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Author(s): Mark Achtman, Barica Kusecek, Giovanna Morelli, Kirsten Eickmann, Wang Jianfu, Brian Crowe, Robert A. Wall, Musa Hassan-King, Patrick S. Moore and Wendell Zollinger

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A Comparison of the Variable Antigens Expressed by Clone IV-1 and Subgroup III of *Neisseria meningitidis* Serogroup A

Mark Achtman, Barica Kusecek, Giovanna Morelli, Kirsten Eickmann, Wang Jianfu, Brian Crowe,* Robert A. Wall,* Musa Hassan-King, Patrick S. Moore,* and Wendell Zollinger

Max-Planck Institut für molekulare Genetik, Berlin, Germany; Medical Research Council, Fajara, The Gambia; Meningitis and Special Pathogens Branch, Centers for Disease Control, Atlanta, Georgia; Department of Bacterial Diseases, Walter Reed Army Institute for Research, Washington, DC

Serogroup A *Neisseria meningitidis* of subgroup III has caused two pandemics of meningococcal meningitis since 1966 and recently spread to East Africa. The last epidemics in West Africa in the early 1980s were caused by clone IV-1. Surface antigens of clone IV-1 strains from West Africa and subgroup III strains from both pandemic waves were analyzed. Lipopolysaccharide was stable within clone IV-1 but variable in subgroup III. Pili from clone IV-1 possessed class I epitopes, while those from subgroup III also possessed class IIa epitopes. Certain class 5 protein variants were expressed by both bacterial clones, possibly reflecting either inheritance of primeval genes or horizontal transmission. Exposure of Gambians to clone IV-1 bacteria stimulated production of bactericidal antibodies cross-reactive with subgroup III bacteria in some individuals but of type-specific antibodies in others. Gambians without bactericidal antibodies usually became healthy carriers rather than developing meningococcal disease on exposure to virulent meningococci.

Variation in electrophoretic migration of cytoplasmic isoenzymes and outer membrane proteins can be used to estimate the genetic relatedness between bacterial strains and to subdivide them into clones of common ancestral descent (clonal analysis) [1, 2]. Using clonal analysis, four subgroups (I–IV) containing 21 distinct clones (I-1 to IV-4) were elucidated among serogroup A meningococci from diverse epidemics and from endemic situations [3]. Most isolates from any one epidemic were homogeneous by clonal analysis, and concurrent epidemics in neighboring countries (epidemic waves) were often caused by the same clone or subgroup [3].

Epidemic waves of meningococcal disease have occurred about every 10 years in the African meningitis belt [4, 5] and in China [6]; recent information has placed some of these waves within a global context. The African wave of 1967–1974 was part of a pandemic caused by clone I-1 [3, 5]. The subsequent African wave of 1981–1983 was caused by clone IV-1. Analysis of numerous serogroup A meningococci isolated from diseased patients and from healthy carriers in The Gambia between 1982 and 1988 showed that they all belonged to clone IV-1 [7]. Clone IV-1 has also been repeatedly isolated in West Africa during interepidemic and epidemic periods since the early 1960s but has not been of importance outside of Africa [3].

A series of recent independent reports indicates that the epidemics since 1988 in East Africa are best regarded as an extension of the pandemic spread of clone III-1 and related subgroup III bacteria (figure 1). The earliest documented epidemic caused by clone III-1 bacteria [8] began in China in 1966 with several million registered cases of meningococcal meningitis [6]. A large clone III-1 epidemic began in Russia [9] in 1969 concurrently with a III-1 outbreak in Norway [10]. They were followed in 1973 by an epidemic in Finland [3, 11]. Although Sweden did not suffer an epidemic, most serogroup A bacteria isolated there between 1973 and the mid-1980s were clone III-1 [12]. We have depicted an epidemic in Brazil in the late 1970s [3, 13, 14] as being part of this pandemic wave because of the timing, although epidemiologic links are lacking. Because rare bacteria belonging to subgroup III but distinct from clone III-1 were also isolated from these sources [3], this pandemic is concluded to have been caused by subgroup III even though most cases were caused by clone III-1 per se.

In the early 1980s, an epidemic in Nepal [15] and a rise in morbidity in China [8] were both caused by clone III-1. We believe that this represents the beginning of a second pandemic, because a major Chinese epidemic in the mid-1970s was caused by unrelated bacteria of subgroup V [8]. No bacteria have yet been tested from a major epidemic in northern India that occurred in 1985 [16]; the next proven III-1 outbreak was during the annual pilgrimage (Haj) to Mecca of

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Reprints or correspondence: Dr. Mark Achtman, Max-Planck Institut für molekulare Genetik, Ihnestr. 73, D-1000 Berlin 33, FRG.

* Present addresses: Immuno AG, Orth-Donau, Austria (B.C.); Division of Communicable Diseases, Clinical Research Centre, Northwick Park Hospital, Harrow, UK (R.A.W.); Center for AIDS Prevention Studies, University of California, San Francisco (P.S.M.).

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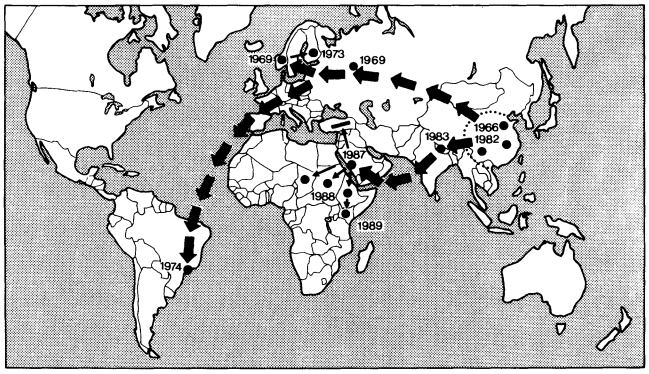

Figure 1. Two waves of pandemic spread of epidemics caused by subgroup III meningococci. Dates shown are beginnings of epidemics and outbreaks caused by clone III-1; arrows show our interpretation of pattern of spread of these bacteria. Additional epidemics that coincide temporally and geographically began in Rumania in 1969, in India in 1985, and in eastern Africa in 1989, but no bacteria have been tested. Bar terminating northwest arrow from Saudia Arabia indicates spread of bacteria without spread of epidemics to United Kingdom, United States, and probably most countries.

1987 [16, 17]. Numerous pilgrims carried clone III-1 serogroup A meningococci back to their countries of origin, including the United States [16] and United Kingdom, where 33 cases were noted among pilgrims and their contacts [18], but the importation of these bacteria did not result in epidemics outside of East Africa. Epidemics caused by clone III-1 bacteria began in Chad [17], Sudan [12], and Ethiopia [19] in 1988 and spread to Kenya in 1989 [18, 20]. Epidemics for which causative bacteria have not yet been tested began in 1989 in Tanzania and Uganda. Before 1988, clone III-1 had never been isolated from Africa [3].

In addition to the capsular polysaccharide, other antigenic cell surface components are expressed in large amounts by meningococci [3, 21]: lipopolysaccharide (LPS), pili, and the major outer membrane proteins termed H.8, class 1, class 2/3, class 4, class 5, and class 6. Some of these components, can differ in molecular weight and antigenicity between different meningococci. Furthermore, the degree of variability can differ between individual clonal groupings. The class 4 protein is electrophoretically invariant [3, 21] among different meningococci. Some serogroup A clones express a class 6 protein, whereas others do not [3]; no electrophoretic differences were observed between the class 6 proteins from different clones. Monoclonal serotyping reagents specific for the class 2/3 protein, serosubtyping reagents specific for the class 1 protein, and immunotyping reagents specific for LPS have been and are continuing to be developed [21, 22]. Almost all serogroup A meningococci express a class 3 protein that reacts with serotype 4 and 21 reagents [7, 21, 23], and none expresses a class 2 protein [3]. Seven electrophoretic variants of the class 1 protein were associated with distinct clones of serogroup A strains [3], and these variants fell into five serosubtype specificities [5, 24].

LPS immunotypes L4, L6, L7, L8, L9, L10, L11, and L12 have been described in various combinations within serogroup A bacteria [25, 26]. Unlike the clonal associations for the class 1 protein, no correlation was found between LPS immunotyping and clonal analysis [3]. Among the highly related clone IV-1 bacteria isolated in The Gambia, most isolates expressed L9 LPS, but 9 of 105 expressed LPS with different serologic reactivities [7].

In contrast, variation in the electrophoretic migration of pilin and of class 5 proteins was highly dramatic within the Gambian clone IV-1 bacteria. Each strain expressed no to two pilin electrophoretic variants and no to four distinct class 5 protein variants [7]. However, despite the variation from isolate to isolate, only five class 5 protein electrophoretic variants, called 5a–5e, were found among >300 Gambian bacteria, as if these bacteria possessed only a limited genetic repertoire [27]. Analysis of clone IV-1 strains isolated from other African countries between 1963 and 1982 identified three more class 5 protein electrophoretic variants, called 5f, 5g, and 5h [28]. These eight class 5 proteins were assigned to three seroclasses called A (protein 5a), B (5b, 5d, 5e, and 5g), and C (5c) on the basis of reactivity with a limited selection of monoclonal antibodies (MAbs); the serologic reactivity of 5f and 5h remained undefined [28]. Tests with other serogroup A bacteria and with meningococci of other serogroups revealed that the 5c protein was widespread and that seroclass A proteins variable in electrophoretic migration were found in diverse bacteria [28]. Seroclass B was found only in clone IV-1 [28]. Expression of most of these proteins is subjected to an on-off mechanism, whereas that of 5c is tripartite: off, expressing small amounts (5c), or expressing large amounts (5C) [28]. A common epitope on all gonococcal P.II and meningococcal class 5 proteins other than 5c/C is recognized by MAb 4B12/C11, but most specific rabbit and mouse antibodies recognized certain seroclass-specific epitopes [28]. The significance of these observations remained unclear, and it was unclear whether the observations with the Gambian isolates could be extrapolated to other meningococci.

Although current meningococcal vaccines consist of purified capsular polysaccharide, natural immunity to infection seems to be directed primarily against noncapsular components [29, 30]. Sera from patients recovering from meningococcal disease or from volunteers vaccinated with an experimental outer membrane vaccine possessed elevated levels of antibodies against the class 1, 2, and 5 proteins, the pili, and the LPS of the infecting strain [31, 32]. Nasopharyngeal carriage of clone IV-1 bacteria was common within the healthy Gambian population during the epidemic wave in the early 1980s [33], and all serogroup A meningococci isolated during that period from epidemics in West Africa were clone IV-1. Did a large part of that population develop pro-

tective immunity via such carriage? Was that presumptive immunity directed against the variable antigens just described? And can such immunity protect against epidemic disease caused by clone III-1? Because the Gambian bacteria might not reflect the diversity of clone IV-1, we extended our former analyses to all the (African) clone IV-1 bacteria in our strain collection. We also investigated the electrophoretic and serologic properties of the outer membrane components expressed by subgroup III bacteria isolated from both pandemic waves. These analyses allow a comparison of the variability of such outer membrane components within two marginally related bacterial groups as well as enabling an estimate of whether residual herd immunity in West Africa engendered by contact with clone IV-1 might be protective against clone III-1 epidemics.

Materials and Methods

Media and buffers. PBS consisted of 0.14 *M* NaCl, 1.5 m*M* KH_2PO_4, 20 m*M* Na_2HPO_4, and 2.7 m*M* KCl. Supplemented GC agar [3] and Gey's balanced salts solution with gelatin (GBSS) [7] were as described. Diethanolamine buffer, pH 9.8, contained 97 ml of diethanolamine, 0.2 g of NaN_3, and 0.1 g of $MgCl_2 \cdot 6H_2O$ per liter. ELISA substrate consisted of 1 mg/ml 4-nitrophenyl phosphate in diethanolamine buffer.

Bacterial strains. For clone IV-1, 73 reference strains were from The Gambia, 1982–1988 [7], and 60 from other African countries: Niger, 1962–1965, 24 strains; Burkina Faso, 1963–1983, 19; Cameroon, 1966, 4; Ghana, 1970–1973, 9; Mali, 1981, 1; and Sudan, 1985, 3 [3, 12]. For subgroup III, 101 strains of clone III-1 isolated from epidemics and cases related to the Mecca outbreak of 1987 were from the following countries: Saudi Arabia, 1987–1988, 4 [16, 17]; United Kingdom, 1987–1988, 28 [18]; the United States, 1987, 13 [16]; Chad, 1988, 31 [17]; and Sudan, 1988, 25 [12]. Of these, 18 were from healthy carriers and the rest from diseased patients. The English strains were confirmed as being clone III-1 by D. A. Caugant (National Institute of Public Health, Oslo) with the use of 13 isoenzymes. The strains from Sudan had been stored in liquid nitrogen at −70°C since shortly after isolation; the other strains had been maintained in transport medium for an undetermined time before isolation and freezing. There were 121 subgroup III strains from other sources: China, 1966–1987, 45 [8]; Moscow, 1969–1977, 11; Norway, 1969–1973, 14 [10]; Finland, 1973–1982, 15 [3]; Holland, 1973, 1 [3]; Denmark, 1974, 3 [3]; Sweden, 1973–1987, 22 [12]; Brazil, 1974–1976, 5 [3]; and Nepal, 1983, 5 [3]. Nine of the strains from Moscow, isolated between 1969 and 1971, were obtained from N. Kostyukova (Gamaleya Institute of Epidemiology and Microbiology) and have not been described, whereas the other two were described in [3]. Of the 121 strains, 108 were indistinguishable by isoenzyme analysis from reference clone III-1 strains. The other 13 belonged to other clones of subgroup III, namely, 1 clone III-2 strain from Finland, 2 III-3 strains from Nepal, and 2 III-4 strains from China and Brazil [3], as well as 2 strains from Sweden [12] and 6 from China [8] whose exact clonal designation will be presented elsewhere. The strains from Sweden were stored at −70°C, and the strains from Norway were lyophilized shortly after isolation; storage conditions for the other strains are unknown.

All strains were restreaked to single colonies and stored at −70°C before making membrane preparations [7] and extracts for whole cell ELISA.

Polyclonal antibodies. The original hyperimmune rabbit sera generated for LPS immunotyping [25] were retested by Western blotting against the original immunotyping reference strains and against a selection of the bacteria described here. Twenty other hyperimmune rabbit sera [28] from rabbits immunized intravenously with different live clone IV-1 bacteria were also screened in Western blots against LPS reference strains. Alkaline phosphatase–conjugated, affinity-purified rabbit immunoglobulins to mouse immunoglobulins (Dakopatts, Copenhagen) were used in whole cell ELISA (diluted 1:500) and Western blots (1:1000) to detect binding of MAbs. Antibody to rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma Chemical, St. Louis) was used at a dilution of 1:1000 in Western blots to detect binding of rabbit antibodies to LPS.

Monoclonal antibodies. Table 1 lists the Mabs used. Antibodies generated in the course of this study are cited as "Berlin" and were generated as follows. BALB/c mice were immunized with heat-inactivated whole bacterial preparations (10^9 cells/ml in PBS, 56°C, 1 h): 0.1 ml was injected intraperitoneally on days 0, 4, 8, and 12 and the mice were sacrificed 3 days after a similar booster immunization between days 16 and 40. After polyethylene glycol (PEG)–induced fusion of spleen cells with the cell line P3-X63-AG 8.653 [38], hybridomas secreting MAbs of interest were detected by screening microwell supernatants. Initial screening was by whole cell ELISAs, and positive wells were screened by Western blotting against bacterial membrane preparations. MAbs whose designation begins with O or P were from mice immunized with a Gambian clone IV-1 strain (C623) expressing class 5 proteins 5a, 5b, 5C, and 5d. The others (except for AB419) were from one of nine fusions from mice immunized with different clone III-1 strains expressing class 5 proteins or LPS species of interest. For some of these latter fusions, cell extracts from two strains expressing the same antigen were mixed before immunization.

Class 5h protein was purified according to the protocol described for 5h purification from clone IV-1 strains [28] from a clone III-1 strain, Z3714, isolated in Sweden in 1976. This strain expresses no class 5 proteins other than 5h. A mouse was injected subcutaneously on day 0 with 100 μg of protein in incomplete Freund's adjuvant and intraperitoneally on days 10 and 24 with 100 μg of protein in water. The mouse was sacrificed on day 27, and hybridomas were screened using the whole cell ELISA method. The hybridoma cell line AB419 producing MAbs specific for 5h was recognized as described above.

All cell lines were cloned twice by limiting dilution. Antibodies were concentrated from culture supernatants by ultrafiltration or ammonium sulphate precipitation; in some cases they were purified by protein A–sepharose affinity chromatography before use. Antibodies not generated in Berlin were obtained, usually as ascites fluid, from the sources listed.

The antigen specificity for each MAb was determined by Western blotting. All antibodies were then used at a dilution determined to be optimal for a specific reaction with the antigen

Table 1. Monoclonal antibodies and specificities.

Antigen, specificity	Designation	Source
Pili		
Class I	SM1	J. Heckels [34]
Class IIa	U101, (T613)	Berlin
LPS		
L8	2-1-L8	W. Zollinger [26]
L8	A'dam 2, A'dam 7	J. Poolman
L10	MCA14.2	R. Sugasawara [35]
L10	14-1-L10	W. Zollinger
L10	CL135/110	H. Peters
L10	R815	Berlin
L11	W314/E12	Berlin
Class 1 protein		
P1.5	MN22A9.19	J. Poolman (unpublished)
P1.7	A'dam 1	J. Poolman [22]
P1.9	MN16E17G4	J. Poolman [22]
P1.9	R523, S112, T102, W316	Berlin
P1.x	V502	Berlin
Class 3 protein		
T4	MN14G21.17	J. Poolman [22]
T21	14-1-P21	W. Zollinger
Class 4 protein		
General	SM50	J. Heckels [36]
General	U406, V414	Berlin
Class 5 protein		
Seroclass A	P219, P322, P414	Berlin
Seroclass A	W104, W124, W320/15	Berlin
Seroclass B	O516/2, P110, P112	Berlin
Seroclass B	P416, P514, P515	Berlin
Seroclass C	B306	[28]
Seroclass F	U205	Berlin
Seroclass H	AB419	Berlin
Seroclass I	T116, U106, U214, W320/16	Berlin
Panreacting (except 5c)	4B12/C11	M. Blake [28]
H.8 protein		
General	SM70	J. Heckels [37]

NOTE. LPS, lipopolysaccharide; seroclasses A, C, F, H, and I contain the class 5 proteins designated 5a, 5c, 5f, 5h, and 5i, respectively. Seroclass B contains the class 5 proteins designated 5b, 5d, 5e, and 5g.

under test (1:500–1:50,000 for Western blots; usually 10-fold more dilute for whole cell ELISA). Most results herein are based on whole cell ELISAs except for B306 (specific for class 5c; used in dot blots and Western blots to distinguish 5c from 5C), 4B12/C11 (used in Western blots to determine the number of different class 5 proteins other than 5c), and SM70 (specific for H.8 protein; used in Western blots). Most of the antibodies reacted only with the specified antigen in Western blots, but B306 and SM70 each bound weakly to a second unknown protein band and all the anti-LPS antibodies reacted with numerous protein bands in addition to the LPS bands.

Human sera. The epidemiology and sources of human sera from The Gambia have been described [7, 27]. They consisted of 200-μl samples taken during the acute and convalescent phases from patients with meningococcal disease or taken from villagers tested for nasopharyngeal carriage on three occasions. One of the villagers carried meningococci of serogroup 29E, which were ignored for this analysis; all other meningococci were serogroup A, clone IV-1. The sera have been stored at −30°C since 1983 and thawed fewer than five times. The sera from Finland were obtained from H. Käyhty (Public Health Institute, Helsinki) [39]. They were taken from patients during the Finnish epidemic in the mid-1970s. Although the endogenous bacteria were not available for most of these patients, all strains tested from Finland were subgroup III and most were clone III-1 [3]. Human complement was from a rare donor whose serum is not bactericidal for serogroup A strains at concentrations up to 50% [7].

SDS-PAGE, Western blots, and dot blots. Except for the following details, all procedures and buffers have been described elsewhere [27, 28]. Membrane samples were diluted 1:8 in gel sample buffer [28] and boiled for 2 min before SDS-PAGE analysis.

Electrophoretic migration of major outer membrane proteins was analyzed on duplicate large gels containing 11% acrylamide (acrylamide-to-BIS ratio, 30:0.8) and either 3 or 4 *M* urea [28] followed by staining with Coomassie blue. For Western blotting, small gels containing 11% acrylamide and 3 *M* urea were transferred to nitrocellulose in TRIS-glycine buffer as described [28].

For H.8 protein and pili, small gels containing 15% acrylamide and no urea were used, and transfer to nitrocellulose was in 25 m*M* PO_4^-, pH 7.5.

Large gels containing 15% acrylamide and no urea were used for analyzing electrophoretic migration of LPS (after staining with silver nitrate [40]) and for Western blotting.

Whole cell ELISA. The method described by Abdillahi and Poolman [41] was modified as follows. One-quarter of the growth from a plate of supplemented GC agar incubated overnight at 37°C, 5% CO_2, 90% humidity, was resuspended in 10 ml of PBS. The suspension was incubated 1 h at 56°C and allowed to cool to room temperature (RT); sodium azide was added to a concentration of 0.02% (wt/vol). The suspension was stored at 4°C for at least 24 h to allow larger particles to settle, and only the soluble supernatant was used. Quantitatively comparable results were obtained when such preparations were retested after storage at 4°C for 9 months. Polystyrene flat-bottomed microwell plates (Nunc, Copenhagen) were coated with 50 μl of the solution per well and allowed to dry at 37°C overnight. These plates could be stored for several weeks at 37°C with no quantitative difference in results. Before use, the plates were washed twice with PBS with 0.05% Tween 80 (PBST) and blocked with 200 μl/well of PBST containing 10% fetal calf serum (FCS) for 1 h at RT. The plates were washed three times as above, and 100 μl of MAbs diluted in PBST containing 2% FCS were added. The plates were incubated for 1 h at RT with rapid shaking (600 rpm; Easyshaker; SLT Labinstruments, Grödig, Austria) and washed three times as above. Next, 100 μl of conjugate diluted in PBST with 2% FCS and 4% PEG 6000 was added, and the plates were shaken rapidly for 1 h at RT. In the absence of rapid shaking, 4-h incubation steps were needed to attain the same quantitative results as with shaking. The plates were washed four times, and ELISA substrate prewarmed to 37°C was added

(100 μl/well). After 30 min at 37°C, 100 μl of 1 *N* NaOH was added to stop the reaction, and the OD at 405 nm was measured.

The dilutions of the MAbs were chosen such that a positive reaction yielded an optical density >1.0 and a negative reaction yielded an OD <0.05. Other antibodies did not give such clear reactions and were excluded from the analysis. For each experimental day, several positive and negative reference strains were included. The results were read with a semiautomated microplate reader coupled to an IBM-compatible microcomputer running a program that stored the raw data in ASCII format. The data for each antibody used that day were then converted to a frequency histogram using Excel (Microsoft, Redmond, WA), and cutoff points for + and − values, which included most data points, were visually determined from the histogram. Rare intermediate values were scored as +/−. When several MAbs of the same specificity were tested, usually all yielded the same value for each strain. For the few exceptions in which some antibodies yielded a +/− value and others +, the strain was scored as +. Similarly, when some antibodies yielded +/− values and others −, strains were scored as −. These interpretations were stored together with other strain information in an R:Base (Microrim, Redmond, WA) database and are available on request.

Bactericidal tests. These tests were done in 50 μl of reaction mixture as described [7]. For each endogenous strain, single colonies were reisolated and tested by SDS-PAGE with Coomassie staining as well as by Western blotting with anti–class 5 MAbs to ensure that they still expressed the same class 5 proteins as the original strain. Serum samples were incubated for 30 min at 56°C to inactivate endogenous complement before doing a twofold dilution series in GBSS. The reaction mixtures contained 10 μl of human complement, 12.5 μl of a serum dilution, 10 μl of GBSS containing 500 bacteria diluted from a culture harvested during the exponential growth phase, and 17.5 μl of GBSS. After 30 min at 37°C, duplicate 5-μl samples were plated to determine viable counts. The titer was the highest serum dilution still yielding 50% killing, multiplied by 4 to account for the other components in the reaction mixture. Some serum samples exhibited a prozone effect, in which killing was less dramatic at higher dilutions, and others never achieved complete killing at any dilution. Control experiments done each day with monoclonal anti–class 1 protein MAb always gave complete killing. Repeated experiments with selected sera yielded titers that varied no more than twofold, which were calculated as geometric means; most results are from single tests.

Data reduction and analysis. A bactericidal titer of <4 was converted to the arbitrary value 2 to allow statistical comparisons. The $\log_2$ of the titers were then calculated because such transformed values are better suited for analysis of titers based on exponential dilutions than are untransformed data. Only increases of at least fourfold were considered significant. Tests of significance were done using Statgraphics (STSC, Rockville, MD) and IBM AT–compatible microcomputers and consisted of the χ^2 test and the *t* test for comparison of means of paired samples.

Results

Strain collection and new monoclonal antibodies. A total of 133 strains, 73 reference strains from The Gambia, and all 60 clone IV-1 strains in our collection from West Africa were chosen to represent clone IV-1. A selection of proven subgroup III strains from diverse countries in both pandemic waves was obtained from the laboratories listed in Acknowledgments. On the basis of their P1.9 serosubtype, it seemed likely that serogroup A strains isolated in England after 1987 [18] also belonged to subgroup III, and isoenzyme analyses confirmed that these strains were all clone III-1. Nine of 11 strains isolated in Moscow between 1969 and 1971 also proved to be clone III-1. Thus, a total of 222 subgroup III strains was used for comparison of antigenic properties with those of clone IV-1.

Differences in the electrophoretic migration of major outer membrane proteins, LPS, and pili were determined by subjecting membrane preparations from all bacteria to SDS-PAGE followed by staining with Coomassie blue, by staining with silver nitrate, or by Western blotting with SM1 MAb, respectively. Some of the observed differences in electrophoretic migration correlated with the specific reactivities of MAbs obtained from diverse laboratories, while MAbs correlating with other observed differences were lacking. Therefore, new MAbs were generated after immunizing mice with selected clone IV-1 and subgroup III strains. Hybridomas that yielded specific reactions in Western blots were cloned by limiting dilution and used for the synthesis and purification of MAbs, even when they seemed to have the same reactivity as preexisting MAbs. The antibodies were characterized by testing diverse reference serogroup A, B, and C strains. The resulting collection of MAbs is summarized in table 1. The following conclusions reflect combined observations from SDS-PAGE and from whole cell ELISAs of all 355 strains against all antibodies, and from Western blotting with representative electrophoretic variants.

Stable antigens. As formerly concluded for a subset of the strains tested here [3], all clone IV-1 and subgroup III strains expressed a class 4 and a class 6 protein of uniform electrophoretic migration. The class 4 protein of representative strains reacted in Western blots with three MAbs, one (SM50) generated against gonococci expressing the P.III protein and two others newly generated against subgroup III bacteria. Rare bacteria (figure 2E, lane 10) yielded a double band with these antibodies as though they expressed two class 4 proteins.

A minor electrophoretic difference between the class 3 proteins of clone IV-1 and subgroup III strains has been described [3]. The same difference was seen with all but two of the bacteria tested here. Although the class 3 protein is a major component of the serotype antigen, the minor electrophoretic difference between clone IV-1 and subgroup III was not reflected by reactivity with serotyping MAbs. Like almost all serogroup A bacteria, these strains react in whole cell ELISAs exclusively with MAbs specific for serotypes 4 and 21 (data not shown). No new serotyping MAbs were obtained, possibly because such reagents often react poorly

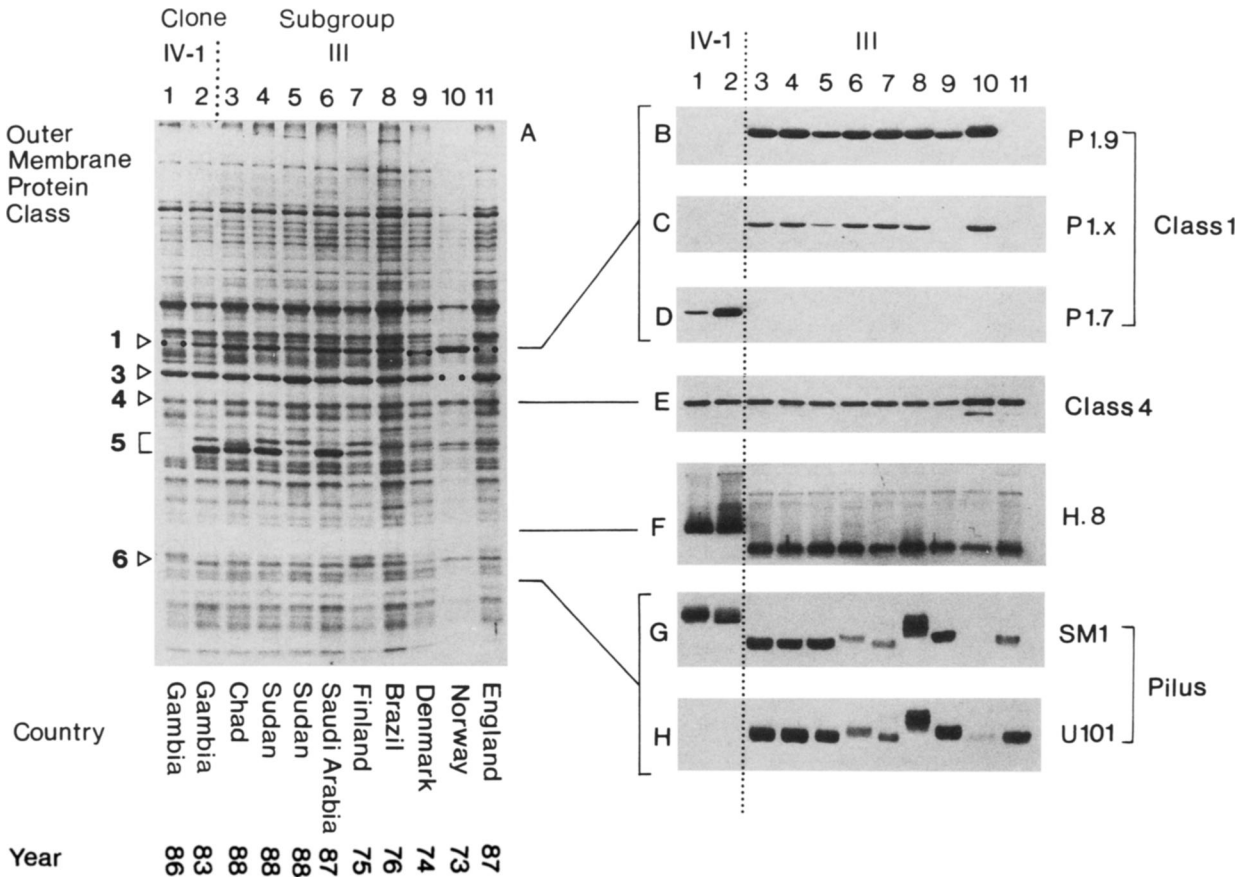

Figure 2. Stable antigens separated by SDS-PAGE. **A**, Staining with Coomassie brilliant blue, constant electrophoretic migration of class 1, 3, and 6 outer membrane proteins observed for most subgroup III strains (lanes 3–8). Paired dots indicate exceptional bands: lane 1, diminished expression of class 1 protein; lane 9, class 1 protein variant 7, which does not react with P1.x antibodies; lane 10, lack of expression of class 3 protein; lane 11, lack of expression of class 1 protein. **B–D**, Class 1 protein: Western blots with W316, V502, and A'dam 1, respectively. Note lack of reaction of clone IV-1 strains (lanes 1, 2) and of the subgroup III strain not expressing the class 1 protein (lane 11) in **B** and **C**, lack of reaction of the subgroup III strains in D, and lack of reaction of electrophoretic variant 7 with P1.x in lane 9. **E**, Class 4 protein: Western blot with SM50. All bands migrated identically except that a double band was seen in lane 10. **F**, H.8 protein: Western blot with SM70. **G**, **H**, Pili: Western blot with MAbs specific for class I (**G**) or Class IIa (**H**) pili. Strains tested by lane: 1, C1419; 2, C623; 3, Z3544; 4, Z3685; 5, Z3696; 6, Z3513; 7, B515; 8, B506; 9, B227; 10, Z3739; 11, Z3778.

in Western blots and we only pursued hybridomas with strong Western blot reactivity. The two exceptional strains consisted of a Gambian strain whose class 3 protein is 1 kDa smaller than normal that reacted only with the serotype 4 reagent [7] and a clone III-1 strain that did not express any detectable class 3 protein (figure 2A, lane 10) and that did not react with either serotype 4 or 21 reagents.

All representative strains reacted in Western blots with an antibody specific for the H.8 protein derived from immunization with a gonococcal strain [37]. The H.8 band in clone IV-1 migrated more rapidly than the band in subgroup III (figure 2F).

Most of the bacteria tested expressed large amounts of a class 1 protein, although diminished (figure 2A, lane 1) or undetectable (figure 2A, lane 11) expression was occasionally found. The class 1 proteins from typical clone IV-1 and clone III-1 strains differ in electrophoretic migration and have been called variant 2 and 4, respectively [3]. One clone III-1 strain expressed a different variant called 7 (figure 2A, lane 9); variant 2 was unique to clone IV-1 and variants 4 and 7 were unique to subgroup III [3]. Preliminary tests had shown that variant 2 reacts with P1.7 serosubtyping MAbs (formerly called P1.1,16), while variants 4 and 7 react with P1.9 MAbs [24]. All but 1 of 133 clone IV-1 strains reacted with P1.7 MAbs in whole cell ELISA tests, and the class 1 protein of representative strains reacted in Western blots (figure 2D), even when it was expressed in diminished amounts (figure 2, lane 1). P1.7 is not uniquely associated with clone IV-1; it reacts with protein variant 6 of clone II-3 [24] and with certain new Chinese strains [8].

Of 222 subgroup III strains, 218 reacted with P1.9 MAbs in whole cell ELISAs, and the class 1 protein of representatives also reacted in Western blots (figure 2B). All strains that did not react also did not express any detectable class 1

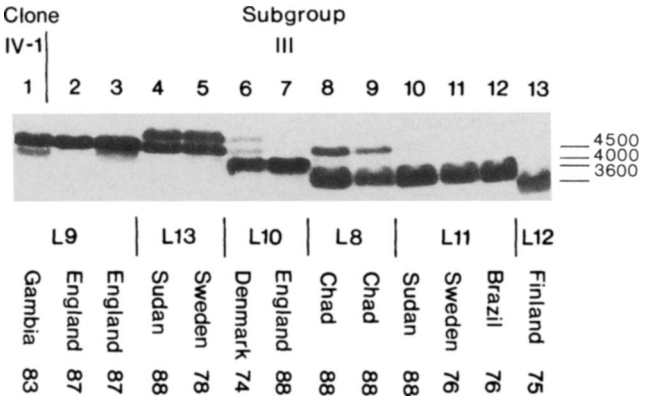

Figure 3. Lipopolysaccharide (LPS) from representative subgroup III and clone IV-1 strains as revealed by SDS-PAGE followed by silver nitrate staining (part of gel containing LPS bands is shown). Assignments reflect major bands only; thus, L9-like component in L8 bands is ignored. Molecular weights for the L8 or L11 bands (3600), L10 band (4000), and L9 band (4500) are shown at right. Molecular weights for L12 and L13 doublet were not calculated. Strains by lane: 1, C623; 2, Z3787; 3, Z3788; 4, Z3696; 5, Z3717; 6, B227; 7, Z3790; 8, Z3528; 9, Z3549; 10, Z3756; 11, Z3716; 12, B506; 13, B95.

protein (figure 2, lane 11). Comparable reactions were observed with preexisting and newly generated P1.9 MAbs. However, one newly isolated MAb, V502, reacted with all 217 subgroup III strains that expressed electrophoretic variant 4 and were P1.9 but did not react with reference P1.9 serogroup B strains nor with the strain expressing the class 1 protein variant 7 (figure 2C). V502 also did not react with any other serogroup A class 1 protein electrophoretic variant. We assign the designation P1.x to this serosubtype specificity and conclude that 98% of subgroup III strains are serosubtype P1.9,x. Protein variant 7 reacted with MAb to P1.5 and was therefore designated P1.5,9 (data not shown). Thus, clone IV-1 and subgroup III differed in one of the primary cell surface protein antigens. Furthermore, we could not detect any consistent antigenic difference that distinguished individual clones within subgroup III.

LPS. The original immunotyping scheme for LPS of serogroup A was based on inhibition assays using specific hyperimmune rabbit sera that distinguished immunotypes L8 through L11 [25]. More recently, three distinct electrophoretic patterns were found to be characteristic of the immunotypes L9, L10, and L8/L11, and MAbs specific for L10 or L8/L11 were described [26]. Because L8 and L11 LPS bands comigrate and certain MAbs react with both, it remained unclear whether L8 and L11 were distinct LPS species. However, one MAb reacting only with L8 has been described. L9 LPS contained several electrophoretic bands and no specific MAbs have been described [26].

Typical electrophoretic patterns of the LPS from the 355 strains tested here are shown in figure 3, corroborating and expanding the conclusions in [26]. The major band in L8 and L11 LPS did comigrate, but L8 LPS also expressed an additional minor band. L12 and L13 are new designations we assigned to LPS species of unique electrophoretic mobility. Western blot analysis with the hyperimmune rabbit sera used in the original immunotyping scheme [25] confirmed that L8, L9, L10, and L11 were serologically unique (data not shown) and that none of these rabbit sera reacted with L12 or L13 LPS.

We also tested 20 different hyperimmune rabbit sera raised against live clone IV-1 bacteria to determine whether there was extensive cross-reactivity with lapine antibodies. These sera were used in Western blots against strains of the different immunotypes. One-third of the rabbit sera reacted strongly with L9 LPS, but none reacted with any of the other immunotypes found in subgroup III. The MAbs formerly described [26] as well as others supplied by Jan Poolman (RIVM, Bilthoven, Netherlands) and H. Peters (Behring Werke, Marburg, Germany) were screened in whole cell ELISAs, and the quantitative results were compared with the LPS electrophoretic patterns. Most of the antibodies did not yield results that correlated well with electrophoretic migration, but additional MAbs specific for L8 and L10 were identified. Furthermore, new MAbs specific for L10 and L11 were isolated (table 1). The combined results indicate that L8 and L11 are distinct, although their major bands do comigrate and they do share some epitopes.

Western blot reactions with these MAbs to LPS were weak, and numerous protein bands reacted as well. Furthermore, no MAbs were found or generated that allowed the unique recognition of L9, L12, or L13. Thus, at the moment, a complete immunotyping analysis is not possible on the basis of MAbs alone. For the purposes of the following analysis, the designations L9, L12, or L13 were applied to strains whose LPS migrated identically with that of reference strains of those immunotypes, and the designations L8, L10, or L11 were applied to strains that reacted strongly in whole cell ELISAs with appropriate reagents and whose LPS banding pattern was consistent with that assignment. Weak serologic reactions and weak additional bands were ignored but would lead to multiple immunotype reactions in a complete scheme. Furthermore, a few of the strains, especially the older isolates and all clone IV-1 strains not expressing L9 LPS, expressed LPS bands that did not fit into these designations; these LPS species were not given any new designation because of their rare occurrence.

The results confirm the former conclusion with Gambian isolates [7] that almost all clone IV-1 strains express L9 LPS (table 2). In contrast, although most subgroup III strains were of the L10 immunotype, they were highly variable in their LPS, and all the LPS immunotypes were found within this subgroup. There was no obvious correlation between source and immunotype, and even bacteria isolated from a single city within a few weeks of each other (in Chad and Sudan) expressed the full gamut of different immunotypes.

Table 2. Immunotype frequencies within clone IV-1 and subgroup III.

	Subgroup III		Clone IV-1	
Immunotype	Mecca-related, 1987–1988 (n = 101)	Other, 1966–1987 (n = 121)	The Gambia, 1982–1988 (n = 73)	Other, 1962–1985 (n = 60)
L8	11	3		
L9	2	10	91	98
L10	62	49		
L11	15	28		
L12	0	1		
L13	6	2		

NOTE. Data are percentage of strains with indicated immunotype.

Thus, LPS immunotypes must be considered a variable property in this subgroup even though the immunotype of clone IV-1 is semi-stable.

Pili. MAb SM1 recognizes an epitope located in an invariable NH_2-terminal domain of pilin from gonococci and many meningococci [42]. Pili containing this epitope have been called class I pili. Six electrophoretic pilin variants were distinguished by Western blots with MAb SM1 within Gambian clone IV-1 bacteria [7]. Each of those strains expressed no to two distinct pilin bands and variation from expression to nonexpression in electrophoretic migration was common [7] (unpublished data). We screened all the other strains described here in similar Western blots (figure 2G) and found that the pilin bands of subgroup III strains also reacted with MAb SM1, usually migrated at lower apparent molecular weights than those of clone IV-1, and exhibited less electrophoretic variation (figure 2G). Whereas only 69% of Gambian clone IV-1 strains and 57% of other clone IV-1 strains were piliated, 96% of subgroup III strains reacted in Western blots with SM1. Thus, piliation seems to be a more stable property in this subgroup.

Two new anti-pilus MAbs were isolated that reacted in whole cell ELISAs with all piliated subgroup III strains and in Western blots with pilin (figure 2H). They also reacted with the serogroup C strain MC54 from J. Heckels (University of Southampton, UK), which does not react with MAb SM1 and whose pili have been referred to as class II pili [43]. We refer to the epitope on pili that reacts with U101 as IIa, because most class I pili (including those from clone IV-1) did not react with that antibody (figure 2H), most class II pili that react with U101 did not react with SM1, and some class II pili did not react with either antibody but do react with other antibodies that define the class IIb epitope [44]. According to this classification, clone IV-1 strains expressed class I pili, while subgroup III strains are unusual in that they expressed pili containing both the class I and class IIa epitopes. The other new monoclonal reagent, T613, reacted extremely weakly with class I pili and is therefore not suitable for differentiating pilus classes. The distribution of these serologic classes of pili in other meningococci will be presented elsewhere. We conclude that the pili of subgroup III and clone IV-1 differed antigenically for at least one conserved, surface-exposed epitope.

Class 5 proteins. Two of the mice from which hybridomas were isolated had been immunized with clone IV-1 bacteria from The Gambia expressing the class 5 proteins 5a, 5b, 5C, and 5d. Most of these hybridomas were specific for the class 5 protein seroclasses A (5a) or B (5b, d, e, or g), and none reacted with the serotype, serosubtype, or immunotype antigens. In contrast, anti–class 5 MAbs were rare in nine fusions using mice immunized with subgroup III bacteria expressing 5a, 5c, 5f, 5h, or a new protein 5i, and most of the MAbs isolated were directed against other cell surface components (table 1). However, we did isolate MAbs that reacted specifically with the class 5 proteins 5f or 5i and that define the seroclasses F (5f) and I (5i), as well as additional MAbs directed against seroclass A. To isolate MAbs specific for the 5h protein, 5h protein purified from a subgroup III strain was used to immunize a mouse. One MAb, AB419, was isolated that defines the seroclass H (5h). In addition to these MAbs, a few others were isolated whose specificities did not fit into the seroclasses A, B, C, F, H, or I but that reacted with other epitopes common to proteins 5d, f, and g (L614), 5a, b, and e (O623, O521), or 5a, 5b, 5e, and i (U506). These results indicate that the epitopes characteristic of seroclasses A and B were among the most immunogenic in clone IV-1 strains, while in subgroup III strains, epitopes on surface components other than the class 5 proteins were highly immunogenic for mice.

The class 5 proteins of the different clone IV-1 and subgroup III strains were initially classified by visual examination of SDS-polyacrylamide gels (figure 4B). Duplicate gels containing 3 *M* and 4 *M* urea were compared because urea alters the migration of certain class 5 proteins, in particular 5c/C and 5f, and using two urea concentrations allows their differentiation from that of other proteins whose electrophoretic migration is unaffected by urea [28]. At 3 *M* urea, the new protein 5i comigrated with 5c/C, while 5a comigrated

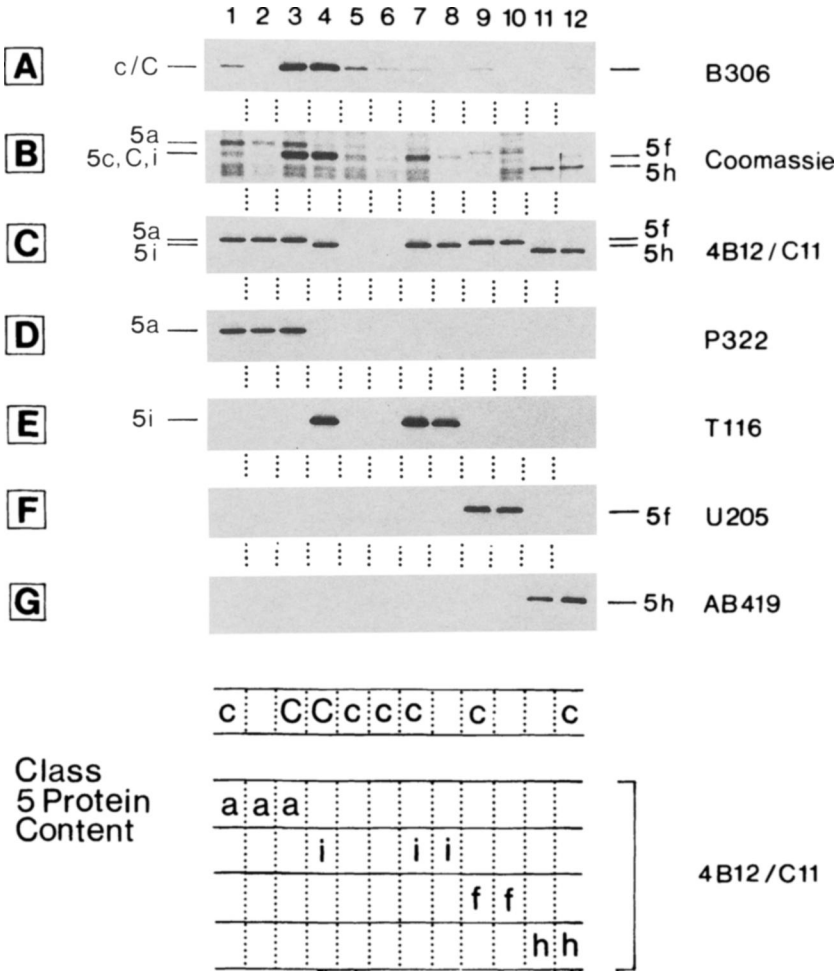

Figure 4. Class 5 proteins. Cell envelopes from subgroup III strains were separated by SDS-PAGE and stained with Coomassie brilliant blue (**B**) or subjected to Western blotting with monoclonal antibodies (**A**, **C–G**) (part of gels containing class 5 proteins is shown). Under conditions used, 5a and 5f comigrate, as do 5c/C and 5i. Thus, band in **B** at 5c,C,i position consists of 5C plus 5i in lane 4 and of 5c plus 5i in lane 7. MAb 4B12/C11 (**C**) reacts with all class 5 proteins other than 5c/C (**A**), including those shown individually in **D–G**. Assignments are shown at bottom of the figure. Strains by lane: 1, Z3696; 2, Z3756; 3, Z3685; 4, Z3513; 5, Z3528; 6, Z3552; 7, Z3790; 8, Z3788; 9, Z3717; 10, B95; 11, Z3714; 12, Z3704.

with 5f (figure 4B). Whole cell ELISAs were done using all the MAbs to class 5 proteins in table 1 against all 355 strains to detect the expression of seroclasses A, B, C, F, H, and I. The distinction between 5c and 5C was achieved by dot blotting because whole cell ELISA results do not distinguish the two. Positive whole cell ELISA reactions were tested by Western blotting with one MAb per seroclass to determine the electrophoretic migration of the reacting protein(s) (figure 4A, D–G). Finally, all the subgroup III strains were tested by Western blotting against the panreacting (other than 5c) MAb 4B12/C11 (figure 4C) to detect additional class 5 proteins that did not react with the specific MAbs available.

All class 5 proteins in the 355 strains were indistinguishable from one of the reference proteins 5a–5i by both electrophoretic migration and serologic reactivity, except for the following. Three clone IV-1 strains isolated in Sudan in 1985 expressed a 5a variant of unusually fast electrophoretic migration [12]. MAbs W104, W124, and W320/15 apparently have such a high affinity for seroclass A that strains producing only low amounts of the 5a protein and that were negative with P322 in whole cell ELISAs were positive in such tests with these MAbs. Two proteins had been designated 5e according to electrophoretic migration (clone IV-1 strains B274 and B336 from Niger, 1963 and 1964); they reacted with MAb U506 (5a, 5b, 5e, or 5i) but not with MAbs to seroclass B, whereas the reference 5e protein from a strain from The Gambia did react with those MAbs, and these proteins were excluded from the present analysis. In four subgroup III strains, class 5 proteins that differed in electrophoretic migration were detected by Coomassie blue staining; they did not react with any serologic reagent except the panreacting MAb 4B12/C11.

The results (table 3) showed that proteins 5a, 5c, 5C, 5f, and 5h were expressed by both clone IV-1 and subgroup III, although their frequencies differed to some extent with the source of isolation within each group of bacteria. In contrast, seroclass B was absent from subgroup III and seroclass I was absent from clone IV-1. Furthermore, the results suggested evolution of certain proteins with time. 5h was present in subgroup III strains from the older pandemic wave but never found among the newer Mecca-related strains. 5i was never found in older subgroup III strains but was common among the newer Mecca-related strains. The one strain expressing 5i

Table 3. Frequencies of class 5 proteins within clone IV-1 and subgroup III.

Class 5 protein (seroclass)	Subgroup III: Mecca-related, 1987–1988 (n = 101)	Subgroup III: Other, 1966–1987 (n = 121)	Clone IV-1: Gambia, 1982–1988 (n = 73)	Clone IV-1: Other, 1962–1985 (n = 60)
5a (A)	40	17	45	47
5b (B)	0	0	58	5
5d (B)	0	0	16	0
5g (B)	0	0	0	7
5c (C)	39	26	58	50
5C (C)	11	7	14	17
5f (F)	49	66	0	35
5h (H)	0	17	0	5
5i (I)	38	1	0	0

NOTE. Data are percentage of strains expressing indicated class 5 protein. Proteins are sorted by seroclass to dramatize lack of seroclass B in subgroup III. Because each strain can express up to four class 5 proteins, percentages do not total 100.

that was not obviously Mecca-related was isolated in Sweden from a Danish patient 1 month after the Mecca outbreak of 1987 and might be related to that outbreak even in the absence of epidemiologic evidence. Similarly, 5f was common among clone IV-1 strains isolated outside The Gambia but was not expressed by any of the hundreds of Gambian isolates [7]. Because the frequencies of proteins 5g and 5h were so low, it is unclear what significance should be attached to their exclusive isolation in clone IV-1 strains isolated outside the Gambia; however, 5d was relatively frequent in The Gambia but never isolated among the other clone IV-1 strains.

We previously reported that class 5 proteins of varying electrophoretic migration that reacted with the sole available MAb to 5a were expressed by serogroup A meningococci unrelated to clone IV-1 or to subgroup III [28]. We repeated these tests using 74 representative strains of subgroups I, II, V, and VI and all the MAbs to class 5 proteins listed in table 1. None of these strains expressed a class 5 protein identical to the reference proteins described here except 5c/C. Proteins reacting with some MAbs to seroclasses A or B did not react with other MAbs. Proteins reacting with the sole MAbs to seroclasses F (one strain) or H (four strains) did not comigrate with 5f or 5h. Proteins reacting with three or four MAbs to seroclass I (three strains) did not comigrate with 5i. Thus, the expression of seroclass B was restricted to clone IV-1 and the expression of 5a, 5f, 5h, and 5i to clone IV-1 and subgroup III. Only the class 5C protein is expressed by meningococci of diverse serogroups [28].

Bactericidal tests. Do antibodies stimulated by exposure to clone IV-1 bacteria protect against disease caused by subgroup III bacteria? Before the large epidemics caused by clone III-1 in 1988, Chad, Sudan, and Ethiopia suffered epidemics during the early 1980s, a period when all isolates from West Africa were clone IV-1. Furthermore, three interepidemic strains isolated from Sudan in 1985 were all clone IV-1 [12]. This observation might indicate that exposure to clone IV-1 did not provoke herd immunity against disease due to clone III-1.

We tested selected serum pairs obtained in The Gambia in 1983 for bactericidal antibodies against a representative Gambian clone IV-1 isolate (strain C12: L9, P1.7, class I pili, class 5 proteins 5a and 5c) and a matched representative clone III-1 isolate from Chad, 1988 (strain Z3545: L10, P1.9,x, class I, IIa pili, class 5 protein 5a). For these tests, the endogenous complement in the sera was heat inactivated, and we added a heterologous human complement source (final concentration, 20%) obtained from a rare donor whose serum is not bactericidal for serogroup A strains [7]. Nine serum pairs were obtained from healthy Gambians who had become nasopharyngeal carriers of clone IV-1 meningococci between the two samplings. The strain isolated from the nasopharynx from each was also tested against the homologous serum pair (table 4). Only one precarriage serum sample demonstrated bactericidal activity against any of the strains. Four of the nine postcarriage sera demonstrated a significant increase in bactericidal activity against the representative clone IV-1 strain and six against the representative clone III-1 strain, as did all six tested against the endogenous strain from that carrier (insufficient serum was available from the other three pairs). These results show that antibodies stimulated by clone IV-1 were bactericidal against subgroup III; the subgroup III strain was more sensitive to killing than the clone IV-1 strain (geometric mean increase, 2.78 dilution steps for clone III-1 vs. 1.89 for clone IV-1); and the antibodies were partially strain-specific because the titers with the endogenous strain were higher than for the two other strains and the two sera that were not bactericidal for the other strains were bactericidal for the endogenous strain. Hyperimmune lapine sera obtained after immunization with clone IV-1 bacteria were also bactericidal against diverse subgroup III strains, and the results confirmed that subgroup III strains were generally more sensitive to bactericidal killing than were clone IV-1 strains (data not shown).

Ten other serum pairs were tested from Gambians who were nasopharyngeal carriers at the time of the first serum sample (table 4). Five had bactericidal antibodies against C12 on the first occasion and another two developed such antibodies by the second sample. The titer and the number of positive reactions was slightly higher against the endogenous strain. Of note, two pairs demonstrated a drop in bactericidal titer with time against each of the strains, suggesting that protection due to natural immunization might be of short duration in the African environment.

The average rise in bactericidal titer was significantly higher against the endogenous strain than against the representative strain for all 16 serum pairs in table 4 for which the

Table 4. Changes in serum bactericidal titers due to nasopharyngeal carriage in healthy Gambians.

Serum source, code	Days between pairs	C12 Titer	C12 Log_2 rise	Z3545 Titer	Z3545 Log_2 rise	Endogenous strain Titer	Endogenous strain Log_2 rise
Nasopharyngeal carriers before and after acquisition							
1414	42	8	1	16	1		
1604	47	<4	0	<4	3		
1635	47	<4	4	<4	3	<4	4
1805	47	<4	3	<4	5		
2203	66	<4	4	<4	5	<4	5
2204	66	<4	5	<4	6	<4	8
2617	118	<4	0	<4	0	<4	5
2644	118	<4	0	<4	2	<4	5
2722	105	<4	0	<4	0	<4	5
Geometric mean		<4	1.89	<4	2.78	<4	5.33
Number positive		1/9	4/9	1/9	6/9	0/6	6/6
Gambians who were persistent carriers							
1619	47	<4	2			16	1
1624	47	<4	0			4	−1
2207	66	64	−3			256	0
2208	66	<4	5			<4	7
2211	66	<4	1			4	0
2212	66	32	1			16	3
2218	66	32	0			512	−2
2225	66	28	0.19			64	−1
2231	66	<4	0			16	2
2415	52	32	−4			<4	0
Geometric mean		8.5	0.02			17.2	0.89
Number positive		5/10	2/10			6/10	3/10

NOTE. A serum pair was considered to have demonstrated a rise in titer only when the titer of the second was at least fourfold higher ($log_2 > 1$) than the first. C12, representative Gambian strain; Z3545, representative III-1 strain.

endogenous strain was tested (median, 2 dilution steps, $P = .02$, double-sided t test for comparing mean values). Similarly, the mean log_2 titer of the second serum from these 16 carriers was also significantly higher ($P < .001$) against the endogenous strain than against the representative strain.

These results were extended by testing paired acute- and convalescent-phase sera from Gambians infected with clone IV-1 in 1983 and from Finns infected with clone III-1 during the epidemic of 1974–1976 (table 5). One of the 12 Gambian sera had bactericidal activity against the representative clone IV-1 strain during the acute phase, whereas none was bactericidal against the endogenous strain. Three of the 4 convalescent-phase sera that showed an increase in bactericidal activity against the endogenous strain also increased in activity against C12, indicating that these antibodies are usually not strain-specific. Four of the 10 Finnish acute-phase sera had bactericidal activity against the clone III-1 strain, and 3 others increased in bactericidal activity on convalescence. Furthermore, 3 of the 4 bactericidal acute-phase sera were also bactericidal against the clone IV-1 strain, and 2 of the convalescent-phase sera showed an increase in titer against that strain. These results show that bactericidal antibodies stimulated by clone III-1 could cross-react with clone IV-1. The endogenous strains were not available for the Finnish patients so that we could not test whether these sera had even higher strain-specific antibody titers.

Discussion

The results presented here characterize the widespread descendants of two bacterial strains that have caused millions of cases of meningococcal disease on diverse continents. Their genetic relationships have been measured by clonal analysis and their historical spread can be inferred from epidemiologic data on epidemics and outbreaks. Clone IV-1 has been routinely isolated from West Africa since the early 1960s, even during epidemic outbreaks caused by other bacteria. We have recently confirmed that four strains obtained from B. Bjorvatn (Haukeland Hospital, Bergen, Norway) and isolated in India in the 1980s were also clone IV-1, but

Table 5. Bactericidal titers of paired acute- and convalescent-phase sera from The Gambia and Finland.

Source, code	Days between pairs	C12 Titer	C12 Log_2 rise	Z3545 Titer	Z3545 Log_2 rise	Endogenous strain Titer	Endogenous strain Log_2 rise
The Gambia							
29	148	<4	0			<4	0
45	118	<4	5			<4	4
415	35	<4	0			<4	0
513	57	<4	1			<4	0
715	62	<4	0			<4	0
830	51	<4	0			<4	0
1106	50	<4	0			<4	0
1719	48	<4	2			<4	2
2241	66	<4	0			<4	3
2427	52	<4	0			<4	0
2514	52	<4	0			<4	1
54	25	8	2			<4	4
Geometric mean		<4	0.8			<4	1.2
Number positive		1/12	3/12			0/12	4/12
Finland							
4001		<4	0	<4	0		
4002		32	−1	256	−2		
4003		<4	0	<4	7		
4004		<4	0	<4	6		
4005		32	1	256	0		
4006		<4	1	<4	4		
4009		<4	0	4	−1		
4010		<4	0	16	1		
4011		<4	0	<4	0		
4012		8	1	128	1		
Geometric mean		4	0.2	10.6	1.6		
Number positive		3/10	0/10	4/10	3/10		

NOTE. A serum pair was considered to have demonstrated a rise in titer only when the titer of the second was at least fourfold higher ($log_2 > 1$) than the first. C12, representative Gambian strain; Z3545, representative III-1 strain.

these bacteria have not yet been described from other countries. Subgroup III bacteria have caused one pandemic wave spreading from China to Russia, Scandinavia, and Brazil and a second wave spreading from Asia to East Africa (figure 1). These bacteria had not been isolated in Africa before 1988, and the two groups of bacteria have probably not had extensive opportunities for direct genetic exchange in the recent past.

Clonal stability of cell surface antigens. The cell surface antigens were analyzed for 133 clone IV-1 strains isolated in West Africa and Sudan between 1962 and 1988 and for 222 subgroup III strains isolated in diverse countries from both pandemic waves. These antigens could be subdivided into stable antigens characteristic of most serogroup A bacteria (H.8 antigen, class 4 protein, serotype 4/21 class 3 protein) or of major subgroups (class 6 protein), clonal antigens that are characteristic of most members of a clone (the P1.7 serosubtype class 1 protein for clone IV-1 vs. the P1.9,x serosubtype for subgroup III), and hypervariable antigens (pili, class 5 proteins). These results resemble those found with a subset of these strains consisting of clone IV-1 bacteria isolated between 1982 and 1988 from patients and healthy carriers in The Gambia [7]. However, the stability of expression of certain antigens can apparently vary between different clones, because most clone IV-1 strains expressed L9 LPS while all LPS immunotypes known to be expressed by serogroup A were found within subgroup III, even among strains isolated in one locale and at one time. A similar lack of correlation between LPS immunotype and clonal grouping has formerly been described for clone I-1 [3].

Several minor exceptions to these generalizations were observed: the class 3 protein differed dramatically in electrophoretic migration in one clone IV-1 strain, and one subgroup III strain did not express detectable class 3 protein or serotype antigen, the first such natural isolate to our knowledge. Rare bacteria expressed two protein bands reacting with MAbs to

the class 4 protein. These might represent mutational events due to deletion or other genetic changes. Although indicating that stable properties are not immutable, the exceptions do not affect the generalization that essentially all related bacteria were uniform for the expression of stable cell surface antigens. The H.8 protein differed in electrophoretic migration between clone IV-1 and subgroup III; further analyses of other clonal groups would be needed before concluding that such differences in migration can be used as a marker for clones or subgroups. It also remains unclear whether this electrophoretic difference is reflected by serologically distinguishable H.8 epitopes.

Somewhat surprisingly, clonal associations were seen even for the expression of hypervariable properties that can differ from strain to strain. Although the expression and electrophoretic migration of pilin is highly variable within each strain, all clone IV-1 pili reacted with the class I pilus–specific MAb SM1 and all subgroup III pili reacted with both the class I MAb and a newly isolated MAb to class IIa pili. Thus, apparently most members of a clonal group conservatively inherit the nucleotide sequences encoding these epitopes, even though other parts of the pilin cistron can vary dramatically. Similarly, only a limited number of class 5 proteins were expressed by any one clone although the expression of individual proteins by any one strain was highly variable. With very few exceptions, all class 5 proteins found in clone IV-1 were indistinguishable serologically and by electrophoretic migration from one of the reference proteins designated 5a, 5b, 5c/C, 5d, 5e, 5f, 5g, or 5h; similarly, almost all class 5 proteins from subgroup III were indistinguishable from the proteins 5a, 5c/C, 5f, 5h, or 5i. Data have indicated that each meningococcus possesses four *opa* genes encoding class 5 proteins [27, 45], demanding an explanation of how the different members of a clone can collectively express five (subgroup III) or even eight (clone IV-1) distinct class 5 proteins.

We propose that the genes for 5a and 5c/C are common to both groups of bacteria and the additional proteins reflect the evolution of *opa* genes with time. The class 5h protein was never found among subgroup III strains related to the Mecca outbreak, while the class 5i protein was found only among those strains, suggesting that the gene encoding 5i has replaced the 5h gene. 5f was expressed by bacteria from both pandemic waves, and it is possible that this subgroup has a basic complement of three *opa* genes encoding the 5a, 5c/C, and 5f proteins and a replaceable gene for 5h or 5i. For the clone IV-1 bacteria, analyses with >300 strains from The Gambia have indicated that only the four proteins 5a, 5b, 5c/C, and 5d were common and that the bacteria expressed a maximum of four class 5 proteins simultaneously [27]. 5d was never found among clone IV-1 bacteria isolated outside of The Gambia, and 5f, which was common among the latter, was not found among Gambian strains. It is possible that clone IV-1 has a basic complement of two *opa* genes encoding 5a and 5c/C and two other genes from the pool of those encoding 5b, 5d, 5f, 5g, and 5h, all of which were expressed only by regionally distinct isolates. Further genetic analyses are needed to resolve these questions.

Given that clone IV-1 and subgroup III were predominantly isolated from nonoverlapping geographic locations during the period 1962–1987, it is surprising that 5f and 5h were expressed by both bacterial groups, although the expression of 5f and 5h was regionally variable within clone IV-1 and that of 5h within subgroup III. To determine whether other meningococci express these proteins, we tested 74 additional strains of subgroups I and II and the new subgroups V and VI. None of the class 5 proteins was identical to the proteins found in subgroup III and clone IV-1 except for 5c/C. It is possible that the 5a, 5f, and 5h proteins differ between clone IV-1 and subgroup III. Indistinguishable electrophoretic migration does not prove sequence identity, and the indistinguishable serologic reactivity was demonstrated with only one MAb per protein for 5f and 5h. Thus, the apparent identity of these proteins might reflect convergent evolution and they might well differ in sequence and serologic reactivity. However, the 5h protein from clones IV-1 and subgroup III did behave identically during purification procedures that normally distinguish distinct proteins. For the proteins 5a and 5c/C the evidence for identity is stronger, because several distinct antibodies each reacted with these proteins from both groups of bacteria. Again, further analyses are indicated, but we consider it possible that all proteins present in both clonal groups reflect either the inheritance of *opa* genes from a distant mutual ancestor or horizontal transmission of these genes via another bacterial vector. Regardless of which mechanism is responsible, these data suggest strong selective advantages for the expression of certain widespread class 5 proteins.

Natural immunity. The experiments described here were initiated to evaluate whether West African countries might be protected against potential future epidemics caused by clone III-1 through their extensive exposure to clone IV-1 during the epidemics of the early 1980s. East African countries have experienced recent severe epidemics due to clone III-1 despite epidemics in the early 1980s, but bacteria from those earlier epidemics have not been subjected to clonal analysis, and it remains possible that the epidemics were caused by bacteria that did not induce cross-reactive immunity. It is also possible that the carriage rate may not have been as high or as widespread as in The Gambia.

Our antigenic analyses have shown that clone IV-1 bacteria expressed several antigens common to clone III-1, namely the capsular polysaccharide, the H.8 antigen, the class 4 protein, the T4/21 serotype antigen, and the class I pilus epitope. Elsewhere we showed that despite extensive strain-to-strain variation of expression, prolonged carriage will result in exposure to most of the class 5 protein seroclasses potentially expressed by a bacterial strain [27]. Thus, effective herd immunity might even be based on antibodies

against the class 5 seroclasses A (5a), C (c/C), and F (5f), which were also found in subgroup III bacteria in East Africa. Finally, although most clone IV-1 strains expressed L9 LPS and most subgroup III strains expressed other LPS immunotypes, protective human anti-LPS antibodies might be induced by chemical components common to these different molecules. These considerations led to several questions that are partially answered by our data herein.

Do carriage of meningococci and subclinical disease lead to the development of long-lasting protective antibodies? The data in table 4 show that all Gambians who became healthy carriers also developed bactericidal antibodies against their endogenous strain. The production of these antibodies can explain why these carriers did not develop clinical disease. Prolonged carriage stimulated antibody production in some individuals who did not react at first but led to the loss of bactericidal antibodies in others who responded quickly (table 4). This suggests that although immunity is stimulated by a high carriage rate, protection might not be long-lasting in the African environment.

Are antibodies stimulated by clone IV-1 potentially protective against clone III-1? The results in tables 4 and 5 demonstrate that a rise in bactericidal activity stimulated by exposure to clone IV-1 was accompanied by a rise in bactericidal antibodies against clone III-1. In fact, strains of clone III-1 seem to be more sensitive to bactericidal killing than are strains of clone IV-1. Similar results were obtained with hyperimmune lapine sera. Therefore, if protective antibodies stimulated by extensive carriage of clone IV-1 meningococci are still prevalent among the population exposed at that time in West Africa, they should provide some protection against disease caused by clone III-1, and any future epidemics should predominantly affect children born after 1982 who were not exposed. However, if protection were not long-lasting, the whole population might now be at risk.

What is the antigenic specificity of natural immunity? Our results are apparently paradoxical. Despite the cross-reactivity just described, the bactericidal antibody response stimulated by carriage was significantly greater against the endogenous strain isolated from that carrier than against a representative clone IV-1 strain; that is, it seems partially or predominantly strain-specific. In some cases no bactericidal activity was seen against a representative strain, although a high titer was measured against the endogenous strain. Because diverse Gambian bacteria were roughly comparable in sensitivity to bactericidal killing stimulated by monoclonal anti-serosubtype or anti-LPS antibodies [7], these results imply that the different Gambian bacteria varied in expression of important cell surface antigens. The only variable antigens currently recognized for these strains are the class 5 proteins and pili. Whereas some of the sera with predominant strain-specific bactericidal killing were from Gambians exposed to bacteria expressing the 5b or 5d antigens (lacking in the representative strain), there was no unique correlation between class 5 protein expression and strain specificity (data not shown), and further analyses are necessary to determine the antigenic basis of strain-specific bactericidal killing.

Disease versus carriage. Studies by Goldschneider et al. [29, 30] described a high frequency of meningococcal disease in army recruits who carried meningococci but did not have bactericidal antibodies. Subsequent analyses have interpreted the lack of bactericidal killing in acute-phase sera from epidemic disease as reflecting inhibition by IgA of complement activation by low levels of existing IgG and IgM [46–48]. However, these analyses are based on data from the United States, and they differ from our results obtained with sera from an African epidemic. Although Gambian acute-phase sera did not possess bactericidal activity, neither did sera from Gambians who went on to become healthy carriers rather than becoming sick (tables 4, 5). Thus, the lack of bactericidal antibodies before exposure to virulent meningococci had no predictive value, and given the 15-fold higher frequency of carriage than disease in The Gambia, it can be extrapolated that almost all Gambians lacking bactericidal antibodies became healthy carriers on initial exposure to clone IV-1 rather than developing meningococcal disease. Studies have indicated that epidemic disease may be associated with coinfection with meningococci plus a second independent organism [49, 50]. In that case, the critical factor for disease development may be acquisition of the second organism; in its absence, the carrier may begin synthesis of protective antibodies and never develop disease. We were surprised to note that only few of the patients with meningococcal disease developed bactericidal antibodies after convalescence. Most carriers developed such antibodies to their endogenous strain, suggesting that one factor associated with disease may be a lessened antibody response or the production of antibodies that do not activate complement effectively. Because other analyses have demonstrated a rise of specific (but not necessarily bactericidal) antibodies in most convalescent-phase sera (unpublished data), the latter possibility may be more likely. In that event, disease might reflect the inability of the colonized individual to quickly mount a protective antibody response.

Are epidemics caused by clone III-1 imminent in West Africa? In addition to being affected by the antigenic and serologic factors discussed above, epidemics can first occur when the colonization rate is high. Although clone III-1 bacteria were certainly exported to all countries, including those of West Africa, by pilgrims returning from the 1987 Haj, neither epidemics nor extensive disease have resulted in most countries. Of 1000 returning American pilgrims, 10% carried clone III-1 bacteria [16], but no cases of III-1 disease from bacteria acquired in the United States have yet been reported. Ten thousand English pilgrims returned from the Haj in 1987; over the next few months, 33 cases of III-1 disease were reported among primary and secondary con-

tacts and among 6 others without epidemiologic connection to the pilgrimage. Since then, clone III-1 disease has again become rare in England. Thus, import of these bacteria need not lead to extensive spread or epidemic disease. The same conclusion may be reached from the observation that most cases of meningococcal disease in Sweden in the mid-1970s were caused by clone III-1, although there was no outbreak in that country [12]. Although the recent isolation of clone III-1 bacteria from The Gambia has been reported [19], no epidemics have been reported from West Africa. Furthermore, none of 45 meningococci isolated from disease during 1990 in Bamako, Mali, were clone III-1 [44]. Thus, probably these bacteria are currently rare in West Africa and have not succeeded in widespread colonization. Should such widespread colonization spread from East African epidemics, our data suggest that epidemic disease in West Africa might predominate among younger children due to residual immunity. However, these optimistic conclusions must be tempered by caution, as we are still unable to accurately predict the course of meningococcal epidemics.

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