L Form of Neisseria gonorrhoeae

RICHARD B. ROBERTS

Department of Bacteriology, Division of Communicable Diseases and Immunology, Walter Reed Army Institute of Research, Washington, D.C.

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ABSTRACT

ROBERTS, RICHARD B. (Walter Reed Army Institute of Research, Washington, D.C.). L form of Neisseria gonorrhoeae. J. Bacteriol. 92:1609–1614. 1966.—L forms were produced by the penicillin gradient plate technique from a recently isolated strain of Neisseria gonorrhoeae. To date, these L forms have had 30 serial passages on medium containing penicillin. Stabilized L forms developed on penicillin-free medium after 10 or more passages in the presence of penicillin. Morphological characteristics of these organisms were identical to L forms of meningococci. Medium and environmental conditions necessary for optimal growth included: Brain Heart Infusion of pH 7.2 to 7.4, 1.1 to 1.3% agar, 10 to 20% sucrose, 10 to 20% horse serum, temperature at 35 to 36 C, and increased CO₂ tension (candle jar). L forms were more resistant than the parent gonococcus to penicillin, ampicillin, methicillin, cycloserine, and cephalothin, whereas both organisms had similar sensitivities to bacitracin, vancomycin, ristocetin, novobiocin, tetracycline, and erythromycin. Revertant gonococci were produced on penicillin-free medium from L forms which had had 1, 5, and 10 serial passages. Morphology and fermentative reactions of revertant strains were identical to those of the parent gonococcus. Revertant strains produced L forms more readily than the parent organism; in fact, L forms from certain revertants did not require penicillin, but only serum and sucrose for their production and propagation on artificial medium.

The in vitro production of L-type growth from cultures of Neisseria gonorrhoeae has been described in previous reports (1, 4, 5, 12). However, successful serial propagation of gonococcal L forms has not been reported; thus, optimal growth conditions, as well as morphological and biochemical properties of these organisms, are not known. Knowledge of these factors is essential for logical investigation of the possible role of gonococcal L forms in certain disease states.

With the application of techniques previously described in the study of L forms of Neisseria meningitidis (11), four recent isolates of N. gonorrhoeae were tested in vitro for L-form production. Gonococcal L forms were produced from one of the four strains. This report describes the methods of production and propagation, and certain properties of these L forms. Characteristics of L forms of N. gonorrhoeae are compared with those of N. meningitidis.

MATERIALS AND METHODS

Bacteria. N. gonorrhoeae strain 424, from the Department of Bacteriology, Walter Reed Army Institute of Research, was a recent isolate (from a patient with acute Bartholin's abscess) which produced acid from glucose, but not from maltose or sucrose.

Media and growth conditions. Parent and revertant gonococci were subcultured either in Eugonbroth (BBL) or on Mueller Hinton chocolate-agar (Difco). Broth cultures were placed on an Eberbach reciprocating shaker (6,500 rev/hr) and incubated aerobically at 37 C for 8 to 12 hr. Cultures on agar were incubated at 37 C in the presence of moisture and CO₂ (candle jar) for 18 to 24 hr.

The medium used for the production and propagation of L forms was Brain Heart Infusion (Difco) to which was added 1.2% agar, 10% sucrose, 0.5% yeast extract, and 10% inactivated (60 C for 30 min) horse serum. This medium is designated BRHIA. Benzylpenicillin, 100 or 1,000 units per ml of complete medium, was added to BRHIA for serial propagation of L forms. All plate cultures were incubated at 35 to 37 C in the presence of moisture and CO₂ (candle jar).

Production and propagation of L forms. The methods for production and propagation of L forms were similar to those previously described (11), except that gradient plates were examined for a period of 30 days and cultures were serially transferred every 4 to 6 days.

Morphology of L forms. The morphological characteristics of L-form colonies were studied by ex-
amination of unstained colonies with a colony micro-
scope (magnification × 45), and by the stained-agar
method of Dienes (10) with phase-contrast
microscopy.

Relative antibiotic sensitivities of L forms and parent
gonococci. Relative antibiotic sensitivities of parent
strain 424 and its L form to penicillin, methicillin,
ampicillin, cephalothin, cycloserine, vancomycin,
ristocetin, bacitracin, novobiocin, tetracycline, and
erthyromycin were determined. Serial 10-fold dilutions
of each antibiotic (in distilled water) were added to
BRHIA. These plates were inoculated either with
0.2 ml of a 12-hr broth culture of the parent gono-
coccus (10⁴ organisms per milliliter) or with agar
blocks containing a 4- to 6-day growth of L-form
colonies. All plates were read on the 2nd, 5th, and 8th
day after inoculation.

Reversion of L forms. Following the 1st, 5th, and
10th serial passage of L forms on medium containing
penicillin, L-form colonies were transferred to BRHIA
without penicillin and were serially subcultured until
bacterial colonies appeared. These revertant or-
ganisms, designated R1, R5, and R10, were trans-
ferred to Mueller Hinton chocolate-agar, examined
by Gram stain, and tested for fermentative reactions

RESULTS

Production and propagation of L forms. Fol-
lowing the inoculation of penicillin gradient
plates with a broth culture of the parent gono-
coccus (10⁶ organisms per milliliter), four L-form
colonies appeared in 8 to 10 days. An agar
block containing these colonies was transferred
on the 14th day to BRHIA with penicillin.
Because no growth was observed after 20 days
of incubation, the agar block was restreaked
on the surface of the same plate. Four days later,
revertant gonococci appeared on the streaked
surface.

Gradient plates were then inoculated with a
broth culture of revertant organisms, and, within
6 days, 20 L-form colonies appeared. These
colonies were transferred to BRHIA containing
penicillin; after 25 days of incubation, growth
did not occur. Again the agar block was re-
streaked, and, within 3 days, L-form colonies
grew along the streaked pathway. Initial growth
of these colonies was slow, but growth markedly
improved after three serial passages. After seven
passages, L forms were routinely transferred
every 4 to 6 days and, to date, have had 30 serial
passages on BRHIA containing penicillin.

Optimal growth conditions for L forms. Optimal
growth of strain 424 L forms occurred on Brain
Heart Infusion (pH 7.2 to 7.4) containing 1.1 to
1.3% agar, 10 to 20% sucrose, and 10 to 20% horse
serum which was incubated at 35 to 36 C in
the presence of moisture and CO₂ (candle jar).
L-form growth was not observed on medium of
pH less than 7.0 or containing 2 or 4% NaCl,
10% rabbit and human serum, or 1 to 5% horse
serum. In addition, growth did not occur when
plate cultures were incubated in either an aerobic
or anaerobic environment or when temperatures
were less or greater than 34 to 38 C.

Morphology of L forms. Two types of L-form
colonies were observed. The first type had large
cores, little or no periphery, and was associated
with poor, if any, growth. These colonies were
seen on gradient plates inoculated with the parent
bacterium. The second type of colony had small,
well-demarcated cores and well-defined peripher-
ies. These colonies propagated well and are
characteristic of those seen in our present cul-
tures. Figures 1 and 2 demonstrate the appearance
of these colonies after 5 and 8 days of growth,
respectively. With prolonged incubation, colonies
enlarged in size, the central core darkened, and
propagation occurred less readily. When L-form
colonies were stained and examined under higher
magnification, structural elements in the periph-
ery became apparent (Fig. 3). These structures
included granules and vacuolated large bodies,
both of which have been observed in L-form
colonies of other bacteria. With phase-contrast
microscopy (Fig. 5), these structures were seen
not only in the periphery, but also in the central
core area. In addition, small granules were char-
acteristically located either between or along
the boundary of vacuolated large bodies (Fig. 6).
Intermediate-sized phase-dense bodies and non-
vacuolated large bodies were also present (Fig.
4).

Relative antibiotic sensitivities of L forms and
parent gonococci. The relative antibiotic sensitivi-
ties of parent strain 424 and its L form are shown
in Table 1. The greatest difference in sensitivities
was observed with penicillin, though at least a
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FIG. 5. A 4-day growth of an L-form colony containing intermediate-sized phase-dense bodies, small granules, and vacuolated large bodies. The two latter structures are seen within both the central core and periphery. Dienes' stain with phase microscopy. × 1,200.

FIG. 6. A 4-day growth of L-form colonies. Peripheries approximate each other and demonstrate small granules located either between or along the boundaries of vacuolated large bodies (arrows). Dienes' stain with phase microscopy. × 1,600.

TABLE 1. Relative antibiotic sensitivities of a parent gonococcus and its L form

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Strain 424</th>
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<tbody>
<tr>
<td></td>
<td>Parent</td>
</tr>
<tr>
<td>Penicillin (units/ml)</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Bacitracin (units/ml)</td>
<td>1.0</td>
</tr>
<tr>
<td>Ampicillin (µg/ml)</td>
<td>1.0</td>
</tr>
<tr>
<td>Methicillin (µg/ml)</td>
<td>1.0</td>
</tr>
<tr>
<td>Cephalothin (µg/ml)</td>
<td>0.1</td>
</tr>
<tr>
<td>Cycloserine (µg/ml)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Vancomycin (µg/ml)</td>
<td>1.0</td>
</tr>
<tr>
<td>Ristocetin (µg/ml)</td>
<td>100</td>
</tr>
<tr>
<td>Novobiocin (µg/ml)</td>
<td>0.1</td>
</tr>
<tr>
<td>Tetracycline (µg/ml)</td>
<td>0.1</td>
</tr>
<tr>
<td>Erythromycin (µg/ml)</td>
<td>0.1</td>
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</tbody>
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* Minimal inhibitory concentration.

1,000-fold difference was also seen with ampicillin, methicillin, cephalothin, and cycloserine. The sensitivities of parent strain and L form to bacitracin, vancomycin, ristocetin, novobiocin, tetracycline, and erythromycin were similar.

Reversion of L forms and properties of revertant gonococci. When serially transferred on penicillin-free BRHIA, L-form colonies which had had one or five serial passages on medium containing penicillin readily reverted to bacteria. Relatively few L-form colonies from the 10th serial passage reverted, although these L forms had 20 serial passages on penicillin-free BRHIA. In an attempt to induce reversion, these latter L forms were also transferred to medium containing either 2.0% or 2.4% agar (8) or 20% gelatin. Though L-form growth was observed on these media, reversion to the bacterial form did not occur.

When the cells were Gram-stained, revertant gonococci (R1, R5, and R10) demonstrated typical gram-negative diplococci and produced acid from glucose but not from maltose or sucrose.

All revertant gonococci, especially those from L forms which had had 10 serial passages (R10), produced L forms more readily than the parent strain. During these studies, it was observed that, following the inoculation of gradient plates with
revertant organisms (R10), L-form growth was observed. These L forms, produced without penicillin, did not revert even after 10 serial passages on penicillin-free BRHIA.

Studies comparing the medium constituents necessary for L-form production from *N. gonorrhoeae* and *N. meningitidis* demonstrated that sucrose and serum, but not penicillin, were necessary for the in vitro production of gonococcal L forms, whereas L-form production from revertant meningococci depended on sucrose, serum, and penicillin.

**DISCUSSION**

The successful production and propagation of gonococcal L forms depended on the inoculation of gradient plates with revertant organisms and the restreaking of previously inoculated agar blocks. These factors were also important in the initial production of propagating L forms of meningococci (11). Modifications in culture techniques, such as the addition of 100 units of benzylpenicillin per ml of complete medium and incubation of plates at 35 to 36 C, improved the growth of gonococcal L forms so that the duration of incubation between subcultures was decreased.

The conditions for optimal growth of gonococcal L forms described in this report differ from those suggested by Dienes, Bandur, and Madoff (5). The observations that gonococcal L forms do not propagate in (i) a medium without an osmotic stabilizing agent, (ii) a medium with a pH less than 7.0, (iii) an environment without CO₂, and (iv) a temperature less than 34 C, may explain why serial cultivation of L-type growth by these investigators was unsuccessful.

Significant differences in growth conditions of L forms of gonococci and meningococci were apparent (11), since gonococcal L forms did not grow in the presence of 2% NaCl, 1% and 5% horse serum, or in an aerobic environment. In addition, the range of temperature (34 to 38 C), within which growth was observed, was narrower than that determined for the growth of meningococcal L forms. Gonococcal L forms did not grow on media containing rabbit or human serum, whereas meningococcal L forms initially grew on these media with some difficulty. Since cultures were transferred from a medium with horse serum, difficulty in adaptation of these organisms to media with different sera could explain this finding.

Certain conditions for optimal growth of gonococcal L forms and parent gonococci were similar: the presence of serum in the medium, a CO₂ environment, and a temperature range of 35 to 36 C (14).

At the present time, there are no methods for specific identification of gonococcal L-form colonies. Reversion experiments have shown that L forms may become stable after repeated transfer on medium containing penicillin. Colony morphology of these organisms, examined by colony or phase-contrast microscopy, was identical to that of L forms of meningococci. The indophenol oxidase test by L forms of both pathogenic *Neisseria* species was positive. Determination of carbohydrate fermentations by the method used for meningococcal L forms (6) was unsuccessful, because gonococcal L-form growth was inhibited by the addition of 2% NaCl to the medium.

Certain serological techniques, such as immunofluorescence, have been used to identify L-form colonies of group A streptococci (7). Though fluorescent-antibody methods have been used in identifying parent gonococci, cross-reactions with other *Neisseria* species are commonly seen (2, 3). Absorption of fluorescein isothiocyanate labeled antigenococcus globulin with group A or C meningococci eliminated these cross-reactions (3). These findings suggest a possible serological technique for the identification of gonococcal L forms.

Antibiotic sensitivity determinations of gonococcal L forms and the respective parent strain demonstrated a resistance of L forms to four of the eight antibiotics that inhibit bacterial cell wall synthesis. The sensitivity levels of both organisms to other antibiotics (novobiocin, tetracycline, and erythromycin) were similar. The sensitivities of the parent gonococcus, determined by serial 10-fold dilutions of each antibiotic, are comparable to gonococcal sensitivities previously reported (9, 13). Previous studies (to be published) have shown that meningococcal L forms were more resistant than their parent strain to the eight antibiotics that inhibit cell wall synthesis, except ristocetin. Furthermore, L forms from revertant meningococci were produced by each of these antibiotics. Similar studies were not performed with revertant gonococci, because these organisms produced L forms when exposed to control media without antibiotics (see below).

Revertant gonococci were produced from L forms which had had 1, 5, and 10 serial passages in the L-form state. Morphological and fermentative properties of these organisms were identical to those of the parent gonococcus. The only difference observed between the two organisms was the property of revertant gonococci to produce L forms more readily than the parent strain. Though this characteristic has also been observed with meningococci, penicillin or an antibiotic having a similar antibacterial action was necessary for meningococcal L-form induction (to be published). In the present study,
gonococcal L forms were produced from revertant organisms in a suitable medium containing only sucrose and serum. This finding emphasizes the need for controlled studies when L-form production by different substances is examined.

ACKNOWLEDGMENTS

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LITERATURE CITED