THE SYSTEMATIC SEROLOGY OF *NEISSERIA GONORRHOEAE*: ANTIGENS ASSOCIATED WITH PATHOGENESIS IN *NEISSERIA* SPP. FROM MAN

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SUMMARY. Sonicates of eight *Neisseria* species from man were analysed in a micro-Ouchterlony double-diffusion absorption assay in comparison with a gonococcal reference antiserum-antigen system. Five major gonococcal precipitin zones were identified which comprised genus-, species- and type-specific components. One antigen was found in all strains of three species with pathogenic capability—*N. gonorrhoeae*, *N. meningitidis* and *N. flavescens*. It was not detected in *N. lactamica*, *N. pharyngis*, *N. elongata*, *N. cinerea* or *N. catarrhalis*.

INTRODUCTION

In gonococcal research, the quest for specific antigens for use in serodiagnosis, prophylaxis and serotyping has a long history. The trial and error approach to these problems has failed, yet there have been relatively few efforts to obtain systematic serological information about the genus *Neisseria*. Early work was compromised by the instability of the gonococcus in culture. Intracellular genus-specific antigens (Danielsson, 1965*a*), both surface (Johnston, Holmes and Gotschlich, 1976) and internal (Geizer, 1975) type-specific antigens and also surface-located subgeneric antigens (Johnston *et al.*, 1976) have been described. Since about 1970 there have been great improvements in methods for the culture of stable gonococci of known infectivity (Kellogg *et al.*, 1968) which may be considered antigenically "complete". Taking advantage of these techniques we undertook a systematic investigation of the *Neisseria* spp. from man, with a view to generating basic serological information about the gonococcus.

MATERIALS AND METHODS

Bacterial strains. The organisms used in this study were: (a) 11 representative gonococcal strains from local and international sources; local strains were selected on the basis of their susceptibility to nine antibiotics (Gupta *et al.*, 1982) because this is known to correlate with other epidemiological markers (Bygdeman, 1981); (b) NCTC reference strains of four serogroups of *N. meningitidis*; (c) six NCTC strains of human commensal *Neisseria* (National Collection of Type Cultures, 175 Colindale Avenue, London NW9 5HT).

Identity of strains was confirmed by standard bacteriological methods including sugar

utilisation tests (Young, Peterson and McDonald, 1976), polysaccharide production from sucrose (Catlin, 1970) and immunofluorescence assay with FA *N. gonorrhoeae* antiserum (Difco). The cultures were grown routinely in GC Medium agar base (Difco) with defined supplement (Kellogg *et al.*, 1963) for 20 h in a humid candle jar at 36°C. Colony morphology was typed and cultures were lyophilised by methods described previously (Saikh and Bhattacharyya, 1983).

Antigens and antisera. Cells were harvested in cold 50 mM Tris-HCl buffer pH 7.5 to a turbidity equivalent of 2000 Klett units (540 nm filter). Antigens for immunodiffusion were cells which had been lysed in the MSE 100 Ultrasonic Disintegrator for a total of 10 min in ice at amplitude 7 and a frequency of 20 Kcycles/s. Sonicates were preserved in divided portions at -18°C. Cell suspensions fixed with formalin 0.5% v/v and diluted 1 in 10 in phosphate-buffered saline pH 7.0, were used to raise antisera in rabbits. A total volume of 8 ml of suspension was administered in eight graded, bi-weekly injections, initially by the intramuscular route and subsequently by the intravenous route. Preimmune serum was obtained from each animal.

Immunodiffusion (ID). Gels of 2 mm thickness were set and cut on microscope slides using the Gelman ID kit with 3 mm cutters. The gel was Oxoid Agar No. 1, 1% w/v; barbital buffer pH 7.4, ionic strength 0.15; polyethylene glycol 6000, 1.5% w/v; sodium azide 0.2% w/v. The wells were filled with reactants and the slides incubated at 36°C for 48 h in humidity chambers before recording the reactions. After development, the slides were washed, dried and stained with amido black (Crowle, 1973).

The antigen and antiserum preparations were tested by direct comparison and also by absorption analysis. Absorption of antisera with intracellular as well as cell-surface components was done by prediffusion of absorbing sonicate in the antiserum well for 2 h before adding the reactants. A double application of the absorbing antigen was done if absorption was found incomplete. Absorption of antibody to only the cell-surface antigens was done with formalin-fixed cells: 1 g wet weight of cells was mixed with 1 ml of antiserum for 5 h at 4°C (Danielsson, 1965b). The serum was then clarified by centrifugation.

RESULTS

Rabbit response to immunisation

Individual sera from ten rabbits were tested before immunisation and were found to be unreactive in ID with gonococcal sonicates. Considerable variation was observed

FIG. 1.—Variation in rabbit response to immunisation with gonococcal whole-cell antigen demonstrated by immunodiffusion analysis: R₁, R₂, R₃, R₄, R₅=antisera to gonococcal strain N133 from five different rabbits; RS=N133 pooled reference antiserum; RA=N133 reference antigen sonicate; 1,2,3,4 and 5=designation of precipitin bands in reference pattern.

in the rabbit antibody response to formalin-fixed gonococcal cells with the four gonococcal strains investigated. Five rabbits were used for antiserum preparation against the gonococcal reference strain N133. Their antisera showed two kinds of response when examined by double-diffusion analysis with the N133 reference sonicate antigen (RA, fig. 1). Precipitin lines which were prominent with some sera were absent in others; e.g., antisera from rabbits R1, R2 and R4 gave precipitin reactions with only two of the five antigen complexes detected by sera from rabbits R3 and R5. This response was mainly to the type-specific complex 2. Because of this variability a N133 reference antiserum (RS) was prepared by pooling sera to gain maximum resolution of precipitin bands. These bands were designated 1 to 5 on the basis of their proximity to the antiserum well. Band position, particularly of component 2 was subject to some variation because of slide variables such as thinness of the agar at the slide edge (compare fig. 3 and fig. 5).

Analysis of gonococcal sonicates

Gonococcal sonicates gave reproducible precipitin patterns in ID irrespective of colony type and preparation batch. Although pure virulent-colony-type preparations (T1 or T2) cannot be obtained because of the high degree (c. 20%) of segregation on subculture, T1- and T2-enriched sonicates were compared with sonicates of pure avirulent cultures (T3 and T4). No qualitative differences in band patterns were detected. Where possible, mixed sonicates of all four colony types were used; they were obtained by plating each type separately and harvesting them in equal proportions. Avirulent type 5- segregants (Jephcott and Reyn, 1971) and opacity variants (Swanson, 1978) were not examined.

All the precipitin bands except for band 3, were found to consist of multiple components in some reaction conditions, e.g., in reactions with other sera and in absorption analysis (see below). The precipitin bands are, therefore, referred to as zones. When sonicates from several gonococcal isolates were compared with the N133 reference system, some quantitative and qualitative differences were evident (fig. 2),

FIG. 2.—Reactivity of gonococcal sonicates with reference anti-serum N133: Outer wells = antigen sonicates of Calcutta isolates N123, N125 and N131 and international strains F62, GC9, WHO VII and 5731.

FIG. 3.—Type-specific gonococcal antigens shown by pre-diffusion absorption with gonococcal sonicate: N131, N123, F62 = gonococcal antigen sonicates; RSg = reference antiserum absorbed by prediffusion of N131 sonicate; 5' = strain specific component of band 5.

e.g., N133 (RA) was not a good producer of zone 5. The distribution of the five zones among gonococci is compiled in table I. Zones 1, 3, and 4 showed complete identity in all gonococci (fig. 2). The prominent zone 2 was strain-specific, and occurred irrespective of antibiotic-resistance type. The majority of the Calcutta strains contained this component whereas only one of five international strains did so (fig. 2; table I). These results were confirmed by intragel absorption analysis where the

TABLE I

Cross-reactivity of the N133 gonococcal reference antiserum with sonicates of 11 gonococcal strains

Test antigen			Precipitin bands formed with N133 reference antiserum				
			Inner zone (anti-serum well)				Outer zone (antigen well)
			1	2	3	4	
Strain	Source	Antibiotic resistance type* and other characteristics†					5
N123	Calcutta	Ery, Pc, Cl, Tc, Sm, Su, Cm; T1-T4	+	+	+	+	+
N125	Calcutta	Amp, Pc, Cl, Sm; T4	+	—	+	+	+
N131	Calcutta	All sensitive; T1-T4	+	+	+	+	+
N133	Calcutta	Ery, Pc, Cl, Tc, Sm; T1-T4	+	+	+	+	+
N179	Calcutta	Amp, Pc, Cl, Tc, Sm, Su; T4	+	+	+	+	+
N213	Calcutta	Ery, Pc, Cl, Amp, Tc, Sm, Su; T4	+	—	+	+	+
F62	USA	All sensitive; T1-T4; Pro	+	—	+	+	+
GC9	USA	Not done; T1-T4; Dark	+	—	+	+	+
H 8793	Britain	Ery, Pc, Cl, Amp, Tc, Sm, Su; T4; β	+	+	+	+	+
5731	Sweden	Pc, Amp, Cl, Sm; T4; β	+	—	+	+	+
WHO VII	Sweden	All sensitive; T4	+	—	+	+	+

* Resistance to erythromycin = Ery; penicillin = Pc; cloxacillin = Cl; ampicillin = Amp; tetracycline = Tc; streptomycin = Sm; sulphamethoxazole = Su; chloramphenicol = Cm.

† T1-T4 denotes the mixture of the four different morphological colony types in the sonicates. Pro = proline auxotroph; Dark = dark colony variant; β = presence of β -lactamase.

TABLE II

Cross-reactivity of N133 gonococcal reference antiserum with sonicates of other Neisseria spp.

Test antigen		Precipitin bands formed with N133 reference antiserum				
		Inner zone (antiserum well)				Outer zone (antigen well)
		1	2	3	4	5
<i>N. gonorrhoeae</i>	N133	+	+	+	+	+
<i>N. meningitidis</i> (Group A)	NCTC10025	+	—	+	+	+
<i>N. meningitidis</i> (Group B)	NCTC10026	+	—	+	+	+
<i>N. meningitidis</i> (Group C)	NCTC8554	+	—	+	+	+
<i>N. meningitidis</i> (Group D)	NCTC9714	+	—	+	+	+
<i>N. lactamica</i>	NCTC10617	+	—	+	—	—
<i>N. flavescens</i>	NCTC8263	+	—	+	+	—
<i>N. pharyngis</i>	NCTC4590	+	—	+	—	—
<i>N. elongata</i>	NCTC10660	+	—	+	—	—
<i>N. cinerea</i>	NCTC10294	+	—	+	—	—
<i>N. catarrhalis</i>	NCTC4103	—	—	—	—	—

gonococcal sonicate was allowed to diffuse from the antiserum well before the other reactants were added. Strain-specific components were also revealed in zone 5 (fig. 3).

Prediffusion of gonococcal sonicate N131 prevented the subsequent formation of precipitin bands with sera N131 and N123 but not with N133 (RA) and F62 with which residual reactions in zone 5 were obtained.

Absorption of the reference serum with intact cells of strain N133 removed precipitins of only zone 5, indicating that components of zones 1–4 were located internally in the cell of the reference strain.

*Cross-reactivity of other Neisseria spp. with the
N133 gonococcal reference system*

Of the 10 strains of *N. meningitidis* and commensal *Neisseria* spp. tested, various degrees of cross-reactivity with the N133 gonococcal reference system were seen. *N. catarrhalis* did not react. Zones 1 and 3 were present in all the other *Neisseria* spp, but zone 2 was missing (table II). The presence of zones 1 and 3 was confirmed by intragel absorption. For example, when the gonococcal reference serum (RS) was absorbed by prediffusion with a sonicate of *N. lactamica*, only zones 2, 4 and 5 of the reference system remained (fig. 4). No precipitin bands were seen with the *N. pharyngis* sonicate whereas zone 4 was formed with the *N. flavescens* sonicate. Zone 4 was also shared by all four serogroups of meningococci in addition to *N. flavescens* and it was absent from the other *Neisseria* species (table II). This finding was confirmed by the absorption test (figs 4 and 5). Cross-reactivity in zone 5 was restricted to meningococci and was only partial. These results were confirmed by absorption (fig. 5). In this experiment, prediffusion of *N. meningitidis* serogroup A sonicate prevented the formation of precipitin bands with all the meningococci, whereas residual activity in the gonococcus N133 (RA) was seen in zones 2 and 5. In zone 5, in addition to strain-specific activity, a minor late-developing component remained in all the gonococci tested.

The heterogeneous nature of zones 2 (fig. 5), 4 (fig. 4) and 5 (fig. 3 and 5) was evident in these slides.

FIG. 4.—Subgeneric group antigens of *Neisseria* spp. shown by prediffusion absorption of gonococcal reference antiserum with *N. lactamica*: F, P, L and RA=antigen sonicates of *N. flavescens*, *N. pharyngis*, *N. lactamica* and gonococcal reference strain; RSL=reference antiserum absorbed by prediffusion with sonicate of *N. lactamica*.

FIG. 5.—Specific gonococcal antigens shown by prediffusion absorption analysis of gonococcal reference antiserum with *N. meningitidis* serogroup A: B, D, A=antigen sonicates of *N. meningitidis* serogroup B, D and A respectively; RSM=reference antiserum absorbed by prediffusion of *N. meningitidis* serogroup A sonicate; 5'=gonococcus-specific component of band 5.

DISCUSSION

With the N133 gonococcal reference serum, it was possible to detect several fundamental inter-relationships between the gonococcus and other *Neisseria* spp. All the species could be grouped into several hierarchical categories which is in agreement with current views on their classification. *N. catarrhalis*, now classified in the genus *Branhamella*, was found not to be related to the gonococcus in this assay. Antigens 1 and 3 appeared to be intracellular and present in all the "true" *Neisseria* spp. tested; they were, therefore, group antigens at the generic level.

Major components of zone 5, which are located at the cell surface, were subgeneric, being common to the pathogens *N. gonorrhoeae* and *N. meningitidis*. Also of interest was subgeneric zone 4, the components of which were intracellular. Zone 4 was not

only a feature of *N. gonorrhoeae* and *N. meningitidis* but also *N. flavescens*. This NCTC strain had been the cause of an epidemic of meningitis (Branham, 1930). However, *N. flavescens* is rarely isolated in health or disease; the use of selective media, on which it does not grow, may be a reason for this. Furthermore, it is not easily distinguished from other asaccharolytic species, *N. cinerea* and *B. catarrhalis* (Knapp *et al.*, 1984), making interpretation of subsequent reports on the isolation of these species unreliable. The strain of *N. flavescens* used in this study, however, was known to have pathogenic capability. It was also investigated in immunoelectrophoresis by Høff and Høiby (1978) and was shown to have a meningococcal component, arc 55, in common with gonococci. Cannon and Black (1982) have also reported a neisserial component, which they associated with pathogenicity, by the use of monoclonal antibody. Pathogenesis in the neisseriae is associated with factors, e.g., the exoenzyme IgA protease, not found in the commensals (Mulks and Plaut, 1978). Zones 4 and 5 may represent similar components which play a role in pathogenesis.

In addition to generic and subgeneric antigens, gonococcal type-specific components were detected in zones 2 and 5. The geographical distribution of the internal cell components in zone 2 amongst gonococci from local and international sources was clear. This class of antigen may prove a useful addition to current serotyping systems (Johnston *et al.*, 1976; Danielsson and Sandstrom, 1979). In future studies, it will be important to identify responder rabbits for raising antisera because, in our series, only two of five animals produced high levels of antibody to the 2 complex, to the exclusion of several other precipitins. The distribution of type-specific elements of zone 5 was less frequent and could not be assessed for epidemiological significance in this limited study. This complex appeared to be similar to the type antigens of Johnston *et al.* (1976) in cell-surface location, diffusion characteristics and in possessing group and type specificities. A gonococcal species-specific component was also apparent in this zone on prolonged incubation. Further work with cell-wall preparations is necessary to resolve this potentially important antigen complex.

There have been few investigations of whole-cell antigens of the neisseriae, and only those of Danielsson (1965*a* and *b*) were comparable in scope to ours. Our study differed in the use of colony type-defined cell suspensions and in the method of absorption. Danielsson was unable to detect antigens which could be associated with pathogenesis or serotype. Two important reasons for this may have been failure of his absorption technique to remove precipitins to intracellular components, leading to obscurity of reaction patterns, and the over-riding factor of rabbit response to immunisation which was not recognised. The rabbit antibody response was highly variable and was the single most important determinant of antigen resolution in our analysis.

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