COLONY VARIANTS OF NEISSERIA MENINGITIDIS STRAIN 2996 (B:2b:P1.2): INFLUENCE OF CLASS-5 OUTER MEMBRANE PROTEINS AND LIPOPOLYSACCHARIDES

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SUMMARY. Different colonial morphologies were found among colonies of Neisseria meningitidis strain 2996 (B:2b:P1.2). Examination of cultures, selected on the basis of colony transparency or opacity, revealed that both lipopolysaccharides (LPS) and class-5 outer membrane proteins (OMP) are associated with differences in colonial morphology. Among 13 variants, four LPS variants and two class-5 OMP variants were recognised. All variants were non-fimbriate. The LPS variations were confirmed by immunoprecipitation. In addition to these qualitative variations of LPS, meningococci synthesise LPS of different molecular size depending upon growth phase; larger LPS molecules were found after analysis of stationary-phase cultures than with exponential-phase cultures. These changes did not cause a change in serotyping characteristics. The recognition in this study of intra-strain heterogeneity of meningococcal LPS and class-5 OMPs is important for the understanding of meningococcal pathogenicity. This heterogeneity was also detected in simultaneous isolates from different sites of a patient.

INTRODUCTION

Pathogenic bacteria grown in vitro on agar plates often show various colonial morphologies that are associated with changes in cell envelope components such as capsular polysaccharides, lipopolysaccharides (LPS), fimbriae, flagella and outer membrane proteins (OMP). Examples of these are flagellar phase variation in Salmonella, LPS variation in Salmonella (smooth-rough), fimbriae and OMP variation in Neisseria gonorrhoeae, capsular polysaccharide variation in Streptococcus pneumoniae (Austrian, 1953; Nikaido et al., 1964; Silverman et al., 1979; Swanson, 1982; Swanson and Barrera, 1983). Some of these changes only occur in vitro and are associated with a loss of virulence (e.g., pneumococcal capsular polysaccharide; Salmonella LPS). Others, however, apparently also occur in vivo and are not associated with a loss in virulence (e.g., Salmonella flagellar phase variation; fimbriae and certain OMP variations in gonococci). The latter changes supply the bacteria with means to
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adapt themselves to varying conditions within the host, such as the presence of inhibitory antibodies. Meningococci show intra-strain variation among class-5 OMPs (Poolman, de Marie and Zanen, 1980a), and these are associated with colonial morphology, although this association is not conclusive (Stephens and McGee, 1983). The nomenclature of meningococcal OMPs has been suggested by Tsai, Frasch and Mocca (1981). Variation in meningococcal fimbriation does not cause reproducible differences in colonial morphology (Froholm, Jyssum and Bøvre, 1973; DeVoe and Gilchrist, 1978; McGee et al., 1979). Meningococci reveal phenotypic changes of their LPS which depend upon growth phase and growth rate (Tsai, Boykins and Fransch, 1983).

In this report we describe the colonial characteristics of *N. meningitidis* strain 2996 (B:2b:P1.2, according to a recent nomenclature proposal by Frasch, Zollinger and Poolman, in press). Colonial morphology appeared to be associated with rapid changes in LPS and class-5 OMPs. It will be shown that these changes in LPS are different from the phenotypic variations described previously.

**MATERIALS AND METHODS**

*Growth conditions and colonial morphology.* *N. meningitidis* strain 2996 (B:2b:P1.2; Poolman, Hopman and Zanen, 1980b) was grown in Trypticase Soy Broth (TSB) (Difco) without shaking at 35°C (Froholm et al., 1973). The pellicle was transferred to tubes of fresh TSB every 2 days and this procedure was continued for 4 weeks. The cultures were then inoculated on to plates of clear gonococcal colony-typing medium (Swanson, 1982) and after 18 h at 37°C the colonies were examined with a Zeiss stereomicroscope with transmitted light from a FiL 151-beam lighting system. Photographs were taken of the various colonies. Typical colonies were purified several times by single colony passage. Agar-plate cultures consisting of a single colony type (>95%) were used to inoculate 200 ml of TSB in flasks. These cultures were grown for either 10 or 16 h at 37°C and 150 rpm, on a gyrotory shaker (New Brunswick Scientific). Colonial morphology was then checked by streaking samples on agar plates.

Outer membrane complexes were isolated from these cultures as described previously (Poolman et al., 1980b).

*SDS-polyacrylamide gel electrophoresis (SDS-PAGE).* The Laemmli system was used as previously described (Poolman et al., 1980b) and 4 M urea was included in the separating gel (15% acrylamide) to give better resolution of LPS. The gels were stained by Coomassie Brilliant Blue (Poolman et al., 1980b) and by silver staining for LPS (Tsai et al., 1983).

*LPS serotyping.* Meningococcal LPS antisera were prepared in rabbits and Ouchterlony microprecipitation in agar on glass slides was performed as described by Poolman et al. (1980b). The various outer-membrane preparations were serotyped for the presence of the ten LPS serotypes described previously (Poolman, Hopman and Zanen, 1982).

**RESULTS**

*Colonial morphology*

Several different colonial morphologies could be recognised in the agar cultures. Examples of a mixed culture and two pure cultures are shown in fig. 1.

Thirteen colony types were chosen for investigation of OMP and LPS characteristics. The thirteen colony types came from two TSB cultures passaged for one month and then streaked on clear agar plates; they are described in the table. There were subtle differences between the colony types that cannot be described adequately in words (see fig. 1).
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Fig. 1.—Colonial morphology of \textit{N. meningitidis} strain 2996 (B:2b:P1.2): (a) mixed culture; (b) variant 4, transparent (intermediate); (c) variant 6, very opaque.

Fig. 2.—SDS-PAGE of outer membranes of \textit{N. meningitidis} strain 2996 colony variants (15\% polyacrylamide with 4\% urea) stained by silver staining: \(a\) = variant \(a\), logarithmic phase; \(\bar{a}\) = variant \(\bar{a}\), stationary phase; \(M\) = mother culture (unselected strain 2996); \(S\) = mol. wt standards.
### Characteristics of *N. meningitidis* strain 2996 colony types

<table>
<thead>
<tr>
<th>Variant no.</th>
<th>Colonial morphology</th>
<th>Class-5 OMP (mol wt)*</th>
<th>LPS profile (SDS-PAGE)</th>
<th>LPS serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>very opaque</td>
<td>—</td>
<td>III</td>
<td>(1),8</td>
</tr>
<tr>
<td>2</td>
<td>opaque (intermediate)</td>
<td>—</td>
<td>III</td>
<td>(1),8</td>
</tr>
<tr>
<td>3</td>
<td>transparent (intermediate)</td>
<td>—</td>
<td>I</td>
<td>NT</td>
</tr>
<tr>
<td>4</td>
<td>transparent (intermediate)</td>
<td>—</td>
<td>II</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>very transparent</td>
<td>25 000</td>
<td>II</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>very opaque with rough edges</td>
<td>—</td>
<td>III</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>very opaque</td>
<td>—</td>
<td>III</td>
<td>8</td>
</tr>
<tr>
<td>a</td>
<td>very transparent</td>
<td>—</td>
<td>I</td>
<td>NT</td>
</tr>
<tr>
<td>b</td>
<td>transparent (intermediate)</td>
<td>—</td>
<td>I</td>
<td>NT</td>
</tr>
<tr>
<td>c</td>
<td>transparent (intermediate)</td>
<td>28 000</td>
<td>I</td>
<td>NT</td>
</tr>
<tr>
<td>d</td>
<td>very transparent</td>
<td>—</td>
<td>I</td>
<td>NT</td>
</tr>
<tr>
<td>e</td>
<td>very opaque</td>
<td>28 000</td>
<td>I</td>
<td>NT</td>
</tr>
<tr>
<td>f</td>
<td>opaque (intermediate)</td>
<td>28 000</td>
<td>I</td>
<td>NT</td>
</tr>
</tbody>
</table>

NT = nontypable.
(1) = weak reaction with LPS 1.
*— = Class-5 OMP not present.

### SDS-PAGE analysis of outer membranes

SDS-PAGE analysis and silver staining showed that the LPS composition of the various colony types was quite different (fig. 2). Variants a–f showed similar LPS profiles (LPS profile I), with clear differences between preparations from exponential- and stationary-phase cultures. At least four bands were recognised in the stationary-phase preparations, whereas sometimes only one clear band was present in exponential-phase preparations. Some apparently exponential-phase cultures might have been in early stationary phase, thus explaining the differences between the various preparations. Indications for the existence of even more bands, representing longer LPS molecules in stationary-phase preparations of variants c and d, can be found in the gel (see arrow). Variant 3 was similar to variants a–f (LPS profile I). Variants 4 and 5 showed LPS patterns similar to the outer-membrane preparation of the parent culture (strain 2996 culture without colony selection) (LPS profile II). A typical broad, brown band was apparent in the preparations from the parent culture and in those from variants 4 and 5, contrasting with the mostly black bands (one brown band occurred, but in a different place) in preparations from variants 3 and a–f (LPS I). The stationary-phase preparations of variants 4 and 5 showed “tailing” towards higher mol. wts (brown-yellow colour). Finally, preparations from variants 1, 2, 6 and 7 revealed two LPS bands (an upper brown one and a lower black one) without an alteration in band patterns between exponential- and stationary-phase preparations (LPS profile III). Coomassie Blue staining of another gel (12.5% polyacrylamide without urea) showed that variations in OMP composition were also present among the colony variants. Preparations from variants c, e and f showed the presence of a 28 000-mol. wt class-5 OMP and those from variant 5 showed a 25 000-mol. wt class-5 OMP (fig. 3). The LPS:OMP ratio in the outer membrane was higher in stationary-phase cells than in exponential-phase cells, i.e., there was more LPS per unit of surface area.
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Fig. 3.—SDS-PAGE of outer membranes of *N. meningitidis* strain 2996 colony variants 1–7 and a–f (12.5% polyacrylamide) stained by Coomassie Blue.

LPS serotyping

Fig. 4 shows the microprecipitation patterns of anti-LPS 1 and anti-LPS 8 against outer membranes of variants 1–7. Outer membrane preparations of variants 1 and 2 reacted with both LPS 1 (weakly) and LPS 8. Preparations from variants 4 and 5 reacted with LPS 1 and those from variants 6 and 7 reacted with LPS 8. Preparations from variant 3 did not react with LPS 1 or 8, like those from variants a–f (b–f not shown).

The LPS serotyping patterns correlated well with the silver stain patterns in the polyacrylamide gel (fig. 2 and table) (see below).

Correlation between colonial morphology, LPS and class-5 OMP composition of strain 2996

In the table, the characteristics of the 13 colony types are given with their LPS and class-5 OMP composition. In variants c, e and f, which have similar LPS, the presence of a 28 000-mol. wt class-5 OMP appears to be associated with opaque colonies, whereas variants a, b and d, which do not have a class-5 OMP, are transparent. In variants 1, 2, 6 and 7, the presence of LPS serotype 8 appears to be associated with colony opacity. LPS-1 variants (4 and 5) appeared to be transparent, despite the presence of a 25 000-mol. wt class-5 OMP. Variant 3 was similar to variants a, b and d.
FIG. 4.—LPS serotyping of *N. meningitidis* strain 2996 colony types by Ouchterlony microprecipitation. R = reference preparations (LPS 1 and LPS 8, respectively); A = antiserum.

**DISCUSSION**

In this study, we provide evidence for intra-strain variation of meningococcal class-5 OMPs and LPS. The variability of class-5 OMPs had been shown before (Poolman *et al.*, 1980a; Ashton *et al.*, 1983). It is a property that may be associated with the immunogenicity of these proteins in patients and an antigenic drift mechanism (Poolman, Hopman and Zanen, 1983).

Meningococcal colonial morphology is associated with both LPS and class-5 OMPs. The presence of class-5 OMPs caused strain 2996 colony types to be opaque, as does gonococcal protein I1 (Swanson, 1982). This is in contrast to the findings of Stephens and McGee (1983). The above associations may be strain-dependent. All the variants studied by us were non-fimbriate as judged by electronmicroscopy. The intra-strain LPS variations observed in this study are clearly different from the phenotypic changes described previously (Tsai *et al.*, 1983). We were able to confirm these growth-phase-dependent changes but the colony-type-associated LPS variants were qualitatively different as shown by SDS-PAGE, silver staining and LPS serotyping. The molecular mechanisms underlying these two types of LPS changes are unknown but the meningococcus appears to have the capacity to change the molecular length as well as the chemical (sugar) composition of its LPS. Such changes are likely to have a considerable effect on the interaction with the human host, including human antibody effectiveness and bacterial endotoxin liberation (Andersen and Solberg, 1984).

LPS serotypes 1 and 8 both occur in patients without colony selection and the analysis of strains isolated from various sites of one patient also revealed LPS variations (data not shown) (Poolman *et al.*, 1980a; Poolman *et al.*, 1982). This indicates that the LPS variations described also occur *in vivo*, as has been shown for meningococcal class-5 OMPs.
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