L Form of *Neisseria gonorrhoeae*

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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-

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amination of unstained colonies with a colony microscope (magnification $\times 45$), and by the stained-agar technique 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 erythromycin 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 gonococcus (10^4 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 organisms, designated R1, R5, and R10, were transferred to Mueller Hinton chocolate-agar, examined by Gram stain, and tested for fermentative reactions (Evans et al., *Bacteriol. Proc.*, p. 56, 1964).

RESULTS

Production and propagation of L forms. Following the inoculation of penicillin gradient plates with a broth culture of the parent gonococcus (10^8 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 restreaked, 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 peripheries. These colonies propagated well and are characteristic of those seen in our present cultures. 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 periphery 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 characteristically 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 sensitivities 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

FIG. 1. A 5-day growth of gonococcal L forms. Colonies are small, with well-defined central cores and peripheries. $\times 38$.

FIG. 2. An 8-day growth of gonococcal L forms. Colonies are larger and have dark cores and peripheries which vary in width. $\times 38$.

FIG. 3. An L-form colony with a double core and peripheral vacuoles. Dienes' stain with light microscopy. $\times 320$.

FIG. 4. A 4-day growth of an L-form colony. Periphery in focus demonstrating intermediate-sized phase-dense bodies, as well as vacuolated and nonvacuolated large bodies. Dienes' stain with phase microscopy. $\times 1,200$.

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. $\times 1,200$.

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. $\times 1,600$.

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

Antibiotic	Strain 424	
	Parent	L form
Penicillin (units/ml).....	<0.01 ^a	10,000
Bacitracin (units/ml).....	1.0	1.0
Ampicillin (μ g/ml).....	1.0	10,000
Methicillin (μ g/ml).....	1.0	10,000
Cephalothin (μ g/ml).....	0.1	1,000
Cycloserine (μ g/ml).....	<0.1	1,000
Vancomycin (μ g/ml).....	1.0	1.0
Ristocetin (μ g/ml).....	100	100
Novobiocin (μ g/ml).....	0.1	0.1
Tetracycline (μ g/ml).....	0.1	0.1
Erythromycin (μ g/ml).....	0.1	0.1

^a 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.

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