Expression of *Neisseria meningitidis* Iron-Regulated Outer Membrane Proteins, Including a 70-Kilodalton Transferrin Receptor, and Their Potential for Use as Vaccines

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Invasive human pathogens must acquire iron for growth from host iron-binding proteins. In the mammalian host, there is essentially no soluble iron, because the iron is complexed with different iron-binding proteins, such as hemoglobin, transferrin, and lactoferrin. The latter two proteins have very high binding affinities for iron. Nonpathogenic or noninvasive bacteria do not have the ability to acquire the needed organic iron (14). There is now considerable evidence suggesting that the ability of a variety of human pathogens to produce disease is directly related to their ability to obtain iron (24). Pathogens have evolved a number of mechanisms for acquiring iron from the host. Some, like *Escherichia coli* and *Salmonella* spp., produce soluble factors called siderophores to chelate iron from the medium or host (12, 14). Others, like the pathogenic neisseriae, are able to acquire iron from the host by other mechanisms. The neisseriae elaborate a number of new proteins on their cell surface when grown under conditions of iron limitation (4, 25). These proteins are referred to as iron-regulated proteins (IRPs).

The neisserial IRPs range in molecular mass from 36 to over 100 kilodaltons (kDa) (2, 13). The role these proteins play in the organism's ability to survive in the host has not been determined. Simonson et al. (19) found that a 36.5-kDa protein firmly bound radioactive iron. This protein may be involved in iron transport. It is known that neisseriae can acquire iron from human transferrin and lactoferrin but are unable to acquire iron from bovine transferrin and lactoferrin (15) or the closely related chicken protein conalbumin (9). In addition, neisseriae cannot take up the transferrin-bound iron unless they are in direct contact with the protein (1). These data suggest that neisseriae have a specific transferrin-binding protein. Iron-starved meningococci express a cell surface receptor(s) for acquisition of iron from transferrin (19). The receptor was specific for transferrin and was saturable. Dyer et al. (4) isolated a mutant of meningococcal strain M986-NCV-1 (M986) that lacked a 70-kDa iron-regulated outer membrane protein that was unable to take up iron from either transferrin or lactoferrin. However, they later isolated revertants still lacking the 70-kDa protein but able to bind transferrin normally (25). Schryvers and Morris (18) described a 71-kDa meningococcal transferrin receptor protein; however, additional studies with affinity purification of chemically modified transferrin suggested that the receptor may be a complex of two proteins, including a 98-kDa protein (16, 17).

The present studies were initiated to study the outer membrane protein composition of *Neisseria meningitidis* grown under different simulated in vivo conditions so as to identify proteins that may not be seen in cells grown under the usual laboratory conditions. Ideally, an outer membrane protein vaccine should contain those proteins that are expressed during disease. We present data on the outer membrane proteins induced as a result of growth under various iron-limited conditions. Outer membrane protein vaccines were prepared from two meningococcal strains grown to express IRPs.

**MATERIALS AND METHODS**

**Meningococcal strains.** Capsule-deficient mutants of the group B *N. meningitidis* strains M986 (2a:P1.2) and 44/76 (B:15:P1.16), designated M986-NCV-1 and 44/76-M25, respectively, were used for most of these studies. Also used were four other class 2 protein-containing strains, BB-567, BB-569, M992, and 3006, and five class 3 protein-containing strains, M981, H355, S3032, M978, and M1080. All strains were from a collection maintained by C. Frasch.
Growth conditions and induction of IRPs. The primary medium used was Catlin medium without added FeCl₃ (3). Deionized water was used for medium preparation, and the glassware was acid washed. Desferal (desferrioxamine mesylate; Ciba Pharmaceutical Co., Summit, N.J.) was added to the medium to chelate free ferric iron and induce formation of the IRPs in the outer membrane. Since Desferal inhibited growth, the bacteria were first grown overnight in Catlin medium without added iron on a gyratory shaker at 37°C. The low level of iron remaining in the medium permitted good yields of cells. The cells were then transferred to 3 volumes of fresh Catlin medium with 50 μM Desferal (final concentration). The organisms were returned to the shaker for an additional 4 to 6 h to induce IRPs.

Human transferrin (Sigma Chemical Co., St. Louis, Mo.) was made 30% iron saturated as described previously (22), sterile filtered, and added to the Catlin medium as a source of iron during active growth. Similarly, bovine hemoglobin was dissolved in deionized water, sterile filtered, and added to the medium. The organisms were grown overnight as before and harvested by centrifugation for preparation of outer membrane vesicles (OMV).

To simulate in vivo conditions, strain M986-NCV-1 was grown in 200 ml of the tissue culture medium RPMI-1640 containing 20% fetal calf serum in tissue culture flasks in a 37°C incubator with 5% CO₂ for 72 h. The cells were harvested and used to prepare membrane vesicles.

Preparation of OMV. The OMV were prepared by lithium chloride extraction as described previously (11).

SDS-PAGE and immunoblotting. The OMV were examined by 10% alkaline sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with the discontinuous buffer system of Laemmli (7). From 10 to 20 μg of protein was heated in sample buffer at 37°C for 20 min or boiled for 5 min. After electrophoresis, the gels were either stained with Coomassie brilliant blue R-250 or used for transfer of the separated proteins onto nitrocellulose. The transfer was achieved by using the Trans-Blot chamber from Bio-Rad Laboratories (Richmond, Calif.) with 25 mM Tris–129 mM glycine buffer (pH 8.3) without methanol at 100 mA overnight with cooling. In a variation of the immunoblot, the untreated OMV were dot-blotted directly onto the nitrocellulose with 2 μl of a 1- to 2-mg/ml protein solution.

The immunoblots and dot-blot were probed either with antibodies to human transferrin or with mouse immune sera. The antibodies were visualized by using horseradish peroxidase–anti-immunoglobulin conjugates, followed by the substrate 4-chloro-1-naphthol (21).

Detection of transferrin binding. Native human transferrin bound either to washed whole meningococcal cells or to isolated OMV was detected by dot-blot. The cells or the OMV extracted from cells recovered after growth of meningococci in Catlin medium with 30% iron-saturated transferrin were applied to nitrocellulose, blocked with gelatin as described before (21), and then treated with antitransferrin antibody (Boehringer Mannheim, Indianapolis, Ind.). For some experiments, OMV from cells grown either with sufficient soluble iron or under iron-limited conditions were blotted onto the nitrocellulose and then incubated with 5 to 10 μg of 30% iron-saturated transferrin per ml in Tris-buffered saline (pH 7.4) containing 1% soluble gelatin (Bio-Rad) or the nitrocellulose was blocked for 30 min in the gelatin solution. Excess transferrin was removed by two 10-min washes with Tris-buffered saline containing 0.05% Tween 20. The transferrin was detected as for the immunoblot.

The completed dot-blot and immunoblots were scanned with a laser densitometer (Pharmacia LKB, Piscataway, N.J.), and the data were integrated to quantitate the amount of transferrin bound.

Iodination of human transferrin. Transferrin was labeled with 125I by using Iodobeads (Pierce Chemical Co., Rockford, Ill.). A 1 mg/ml solution of transferrin was prepared in 40 mM Tris hydrochloride (Tris-HCl)–20 mM sodium bicarbonate (pH 7.5), and 500 μg was added to the Iodobeads containing 500 μCl of 125I solution. After 15 min of incubation, the reaction mixture was transferred to a Sephadex G-25 column to separate labeled protein from free iodide.

Fractionation of outer membrane proteins by gel filtration. An OMV preparation from strain M986-NCV-1, grown to express the IRPs, was solubilized for 2 h at 4°C in 0.1 M Tris-HCl buffer, pH 7.0, containing 1% Sarkosyl (Sigma). Insoluble material was removed by centrifugation for 2 h at 100,000 × g. The supernatant, shown by dot-immunoblot to contain most of the transferrin-binding activity, was applied to a column (1.5 by 100 cm) containing Sephacryl S-300 (Pharmacia LKB). The elution buffer was the same as the solubilization buffer. Four volumes of 95% ethanol were added to each fraction to recover protein. The precipitates were dissolved in water. Each fraction was examined by SDS-PAGE and by a dot-blot transferrin-binding assay. Fractions shown to contain transferrin-binding activity by dot-blot were incubated for 20 min at 37°C in SDS-PAGE sample buffer and then separated by SDS-PAGE and electroblotted onto nitrocellulose. The nitrocellulose was blocked with 3% gelatin in Tris-buffered saline with Tween 20 and then incubated with 125I-labeled transferrin for 5 h. After several washes in Tris-buffered saline with Tween 20, the nitrocellulose was exposed to X-ray film for autoradiography.

Vaccine preparation and immunization of mice. Outer membrane protein vaccines were prepared from the nonencapsulated variants M986-NCV-1 (M986) (—:2a:P1.2) and 44/76-M25 (44/76) (—:15:P1.16). The M986 variant was selected as a naturally occurring capsuleless mutant by using group B meningococcal antiserum agar. The 44/76 variant was selected on antiserum agar following ethyl methanesulfonylate mutagenesis.

Vaccines were prepared from the type 2 and type 15 strains grown with induced IRPs. The OMV were prepared from the cells as described above and then extracted with 2% (wt/vol) sodium deoxycholate (Sigma) in 30 mM Tris-HCl–2 mM EDTA (pH 8.5) at room temperature for 30 min. The LPS-depleted vesicles were pelleted by centrifugation at 150,000 × g and washed twice with water to remove the detergent. The protein content of the treated vesicles was measured by the method of Lowry et al. (8), and the LPS content was measured by an SDS-PAGE method (21).

LPS-depleted OMV were mixed in a ratio of 1:100 with aluminum hydroxide. The protein was also prepared in phosphate-buffered saline without adjuvant. Groups of 10- to 6-week-old female NIH general purpose mice were immunized with a single 5-μg intraperitoneal dose of the vaccine. Mice were bled 3 weeks later.

Mouse sera were examined for anti-outer membrane antibodies by enzyme-linked immunosorbent assay (ELISA) (6) with OMV purified from the vaccine strains grown with sufficient iron or under iron-limited conditions. The OMV were coated onto the polystyrene plates at 4 μg of protein per ml. The A₅₀₅ was measured after 20 to 50 min and extrapolated to 100 min. The antibody was expressed as units of antibody in reference to a standard serum. The sera
were also examined for bactericidal antibodies by using baby rabbit sera as the source of complement (5).

RESULTS

Induction of IRPs. Removal of free ferric ions from the growth medium caused the appearance, as expected, of new proteins of between 70 and 108 kDa in the outer membrane of meningococcal strain M986-NCV-1 (Fig. 1). Cells grown in the complete Catlin medium showed three high-molecular-mass bands above 70 kDa. Addition of 50 μM Desferal resulted in expression of four new high-molecular-mass proteins. The two prominent high-molecular-mass proteins seen in the medium with sufficient iron appeared to be downregulated.

IRPs could be induced by growing M986-NCV-1 in Catlin medium containing 30% iron-saturated human transferrin (Fig. 2A, lane 3). Examining the doublet protein bands at about 85 kDa, it is evident that the upper band was not induced as occurred with Desferal. Essentially the same results were seen when the strain was grown with hemoglobin as the only source of iron (Fig. 2A, lane 4). Only partial induction of IRPs occurred when 20% fetal calf serum was added to Catlin medium without added iron (Fig. 2B, lane 4).

To simulate in vivo conditions, strain M986-NCV-1 was grown in stationary culture in RPMI-1640 containing 20% fetal calf serum, and under these conditions IRPs were expressed (Fig. 2B, lane 5). An additional 51-kDa protein was induced in the tissue culture medium independent of iron levels when the organisms were grown in stationary cultures and when grown under low pH and is probably a stress protein (data not shown). Additional studies are planned, as it is likely that this protein is also expressed in vivo under lower oxygen levels.

Meningococci can be divided into two populations based on expression of a class 2 or class 3 major outer membrane protein. To determine whether these two different popula-

FIG. 1. Iron-regulated outer membrane proteins of meningococcal strain M986-NCV-1. Lane 1, Membranes from cells grown in iron-deficient Catlin medium containing 12.5 μM Desferal; lane 2, membranes from cells grown in normal Catlin medium. Sizes are shown in kilodaltons.

FIG. 2. Effect of iron limitation (A) and growth conditions (B) on the expression of IRPs by meningococcal strain M986-NCV-1. (A) Lane 1, 12.5 μM Desferal; lane 2, 50 μM ferric iron; lane 3, 2.5 μM human transferrin; lane 4, 10 μM bovine hemoglobin; lane 5, same as lane 2. (B) Lane 1, Molecular mass markers (116, 84, 58, and 48 kDa) (Sigma); lane 2, normal Catlin defined medium; lane 3, Catlin with 12.5 μM Desferal; lane 4, Catlin with 20% fetal calf serum; lane 5, RPMI-1640 with 20% fetal calf serum.

FIG. 3. Meningococcal strains expressing the class 3 porin protein grown in iron-complete and iron-deficient Catlin medium. For each pair of lanes, membranes from iron-sufficient and then iron-deficient medium are shown. The strains are: lanes 1, M981 (B:1); lanes 2, H355 (B:15;P1.15); lanes 3, S3032 (B:12;P1.16); lanes 4, M1080 (B:1); and lanes 5, M978 (B:8). The molecular size standards are the same as in Fig. 2.

Additional IRPs were detected in the PAGE gel by using an antitransferrin dot-blot immunoassay (Fig. 4). The 36-kDa protein described by Meitzner et al. (10) was not apparent in our SDS-PAGE gels as visualized by Coomassie brilliant blue staining.

Transferrin binding to the cell surface. Transferrin binding to isolated outer membranes could be detected and relatively quantitated by an antitransferrin dot-blot immunoassay (Fig. 4). Cells exposed to increasing levels of the iron chelator Desferal showed a progressive increase in the amount of transferrin bound. Thus, we were able to measure variable
amounts of receptor activity by dot-blot by using native transferrin, which led to the following experiment.

**Solubilization and identification of the transferrin-binding protein.** Different detergents were compared for their ability to extract the transferrin-binding protein from purified outer membranes. Sarkosyl solubilized over 90% of the transferrin-binding activity from the membranes, determined by comparison of supernatant and pellet after ultracentrifugation. The Sarkosyl-soluble material was separated by gel filtration on Sephacryl S-300, and each fraction was examined for transferrin-binding activity by dot-blotting onto nitrocellulose. Fifteen consecutive fractions were selected for SDS-PAGE analysis (Fig. 5A) based on measurement of transferrin binding on the dot-blot. The amount of transferrin bound was semiquantitatively measured by densitometry (Fig. 5B). The arrows on the SDS-PAGE gel denote the two proteins whose elution profiles appeared to correspond to the peak transferrin-binding activity in fractions 9 and 10, suggesting that one or both of these proteins could have transferrin receptor activity. To determine which protein would bind transferrin, fraction 10 was blotted (Western blot) as described in Materials and Methods. Only the higher-molecular-mass band bound 125I-labeled human transferrin (data not shown). Thus, the minor 70-kDa protein below the major 74-kDa protein appears to be a transferrin receptor.

**Outer membrane vaccines containing IRPs.** We found that the organisms could be grown in the defined medium to high yields in the presence of minimal iron and then transferred to fresh medium with high levels of Desferal to induce expression of the IRPs. Thus, we were able to prepare large amounts of cells having expressed IRPs for outer membrane protein vaccine preparation. Vaccines from M986-NCV-1 (−: 2a: P1.1) and 44/76-M25 (−: 15: P16) were prepared and used to immunize adult mice with and without aluminum hydroxide adjuvant. A single 5-μg injection of vaccine with or without adjuvant elicited good immune responses as measured by ELISA (Table 1). The antibody response was measured against outer membranes from the vaccine strains with and without IRPs expressed. All sera gave significantly...

**FIG. 4.** Relative quantitation of transferrin receptor activity by dot-immunoblot. Membranes from cells incubated with 0, 2, 4, or 50 μM Desferal were reacted with human transferrin and then spotted onto nitrocellulose membranes. Laser densitometer peak heights are shown.

**FIG. 5.** Identification of the transferrin receptor protein of meningococcal strain M986. (A) SDS-PAGE analysis of Sarkosyl-soluble outer membrane proteins after separation by gel filtration on Sephacryl S-300. Arrowheads show the two proteins coeluting with the peak transferrin-binding activity. (B) Transferrin binding was measured by densitometry of dot-bLOTS of Sephacryl S-300 column fractions. Only those fractions eluting around the transferrin receptor-positive fractions are shown.
higher antibody binding to membranes containing the IRPs. The adjuvant had little effect (data not shown).

The specificity of the antibodies was evaluated by immunoblotting (Fig. 6). The sera were reacted against SDS-PAGE-separated M986 and 44/76 outer membranes prepared from cells grown under iron-sufficient and iron-limited conditions. It can be seen that three IRPs in M986 were immunogenic in almost all the mice, and one of these proteins had common epitopes with those expressed by 44/76. The 44/76 vaccine-induced antibodies were reactive by immunoblot with only one of the IRPs present in this strain. An approximately 60-kDa non-iron-regulated protein was common to both M986 and 44/76 and was strongly immunogenic in all the mice. A 180-kDa protein, common to most of the serotype 2 strains, was also strongly immunogenic. Of the class 1 through 5 proteins, only the class 1 and 5 proteins induced antibodies reactive by immunoblot.

To obtain a preliminary indication of whether the antibodies to the IRPs detected by immunoblotting were also bactericidal, the mouse sera prepared to the M986 vaccine were examined for the ability to kill M986 grown with sufficient iron or under iron-deficient conditions (Fig. 7). The bactericidal activity of the sera was shifted to higher titers against the organisms expressing IRPs. Similar experiments were done with strain 44/76 and the 15:P.16 vaccine serum, but 44/76 was killed at bactericidal titers in excess of 1:640 by many of the sera with or without the IRPs expressed.

DISCUSSION

Induction of new proteins under conditions of iron limitation is a well-known phenomenon in different microorganisms (12). The meningococcus is obligately dependent on iron for growth and can obtain the iron from a number of sources. In its normal environment, most of the iron is complexed with protein. Therefore, a set of IRPs are induced under conditions of very low free iron (2, 13). In presence of 50 μM Desferal, an iron chelator, meningococci

![FIG. 6. Immunoblotting of mouse sera following immunization with outer membrane protein vaccines from strains M986 and 44/76 grown to express IRPs. Ten mice were immunized in each group. Each blot contains (lanes 1) anti-M986 sera and (lanes 2) anti-44/76 sera. The blots are: (A) M986 outer membranes from cells grown under iron-sufficient conditions; (B) M986 outer membranes, IRPs expressed; (C) 44/76 outer membranes from cells grown under iron-sufficient conditions; (D) 44/76 outer membranes, IRPs expressed. Three IRPs are seen in B and one IRP is seen in D.](http://iai.asm.org/)

![FIG. 7. Homologous bactericidal activity of 10 individual mouse antisera prepared to an M986 outer membrane protein vaccine containing IRPs. The sera were examined for their ability to kill M986 cells grown under iron-sufficient and iron-deficient conditions.](http://iai.asm.org/)
were able to fully induce their IRPs but were unable to grow. In contrast, when presented with their normal in vivo iron sources, transferrin and hemoglobin, the IRPs were induced and the bacteria grew well. The transferrin must be human, as bovine transferrin or conalbumin will not substitute (9). However, we observed that the organisms were able to use bovine hemoglobin as an iron source.

To simulate in vivo growth conditions, we grew the meningococci under conditions of both low iron and lower oxygen levels in the tissue culture medium RPMI-1640 supplemented with 20% fetal calf serum. Under these conditions, growth occurs and IRPs are induced. Studies on the antibody response of meningococcal patients to outer membrane proteins also indicate that the IRPs are expressed in vivo (2).

For the IRPs to be useful as potential vaccine candidates, antigenically related IRPs must be widely shared. We compared the IRPs of five class 2 and five class 3 strains to see the variability of proteins induced as a result of iron limitation. All strains had three or four high-molecular-weight IRPs. As with other meningococcal outer membrane proteins, there was some degree of variation in protein expression and molecular weight, suggesting antigenic differences. Black et al. (2) studied the IRPs in strains isolated from a number of patients and found that some IRPs were shared among strains.

West and Sparling (25) found that expression of IRPs in gonococci varied depending upon the gonococcal strain and the source of iron. The functional significance of these proteins is only now becoming evident (4, 17, 18). A 36- to 37-kDa protein, which occurs as a structurally conserved and widely cross-reactive component of the outer membrane, may function as an iron uptake protein (10). Schryvers et al. reported that the transferrin-binding protein is either a minor IRP of about 70 kDa or a complex of this protein and a 98-kDa protein, while the lactoferrin-binding protein is a 105-kDa IRP (16-18).

We have also determined the 70-kDa outer membrane protein to be a transferrin receptor, as recently reported by Schryvers and Morris (18). By sequential analysis of column fractions containing IRPs by SDS-PAGE, different proteins eluted with peak concentrations in different fractions, and through comparison of the SDS-PAGE results with transferrin binding by dot-blot, we were able to locate a 70-kDa protein that correlated well with the transferrin-binding activity. When the proteins from gel filtration column fraction 10, containing peak binding activity, were dissolved in the SDS digestion mixture, incubated at 37°C, and run directly on the PAGE gel, a protein of approximately 70 kDa bound radiolabeled transferrin. Samples that were boiled in the SDS digestion mix failed to bind transferrin when electrobotted.

Meningococci prefer to use soluble iron. In the presence of 10 μM ferric iron, the IRPs were not expressed, and the cells failed to bind appreciable amounts of the 30% iron-saturated transferrin. When 2 μM Desferal was added to the Catlin medium, the membranes bound appreciably more transferrin. The amount of transferrin receptor expressed was progressively upregulated by decreasing amounts of available inorganic iron, as evidenced by the progressive increase in bound transferrin with decreasing availability of free iron (Fig. 4).

An effective vaccine against group B meningococcal disease will have to contain outer membrane proteins, because the group B polysaccharide, in contrast to the other meningococcal polysaccharides, does not stimulate protective antibodies. However, outer membrane protein vaccines are in general too serotype specific and do not contain those outer membrane proteins normally expressed only under in vivo conditions. The outer membrane IRPs are critical cell surface proteins, and it is possible that antibodies against these proteins either are bactericidal or will block invasion.

Expression of the IRPs can be induced without cell division. Meningococci grown with relatively low iron levels and then transferred to fresh medium containing 50 μM Desferal did not divide but expressed the IRPs, including the 105-kDa lactoferrin-binding protein (17), shown in Fig. 1 as the 108-kDa band. This allowed us to obtain sufficient cell yields to prepare serotype 2 and serotype 15 outer membrane protein vaccines containing expressed IRPs.

There was little cross-reactivity in the mice between the serotypes 2 and 15, unless the IRPs were also present. The immunogenicity of the IRPs in mice was confirmed by Western blot analysis. An important finding was that the major 74-kDa IRP appeared to be antigenically the same in the two quite divergent serotypes.

The vaccines were administered with and without adsorption to aluminum hydroxide. In contrast to our previous vaccines, the present vaccines did not contain capsular polysaccharide. The aluminum hydroxide had no demonstrable effect on the antibody response to the outer membrane proteins, in marked contrast to earlier studies in mice with vaccines containing aluminum hydroxide and polysaccharide and lipopolysaccharide-depleted outer membranes (6, 22).

There is good evidence that the IRPs are expressed in vivo and that they induce detectable antibody levels as a consequence of infection in children (2). These proteins are critical to the survival of the organisms in vivo, are immunogenic, and become major outer membrane proteins with iron limitation. Meningococcal outer membrane protein vaccines should therefore contain these proteins.

LITERATURE CITED

MENINGOCOCCAL IRON-REGULATED PROTEINS

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