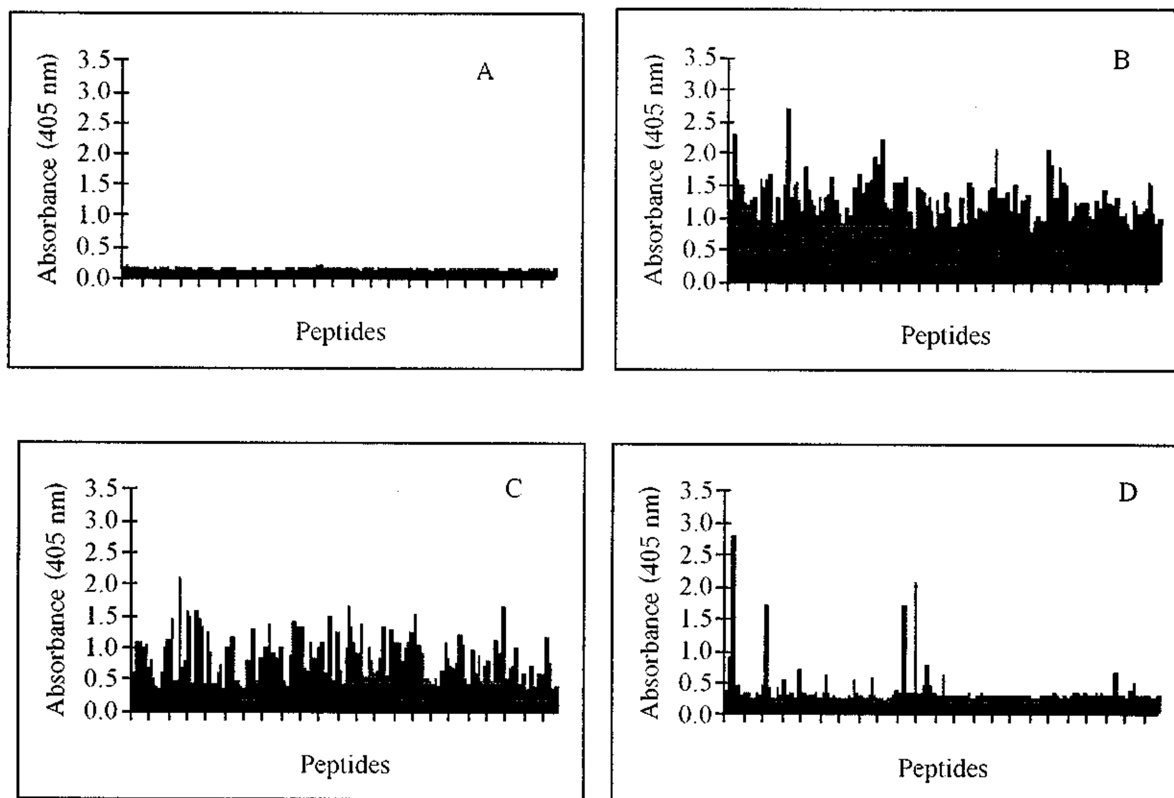

FIG. 2. ELISA reactivities of polyclonal antisera raised to TbpB versus the 7-mer overlapping peptides spanning the whole TbpB M982. The histograms show the Pepscan analysis of rabbit polyclonal antisera raised against *E. coli* control protein (A), full-length TbpB (rTbp2-100%) (B), TbpB in which four stretches in the central domain were deleted (rTbpB-80%) (C), or the N-terminal part of TbpB (rTbpB-53%) (D). The absorbance at 405 nm is plotted for each 7-mer peptide spanning the total TbpB M982 protein.

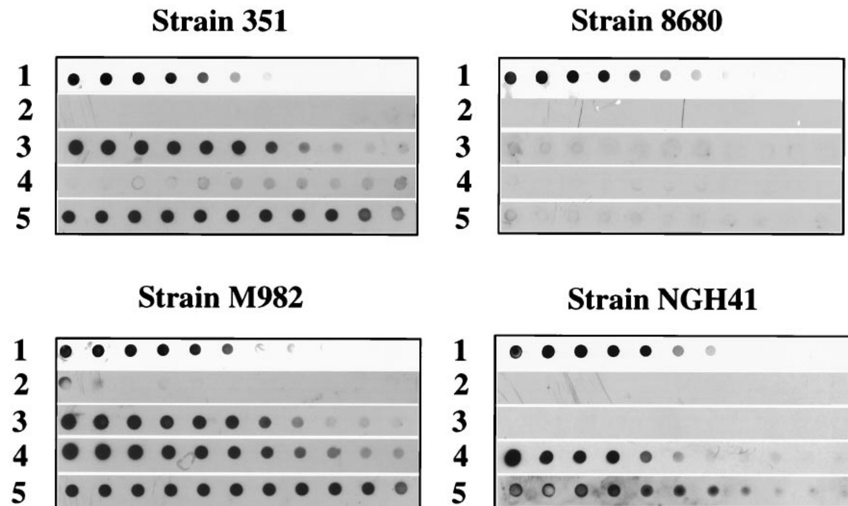

FIG. 3. Reactivity of anti-rTbpB M982 sera on different strains of *N. meningitidis*. Iron-starved whole cells were prepared from group B *N. meningitidis* M982, 351, 8680, and NGH411. Twofold serial dilutions (ranging from 1×10^8 to 5×10^2 CFU) were spotted onto five different nitrocellulose filters. The filters were incubated with hTf-peroxidase (1 μg/ml) (lane 1), a negative *E. coli* control serum (lane 2), anti-rTbpB-100% (lane 3), anti-rTbpB-80% (lane 4), and anti-rTbpB-53% (lane 5). The dilution of sera was 1/2,000. The filters corresponding to lanes 2 to 5 were then incubated with a goat-anti rabbit IgG horseradish peroxidase conjugate (1/1,000; Zymed). The substrate of peroxidase was either 4-chloro-1-naphthol (lanes 1) or chemoluminescent ECL substrate (Amersham) (lanes 2 to 5).

EG 329, BZ83, NGPB37, NGP355, NGPB24, and 84250), and they were all recognized by anti-rTbpB-80% and anti-rTbpB-53%. This observation indicates that the ET-5 complex is heterogeneous as far as TbpB proteins are concerned.

Bactericidal activity of anti-recombinant TbpB antisera. Purified IgGs corresponding to sera raised against the three variants of rTbpB and to control sera were tested for their ability to kill the homologous strain M982 and 14 strains whose TbpB is encoded by a 2.1-kb gene (Table 2). These strains were chosen from the 58 available strains on the basis of their level of reactivity with three anti-rTbpB sera in order to represent a large range of reactivity as determined by the dot-blot assay (normalized value, 0 to 2). Strain B16B6 was included as a negative control representative of strains having a 1.8-kb *tbpB* gene. All strains were tested for their sensitivity to complement alone. For some strains like NGPB37 or 8680, complement

TABLE 2. Bactericidal activity of purified IgG specific for different forms of rTbpB M982 and Tbp complex against group B *N. meningitidis*

Strain[a]	Characteristic of strains				Bactericidal titer[d]						
	Dot blot reactivity[b]			Complement sensitivity[c]	Anti-88 kDa-protein		Anti-67-kDa protein		Anti-43-kDa protein		Anti-Tbps complex
	Anti-rTbp2-100%	Anti-rTbp2-80%	Anti-rTbp2-53%		Control	rTbp2-100%	Control	rTbp2-80%	Control	rTbp2-53%	
BZ83	0.031	0.125	0.5	30	(8)	32	<4	<4	<4	(8)	128
NGPB37	0.031	0.0625	0.25	**35**	<4	128	<4	<4	8	8	256
8680	0	0	0	**51**	<4	<4	<4	<4	<4	<4	<4
NGP355	0	1	0.25	**26**	<4	<4	<4	<4	<4	<4	128
EG328	0.5	1	1	15	<4	16	<4	16	<4	64	<4
1000	0.25	0.5	2	25	<4	64	<4	8	<4	<4	32
NGH38	0.25	0.125	0.031	17	<4	256	<4	32	<4	<4	32
B16B6	0	0	0	0	<4	<4	<4	<4	<4	<4	<4
SWZ107	1	0.0625	0.5	30	<4	(64)	<4	8	<4	<4	8
351	0.5	0	2	15	<4	64	<4	128	<4	256	<4
NGG40	0.5	0.25	0.5	**17**	<4	64	<4	64	<4	512	<4
NGH41	0	1	0.25	20	<4	64	<4	16	<4	(8)	32
BZ157	0	0	1	12	<4	<4	<4	<4	<4	<4	<4
M982	1	0.5	1	0	<4	256	<4	32	<4	256	512
6940	0.0625	0.5	0.25	**8**	<4	16	<4	16	<4	64	ND[e]
M978	0.5	0.25	0.125	0	<4	32	<4	8	<4	8	8

[a] Strains were ordered as in Table 1 (strains with an asterisk).

[b] The values of the dot blot reactivity of sera raised against rTbpB-100%, rTbpB-80%, or rTbpB-53% correspond to normalized values (titer of antiserum/hTf-binding titer).

[c] The sensitivity of strains to complement corresponds to the percent reduction in the viable count when only complement without test serum is added. Boldface indicates sensitivity of strains to complement after adsorption of the complement on the homologous strain grown with iron.

[d] Bactericidal titers are expressed as the last dilution of purified IgG in the presence of which the viable count was reduced by 50% compared to the complement control. Titers are given for a control serum which is raised against a protein of *E. coli* with the same molecular mass as the purified rTbpB (control) and for sera raised against rTbpB-100% (anti-88 kDa), rTbpB-80% (anti-67 kDa), and rTbpB-53% (anti-43 kDa). Bactericidal titers are also given for purified IgG raised against Tbp complex purified from strain M982. Numbers in parentheses correspond to incomplete killing, meaning that whatever the dilution of serum used, the bactericidal effect was not total: a few colonies were observed with lower serum dilutions. Protein concentrations in IgG solution ranged from 8 to 15 mg/ml.

[e] ND, not done.

killing was high and was not reduced by adsorbtion of the complement on the strains grown with iron. Because whole rabbit sera present nonspecific bactericidal activity, IgGs were purified from each serum on a protein-G Sepharose column.

The results presented in Table 2 show the following. First, purified IgGs from the control sera (anti-88 kDa, anti-67 kDa, and anti-43 kDa) were not bactericidal against all the strains tested. Second, all three antisera specific for rTbpB-100%, rTbpB-80%, or rTbpB-53% were bactericidal for the parent strain (M982), demonstrating that the purification procedure used (preparative SDS-PAGE) allowed us to produce the recombinant molecules in a conformation able to induce bactericidal antibodies. It should be noted that when denatured TbpB (denatured by 30 min of boiling in the presence of urea as described by Vonder Haar et al. [51]) is used to immunize animals, specific antibodies are induced but the antisera do not display bactericidal antibodies against the parent strain (data not shown). Third, the IgG corresponding to rTbpB-100% killed 12 of 15 strains tested; 10 of 15 strains were killed with anti-TbpB 80%, and 9 of 15 were killed with anti-rTbpB-50%. None of the sera killed the B16B6 strain, as expected. One of two strains of the A4 cluster (NGH41) was killed by all the sera, while the other one (BZ157) was killed by none of them; these two strains were recognized differently by the antisera in a dot blot assay. Strain 8680 (ET-5 complex strain), which was not recognized by any of the sera in a dot blot assay, was not killed by any of these sera. Together, these results indicate that a serum raised against the full-length TbpB (rTbpB-100%) induces a large cross-bactericidal activity. This activity was reduced if the TbpB protein was truncated. Strain 8680 expressed a TbpB that seemed, on the basis of the two assays used in this study, to be different from the M982 TbpB. Moreover, this protein was also different from the B16B6 TbpB, because an anti-TbpB from B16B6 did not kill strain 8680 (data not shown).

Comparison of the bactericidal activity of antisera raised to recombinant TbpB and to the meningococcal TbpA-TbpB complex. The bactericidal activity of IgG purified from an antiserum raised to the Tbp complex isolated from *N. meningitidis* M982 was determined on 14 of the 15 strains selected. The results (Table 2) show that the IgG preparation was bactericidal for the homologous strain M982 and for 9 of the 14 strains tested expressing a TbpB of approximately 85 kDa. As expected, the IgG preparation did not kill strain B16B6 (TbpB of 68 kDa); this result was consistent with previous data showing that the specific antiserum to TbpB M982 did not react with TbpB B16B6 and vice versa (40). Of the five strains with a 2.1-kb *tbpB* gene resistant to killing with an anti-Tbp complex, two (strains 8680 and BZ157) were also resistant to killing by rTbpB antisera. These strains appear as true TbpB variants, although this remains to be demonstrated by sequencing their *tbpB* gene. The other three strains resistant to killing with the anti-TbpB complex (strains 351, EG328, and NGG40) were killed with all three rTbpB antisera. This result suggests that antibodies directed to bactericidal epitopes present in the N-terminal half of TbpB molecules may be more abundant in sera obtained by immunization with rTbpB rather than immunization with the complex. Finally, one strain (strain NGP355) was not killed by the anti-rTbpB antisera, while the anti-Tbp complex was bactericidal against this strain. To investigate whether anti-TbpA response could contribute to this cross-reactivity, we tested the bactericidal activity of anti-meningococcal TbpB on this strain; this serum also killed strain NGP355 (titer, 512), suggesting that TbpA antibodies were not responsible for the differences observed between anti-Tbp complex and anti-rTbpB (data not shown). This result suggests that some important conformational epitopes may have been destroyed during the purification process of rTbpB, although this seems specific to this particular strain.

Overall, it appeared that anti-recombinant TbpB-100% serum was more cross-bactericidal than anti-Tbp complex (80% versus 60% of strains killed).

DISCUSSION

As recently reviewed (1), Tbps from meningococci have been proposed as good vaccine candidates (2, 13, 19, 28), although TbpB has been reported to be variable among strains (16, 17, 20, 40). To be a good vaccine candidate, these proteins should induce broadly cross-reactive antibodies to many different isolates, and this remains somewhat controversial. In our laboratory, we have shown that meningococcal strains could be classified into two families (B16B6-like strains displaying a 1.8-kb *tbpB* gene, and M982-like strains displaying a 2.1-kb *tbpB* gene), based on the antigenic and genomic features of TbpB (40). It was demonstrated that an antiserum raised to a TbpA-TbpB complex composed of a 68-kDa TbpB can kill, in the presence of complement, strains which express a 68-kDa TbpB but not strains which express TbpB with a higher molecular mass (13). This was contradicted recently by Ala'Aldeen and Borriello, who have demonstrated that an antiserum raised to TbpA-TbpB complex from strain SD was broadly cross-reactive in the bactericidal assay against different strains expressing either a 68- or an 85-kDa TbpB (2). In the present study, the TbpA-TbpB complex was purified from *N. meningitidis* M982 and specific antiserum was produced; this antiserum was bactericidal to the parent strain but not to strain B16B6 (Table 2), in agreement with our previous results demonstrating the absence of cross-reactivity between TbpB of the two strains (13, 40); it remains possible that the affinity purification scheme used to purify the TbpA-TbpB complex partly denatured important epitopes, as suggested previously (4, 28). Within the strains displaying a TbpB whose molecular mass is in the range of 85 kDa, a specific antiserum raised to the TbpA-TbpB complex isolated from strain M982 yielded only partial cross-bactericidal activity on the different strains tested (Table 2, right-hand column: 9 strains were killed of 14 tested). This result was comparable to what has been described when pigs were immunized with recombinant TbpB from *A. pleuropneumoniae*, where protection was observed against a challenge with *A. pleuropneumoniae* serotype 7 and not with serotype 1 (42). Nevertheless, to determine if this cross-reactivity could be increased and at the same time to develop a scheme to make TbpB in quantities compatible with an eventual vaccine development, we produced TbpB in *E. coli*. The production of recombinant TbpB from *N. meningitidis*, *A. pleuropneumoniae*, and *H. influenzae* in *E. coli* has been described previously and was shown to yield proteins that retained the ability either to bind hTf or to induce protective antibodies in animals (18, 26, 29).

Full-length lipidated TbpB from strain M982 (aa 1 to 691), referred to as rTbpB-100%, was produced in *E. coli* by using the arabinose-inducible expression vector (26) along with two other variants designed to determine which part of TbpB molecule is essential in the induction of cross-bactericidal antibodies. One variant represented approximately the N-terminal half of the protein (aa 1 to 350), referred to as rTbpB-53%, corresponds to the minimal hTf-binding domain (51). This variant was totally devoid of the central immunodominant, hypervariable region (39). The N-terminal domain is thought to be surface exposed since it binds hTf. Because we are aware that TbpB is a highly structured protein (51) and is likely to be symmetrical, organized in two independent lobes as suggested

by Mazarin et al. (31), we suspected that totally removing the C-terminal half might influence the conformation of the N-terminal half and modify important epitopes. Therefore, we designed another variant in which the N-terminal half was present and where the central region had four stretches, based on alignments between TbpB M982 and B16B6, deleted (25). The rationale for this approach was that the absence of these stretches in TbpB B16B6 did not alter the iron acquisition pathway in this strain. The choice of these deletions may be questioned, since we have subsequently generated more sequence data for the central part of TbpB molecules (39), which suggest different deletion strategies for this central region. The three variants, rTbpB-100%, rTbpB-80%, and rTbpB-53%, were produced in a lipidated form in *E. coli* and purified by preparative SDS-PAGE. Such a denaturing method of purification was chosen because it has been shown that TbpB retains a highly structured conformation that confers the hTf-binding specificity even after SDS-PAGE and Western blotting (51). Moreover, preparative SDS-PAGE allowed us to use the same purification scheme for all rTbpB variants produced while more conventional chromatography methods would have involved slightly different processes, and such differences may have influenced the results obtained. Specific antisera were produced to the proteins as well as to products obtained from mock purifications of control culture for each variant. All three proteins were able to bind hTf (Fig. 1A), and the antisera were all able to kill the parent strain in the presence of complement in the bactericidal assay (Table 2). The fact that the antisera were bactericidal against the homologous strain again shows that important conformational epitopes were maintained in the rTbpB proteins purified by preparative SDS-PAGE.

To address the key question of broad cross-reactivity among different strains, representative strains of serogroup B *N. meningitidis* were collected based on their different multilocus genotypes. These strains were representative of serogroup B meningococci (45). Most serogroup B disease isolates from outbreaks have been assigned to four genetically distinct lineages, cluster A4, lineage III, the ET-37 complex, and the ET-5 complex (8–10, 52), and these four lineages were represented in the collection used in this study. The strains were genetically diverse and originated from different countries and continents. All the isolates analyzed expressed hTf-binding activity. Only five strains displayed a *tbpB* gene of 1.8 kb, and all four strains of the ET-37 complex in our collection had a 1.8-kb *tbpB* gene. Further studies on a larger number of ET-37 complex strains will be required to assess whether all the ET-37 complex strains express a 67-kDa TbpB protein like strain B16B6. The 58 strains displaying a 2.1-kb *tbpB* gene were further studied for their ability to react with the different antisera in a dot blot assay. The dot blot assay was developed to allow the study of many isolates and to maintain the outer membranes in a state as native as possible. In this particular solid-phase assay, the bacteria were exposed only to air drying, in contrast to the solid-phase binding assay used for serotyping and subtyping, which uses heat inactivation (38). In parallel, the bactericidal assay was performed on 15 strains with different dot reactivity; as most isolates were sensitive to whole rabbit serum, IgG had to be purified from the antiserum. When the reactivity of the antisera was compared in both types of assays, the results were apparently contradictory. The antiserum raised to the N-terminal hTf-binding domain (rTbpB-53%) reacted with 57 of 58 strains tested in the dot blot assay but killed only 9 of 15 strains in the bactericidal test. Inversely, the antiserum raised to the full-length TbpB (rTbpB-100%) reacted with 46 of 58 strains in the dot blot assay but killed 12 of 15 strains tested. In both types of assays, the antiserum raised to rTbp-80% gave intermediate results. The difference in the results may occur because in the dot blot assay, which is done in the solid phase and includes several washing steps, only high-affinity antibodies may be detected. Moreover, it cannot be excluded that the drying process influences the conformation of TbpB at the surface, as was suggested by Cornelissen and Sparling when studying mutants, i.e., that TbpB in the absence of TbpA was more susceptible to the drying process used in the dot assay (12). In the bactericidal assay, bacterial cells were incubated directly with the Igs, and it is very likely that even low-affinity IgG would interact with the meningococci.

The absence of correlation between the two assays is evident if one looks at positive reactivity, i.e., the number of strains reacting in a dot-blot assay versus the number of strains killed in the bactericidal assay. However, if one looks at nonresponders, i.e., strains not reacting in the dot blot assay and not killed in the bactericidal assay, a good correlation is observed. Strains not reacting in the dot blot assay with the three rTbpB antisera (strains 8680 and B16B6) or with both anti-rTbpB-100% and anti-rTbpB-80% (strain BZ157) were not killed by any M982-derived Tbp-specific antiserum, whether antigens used to produce the antisera were recombinant or extracted from meningococcal cells (Table 2). If this observation is extended to the entire collection of strains studied, it would appear that theoretically, 5 strains of the 58 exhibit such behavior and should be resistant in the bactericidal assay (strains 315, 8680, BZ157, NGE28, and NGPB24). However, when reactivity in the dot-blot assay is absent with only one of the three sera, the sensitivity in the bactericidal assay is difficult to predict. The absence of correlation between the bactericidal activity of an antiserum specific for the N-terminal hTf-binding domain and its reactivity among strains suggests that the presence of the C-terminal domain in the immunizing antigen may be important for an antiserum to the complement-mediated killing. Two hypotheses can be proposed to explain the role of the C-terminal domain: either antibodies directed to epitopes in the C-terminal domain are themselves bactericidal, or the C-terminal domain contributes to maintaining the structure of the N-terminal domain and conformational epitopes in the N-terminal domain are required to induce bactericidal antibodies.

Moreover, we should note that among ET-5 complex and A4 cluster strains, two of four and one of two, respectively, were killed with the antiserum raised to TbpB, indicating that TbpB proteins may be heterogeneous among these clusters. Further sequence data may be required to see whether some link may be found between susceptibility to anti-TbpB antiserum and *tbpB* nucleotide sequence variability. Notably, the ET-5 complex strain 8680 (B:15:P1.3), isolated in Chile in 1987, failed to react with all three antisera in the two assays used. This strain will be further analyzed, and its TbpB protein will be characterized.

Overall, in this study, we have shown that a full-length recombinant TbpB from strain M982 produced in *E. coli* with yields compatible with industrial development was able to induce bactericidal antibodies against 80% of the strains tested. Antisera specific for recombinant TbpB seemed to be at least as cross-reactive as an antiserum specific to the TbpA-TbpB complex. In the course of this study, some heterogeneity was observed among strains of the ET-5 complex and of cluster A4. Specific studies of TbpB among strains of these particular complexes are under way in our laboratory to define more precisely the extent of the variability.

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