Production of L Forms of Group A Streptococci in Mice.* (30125)

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Sharp(1) reported that strains of group A streptococci were converted to the L form *in vitro* by inoculating streptococci onto solid nutrient agar containing horse serum, a high concentration of electrolyte and a gradient of penicillin. L colonies appeared after 3 or more days' incubation under anaerobic conditions. These L forms propagated aerobically on similar media containing high concentrations of penicillin and often reverted to the bacterial form following withdrawal of penicillin. Others(2) have confirmed these observations. The L form of the group A streptococcus has been shown to retain many of the biochemical characteristics of the original streptococcus(3), but the ability to construct a cell wall has been lost. Because the streptococcal L form has not heretofore been recovered from human or animal hosts, the role of this variant of the organism in the natural history of streptococcal disease is unknown. The following observations indicate that L forms of group A streptococci occur during experimental group A streptococcal infections in mice.

Material and methods. Adult white Swiss mice (Harvard strain) were inoculated intraperitoneally with 0.1 ml of a 7 to 8 hour culture of type 3 (Richards strain) or type 14 streptococci in Todd-Hewitt blood broth. At autopsy after death swabs of peritoneal exudate and heart blood were inoculated onto 5% sheep blood agar plates and onto a solid L form medium. This medium contained brain-heart infusion broth (Difco), 1.2% agar (Difco), 2% sodium chloride, 10% horse serum, 0.5% yeast extract (Oxoid), cyclohexamide (Upjohn) 0.01%, and penicillin (1,000 U/ml). The sheep blood agar plates were observed for *beta* hemolytic streptococci and other organisms after 18 to 24 hours incubation at 37°C, and the plates of L form medium were examined with a colony microscope (6.6 power) for L-type colonies after aerobic incubation at 37°C for 1 week. Agar blocks containing L colonies were cut out, inverted and transferred onto fresh plates of identical medium to propagate L forms.

Three methods were employed to determine whether the L forms were derived from the original group A streptococcus. First, the production of *beta* hemolysis was evaluated after transfer of agar slabs containing L colonies to L form medium to which 5% sheep blood had been added, or by incorporating blood and fragmented L colonies into pour plates of the same medium. Secondly, attempts were made to demonstrate that the L strains produced M protein of the same serologic type as that of the streptococcus originally inoculated into the mice. The method employed was a modification of a technique(3) in which L forms were grown in towers of agar on immunodiffusion plates. These agar towers were adjacent to wells which contained homologous and heterologous type-specific, absorbed anti-M sera(4). Lastly, in an effort to induce reversion to the bacterial form, L forms recovered from mice were transferred serially to L form medium free of penicillin and eventually to medium that contained no horse serum. Streptococci obtained by reversion of L strains were grouped and typed by standard techniques(4).

Results. Sixty mice succumbed to infection with the type 3 streptococcus (Richards strain) and 34 died after inoculation with the type 14 organism. Numerous streptococci were recovered from peritoneal exudate and

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TABLE I. Relation of Virulence for Mice of Inoculated Streptococci of Types 3 and 14 and Recovery of L Forms.

Maximum lethal dilution*	Dead mice	L forms recovered	
		No.	%
10^{0} to 10^{-2}	36	15	42
10^{-3} to 10^{-6}	58	2	3

* Greatest 10-fold dilution of 7 to 8 hr broth culture that was lethal to mice.

heart blood of all 94 mice. Typical L-type colonies, presumably derived from the original streptococcus, were recovered from 17 (18%) of the 94 mice after culture of peritoneal exudate and heart blood on L form medium. Ten isolations of L forms were from mice that were inoculated with type 3 streptococci and 7 from mice that received the type 14 strain. Nine isolations were from heart blood and 8 from peritoneal exudate. Usually 5 to 20 L-type colonies appeared; the maximum number of L colonies obtained from a single mouse was 60. These L strains propagated readily on transfer to L form medium with the development of typical macroscopic "fried egg" colonies, best observed with a colony microscope as described by other investigators(3). L forms which were not streptococcal in origin were recovered from 3 additional mice; cultures from these mice on sheep blood agar yielded Gram-negative bacilli in addition to streptococci. These L strains were non-hemolytic, were not shown to produce M protein by immunodiffusion and did not revert to group A streptococci.

Further examination of the data has indicated that repeated mouse passage of the streptococci and consequent enhancement of virulence were associated with a decreased rate of recovery of L forms. Table I shows the relationship between the recovery rate of L forms and the virulence of the injected organism as estimated by the greatest dilution of the 7 to 8 hour culture which was lethal for mice. L forms were recovered from only 2 mice succumbing to a streptococcal inoculum sufficiently virulent to kill at a dilution of more than 10^{-2}. Neither of these L strains was satisfactorily identified as a group A streptococcal L form.

Twelve of the 17 strains of L forms were shown to be derived from the original group A streptococci; 5 strains were lost due to contamination before all studies could be completed, although 2 of these were shown to be *beta* hemolytic. The conclusion that the remaining 12 L strains were derived from the original group A streptococcus was based on the following evidence. First, all 12 strains produced *beta* hemolysis. Secondly, a band of precipitate appeared by immunodiffusion against antisera to the M protein of the type of streptococcus originally inoculated, and not against heterologous types of anti-M sera. Lines of identity were observed between colonies of L forms derived from different mice injected originally with streptococci of the same type, including those L colonies that eventually reverted to group A streptococci. Finally, 3 of these 12 L strains isolated from mice reverted to group A streptococci after repeated passage on penicillin-free L form medium. Two of these strains, from mice inoculated with type 3 streptococci, were readily identified as type 3 organisms. The third strain, recovered from a mouse inoculated with type 14 streptococci, was also shown serologically to be derived from the original organism. This type 14 strain, designated PCA, was employed in subsequent mouse inoculation experiments and exhibited a remarkable propensity to assume the L form.

In contrast to these observations of the recovery of L forms from 17 of 94 mice inoculated with streptococci, L colonies did not appear on 22 plates of L form medium inoculated directly with the same broth cultures employed for inoculation of mice. On 6 occasions simultaneous inoculation of aliquots of a broth culture of streptococci into mice and onto L form medium resulted in L forms from the mice after death but not from direct inoculation of the medium.

Eighteen other strains of group A streptococci of various types were also tested for production of L forms by inoculation into mice. In experiments with 7 of these streptococcal strains, L forms were recovered from the first mouse inoculated; 3 other strains subsequently yielded L forms after several

attempts with different mice. A relationship between the colonial characteristics of the streptococci on sheep blood agar plates and the readiness with which L forms appeared was noted. Strains that produced glossy colonies were converted to L forms more frequently in mice than those that assumed matt characteristics. Streptococci producing glossy colonies were also converted to L forms more readily by the penicillin gradient technique (1).

It was necessary to obtain evidence as to whether streptococci were converted to L forms in the mouse, or whether L forms were induced *in vitro* by inoculation of large numbers of streptococci in blood and exudate onto penicillin-containing medium. Inoculation of blood and peritoneal exudate from mice dying of streptococcal infection onto L form medium free of penicillin yielded confluent streptococcal growth. Infrequently, small L-type colonies situated beneath the surface growth of streptococci were observed with the colony microscope. Although these L-type colonies propagated following transfer of slabs of agar containing them, it was necessary to transfer the slabs to L form medium to which penicillin had been added in order to free the preparations of streptococci. Accordingly, it was impossible to be certain that the L colonies that appeared on the recipient plate did not result at the time of transfer from conversion of surface streptococci by penicillin in the recipient medium.

More convincing evidence that the streptococcus was modified to the L form in the mouse was obtained by determining numerical ratios of L forms to streptococci recovered from mice and comparing these to similar conversion ratios obtained by simultaneous inoculation of broth cultures. These experiments were conducted employing the type 14 PCA strain that had been obtained in previous experiments by reversion of a mouse-induced L form. To obtain ratios of L forms to streptococci, heart blood and peritoneal exudate from mice succumbing to streptococcal infection with this strain were suspended in Todd-Hewitt broth and serial 10-fold dilutions made in the same broth. The initial suspension and the first dilution were in broth containing 2% sodium chloride to afford protection against osmotic lysis of L forms. Plates of L form medium were inoculated with 0.1 ml from each of the first 2 tubes, and 0.1 ml aliquots of higher dilutions were incorporated into pour plates containing trypticase soy broth (Difco) and 1.2% agar for counting of streptococci. Broth cultures of the same streptococci were simultaneously and identically diluted and inoculated onto the same media. Eight mice were studied; all yielded streptococci in large numbers from both peritoneum and heart blood at autopsy. L forms were recovered from the peritoneal exudate of all 8 mice inoculated and from the heart blood of 6. Table II shows the ratio of L forms to streptococci. The maximum rate was one L form per 750 streptococci from the heart blood of mouse 7, and the minimum rate (excluding the 2 specimens of heart blood that failed to yield L forms) was 1 per 3,000,000 streptococci from the peritoneal exudate of mouse 5. In contrast, direct inoculation of broth cultures onto 32 plates of L form medium resulted in no L colonies. The numbers of streptococci in each inoculum from the broth cultures varied from 6.5×10^6 to 6.3×10^7. These results suggested that the L forms from the mice were not produced by spontaneous variation alone.

TABLE II. Number of Streptococci Per Each L Form Recovered from Heart Blood and Peritoneal Exudate of Mice.

Mouse	No. of streptococci per L form	
	Heart blood	Peritoneal exudate
1	5,000	80,000
2	100,000	13,000
3	38,000	2,500
4	1,200	14,000
5	*	3,000,000
6	*	37,000
7	750	170,000
8	1,150	74,000

* Streptococci but no L forms recovered.

To obtain better substantiation that conversion of the streptococci took place in the mice and not as a result of penicillin contained in the L form medium, it was necessary to find a means by which the bacterial forms could be separated from the L forms,

TABLE III. Recovery of L Forms From Filtrates* of Blood and Exudate from Mice Inoculated with Type 14 PCA Streptococci.

Filter pore size	L forms recovered/mice cultured: Penicillin in medium	No penicillin in medium	Total
.80 μ	1/3	1/3	2/3
.65 μ	3/6	3/6	4/6
.45μ	0/5	1/5	1/5
Total	4/14	5/14	7/14

* No streptococci recovered from filtrates.

other than by killing the bacteria with penicillin. If separation could be achieved by other means, it was anticipated that L forms might be recovered on L form medium free of penicillin. To accomplish this, advantage was taken of the relatively small size and plasticity of reproducing L particles(5). Preliminary experiments with laboratory-induced L forms indicated that these organisms would pass through millipore membrane filters with pore sizes as small as 0.45 μ, although approximately 99% were lost in the process. In contrast, streptococci were completely retained by pores of 0.65 μ, and often by pores of 0.80 μ.

Peritoneal exudate and heart blood from 14 mice succumbing to infection with type 14 streptococci (PCA strain) were suspended in 5 ml of Todd-Hewitt broth containing 2% sodium chloride and 10% horse serum. After each suspension was passed through the sterile membrane filter, 0.1 ml aliquots of filtrate were inoculated onto a sheep blood agar plate and each of 4 L form plates, 2 containing penicillin and 2 without penicillin. On inoculation of media prior to filtration streptococci were recovered from both sites of all 14 mice; L forms were recovered in profusion from all unfiltered samples of peritoneal exudate and from the unfiltered heart blood of all but 1.

Table III shows the results of the cultures of the filtrates of peritoneal exudate and heart blood. No bacterial forms appeared on the sheep blood agar plates or on the penicillin-free L form plates on cultures from any of the mice. However, from 5 of these mice typical L form colonies but no streptococci were recovered on medium without penicillin; 2 other mice yielded L forms on penicillin-containing plates only. These L forms were recovered from the peritoneal exudate of 5 mice and from the heart blood of 3; 1 mouse yielded L forms from both sites. The numbers of colonies isolated from the 0.4 ml of filtrate inoculated from each site varied from 1 to 18. Broth cultures of the original organism, similarly suspended, filtered and inoculated, failed to yield L forms.

Identification of the L forms recovered from the mice was achieved in 2 ways after serial propagation. First, all 7 strains were shown to produce type 14 M protein by immunodiffusion. Secondly, reversion of L forms to streptococci was accomplished by immersing slabs of solid L form medium containing large numbers of L colonies in tubes of Todd-Hewitt broth to which 2% sodium chloride but no horse serum had been added, and incubating the tubes in a water bath at 37°C. The tubes of broth were observed daily and subcultures were made onto sheep blood agar when cloudiness suggestive of bacterial growth appeared. Preparations of all 7 strains recovered from filtrates yielded typical *beta* hemolytic colonies that were identified serologically as group A type 14 streptococci. In addition, several of the filtered L strains that were recovered on penicillin-free medium were propagated on medium with penicillin as well as medium without penicillin. After several passages on penicillin-containing medium to ensure that no unrecognized streptococci had been carried along, they were grown in the absence of the antibiotic and reversion to the bacterial form was attained.

Discussion. It is logical to conclude from the observations reported in these studies that group A streptococci were converted to the L form *in vivo* in the mouse. This conclusion is based on the recovery of L colonies on medium free of penicillin after separation from the streptococcus by filtration. The L forms isolated from mice exhibited typical L-type colonial characteristics, propagated readily in penicillin-containing medium, and eventually reverted to the bacterial form. In contrast, repeated inoculation of strains of streptococci in broth

directly onto L form medium failed to yield L forms.

Two observations indicate that the readiness with which L forms were produced was related inversely to the virulence of the streptococcal strain. First, L forms were recovered most frequently from mice injected with strains that require a large inoculum to produce a fatal infection. Secondly, strains of streptococci that grew on sheep blood agar as small, glossy colonies were more likely to yield L forms than those that grew as matt colonies. Because streptococcal virulence is related to the amount of M protein in the cell wall(6), it is possible that streptococci of low virulence have partially defective cell walls and are more easily stripped of the remainder of the wall.

Although these experiments indicate that the streptococcus was converted to the L form in the mouse, the observations do not demonstrate a mechanism by which conversion to the L form occurred, nor do they explain how these forms might escape phagocytosis or osmotic lysis. It is not known whether they persist or whether their presence might simply represent a transient stage in the destruction of the bacteria by the host.

It must also be considered that the appearance of L forms *in vivo* in the mouse does not require some active process on the part of the mouse. It is possible that rapidly-multiplying streptococci yield occasional L-type variants. In the usual hypotonic liquid or solid media these variants would promptly undergo osmotic lysis and thus fail to survive. *In vivo,* such variants might be defended against osmotic lysis by proteins or other substances that could provide protective coating for the organisms.

However, whether the streptococcus is actively converted to the L form by the mouse or whether the L form appears spontaneously and is simply protected against lysis by substances within the mouse, these observations do indicate that group A streptococcal L forms exist *in vivo* and that they may therefore play a role in the natural history of streptococcal disease.

Summary. Mice, inoculated intraperitoneally with lethal doses of group A streptococci, yielded L forms as well as streptococci from peritoneal exudate and heart blood at death. These L forms propagated readily and were recovered on penicillin-free medium after separation from the streptococci by differential filtration. They were shown to be derived from the original streptococcus, and usually appeared in mice inoculated with streptococci of relatively low virulence.

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