Experimental Infection with Parent and L-Phase Variants of *Neisseria meningitidis*

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Thirty-six strains of *Neisseria meningitidis*, including groups A, B, and C, produced L forms in vitro in the presence of an osmotic stabilizer and high concentrations of horse serum using penicillin as the transforming agent. Transformation to L growth occurred most readily among strains recently isolated from patients, and an unusually high rate of transformation was observed in 7 of the 36 strains. Revertant L strains developed diplococcal colonies on blood-agar and L colonies on sucrose-serum-penicillin-agar—always in a ratio of approximately 10 to 100 diplococcal colonies to 1 L colony. Using mucin as a host depressant, comparison was made between parent and revertant L strains of their initial pathogenicity and development of virulence by serial mouse passage. In general, revertant L strains showed the same pathogenic characteristics as the parent. Heart blood cultures from mice dying of infection with revertant L strains retained their ability to grow as L forms on penicillin media. Three stable L strains were completely avirulent for mice, although persistence of L forms could be demonstrated in peritoneal exudate for 6 days after inoculation.

The increased incidence of sulfonamide-resistant strains of all serotypes of *Neisseria meningitidis* among military (12) and civilian (3, 10) populations has limited the value of the sulfonamide drugs in the treatment of meningococcal disease and carriers of meningococcus. Moreover, failure of prophylactic treatment with penicillin and other antibiotics (1, 12) to eliminate meningococci permanently from the nasopharynx has suggested the possibility that penicillin-insensitive strains may also be present. Although the carrier rate is temporarily decreased during medication, it promptly returns to pretreatment levels after prophylaxis, despite the fact that the organisms recovered remain sensitive to the drug used as demonstrated by ordinary methods of in vitro assay. This investigation was undertaken to study the behavior of meningococcal L-phase growth in vitro and in vivo in an effort to determine whether transformation to L forms might be one mechanism which permits meningococci to survive during prophylactic therapy with penicillin.

MATERIALS AND METHODS

Strains of *N. meningitidis*. A total of 36 strains of meningococci were examined for their ability to undergo L transformation. Thirty-three were repre-

sentatives of groups A, B, and C, including fresh isolates from patients in the University of Chicago Hospitals and also carrier and stock strains obtained elsewhere. The remaining three strains included one each of the serotypes Boschard (20) and Slaterus' X and Y (21).

Media. The medium employed for the isolation and maintenance of *N. meningitidis* parent strains was Trypticase Soy Agar (BBL) enriched with 5% defibrinated sheep blood. Stock cultures were transferred weekly to freshly prepared blood-agar slants, incubated aerobically at 37 C for 18 hr, and then kept under refrigeration. The basic medium for the isolation and maintenance of L-phase variants was Trypticase Soy Broth (BBL), supplemented with 10% sucrose and, for solid medium, with 1.25% agar. After sterilization, the medium was completed by the addition of 20% unheated horse serum (Grand Island Biological Co., Grand Island, N.Y.) and 100 to 500 µg of benzyl penicillin per ml. Small plastic dishes (50 × 12 mm, Falcon Plastics, Los Angeles, Calif.) with box-type lids were used to prevent dehydration during prolonged incubation.

Mucin. A 4% suspension of hog gastric mucin (Wilson Laboratories, Chicago, Ill.) was prepared by the method of Miller and Bohnhoff (14) with slight modification. Just before use, it was adjusted to pH 7.2 to 7.4 with 1 N NaOH and diluted by 10% with sucrose-serum broth.

Mice and inoculation. CF₁ (Carworth Farms, New City, N.Y.) female mice, 9 to 10 weeks old, were used throughout this series of experiments. Inoculations of

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microorganisms suspended in mucin were made by intraperitoneal injection in 1-ml volumes.

RESULTS

Isolation of L-phase variants from parent strains of N. meningitidis. After many preliminary trials, the following method was found to be the most satisfactory for inducing transformation and maintaining growth in the L phase. A large loopful of the parent strain taken from an 18-hr blood-agar plate was streaked over the surface of sucrose-serum-penicillin-agar. The inoculum was then overlaid with 0.5 ml of the medium diluted 1:1 with sucrose-serum broth. The plates were incubated in a candle jar at 37 C. Cultures were examined carefully each day under 27 and 9 × magnification using transmitted light. Most strains of meningococcus produced classical L colonies after 3 to 5 days of incubation—usually 10 to 50 in number. A few strains, however, showed confluent L growth within 36 to 48 hr, whereas others required 7 to 10 days of incubation before colonies could be distinguished. Primary growth of L colonies always occurred in the semisolid overlay. Colonies in the overlay were transferred to fresh medium and disintegrated with the point of a sterile blood lancet; the inoculum was again covered with 0.5 ml of semisolid sucrose-serum-agar. After 5 to 10 subcultures, most L strains multiplied rapidly and luxuriantly, growing both in the semisolid overlay and also into the solid medium beneath. When growth had developed sufficiently, the cultures were removed from the incubator and kept under refrigeration in a candle jar. L strains were transferred to fresh medium at weekly intervals.

Ease of L transformation from strains of meningococcus. Transformation to L-phase growth could be induced in all strains of *N. meningitidis*, but the ease with which it occurred seemed to depend upon recent isolation from human material. All of the 12 strains cultured from patients in the University of Chicago Hospitals and examined within a few days of their isolation showed L-phase growth on primary exposure to penicillin (Table 1). This was also true of five fresh isolates received from the Great Lakes Naval Hospital. Of the remaining 19 strains which had been carried as stock cultures for unknown periods of time before they were received, approximately two-thirds produced L-phase growth on primary exposure to penicillin. Transformation to L phase was demonstrated in the remainder after repeated subcultivation of large inocula onto sucrose-serum-penicillin-agar.

Reversion of L colonies to the diplococcal form. When L colonies were transferred onto the surface of sucrose-serum-agar containing 1,000 µg of

TABLE 1. Occurrence of L transformation during primary exposure to penicillin among freshly isolated and stock strains of *Neisseria meningitidis*

Source of strain	Incidence of L transformation by group
Fresh isolates	
University of Chicago Hospitals	10/10 Group B
Great Lakes Naval Hospital, Ill. ^a	2/2 Group C
	2/2 Group B
	2/2 Group C
	1/1 Boschard strain
Stock cultures	
University of Chicago Hospitals	0/1 Group A
Naval Medical Research Unit #4, Great Lakes, Ill. ^a	2/2 Group A ^b
	3/4 Group B
	1/2 Group C
	0/1 Slaterus type X
	1/1 Slaterus type Y
National Communicable Disease Center, Atlanta, Ga.	5/8 Group A

^a Kindly supplied by D. W. Beno, Naval Med. Res. Unit #4, Great Lakes, Ill.

^b Sulfonamide-resistant strains isolated in Morocco.

^c Kindly supplied by D. Ivler, Infectious Disease Laboratory, University of California, Los Angeles, Calif.

penicillinase per ml, they reverted to growth containing gram-negative diplococcal cells. After 24 hr of incubation, the periphery of the L colonies developed a translucent type growth, which consisted of many swollen gram-negative cocci and diplococci, together with amorphous material and intensely stained round bodies.

Macroscopic slide agglutination tests (serum obtained from the National Communicable Disease Center, Atlanta, Ga.) were frequently done on revertant and stable L-phase growth. In all instances, the serotypes were identical to those of the parent strains.

Nonreverting L strains. Three L strains derived from group B meningococci stabilized after 30 to 35 weekly transfers on penicillin media; i.e., subcultures to penicillinase media failed to revert to diplococcal cells, and growth continued only in the L phase. Conversion to stable L growth in all three strains was characterized by increasingly rapid and luxuriant multiplication for several transfers preceding stabilization, whereas reversion to diplococcal growth on penicillinase media was delayed for 48 to 72 hr. One of the three stable strains grew equally well on the surface

of blood-agar as in sucrose-serum agar, although the L colonies developed slowly, requiring 4 to 7 days of incubation in a candle jar. The other two stable strains grew very poorly on blood-agar, producing sparse microcolonies after 10 days of incubation.

Growth characteristics of revertant L strains. Populations derived from reverted L-phase growth differed from the parent strain by retaining their ability to multiply as L colonies on sucrose-serum-penicillin-agar and by requiring incubation in a candle jar to initiate growth on blood-agar. When equal inocula of revertant L strains were subcultured onto blood-agar and sucrose-serum-penicillin agar, the numbers of colonies developing on each medium were always in approximately the same ratio; i.e., 10 to 100 on blood-agar to 1 on penicillin-agar.

Revertant L growth from penicillinase agar was carefully suspended in sucrose-serum broth. The suspension was diluted to a standard density containing approximately 10^8 cells/ml and titrated by 10-fold dilution through 10 tubes of broth. Appropriate dilutions were inoculated (0.1 and 0.05 ml) onto the surfaces of standard-sized blood-agar plates and sucrose-serum-penicillin-agar. The agar plates were incubated in a candle jar and the broth dilutions in an air-tight Mylar bag (Vac Pac, Inc., Baltimore, Md.), which had been filled with a mixture of 5% CO_2 in air. After 24 hr of incubation, samples from the last six dilution tubes were titrated and plated to determine the numbers of microorganisms in each.

The ratio of the numbers of organisms/ml, as determined by colony counts on blood-agar and sucrose-serum-penicillin-agar, remained approximately the same irrespective of the inoculum (Table 2). At zero-time, it was calculated that the 10^{-5} dilution tube contained 2.0×10^8 viable colony-forming units/ml, of which 2.6×10^2 were capable of growing as L colonies in the presence

of penicillin—a ratio of 1 in 7.7 of the total inoculum. This ratio did not change significantly throughout the dilution series after 24 hr of multiplication in broth. The final broth dilution showed no growth after 7 days of incubation, indicating that the actual inoculum was <1 viable cell in the total volume of 9 ml.

Similar results were obtained from standard growth curve studies in which the rates of multiplication from small inocula were determined. Suspensions of parent and revertant L strains were prepared as described and titrated by 10-fold dilution in sucrose-serum broth. The dilution tubes, containing approximately 10^8 colony-forming units/ml, were incubated under 5% CO_2 . Samples were removed at intervals during incubation, and the numbers of microorganisms were determined by plating appropriate dilutions in duplicate on blood-agar and sucrose-serum-penicillin-agar.

The rate of multiplication of the parent strain was compared with its revertant L strain (Fig. 1). The inocula/ml at zero-time were 2.09×10^8 for the parent strain and 1.43×10^8 for the revertant L strain. Of the latter, 1.44×10^2 grew as L colonies in the presence of penicillin—a ratio of 1 in 9.9. As shown by the stippled bars, this ratio did not change significantly throughout the 32-hr observation period. Comparison of the curves also shows that the generation time of the revertant L strain was somewhat greater than that of the parent strain. From these results, it seems evident that cultures of meningococcus, once having undergone L transformation, do not revert completely to the low potential for L-phase growth characteristic of the parent strain.

Comparative pathogenicity of parent and revertant L strains. Virulence tests with strains of *N. meningitidis* were made in mice, using 4% gastric mucin to depress host resistance. All mice which died during the working day were autopsied im-

TABLE 2. Comparison of numbers of colonies on blood-agar (diplococcal) and sucrose-serum-penicillin-agar (L form) after 24 hr of growth of revertant L strain in liquid media^a

Estimated inocula/ml ^b		No. of colony-forming units/ml on		Colony ratio, no. diplococcal to one L form
Blood-agar	Sucrose-serum-penicillin-agar	Blood-agar	Sucrose-serum-penicillin-agar	
2.0×10^8	2.6×10^2	7.5×10^7	0.47×10^7	15.9
2.0×10^2	36.0	6.8×10^7	0.6×10^7	11.3
20	2.6	6.3×10^7	0.34×10^7	18.5
2.0	0.26	6.8×10^7	0.36×10^7	18.9
0.2	0.026	5.7×10^7	0.1×10^7	57.0
0.02	0.0026	0	0	—

^a Trypticase Soy Broth supplemented with 10% sucrose and 20% unheated horse serum.

^b From colony counts on blood-agar and sucrose-serum-penicillin-agar at zero-time.

FIG. 1. Multiplication of parent and revertant L strain in serum-sucrose broth. Solid line plots growth of parent strain determined by colony counts on blood-agar. Dotted lines plot growth of revertant L strain on two media: diplococcal colonies on blood-agar (dashed-dotted lines) and L colonies on serum-sucrose-penicillin-agar (dotted lines). The stippled bars represent the ratio between the two counts at each observation time.

mediately, and cultures were made of heart blood and occasionally of spleen and peritoneal exudate on blood, eosin-methylene-blue-agar and sucrose-serum-penicillin-agar. The initial challenge inocula for parent strains were prepared to contain approximately 10^7 , 10^6 , and 10^5 diplococci and for revertant L strains 10^8 , 10^6 , and 10^4 colony-forming units. The actual numbers of microorganisms were determined from colony counts on blood-agar and sucrose-serum-penicillin-agar. Unless otherwise indicated, experiments were terminated after 3 days. Succeeding mouse passages were designed to provide a range of inocula that would kill all or most of the mice injected with the lowest dilution and permit survival with the highest. Subcultures for inoculations were always taken from several heart blood cultures and, in the case of revertant L strains, from growth on penicillin media.

A comparison has been made of the initial pathogenicity and development of virulence between 17 strains of *N. meningitidis* and their revertant or stable L variants. Table 3 indicates the serotype and history of the strains so far as they are known, and Table 4 summarizes the results of mouse inoculation tests. The most pathogenic strains were of serologic groups A and

C, and, in the case of the latter, despite the fact that three of the four were carrier strains. The initial challenge inoculum required to kill 100% of the mice with parent strains of group B ranged from 10^8 to 10^7 meningococci. Virulence of most strains could be increased by serial inoculation of heart blood cultures, and, in general, each successive passage increased virulence approximately 10-fold. Maximal virulence was defined as 100% mortality after inoculation with (i) 10^2 diplococci of parent strains within a mean death-time of 25 hr, and (ii) 10^3 colony-forming units of revertant L strains within a mean death-time of 30 hr.

The initial pathogenicity of revertant L strains depended upon that of the parent strain at the time of its isolation, and, in most instances, increase in virulence if it occurred was similar to the parent. The correlation of pathogenicity between parent and revertant L strains was demonstrated with strain B89 from which two L revertants were tested. Table 5 gives the results of inoculations, illustrating in detail the methods used during serial mouse passage. The parent strain attained maximal virulence at the 3rd passage, killing all mice injected with 1.1×10^2 diplococci within a mean death-time of 25 hr. The first L-strain isolate, b89₁, was obtained from the parent before mouse passage, and its pathogenicity remained unchanged during seven serial transfers in vivo. A second L variant, b89₂, isolated from the parent after its 9th passage in mice, showed a virulence comparable to the strain from which it was derived.

Strain B72 was exceptional in that the parent strain agglutinated with anti-B serum, but its L variant with anti-C. At the time, it was the only strain in our laboratory, and it was reported by two independent investigators to be group B and group C. When we finally obtained typing sera, we found it to be group B. In view of these limited observations, we offer the explanation that the original culture was a mixture of groups B and C microorganisms and that the primary L-colony isolate happened to be group C. All subsequent L isolates from the parent were group B. Cultures containing two or more serotypes have been observed by Slaterus et al. (21) and by Beno (*personal communication*) in nasopharyngeal samples taken from military recruits.

Pathogenicity of stable L cultures. Stable L strains b177, bKN, and b291 required inocula of 10^8 or greater to kill mice. Rapid serial passage of strain b177 through 18 subcultures in mice failed to increase its virulence, although persistence of the microorganism could be demonstrated in the heart blood for 3 days and in peritoneal fluid and spleen for 6 days after inoculation. Such prolonged survival of inocu-

TABLE 3. *History of strains of Neisseria meningitidis tested in mice*

Group	Strain no.	Source	Diagnosis	No. of weekly subcultures between L transformation and mouse inoculation
A	22	Unknown ^a		6
	25	Unknown ^a		4
	525	Unknown ^a		20
	565	Unknown ^a		60
B	72	Cerebrospinal fluid	Meningitis	10
	88	Cerebrospinal fluid	Meningitis	25
	89	Cerebrospinal fluid	Meningitis ^b	25
	94	Cerebrospinal fluid	Meningitis ^b	25
	KN	Cerebrospinal fluid	Meningitis	44 ^c
	91	Blood	Chronic meningococemia	2
	177	Nasopharynx	Carrier	55 ^c
	179	Nasopharynx	Carrier	55
	291	Nasopharynx	Carrier	80 ^c
C	95	Blood	Meningococemia	4
	1	Nasopharynx	Carrier	20
	2	Nasopharynx	Carrier	10
	158	Nasopharynx	Carrier	25

^a Stock strains from National Communicable Disease Center.^b Fatal meningitis.^c Stable L strains.TABLE 4. *Comparison of initial pathogenicity and development of virulence by serial mouse passage of parent and revertant L strains of Neisseria meningitidis*

No. of serial mouse passages to attain maximal virulence ^a	Initial challenge inocula ^b						
	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷	10 ⁸
Initial	A22 C1 C158 b89 ₂ A25	a565 c1					
1st		A525 A565 C95 c95 B177 BKN B94					
2nd			a22	a25			
3rd			B89	a525 B88 b88	B72 B291 c72		
4th					B91 b94		
6th					b91		
Some increase in virulence							
No increase in virulence						B179 C2 b179 c2	b89 ₁ c158 b177 ^c bKN ^c b291 ^c

^a Maximal virulence defined for parent strain (A, B, C), 100% mortality with 10³ organisms within a mean death-time of 25 hr; revertant L strain (a, b, c), 100% mortality with 10³ organisms within a mean death-time of 30 hr.^b Least number of organisms resulting in 100% mortality within 3 days.^c Stable L strains.

TABLE 5. *Initial pathogenicity and development of virulence by parent strain B89 and two revertant L-strain isolates^a*

Strain	No. of mouse passage	No. of organisms inoculated	Log of inocula							
			10	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷	10 ⁸
B89	Initial	2.4			2/5 (35)		5/5 (21)		5/5 (17)	
		1.4 ^b		1/3 (42)		3/3 (28)				
	1st	1.5		4/4 (32)	4/4 (30)	4/4 (28)				
	2nd	1.8	3/3 (31)	3/3 (28)	3/3 (24)					
	3rd	1.1	3/3 (38)	3/3 (25)	3/3 (21)					
	9th	2.1	3/3 (24)	3/3 (22)	3/3 (20)					
b89 ₁	Initial	3.3				0/5		0/5		5/5 (20)
	1st	2.1						0/4	0/4	4/5 (22)
	2nd	1.1							0/4	2/4 (37)
	3rd	2.7							2/6 (32)	5/6 (21)
	7th	3.0						0/6	1/6 (20)	5/6 (23)
b89 ₂	Initial	2.0				4/4 (22)		4/4 (15)		4/4 (15)
		2.3 ^b	4/4 (26)	4/4 (22)	4/4 (21)					

^a Results are expressed as number of mice that died/number inoculated, with the mean time to death (in hr) given in parentheses.

^b Repeat inoculation.

lated organisms did not occur with the avirulent parent strain B179. Mice injected with 10⁶ diplococci, a 10-fold dilution of the fatal inoculum, were killed for culture at daily intervals. The results showed that circulating blood was cleared of diplococci within 15 hr and the peritoneal exudate and spleen within 36 hr after inoculation.

There is some evidence that "stability" may be reversed in vivo after intraperitoneal inoculation of large numbers of L microorganisms. Strain bKN, which had been transferred eight times on penicillinase media without evidence of reversion in vitro, was inoculated into mice in the customary manner. On the 2nd mouse passage, one of six animals inoculated with 10⁶ cells and killed for culture of heart blood and peritoneal fluid 48 hr later showed a few revertant colonies on blood-agar. Cultures of heart blood and peritoneal fluids of the remaining five mice contained only the stable variant.

Growth of revertant L strains in vivo. Although revertant L strains multiplied in vivo as the diplococcal form, they retained their ability to grow on penicillin media. Cultures of heart blood were always positive on penicillin-agar if the inoculum contained sufficient numbers of microorganisms. Equal inocula of heart blood made on blood-agar and sucrose-serum-penicillin-agar showed that approximately 10 to 100 times as many colonies grew on blood-agar as on penicil-

lin-agar, a ratio comparable to that obtained during growth in vitro.

Growth of revertant L strains through bacteria-tight membranes. In a few experiments, cultures of heart blood were made on the surfaces of sterile bacteria-tight membranes (Millipore HA filter, 47 mm, 0.45 μ). At autopsy, a sterile membrane was laid on the surface of penicillin-agar and several drops of heart blood were placed in the center. Approximately equal inocula were also cultured on blood-agar, EMB agar, and sucrose-serum-penicillin-agar. After 5 days of incubation, the membrane was removed and the surface of the inoculated area was overlaid with semisolid agar containing penicillinase. The plates were reincubated and examined the following day for growth. In about half of such cultures, revertant L colonies developed at the site of the heart blood inoculum, demonstrating their ability to grow through a bacteria-tight membrane when they were in the L phase of multiplication. This phenomenon did not occur when such cultures were growing in the diplococcal phase, that is, when similar inocula were made on membranes covering sucrose-serum agar without penicillin. Nor did it occur with parent strains which had never been exposed to penicillin.

DISCUSSION

Transformation to L-phase growth occurs readily among all groups of *N. meningitidis* in the

presence of penicillin, an osmotic stabilizer, and high concentrations of horse serum. The medium and method of isolating L variants used differ from those of Roberts and Wittler (18), who employed a penicillin-gradient plate technique to isolate L forms from four strains of group B meningococci. More recently, D. W. Beno and Y. E. Crawford (Bacteriol. Proc., p. 70, 1967) reported the successful isolation of L colonies from 95% of 86 strains of *N. meningitidis*, using essentially the same techniques described herein.

We have shown that revertant L strains of meningococci obtained by subculturing L-phase growth in the presence of penicillinase have the potential to grow either on blood-agar as diplococcal colonies or on sucrose-serum-penicillin-agar as L colonies—always in the ratio of approximately 10:1 to 100:1. Among the strains examined in this study, seven isolated from patients produced innumerable L colonies within 36 to 48 hr after subculture on penicillin agar. It is tempting to speculate that these strains were actually L-form revertants, since their incidence of transformation to L colonies was so high, approaching the rate of occurrence observed in vitro with laboratory revertant strains. The possibility that such revertant L strains exist in nature becomes more likely, in view of the fact that transformation to L-phase growth can result from many and diverse influences (2, 5). It has been shown in mice, for example, that L transformation occurs in vivo after inoculation with group A streptococci (16) and *Bordetella pertussis* (22). L-phase variants have also been implicated in chronic staphylococcal (4) and recurrent urinary-tract infections (6).

Among meningococci, revertant strains produced in vitro do not lose their increased potential to propagate in the L phase even after subcultivation in the absence of conditions favorable to L-phase growth. Revertant L strains of *N. meningitidis* have never been observed to revert to cultures showing the same low incidence of transformation to L colonies demonstrated by the parent strain. It has been shown both in vitro and in vivo that approximately 1 of every 10 to 100 revertant cells of meningococci is capable of multiplying in the L phase. This ratio varies with the media and methods of cultivation employed, suggesting that the most suitable conditions for L-phase multiplication of *N. meningitidis* have not yet been determined.

The observation (13, 15) was confirmed that virulence for mice among meningococcal strains is not associated with either the source of isolation or the number of subcultivations on artificial media before experimental infection. In general, strains of groups A and C were the most virulent

despite the fact that most had been subcultured in vitro many times between isolation and mouse inoculation tests, and all but one of the group C strains had been isolated from the nasopharynx.

Although the mode of action by which gastric mucin interferes with the host's defense mechanism is still unclear (11, 17), its effect in potentiating meningococcal infection in mice is only transitory, diminishing 3 to 5 hr after injection into the peritoneal cavity (15). For this reason, pathogenicity of meningococcal strains which have a slow rate of multiplication, especially revertant L strains, cannot be so readily demonstrated. Nevertheless, with few exceptions, the initial pathogenicity and development of virulence during serial mouse passage by revertant L strains closely paralleled that of the parent.

Three L strains which had stabilized after prolonged cultivation in the presence of penicillin were completely avirulent for mice, although persistence of the microorganisms could be demonstrated in peritoneal exudate, spleen and blood. Deaths occurred only after injection of 10^8 cells and could be ascribed to endotoxin rather than infection. However, persistence of stable L strains within the peritoneal cavity indicates that the host's cellular defense mechanism is either inadequate or not responsive to this type of invasion. Similar experiments with an avirulent strain of parent meningococci showed that both the circulating blood and peritoneal cavity were cleared of diplococci within 36 hr and could not be isolated thereafter. Schmitt-Slomska et al. (19) described persistence of stable L forms of group A streptococci in blood and peritoneum of mice for 25 days after intraperitoneal inoculation.

None of the stable L strains of *N. meningitidis* has been shown to revert in vitro during routine transfer on sucrose-serum agar without penicillin. One of the strains, however, did revert in vivo and was isolated from cultures of heart blood and peritoneal fluid on blood-agar 48 hr after inoculation. This observation is similar to that of Schmitt-Slomska et al. (19), who were able to detect cocci in smears of blood and peritoneum by direct microscopy many days after intraperitoneal inoculation of group A streptococcal L forms. They were unable, however, to subculture these revertant cells on any of the ordinary media and could isolate viable cocci only after inoculation on yolk sacs of unembryonated hens' eggs. L variants of *Proteus mirabilis* cultured from the urine of a patient suffering from pyelonephritis reverted to the bacillary form only after subpassage in rats (6).

Rare reversions to gram-staining cells do occur spontaneously in vitro among stable L strains (2, 8), and alteration of the chemical and physical

environment influences their stability (9). The results described here and elsewhere seem to indicate that unknown conditions favorable to reversion were obtained *in vivo*, although in the case of group A streptococci the revertant cells have become much more exacting in their growth requirements (19).

The facility with which freshly isolated strains of *N. meningitidis* undergo L transformation *in vitro* and the observation that 20% of the strains examined showed an exceptionally high rate of transformation suggest that L-phase growth can occur *in vivo*. The possibility must be considered, therefore, that failure to eradicate meningococci from the nasopharynx by prophylactic treatment with penicillin may be due to L transformation. In a recent study of "penicillin-resistant" gonorrhea in man (7), transformation to L-phase growth has been proposed as an explanation for persistence of residual postgonococcal urethritis.

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