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Human Immune Response to Iron-Repressible Outer Membrane Proteins of *Neisseria meningitidis*

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***Neisseria meningitidis* grown under iron-limiting conditions in vitro expresses additional iron-repressible outer membrane proteins (FeRPs). To see which FeRPs were expressed and immunogenic in human infection, we examined purified membranes from four meningococcal disease isolates with Western blotting of patient sera. Convalescent serum from each patient contained immunoglobulin G (IgG) and IgM antibodies against the homologous 70-kilodalton (kDa) FeRP and IgG antibody to the homologous 94-kDa FeRPs. Three other immunoreactive FeRPs were identified in two or more strains. Neither acute-phase sera nor pooled normal human sera contained appreciable levels of these antibodies. Antigenic cross-reactivity among FeRPs was suggested by the observation that the convalescent sera of two patients contained IgG antibodies reactive with the 70- and 94-kDa FeRPs and IgM antibodies reactive with the 70-kDa FeRPs from all four strains. Additionally, rabbit antiserum against the 70-kDa FeRP from one of these disease isolates contained IgG and IgM antibodies that reacted in Western blots with the 70-kDa FeRPs in all four strains. These results demonstrate that meningococcal FeRPs are expressed and immunogenic in vivo and that certain of these proteins are immunologically cross-reactive.**

Neisseria meningitidis is second only to *Haemophilus influenzae* as a cause of bacterial meningitis in the United States and is a major human pathogen worldwide (6). Polysaccharide vaccines offer protection against infection by organisms from the A, C, Y, and W-135 capsular serogroups, but no effective vaccine yet exists for serogroup B meningococci. Serogroup B organisms are currently responsible for the majority of meningococcal diseases in the United States and Western Europe (6). Moreover, in the age group (under 2 years) most susceptible to disease, the immune response to serogroup A and C polysaccharides is poor (9). Consequently, in addition to group-specific polysaccharides, other antigens must be identified to achieve broadly effective immunoprophylaxis against meningococcal disease. Several recent observations suggest that iron acquisition mechanisms of meningococci might provide such targets.

Many studies suggest that iron acquisition is important for bacterial pathogens, including *N. meningitidis* (5, 27, 28). Holbein (13) showed that the use of transferrin (TF) iron is a critical factor in maintaining meningococcemia in experimentally infected mice. Most bacteria manufacture iron-chelating compounds (siderophores) to acquire essential iron from their environment (22). Siderophore production enhances the virulence of many bacteria, probably by increasing the availability of iron bound to host proteins such as TF and lactoferrin (LF). These two iron-binding proteins are thought to be the primary iron sources available to bacterial pathogens in plasma and on mucosal surfaces, respectively (27). Although Yancey and Finkelstein (32) reported that meningococci produce minute quantities of a hydroxamate siderophore, other studies have failed to detect meningococcal siderophores (1, 26, 29). Nevertheless, all meningococci can utilize partially saturated TF and LF as sole sources of iron for growth (19, 20, 26). In contrast, most nonpathogenic *Neisseria* spp. cannot use TF or LF as an iron source (19, 20). The mechanism by which meningococci acquire iron from TF is unclear, but it is known that TF is not internalized as iron is removed and that digestion of the meningococcal cell surface with trypsin inhibits iron uptake from TF (26). These observations imply that the meningococcus may possess cell surface receptors involved in acquisition of iron from TF and perhaps from other iron-binding proteins.

Iron-limited growth of many bacteria derepresses the synthesis of a variety of outer membrane proteins, many of which are critically involved in iron uptake (23). For example, in *Escherichia coli* iron-repressible proteins (FeRPs) varying in molecular mass from 74 to 81 kilodaltons (kDa) are outer membrane receptors for various ferri-siderophore complexes (11, 12, 18, 30). These FeRPs are expressed in the membranes of *E. coli* isolated from experimentally infected guinea pigs (10), suggesting their importance during infection. Meningococci also possess outer membrane FeRPs (Fig. 1), and Brener et al. (3) found that increased synthesis of one of these FeRPs was associated with enhanced virulence of meningococci in experimentally infected mice. Taken together, these observations suggest that meningococcal FeRPs may be involved in iron acquisition and in the pathogenesis of meningococcal disease.

Consequently, we were interested in whether meningococcal FeRPs (seen in vitro only under iron-limited growth conditions) are expressed in vivo and whether they are immunogenic. Therefore, we studied sera from four patients with meningococcal disease by Western blotting for evidence of an immune response to meningococcal FeRPs. We also examined the antigenic relatedness of individual FeRPs by probing membranes from all four strains with sera from two patients and with rabbit antiserum directed against the 70-kDa FeRP from one of these strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The meningococcal strains used in this study were all recent clinical disease

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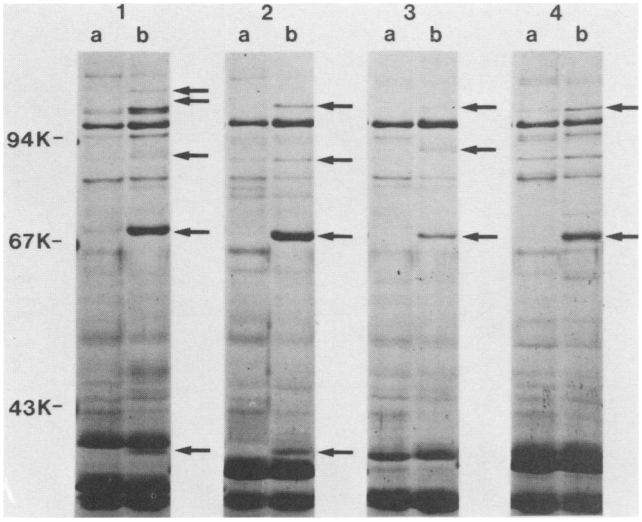

FIG. 1. Meningococcal membranes analyzed by SDS-PAGE (Coomassie blue stain). In each numbered pair of samples, lane a designates iron-sufficient conditions, and lane b designates iron-limited growth. Samples: 1, JB501; 2, JB502; 3, JB503; 4, FAM20. FeRPs are identified with arrows. Molecular weights are indicated in thousands (K).

isolates (blood or cerebrospinal fluid) obtained from the North Carolina Memorial Hospital and identified as *N. meningitidis* by Gram stain, oxidase reaction, carbohydrate utilization, and reaction with meningococcal serogroup-specific antiserum (Burroughs Wellcome Co., Research Triangle Park, N.C.). Strains JB501, JB502, and FAM20 were serogroup C, while JB503 was serogroup B. FAM20 was serotyped as 2a, according to the scheme of Frasch et al. (8). JB501, JB502, and JB503 were nontypable but displayed very different protein profiles after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). Upon isolation, each strain was passed only once on GCB agar (Difco Laboratories, Detroit, Mich.) with Kellogg supplements (14); the organisms were then frozen (−70°C) in GCB broth plus 20% sterile glycerol for further use.

Membrane preparation. Bacteria were grown to late log phase in the iron-limited defined liquid medium described by Mickelsen and Sparling (20); iron-replete cells were grown in defined medium containing 2 μM added iron [as $Fe(NO_3)_3$]. Cells were harvested by centrifugation at 12,000 × *g*, suspended in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7.2, and disrupted by passage through a French pressure cell at 14,000 to 16,000 lb/in^2. The lysate was centrifuged at 12,000 × *g* to remove unbroken cells, and the supernatant was collected; the membranes were pelleted and washed by centrifugation at 102,000 × *g* for 1 h. The protein concentration of each membrane preparation was determined as described by Markwell et al. (17).

Sera. Human sera were obtained from the patients from whom JB501, JB502, JB503, or FAM20 was isolated. Blood was collected by venipuncture within 24 h of the onset of symptoms (acute sera) and between 4 days and 4 weeks following onset (convalescent sera). Only convalescent serum was available from the patient whose culture yielded strain JB503, as this individual suffered from chronic meningococcemia. Normal human serum was obtained from a pool of individuals without a prior history of overt meningococcal or gonococcal infection.

Rabbit antiserum directed against the 70-kDa FeRP of strain FAM20 was prepared essentially as described by Payne and Collins (Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, K199, p. 204). A 500-μg sample of outer membranes from iron-limited cells was electrophoresed on a 10% SDS-polyacrylamide gel, and the 70-kDa FeRP was identified by staining with Coomassie blue. The protein band was excised from the gel, electroeluted into 25 mM Tris–192 mM glycine–0.1% SDS (pH 8.3), and dialyzed against 2 mM Tris–30 mM NaCl (pH 7.4). The protein was then concentrated by lyophilization, dissolved in a small volume of sterile water, and emulsified with an equal volume of complete Freund adjuvant. The mixture was then injected subcutaneously into a young (6-week-old) New Zealand White female rabbit; booster injections followed at 2-week intervals, with antigen emulsified in incomplete Freund adjuvant.

SDS-PAGE and Western blotting. SDS-PAGE was performed in 10, 15, or 4 to 30% linear-gradient acrylamide gels, using the discontinuous buffer system of Laemmli (15). Outer membrane preparations (10 μg) were mixed with an equal volume of sample buffer (0.125 M Tris hydrochloride [pH 6.8], 4% SDS, 20% glycerol, 0.004% bromophenol blue) and solubilized at 37°C for 60 min or 100°C for 5 min. The solubilization conditions used did not affect the mobility of any FeRPs on SDS-PAGE. Electrophoresis was carried out at 30 mA constant current. Gels were stained by the procedure of Wray et al. (31) or used for Western transfer.

For Western blots, electrophoretically separated proteins were transferred to nitrocellulose (Millipore Corp., Bedford, Mass.), using the phosphate buffer system of Batteiger et al. (2). Transfer was performed in a Transblot device (Bio-Rad Laboratories, Richmond, Calif.) with a current of 75 mA for 16 h. Nitrocellulose blots were then probed with acute or convalescent patient sera, with pooled normal human sera, or with immune or pooled normal rabbit sera, diluted in 10 mM Tris hydrochloride (pH 7.4)–0.9% NaCl–5% bovine serum albumin or in phosphate-buffered saline (4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl [pH 7.3]) plus 0.05% (vol/vol) Tween 20. Resulting antigen-antibody complexes were identified by ^{125}I-protein A for immunoglobulin G (IgG) (Amersham Corp., Arlington Heights, Ill.) or goat anti-human or anti-rabbit IgM (Capell, Malvern, Pa.) that were labeled by the procedure of Markwell and Fox (16). Blots were then exposed to Kodak X-OMAT AR-5 X-ray film for 24 to 72 h at −70°C, with intensifying screens before developing.

RESULTS AND DISCUSSION

When grown under iron-limiting conditions, membranes of the four meningococcal strains contained FeRPs whose apparent molecular weights were identical or very similar in all strains, as well as other FeRPs not shared by all strains (Fig. 1). SDS gels stained with Coomassie blue showed FeRPs of about 70 and 105 kDa in all strains (Fig. 1). SDS gels stained with silver showed an FeRP of about 94 kDa in all strains and an FeRP of about 88 kDa in all strains except JB503. Other FeRPs in the size range of 84 to 110 kDa were seen in strains JB501, JB502, and JB503. Mietzner et al. (21) recently described a 37-kDa FeRP in *N. meningitidis*, using 9.5 to 12.5% SDS-PAGE gradient gels containing 70 mM NaCl. However, this protein does not appear to be expressed in all strains of meningococci grown in our defined, iron-limited medium (Fig. 1; D. Dyer and P. F. Sparling, personal communication).

Acute-phase serum did not show binding in Western blots to meningococcal FeRPs from these strains at dilutions used

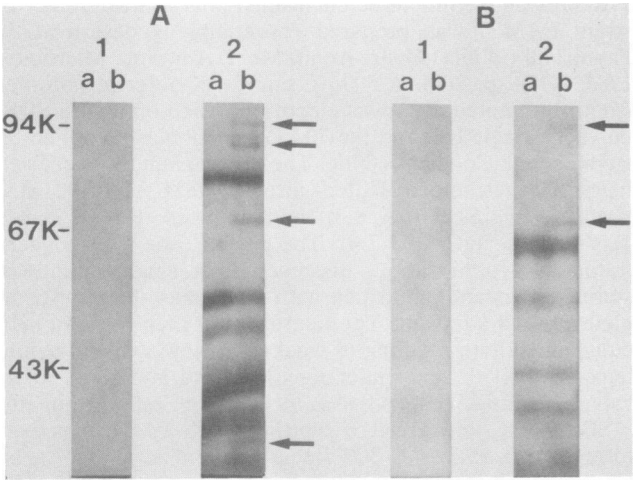

FIG. 2. Western blots of meningococcal membranes grown with (a) and without (b) added iron. These autoradiograms show the IgG antibody response (A) and IgM antibody response (B) in acute (1) and convalescent (2) sera from the patient with strain JB502 to proteins from the homologous infecting strain JB502. Immunoreactive FeRPs are marked with arrows. Sera were diluted 1:200 (A) or 1:50 (B). Molecular weights are indicated in thousands (K).

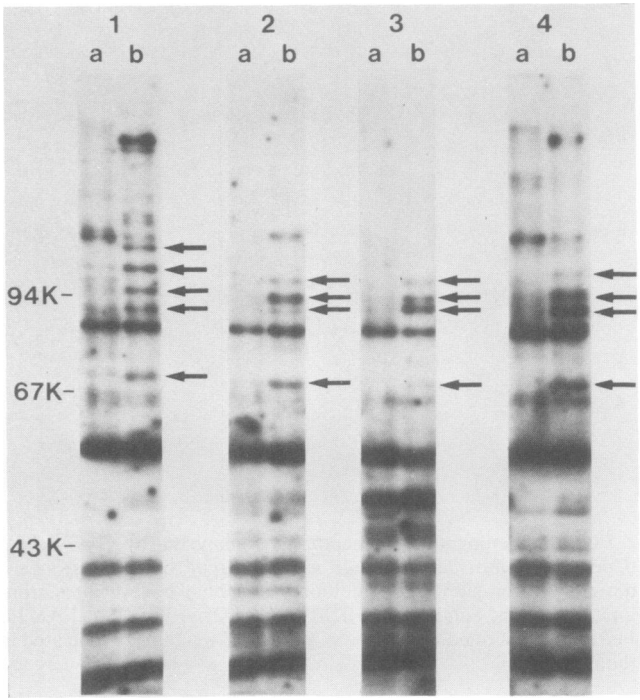

FIG. 3. Western blot showing IgG response in convalescent serum from the patient with strain JB501 to individual proteins from membranes of four meningococcal isolates. In each pair of samples, lane a designates iron-sufficient medium, and lane b designates iron-limited conditions. Samples: 1, JB501; 2, JB502; 3, JB503; 4, FAM20. Immunoreactive FeRPs are identified with arrows. Serum was diluted 1:200. Molecular weights are indicated in thousands (K).

for convalescent serum (1:200 for IgG antibody experiments and 1:50 for IgM antibody experiments); similar dilutions of pooled normal human serum also lacked reactivity. Convalescent serum from each patient contained IgG and IgM antibodies that bound to the 70-kDa FeRP and IgG antibody that bound to the 94-kDa FeRP from the homologous isolate (Fig. 2; other data not shown). Convalescent sera from all patients except the patient whose culture yielded strain JB503 contained IgG antibody that bound to the 88-kDa FeRP from the homologous isolate; strain JB503 did not make a significant amount of an FeRP of this molecular weight (Fig. 1). Although IgG or IgM antibodies against other FeRPs were seen in individual homologous sera, as for instance the 37-kDa FeRP in JB502 (Fig. 2A, lane 2b), the only consistent immunoreactivity was directed against the 70-, 88-, and 94-kDa FeRPs. Very high-molecular-weight FeRPs bound antibody in some experiments (Fig. 3, lanes 1b and 2b), but these were not always reproducible and might represent incomplete denaturation of lower-molecular-weight FeRPs. These results indicate that meningococcal FeRPs are expressed in vivo, since they elicit an antibody response in convalescing humans. Immune responses to FeRPs have been seen in naturally occurring or experimental infections caused by *E. coli* (10), *Pseudomonas aeruginosa* (4), *Vibrio cholerae* (24), *Klebsiella pneumoniae*, and two species of *Proteus* (25).

Antigenic cross-reactivity of meningococcal FeRPs was investigated by probing membranes from all four strains with convalescent sera from the two patients whose cultures yielded JB501 and JB502 (Fig. 3; other data not shown). These two patients produced IgG antibodies that bound to the 70- and 94-kDa FeRPs in all strains and IgM antibodies that bound to all 70-kDa FeRPs, suggesting that these proteins share common immunoreactivity. Antibody from each patient also bound to certain other heterologous FeRPs (Fig. 3).

We were particularly interested in the observation that the 70-kDa FeRP in these strains might be cross-reactive, since a meningococcal mutant lacking the 70-kDa FeRP has been isolated. This mutant is unable to internalize appreciable amounts of ^{55}Fe bound to TF or LF (7). Therefore, we raised monospecific rabbit antiserum against purified 70-kDa FeRP isolated from strain FAM20 and used this to probe membranes of all four strains (Fig. 4). This rabbit antiserum contained both IgG and IgM antibodies that reacted strongly with the 70-kDa FeRP in FAM20 (the homologous strain) as well as JB501, JB502, and to a lesser extent, JB503. This rabbit antiserum also reacted with analogous 70-kDa FeRPs in each of two gonococcal strains tested (data not shown). We realize that Western blots provide only a qualitative measure of the cross-reactivity among 70-kDa FeRPs of different strains. However, these results indicate a certain

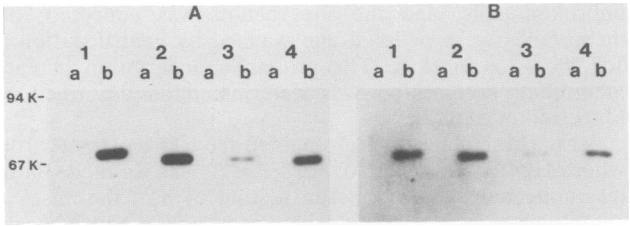

FIG. 4. Western blot of meningococcal membranes showing cross-reactive IgG (A) and IgM (B) immune responses to 70-kDa FeRPs in serum from a rabbit immunized with the 70-kDa FeRP from strain FAM20. Lanes: a, iron-sufficient growth; b, iron-limited growth. Samples: 1, JB501; 2, JB502; 3, JB503; 4, FAM20. Serum was diluted 1:1,000. Molecular weights are indicated in thousands (K).

degree of conservation of antigenicity of 70-kDa FeRPs in these strains. The observation that a meningococcal mutant lacking this protein no longer uses TF or LF for growth (7) makes the 70-kDa FeRP an especially attractive target for future studies on both its function and its possible utility in meningococcal prophylaxis.

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