Acquisition of new genes by oral *Neisseria*

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SUMMARY It is suggested that part of the increased pharyngeal carriage of meningococci reported in patients with gonorrhoea is due to misidentification of gonococci which have been transformed to maltose fermenters by DNA from normal throat flora. The distribution of specific aminopeptidases in strains of gonococci, meningococci isolated from the throat and meningococci from systemic infections is consistent with this view. Gonococci oxidising maltose and gonococci with γ -L-glutamyl aminopeptidase activity, both factors regarded as typical of *Neisseria meningitidis*, can be produced in vitro by transformation with DNA from *N lactamica* and *N meningitidis*. The clinical and theoretical implications of such changes are discussed.

The unduly high frequency of meningococcal carriage in patients with gonorrhoea noticed by Willcox *et al*¹ was confirmed by Rufli² and Young *et al*,³ but not by Noble *et al*.⁴ Differences in reported results may be related to the type of population studied. Most of the patients studied in the sexually transmitted disease (STD) clinic at St Mary's were male homosexuals with a recent history of oral sexual intercourse. Turgeon *et al*⁵ reported meningococcal carriage in 41% of homosexual men compared with 17% in heterosexual men and 11% in women. Two explanations given for these findings are, promiscuity favouring the spread of both meningococci and gonococci and undue susceptibility of some individuals to both these species of *Neisseria*. Evidence for a third possibility is given below.

In most clinical laboratories the definitive distinction between *Neisseria gonorrhoeae* and *Neisseria meningitidis* is the ability of the latter to oxidise maltose as well as glucose. It is conceivable that DNA from normal mouth flora may transform some strains of *N gonorrhoeae* so that they acquire the ability to oxidise maltose and are subsequently misidentified as *N meningitidis*.^{6,7}

Neisseria gonorrhoeae and *N meningitidis* also differ in their aminopeptidase (AP) profiles,^{8,9} in asparaginase¹⁰ and glutaminase activity and in sensitivity to oleic acid.¹¹ These criteria have been used to examine the relation of throat meningococci to systemic meningococci and gonococci. Lastly, the hypothesis has been tested by transformation experiments in vitro using DNA from *Neisseria* spp found

in the throat with a proline auxotrophic strain of *N gonorrhoeae* as the recipient.

Material and methods

STRAINS OF BACTERIA

A bank of strains was established as described below. Not all the tests were done on all strains as some died out during the period of the experiments.

Neisseria gonorrhoeae

Fifty-two strains were all isolated in the diagnostic laboratory from the urethra, rectum, or cervix of patients attending the sexually transmitted diseases (STD) clinic.

Neisseria meningitidis

Fifty strains, all isolated from the blood or cerebrospinal fluid of patients with systemic infections, were provided by Dr AG Taylor (Central Public Health Laboratory, Colindale). A further 120 strains were isolated from the throats of male patients recently attending the STD clinic with a history of oral sexual intercourse.

CULTURE AND IDENTIFICATION

Strains were initially isolated on GC agar base + 1% Isovitalex made selective by the addition of vancomycin, colistin, trimethoprim and nystatin,¹² and incubated at 36°C in 7% CO₂. All further subcultures were made on the same medium without antibiotics and were all oxidase positive, Gram-negative cocci. Strains were identified as *N gonorrhoeae* if they produced acid from the oxidation of glucose only. Strains

were identified as *N meningitidis* if they produced acid from glucose and maltose, but not from sucrose and lactose. After identification each isolate was suspended in 10% glycerol and stored in ampoules in liquid nitrogen.¹³

QUANTITATIVE AMINOPEPTIDASE ASSAY

Each strain was grown overnight on GC agar + 1% Isovitalex and a heavy suspension made in saline. The absorbance was adjusted to 1.0 ($\lambda = 540\text{nm}$) for the glutamyl aminopeptidase assay and to 5.0 for the specific hydroxyproline aminopeptidase assay. The protein concentration of the adjusted suspensions were estimated by the method of Lowry *et al.*¹⁴

Assays were done in duplicate in flat-bottomed microtitre plates (Dynatech). Suspension (50 μl) was tested with specific substrate (50 μl) and a buffer control. After three hours incubation in moist conditions at 37°C, the colour was developed by the addition of 50 μl each of 0.08% sodium nitrite, 0.4 N hydrochloric acid, 2% ammonium sulphamate and 1.6% naphthylethylene diamine (in methanol) in that order and the absorbance measured immediately. Two specific substrates were used, 1 mmol L- γ -glutamyl-4-nitroanilide (BDH) in 0.2 M TRIS pH 7.6, and L-4-hydroxyprolyl- β -naphthylamide (Koch-Light) 0.2 M TRIS pH 7.2.

The absorbance was read at 550 nm using a Titer-tek Multiskan (Flow Labs), and compared to the appropriate standard curve using either *p*-nitroaniline (BDH), or β -naphthylamine (LAP calibration solution, Sigma) 0.250 nmol/ml. The specific enzyme activity was expressed per mg protein of the original suspension.

ESTIMATION OF ASPARAGINASE AND GLUTAMINASE

Bacterial suspensions were prepared as above. The substrates used were 50 μl of 5 mmol L-asparagine or 5 mmol L-glutamine in 0.05 M borate buffer pH 8.0. After two hours incubation at 37°C, the ammonia released was measured using Nessler's reagent.¹⁵ The enzyme activity was expressed as mmol NH_3 released per mg protein; ammonium chloride, 0.30 mmol was used for the standard curve.

SENSITIVITY TO OLEIC ACID

Agar plates were prepared containing GC base agar + 1% Isovitalex with the addition of Dubos broth base with and without 0.02% (wt/vol) oleic acid (Gibco Diagnostics). Bacterial suspensions were adjusted to absorbance 1.0 ($\lambda = 540\text{ nm}$) and also diluted 1/100. They were inoculated on to both media using a multipoint inoculator (Denley). After incubation at 36°C for 48 h in 7% CO_2 , the presence or absence of growth was assessed.

TRANSFORMATION

DNA isolated from strains of *N meningitidis* and *N lactamica* (NCTC 10617) was used in transformation experiments using the rapid method described by Janik *et al.*¹⁶ The recipient was a strain of *N gonorrhoeae* (colony type 1) which requires proline for growth. Pro⁺ transformants were detected on chemically defined media.¹⁷ Pro⁺ colonies were transferred to maltose-containing plates¹⁸ using the replica technique with velvet pads. Some transformants were tested for aminopeptidase activity by the quantitative method described earlier.

Results

AMINOPEPTIDASE ACTIVITY

Specific γ -L-glutamyl and L-hydroxyproline aminopeptidase activity was measured quantitatively in all strains. The significance of differences between means was estimated by student's *t* test.

The mean specific γ -L-glutamyl AP activity of the *N gonorrhoeae* strains is one tenth that of both groups of *N meningitidis* ($p < 0.001$). Meningococci isolated from the throat have significantly less activity than systemic meningococci ($p < 0.001$).

The mean specific L-hydroxyproline AP is significantly higher in *N gonorrhoeae*, tenfold that of the meningococci taken as a whole ($p < 0.001$). Within the meningococci activity was significantly higher in the throat than in the systemic strains ($p < 0.02$).

The relation of the two specific aminopeptidases in individual strains can be seen in the Figure (a, b, c). The difference between *N gonorrhoeae* and systemic meningococci is clear. The throat meningococci are a more heterogeneous group. They have a lower mean glutamyl and higher mean hydroxyproline AP activity than the systemic strains. If the throat strains are divided into those with and without hydroxyproline AP activity, the mean glutamyl AP activity is lower in those with hydroxyproline AP, but the difference is not statistically significant.

ASPARAGINASE AND GLUTAMINASE

The mean values for each group of strains tested are shown in Table 1. *Neisseria gonorrhoeae* differ significantly from *N meningitidis*, having one-third of the asparaginase and one tenth of the glutaminase activity. However, the two groups of meningococci do not differ significantly from each other.

OLEIC ACID SENSITIVITY

Neisseria gonorrhoeae are significantly more sensitive to oleic acid than *N meningitidis* (Table 2). Although throat meningococci appear more sensitive than systemic strains, the difference is not statistically significant.

Table 1 *Asparaginase and glutaminase activity in Neisseria gonorrhoeae and N meningitidis*

	No tested	mmol NH ₄ released/mg protein		
		Substrate		
		<i>L</i> -asparagine Mean (SE)	<i>L</i> -glutamine Mean (SE)	Asp:glut ratio
*† <i>N gonorrhoeae</i>	52	1.99 (0.2)	0.29 (0.04)	6.9
*‡ <i>N meningitidis</i> (throat)	80	6.29 (0.22)	2.33 (0.12)	2.7
†‡ <i>N meningitidis</i> (blood/CSF)	42	5.52 (0.23)	2.09 (0.14)	2.6

*†*p* < 0.01 (Student's *t* test).‡NS (Student's *t* test).

TRANSFORMATION

When DNA isolated from various strains of *N meningitidis* and *N lactamica* was used, the pro⁺ gene was transferred to the proline-requiring recipient in

approximately 1 in every 100 colony forming units (CFU) tested. Of the pro⁺ transformants made with meningococcal DNA and tested for glutamyl AP activity (Table 3), 13 (4%) had gained the ability to

make this enzyme with activities at levels equivalent to that of the donor. The ability to oxidise maltose was not detected in these transformants. This is partly a reflection of the small number tested, but it may be that the maltose and proline genes are far apart. When *N lactamica* was used as the donor, the ability to oxidise maltose was found in 20 (0.2%) of pro⁺ transformants tested. They had all retained the ability to oxidise glucose, and had not acquired the ability to oxidise lactose. The pro⁺ gene has also been transferred to *N gonorrhoeae* using DNA from *N pharyngis* and *E coli*.

Table 2 Sensitivity of *N gonorrhoeae* and *N meningitidis* to oleic acid

	No tested	% inhibited at	
		Abs 1.0	Abs 0.01
† <i>N gonorrhoeae</i>	25	80	96
*† <i>N meningitidis</i> (throat)	96	9.3	20.8
*† <i>N meningitidis</i> (blood/CSF)	46	6.5	17.4

Oleic acid was used at 0.02% wt/vol. Bacteria were plated at absorption = 1.0 and 0.01.

*NS (χ^2 test).

†p < 0.01 (χ^2 test).

Table 3 Phenotypic changes in transformed *N gonorrhoeae*

Donor DNA	No pro ⁺ transformants tested	No positive for	
		γ -glutamyl AP activity	Maltose fermentation
<i>N meningitidis</i>	331	13	0
<i>N lactamica</i>	8 340	NT	20

Discussion

The aminopeptidase results confirm the clear distinction between *N gonorrhoeae* and *N meningitidis* described previously;^{8,9} *N gonorrhoeae* showing hydroxyproline AP activity only and meningococci showing glutamyl AP activity only. However, the throat meningococci can be divided into three populations, 66% of strains giving the typical meningococcal pattern, 30% which also had some hydroxyproline AP, and lastly three strains with no glutamyl AP but hydroxyproline AP at levels similar to those of gonococci.

The group showing both enzyme activities would be consistent with gonococci that have gained DNA derived from normal mouth flora and consequently gained glutamyl AP and the ability to oxidise maltose, while retaining hydroxyproline AP. However, particularly in view of their relatively low hydroxyproline AP activity, they could be meningococci which had gained some DNA from gonococci. The

possibility that hydroxyproline AP may be repressed when glutamyl AP is acquired, was not confirmed by quantitative estimations on the transformants.

The three strains with no glutamyl AP could be gonococci which had gained a maltose-oxidising enzyme. The possibility that they are meningococci which have gained hydroxyproline AP and lost glutamyl AP seems less likely. Moreover, there is no evidence to suggest that throat rather than systemic strains should lose DNA, whereas gonococci are known to be highly competent to accept DNA.⁷ Changes in phenotype are probably not limited to the markers we have used and this could explain differences in auxotype between strains of gonococci isolated from different sites on the same patient.¹⁹

Whatever the nature and frequency of maltose-positive *Neisseria* in throats of STD patients, maltose oxidation alone appears to be an inadequate method of identification of meningococci. Previously some 80% of sulphadiazine-resistant meningococci were found to be maltose-negative.²⁰ Reports of the greater sensitivity of gonococci to oleic acid¹¹ need to be interpreted with caution as the method is difficult to quantify and some strains are anomalous. A combination of tests such as aminopeptidases and carbohydrate fermentation may well be required to give the best differentiation.

DNA hybridisation experiments have shown that meningococci and gonococci are homologous for about 80% of their genome.²¹ It is therefore not surprising that they have so many properties in common. Recent experience of plasmid spread within and between species has led to a renewed appreciation of bacterial variability. We should give more attention to the possibilities of chromosomal gene exchange. These results give preliminary evidence for such exchange between *N gonorrhoeae* and normal mouth flora, though it probably occurs in only a small number of strains. Nevertheless gene exchange may be another factor besides increased acquisition and undue susceptibility contributing to the increased prevalence of meningococcal carriage by STD patients. Further investigations on DNA hybridisation and on the presence of plasmids are in progress.

There are frequent references in published reports to the isolation of either meningococci or gonococci from sites and under circumstances regarded as typical of the other—for example, gonococcal meningitis and meningococcal urethritis²²⁻²⁴ and these would support the theory of genetic interchange leading to misdiagnosis. There does, however, exist the possibility that meningococci and gonococci belong to one species adapting to different environments.

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