Molecular polymorphism and epidemiology of *Neisseria meningitidis* immunoglobulin A1 proteases

(population genetics/immunoglobulin A/enzyme polymorphism/serology)

HANS LOMHOLT*, KNUD POULSEN*, DOMINIQUE A. CAUGANT†, AND MOGENS KILIAN*‡

*Institute of Medical Microbiology, The Bartholin Building, University of Aarhus, DK-8000 Aarhus C, Denmark; and †Department of Bacteriology, National Institute of Public Health, Geitmyrsveien 75, Oslo 4, Norway

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ABSTRACT *Neisseria meningitidis* is one of several important bacterial pathogens that secrete a specific protease capable of cleaving human immunoglobulin A1 (IgA1) in the hinge region. To obtain further information on this putative virulence factor, we examined the IgA1 protease and *iga* gene region of 133 isolates of *N. meningitidis* assigned to 88 multilocus enzyme genotypes and representing major epidemics and carrier strains from 19 countries. Of the two IgA1 cleavage specificities previously observed, isolates associated with epidemics of meningococcal disease showed exclusively type 1 IgA1 protease activity. Considerable heterogeneity of the *N. meningitidis* IgA1 protease was demonstrated at both the protein and gene levels. Thus, five different forms of IgA1 protease were detected with enzyme-neutralizing antibodies raised in rabbits. An antiserum raised against a single type 2 IgA1 protease inhibited the enzyme activity of all strains examined, a finding of potential significance for the possible application of IgA1 protease in a vaccine against meningococcal disease. Examination of the *iga* gene region with restriction endonucleases revealed a high degree of polymorphism among strains belonging to some multilocus enzyme genotypes. The different *iga* gene types did not correlate with cleavage type or inhibition of the IgA1 protease. Our findings indicate that horizontal genetic exchange occurs *in vivo* with considerably different frequency in different clones of meningococci.

The immunoglobulin A1 (IgA1) proteases are highly specific bacterial enzymes that cleave certain peptide bonds in the hinge region of human IgA1. They are excreted by pathogenic members of the genera *Neisseria*, *Haemophilus*, *Streptococcus*, *Prevotella* (*Bacteroides*), and *Capnocytophaga* and are considered important factors of virulence (1–3). The only known alternative substrates of the *Neisseria* and *Haemophilus* IgA1 proteases are autocatalytic sites inside the protease precursor molecule (4, 5). In gonococci, these autocatalytic sites separate the extracellular mature protease from a released α protein and an outer membrane-associated β protein required for extracellular secretion (4).

Homologies among the nucleotide sequences have been shown for the IgA1 protease genes (*iga*) of *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Haemophilus influenzae* (6), suggesting a common evolutionary origin. Despite this overall similarity, a surprising degree of heterogeneity has been found, even among the IgA1 proteases secreted by members of a single species. The enzymes differ in the exact position of cleavage in the human IgA1 hinge region. Proteases cleaving one of several prolyl-seryl bonds are called type 1 IgA1 proteases, and those attacking one of several prolyl-threonyl bonds are designated as type 2 IgA1 proteases (7–9). Furthermore, among IgA1 proteases released by *H. influenzae* strains, at least 15 different antigenic types have been

detected by the use of enzyme-neutralizing antisera, and a correlation between these “inhibition types” and capsular serotype has been observed (8). Restriction fragment length polymorphism (RFLP) analysis of the *iga* gene region of *N. gonorrhoeae* strains has revealed similar heterogeneity. Thus, eight different *iga* gene region types have been detected among gonococci that produce a type 2 IgA1 protease, as opposed to only a single gene type coding for the type 1 protease (10).

To obtain further information concerning the epidemiology and molecular biology of the IgA1 proteases, we have examined the *iga* gene and its product in a large collection of *N. meningitidis* strains. The specificity and antigenicity of the protease and the RFLP of the corresponding *iga* gene were compared to the epidemiology, serology, and electrophoretic type (ET) of the isolates. We show that the epidemic clones of meningococci are characterized by a single common cleavage type of IgA1 protease. Polymorphism both in the *iga* gene and in the antigenic properties of the IgA1 protease was seen among strains belonging to single ETs and clusters of ETs, suggesting frequent horizontal genetic exchange *in vivo*.

MATERIALS AND METHODS

Bacterial Isolates. A collection of 133 *N. meningitidis* isolates recovered during the period 1917–1988 from 97 patients and 36 healthy carriers in 19 countries was examined. The sources, serogroups, and serotypes of 113 of the isolates have been presented (11, 12). Additional strains were chosen from the authors' collections to increase the representation of three ETs responsible for epidemics in several countries.

The 133 isolates were assigned to 88 multilocus enzyme genotypes on the basis of electrophoretically detectable allelic variation in 15 metabolic enzymes. Ten of the 14 main lineages of *N. meningitidis* were represented in the strain collection examined, and isolates belonging to 35 of the 61 clusters described (11) were included. The isolates were serologically examined and assigned to serogroups and serotypes as described (11).

IgA1 Protease Activity. Each strain was examined for IgA1 protease activity by overnight incubation in a solution of human IgA1 paraprotein (1.5 mg/ml) purified from the plasma of a patient with myelomatosis (13) in Tris-HCl, pH 7.4. IgA1 cleavage was detected by immunoelectrophoresis and SDS/PAGE as described (13). Two prototype strains of *N. gonorrhoeae*: MS11 (IgA1 protease type 2) and NG74 (IgA1 protease type 1) (14) received from Thomas F. Meyer (Max-Planck Institut für Biologie, Tübingen, F.R.G.) were used as reference in SDS/PAGE for determining the cleavage type of IgA1 proteases.

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Abbreviations: ET, electrophoretic type; RFLP, restriction fragment length polymorphism.

‡To whom reprint requests should be addressed.

Serological Typing of IgA1 Proteases. Four strains of meningococci, VK3 (serogroup W135, IgA1 protease type 1), BB353 (serogroup C, IgA1 protease type 2), HF13 (serogroup Y, IgA1 protease type 2), and NG117 (serogroup B, IgA1 protease type 2), representing different *iga* gene RFLP patterns, were selected for preparation of antisera to IgA1 proteases. IgA1 proteases were prepared and used for s.c. immunization of rabbits as described (8). Immunoglobulins were isolated from rabbit blood and redissolved in 0.05 M Tris-HCl, pH 7.4. IgA1 proteases from 32 selected meningococcal strains and the two gonococcal strains were prepared, and the enzyme activity was adjusted so that it was twice the lowest concentration that would induce complete cleavage of 0.5 mg of IgA1 per ml in 1 hr at 35°C in a reaction mixture of 1 vol of enzyme preparation and 1 vol of substrate solution. The ability of the four antisera to inhibit IgA1 cleaving activity in each of the 32 adjusted protease preparations was examined by determining the antiserum dilution that caused 50% inhibition of the protease. Fifty microliters each of protease preparation and respective 2-fold serum dilutions were mixed and incubated for 1 hr at room temperature. Fifty microliters of IgA1 substrate (1.5 mg/ml in Tris-HCl, pH 7.4) was added, and cleavage was detected by immunoelectrophoresis after incubation of the reaction mixture for 18 hr (13). The IgA1 proteases of the remaining 98 meningococcal strains were examined by suspending a loop full of bacteria from a chocolate agar culture in 40 μ l of undiluted antiserum. After a 1-hr incubation at room temperature 40 μ l of substrate was added, and the activity was determined as described.

Preparation and Analysis of Chromosomal DNA. Bacteria were harvested from chocolate agar after 24 hr and were lysed with lysozyme, EDTA, and Triton X-100. Proteins were degraded by incubation with proteinase K (15), and DNA was purified from the suspension with Qiagen-tube 20, as described by the manufacturer (Diagen, Düsseldorf, F.R.G.). Restriction endonucleases were purchased from Boehringer Mannheim and used according to the manufacturer's instructions. The resulting DNA fragments were separated by 0.8% agarose gel electrophoresis and were subsequently blotted onto nylon membranes (Nytran-NY 13N, Schleicher & Schuell) (15). The DNA probe for hybridization was isolated and labeled with [³²P]dATP (Amersham) incorporated by nick-translation as described (15). Hybridization was done under stringent conditions (posthybridization wash: 0.5× standard saline citrate/0.1% SDS at 60°C) (15). As probe we used a fragment of the *iga* gene from *N. gonorrhoeae* strain F62 (Fig. 1); it was isolated as a 4.2-kilobase (kb) *Hind*III fragment from the recombinant *Escherichia coli* plasmid pVD105 (16) donated by J. M. Koomey (The Rockefeller University, New York).

RESULTS

IgA1 Protease Activity and Enzyme Type. Of the 133 *N. meningitidis* strains analyzed, 130 had detectable IgA1 protease activity. The three negative strains had all been isolated from patients with meningococcal disease, and two of these strains were assigned to the same electrophoretic type (ET71). Nine other strains of the same ET all showed IgA1 protease activity.

The majority (70%) of the IgA1 protease-producing strains showed protease activity of cleavage type 1. All 18 serogroup A strains possessed a type 1 protease, but no relationship was found between protease cleavage type and other serogroups (data not shown). All serotype 2a, 2b, and 21 and all serotype 15:P1.16 strains produced a type 1 protease, whereas all serotype 2c strains produced a type 2 protease.

Fifty-eight of the IgA1 protease-producing strains belonged to clones that have caused epidemics (ET clusters A4, B2, E1, E2, and I1) (11). All these strains, except the strain

FIG. 1. Variation in *iga* gene region among 133 *N. meningitidis* strains as revealed by *Pst* I restriction endonuclease digestion. (1) Broken line represents 4.2-kb *Hind*III fragment of *N. gonorrhoeae* strain F62 *iga* gene (16) used as hybridization probe. (2) Linear map of *iga* gene encoding N-terminal leader peptide (L), excreted IgA1 protease, and helper region consisting of α protein and β transmembrane pore structure (β protein) (4). Vertical arrows indicate *Pst* I cleavage sites. (3) Horizontal arrows represent three major *Pst* I fragments detected by hybridization with *iga* gene probe. (4) Different sizes of each of the three fragments. Figures in parentheses indicate weakly hybridizing fragments. An additional small hybridizing fragment of $\approx$ 600 bases was regularly detected.

of cluster I1, ET76, which was exceptional in several ways (see below), produced a type 1 protease. In contrast, among 72 strains belonging to clones not known to be associated with epidemics, type 1 and type 2 protease production was evenly distributed. Thus, the results show a strong correlation between type 1 protease production and epidemic pathogenicity.

Serological Analysis of IgA1 Proteases. Sera from rabbits immunized four times with selected meningococcal IgA1 proteases inhibited the IgA1-cleaving activity of the homologous enzyme at a titer between 256 and 4096. The IgA1 proteases of all 130 strains examined were inhibited by one or more of the four antisera produced. Two of the antisera (anti-BB353 and anti-HF13) showed the same inhibition spectrum. The inhibition pattern seen with the antisera against proteases from VK3, HF13, and NG117 allowed distinction between three different inhibition types among type 1 proteases (inhibition types a, b, and c) and two different patterns among type 2 proteases (inhibition types d and e) (Table 1).

The antiserum raised against cleavage type 2 IgA1 protease HF13 inhibited all 130 meningococcal and the two gonococcal IgA1 proteases examined, irrespective of cleavage specificity. In contrast, the antisera VK3 and NG117 inhibited proteases only of the corresponding cleavage type except for the 26 type 1 proteases inhibited by anti-NG117 (Table 1).

The five inhibition types identified showed no correlation with serogroup or serotype of the strains. However, in most cases strains assigned to a single ET cluster produced IgA1 protease of a single inhibition type. Exceptions were clusters encompassing both type 1 and type 2 protease-producing strains (Table 2). In addition, strains being exceptional within the cluster because of their *iga* gene RFLP pattern often produced an IgA1 protease of exceptional inhibition type [e.g., the strain of cluster E1, ET64 (Table 2), and two other examples not shown]. All three type 1 inhibition types (a, b, and c) were represented among proteases of epidemic strains.

RFLP. The *N. gonorrhoeae* *iga* gene probe (Fig. 1) gave positive hybridization signals on all Southern blots of genomic DNA from the 133 *N. meningitidis* strains examined,

Table 1. Antibody-induced inhibition patterns of five IgA1 protease inhibition types detected among 127 strains of *N. meningitidis*

Inhibition type	Cleavage type	IgA1 protease inhibition titer with antibodies against IgA1 protease			Strain, no.
		VK3(1)*	HF13(2)	NG117(2)	
a	1	128–512	512–2048	256–512	26
b	1	256–512	2048	0	42
c	1	64–256	128–512	0	20
d	2	0–4	4096	1024–2048	17
e	2	0–4	2048	128–256	7
d or e	2	–†	+†	+†	15

Three additional IgA1 protease-positive strains could not be classified with certainty.

*Figures in parentheses indicate cleavage type of IgA1 protease used for immunization.

†IgA1 protease activity was not titrated against the antisera. + and – indicate presence and absence of protease inhibition, respectively.

including the 3 strains without detectable IgA1 protease activity. Genomic DNA from all isolates could be digested with the 6-base recognition sequence restriction endonucleases *Pst* I, *Eco*RI, and *Bam*HI. *Pst* I digestion revealed at least one cleavage site inside the *iga* gene of all strains as opposed to *Eco*RI and *Bam*HI, which cleaved within the *iga* gene of only 8 and 2 of the 133 strains, respectively. Size variation of hybridizing DNA fragments detected after *Pst* I digestion (Fig. 1) show that the *iga* gene is a single-copy gene.

The total number of RFLP variants detected among the 133 strains after digestion by the three 6-base restriction enzymes added up to >20. Table 2 details the data for 81 strains assigned to seven ET clusters. For further evaluation of the extent of variation among the meningococcal *iga* genes, DNA from 24 strains from two epidemic clones that belonged to ET clusters E2 and I1 were digested with the 4-base restriction endonuclease *Hae* III. This procedure revealed an additional six *iga* gene types among 13 strains assigned to cluster I1, five of these types were detected among 11 strains of a single ET (ET71). In contrast, the 11 strains of ET-cluster E2 remained homogeneous (Table 2).

Serogroup A strains were very homogeneous with respect to *iga* gene RFLP pattern. All 18 isolates showed the hybridizing *Pst* I fragment of 4.1 kb. An *iga* *Pst* I fragment of the same size was seen in only four strains of other serogroups. Within each of the serotypes 2a, 2b, 15:P1.16, and 21, which mainly included isolates of related enzyme genotypes, only minor differences in RFLP patterns were found, and all three serotype 2c strains showed identical patterns.

No specific RFLP pattern was associated with carriage versus infection, place of isolation, serotype, or IgA1 protease cleavage type. Most ET clusters showed an identical *iga* RFLP pattern. However, considerable variation was found in some ET clusters and even within single ETs (e.g., A4, 7 and I1, 71; Table 2).

The RFLP analyses demonstrate a high degree of variation in the *iga* gene region among *N. meningitidis* isolates. Combined with the ET-typing results these findings indicate that the meningococcal population is composed of a large number of clones with different genetic stability.

DISCUSSION

This study allows comparison of the overall polymorphism in the meningococcal genome, as reflected in 15 randomly selected intracellular enzymes, with the polymorphism of an extracellular putative virulence factor (IgA1 protease) exposed to immunologic selection pressure. Several studies

using multilocus enzyme electrophoresis have shown that *N. meningitidis* populations are strongly heterogeneous and that the genetic diversity in the species exceeds that of any other bacterial species analyzed (11). A total of 88 ETs from 10 of 14 main lineages of *N. meningitidis* (11) were represented among the 133 strains studied. The collection comprised strains from both healthy carriers and from major outbreaks of meningococcal disease in Europe, the Americas, Australia, and Africa. The heterogeneity was also evident in the IgA1 proteases of the strains examined. Our methods generated data at the protein level, by analyses of cleavage specificities and antigenic properties of the IgA1 proteases, and at the DNA level, by RFLP analysis of the *iga* gene and flanking regions of the chromosome.

N. meningitidis IgA1 proteases may cleave either of two peptide bonds in the hinge region of human IgA1 (7). Not surprisingly, both cleavage types 1 and 2 IgA1 proteases were seen among the meningococcal strains examined. However, interestingly all but 1 of 58 isolates from clones of meningococci associated with epidemics (ET clusters A4, B2, E1, E2, and I1) produced a type 1 protease. IgA1 protease of cleavage type 1 is also produced by all strains of *H. influenzae* serotype b, the serotype responsible for virtually all cases of invasive disease from this organism, and by *N. gonorrhoeae* strains of the AHU auxotype, which is associated with disseminated gonococcal disease (8, 9). Why most pathogenic clones of *Haemophilus* and *Neisseria* are associated with IgA1 proteases of type 1 cleavage specificity is unclear. The dipeptide Thr-Pro that separates type 1 and type 2 cleavage sites in the hinge region probably does not determine the biological effect of the IgA1 cleavage. An alternative explanation is that the two types of proteases differ in other as-yet-unrealized inherent properties, such as the ability to cleave other substrates involved in pathogenicity.

The serological analysis on the meningococcal IgA1 proteases revealed variations in epitopes involved in antibody-induced enzyme neutralization. The actual epitopes, with which these antibodies react, are yet unknown. Probably they involve the catalytic site and/or the substrate-recognition site of the protease. With the antisera used only five inhibition types were detected among the 130 protease-producing strains. Only a partial correlation between inhibition type and cleavage type was found (Table 1), in contrast to our previous findings for *H. influenzae* IgA1 proteases (8). Thus, only one of the antisera (VK3) exclusively inhibited type 1 proteases, whereas another antiserum (HF13) inhibited all meningococcal proteases, regardless of cleavage type. If IgA1 proteases prove to be an important virulence factor, the latter protease type would be an obvious candidate for vaccine use.

The variation at the protein level was also detected by RFLP analyses of the *iga* gene region. Among the three 6-base restriction enzymes used to examine all 133 strains, *Pst* I cleaved the *iga* gene at three sites (Fig. 1), in agreement with published physical maps of gonococcal and meningococcal *iga* genes (10, 14, 17). More than 20 distinct arrangements of *Pst* I recognition sites were detected in the *iga* gene region. The size variation of hybridizing *Pst* I fragments representing the 5' and 3' ends of the *iga* gene and flanking sequences (Fig. 1) suggests that the meningococcal *iga* gene is located in a highly variable part of the chromosome.

By comparison with these published maps, it can be deduced that the internal *Pst* I sites of the *iga* gene are all located in the helper domain (Fig. 1). Halter *et al.* (14) recently differentiated gonococcal *iga* genes into two types, H1 and H2, on location of the autoproteolytic sites and by size of the α and β proteins (Fig. 1). The H1 type has a characteristic *Pst* I site in the α domain region. Our observations on *Pst* I recognition sites indicate that all 133 meningococcal strains examined had *iga* gene helper domains

Table 2. Characteristics of seven selected ET clusters of *N. meningitidis* represented by 81 isolates

iga cleavage fragment size, kb or bases (b)													
ET cluster	ET*	n [†]	Serogroup [‡]	Sertotype [‡]	IgA1 protease type	B or E [§]	Pst I		Hae III				Inhibition type
							kb	b	kb	b	b	b	
A3	28–30	3	X	14	1	–	17	1200					b
	33, 37	4	E, Y(2), W	2c(2), 2c:–, 14	2	–	>20	1050					e
	34	1	NG	14	2	–	12	1050					e
	35	1	Y	14	1	–	4.1	1050					b
	36	1	E	14	1	–	>20	1050					b
A4	7	5	B(4), C	2b, 2b:–, 2b:P1.2 2b:P1.Ham, 2b:p1.15	1	–	17	1000					b
	7	3	B	2b:–, 2b:P1.1, 2b:P1.2	1	E	17	1000					b
	7–8	3	B	2b, 2b:P1.2(2)	1	–	>20	1000					b
	32	1	E	NT	1	–	17	1150					NC
	B2	1–3, 6	11	B(3), C(5), W(3)	2a(8), 2a:P1.2(2), NT	1	–	17	1050				
4		1	W	2a	1	E	17	1050					b
5		1	B	NT	1	–	>20	1050					b
E1	58–63, 67	7	A	21:–(4), NT, 21:P1.3, NC	1	–	4.1	1200					b
	64	1	A	21:–	1	E	4.1	1200					a
E2	65	10	A(9), NG	21P1.9(8), NT(2)	1	–	4.1	1200	3.7	1300	740	560	a
	66	1	A	NT	1	–	4.1	1150	3.7	1300	740	560	a
E3	11	1	X	NT	1	–	12	1150					a
	47	1	E	4:P1.15	2	–	>20	1200					d
	48	3	NG	NT:–	2	–	>20	1150					d
	49–53	5	NG(4), E	NT:–(2), NT:P1.2(2), NT:P1.16	1	–	>20	1200					a
I1	71	1	B	15:P1.16	1	–	>20	1000	6.1			740	c
	71	6	B(5), C	15P1.16(3), 15:–, 15:P1.3, NT:–	1	–	>20	1200	3.7		1300	740	c
	71	1	B	15:P1.16	–	–	>20	1200	3.7		1300	740	c
	71	1	B	NT:P1.15	1	–	>20	1200	3.7	1300	1100	740	c
	71, 76	1	B	15:–, 15:P1.16	1	–	>20	1200	3.7	3400	2600	740	c
	71	1	B	15:P1.16	–	–	>20	1200		2100	1300	740	c
	72–75	4	B(3), C	15:P1.16(2), NT:–, NT:P1.15	1	–	>20	1200					c
	76	1	B	15:P1.2	2	B	>20	1200		2500	1300	740	d

Detailed information about individual strains is available on request. NC, not classified; NT, nontypeable; NG, nongroupable.

*ET and cluster are based on multilocus enzyme electrophoresis analyses (11).

[†]n, number of isolates.

[‡]Number in parentheses indicate number of strains with the same serogroup, serotype, or outer membrane protein pattern.

[§]B, internal *Bam*HI site; E, internal *Eco*RI site.

similar to the H1 type in gonococci, which is characterized by a small α protein. In contrast to observations for gonococci (10), the meningococcal *iga* gene helper domain existed in at least four different versions characterized by inserts or deletions of ≈ 50 –200 base sequences between two of the *Pst* I recognition sites in the helper domain (Fig. 1). Each type of helper domain was found in strains of widely different ETs and was not correlated with any particular protease cleavage or inhibition types. Furthermore, no correlation with any known phenotypic trait was observed.

Isolates of the same ET may show considerable polymorphism of the IgA1 protease genes. As an example, RFLP analyses of the 17 strains of ET cluster I1 [previously designated ET-5 complex (11)] showed at least six different *iga* gene types by *Hae* III digestion (Table 2). Five of them were found among 11 strains of a single ET (ET71). That two ET71 strains isolated in Norway during the B.15:P1.16 epidemic of the late 1970s and early 1980s differed in *iga* gene type indicates that changes in the *iga* gene occurred during the epidemic. However, this gene change was not reflected in any altered inhibition type of IgA1 protease. A similar high degree of polymorphism was shown among the 10 ET7 strains. Thus, three different *iga* gene region structures were detected with only two restriction enzymes (Table 2).

In agreement with previous findings for *H. influenzae* and *N. gonorrhoeae* (4, 5), this study demonstrates that the meningococcal *iga* gene is a single-copy gene. This finding excludes the possibility of changing the protease by intrage-

nomic recombination. Therefore, the observed high degree of polymorphism of the *iga* gene, even within single ETs combined with the lack of correlation between helper gene type, protease cleavage type, and inhibition type, indicate that frequent interstrain recombination processes occur *in vivo*. This conclusion is consistent with the mosaic-like structure of the *iga* gene seen in both gonococci (14) and *H. influenzae* (18). Interstrain gene transfer is likely to result from transformation via specific DNA uptake followed by homologous recombination between clones simultaneously colonizing the host. However, our data suggest that recombination occurs with considerably different frequency in different clones of meningococci. Thus, ET65, which is composed of group A strains, appeared entirely homogeneous with regard to their IgA1 protease, although the strains spanned 20 yr in isolation and varied widely in geographic origins. Among all 18 serogroup A strains examined (ET clusters E1 and E2) the only exceptional one by its IgA1 protease, was ET64, the reference strain for serotype A, which was isolated in 1937 (Table 2). These findings agree with the previous observations that differentiation of serogroup A strains by outer membrane protein and lipopolysaccharide composition is notoriously difficult due to limited diversity (19–21). The reason for the apparent genetic stability of some parts of the meningococcal population, and not others, is not known, but this variation may reflect differences in transformation competence.

It is tempting to speculate that transformation-mediated recombination in the meningococcal *iga* gene serves an

immune-escape purpose, as appears true for several other virulence factors in gonococci and meningococci (22). However, the surprisingly small number of IgA1 protease inhibition types detected seems to limit the immune-escape value of these variations, particularly as most meningococcal IgA1 proteases shared "inhibitory" epitopes (Table 1).

This study provides information on the worldwide variation among meningococcal IgA1 proteases and shows that clones known to cause epidemics produce almost exclusively the type 1 enzyme. The observations reveal strong evidence for frequent horizontal gene transfer involving the *iga* gene region in some parts of natural populations of meningococci.

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