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Immune Responses in Humans and Animals to Meningococcal Transferrin-Binding Proteins: Implications for Vaccine Design

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The results reported here show that the two meningococcal transferrin-binding proteins (TBP1 and TBP2) generate different immune responses in different host species and that there is variation in response dependent on the method of antigen preparation and possibly the route of administration. Mice immunized with either whole cells of *Neisseria meningitidis* SD (B:15:P1.16) or the isolated TBP1-TBP2 complex from the same strain produced antisera which, when tested against a representative panel of meningococcal isolates by Western blotting (immunoblotting), recognized some but not all heterologous TBP2 molecules. In contrast, rabbit antisera raised to the same preparations were cross-reactive with almost all the TBP2 molecules. The immune response to TBP1 was also host species dependent. Western blot analysis with denatured TBP1 failed to detect antibodies in antisera raised in mice to whole cells or in a rabbit to the TBP1-TBP2 complex but detected broadly cross-reactive antibodies in mouse anti-TBP1-TBP2 complex sera and strain-specific antibodies in rabbit anti-whole-cell serum. Human convalescent-phase sera obtained from five patients infected with meningococci of different serogroups and serotypes contained fully cross-reactive antibodies to TBP2 but no anti-TBP1 antibodies, when examined on Western blots. However, on dot immunoblots, the same patients' sera, as well as the mouse anti-whole cell and the rabbit anti-TBP1-TBP2 complex sera, reacted with purified biologically active TBP1 of strain SD. This indicates that native TBP1, a protein which loses its biological and some of its immunological activities when denatured, is immunogenic and that humans generate cross-reactive antibodies to native epitopes. These observations have important implications for assessing the vaccine potential of TBPs and other meningococcal antigens. Conclusions regarding the usefulness of TBPs as candidate components of meningococcal serogroup B vaccines based on results from certain animal species such as mice, or on methods such as Western blotting, may have little bearing on the situation in humans and may lead to some potentially useful antigens being disregarded.

Neisseria meningitidis is the second most common cause of meningitis in children, the most common overall cause of pyogenic meningitis, and the only bacterium capable of generating epidemic outbreaks of meningitis. Meningococci also cause septicemia which can kill within several hours of the appearance of the first symptoms. Despite extensive studies, the mechanisms responsible for the development of natural immunity to meningococcal disease remain unclear, although it is known that antibodies to the polysaccharide capsule of serogroups A, C, Y, and W135 of *N. meningitidis* offer good protection against infection. Vaccines consisting of these polysaccharides are available, but protection is group specific and the vaccines are not effective in children under 2 years old, the most susceptible age group (13, 27). Furthermore, the capsular polysaccharide of serogroup B meningococci, which are responsible for the majority of cases of meningococcal disease in Europe and America, is only very poorly immunogenic in humans (15). In view of the problems associated with polysaccharide vaccines, many investigators have turned their attention to other meningococcal antigens. Particular attention has been given to outer membrane proteins, especially to the class 1 proteins; and vaccine preparations enriched in the class 1 and serotype antigens, sometimes in the form of noncovalent complexes with capsular polysaccharides and combined with aluminum hydroxide, have been tested clinically. Various degrees of efficacy have been reported for these preparations (7, 11, 31, 34). It is important to note, however, that although these types of vaccines are enriched for the class I and serotype antigens, they contain significant amounts of other membrane proteins and this complicates the search for the component(s) relevant to protection in humans (29). It also makes it more difficult to standardize preparations for future use. Another major problem, if protection in humans is based on the class 2/3 or class 1 proteins, is that these vaccines may be serotype and/or subtype specific (15). The antigenic variability of the class 1 proteins, for example, is well established, and recent sequencing work has identified the variations in the primary structures that account for the antigenic differences between the proteins (22, 23). Furthermore, the evidence suggests that the antigenic heterogeneity of the class 1 proteins may be increased by horizontal genetic exchange (14).

An ideal meningococcal vaccine would be immunogenic in all age groups and protect against all serogroups and serotypes of *N. meningitidis*. The search for alternative subcapsular vaccine candidates includes the consideration of environmentally regulated antigens. Most investigations of bacterial pathogens, including *N. meningitidis*, are carried out with organisms grown in vitro under conditions that do not necessarily reflect

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TABLE 1. Serogroup, serotype, and site of isolation of each *N. meningitidis* strain and clinical diagnosis of each patient

Patient no.	Strain	Group and type	Site of clinical isolation	Diagnosis	Approximate molecular mass of TBP2 (kDa)
1	AS	A:4:P1.7	CSF[a]	Meningitis	80
2	BT	A:4:P1.9	Blood	Septicemia	75
3	SD[b]	B:15:P1.16	CSF	Meningitis	78
4	44/76	B:15:P1.7,16	NA[c]	NA	78
5	B16B6	B:2a:P1.2	NA	NA	68
6	OR	C:2a:P1.10	Blood	Acute coccemia	85
7	GN	C:NT	Blood	Acute coccemia	80
8	JB	B:NT	Blood	Meningitis	92
9	K1041	B:15:P1.7,16	NA	NA	78
10	K995	B:15:P1.7,16	NA	NA	78
11	J129	B:NT:P1.15	Blood	NA	78
12	K153	B:2a	NA	NA	78

[a] CSF, cerebrospinal fluid.
[b] Strain SD is also known as 70942 (19).
[c] NA, information not available.

those in vivo. That this is likely to give at best only a partial picture of bacterial characteristics associated with virulence and with immune responses important for protection is now well recognized (18). The extremely low availability of iron in mammalian tissue fluids constitutes one major difference between the usual in vitro growth conditions and those found in vivo. Pathogens which need to multiply in vivo to establish an infection adapt to this iron-restricted environment by expressing new iron-regulated outer membrane proteins which are involved in assimilating protein-bound iron or in acquiring it from liberated hemoglobin or heme (9). During iron-restricted growth, *N. meningitidis* expresses several new proteins, two of which are transferrin-binding proteins, TBP1 and TBP2, with molecular masses of approximately 98 and 65 to 90 kDa, respectively (3, 19, 30, 32); these are believed to be involved in the acquisition of iron from human transferrin during growth in vivo, with TBP1-TBP2 forming part or all of the meningococcal transferrin receptor (5). There is growing interest in the incorporation of such iron-regulated proteins in future meningococcal vaccines (6), and the TBPs are obvious candidates. However, if these proteins are to play a useful role in such vaccines, then their antigenicity and expression need to be better understood. Work with polyclonal antibodies raised in mice to whole cells of iron-restricted *N. meningitidis* showed that TBP2 exhibited antigenic heterogeneity, which seemed to make it a less than promising vaccine candidate (19). More recently, studies carried out in rabbits with purified denatured TBPs showed a higher degree of cross-reactivity among strains (28, 32). However, the immunological properties of the native TBPs have not been fully explored, primarily because of the previous lack of purified and separate biologically active TBP1, and it was unclear how either of them behaved immunologically in different animal species or in humans or whether antibodies against these proteins have the potential to confer protection. Stevenson et al. (32) showed that common antigenic domains exist in the TBP2 molecules of all meningococcal isolates examined, as well as in the analogous TBP2 protein of *Haemophilus influenzae* type b. Given that in both pathogens these TBPs have to recognize and bind to the same human serum protein, transferrin, they offer considerable interest as vaccine components which could conceivably confer protection against both haemophilus and meningococcal infection.

We have examined the immunogenicity of transferrin receptors in humans following infection and in two animal species commonly used to assess immunological characteristics of various meningococcal antigens, rabbits and mice, following challenge with infective organisms or purified native transferrin receptors.

(Part of this work was reported at the 8th International Pathogenic Neisseria Conference, Cuernavaca, Mexico, October 1992.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. Meningococcal isolates used in this study are representative of different serogroups and serotypes and are listed in Table 1. Bacteria were grown in Mueller-Hinton broth (Oxoid) alone or in Mueller-Hinton broth containing either 25 μM desferrioxamine (3, 4) or 40 μM ethylenediaminedihydroxyphenylacetic acid (EDDA; Sigma) (19) to limit available iron.

Membrane protein preparation and purification of the TBPs. Total membrane proteins were prepared by the lithium acetate-chloride extraction method described previously (3, 4). For use in Western blot (immunoblot) experiments, TBPs (TBP1 and TBP2) were affinity purified from whole-cell envelope preparations as TBP1-TBP2 complexed to human transferrin on streptavidin-agarose beads as described by Schryvers and Gonzalez (30) and modified by Stevenson et al. (32).

Because TBP1 loses its biological activity following the denaturation involved in Western blotting, biologically active TBP1 (separated from TBP2) was prepared for use in dot immunoblots, by affinity chromatography followed by chromatofocusing. Initially, TBPs were purified from iron-depleted cells as described previously by Gorringe et al. (16). Briefly, TBPs were solubilized from whole bacteria with 2% Elugent (vol/vol) (Calbiochem) in phosphate-buffered saline (PBS). The suspension was incubated for 10 min, cells were removed by centrifugation at 27,500 × *g* for 45 min, and EDTA and Sarkosyl were added to 10 mM and 0.5% (wt/vol), respectively. After further centrifugation, as above, the supernatant was applied to a column of transferrin-Sepharose, prepared by coupling 0.24 g of human transferrin to 10 g of CNBr-Sepharose. The column was then washed with 2% Elugent–PBS, and TBPs were then eluted with 50 mM glycine (pH 2.0), also containing 2% (vol/vol) Elugent. The two TBPs were then separated by chromatofocusing, which was carried out on a column (1 by 4 cm) of Polybuffer exchanger (PBE 94; Pharmacia). The column was washed overnight (12 ml/h) with 0.025 M imidazole buffer (pH 7.4) containing 0.1% (vol/vol) Triton

X-100. Affinity-purified TBPs in 2% Elugent–PBS were applied directly to the column, washed in the above buffer, and eluted with 32 ml of a 1-in-8 dilution of Polybuffer 74 (Pharmacia)–0.1% (vol/vol) Triton X-100 adjusted to pH 3.5. TBP1 eluted at the start of the pH gradient and TBP2 eluted at the acid end of the gradient. Biological activity (human transferrin binding) of both proteins after separation was confirmed on dot immunoblots as described below.

Sera. Human convalescent-phase sera were taken from patients with meningococcal disease (Table 1). Early convalescent-phase sera were taken up to 7 days after admission (patients 2, 6, and 7), and late convalescent-phase sera were taken 4 or more weeks after recovery (patients 3 and 8). Normal human serum was obtained from an individual with no prior history of infection with *N. meningitidis*. Sandoglobulin (3% [wt/vol]) (Sandoz Pharmaceuticals), a purified immunoglobulin G preparation obtained from pooled normal human sera, was also used.

Rabbit polyclonal antiserum against the biologically active TBP1-TBP2 of strain SD was raised by injecting a New Zealand White male rabbit (Interfauna) subcutaneously with five doses of the streptavidin-agarose–TBP1-TBP2 complex (mixed with Freund's complete and incomplete adjuvants) as described previously (5). Rabbit antiserum raised against live cells of strain SD was as described previously (4). Preimmune normal rabbit serum was also obtained.

Mouse polyclonal antibodies against the affinity-purified TBP1-TBP2 complex of strain SD were raised by injecting four BALB/c mice (Ola) intraperitoneally with four doses of the streptavidin-agarose–TBP1-TBP2 complex suspended in 150 μl of PBS mixed with an equal volume of Freund's complete (initial injection) or incomplete (subsequent weekly boost injection) adjuvant. Each dose consisted of the pellet obtained following the affinity purification from 1 ml (1 to 3 mg of protein) of a suspension of cell envelopes of strain SD. Mouse immune sera against live cells of strains SD and B16B6 were raised previously by Griffiths et al. (19), following intraperitoneal injection of female BALB/c mice (Ola), and the sera were taken 7 days after the second injection.

SDS-PAGE and Western blotting. Membrane proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described elsewhere (3, 4). The affinity-purified TBP1-TBP2 protein complex was solubilized at 37°C for 30 min in sample buffer without mercaptoethanol reduction and analyzed by SDS-PAGE as described by Stevenson et al. (32). Electrophoresis was carried out for an extended period of time to improve the resolution of the iron-regulated membrane proteins (3, 20). Following electrophoresis, the proteins were transferred to nitrocellulose paper by electroblotting as described previously (3, 4). Blots were used for the detection of antibody-antigen interactions or of the human transferrin-binding protein with labelled transferrin, or they were stained overnight in undiluted AuroDye (AuroDye forte kit; Janssen Life Science Products) to detect the transferred proteins. For immunoblotting, the human sera were used at dilutions of 1:200 (unless stated otherwise), and the rabbit and murine antisera were used at dilutions of 1:500 to 1:1,000. The nitrocellulose blots were incubated at room temperature overnight in the sera. The antigen-antibody interactions were detected as described previously (3, 4, 19) with peroxidase-conjugated anti-human immunoglobulin G (Dakopatts) or goat anti-rabbit (Bio-Rad) or rabbit anti-mouse serum (Dakopatts) as the second antibody as appropriate. Peroxidase-conjugated human transferrin (Jackson Immunoresearch Laboratories) was used at a 1-μg/ml final dilution to detect the human transferrin-binding protein as described previously (5, 32).

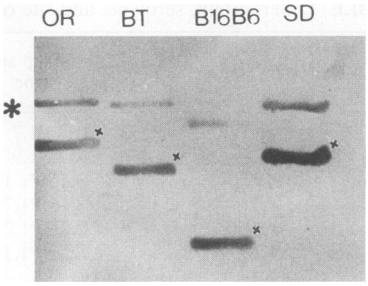

FIG. 1. Purified TBP1-TBP2 complexes of different meningococcal strains electrophoresed on SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and stained with AuroDye. Only the region of the gel containing the TBPs is shown. *, TBP1; x, TBP2.

Dot immunoblots. Prior to dot immunoblotting, the purified native TBP1 preparation (100 μg/ml) was diluted 1:2 either in PBS (pH 7.3) or in denaturing solution consisting of PBS containing 1% SDS. The latter was incubated at 37°C for 30 min. Portions (5 μl) of each mixture were fixed on nitrocellulose strips (24), which were then probed with primary antibodies at dilutions used for Western blots (except for human sera, which were used at 1:20) followed by the appropriate secondary antibodies. Primary antibodies included the human convalescent-phase sera, normal human serum, rabbit anti-TBP1-TBP2 complex, rabbit preimmune serum, mouse anti-whole-cell serum, and mouse preimmune serum. In addition, one membrane strip was probed with perioxidase-conjugated human transferrin and developed as for Western blots.

RESULTS

To screen the different sera by immunoblotting for the presence of antibodies to the TBPs, meningococcal isolates of patients 1 to 8 (Table 1) were chosen for their diversity in terms of serogroups, types, and subtypes and for the heterogeneity of the molecular masses of their TBP2 molecules. Figure 1 shows a typical range of TBP1 and TBP2 molecules obtained from strains by affinity purification. However, for further examination of sera obtained from mice and rabbits which were vaccinated with purified TBP1-TBP2 complex, another four isolates were also examined (isolates from patients 9 to 12). The TBP2 molecules of the latter strains are almost identical to that of strain SD (approximate molecular masses of the variable TBP-2s for all the strains are listed in Table 1).

Western blot examination of the animal antibody response to the TBP1-TBP2 vaccine. Table 2 summarizes the results, highlighting the differences in immune responses to the TBPs according to differences in species, immunogen, and route of infection and immunization. In response to vaccination with purified TBP1-TBP2 complex derived from strain SD, all four mice responded with broadly, but not fully, cross-reactive antibodies to denatured TBP1 and with antibodies of restricted cross-reactivity to denatured TBP2 (representative reactions are shown in Fig. 2). These sera recognized both the denatured TBP1 and TBP2 molecules of the homologous SD strain and those of strains J129, K995, and K1041, which have TBPs of molecular masses indistinguishable from those of strain SD. They also recognized TBP1 and TBP2 of strain GN. However, these sera recognized TBP1 but not TBP2 of strains AS, BT, OR, JB, and 44/76. The latter strain has the same serogroup,

TABLE 2. Comparison between antibody responses of different host species to TBP1 and TBP2 as detected by Western blots[a]

Immunogen	Host	Route[b]	Total no. of strains examined	Reaction with homologous:		No. of strains reacting with heterologous TBPs			
				TBP1	TBP2	TBP1 only	TBP2 only	Both TBPs	Neither
TBP1-TBP2	Mouse	i.p.	12	+	+	5	0	4	2
TBP1-TBP2	Rabbit	s.c.	12	−	+	0	11	0	0
Live cell	Mouse	i.p.	8	−	+	0	1	0	6
Live cell	Rabbit	i.v.	8	+	+	0	6	0	1
Live cell	Human[c]	n.f.	8	−	+	0	7	0	0

[a] A rabbit and mice were immunized with live meningococci of strain SD or with affinity-purified native TBP1-TBP2 complex derived from this strain. All sera were examined with TBPs extracted from a homologous strain and a number of heterologous strains.
[b] i.v., intravenous; n.f., nasopharynx (assumed port of entry on the basis of the accepted mechanism of meningococcal acquisition).
[c] Convalescent-phase serum of patient 3.

serotype, and serosubtype antigens as strain SD. These sera failed to recognize both TBP1 and TBP2 of strains B16B6 and K153. The TBP1 and TBP2 molecules of the latter strain are almost identical to those of strain SD.

In contrast to the situation with the mouse immune response, no anti-TBP1 antibodies were detected on Western blots with the rabbit antiserum raised to the purified TBP1-TBP2 complex. However, this antiserum was fully cross-reactive with TBP2 of each of the 12 examined isolates (typical examples are shown in Fig. 3). Reactions varied in strength between strains, and the reaction against strain B16B6, whose TBP2 has the lowest molecular weight, was the weakest.

Western blot examination of animal and human antibody responses to infection with strain SD. In response to infection with live meningococci (strain SD), the mouse failed to produce detectable antibodies to denatured TBP1 but generated a strong antibody response to the homologous TBP2 and showed a very weak cross-reaction with TBP2 of only one (strain OR) of seven heterologous strains. Some of these reactions are shown in Fig. 4. In contrast, the rabbit responded to infection with live organisms of the same strain with a highly strain-specific antibody reaction against denatured TBP1 and a broadly, but not fully, cross-reactive antibody response against TBP2 (representative reactions are shown in Fig. 5). It reacted with the TBP2 molecules of homologous strains and of six of seven heterologous strains, including those with molecular masses different from that of the homologous strain. The reaction varied in strength and was weakest against the TBP2s of strains B16B6 (whose TBP2 has the lowest molecular weight) and OR (whose TBP2 has a high molecular weight). No reaction was detected against strain GN, which expresses a TBP2 of high molecular weight (3) (data not shown). Note that both rabbit sera (obtained following immunization with purified TBP1-TBP2 complex or whole cells) reacted with a protein migrating close to TBP2, as well as a number of proteins of smaller molecular weight, mainly of the homologous strains; these could be breakdown products of TBP2. Mouse and rabbit preimmune sera did not react with TBP1 or TBP2.

In sharp contrast to the work with animal sera, all of the five human convalescent-phase serum samples examined (obtained from patients 2, 3, 6, 7, and 8; Table 1), failed to react with denatured TBP1 of homologous or heterologous strains when examined on Western blots. However, they all reacted with the TBP2 of the eight strains examined (strains isolated from patients 1 to 8). Figure 6 shows the reaction of the convalescent-phase serum from patient 3 with the homologous strain SD and three representative heterologous strains. Results remained unchanged when sera were used at higher concentrations (1:20) (data not shown). Late-convalescent-phase sera from patients 3 and 8 showed stronger reactions than the early-convalescent-phase sera of patients 2, 6, and 7 (data not shown). Normal human serum or a human immunoglobulin G population (Sandoglobulin) failed to react with either TBP1 or TBP2 from any of the eight strains examined by Western blotting (data not shown).

Dot immunoblots. It is evident that detection of antibodies to TBP1 by the Western blot technique is more difficult than detection of antibodies to TBP2. TBP1 is known to lose its biological activity following SDS-PAGE (i.e., it fails to bind human transferrin on Western blots). It was not clear, how-

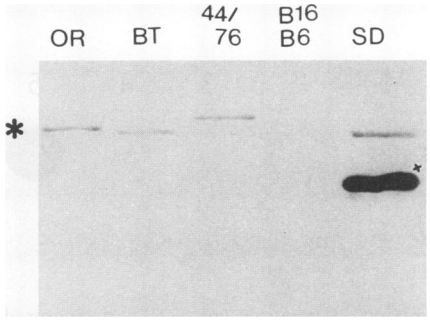

FIG. 2. Purified TBP1-TBP2 complexes of five different meningococcal strains (see Table 1 for strain details) Western blotted with mouse antiserum raised to the TBP1-TBP2 complex of strain SD. Only the region of the gel containing the TBPs is shown. *, TBP1; x, TBP2.

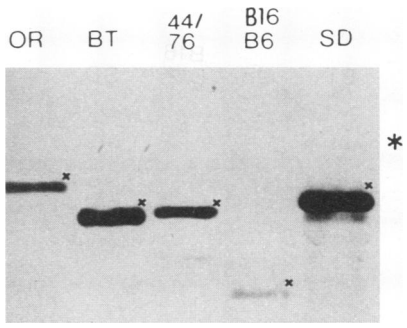

FIG. 3. Purified TBP1-TBP2 complexes of five different meningococcal strains (see Table 1 for strain details) Western blotted with rabbit antiserum raised to the TBP1-TBP2 complex of strain SD. Only the region of the gel containing the TBPs is shown. *, the place where TBP1 would be expected; x, TBP2.

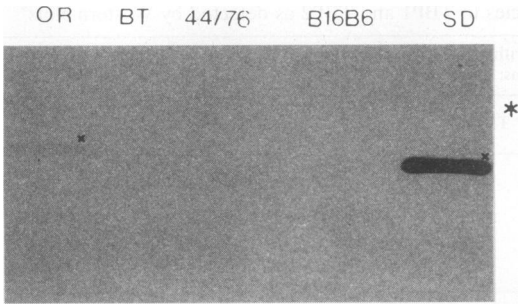

FIG. 4. Purified TBP1-TBP2 complexes of five different meningococcal strains (see Table 1 for strain details) Western blotted with mouse antiserum raised to live organisms of strain SD. Only the region of the gel containing the TBPs is shown. *, the place where TBP1 would be expected; x, TBP2.

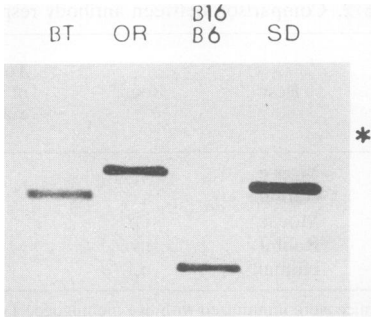

FIG. 6. TBP1-TBP2 complexes of four different strains (see Table 1 for strain details) Western blotted with human convalescent-phase serum of patient 3, from whom strain SD (B:15:P1.16) was isolated. Only the region of the gel containing the TBPs is shown. All visualized bands are TBP2. *, the place where TBP1 would be expected.

ever, whether the failure to detect anti-TBP1 in some of the examined sera was due to the absence of TBP1-specific immunoglobulin G antibodies in these sera or due to failure of the system to detect antibodies to conformational epitopes which may have been lost following exposure to SDS during SDS-PAGE. Therefore, special attempts were made to separate native TBP1 from the outer membrane of strain SD, which was then dot immunoblotted against the convalescent-phase sera of patients 2, 3, 6, 7, and 8 and against those animal antisera which failed to produce any reaction to the homologous TBP1 on Western blots, i.e., rabbit anti-TBP1-TBP2 complex and the mouse anti-whole cells. Results showed that the native TBP1 reacted weakly with the homologous and heterologous convalescent-phase sera. Reactions with the late-convalescent-phase sera from patients 3 and 8 (Fig. 7) were stronger than those with the early convalescent-phase sera from patients 2, 6, and 7 (data not shown because they were not photoreproducible). These anti-TBP1 reactions were lost following denaturation of the protein by prior incubation of the native TBP1 in SDS for 30 min at 37°C. Anti-TBP1 response was also clearly detectable in the rabbit anti-TBP1-TBP2 complex serum and mouse anti-whole cell serum (Fig. 7). These reactions were virtually lost following denaturation of the protein. All normal sera (human and animal) failed to react with either native or denatured TBP1 (not shown). The purified TBP1 was also able to react with peroxidase-conjugated human transferrin and lost this ability following exposure to SDS (Fig. 7).

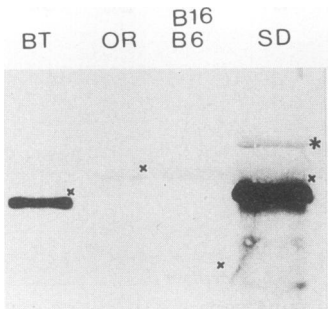

FIG. 5. Purified TBP1-TBP2 complexes of four different meningococcal strains (see Table 1 for strain details) Western blotted with rabbit antiserum raised to live organisms of strain SD. Only the region of the gel containing the TBPs is shown. *, TBP1; x, TBP2.

DISCUSSION

Following the failure of the capsular antigens of meningococci to provide an ideal vaccine, a great deal of attention has been focused on the outer membrane proteins, including iron-regulated proteins (such as TBPs), in the search for a vaccine that would protect against all serogroups and serotypes. The most widely reported method of studying the immunogenicity and potential usefulness for vaccination of meningococcal antigens is that of injection into mice (intraperitoneally [i.p.]) or rabbits (intravenously or subcutaneously [s.c.]) (3, 5, 6, 12, 19, 21, 28, 32). Also, the characterization of the antibody response (human or animal) is often done on Western blots (2, 3, 5, 6, 8, 19, 28, 32, 33). These two common practices are clearly associated with inaccurate extrapolation from animal results, which may not reflect the natural situation in humans.

Very little is known about the immunogenicity of the two major components of the meningococcal transferrin receptor (TBP1 and TBP2) and their behavior in humans and animals following infection with live cells or vaccination with purified proteins. In this study, we used the standard conventional methods of assessment to look at the immunogenicity of the TBPs as expressed on live infective organisms in vivo in humans, rabbits, and mice. Also, we looked at the immune responses of the two animal species to natively purified proteins (complexed to transferrin). Studies published to date on the immunogenicity of the TBPs have all been carried out with denatured TBPs (10, 28, 32). In the present study, however, particular attention was given to preserving, as well as possible, the native conformation of the transferrin receptors, particu-

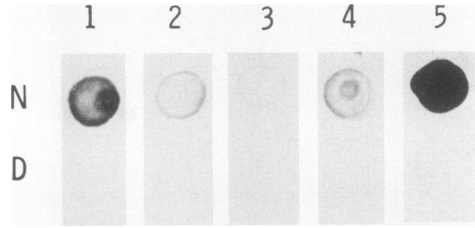

FIG. 7. Equimolar amounts of native (N) and denatured (D) TBP1 protein dot immunoblotted against peroxidase-conjugated human transferrin (lane 1), convalescent-phase serum of patient 3 (lane 2), convalescent-phase serum of patient 8 (lane 3), mouse anti-whole-cell serum (lane 4), and rabbit anti-TBP1-TBP2 complex (lane 5).

larly TBP1, which is known to lose its biological activity when subjected to SDS-PAGE conditions. Attempts to elute TBPs from transferrin-streptavidin complex under nondenaturing conditions, including low pH, failed to yield significant quantities of undenatured and stable proteins (data not shown). Therefore, in an attempt to preserve the stable native structure of TBP1 and TBP2, the animals were vaccinated with purified TBPs bound to the affinity purification complex, which consists of transferrin, biotin, and streptavidin-agarose beads.

Rokbi et al. (28) raised antisera in rabbits with a purified denatured TBP1 and TBP2 mixture (injected s.c and intramuscularly) and found full cross-reaction on Western blots between the TBP1s of all strains examined. However, on the basis of cross-reactivity of antisera with TBP2 molecules, they classified strains of *N. meningitidis* into two mutually exclusive groups. In contrast, the present study with rabbit antiserum raised to the native TBP1-TBP2 complex (injected s.c) showed no reaction on Western blots to TBP1 at all and showed full cross-reactions with all TBP2 molecules tested, although the strength of the reactions varied. This is in keeping with the full cross-reactivity of rabbit antibodies to reduced and denatured TBP2 (of strain B16B6) demonstrated by Stevenson et al. (32). Explanation of the discrepancy between the two results is only speculative. However, it is likely that the TBP1, when denatured, reveals conserved and strongly immunogenic epitopes (2, 10) which are detectable on Western blots, whereas the native protein conceals such epitopes and is capable of generating antibodies to conformational epitopes that would not be detectable on Western blots. It is more difficult to explain the difference between results of the two studies regarding immune reaction to TBP2. There are some obvious differences between the experimental designs, which include antigen preparation, route of injection, processing of the primary antibodies (Rokbi et al. [28] adsorbed serum with meningococci grown under iron-sufficient conditions), and incubation times of the primary and secondary antibodies.

Intraperitoneal injection of the TBP1-TBP2 complex in mice yielded a broadly cross-reactive antibody response to TBP1 and a much less cross-reactive antibody response to TBP2, which was limited to TBP2 molecules of almost identical molecular masses. These results are much more in keeping with those of Rokbi et al. (28) with rabbit antisera raised to denatured TBP1 and TBP2. The difference between murine and rabbit immune responses to the native TBP1-TBP2 complex could be attributed to the difference in animal species and/or differences in routes of vaccination.

The animal immune responses to live organisms were rather surprising. On Western blots, no anti-TBP1 antibodies were detected in the mouse antiserum (injected i.p.), and only strain-specific anti-TBP1 antibodies were detected in the rabbit antiserum (injected s.c.). In both animal species, the response to TBP2 was similar to that generated by the TBP1-TBP2 complex in the respective animals.

Of particular importance is the observation that humans respond to live meningococcal organisms by producing fully cross-reactive antibodies to TBP2, but no reaction against TBP1, as examined by Western blotting. The use of biologically active TBP1 in blotting experiments showed that this was not due to the lack of anti-TBP1 antibodies. Homologous and heterologous human convalescent-phase sera reacted weakly with purified TBP1, which had clearly retained its native biologically functional structure. These reactions were lost following denaturation with SDS. The results presented in this study confirm that both TBP1 and TBP2 are immunogenic in humans and that antibodies to TBP1 are generated but seem to be directed to conformational epitopes. Antibodies to TBP2 might also be directed against conformational epitopes, but TBP2 may renature sufficiently after SDS-PAGE to allow interaction with transferrin.

These data indicate that the native structure of the transferrin receptor of meningococci differs from that of transferrin receptors of eukaryotic cells; otherwise, one would have expected either tolerance of and inability to generate antibodies against this protein or the development of postinfection autoimmune diseases in most patients, which is not the case. Further evidence for differences between these functionally similar antigens is provided by the fact that rabbit anti-TBP1-TBP2 complex does not recognize human lymphocytes (1) and polyclonal monospecific rabbit anti-TBP2 does not recognize purified human (placental) transferrin receptors on immunoblots (32).

The reasons for differences noted here in the immune responses of the different hosts to the same antigens are unknown. However, the form and route of antigen presentation in a vaccine, or during infection, may play a part, as well as differences between the immune systems of the host species. Similar results have been seen with the meningococcal iron-regulated 70-kDa (FrpB) protein, such that humans, rabbits, and mice responded differently to the same antigen (2, 4, 8, 10, 25). It is likely that the different host species' immune systems respond to different immunodominant epitopes and that inbred mice respond to a more limited number of epitopes (on TBP2) than do humans. The possibility that other strains of mice might respond to different sets of epitopes must also be considered.

Although Western blots are generally considered to detect antibodies reacting with linear epitopes, there is no reason to suppose that differences similar to those reported here will not occur in the recognition of conformational epitopes. Thus, these observations have important implications for the initial assessment of the vaccine potential of TBPs and indeed of other meningococcal antigens. Conclusions regarding the usefulness of TBPs as candidate antigens based on results from mice may have little bearing on the situation in humans. In the present study, we demonstrated that both TBP1 and TBP2 are immunogenic in humans and generate cross-reactive antibodies. Previously reported data suggest that anti-TBP1-TBP2 complex can interrupt transferrin binding by live organisms (5) and can kill homologous and many, but not all, heterologous strains from various serogroups and types (reference 1a and unpublished data). Whilst it remains to be shown that TBP2 elicits good cross-protective antibodies in humans, previous conclusions that TBP2 was a less than promising vaccine candidate, because of its antigenic heterogeneity (19), are incorrect. Similar reservations regarding the usefulness of FrpB (2, 8, 17, 26) may also prove unfounded, since they also are based on results obtained with murine antibodies. Similarly, the relevance to human protection of the subtyping epitopes of the class 1 protein, which have been investigated in detail with murine monoclonal antibodies, might be questioned; the protective epitopes recognized by human antibodies may be different. A note of caution is therefore recommended when extrapolating data produced in animals, especially mice, to the situation in humans; otherwise, potentially useful antigens might be disregarded. The results presented here on the human immune response to the TBPs further support their vaccine candidature.

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