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Monoclonal Antibodies against the 70-Kilodalton Iron-Regulated Protein of *Neisseria meningitidis* Are Bactericidal and Strain Specific

ANNIKA PETTERSSON,[1,2,*] BETSY KUIPERS,[1] MARGA PELZER,[1] ELLY VERHAGEN,[1] RUDY H. TIESJEMA,[1] JAN TOMMASSEN,[2] AND JAN T. POOLMAN[1]

Laboratory of Bacterial Vaccines, National Institute for Public Health and Environmental Protection, 3720 BA Bilthoven,[1] and Department of Molecular Cell Biology, University of Utrecht, Padualaan 8, 3584 CH Utrecht,[2] The Netherlands*

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When grown under iron limitation, *Neisseria meningitidis* expresses a number of outer membrane proteins (OMPs), one of which is a 70-kilodalton (kDa) major OMP. After immunization of mice with outer membrane preparations of iron-depleted cells of strain H44/76 (B:15:P1.7,16), hybridoma cell lines producing monoclonal antibodies against the 70-kDa OMP were obtained. Some of these monoclonal antibodies demonstrated strong bactericidal activity against the homologous strain H44/76 in the presence of human complement, suggesting potential application of the 70-kDa OMP as a vaccine component. However, none of the 10 selected monoclonal antibodies was able to recognize the corresponding protein from five heterologous strains of various serosubtyping characteristics. A polyclonal anti-70-kDa OMP serum also did not react with the other strains. This result shows that immunodominant surface-exposed epitopes of the meningococcal 70-kDa iron-limitation-inducible OMP are strain specific.

Iron is an essential nutrient for bacterial cell growth, and efficient iron acquisition seems to be an important virulence factor for many pathogens (6, 11, 31, 32). In the extracellular compartments of the human host, most iron is bound to transferrin in serum (7) and to lactoferrin on mucosal surfaces (17). Consequently, the amount of free iron is too low to support the growth of bacteria. Most bacterial pathogens can overcome this limitation by synthesis of iron-chelating agents, called siderophores, which have the capability to compete with the iron-binding proteins of the host (21). The iron-siderophore complexes are bound by outer membrane receptors of the bacteria, and the iron is internalized. In contrast, *Neisseria meningitidis* and *Neisseria gonorrhoeae* seem to be incapable of synthesizing siderophores (3, 28, 33). They are able to sequester iron directly from human transferrin (19) and lactoferrin (18).

The acquisition of iron from transferrin and lactoferrin by *N. meningitidis* seem to be receptor-mediated processes (25–27). The lactoferrin-binding protein has been identified as a 105-kilodalton (kDa) iron-limitation-inducible outer membrane protein (OMP) (25, 27). Competition binding assays have shown that the binding of lactoferrin is specific for human lactoferrin. Neither bovine lactoferrin nor human transferrin is capable of blocking the receptor (27). Acquisition of iron from transferrin by *N. meningitidis* requires direct contact with the bacterial surface (3, 28), since meningococci are unable to use transferrin as a sole source of iron when the transferrin is sequestered in a dialysis bag. Experiments with ^{125}I-labeled transferrin have shown that transferrin itself is not internalized (28). The character of the binding is saturable (26, 30) and specific for human transferrin. Attempts have been made to identify the transferrin receptor. In experiments with peroxidase-conjugated human transferrin, a 71-kDa iron-regulated protein was identified as the transferrin receptor (26). In another study using affinity isolation of biotinylated human transferrin (25), two or more proteins in many different strains were identified. A protein of about 98 kDa and a smaller protein, which varied in size between 68 and 86 kDa but was not identical to the 71-kDa protein, were observed in all strains tested. The discrepancy between these results is unexplained.

The 71-kDa protein is probably identical to the 70-kDa common, iron-regulated surface-exposed antigen described by Dyer et al. (9), which varies slightly in size between different strains. A mutant (FAM11) that lacks this protein is unable to use transferrin or lactoferrin as a sole source of iron. Since this mutant is able to bind transferrin (30), it is unlikely that the 70-kDa protein is the transferrin receptor. Nevertheless, it seems to be essential for the uptake of iron from both transferrin and lactoferrin in an unknown manner.

Experiments with human convalescent-phase sera or rabbit antisera have indicated that the 70-kDa protein is antigenically stable (5, 9). This characteristic would make it a possible candidate for a vaccine against *N. meningitidis*. No effective vaccine exists for serogroup B meningococci, which have caused meningococcal infections during nonepidemic periods (22). Capsular polysaccharide vaccines are used against meningococci of serogroups A, C, Y, and W135. The capsular polysaccharide of serogroup B is a poor immunogen (34), and it shows cross-reactivity with human neonatal brain tissue (12). Therefore, its use as a vaccine has been excluded.

To further investigate the function of the 70-kDa protein and to assess its potential as a vaccine, we have raised monoclonal antibodies against this protein. The bactericidal activity and strain specificity of the antibodies were determined. The results were contradictory to those of earlier reported results (5, 9) in that the antibodies were strictly strain specific. This finding indicates that the surface-exposed epitopes of the 70-kDa protein show antigenic variability.

* Corresponding author.

TABLE 1. Meningococcal strains used

Strain	Serotype	Subtype	Serogroup	Source[a]
H44/76	15	P1.7,16	B	1
2996	2b	P1.2	B	2
MC51	NT[b]	P1.15	C	3
S3032	12	P1.12,16	B	4
M990	6	P1.6	B	4
H355	15	P1.15	B	4

[a] 1, E. Holten, Central Hospital of Abershus, Nordbyhagen, Norway; 2, J. T. Poolman. The Netherlands; 3, J. E. Heckels, Southampton General Hospital, Southampton, England; 4, C. E. Frasch, Food and Drug Administration, Bethesda, Md.

[b] NT, Not typed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *N. meningitidis* strains used are described in Table 1. Strains FAM11 and M986 were kindly supplied by P. F. Sparling, University of North Carolina. For cross-reactivity studies, a collection of prototype and nontypable strains was used (2). Bacteria were cultured on GC agar plates (Difco Laboratories) at 37°C overnight in a humid 5% CO_2 atmosphere. For preparation of outer membranes and for bactericidal assays, strains were grown in 40-liter fermentors in adapted Frantz medium (4) with pH and pO_2 control to the late exponential phase (8 h, iron starvation conditions). Iron-sufficient conditions were obtained by adding 150 μM $Fe(NO_3)_3$ to the medium. Fermentor cultures with and without $Fe(NO_3)_3$ were checked for the production of iron-limitation-inducible OMPs at various time intervals of the growth cycle. For screening of different strains in whole-cell enzyme-linked immunosorbent assays (ELISAs) and immunoblotting, bacteria were inoculated into 5 ml of adapted Frantz medium supplemented with 150 μM $Fe(NO_3)_3$ and were allowed to grow to the exponential phase with shaking at 37°C in an atmosphere of 5% CO_2. To obtain expression of the 70-kDa OMP, the culture was diluted 1:100 into 50 ml of the same medium without $Fe(NO_3)_3$ but containing a 20-μg/ml concentration of the iron chelator ethylenediamine di-*o*-hydroxyphenylacetic acid (Sigma Chemical Co.). The bacteria were grown overnight.

Preparation of outer membranes. Crude bacterial outer membranes were prepared as follows. The bacteria, grown in fermentors, were inactivated at 56°C for 30 min. The cells were harvested by centrifugation at 3,000 × *g* for 60 min, and the pellet was suspended in phosphate-buffered saline and lyophilized. Lyophilized cells were suspended in 10 mM Tris, pH 8.0. The suspension was ultrasonicated on ice for 15 min. After centrifugation at 10,000 × *g* for 10 min to remove cell debris, the supernatant was ultracentrifuged at 50,000 × *g* for 75 min at 4°C. To extract cytoplasmic membrane proteins from the crude outer membranes, a solution of 1% sodium-lauroyl sarcosinate (Sarcosyl; Sigma) was added to the pellet (10). The suspension was centrifuged at 10,000 × *g* for 10 min to remove aggregates, and the supernatant was ultracentrifuged at 50,000 × *g* for 75 min to pellet the outer membranes. The crude membranes were suspended in 10 mM Tris, pH 8.0. Protein concentration was measured with the BCA protein assay reagent (Pierce Chemical Co.), using bovine serum albumin as a standard.

Production of monoclonal antibodies. BALB/c mice were immunized intraperitoneally with 20 μg of outer membranes from bacteria grown under iron limitation together with 0.5 mg of $AlPO_4$. The injections were given four times at weekly intervals. Final boosters of 20 μg were given 10 and 3 days before fusion. The spleen cells were fused with mouse myeloma Sp2/0 cells as described previously (2, 14). Interesting hybridomas were selected by testing culture supernatants in ELISAs and by immunoblotting. Hybridomas corresponding to culture supernatants that reacted with outer membranes from bacteria grown under iron limitation, but not with outer membranes from bacteria grown with excess iron, were cloned with the limiting-dilution method. Clones were expanded and cultured in the peritoneal cavities of Pristane-primed mice to obtain ascites fluid, which was used for further experiments.

ELISA. For ELISA, 100 μl of a 2-μg/ml concentration of outer membranes in phosphate-buffered saline was dispensed into the wells of round-bottom polyvinyl chloride microtiter plates (Flow Laboratories) and were allowed to adsorb for 16 h at room temperature. The plates were washed twice with tap water–0.02% Tween 80 (Merck & Co., Inc.). The reactions were done as described previously (1), with some modification: 0.5% Protifar (Nutricia) was used instead of casein to block unspecific binding. Protein A-peroxidase conjugate was prepared from protein A (Pharmacia) and horseradish peroxidase (Sigma type VI, RZ 3.0) as described previously (20) and used at a working dilution of 1:10,000. The results were quantified by reading the optical density at 450 nm, using a Titertek Multiscan (Flow Laboratories).

Whole-cell ELISAs were performed as described previously (1), with the modifications described above.

SDS-polyacrylamide gel electrophoresis (PAGE). Proteins from whole cells or outer membranes were separated on sodium dodecyl sulfate (SDS) polyacrylamide gels by the method of Laemmli (15). The stacking gel contained 5% acrylamide, and the resolving gel contained 11% acrylamide.

Samples of whole cells, used for immunoblotting, were prepared as follows. Bacteria from a 3.5-ml suspension with an A_{620} of 1 were harvested and suspended in 70 μl of water and 130 μl of sample buffer (16). The sample was heated at 95°C for 5 min, applied to a preparative well (12.3 cm) of a gel (10 cm by 15 cm by 1 mm), and run at a constant current of 40 mA.

Immunoblotting. Proteins from SDS-polyacrylamide gels were transferred electrophoretically to nitrocellulose paper (pore size, 0.45 μm; Bio-Rad Laboratories) by a modification of the method of Towbin et al. (29). The transfer was performed at 140-mA constant current for 1 h on an Ancos Semi-Dry Electroblotter as specified by the manufacturer. After transfer, the nitrocellulose was washed for 30 min in Tris-saline buffer (150 mM NaCl, 10 mM Tris, pH 7.4) containing 0.5% Tween 80 at 37°C. Immunoassay was carried out as follows. Nitrocellulose was washed for 30 min in Tris-saline buffer with 0.5% Tween 80, whereafter it was incubated for at least 1 h with antibodies appropriately diluted in the same buffer. The paper was washed three times in Tris-saline-Tween 80 and incubated with 0.5% Protifar in Tris-saline-Tween 80 for 10 min to block nonspecific protein-binding sites. Nitrocellulose was then incubated for 1 h with protein A-peroxidase in Tris-saline-Tween 80 containing 0.5% Protifar. The filter was washed three times with Tris-saline-Tween 80 and three times with water and then incubated with peroxidase substrate containing 3,3′,5,5′-tetramethylbenzidine (Sigma), dioctyl sulfosuccinate (Sigma), and hydrogen peroxide, prepared by dissolving 24 mg of 3,3′,5,5′-tetramethylbenzidine and 80 mg of dioctyl sulfosuccinate in 10 ml of 96% ethanol. This solution was added to 30 ml of a 10 mM Na_2HPO_4–5 mM citric acid buffer, pH 5.0, and 20 μl of 30% H_2O_2 was added.

Determination of IgG isotype and competition ELISA.

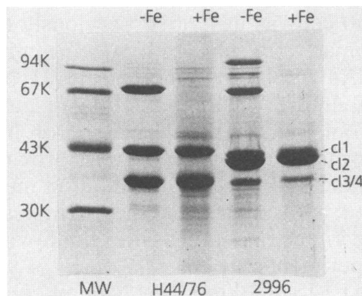

FIG. 1. SDS-PAGE analysis of OMPs from strains H44/76 (B:15: P1.7,16) and 2996 (B:2b:P1.2) after growth to stationary phase in 40-liter fermentor cultures with and without 150 μM $Fe(NO_3)_3$. Positions of the class 1, 2, 3, and 4 proteins are indicated on the right. The class 5 protein is not expressed. Additional OMPs are visible in the outer membrane preparations of the cultures without $Fe(NO_3)_3$. MW, Molecular weight standard proteins (positions indicated on the left in thousands [K]).

Determination of immunoglobulin G (IgG) isotype was carried out in ELISA as described above, using microtiter plates coated with outer membranes from bacteria grown under iron limitation. Since the hybridomas were selected by using protein A-peroxidase conjugate, the only possible isotypes were IgG2a, IgG2b, and IgG3. The anti-IgG antibodies (Bionetics) were conjugated to horseradish peroxidase (Sigma type VI, RZ 3.0) as described previously (20). Sheep anti-mouse immunoglobulins (National Institute for Public Health and Environmental Protection) conjugated to peroxidase were used as a positive control.

For competition ELISAs, the limiting dilutions of the monoclonal antibodies were determined. The limiting dilution is defined as the highest dilution of an antibody preparation that still shows the maximum optical density value in ELISAs after incubation with the appropriate isotype-specific anti-IgG antibody–peroxidase conjugate. This dilution was used in the competition assay for the primary antibody. Microtiter plates, coated with outer membranes from bacteria grown under iron limitation, were incubated with this primary antibody and simultaneously with increasing concentrations of a second antibody of a different IgG isotype. The inhibitory effect of the second antibody on the binding of the primary antibody was determined by incubation with anti-IgG antibody–peroxidase conjugate specific for the isotype of the primary antibody.

Bactericidal assay. Bactericidal titers of the monoclonal antibodies were determined as described previously (24), using human complement from an immunoglobulin-deficient patient (courtesy of M. van Tol and J. Labadie, Academic Hospital, Leiden, The Netherlands).

RESULTS AND DISCUSSION

Expression of the 70-kDa protein in fermentor cultures. When OMPs from strains H44/76 (B:15:P1.7,16) and 2996 (B:2b:P1.2) grown to stationary phase in fermentors were analyzed by SDS-PAGE, the protein profiles of both strains revealed proteins in addition to the well-characterized class 1, 2/3, 4, and 5 OMPs (13) (Fig. 1). The major additional protein expressed is a 70-kDa OMP, suggestive of iron-limiting growth conditions (5). Expression of the additional OMPs takes place in the late exponential to early stationary phase of the fermentor culture. Addition of 150 μM $Fe(NO_3)_3$ to the fermentor medium allowed for a faster growth rate and higher end culture density (Fig. 2) and prohibited expression of the additional OMPs such as the 70-kDa protein (Fig. 1). Strain 2996 also expresses a 98-kDa protein under iron limitation.

Monoclonal antibodies against the 70-kDa protein. Mice were immunized with outer membranes from strain H44/76 grown under iron limitation. Ten monoclonal antibodies that reacted well in ELISAs with outer membranes from bacteria grown under iron limitation, but not with outer membranes from bacteria grown with excess iron, were chosen for ascites production (Table 2). These monoclonal antibodies were selected from three different fusions out of a total of 45 monoclonal antibodies. Figure 3 shows the results of a Western immunoblot of OMPs from cells grown with (Fig. 3B) or without (Fig. 3A) iron with the different antibodies. All of the monoclonal antibodies except mn70K4 reacted strongly with the 70-kDa protein. The fact that mn70K4 did react strongly in the ELISA suggests that this antibody recognized a conformational epitope that was destroyed during sample preparation.

Characterization of the monoclonal antibodies. Four of the monoclonal antibodies were of isotype IgG2a, three were of

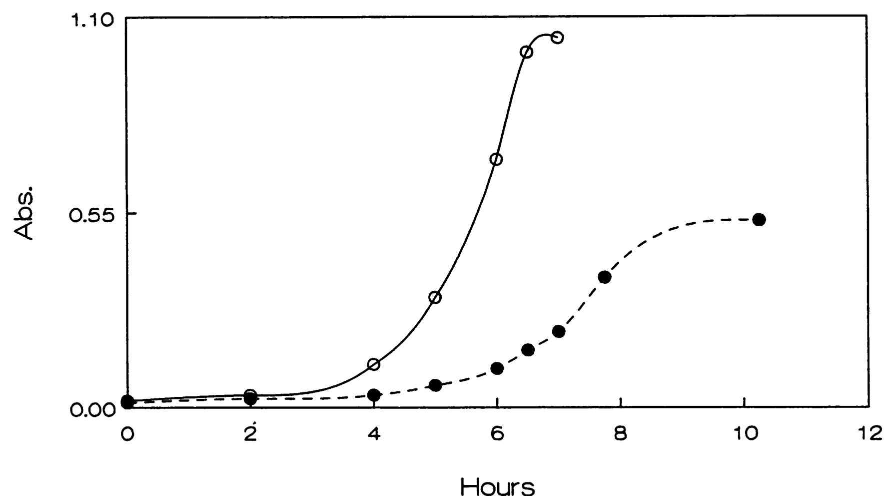

FIG. 2. Growth curves of 40-liter fermentor cultures of strain H44/76 in adapted Frantz medium. Symbols: ○, with 150 μM $Fe(NO_3)_3$; ●, without $Fe(NO_3)_3$.

TABLE 2. Characterization of monoclonal antibodies

Monoclonal antibody	ELISA[a]		Immunoglobulin isotype	Bactericidal titer[b]
	+Fe	−Fe		
mn70K1	0.07	1.63	IgG2a	6,400
mn70K2	0.04	1.52	IgG2a	3,200
mn70K3	0.02	1.47	IgG2a	3,200
mn70K4	0.04	0.76	IgG3	–
mn70K5	0.03	1.50	IgG3	3,200
mn70K6	0.03	1.50	IgG3	1,600
mn70K7	0.05	1.64	IgG2a	800
mn70K8	0.02	1.62	IgG2b	–
mn70K9	0.02	1.54	IgG2b	–
mn70K10	0.04	1.25	IgG2b	–

[a] The plates were coated with outer membranes of bacteria grown with additional iron (+Fe) or under iron limitation (−Fe). Optical densities of the ELISA reactions were measured at 450 nm.

[b] Dilution of antibody that killed half of the meningococcal cells. –, No bactericidal activity.

isotype IgG2b, and three were of isotype IgG3 (Table 2). The bactericidal activities of the antibodies were determined by using human complement. Six antibodies were bactericidal (Table 2) against strain H44/76, indicating that antibodies against this protein may be involved in the immunological defense mechanism against meningococci in vivo. The bactericidal activities of these antibodies were investigated against strain 2996, which is of different serotype and subtype (Table 1). None of the antibodies was bactericidal (data not shown). This finding suggests that the 70-kDa proteins from the two different strains expose different epitopes on the cell surface; i.e., the protein shows antigenic variation. This result would argue against previous observations (5, 9).

To investigate the antigenic variability of the 70-kDa protein, six different meningococcal strains (Table 1) were grown under iron limitation in batch cultures and tested in whole-cell ELISAs, using anti-70 kDa protein monoclonal antibodies raised against H44/76. All of the strains produced a 70-kDa protein when grown under iron limitation (data not shown). The antibodies reacted strongly only with strain H44/76. Some very weak reaction was observed with strains M990 and H355, but a similar reaction was also seen with these bacteria grown with excess iron (data not shown). Therefore, we conclude that this binding was aspecific. The other strains did not react at all. To test these results further, immunoblotting experiments were performed. No binding to the 70-kDa protein could be seen except to the 70-kDa protein of strain H44/76 (data not shown).

As described above, a monoclonal antibody directed against the 70-kDa protein of strain 2996 was obtained. In immunoblotting experiments, this antibody did not react with the 70-kDa protein of strain H44/76 (results not shown).

To facilitate the screening of a large number of strains, we made use of the observation that bacteria grown on agar plates are limited for iron (8). ELISAs and immunoblotting experiments confirmed that strains H44/76 and 2996 produced the 70-kDa protein when grown on GC agar plates. Whole-cell ELISAs were performed with 74 different meningococcal strains grown on GC agar plates. The monoclonal antibody against the 70-kDa protein of strain 2996 reacted with four of these strains. A monoclonal antibody against the

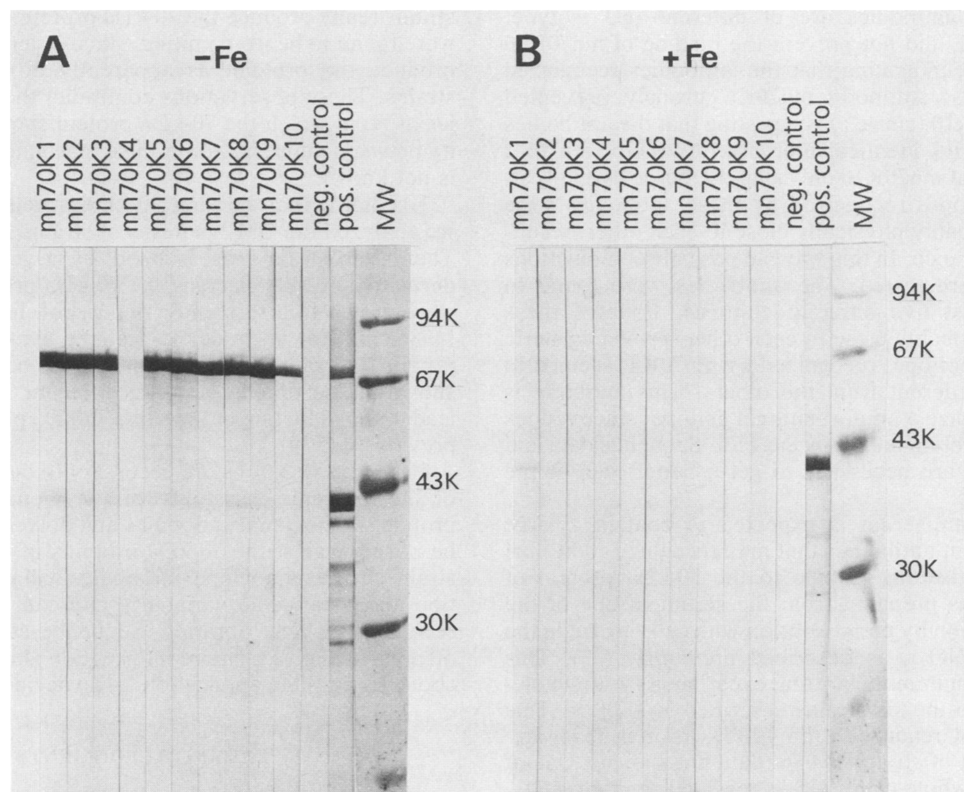

FIG. 3. Immunoblots of OMPs with monoclonal antibodies. (A) OMPs from H44/76 cells grown under iron limitation; (B) OMPs from cells grown with excess iron. An antiserum from one of the mice used for fusion was used as a positive control. Tris-saline buffer was used as a negative control. The antibodies and the serum were diluted 1:1,000. Positions of molecular weight (MW) markers are indicated on the right in thousands (K).

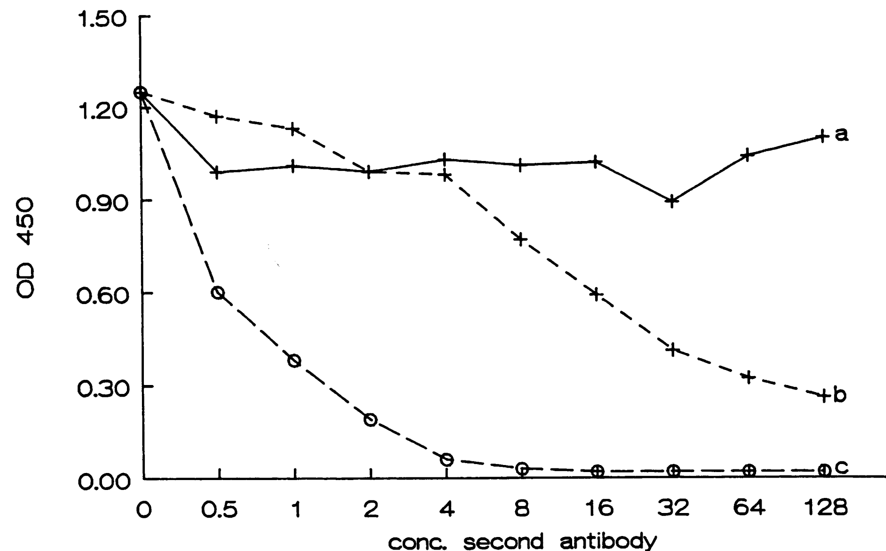

FIG. 4. Competition ELISA experiment. The primary antibody was mn70K10. The second antibodies in curves a, b, and c were mn70K7, mn70K2, and mn70K6, respectively. Concentrations of the second antibodies are given as the multiplicity of the limiting dilution.

70-kDa protein of H44/76 (mn70K7) reacted with five other strains.

These results indicate that the 70-kDa protein exhibits antigenic variability in different strains. However, it can be argued that the monoclonal antibodies recognize only a very limited number of epitopes. To test this possibility, competition ELISAs were performed as described in Material and Methods. The competition assay was based on the fact that the monoclonal antibodies are of different IgG isotype. Antibody mn70K7 did not prevent the binding of mn70K10 (Fig. 4, curve a), indicating that the antibodies recognized different epitopes. Antibody mn70K6 strongly prevented binding of mn70K10 (curve c), suggesting that the antibodies recognized (nearly) identical epitopes. Antibody mn70K2 affected binding of mn70K10 only slightly (curve b), suggesting that the epitopes recognized by these antibodies were different but probably physically close to each other, resulting in steric hindrance. In this way, all possible combinations of antibodies were tested. The antibodies were found to recognize at least five different epitopes. Four of these epitopes were probably close to each other, providing steric hindrance. One epitope, recognized by mn70K4, seemed to be completely different from the others. This antibody is thought to recognize a conformational epitope, since it does not bind to the protein on a Western blot. Sequence data and epitope mapping are necessary to get a better idea of the different epitopes.

A polyclonal antiserum is expected to contain a large amount of different antibodies that may recognize additional epitopes. An antiserum specific to the 70-kDa protein of strain H44/76 was prepared from the serum of one of the mice used for fusion by preadsorption with cells grown in the presence of $Fe(NO_3)_3$ as described previously (23). This serum was used in immunoblotting experiments with whole-cell proteins from the six strains described in Table 1. This antiserum did not react with the 70-kDa protein from any strain except that of strain H44/76 (data not shown). Use of the antiserum in whole-cell ELISAs revealed similar results.

Conclusions. Monoclonal antibodies were raised against cell-surface-exposed epitopes of the iron-limitation-inducible 70-kDa protein of *N. meningitidis* H44/76. Many of them were bactericidal in combination with human complement, which makes the 70-kDa protein a vaccine candidate. However, we observed antigenic variability of the 70-kDa protein. Monoclonal and polyclonal antibodies against the 70-kDa protein of H44/76 did not react with the 70-kDa protein of five other strains. One of the monoclonal antibodies reacted with 5 of 74 meningococcal strains. A monoclonal antibody against the 70-kDa protein of strain 2996 reacted with four other strains. We did not test whether all of these strains really produce the 70-kDa protein, but the conditions were found to be iron limiting, since strains H44/76 and 2996 produce the protein, as apparently do the nine positive strains. These observations contradict those of earlier studies (5, 9) in which the 70-kDa protein showed cross-reactivity between different strains. The reason for this discrepancy is not known.

To confirm that the same 70-kDa protein was under study, we analyzed the OMP patterns of strains M986 and FAM11. The latter strain was isolated by Dyer et al. (9) as a derivative of M986 lacking the 70-kDa protein. Both strains were grown in a fermentor under iron limitation. In strain M986, but not in its derivative, the synthesis of a 70-kDa protein was induced under our culture conditions (data not shown). This finding confirms that our culture conditions lead to the induction of the same 70-kDa protein as described previously (5, 9).

More investigations are necessary to determine the extent of the antigenic variability of the meningococcal 70-kDa protein. Monoclonal antibodies and polyclonal antisera must be raised against the 70-kDa proteins of other serogroup B strains. Such monoclonal antibodies will give more information about antigenic variability and can be used to screen recombinant gene libraries. Sequence studies and epitope mapping will give us more information about the protein and about its possible application as a vaccine component.

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