The L Form of *Neisseria meningitidis*

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SUMMARY

L forms were produced by the penicillin gradient plate technique from four strains of *Neisseria meningitidis*. All strains were group B and had various sulphadiazine sensitivities. One parent strain had been propagated for many years in the laboratory, whereas the other three were isolated from recent cases of meningitis. To date, each L form has had more than 60 serial passages on medium containing penicillin. From two strains stabilized L form variants developed on penicillin-free medium following 30 or 40 such passages. Morphological characteristics of these organisms were similar to L forms of other bacteria. Medium and environmental conditions necessary for optimal growth included: brain heart infusion containing a final agar concentration of 1·2 % (w/v), sucrose, 10 % (w/v), horse serum, 10 % (v/v), pH 7·4, temperature 37° and increased CO₂ tension (candle jar). The L forms were non-groupable and had various fermentative reactions, whereas their sensitivity to sulphadiazine was the same as that of their respective parents. Revertants were produced on penicillin-free medium following every tenth serial passage in the L-form state. Sensitivities to sulphadiazine, fermentative reactions and serological groups of the revertant strains were identical with those of their respective parents.

INTRODUCTION

The induction of L forms by modifications of the gradient plate technique described by Sharp (1954) has been reported by many investigators (Lederberg & St Clair, 1958; Madoff & Dienes, 1958). Penicillin has been the inducing agent most commonly used, though other antibiotics such as bacitracin (Rotta, Karakawa & Krause, 1965), cycloserine (Ward & Martin, 1962) and cephalosporin (Williams, 1963), have also been used. Although L forms from a number of bacteria have been produced by this method, the successful production and propagation of L forms of *Neisseria meningitidis* have not been previously reported. In recent years a high carrier rate and an increasing number of clinical meningococcal infections have been noted in the United States (Bristow, Van Peenen & Volk, 1965; Brown & Condit, 1965; Communicable Disease Center, 1964). Because of the possibility that L forms may persist both during prophylaxis and in recurrent meningococcal disease, the present study was undertaken. This paper describes the *in vitro* production and propagation of L forms from four strains of *Neisseria meningitidis*. Morphological characteristics and optimal growth requirements are described. Serological groups, carbohydrate fermentations, and sulphadiazine sensitivities of the parent organisms are compared with those of their respective L forms and revertants.

METHODS

Organisms. Three of the four strains of *Neisseria meningitidis* used in this study were obtained from the Department of Bacteriology, Walter Reed Army Institute of Research, Washington, D.C., U.S.A. These strains (78-Eur., 79-Eur., 55-III, hereafter designated 78, 79, 55) were recently isolated (April 1965) from the blood of patients with meningitis. Two of the patients were stationed in Europe, the third in the United States. The fourth strain (Ne 15) was kindly sent to us by Dr B. W. Catlin (Marquette University School of Medicine, Milwaukee, Wisconsin). This strain was originally isolated in 1955 from the spinal fluid of a fatal case of non-epidemic meningitis (Catlin & Cunningham, 1961). Since then it has been repeatedly subcultured on artificial media and is now considered to be a 'laboratory strain'. All four strains were group B and had identical fermentative reactions.

Media. The standard medium used for the production and propagation of the L forms was Difco brain heart infusion broth (BRHIB) to which was added Difco agar, 1.2 % (w/v), sucrose, 10 % (w/v), Difco yeast extract, 0.5 % (w/v), and heated (60° for 30 min.) horse serum, 10 % (v/v). Benzylpenicillin (Charles Pfizer and Co., Inc., New York), 1000 units/ml., was added to the complete medium to maintain unstable organisms in the L form. This enriched agar medium containing benzylpenicillin, unless otherwise noted, is designated BRHIA. Penicillin was not added to the medium for the propagation of stable L forms. BRHIA was prepared weekly. To test sterility freshly prepared medium was incubated at 37° for 24 hr before storage at 4°.

To determine range and optimal conditions necessary for growth of meningococcal L forms, colonies were transferred for five serial passages on various media and the degree of growth noted. Growth was tested on three types of base medium; BRHIA, Difco heart infusion agar (HIA) and Baltimore Biological Laboratories (BBL) Trypticase agar (TA). Sodium chloride, 2 or 4 % (w/v), and sucrose, 10 or 20 % (w/v), were compared for osmotic stabilization. The effect on L-form growth of sera from horse, rabbit and man was also compared. Growth was evaluated on agar concentrations of 0.7–2.4 % (w/v), at temperatures of 25–41°, in three gaseous environments (candle jar, aerobic, anaerobic) and in the range pH 5.5–11.0. The pH value of the medium was adjusted by adding either 0.1 N- or 0.5 N-HCl or NaOH.

Parent and revertant meningococci were subcultured either in BBL Eugonbroth or on Difco Mueller–Hinton chocolate agar.

Production and propagation of L forms. As the initial step in the production of L forms, a 12–18 hr growth of meningococci was inoculated into 20 ml. of Eugonbroth. This broth was placed on an Eberbach reciprocating shaker (6500 osc./hr), and incubated aerobically at 37° for 6–8 hr. Penicillin-free BRHIA plates (100 × 15 mm.) in which a 5 × 50 mm. trough had previously been made were inoculated with 0.3 ml. of this broth culture. After distributing the inoculum over the surface of the agar, 0.25 ml. of benzylpenicillin (100,000 units/ml.) was placed in the trough. Plates were incubated at 37° in candle jars containing moist gauze and were examined each day for 10 days under a colony microscope. When L-form growth was present, blocks of agar containing L-form colonies were cut out with a small metal spatula, inverted onto fresh BRHIA plates and streaked with the spatula

or a sterile glass rod. Subsequent cultures were then transferred in duplicate every 2-3 days. For future studies, several agar blocks containing a heavy growth of L-form colonies were frozen with acetone and dry ice following each 10th serial passage and immediately stored at -20° .

Reversion of L forms. Following every 10th serial passage, agar blocks containing L-form colonies were transferred to BRHIA without penicillin and subcultured until bacterial forms appeared. These revertant organisms were then transferred to Mueller-Hinton chocolate agar and subsequently freeze-dried.

Morphology of L forms. The morphological characteristics of L-form colonies were studied by two methods: (1) examination of unstained colonies with the colony microscope (magnification $\times 45$); (2) by the stained agar technique of Dienes (Madoff, 1960) with phase contrast microscopy.

Serological grouping, carbohydrate fermentation and sulphadiazine sensitivity of L forms and revertants. Two methods were used in the preparation of L-form antigens for group determinations. In the first method L-form colonies were cultured on BRHIA containing 10% (v/v) rabbit serum instead of horse serum. After three serial passages, growth was removed from the surface of the agar with a sterile cottonwool swab and suspended in normal saline.

The second method was as follows. After three serial passages on BRHIA containing 10% (v/v) rabbit serum, large agar blocks with L-form growth were transferred to flasks containing 25 ml. BRHIA. The inoculated agar was then overlaid with 10 ml. of BRHIB containing 0.01% (w/v) agar, 10% (w/v) sucrose, 0.5% (w/v) Difco yeast extract and 1000 units benzylpenicillin/ml. Both the agar and broth contained 10% (v/v) rabbit serum. On the third and sixth day after inoculation 0.2 ml. of broth was subcultured on BRHIA. Following incubation for 48 hr, these subcultures were in turn transferred to other flasks with diphasic media. This procedure was repeated until a heavy growth of L-form colonies appeared in the broth. Organisms from the 10 ml. of broth were sedimented by centrifugation, washed once and resuspended in physiological saline. The concentration of the final suspensions prepared by both methods was equivalent to a MacFarland optical density standard no. 3. Difco meningococcus antiserum was used for grouping by the usual macroscopic slide and tube agglutination techniques. Controls included both normal saline and normal rabbit serum.

Carbohydrate fermentations were determined on HIA medium by using the method of Edward (1950). The medium also contained NaCl 2% (w/v), horse serum 10% (v/v), test carbohydrate 1.0% (w/v), phenol red 0.005% (w/v), and benzylpenicillin 1000 units/ml. Carbohydrates were not added to the control medium.

Sensitivity to sulphadiazine was determined on BRHIA without penicillin. Both the L form and its respective parent were subcultured on medium containing various concentrations of sulphadiazine and the minimum inhibitory concentration (m.i.c.) determined.

All revertant meningococci were identified by the following methods: (1) Gram stain; (2) grouping with meningococcus antiserum; (3) fermentative reactions and sulphadiazine sensitivities determined by the method of Evans, Hunter, Cary & Rust (1964).

RESULTS

Production and propagation of L forms

Following the inoculation of penicillin gradient plates with a broth culture of the parent meningococcus, L-form colonies appeared in 3–5 days. The number of colony forming units varied from 1 to 30 per plate. When agar blocks containing colonies were transferred to BRHIA, propagation of these colonies generally did not occur. Organism 78 was an exception, and these L forms propagated well following the first passage.

When growth did not occur in 8–10 days, the previously transferred agar blocks were again streaked across the surface of the same agar plate. Within 4–6 days, growth appeared at the previous site of the block though colonies were not evident along the streak pathway. These L colonies were then transferred to BRHIA without penicillin for reversion. The revertants designated 79 R, Ne 15 R, 55 R were subcultured in Eugonbroth and inoculated onto gradient plates. Within 2–4 days, a heavy growth of L colonies appeared in a band-like pattern between the penicillin trough and bacterial growth (Pl. 1, fig. 1). Non-propagating, small, rough bacterial colonies were also seen in this area (Pl. 1, fig. 2). On subsequent transfer, the L-form colonies propagated well, growth being apparent within 24 hr, and well-developed colonies were present in 48 hr. At the time of writing this report these L forms had had at least 60 serial passages on BRHIA.

In general, all revertant bacteria produced a significantly greater number of L forms than their respective parents. This property was maintained even after repeated subculture ($\times 45$) of the revertants on Mueller–Hinton chocolate agar.

To test L-form stability BRHIA cultures were transferred to penicillin-free medium from every 10th serial passage. Initially all L-form colonies reverted to bacteria. Selected stabilized variants of L forms 78 and 55 first developed in penicillin-free subcultures from the 30th and 40th serial passage, respectively, and at the time of this report these variants had had 30 transfers on BRHIA without penicillin. By the 60th serial passage on BRHIA, all L form colonies of strain 78 were stable following subsequent transfer to penicillin-free medium. To date all L form colonies of organisms 79 and Ne 15 have continued to revert to bacteria by the third passage on penicillin-free medium.

Growth requirements of L forms

Range and optimal conditions necessary for growth of meningococcal L forms are shown in Table 1. Though L forms propagated on all three types of base medium (BRHIA, HIA, TA), optimal growth occurred on BRHIA. Growth was also tested on media that contained final agar concentrations of 0.7–2.4 % (w/v). Growth occurred over a range of 0.8–1.8 % (w/v) agar with optimum growth on 1.0–1.6 % (w/v). On 2.0–2.4 % (w/v) agar medium, L forms reverted to bacteria even in the presence of penicillin.

L forms did not propagate unless an osmotic stabilizing agent was present in the medium. Although propagation occurred with a concentration of 2 % (w/v) NaCl, 4 % (w/v) NaCl completely inhibited L form growth. Sucrose, on the other hand, was equally effective in supporting growth at a 10 % or 20 % (w/v) concentration.

Serum was also necessary for the propagation of L forms. Unheated serum appeared to be as effective as heated serum, and growth occurred equally well on media containing serum derived from horse, rabbit or man. In the first several passages, growth was less abundant when L forms were transferred from medium containing one species of animal serum to another. Horse serum concentrations ranging from 1 to 20% (v/v) appeared to be equally effective in L-form propagation.

Table 1. *Growth requirements for L forms of Neisseria meningitidis*

Requirement	Range	Optimum
Base media	Brain heart infusion Heart infusion agar Trypticase agar	Brain heart infusion
Agar % (w/v)	0.8–1.8	1.0–1.6
Osmotic stabilization	Sucrose NaCl	Sucrose
Serum	Horse Rabbit Human	Horse Rabbit Human
pH	6.0–9.5	7.0–8.5
Environment	CO ₂ (candle jar) Aerobic	CO ₂ (candle jar)
Temperature	29–39°	34–38°

The range of pH values in which growth occurred was pH 6.0–9.5 with an optimum of pH 7.0–8.5. Reversion of L forms was common on media at a pH 9.5–10.5. Since the unaltered pH value of freshly prepared BRHIA was pH 7.3–7.4, no adjustment was necessary for the routine cultivation of these organisms. L form propagation was also tested in three different environments: candle jar, aerobic and anaerobic environment. Growth was superior in the presence of extra CO₂ (candle-jar), and L-form propagation did not take place in an anaerobic environment. The concentration of the CO₂ in candle jars was not measured. Finally, cultures were incubated at temperatures ranging from 25 to 41°. Growth occurred in the range 29–39°, though organisms did not propagate well at either temperature extreme. For optimal growth the temperature range was 34–38°.

Morphology of L forms

Two types of L-form colonies were seen. The first type of colony contained little or no periphery and had a poorly developed core which did not penetrate well into the agar. When transferred, these cores tended to break apart and propagation did not occur. This type of L-form colony was characteristic of those seen on gradient plates inoculated with the parent bacterium.

The second type of L-form colony had a well-demarcated core which penetrated into the agar and a sharply defined periphery (Pl. 1, fig. 3). The core of this type of colony remained intact during agar transfer and colony propagation was excellent. The appearance of these colonies and the relative size of the core to periphery varied widely in the same culture (Pl. 1, fig. 4). Colony size, determined by duration of growth and degree of colony crowding, varied between 50 and 500 μ . When stained and observed under higher magnification (Pl. 2, fig. 5), structural elements were

indistinguishable from those of L form colonies of other bacteria. These structures included granular bodies which characteristically were found in the core of the colony and vacuoles which were primarily seen in the periphery. Phase microscopy defined these structures even more clearly (Pl. 2, figs. 6–8). Granular bodies varied in size (Pl. 2, fig. 7) and were also present both between and within the peripheral vacuoles (Pl. 2, fig. 8). The density of these vacuoles appeared homogenous and their boundaries were distinct (Pl. 2, fig. 8). Intermediate sized, phase-dense bodies were also seen throughout the periphery (Pl. 2, fig. 6).

*Serological grouping, fermentative reactions, sulphadiazine sensitivity
and Gram staining of L forms and revertants*

Comparison of serological grouping, fermentative reactions and sulphadiazine sensitivity of L forms and of their respective parents is shown in Table 2. Suspensions of parent bacteria agglutinated with group B antisera. L form antigens whether harvested from agar or from broth did not agglutinate with polyvalent or groups A, B, or C antisera. Both normal serum and saline controls were negative.

Table 2. *Comparison of parent meningococci with their respective L forms*

Organism no.	Group	Sugar fermentation			Sulphadiazine sensitivity† (mg./100 ml.)*
		Glucose	Maltose	Sucrose	
Parent 78	B	+	+	—	0.05
(1) L form	N-g.†	+	+	—	No change
(2) Stabilized L form	N-g.	+	+	—	No change
Parent 79	B	+	+	—	0.05
L form	N-g.	+	+	—	No change
Parent Ne 15	B	+	+	—	0.05
L form	N-g.	±	±	—	No change
Parent 55	B	+	+	—	4.0
(1) L form	N-g.	+	+	—	No change
(2) Stabilized L form	N-g.	+	+	—	No change

* Minimum inhibitory concentration.

† Non-groupable.

‡ Sensitivities determined on complex media.

All parent meningococci produced acid from glucose and maltose but not from sucrose. L forms from organisms 78, 79 and 55 had fermentative reactions identical to those of their respective parents. These reactions were manifested by a permanent yellow colour change of the entire medium within 2 days after plate inoculation. The L form of Ne 15 produced a colour change only beneath the inoculating agar block. Fermentative reactions were tested with both recently produced L forms and L forms which had had 50 serial passages. The degree of fermentation in each group was the same. All control plates were negative.

Initial sulphadiazine sensitivity values of the parent organisms were determined by the method of Evans *et al.* (1964). These results, expressed as minimal inhibitory dose (m.i.c.) in mg./100 ml., are shown in Table 2. L-form sensitivity values were determined on penicillin-free BRHIA plates which contained various concentrations of sulphadiazine. Each plate was also inoculated with the parent organism.

On this medium the L form and its parent bacterium had identical sulphadiazine sensitivities. A comparison of these three properties in revertant bacteria and parent organisms is shown in Table 3. Serological grouping, carbohydrate fermentation and sulphadiazine sensitivity of the revertant strains were identical to those of their respective parent bacterium.

Bacteria were not seen in repeated Gram stains of L-form colonies. When Gram stains were performed on recently reverted bacteria, these organisms varied in size, shape and intensity of staining. However, following several transfers on Mueller-Hinton chocolate agar, the morphology and staining properties of these organisms were identical with those of typical meningococci.

Table 3. *Comparison of parent meningococci with their respective revertants**

Organism no.	Group	Sugar fermentation			Sulphadiazine sensitivity (mg./100 ml.)†
		Glucose	Maltose	Sucrose	
Parent 78	B	+	+	—	0.05
Revertants	B	+	+	—	0.05
Parent 79	B	+	+	—	0.05
Revertants	B	+	+	—	0.05
Parent Ne 15	B	+	+	—	0.05
Revertants	B	+	+	—	0.05
Parent 55	B	+	+	—	4.0
Revertants	B	+	+	—	4.0

* Revertants were produced from every 10th serial passage of unstable L forms.

† Minimum inhibitory concentration.

DISCUSSION

The successful production of L forms of *Neisseria meningitidis* was achieved in this work by using the penicillin gradient plate technique. Possibly the single most important factor in obtaining augmented production and serial propagation of L forms was the use of revertant meningococci for re-inoculation of gradient plates. This *in vitro* method proved simple and generally effective, and L forms from both old and new isolates were readily obtained. More recent studies in this laboratory indicate that the technique is equally applicable to production of L forms from group A and group C meningococci. Our methods differed widely from those described in previous reports in which L forms of neisseria from clinical sources could not be isolated and subsequently propagated (Dienes & Weinberger, 1951; Dienes, Bandur & Madoff, 1964).

To facilitate biochemical and serological studies of these L forms, optimal growth requirements were determined. In some instances these requirements, such as animal serum, agar concentration, pH of medium and gaseous environment, were similar to those that have previously been described for other L forms. Though studies on nutritional requirements of L forms are few, the need for animal serum for growth of certain L forms has been well established (Edward, 1953) and has also proved necessary for the growth of meningococcal L forms. High concentrations of agar promoting L-form reversion were first described by Landman & Halle (1963). They reported that agar concentrations of 2.0–2.5% (w/v) favoured reversion of

L forms of *Bacillus subtilis*. Dr Gooder (personal communication) has found that reversion of *Streptococcus faecalis* L forms occurs on 2.5 % (w/v) agar. These findings were also characteristic of L forms of meningococci. Edward (1950) reported that L forms of *Streptobacillus moniliformis* grew well on media in the range pH 6.8–9.2; growth on a more acid or alkaline medium was not tested. Smith (1964) stated that loss of L-form viability occurred when the medium was less than pH 7.0 or greater than 9.0. Our results were in general agreement with these two reports, since L-form propagation was observed in a range pH 6.0–9.5 and optimal growth occurred at pH 7.0–8.5. To our knowledge, reversion of L forms on a high alkaline medium (pH 9.5–10.5) has not previously been reported. It has also been stated that the gaseous requirement for L-form growth is the same as that for the parent bacterium (Dienes & Weinberger, 1951). The growth of parent meningococci and their respective L forms was superior in the presence of extra CO₂, whereas neither organism propagated in an anaerobic environment.

Differences in growth requirements between meningococcal and other L forms were also apparent. For growth of certain L forms, media with high concentrations of salt are required (Smith, 1964). NaCl 4 % (w/v) inhibited growth of meningococcal L forms, whereas sucrose supported growth at both concentrations tested. The optimal temperature for growth of other L forms has not been reported, though it has been suggested that the temperature should be the same as that for the parent bacterium (Smith, 1964). Studies in this laboratory demonstrated that parent meningococci grow at temperatures of 25–40°. The range for growth of meningococcal L forms was 29–39°, though optimal growth occurred only at 34–38°. Quantitative studies to define further the growth requirements of meningococcal L forms must await adaptation of these organisms to a broth medium.

Colony morphology, when examined under low magnification ($\times 45$), varied both with L forms derived from different parents and with different L-form colonies from the same parent. Factors such as type of medium employed, age of culture and amount of inoculum were important determinants. Under higher magnification granular bodies, intermediate size bodies and peripheral vacuoles were similar to those seen in other L forms. Since colony appearance varies within a given strain and structural elements are similar to L forms of other bacteria, macroscopic and microscopic colony morphology are not reliable differential tools in the identification of L forms of meningococci.

The specificity of serological grouping of group B meningococci is probably determined by a polysaccharide-polypeptide complex (Menzel & Rake, 1942); however, its site within the bacterium has not been determined. Because L forms of group B meningococci did not agglutinate with known group B antisera, the L form may lack this complex; and since L forms do not have a cell wall, the site of this complex is probably in the cell wall of the parent group B meningococcus. Other immunological studies to define the antigenic complexes of both the parent and the L form of group B meningococci are in progress.

The importance of (1) the use of a simplified medium to exclude sulphadiazine competitors, and (2) the standardization of the inoculum size have been previously emphasized in the determination of sulphadiazine sensitivities of meningococci (Jewell, 1958; Feldman, 1965). Because a simplified medium does not support L-form growth, sulphadiazine sensitivities were determined on penicillin-free BRHIA.

The minimum inhibitory concentrations of the L form and its respective parent were identical on this complex medium. Subsequently the m.i.c., both of the same parent and of the revertant from the respective L form, was determined on Mueller-Hinton agar (Table 3). This value was approximately one-tenth that observed on BRHIA. The discrepancy in these values emphasizes the importance of considering the effects of complex media when determining sulphadiazine sensitivity of L forms. The authors hope that with the application of this information and the future elucidation of more definitive tests for identification, the possible role of these L forms in human meningococcal disease can be defined.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. A penicillin gradient plate showing a band of L-form colonies between the trough and bacterial growth; $\times 0.8$.

Fig. 2. Enlargement of an area of L-form growth on a penicillin gradient plate. Non-propagating, small, rough, bacterial colonies also present; $\times 16$.

Fig. 3. Two-day growth of L-form colonies with well-demarcated cores and sharply defined peripheries; $\times 24$.

Fig. 4. Two-day growth of L-form colonies demonstrating variation in size and structure; $\times 16$.

PLATE 2

Fig. 5. An L-form colony with a double core and peripheral vacuoles. Dienes's stain with light microscopy; $\times 320$.

Fig. 6. One-day growth of an L-form colony. Periphery in focus demonstrating many intermediate size phase-dense bodies. Dienes's stain with phase microscopy; $\times 1600$.

Fig. 7. One-day growth of the same L-form colony shown in Fig. 6. Core in focus demonstrating many granular bodies. Dienes's stain with phase microscopy; $\times 1600$.

Fig. 8. Two-day growth of an L-form colony. Periphery in focus demonstrating well defined vacuoles. Dienes's stain with phase microscopy; $\times 1600$.

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