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Immunogenicity and Antigenic Heterogeneity of a Human Transferrin-Binding Protein in *Neisseria meningitidis*

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Growing *Neisseria meningitidis* on an iron restriction medium induces the synthesis of new outer membrane proteins, some of them true iron-regulated outer membrane proteins (IROMPs) and others synthesized because of the stress produced by the iron restriction. Some of these proteins are antigenic and can be considered for the development of vaccines; this is especially desirable in the case of *N. meningitidis* serogroup B, for which polysaccharide vaccines are not efficient. The antigenicity of *N. meningitidis* 37- and 70-kDa IROMPs has been studied previously; in this work, we studied the immunogenicity and antigenic heterogeneity of another IROMP, the human transferrin-binding protein 2 (TBP2), which seems to be indispensable for meningococcal growth inside the host. Mice were inoculated with purified outer membrane vesicles (blebs) from 5 selected *N. meningitidis* strains, and the five serum samples obtained were analyzed for anti-TBP2 antibodies by using the homologous strain and for cross-reactivity with the TBP2 of the 4 other selected strains and another 35 heterologous *N. meningitidis* strains. The TBP2s of the 5 strains tested were all immunogenic in mice to various degrees depending on the strain, and all five TBP2s shared one or more epitopes with heterologous strains (as shown by the cross-reactivities of the five serum samples), although the number of cross-reacting strains was very variable, ranging from 2 for strain V002 to 35 for strain P391. This suggests that the TBP2 epitopes of different strains differ in nature or in their accessibility to the immune system. Under the iron restriction conditions used, all strains synthesized a non-TBP2 antigenic 56-kDa protein thought to be a stress protein.

Meningococcal meningitis is a major health problem because of its severity, incidence in the infant population, and high mortality rates. The most common causal agents are *Neisseria meningitidis* serogroups A, B, and C. Although an effective vaccine against groups A and C has been developed by combining their purified capsular polysaccharides with those of serogroups Y and W_{135}, this approach has been unsuccessful with serogroup B (33), which is responsible for the meningitis endemic in Galicia (northwest Spain) and certain other countries. Because of the nonexistence of an effective vaccine for serogroup B, the relatively easy control and lesser virulence of infections caused by other serogroups, and the fact that meningitis mainly affects young infants, industrialized countries do not recommend vaccination except for the control of epidemic outbreaks caused by the four serogroups covered by the tetravalent vaccine mentioned above. It has, nevertheless, been suggested that vaccination might be justified at least during epidemics if it effectively prevents serogroup B meningitis (21).

To date, all attempts to develop an effective serogroup B vaccine have used capsular polysaccharide, alone or complexed with outer membrane proteins (OMPs) (13, 18, 23), complete outer membranes without capsular polysaccharide (22, 29), or similar systems (28). In all cases, the protection induced was very limited, as it did not cover heterologous subtypes or serotypes. Recently, Banerjee-Bhatnagar and Frasch (3) suggested that, to obtain an effective vaccine, it might be necessary to grow the meningococci in media in which, as in the host media in which the bacteria are found, iron is present in a complexed form (iron restriction media). The outer membrane composition of bacteria grown in normal culture media, in which iron is freely available, can be significantly different from that of invasive bacteria recovered directly from hosts, especially in the case of *N. meningitidis*. In particular, iron-regulated OMPs (IROMPs), some of which are involved in iron uptake mechanisms (26, 27), are synthesized only under iron restriction (11, 19) and later the antigenicity of the outer membrane of *N. meningitidis* (2–4, 15, 17).

Some of the most important *N. meningitidis* IROMPs are the human transferrin-binding proteins (TBPs), of which two have been found: transferrin-binding protein 1 (TBP1), with a molecular mass about 98 kDa, and transferrin-binding protein 2 (TBP2), with a molecular mass varying from 68 to 85 kDa, the latter being the only one able to bind transferrin after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting (1, 3, 12, 20, 24, 27). These two TBPs could be important for the uptake of the transferrin-bound iron needed by the bacteria for survival and growth, making them good targets for the immune system.

As a step towards the development of a vaccine against *N. meningitidis* serogroup B, we have analyzed the immunogenicity and antigenic variability of TBP2 synthesized under iron-restricted conditions by several *N. meningitidis* strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *N. meningitidis* strains used in this study were taken from our laboratory collection and had all been isolated in our region, 20 carrier strains from the oropharynx of healthy individuals (Table 1) and 20 invasive strains from either cerebrospinal fluid or blood (Table 2). The strains were cultured on Choc-Iso agar plates for 24 h at 37°C in a 5% CO_2 atmosphere, and then one isolated colony was subcultured for 8 h in the same conditions before inoculation in Erlenmeyer flasks containing 100

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TABLE 1. Expression and antigenic cross-reactivity of the human TBP of carrier *N. meningitidis* strains grown under iron restriction conditions

Strain	Serogroup; serotype[a]	Mass (kDa)[b]	Cross-reactivity of human TBP with strain:				
			P391	V021	P000	P636	V002
P000	B;15	83.8	+	+	+	−	−
P009	AA;1	83.4	+	−	−	−	−
P095	B;15	81.9	+	+	+	−	−
P097	B;12	84.0	+	+	+	+	−
P124	AA;NT	81.2	+	+	+	−	−
P136	B;2	81.0	+	+	+	−	−
P139	NG;1, 8	81.4	+	+	+	−	−
P148	B;1	79.5	+	−	−	+	−
P164	B;2	80.1	+	+	+	−	−
P192	B;15, 8	80.9	+	−	−	−	−
P214	AA;NT	80.0	+	−	+	−	−
P224	AA;2	80.1	+	−	+	−	−
P230	AA;2	72.9	−	+	−	−	−
P242	AA;15	80.7	+	−	−	−	−
P346	Y;15	82.9	+	−	+	−	−
P361	AA;15	84.7	+	+	−	−	−
P391	AA;15	84.9	+	+	−	−	−
P536	B;1	88.3	+	+	−	−	−
P608	Y;NT	72.2	+	+	+	−	−
P636	AA;8, 15	86.5	+	−	−	+	−
% Cross-reactivity			95	60	55	15	0

[a] AA, autoagglutinable; NG, nongroupable; NT, nontypeable.
[b] See reference 12.

TABLE 2. Expression and antigenic cross-reactivity of the human TBP of invasive *N. meningitidis* strains grown under iron restriction conditions

Strain	Serogroup; serotype[a]	Mass (kDa)[b]	Cross-reactivity of human TBP with strain:				
			P391	V021	P000	P636	V002
V001	C;2	84.0	+	+	−	−	−
V002	B;15	71.4	−	−	−	−	+
V003	B;15	79.1	+	−	+	−	−
V006	B;15	78.5	+	+	+	−	−
V009	B;12	83.4	+	+	−	−	−
V010	B;NT	80.2	+	+	−	−	−
V011	B;4	80.2	+	+	−	−	−
V012	B;NT	81.1	+	+	−	−	−
V013	B;NT	81.1	+	+	−	−	−
V014	B;NT	79.6	+	+	+	−	−
V015	B;15	78.7	+	+	−	−	−
V019	B;1	78.0	+	+	−	−	−
V021	B;NT	80.7	+	+	−	−	−
V022	B;15	83.5	+	−	−	−	−
V026	B;NT	78.3	+	−	−	−	−
V028	C;2	67.1	−	−	−	−	+
V029	B;NT	81.4	+	−	−	−	−
V036	C;2	68.2	−	−	−	+	+
C019	B;8, 15	81.7	+	−	−	+	−
L341	B;2	78.0	+	−	−	−	−
% Cross-reactivity			85	55	15	10	15

[a] AA, autoagglutinable; NG, nongroupable; NT, nontypeable.
[b] See reference 12.

ml of Mueller-Hinton broth (MH; Oxoid) or iron restriction medium (Mueller-Hinton broth with 39 μM ethylene-diamine-dihydroxyphenyl-acetic acid [MH-EDDA]).

Extraction of OMPs for SDS-PAGE and dot blots. Excreted blebs and bacterial outer membranes were obtained from the cultures as previously described (11). Briefly, the cultures were centrifuged at 48,000 × *g* for 2 h; the pellets were resuspended in 0.1 M acetate buffer (pH 5.8) with 0.2 M lithium chloride; and these suspensions were incubated at 45°C for 2 h in a shaking water bath, passed through a 21-gauge needle, and centrifuged at 10,000 × *g* for 15 min. The pellet was discarded, and membrane fragments were obtained from the supernatant by centrifugation at 48,000 × *g* for 2 h and suspended in 0.5 ml of distilled water. The protein content was determined by the Coomassie blue dye method (5). Aliquots were stored at −20°C until analyzed by SDS-PAGE.

Extraction of blebs for immunization of mice and for absorption of sera. Cells from cultures grown in MH-EDDA for the immunization of mice or in MH for the absorption of sera were centrifuged at 10,000 × *g* for 15 min, and the pellet was discarded. Blebs were obtained from the supernatant by centrifugation at 30,000 × *g* for 15 min and were resuspended in distilled water by two 5-s ultrasonic pulses. The suspension was then centrifuged at 10,000 × *g* for 15 min to remove contaminant cells. The protein content was determined by the Coomassie blue dye method.

Immunization of mice. Mice were immunized by a modification of the method of Brodeur et al. (7). Twelve BALB/c mice were injected intraperitoneally with 20 μg of blebs (total protein) suspended in complete Freund's adjuvant. On day 14, the mice were injected with 20 μg of the same preparation suspended in incomplete Freund's adjuvant, and on day 28, a booster dose of 20 μg in phosphate-buffered saline (PBS) was given intravenously. The mice were bled 3 days later through the retrobulbar (retro-orbital) plexus, and the 12 serum samples were pooled. Serum pools were obtained in this way with each of five strains: P000, P391, P636, V002, and V021.

Titration of sera. A dot blot technique was used for serum titration: 2-μl aliquots of extracts from OMPs of the homologous strain (500 μg/ml) were spotted onto nitrocellulose disks placed inside the wells of polystyrene microtitration plates, incubated with 0.1-ml aliquots of serial 1:5 dilutions of the serum, and then developed as described below for immunoblots.

Absorption of sera. To ensure they reacted only with iron-regulated proteins, mouse sera were absorbed essentially as described by Ala'Aldeen et al. (2). Briefly, 5 mg of blebs (total protein) from the MH-grown homologous strain was suspended in 10 ml of 1:50-diluted decomplemented (56°C, 30 min) serum, and the mixture was incubated overnight at room temperature. Antigen-antibody complexes were removed by centrifugation at 1,200 × *g* for 30 min and filtered through 0.2-μm-pore-size membrane filters.

SDS-PAGE and electroblotting. OMPs were analyzed by SDS-PAGE by using 10% acrylamide gels and the discontinuous buffer system of Laemmli (16). Samples containing 20 μg of protein were either boiled at 100°C for 15 min, heated at 37°C for 20 min, or maintained overnight at room temperature in sample buffer prior to electrophoresis. After the run, proteins were transferred from the gels to nitrocellulose membranes by using a Milliblot-SDE electroblotting system (Millipore) and a low-ionic-strength discontinuous buffer system consisting of two anode buffers (0.3 M Tris–10% methanol [pH 10.4] and 25 mM Tris–10% methanol [pH 10.4]) and one cathode buffer (25 mM Tris, 40 mM 6-aminohexanoic acid, 20% methanol [pH 9.4]) according to the

instructions provided by the manufacturer. Electrotransfer was performed over 30 min at 2.5 mA per cm^2 of gel area.

Immunoblotting. Nitrocellulose membranes were blocked with Tris-buffered saline (TBS) containing 0.5% skim milk, 0.001% antifoam A (Sigma Chemical Co., St. Louis, Mo.), and 0.5 g of Tween 20 per ml (blocking solution); washed twice with TBS; incubated at 37°C for 1.5 h in either normal or absorbed serum diluted with blocking solution; washed twice with TBS; and probed for 1.5 h at 37°C with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (DAKO-Immunoglobulins a/s, Glostrup, Denmark) diluted with blocking solution. The membranes were then washed thoroughly with TBS and developed with 4-chloro-1-naphthol–0.01% hydrogen peroxide (32) for 20 min.

Transferrin binding assay. Transferrin binding assays were performed essentially as described by Schryvers and Morris (25). After electrotransfer (and immunoblotting, in the case of sequential development for the detection of both antigens and TBP2 on the same membrane), membranes were incubated with blocking solution (this step was omitted in the case of sequential development), washed with TBS, incubated for 30 min at room temperature with blocking solution containing peroxidase-conjugated human transferrin (Jackson Immunoresearch Laboratories), washed, and developed as described above.

RESULTS

Immunization with blebs induced high antibody levels against antigenic OMPs, requiring antisera at working dilutions of about 1:6,000.

Strain P000 was the first one used for immunization since it produced a considerable amount of TBP2, as measured by the transferrin binding assay. Strains P636, V002, and V021 were successively selected for inoculation from among the strains that did not cross-react with the preceding serum; an additional criterion that was applied when possible was the possession of TBP2 with a markedly different apparent molecular weight (as estimated by SDS-PAGE) from that of the other strains selected. A fifth strain, P391, was also included for immunization. All 40 strains studied cross-reacted with at least one of the five serum samples (Tables 1 and 2).

Figure 1 shows densitograms of the OMPs present in the blebs obtained from the above strains when grown in normal and iron-restricted conditions. Figure 2 shows the main antigens from blebs obtained under both conditions (immunization was always performed with blebs obtained from iron restriction cultures); TBP2 was one of the major antigens in strains P000, V021, and P391, whereas in strains P636 and V002, it induced a much lower immune response than other outer membrane antigens. P000 was the only strain in which TBP2 was the only IROMP to induce antibodies. In all of the other four strains, one of the non-TBP2 antigenic IROMPs had a molecular mass of 56 kDa, though very little was produced by strain P636.

The absorption of the sera with blebs cultured under normal iron conditions generally produced, at most, low residual levels of antibodies against major normal antigens, the chief exception being the survival of antibodies against an 80-kDa non-IROMP in strains P636 and V002 (Fig. 3). The residual anti-non-IROMP antibodies did not interfere with TBP2 cross-reactivity assays, which always involved electrophoresis and electrotransfer followed by immunoblotting and the subsequent detection of TBP2 with peroxidase-conjugated transferrin.

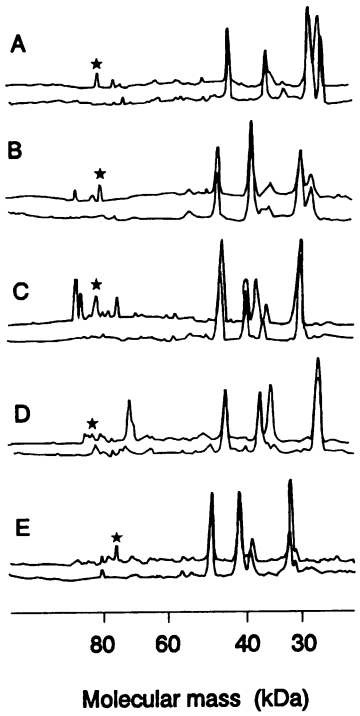

FIG. 1. Laser scanner densitograms of the OMPs of *N. meningitidis* P391 (A), V021 (B), P000 (C), P636 (D), and V002 (E) after SDS-PAGE. For each strain, the lower scan corresponds to bacteria cultured in normal medium and the upper scan corresponds to bacteria grown in iron restriction medium. Stars indicate TBP2.

The TBP2 of 90% (36 of 40) of the strains showed antigenic cross-reactivity with the anti-IROMP serum pool for strain P391, 57.5% (23 of 40) showed cross-reactivity with that for strain V021, and 35% (14 of 40) showed cross-reactivity with that for strain P000. The figures of 12.5% (5 of 40) for strain P636 and 7.5% for V002 may be underestimates due to the presence of the above-mentioned unabsorbed antibody to their 80-kDa antigenic non-IROMP (which cross-reacted with all strains), since it might mask the reaction with anti-TBP2 antibodies in strains with TBP2 of ca. 80 kDa; there were 9 such doubtful strains for V002 and 13 for P636.

Cross-reactivity for the 56-kDa antigen found in four of the strains used for immunization was high: all heterologous strains cross-reacted with strains V002 and P391, 84.6% (33 of 40) cross-reacted with strain P636, and 82% (32 of 40) cross-reacted with strain V021.

DISCUSSION

In recent years, it has become clear that iron plays an important part in bacterial pathogenesis, especially in the case of the meningococcal infections (6, 30, 31). Besides its crucial role in bacterial metabolism, iron influences the composition of the OMPs of many bacteria, including those of the genus *Neisseria*. In human extracellular fluids, iron is strongly chelated by the proteins transferrin and lactoferrin and is thus not readily available to bacteria, which have evolved by developing special iron uptake mechanisms involving the synthesis of IROMPs. Some of these IROMPs, including the receptors for human transferrin and lactoferrin, can be synthesized in large amounts, making them relatively important OMPs (11, 12). The change in outer membrane

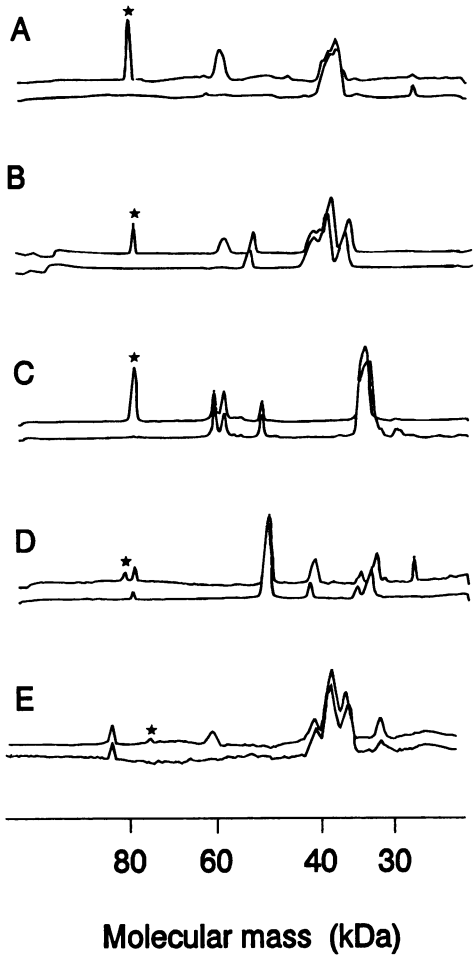

FIG. 2. Laser scanner densitograms of the outer membrane antigens of *N. meningitidis* P391 (A), V021 (B), P000 (C), P636 (D), and V002 (E) after SDS-PAGE, electrotransfer to nitrocellulose membranes, and development with homologous immune serum. For each strain, the lower scan corresponds to bacteria cultured in normal medium and the upper scan corresponds to bacteria grown in iron restriction medium. Stars indicate TBP2. Antisera were obtained by immunization with blebs obtained from iron-restricted growth, which allowed the visualization of both normal and iron-regulated antigens.

composition induced by iron restriction may well be responsible for the failure to obtain effective vaccines against *N. meningitidis* serogroup B and certain other bacteria. It is known, for example, that effective toxoid vaccines against *Corynebacterium diphtheriae* can be obtained only when this microorganism is grown in iron restriction media (14), and recent work on the development of a vaccine for *N. meningitidis* serogroup B (3) has shown that the culture of the bacteria in iron-restricting environments may be crucial to this purpose.

IROMPs are synthesized by *N. meningitidis* in vivo, and some of them can induce the production of immunoglobulin M and immunoglobulin G antibodies (4). Perhaps the most appropriate candidates for effective vaccines are those antigenic proteins whose function for meningococcal metabolism seems to be indispensable since antibodies against such proteins could block their function and disturb bacterial metabolism. Such is the case of the meningococcal transferrin receptors, which are needed by the bacteria to obtain transferrin-bound iron inside the host (9, 10), and in this work, we have accordingly focused exclusively on the antigenic properties of TBP2.

Some workers have reported the existence in all *N. meningitidis* strains of a 98-kDa TBP, named TBP1, detected by silver staining of SDS-PAGE gels after affinity purification by using biotinylated transferrin; however, it is unable to bind transferrin after electrotransfer of the SDS-PAGE gels (20, 22, 24, 25). Others, as was the case in this work, have found another TBP, named TBP2, with a molecular mass between 60 and 90 kDa, depending on the strain, which was able to bind transferrin after SDS-PAGE and electrotransfer (1, 11, 15, 22). The relationship, if any, between these two TBPs remains to be determined.

Dot blot experiments using both intact blebs and whole bacteria (data not shown) probed with peroxidase-conjugated transferrin showed that at least one of the TBPs was surface exposed. Probing the intact blebs and bacteria with adsorbed antiserum suggests that the TBP2 is indeed exposed. The TBP2s of the five strains used for immunization in our work were good immunogens and, in some cases, were among the major OMP antigens, comparable to the serotype antigens produced in normal iron media; in contrast, the TBP2 of other strains induced a much lower antibody response. Although almost all *N. meningitidis* strains synthesize three or four IROMPs (8), only one or two of them (always TBP2) seem to be antigenic in mice. This suggests that some strains are better candidates than others as potential sources of TBP2 for vaccination purposes.

There was great variety among the five inoculated strains with regard to antigenic cross-reactivity between the anti-IROMP sera they raised and the TBP2s of the other strains used in this study. P391 serum cross-reacted with the TBP2s of 90% of the heterologous strains tested, and V002 serum cross-reacted with those of only 7.5% of the strains. This variety suggests that there are several epitopes in the TBP2 molecule and that they can vary from one strain to another. Strain V021, for example, seems to share at least one epitope only with strain P391, strain P148 shares epitopes with strains P391 and P636, and strain P097 shares epitopes with four of the five strains used for immunization, etc. In particular, the fact that none of the three strains cross-reacting with strain V002 reacted with any of the other four strains used for immunization (except for a very slight reaction with strain P636) suggests that the antigenic determinants in the TBP2 of strain V002 are very different from those of the other four strains. This kind of antigenic variability has also been found by other authors (15) and for other IROMPs, including a 70-kDa *N. meningitidis* protein (2).

Interestingly, the serum obtained by immunization with strain P391 reacted with the TBP2s of strains P000 and P636, but not vice versa. It seems possible that the accessibility of epitopes for the immune system may vary among strains. More specifically, we hypothesize that strains may differ not only in the molecular masses of their TBP2s but also in the structural conformation of TBP or its association with other outer membrane structures which might mask one or another antigenic determinant.

The 56-kDa protein induced by the iron restriction medium, which was found to be immunogenic in all the strains used for immunization except strain P000, was detected in all the heterologous strains when probed with the sera from strain V002 or P391 and in more than 80% of the heterologous strains when tested with the sera from strain P636 or

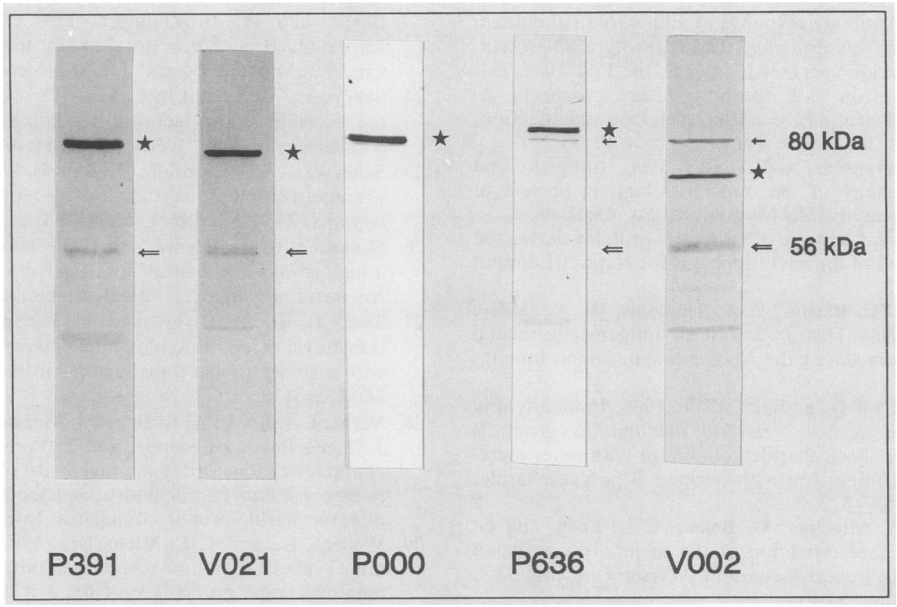

FIG. 3. Iron-regulated outer membrane antigens of *N. meningitidis* P391, V021, P000, P636, and V002 revealed as described by Schryvers and Morris (25). Antisera were obtained by immunization with blebs obtained from iron-restricted cultures and absorbed with blebs from cultures grown in iron-replete medium. ★, TBP2; ←, 80-kDa non-IROMP (not absorbable with normal iron culture blebs); ⇐, 56-kDa IROMP (hardly visible here for strain P636, but visible as a faint band in freshly developed blots). Lower bands correspond to incompletely absorbed major outer membrane antigens.

V021. The importance or involvement of this protein in the iron uptake mechanisms of *N. meningitidis* is not known; nor is it known whether it is really an iron-regulated or a stress-induced protein, but its antigenic homogeneity among strains nevertheless makes it a good candidate for vaccination studies.

The fact that we were unable to rid the immune sera of antibodies against the antigenic 80-kDa non-IROMP shows that this OMP is not exposed on the outer side of the blebs. It is important to keep in mind the existence of antigens of this kind which are detected by SDS-PAGE and immunoblotting but not exposed by the intact bacteria, because even when they are common to many strains (as is the case with this 80-kDa protein), they are unlikely to be effective for vaccination.

We are currently trying to answer some of the questions raised above by continuing our experiments with monospecific sera obtained by immunization with purified TBP2s and with monoclonal antibodies capable of detecting individual epitopes of TBP2 or of the 56-kDa protein.

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