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Evaluation of Transferrin-Binding Protein 2 within the Transferrin-Binding Protein Complex as a Potential Antigen for Future Meningococcal Vaccines

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Because the meningococcal transferrin receptor was shown to elicit bactericidal and protective antibodies in laboratory animals, we undertook a study of the protective role of each of the polypeptides within the Tbp1-Tbp2 complex. We developed a procedure to purify from *Neisseria meningitidis* B16B6 the two proteins in milligram amounts and raised specific antisera in rabbits and mice. Only antisera specific for Tbp2 displayed bactericidal activity against the parent strain. Mice immunized with purified Tbp2 survived a lethal challenge to a similar degree as animals immunized with the Tbp1-Tbp2 complex, demonstrating that Tbp2 played an important role in the protective activity observed with the complex. Both Tbp1- and Tbp2-specific antisera inhibited transferrin binding to the purified receptor in a solid-phase binding assay, suggesting that the antibodies were able to interact with the Tbp1 molecule only when it was removed from its membrane environment. Finally, Tbp2-specific immunoglobulins were able to lower the growth rate of the meningococci when human transferrin was their sole iron source. Therefore, in all four different systems tested, Tbp2 or antibodies specific for Tbp2 displayed biological characteristics close to those of the Tbp1-Tbp2 complex. This suggests that Tbp2 plays an important role in the protective activity of the complex, eliciting antibodies that are not only bactericidal but also inhibitory for meningococcal growth.

Meningitis and meningococcemia caused by *Neisseria meningitidis* continue to be a serious health problem worldwide. The development of vaccines effective against group B meningococci, which is responsible for most of the cases in North America and Europe, would be a major breakthrough in the control and prevention of bacterial meningitis. Aside from more conventional vaccine approaches, including capsular polysaccharide and major outer membrane proteins, there has been growing interest in the inclusion of iron-regulated proteins in future meningococcal vaccines (4). Among iron-regulated proteins, particular interest has been given to transferrin-binding proteins (Tbps). Unlike other gram-negative bacteria such as *Escherichia coli* and *Shigella* and *Salmonella* spp., which acquire essential iron by siderophore-mediated transport systems (6, 9, 26, 29, 31), *N. meningitidis* does not produce siderophores (39, 45). Meningococci acquire iron via a different mechanism which may involve, as a first step, binding of transferrin to the bacterial cells, as shown by earlier studies, direct contact is required between the transferrin and the bacteria (11, 39, 41). The electron microscopy study of Ala'Aldeen et al. demonstrated conclusively that gold-labelled human transferrin (hTf) bound to the surface of meningococci (1). The binding is mediated by a receptor involving two proteins, Tbp1 and Tbp2 (27, 35, 37). To evaluate the potential of the Tbps as meningococcal vaccine candidates, milligram amounts of protein are required. The purification scheme described by Schryvers (34) consistently yields the two polypeptides but in a presumably complexed form. The complex Tbp1-Tbp2 elicited bactericidal and protective antibodies in laboratory animals (10). Two main questions remain. (i) Could both polypeptides confer protection, and, if not, which of the two induces protective antibodies? (ii) What mechanism(s) is involved in protection—simple clearance by common opsonophagocytosis along with complement-mediated killing or interference of the antibodies by iron acquisition, thereby inhibiting or limiting bacterial growth?

We report in the present study a procedure for purifying Tbp1 and Tbp2 independently in sufficient quantities and the evaluation of their immunological properties. The antisera raised against the purified proteins were evaluated for their capacity to mediate killing in the presence of complement, to inhibit transferrin binding to the receptor, and to inhibit bacterial growth. Finally, the protective activity of the protein in mice was evaluated.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *N. meningitidis* B16B6 (B:2a:P1.2) was originally obtained from C. Frasch (Office of Biologics, Bethesda, Md.) and stored lyophilized in our collection. Unless otherwise stated, the strain was grown at 37°C in Mueller-Hinton broth containing 30 μM EDDHA [ethylenediamine di(*o*-hydroxyphenylacetic acid); Sigma].

Protein purification. The Tbp complex was purified from the membrane fraction of *N. meningitidis* as described previously (10). The purified Tbp complex in 50 mM Tris-HCl buffer (pH 8.0) containing 1 M NaCl, 10 mM EDTA, 0.05% Sarkosyl (*N*-lauroylsarcosine; Sigma), and 2 M guanidine hydrochloride was precipitated by the addition of ethanol to 75% (vol/vol) final concentration, centrifuged, and resuspended in 10 mM $NaPO_4$ (pH 7.0) containing 0.5 M NaCl and 5 M guanidine hydrochloride (buffer I). After incubation at 4°C, the mixture was loaded on a phenyl-Sepharose column (Pharmacia) preequilibrated with buffer I. Tbp1 was collected in the flowthrough fraction. After complete elution of Tbp1 by washing the column with buffer I, the column was washed with 10 mM $NaPO_4$ (pH 7.0) to remove any trace amount of guanidine hydrochloride. Tbp2 was eluted with 10 mM $NaPO_4$ containing 0.5% Sarkosyl. Phenylmethylsulfonyl flouride (100 μM) was present in all buffers throughout the purification. Purified Tbp1 and Tbp2 were concentrated, dialyzed, and stored in 100 mM $NaCO_3$ (pH 10.0) containing 0.05% Tween 80 and in 10 mM $NaPO_4$ (pH 7.0) containing 0.05% Sarkosyl, respectively.

Protein characterization. The purified Tbp1 and Tbp2 were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with the buffer system of Laemmli (21). The gel was stained with Coomassie brilliant blue.

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Western blot (immunoblot) was performed on a Multiphore II system (Pharmacia), and the nitrocellulose paper was immunoblotted with either specific anti-Tbp antisera or hTf-peroxidase (Jackson Immunoresearch Laboratory as described previously (10). hTf-binding activity of purified Tbp1 and Tbp2 was also measured on dot blots by simply spotting serially diluted protein solutions onto nitrocellulose. The nitrocellulose was blocked, washed, and incubated with peroxidase-conjugated hTf as described previously (32). Protein concentration was determined by the micro BCA method (bicinchoninic acid protein assay; Pierce) with bovine serum albumin (BSA) as the standard.

Preparation of immune sera and purification of immunoglobulin G (IgG). New Zealand White rabbits were inoculated by subcutaneous and intramuscular routes with three injections of the Tbp complex, Tbp1, or Tbp2 in Freund's adjuvant on days 0, 21, and 42. On day 57, the animals were sacrificed and blood was collected. To ensure their specificity, the antisera were adsorbed onto meningococci grown in the presence of iron as described previously (10). For the bacterial growth experiment, antibodies were affinity purified on protein G-Sepharose 4FF columns (Pharmacia) (19). To produce monospecific mouse sera to Tbp1 and Tbp2, groups of CD1 female mice 6 weeks of age were immunized subcutaneously on days 0 and 21 with different amounts of the purified proteins adsorbed onto aluminum hydroxide (1.5, 6.25, and 25 μg adsorbed onto 100 μg of Al per mouse per injection). Blood was drawn on day 35, and the bactericidal activity of the sera and the specific IgG titers were determined.

Bactericidal assay. The bactericidal activity of rabbit sera was tested as described previously (10). The bactericidal activity of mouse sera was tested in a slightly different system developed to enhance the sensitivity of the assay. The bactericidal activity of mouse sera was tested in 96-well microplates (Nunc). One hundred microliters of serial twofold serum dilutions was incubated with 50 μl of an iron-starved meningococcal suspension adjusted to 10^4 CFU ml^{-1} and 50 μl of baby rabbit complement. After 1 h of incubation at 37°C, 25 μl of the mixture from each well was spread onto Mueller-Hinton agar plates. The plates were incubated overnight at 37°C in 10% CO_2. The colonies were counted, and the bactericidal titer of each serum was expressed as the last dilution of serum at which 50% or greater killing was observed compared with the complement control.

Inhibition of hTf binding to receptor. For binding assays, 96-well plates (Dynatech M129B) were coated with 100 μl of a Tbp solution per well at 6 μg/ml in 50 mM carbonate buffer (pH 9.6). The plates were incubated overnight at 28°C and washed with buffer A (phosphate-buffered saline [PBS] containing 0.05% Tween 20). The wells were saturated by the addition of 250 μl of buffer B (PBS containing 1% BSA), incubated for 60 min at room temperature, and washed with buffer A. Horseradish peroxidase-conjugated hTf (HRP-hTf; Jackson Immunoresearch Laboratory) was serially diluted in buffer C (PBS containing 0.05% Tween 20 and 0.1% BSA), and 100 μl of each dilution ranging from 10^{-6} to 10^{-2} mg ml^{-1} was added to the wells. Each dilution was tested in duplicate. The plates were incubated for 60 min at 37°C and washed with buffer A. The reaction was developed by the addition of 100 μl of substrate solution (100 mM citrate buffer [pH 4.5] containing 1 mg of *o*-phenylenediamine ml^{-1} and 3.5 μM H_2O_2). The reaction was stopped after 20 min of incubation at 28°C by the addition of 50 μl of 10% SDS. The A_{490} was measured, and the concentration of HRP-hTf giving an absorbance of 1.5 (linear portion of the saturation curve) was determined and used for the following inhibition tests. To measure inhibition of binding, the assay was performed as described except that after the coating and washes, 100 μl of twofold serially diluted antisera were added. Each dilution was tested in duplicate. The plates were incubated again for 60 min at 37°C and washed. HRP-hTf was added at the concentration determined above, and the plates were incubated for 60 min at 37°C. The ability of the antisera to inhibit binding of hTf to Tbps was expressed as the reciprocal of the serum dilution which gave 50% inhibition.

Bacterial growth in the presence of anti-Tbp antibodies. A technique to grow the meningococci in the wells of a microtiter plate (Nunc) was optimized in our laboratory. This method enabled growth of cultures as small as 200 μl, and the growth could be monitored easily with an enzyme-linked immunosorbent assay (ELISA) plate reader equipped with an appropriate wavelength filter. To ensure hTf-dependent growth of meningococci, free iron was removed from the Mueller-Hinton broth (MHB) by adsorption onto ovotransferrin (18), and the medium was further chelated with 30 μM EDDHA. Iron-starved meningococcal cells were subcultured in a microtiter plate in this medium containing various amounts of anti-Tbp antibodies. Each antibody concentration was tested in triplicate. hTf was added to final concentration of 5 μM, and the bacterial growth was monitored at various time points with an ELISA plate reader (Bio-Kinetics reader; Bio-Tek Instruments) at 630 nm.

Mouse protection assay. The mouse protection test was based on the model described by Schryvers and Gonzalez (35) with modifications (10). Briefly, groups of 48 mice were immunized subcutaneously on days 0, 21, and 35 with different vaccine preparations containing 5 μg of protein adsorbed onto 0.1 mg of aluminum hydroxide per 0.5 ml; PBS containing aluminum hydroxide and heat-inactivated *N. meningitidis* B16B6 were used as controls. On day 41, mice within a group were subdivided into groups of eight, and each subgroup received by the intraperitoneal route 24 mg of iron-loaded hTf (Sigma) and, immediately after by the intraperitoneal route, 0.5 ml of *N. meningitidis* grown under iron restriction. The bacterial load varied from 10^4 to 10^8 CFU. Mortality rates were measured for 5 days following challenge. For a subgroup of mice, sera were drawn and bactericidal activity was titered as described above. The antibody titers to isolated Tbp2 were determined by an ELISA as described previously (10).

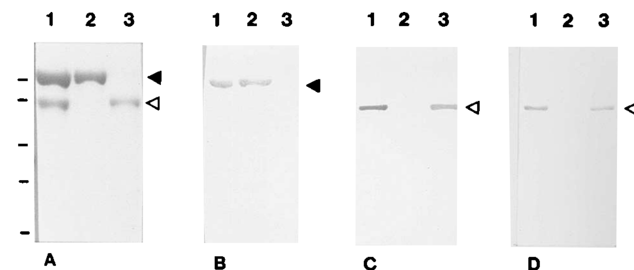

FIG. 1. SDS-PAGE and Western blots of Tbps from *N. meningitidis* B16B6. The proteins were electrophoresed on a 10% minigel; the gels were stained with Coomassie brilliant blue R-250 (A) or electrotransferred onto nitrocellulose and immunoblotted with polyclonal antiserum raised in rabbits to Tbp1 at a 1:5,000 dilution (B) or polyclonal antiserum raised in rabbits to Tbp2 at a 1:10,000 dilution (C). The proteins transferred to nitrocellulose were also incubated with hTf-peroxidase at 2 μg ml^{-1} (D). Lanes: 1, 6 μg of Tbp complex; 2, 2.4 μg of Tbp1; 3, 1.2 μg of Tbp2. The molecular mass markers indicated on the left are 94, 67, 43, 30, and 20 kDa (from top to bottom).

RESULTS

Protein purification and characterization. The Tbp1 and Tbp2 from affinity-purified Tbp complex could be dissociated and isolated individually by the hydrophobic chromatography method described (Fig. 1A). The purified Tbp1 and Tbp2 were shown to be devoid of the other polypeptide chain when reacted with monospecific rabbit polyclonal antibodies (Fig. 1B and C). As observed for the Tbp complex, Tbp2 showed binding activity to hTf-peroxidase on Western blots (Fig. 1D) and dot blots (data not shown), suggesting that the structural or conformational determinants of the hTf-binding domain of Tbp2 is such that it resisted the harsh conditions used in the purification process, including ethanol precipitation and incubation with 5 M guanidine hydrochloride. Tbp1 purified as described displayed no hTf-binding activity on Western blots (Fig. 1D) as was observed for the Tbp1-Tbp2 complex (10). Weak binding activity, however was noted on dot blots (not shown), suggesting that the conformation of the protein may be affected by the purification or the electrophoresis process.

Bactericidal activity of monospecific antisera. Monospecific antisera raised in rabbits and mice against Tbp1 and Tbp2 isolated from *N. meningitidis* B16B6 were tested for their ability to kill the strain in the presence of complement (Table 1). The results show that only anti-Tbp2 antisera were bactericidal for the parent strain. The absence of reactivities of anti-Tbp2 against Tbp1 and of anti-Tbp1 against Tbp2, demonstrated by Western blot in both rabbit sera (Fig. 1B and C) and mouse sera (data not shown), confirmed the specificity of the antiserum. This result is in agreement with those obtained previously with a meningococcal whole-cell dot assay in which only anti-Tbp2 antiserum was able to react with whole meningococcal cells spotted on nitrocellulose (32). The fact that the purification process may have destroyed conformational surface epitopes in Tbp1 cannot, however, be ruled out. The absence of bactericidal activity may also result from the lack of sensitivity of the assay; bactericidal antibodies may be present but in an amount too low to be effective in killing. Nevertheless, it should be noted that Tbp2 in an amount as low as 1.5 μg induced bactericidal antibodies in mice, whereas 25 μg of Tbp1 injected in the same host did not induce antibodies with this property although the level of specific IgG induced was high as measured by the ELISA (Table 1).

TABLE 1. Bactericidal activity of mouse and rabbit antisera specific for Tbp1 and Tbp2

Antigen	Host	Immunizing dose (μg)	Adjuvant	Western blot reactivity		IgG titer[a]		Bactericidal titer[b]	
				Tbp1	Tbp2	Tbp1	Tbp2	Prevaccination	Postvaccination
Tbp1	Mouse	1.5	Aluminum[c]	+	−	3,260	ND[d]	<4	<4
	Mouse	6.25	Aluminum	+	−	5,745	ND	<4	<4
	Mouse	25	Aluminum	+	−	8,580	ND	<4	<4
	Rabbit	25	Freund's	+	−	ND	ND	<8	<8
Tbp2	Mouse	1.5	Aluminum	−	+	ND	1,405	<4	256
	Mouse	6.25	Aluminum	−	+	ND	3,185	<4	1,024
	Mouse	25	Aluminum	−	+	ND	4,483	<4	512
	Rabbit	25	Freund's	−	+	ND	ND	<8	512

[a] IgG titers were determined by an ELISA and are expressed as arbitrary units calculated versus an internal standard.
[b] Bactericidal titers are expressed as the reciprocal of the last serum dilution in the presence of which 50% or more of the initial bacterial load has been killed. The sera from the eight mice within a group were pooled and titered, the sera from two different rabbits were tested, and the titration was performed three times.
[c] Aluminum, proteins were administered adsorbed on 100 μg of aluminum hydroxide.
[d] ND, not determined.

Capacity of antisera to inhibit transferrin binding to receptor. The ability of specific antisera to inhibit transferrin binding to the purified bacterial receptor was determined in a solid binding assay. Peroxidase-conjugated hTf bound to the receptor immobilized on the plate in a saturable manner (Fig. 2A). The ability of the antisera to inhibit this binding was measured four times with antisera obtained from six rabbits (two rabbits each for the Tbp complex, Tbp1, and Tbp2). Similar results were obtained each time, and typical results are shown in Fig. 2B. Polyclonal antisera to the Tbp1-Tbp2 complex showed a strong inhibitory effect on the hTf binding to the receptor, and the extent of inhibition was proportional to the amount of antiserum present. Similarly, but to a lesser degree, both anti-Tbp2 and anti-Tbp1 displayed the same effect while control antiserum was devoid of this inhibitory activity (data not shown). From the graphs, inhibition titers corresponding to the dilution of sera giving 50% inhibition can be calculated. The values are 3,700 for anti-Tbp complex, 1,100 for anti-Tbp2, and 70 for anti-Tbp1.

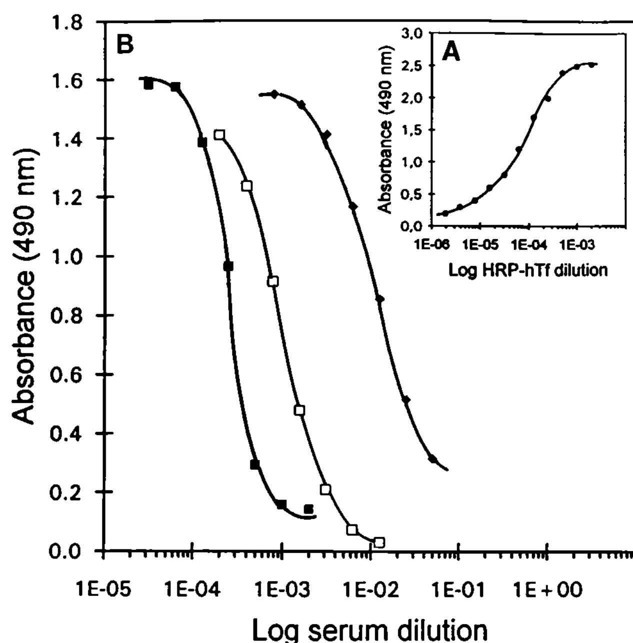

FIG. 2. Demonstration of the inhibitory effect of the anti-Tbp antisera on transferrin binding to the receptor protein. The ELISA was performed as described in Materials and Methods. Microtiter plates were coated with transferrin receptor complex purified from *N. meningitidis* B16B6. (A) Saturation curve of hTf binding to the receptor; (B) inhibition of transferrin binding to the receptor in the presence of serial dilutions of anti-Tbp antisera. Symbols: ■, anti-Tbp complex; ◆, anti-Tbp1; □, anti-Tbp2.

It is clear from Western blot analysis that Tbp2 binds hTf; therefore, antibodies interacting with Tbp2 interfere with this binding activity. The fact that the same antibodies are bactericidal suggests that Tbp2 or a domain of Tbp2 involved in the binding of hTf is surface exposed and accessible to antibodies. On the other hand, it has been demonstrated that Tbp1 has the ability to bind transferrin under certain conditions (7, 28), but it loses this ability after electrophoresis and transfer. The fact that antibodies specific for Tbp1 inhibit to some degree the binding of hTf to the complex indirectly confirms the ability of Tbp1 to bind hTf. However, the discrepancy between the inhibitory effect and the absence of bactericidal activity may be interpreted in two ways: either (i) the level of antibodies directed to native epitopes in Tbp1 is sufficient to inhibit hTf binding but not effective in killing the bacteria because the bactericidal assay is not sensitive enough, as suggested earlier, or (ii) the domain(s) of Tbp1 involved in hTf binding is accessible to antibodies on purified receptor but not on the whole bacteria. The experimental data do not permit a conclusion on this point.

Inhibitory effect of antisera on bacterial growth. To test the hypothesis that antibodies to transferrin receptor could interfere with bacterial growth by limiting access to essential iron, meningococci were grown under strong iron limitation and given hTf as the sole iron source.

The addition of EDDHA to iron-depleted MHB slowed bacterial growth, and as expected, the addition of hTf to the iron-depleted MHB stimulated the bacterial growth to a rate comparable to that of the control in iron-rich MHB (Fig. 3A). Three independent experiments were performed with four to six antibody concentrations in triplicate each time. The results were consistent. In the presence of IgG and the purified Tbp complex, bacterial growth was slowed significantly in a concentration-dependent manner (Fig. 3B1). Complete inhibition was achieved at a twofold dilution (final concentration of IgG, 2.1 mg ml^{-1}). Normal growth was restored when IgG was present at a 1:32 dilution (0.13 mg ml^{-1}), a concentration which was ineffective in interfering with meningococcal growth. A clear inhibitory effect was also observed in the presence of IgG against Tbp2, but to a lesser extent. A normal growth rate was restored with a dilution of 1:16 (0.26 mg ml^{-1}) (Fig. 3B2). The

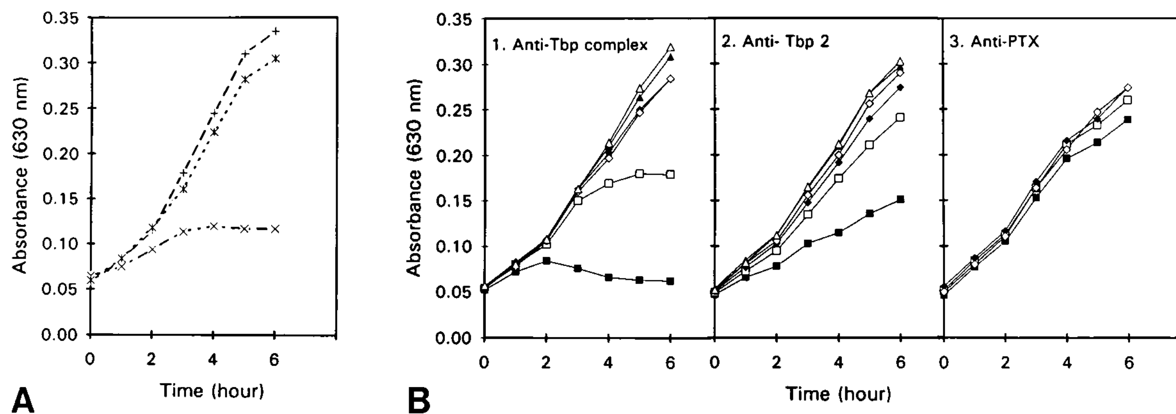

FIG. 3. Demonstration of hTf-dependent growth of *N. meningitidis* B16B6 and its inhibition in the presence of anti-Tbp antisera. Bacteria were grown under conditions described in Materials and Methods. (A) Comparison of meningococcal growth rates in various growth media. Symbols: +, iron-rich MHB; ×, iron-depleted MHB; ⋇, iron-depleted MHB supplemented with 5 μM hTf. (B) Meningococcal growth in the presence of various concentrations of purified IgG to the Tbp complex (panel 1), to Tbp 2 (panel 2); or to an unrelated bacterial toxin (pertussis toxin [PTX]) (panel 3). The concentrations of the antibody used were 2.1 (■), 1.05 μg/ml (□), 0.52 (◆), 0.26 (◇), 0.13 (▲), and 0.06 (△) μg/ml.

effects of IgG to both the Tbp complex and Tbp2 were specific; this was demonstrated by the IgG to a nonrelated bacterial protein (pertussis toxin), in the presence of which bacterial growth was maintained at the level of that of the control at all dilutions tested (Fig. 3B3). These experiments suggest that antibodies directed to the Tbp complex and specifically to Tbp2 interfered with the iron uptake and, as a consequence, slowed bacterial growth.

Protective activity of Tbp2 in mice. Mortality rates of mice immunized with either the Tbp complex or with Tbp2 alone were determined over a period of 5 days after challenge with iron-starved meningococci and compared with those of controls. The results are presented in Table 2. Both the Tbp complex and Tbp2 administered three times subcutaneously at 5 μg per injection conferred protection to the mice. The level of protection was less than, but close to, that observed in mice vaccinated with the heat-inactivated whole cells; in the whole-cell group, mice were protected against approximately 100 50% lethal doses (LD_{50}s), while they were protected against approximately 70 LD_{50}s when immunized with Tbp2 alone. In parallel to this protection, it was shown that mice developed high IgG titers to Tbp2 as well as a significant bactericidal titer (Table 2). There was no clear correlation between the bactericidal titers, the IgG levels, and the level of protection expressed as LD_{50}. This suggests, as in an earlier study (10), that bacterial clearance does not occur solely via complement killing but that opsonophagocytosis may be playing an important role even though it is likely that neutrophil functions are somewhat impaired under such iron-overloading conditions (43). Furthermore, the IgG titers to Tbp2 were not directly proportional to the bactericidal titers; this may be explained by two facts. On the one hand, Tbp2 may be more denatured when it is administered alone than when it is administered in a purified form as the complex Tbp1-Tbp2, suggesting that in part conformational determinants may be important in inducing bactericidal antibodies. On the other hand, it may be that the level of IgG1 induced is significant and measurable in the ELISA but that it does not contribute to the bactericidal activity since this subclass of immunoglobulin in mice does not bind complement.

DISCUSSION

While a growing amount of information on the genes encoding Tbps is becoming available (8, 15, 21), the respective roles of Tbp1 and Tbp2 constituting the hTf receptor and their precise localization in the membrane remain unclear. The surface accessibility of both polypeptides is important in vaccine design. In fact, only easily accessible surface proteins are good vaccine candidates. In the case of Tbps, we know from previous studies that antibodies directed to the complex bound to the bacterial cell and, in the presence of complement, lysed the cell (10). In this study, we undertook to determine what role each polypeptide played in eliciting antibodies reacting to the surface of the bacteria. Transferrin receptor molecules are not abundant in the outer membrane; it has been reported that

TABLE 2. Protection rates and immune responses of mice immunized with Tbp complex or Tbp2

Group	Antigen	Mortality (no. dead/total) after challenge dose[a] of:				Calculated LD_{50} (CFU/mouse)	IgG titer to Tbp2 (arbitrary EU/ml)[b]	Bactericidal titer
		10^5	10^6	10^7	10^8			
1	Tbp complex with Al[c]	0/6	0/7	3/8	7/7	1.5×10^7	3,216	≥2,048
2	Tbp2 with Al[c]	0/8	0/8	0/8	8/8	4.1×10^7	9,615	1,024
3	Buffer with Al[c]	2/8	4/8	8/8	8/8	6×10^5	<5	<4
4	B16B6 cells[d]	0/8	0/8	0/8	6/8	6.4×10^7	544	≥32,000

[a] Challenge dose is expressed as CFU administered intraperitoneally per mouse.
[b] EU, ELISA unit.
[c] Mice were immunized three times with 5 μg of the protein adsorbed onto 0.1 mg of aluminum hydroxide (Al).
[d] Mice were immunized with heat-inactivated meningococcal cells.

there are 2,800 to 2,900 molecules per CFU (5, 41). Consequently, the first step was to develop a purification scheme to acquire separately milligram quantities of the two polypeptides, Tbp1 and Tbp2. The Tbp complex was purified from meningococcal membranes as described previously (10), and several dissociating agents were tested for their ability to separate the two proteins. Only guanidine hydrochloride was effective in dissociating the complex. The lipophilic characteristics of Tbp2 (21, 22) were exploited, and a purification scheme based on hydrophobic interaction chromatography led to the purification of the two separate polypeptides. Under the described conditions, Tbp2, being acylated, was retained on the phenyl-Sepharose column, while Tbp1 came out in the wash. This procedure yielded both polypeptides unlike the procedure described by Ferron et al. (13) in which Triton X-100 treatment followed by ion-exchange chromatography allowed only the purification of Tbp2; in our hands, such treatments left Tbp1 membrane associated (unpublished observations). Once Tbp1 and Tbp2 were purified to homogeneity independently, antisera were raised in rabbits and tested for their ability to kill meningococci in the presence of complement. Under such conditions, only anti-Tbp2 antiserum was demonstrated to be bactericidal. We were not able to demonstrate bactericidal activity of antisera specific for Tbp1. As stated above, because Tbp1 is thought to be membrane associated, it is possible that the purified protein may not conserve all of its conformational epitopes. It is also possible that the topology of Tbp1 in the outer membrane is such that Tbp1 is not easily accessible to antibodies, and yet another explanation may be that the bactericidal assay is not sufficiently sensitive. Conclusions cannot be drawn at this point without further studies on the Tbp1 molecules. The Tbp1-Tbp2 complex elicits bactericidal antibodies in animals (10), and under our experimental conditions, this bactericidal activity could be demonstrated only with rabbit sera specific for Tbp2. Because it has been reported that the immune response to Tbps varies among different animal species (2), the ability of Tbp1 and Tbp2 to induce bactericidal antibodies was also studied in mice. The results obtained were consistent with those obtained in rabbits, and only Tbp2 elicited bactericidal antibodies in mice, while under our experimental conditions, Tbp1 did not. On the basis of these results, mice were immunized with purified Tbp2 or the Tbp1-Tbp2 complex. Immunized mice were challenged with iron-starved meningococci inoculated 2 h after administration of human holotransferrin. Under such conditions, it was observed that mice immunized with Tbp2 or with the complex were protected from the lethal challenge. Although this mouse model does not mimic human disease, i.e., it does not lead to meningitis, it does simulate one of the steps of pathogenesis, namely, bacterial multiplication in the bloodstream. Under the experimental conditions, it is probable that the bacteria multiply successfully not only because a source of iron is available but also because the host system has impaired monocyte, neutrophil, and macrophage functions resulting from iron overload (43, 44). On the basis of the fact that mice immunized with Tbp2 or the complex clearly survived the challenge much better than nonimmune mice, it can be concluded that specific antibodies to Tbps play a protective role. The exact mechanism by which the immunoglobulins exert their protective role remains unclear since many cell functions are impaired. This may be the reason in part why no clear correlations can be observed between the bactericidal titers of the sera, the LD_{50} value, and the specific ELISA titers. Another possibility may be that in the presence of antibodies specific for Tbp2 or the complex, yet another mechanism contributes to the protection observed; antibodies directed to Tbps may interfere with bacterial growth by inhibiting transferrin binding to the receptor and therefore limiting the availability of the essential iron.

To address these two effects, we first developed an hTf binding assay in the solid phase. Under the defined conditions, both antisera directed to the complex and those to Tbp2 had a marked inhibitory effect on transferrin binding to the receptor, while anti-Tbp1 antisera had a lesser effect on this binding. The fact that antibodies to Tbp2 inhibit hTf binding and induce bacterial killing in the presence of complement confirmed that Tbp2 is an easily accessible polypeptide on the bacterial surface, in agreement with previous observations (14, 32). Therefore, antibodies interfering with the binding of hTf to Tbp2 should have an effect on bacterial growth with hTf as the sole iron source. To study this, we developed growth conditions for meningococci in microtiter plates. Preliminary assays performed with the whole specific antisera showed increased bacterial growth independently of the specificity of the antisera (data not shown); presumably, the sera constituted an iron source for the meningococci. To circumvent this, IgGs were purified from each antiserum and used in the assay. Under such conditions, it was clearly demonstrated that IgG specific for Tbp2 inhibited bacterial growth to a similar level as IgG specific for the complex, again illustrating the accessibility of Tbp2. Together, the results obtained with Tbp2 as immunogen are rather consistent. (i) Tbp2 can induce bactericidal antibodies in rabbits and mice. (ii) Tbp2 protected mice from a lethal challenge. (iii) Specific anti-Tbp2 antibodies interfered with hTf binding to the purified receptor. (iv) Specific anti-Tbp2 IgG slowed bacterial growth under conditions where hTf is the sole iron source available to meningococci. In all of the assays, Tbp2 and Tbp2-specific antibodies demonstrated properties similar to that of the Tbp1-Tbp2 complex, indicating that Tbp2 contributes greatly, if not totally, to the protective activity described for the complex.

The contribution of Tbp1 and Tbp1-specific antibodies to the protective activity described for the complex remains unclear from our results. This may be due largely to the fact that Tbp1 could be denatured to some extent by the purification process described in the present study, unlike the Tbp1 and Tbp2 purified by chromatofocusing (2). It is possible that Tbp1 lost most of its native conformation, leading to the observation that polyclonal monospecific antisera raised against Tbp1 were devoid of bactericidal activity. The fact that these same antibodies had the capacity to inhibit hTf binding to the purified Tbp1-Tbp2 complex may indicate that the bactericidal test is not sensitive enough or that certain domains of Tbp1 involved in hTf binding are not accessible on the surface of the bacteria, while they are accessible when the protein is in a purified form, out of its membrane environment. Furthermore, the sequence homology between gonococcal Tbp1 and TonB-dependent outer membrane receptors suggests that Tbp1 is a true membrane protein with transmembrane domains (8, 21). By analogy with meningococcal class 1 proteins (23, 42), one may postulate that only certain loops or domains of Tbp1 are surface accessible. Antibodies directed to these surface loops may be bactericidal. To prove this hypothesis and investigate the protective role played by Tbp1, further studies must be performed with Tbp1 molecules in a state as close to native as possible.

Our results demonstrate that purified Tbp2 displays protective activities similar to those conferred by the complex and could be considered on its own as a vaccine antigen. This observation is in agreement with the results of Rossi-Campos et al., who have demonstrated that recombinant Tbp2 of *Actinobacillus pleuropneumoniae* protected immunized pigs from a challenge (33). Tbp2 thus appears to be an attractive vaccine candidate; it is a surface-exposed molecule that induces anti-

bodies that are not only bactericidal but also inhibitory to bacterial growth. Because *Neisseria* species can utilize transferrin and lactoferrin as their sole iron source (3, 24, 25, 37) by expressing specialized receptor proteins (37, 38), these proteins seem essential for the survival and multiplication of *N. meningitidis* in an iron-restricted environment such as its natural host, the human body. It is tempting to predict that antibodies directed to these receptors could slow bacterial multiplication in the bloodstream, hence slowing the infectious process. Two main issues need to be studied further before vaccines using Tbp2 as antigen are designed. First, the pronounced variability of Tbp2 that exists among different strains is well documented (12, 13, 17, 32, 40) and must be studied further to understand the basis of this heterogeneity in order to circumvent it. Further studies are required to address this issue and are presently under way in our laboratory. Second, as shown in a recent study by Ala'Aldeen et al., the immune response of laboratory animals may be different from that of humans and extrapolating what is observed in laboratory animals to humans may not be obvious (2). Although this statement may a priori hold true for all human vaccine development, it has been shown historically that, in the case of meningococcal vaccines, bactericidal antibodies in humans correlate with protection (16); group C capsular polysaccharides, for example, injected into guinea pigs elicit bactericidal antibodies (unpublished results), indicating that bactericidal antibodies may be considered a marker for predicting the efficacy of a vaccine in the field. It remains clear, however, that no direct correlation can be drawn between the bactericidal titer induced by a vaccine in mice and the percentage of protection observed in efficacy trials (30). This lack of correlation may be due to the design of the bactericidal assay, and clearly, more efforts will be needed to understand this in greater detail.

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