CORRELATION OF CELL-ENVELOPE PHENOTYPES OF *NEISSERIA GONORRHOEAE* WITH SITE OF INFECTION AND SEROGROUP

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SUMMARY. The envelope phenotypes and coagglutination (CoA) W serogroups of 301 unselected clinical isolates of *Neisseria gonorrhoeae* were studied. Of the 287 isolates from infections acquired in the Edinburgh area, 252 (88%) were of the wild-type phenotype, 17 (6%) were of Env phenotype with increased permeability of the cell envelope, and 18 (6%) were of Mtr phenotype with reduced permeability of the cell envelope. Mtr strains were isolated significantly more often from homosexual men than from heterosexual men and women ($p < 0.001$). Of the isolates from homosexual men there were considerably fewer Mtr phenotypes among rectal isolates than among urethral and throat isolates. All isolates from homosexual men, 57% from heterosexual men and 52% from women were of CoA serogroup WII (including WII/WIII). Although the Mtr phenotype was strongly associated with serogroup-WII isolates from homosexual men, there was no such correlation between the Mtr phenotype and serogroup-WII isolates from heterosexual patients. It is suggested that hydrophobic compounds in the rectal environment are not the major factor in selecting serogroup-WII strains of Mtr phenotype associated with homosexually acquired infection. The hypothesis that the Mtr phenotype and serogroup WII are selected independently as a result of a more general selective pressure such as antibiotic usage, common to all infected sites, is discussed.

INTRODUCTION

Cell-envelope mutations of *Neisseria gonorrhoeae* alter the organism's permeability and hence its sensitivity to various hydrophobic agents, including antibiotics (Maness and Sparling, 1973; Maier *et al.*, 1974, 1975*a* and *b*).

Two separate mutations of this sort designated *env* (Maness and Sparling, 1973) and *mtr* (Maier *et al.*, 1974) influence the organism's sensitivity to these agents by altering both the amount of a minor protein (mol. wt 52 000) and peptidoglycan cross-

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linkage in the outer membrane (Guyman *et al.*, 1978). Thus an *env* mutant, which has increased membrane permeability, is markedly more sensitive (hypersensitive) and an *mtr* mutant, which has reduced permeability, is much more resistant than a representative wild-type strain. The *env* mutation suppresses the *mtr* locus if the two occur in the same strain (Sarubbi *et al.*, 1975). Gonococci with *mtr* mutations have been associated with homosexually-acquired infections (Morse *et al.*, 1982).

Gonococci can be divided into three serogroups—WI, WII and WIII—by coagglutination (CoA) serogrouping (Sandstrom and Danielsson, 1980). CoA depends on the principle that immunoglobulin G binds by its Fc portion to protein A on the surface of *Staphylococcus aureus* leaving the Fab site free to bind with the corresponding or related antigen to produce a coagglutination matrix easily visible to the naked eye. In gonococcal serogrouping, specific antibody raised against reference strains of gonococci (Johnston *et al.*, 1976) are selectively absorbed to produce coagglutination reagents which define the three major serogroups, WI, WII and WIII.

Strains of CoA serogroup WII have been associated with homosexually-acquired infection (Bygdeman, 1981*a*; Reid and Young, 1984) and reduced sensitivity to antibiotics (Bygdeman, 1981*b*). Strains isolated from homosexual men often have both the *mtr* and CoA serogroup WII characteristic (Morse *et al.*, 1982). Although both confer resistance to antibiotics they represent two separate loci (Morse *et al.*, 1982).

It has been suggested that the *mtr* mutation and CoA serogroup WII are selected by the rectal environment, which is rich in hydrophobic molecules. Accordingly, the present study was undertaken to increase our understanding of the role of different anatomical sites within the host in the selection of gonococcal strains with particular outer-membrane properties.

MATERIALS AND METHODS

Clinical Isolates. During the period July 1983 to May 1984, 301 isolates of *N. gonorrhoeae* were obtained from patients attending the Department of Genito-Urinary Medicine, The Royal Infirmary, Edinburgh. Of these strains, 14 were from patients who had acquired their infections either elsewhere in Britain or abroad. All isolates were identified as *N. gonorrhoeae* by the rapid carbohydrate utilisation test (Young *et al.*, 1976).

Reference Strains. *N. gonorrhoeae* strains with known genotypes were kindly supplied by Dr P. F. Sparling, University of North Carolina, Chapel Hill, USA. These were FA140 (*pen A2 mtr-2 pen B2*), FA102 (*pen A2*), FA19 (wild type), BR87 (*env-2 str-7 pen A2 mtr-2 pen B2*) and FA136 (*pen A2 mtr-2*). All strains were maintained on MNYC medium (Young, 1978) and envelope phenotypes were determined on the same medium lacking selective antibiotics (SMNYC medium).

Determination of cell-envelope phenotype. Each isolate was tested for sensitivity to the following hydrophobic compounds: erythromycin (Abbot Laboratories Ltd, Queenborough, Kent ME11 5EL), fusidic acid (Leo Laboratories Ltd, Princes Risborough, Bucks), Triton X-100 (Fisons Scientific Apparatus, Loughborough, Leics) and crystal violet (Koch-Light Laboratories, Colnbrook, Berks).

A 19-point hand-held replicator (Mast Laboratories Ltd, Mast House, Serby Road, Bootle, Merseyside) was used to seed separate plates of SMNYC medium containing: erythromycin (doubling concentrations from 0.015 to 2.0 mg/L); crystal violet (doubling concentrations from 0.5 to 4.0 mg/L); fusidic acid (0.03, 0.25 and 0.5 mg/L); and Triton X-100 (0.06, 1.0 and 2.0 g/L). The inoculum was *c.* 10⁵ cfu of an overnight culture of each test strain.

Each batch of plates was set up in duplicate and included the reference strains FA140, BR87, FA19 and FA136. After incubation for 48 h at 37°C in air with CO₂ 10%, plates were examined and the MIC determined as the lowest concentration of each agent that prevented visible growth.

Strains with the following MICs, which were given by the Mtr reference strains FA140 and FA136, were designated the Mtr phenotype: erythromycin ≥ 0.5 mg/L, fusidic acid ≥ 0.5 mg/L, crystal violet ≥ 2.0 mg/L and Triton X-100 ≥ 2.0 g/L. Strains with the following MICs, which were given by the Env reference strain BR87, were designated the Env phenotype: erythromycin ≤ 0.06 mg/L, fusidic acid ≤ 0.03 mg/L, crystal violet ≤ 0.5 mg/L and Triton X-100 ≤ 0.06 g/L. The wild-type reference strain FA19 had the following MICs: erythromycin 0.12 mg/L, fusidic acid 0.25 mg/L, crystal violet 1.0 mg/L, and Triton X-100 1.0 g/L. Test strains with erythromycin MICs of 0.12–0.25 mg/L were designated wild type.

Serogrouping by coagglutination. Coagglutination reagents defining CoA serogroups WI, WII, WIII were prepared and isolates serogrouped as described by Reid and Young (1984).

Statistical analysis. Significance was tested by the χ^2 method with Yates's correction.

RESULTS

Table I shows the distribution of cell-envelope phenotypes among isolates from infections acquired in the Edinburgh area by homosexual contact. There is a highly significant difference in the proportion of wild-type to Env and Mtr phenotypes when gonococcal isolates from 20 homosexual men are compared with those from 116 heterosexual men ($\chi^2 = 23.96$; $p < 0.001$) and 151 women ($\chi^2 = 25.89$; $p < 0.001$). There is no such significant difference when isolates from heterosexual men and women are compared ($\chi^2 = 0.01$; $p = 0.9$). The envelope phenotypes of fourteen isolates from infections acquired outwith the Edinburgh area are given in table II. Seven (50%) of these 14 isolates were of the Mtr phenotype. Of the five isolates linked with Thailand, four were from heterosexual men whereas the two Mtr strains linked with London were from homosexual men.

Table III shows the distribution of envelope phenotype in relation to site of isolate and homosexual or heterosexual acquisition. The proportion of Mtr-phenotype strains among rectal isolates from homosexual men is not significantly different from the proportion of Mtr strains among rectal isolates from women ($\chi^2 = 0.04$; $p > 0.8$). However, a significantly higher proportion of urethral isolates from homosexual men had the Mtr phenotype when compared with genital isolates from heterosexual men and women ($\chi^2 = 21.3$; $p < 0.001$). Likewise, a significantly higher proportion of throat isolates from homosexual men had an Mtr phenotype when compared with throat isolates from heterosexual men and women ($\chi^2 = 10.8$; $p = 0.001$). With the isolates

TABLE I
Distribution of cell-envelope phenotypes among isolates from homosexual and heterosexual patients

Patient Group	Number (and percentage) of isolates from each patient group with the following envelope phenotype			
	Env	Wild type	Mtr	Total
Homosexual men	0(0)	12(60)	8(40)	20
Heterosexual men	9(8)	103(89)	4(3)	116
Women	8(5)	137(91)	6(4)	151
Total	17(5.9)	252(87.8)	18(6.3)	287

TABLE II

Envelope phenotype and serogroup of 14 isolates from male infections acquired outwith the Edinburgh area

Country or area infection acquired	Envelope phenotype	CoA serogroup	Patient group
Thailand	Mtr	WII	Homosexual
Thailand	Mtr	WII	Heterosexual
Thailand	Mtr	WII	Heterosexual
Thailand	Mtr	WII	Heterosexual
Thailand	Mtr	WII/III	Heterosexual
Kenya	Wild type	WII	Heterosexual
Spain	Wild type	WI	Homosexual
Germany	Wild type	WII	Homosexual
London	Mtr	WII	Homosexual
London	Mtr	WII	Homosexual
London	Wild type	WII	Heterosexual
England	Wild type	WII	Heterosexual
Northern Ireland	Wild type	WII	Heterosexual
Inverness	Wild type	WII	Heterosexual

TABLE III

Cell-envelope phenotype in relation to patient group and site of isolation

Patient group	Site of isolation	Number (and percentage) of isolates with the following envelope phenotype			
		Env	Wild type	Mtr	Total
Homosexual Men	U	0(0)	3(50)	3(50)	6
	R	0(0)	8(89)	1(11)	9
	T	0(0)	1(20)	4(80)	5
Heterosexual Men	U	9(9)	90(87)	4(4)	103
	T	0(0)	13(100)	0(0)	13
Women	G	4(4)	98(93)	3(3)	105
	R	2(7)	25(89)	1(4)	28
	T	2(11)	14(78)	2(11)	18
Total		17(5.9)	252(87.8)	18(6.3)	287

U = urethra; G = female genital; R = rectum; T = throat.

from homosexual men there were considerably more Mtr phenotypes among urethral and throat isolates than among rectal isolates ($\chi^2 = 3.71$; $0.1 > p > 0.05$).

Table IV shows the distribution of envelope phenotype in relation to CoA serogroup and patient group. All isolates from homosexual men, 57% from heterosexual men and 52% from women were of serogroup WII (including WII/III). With regard to envelope phenotype, 13 (72.2%) of the 18 Mtr strains and 151 (56.1%) of the 269 non-Mtr strains were serogroup WII; this difference is not significant at the 5% level ($\chi^2 = 1.2$; $p > 0.2$). However, the Mtr phenotype occurred significantly more often among WII isolates from homosexual men than among WII isolates from heterosexual men ($\chi^2 = 14.3$; $p < 0.001$) and women ($\chi^2 = 20.4$; $p < 0.001$). In heterosexual men and women there was no significant difference in the distribution of Mtr

TABLE IV
Relationship of cell-envelope phenotype with patient group and CoA *W* serogroup

Patient group	CoA serogroup	Number (and percentage) of isolates with the following envelope phenotype			
		Env	Wild type	Mtr	Total
Homosexual men	WI	0(0)	0(0)	0(0)	0
	WII or WII/III	0(0)	12(60)	8(40)	20
Heterosexual men	WI	6(12)	43(86)	1(2)	50
	WII or WII/III	3(4.5)	60(91)	3(4.5)	66
Women	WI	4(5.5)	65(89)	4(5.5)	73
	WII or WII/III	4(5)	72(92)	2(3)	78
Total		17(5.9)	252(87.8)	18(6.3)	287

* A total of 17 strains serogrouped as WII/III: 3 from homosexual men; 5 from heterosexual men and 9 from women.

strains ($\chi^2 = 0.005$; $p > 0.9$) or Env strains ($\chi^2 = 0.7$; $p > 0.3$) between serogroups WI and WII (including WII/III).

DISCUSSION

Of 287 unselected gonococcal strains isolated from infections acquired in the Edinburgh area 252 (87.8%) had a wild-type phenotype, 17 (5.9%) had a Env phenotype and 18 (6.3%) had Mtr phenotypes. Morse *et al.*, (1982) found 128 (82.9%) wild type strains, 7 (4.6%) Env strains and 19 (12.5%) Mtr strains among 152 isolates collected during a 5-year period. Although the genotypes of our isolates were not confirmed by transformation experiments, the proportions of Env and wild-type phenotypes are similar in the two studies. In spite of a significantly lower proportion of Mtr strains in our series ($p < 0.05$) we confirm the findings of Morse *et al.* (1982) that Mtr strains are significantly more prevalent among isolates from homosexual men than among those from heterosexual men and women ($p < 0.001$). However, our findings cast doubt on the hypothesis (Morse *et al.*, 1982) that the rectal environment, essential for the transmission of gonococci within the homosexual population, exerts a selective pressure favouring the emergence of the Mtr phenotype. We found Mtr strains considerably more often among urethral and throat isolates from homosexual men than among rectal isolates; and Mtr strains were no more common among male rectal isolates than female rectal isolates ($p > 0.8$).

The lack of association between the Mtr phenotype and male rectal isolates was surprising. Eisenstein and Sparling (1978) reported that the *mtr* mutation consistently resulted in reduced rates of exponential growth in enriched broth cultures, whereas introduction (by transformation) of an *env* mutation resulted in partial restoration to normal growth rates *in vitro*. Therefore, *mtr* mutants may be less able to establish rectal infection as their poorer growth characteristics could make them less capable of competing with the complex microbial flora of the rectum.

In homosexual men, serogroup WII predominates regardless of anatomical site of isolation and has been shown to account for 94%, 90% and 81% of rectal, urethral and throat isolates respectively (Reid and Young, 1984). Although the Mtr phenotype has been reported to be strongly correlated with CoA serogroup WII (Morse *et al.*, 1982), our data (table IV) show that this applies only to WII strains isolated from homosexual men; strains isolated from heterosexuals show no correlation between Mtr and CoA serogroup WII. It would appear, therefore, that CoA serogroup WII and the Mtr phenotype are selected independently in the homosexual population rather than by a close genetic linkage.

Our results suggest that the rectal environment is not responsible for selection of Mtr phenotypes. The report of an Mtr-independent system (McFarland *et al.*, 1983) for resistance to faecal lipids also casts doubt upon the role of hydrophobic compounds in the rectal environment in selecting gonococcal strains with reduced membrane permeability. We consider it likely that the rectal environment is not responsible for selecting serogroup WII but that a more general selective pressure such as exposure to antibiotics common to all infected sites is the driving force for CoA WII and Mtr selection in isolates from homosexual men. The following associations between serogroups and antibiotic sensitivity observed in other geographical areas support our hypothesis.

Gonococcal strains with decreased susceptibility or so-called multi-resistant strains belong to serogroups WII and WIII whereas those highly sensitive to β -lactam antibiotics, tetracyclines and other antibiotics belong to serogroup WI (Bygdeman, 1981*b*). Bygdeman *et al.* (1982) confirmed a genetic linkage between serogroup specificity and antibiotic multi-resistance. Although the resistance pattern of the WII transformants studied was very similar to that resulting from the *mtr* locus, there was no increase in the 52 000-mol. wt protein associated with the *mtr* mutation. Further transformation experiments are required to determine the precise nature of the genetic locus.

Bygdeman *et al.* (1983) considered that greater antibiotic pressure in larger towns than in smaller ones might account for their observed higher incidence of WII strains in larger towns. Isolates from infections acquired in the Far East, where antibiotic pressure is very high because of the lack of antibiotic control, exhibit reduced sensitivity to a wide variety of antibiotics and are predominantly of serogroups WII and WIII (Bygdeman, 1981*b*). Our serogrouping of a limited number of strains supports these findings and demonstrates a high incidence of the Mtr phenotype among heterosexual men (table II).

The similarity in the serogroup and envelope phenotype of isolates from homosexual men and those from the Far East suggests that the same selective pressure may be responsible. Homosexual men experience a wider spectrum and a higher rate of sexually transmitted diseases than heterosexual men (Fluker, 1976; Willcox, 1981). Although difficult to prove, it is likely that overall antibiotic usage is greater among homosexual men than among heterosexuals. Evidence for antibiotic selection independent of serogroup WII was reported by Bygdeman (1981*a*) who found that serogroup-WI isolates from homosexual men were more resistant to penicillin than were unselected WI isolates.

It is not unexpected that such differences which most likely result from antibiotic pressure should also occur within serogroup WI because the different protein antigens

representing serogroups WI, WII and WIII act as "porins", enabling selective passage of molecules across the cell wall (Douglas *et al.*, 1981). However, the proteins IA (representing serogroup WI) and IB (representing serogroups WII and WIII) (Sandstrom *et al.*, 1982) have different structures and orientations in the outer membrane (Barrera and Swanson, 1984) and differ significantly in apparent pore size (Blake, personal communication cited by Barrera and Swanson, 1984). These differences may account for the preferential selection, in response to antibiotic pressure, of protein-IB antigens among isolates from homosexual men.

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