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Group A Streptococcal L Forms

I. Persistence Among Inoculated Mice

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ABSTRACT

L forms induced from two strains of group A *Streptococcus* were inoculated intraperitoneally and intravenously into conventional and germ-free mice. After intravenous injection, streptococcal L forms disappeared very quickly from the blood, whereas, after intraperitoneal injection, it was possible to isolate them as long as 25 days after inoculation. Observations suggest that a certain degree of reversion to a bacterial form may occur spontaneously in animals.

It is now a well-known fact that the L forms of bacteria do not exhibit the same virulence as the original bacteria. However, relatively few studies have been reported concerning the behavior and evolution of L forms experimentally introduced into animals.

The present study was started to investigate the ability of L forms of group A streptococci to live, persist, and possibly evolve in living organisms.

MATERIALS AND METHODS

Strains. Two strains of group A streptococci were used: GL8, unknown type (strain A 4514 of our collection), and Richards G3, type 3 (strain A 392 of our collection).

Media. After several trials, we used as basic medium Tryptic Soy Broth (TSB; Difco), enriched with 1% yeast extract (Difco), to which we added various factors as needed. For solid media (TSA), agar was added to TSB to a final concentration of 0.8%. Todd Hewitt Broth (Oxoid) and Todd Hewitt Blood Agar (1.5% agar, 5% defibrinated horse blood) were used as standard media for routine culture and identification of bacterial forms.

Induction and cultivation of L forms. Our technique for induction of L forms was derived from that of Sharp (5). Group A strains were cultivated in increasing concentrations of NaCl up to 0.61 M. The organism, in logarithmic phase, was then heavily inoculated on TSA petri plates, supplemented with 20% inactivated horse serum and penicillin at a final concentration of 1,000 units per ml. Between 5 and 10 days, subcultures were made daily on fresh media by the "sandwich" technique. After several subcultures, we obtained L forms able to grow and maintain themselves in solid and liquid media without penicillin. It was not always necessary to use anaerobic incubation for this induction.

Reversion of L forms. Neither strain used ever reversed spontaneously to its original bacterial form when cultivated in penicillin-free TSA, even after several subcultures.

Reversions generally occurred after several subcultures on TSB and 0.26 M NaCl, enriched with 10% of an extract made from egg yolk, coagulated (Difco). In some difficult cases, we inoculated yolk sacs of fresh unembryonated hen's egg, according to the findings made by one of us during the isolation and identification of atypical forms of bacteria in blood cultures (4).

Identification of streptococci. Group A hemolytic streptococci were identified by microscopic examination, cultural characteristics on Todd Hewitt Blood Agar, and Lancefield's serological method (2). The direct immunofluorescence technique was also used with anti-group A γ globulins labeled with fluorescein isothiocyanate (FITC).

L-form inocula for experiments. We inoculated 10 ml of TSB, 0.26 M NaCl, 20% horse serum, and 1,000 units per ml of penicillin with several "sandwiches" of a luxuriant L-form culture on TSA. After 3 to 5 days, this culture was used (in 10% volume) as inoculum for a larger amount (100 ml) of the same liquid medium. From 3 to 5 days after this second inoculation, this new culture was checked for the presence of living L forms, for the absence of bacterial streptococci, and for possible other contaminants; it was photometrically adjusted to an optical density of 0.200 (at 650 mμ) by dilution with sterile TSB. This constituted the inoculum for animals.

Mice. In the present study, we used 178 conventional and 29 germ-free Swiss albino mice. Mice, weighing 16 to 20 g, were inoculated either intraperitoneally (ip) with 1 ml or intravenously (iv) with 0.2 ml of the above-mentioned inoculum. Controls were inoculated with the same amount of sterile medium. Animals were sacrificed after intervals varying from 1 hr to 25 days. During autopsy, the following

materials were taken. From mice injected ip, peritoneal exudate, if present, peritoneal membrane (approximately 0.5 cm^2), and blood (approximately 0.5 to 1 ml) were taken. From mice injected iv, blood (0.5 to 1 ml) was taken. The material was spread on slides (for microscopic examination after staining by hot Giemsa and fluorescent serum) and was simultaneously inoculated on the following media: TSA, 0.51 M NaCl, 10% horse serum, and 1,000 units/ml of penicillin; TSA, 0.26 M NaCl, 10% horse serum, 1,000 units/ml of penicillin; TSA and 5% horse blood; TSB, 0.26 M NaCl, and 10% egg yolk; and Todd Hewitt Blood Agar.

Cultures were inoculated at 37 C. Plates were examined microscopically (× 150 and × 200) every day for 3 weeks. Subcultures were made on TSB, with and without penicillin, when an opacity developed in liquid medium or when something appeared on plates. Colonies resembling L forms were stained by Dienes' technique (1).

RESULTS

Lack of virulence. Thirty conventional mice inoculated ip with a dilution of original strains containing approximately 1,000 living organisms were dead or dying after 18 hr. The organisms were isolated from blood in every case.

In contrast, not one of the 126 mice inoculated with L forms induced from these strains, ip as well as iv, died spontaneously, even among the germ-free animals and even after several days.

Isolation of L forms from conventional animals. The results are quite different, varying with the manner of inoculation. When the mice were injected iv, we were able to isolate L forms only during the very early stages of the experiment. Of 30 inoculated mice, L forms were isolated from the blood of only three at 1 hr after injection. No growth occurred when the blood was taken later. No organism resembling L forms of bacteria was recovered from the 10 animals inoculated with sterile TSB as control. No evidence of bacterial streptococci was encountered in any of these cases.

Mice injected ip gave strikingly different results (Table 1). We were able to isolate L forms from almost all of the inoculated animals, and, most of the time, from both locations, the blood and the peritoneum, for as long as we continued our observations, i.e., up to the 25th day.

In the peritoneum, we observed a decrease in the percentage of positive results during the first stage, as well as in the apparent concentration of living L forms isolated. The small quantity of liquid noticeable during the first hours, and probably existing mostly in the rest of the inoculum itself, decreased rapidly. Practically no exudate was taken at 6 hr, after which L forms were isolated only from the cells of the peritoneal membrane. We find it remarkable that L forms were isolated from the blood stream after the 6th hr throughout the study. When freshly isolated from

TABLE 1. *Isolation of L forms from conventional mice after ip injection*[a]

Sacrificed after	Controls	L forms isolated[b]	Inoculation with strain		L forms isolated from		Bacterial forms			
			A 395	A 4514	Peritoneum	Blood	Observed in peritoneum	Identified as group A	Observed in blood	Identified as group A
1 hr	3	0	7		7	0	0		0	
2 hr	3	0	7		6	0	0		0	
4 hr	3	0	7		1	1	0		0	
6 hr	3	0	7		7	5	0		0	
8 hr	2	0	6		6	6	0		2	
1 day	7	0	7	6	13	13	1	1	9	1
2 days	4	0		5	5	5	1	1	3	1
3 days	4	0	2	3	4	4	2		4	
5 days	3	0		5	5	5	1		5	
7 days	3	0	3	2	5	4	2		3	
9 days	3	0		5	4	3	1		4	2
10 days	3	0		5	3	4	0		3	
15 days	3	0		5	4	5	0		2	
20 days	2	0		4	4	4	0		2	
25 days	2	0		4	4	4	0		1	
Total	48	0	46	44	78	66	8	2	36	4

[a] Figures give the number of animals.
[b] From peritoneum and blood.

the blood, they were frequently rather atypical, resembling globular forms or granular heaps, strongly refringent, and deeply embedded in agar (Fig. 1). After several subcultures, their appearance became more typical (Fig. 2), and they finally revealed their familiar appearance (Fig. 3).

Another interesting fact is that, after the 1st day, we were able to notice the presence of bac-

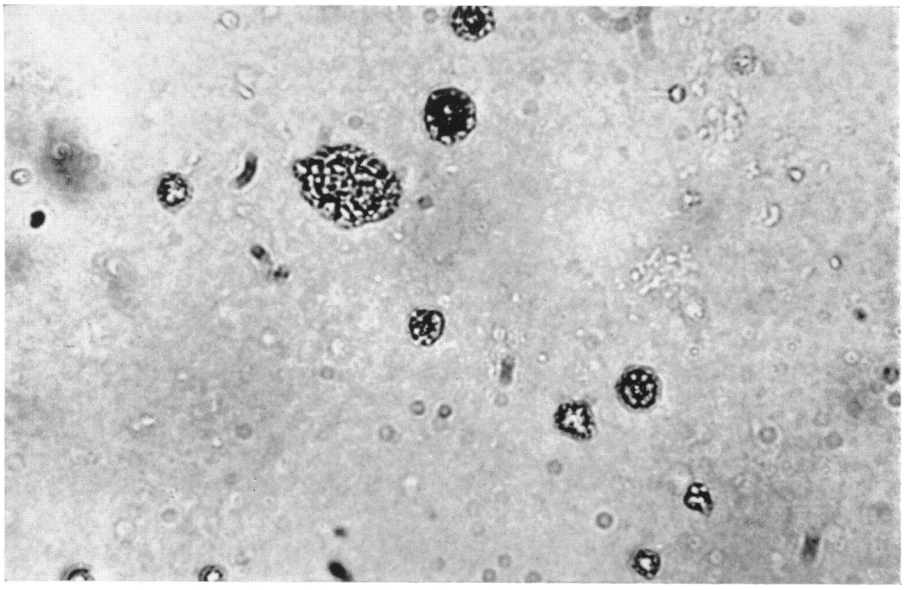

FIG. 1. *Atypical aspect of L forms isolated from mice inoculated ip; after first culture, microcolonies on TSA medium with serum and penicillin.* × *160.*

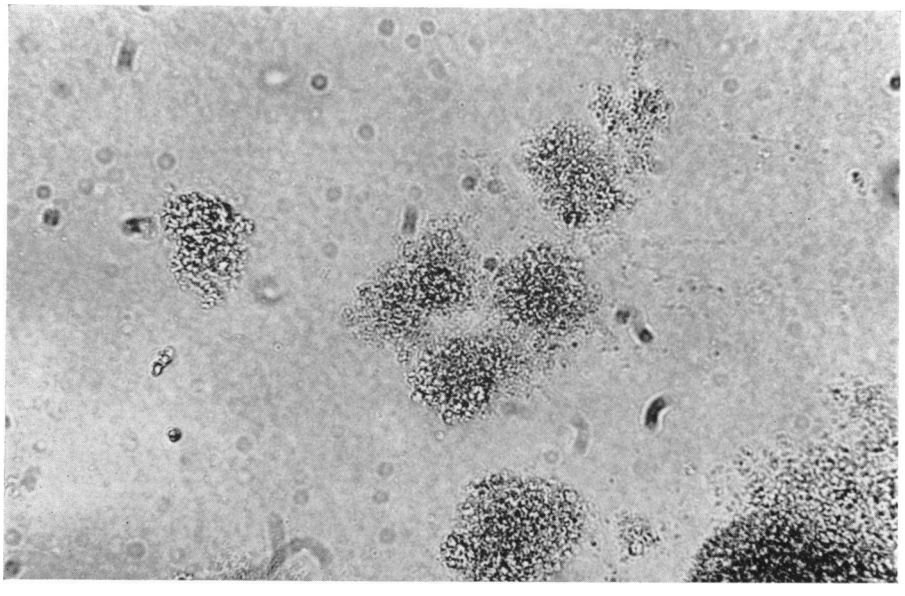

FIG. 2. *Same strain as in Fig. 1 after several subcultures on the same medium. Evolution towards a more typical aspect of the colonies.* × *160.*

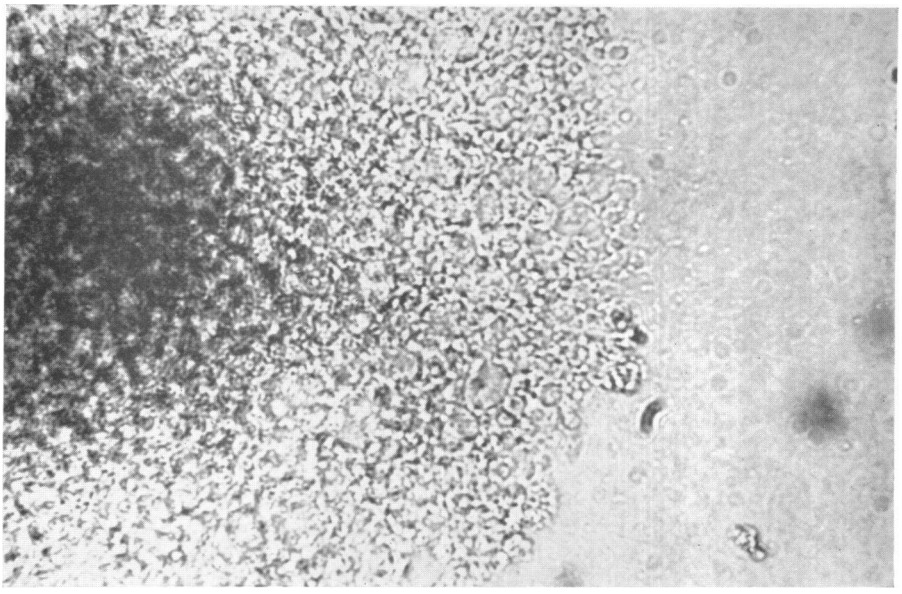

FIG. 3. *Typical appearance of L form colonies after additional consecutive subcultures.* × *160.*

terial cocci in blood as well as on peritoneum. These cocci, arranged sometimes by pairs and frequently in typical chains, were seen on direct slides and more often on enriched TSB petri plates, on which they did not multiply. Attempts to cultivate these bacteria on ordinary media, even enriched with yeast extract, blood, and serum, were negative. Inoculation on yolk sac was the only technique suitable for their cultivation. By this method, these organisms recovered their characteristics after five or six subcultures and were able to grow subsequently on ordinary media. Eight of these strains were treated this way. Two were lost before final identification; the other six showed their typical microscopical aspect and β-hemolysis, they gave specific precipitation with anti-A sera and immunofluorescence with FITC-labeled sera, and they showed the same virulence as the original strain. The number of manipulations involved in this technique did not allow us to try this kind of culture on all the cocci found in these series.

Experiments with germ-free mice. Since we had not yet obtained anti-L form fluorescent antisera, we had no direct proof that the forms we isolated were truly streptococcal. Therefore, one could object that some of our observations may have been due to spontaneous infection by other bacteria, L forms, or *Mycoplasma*, a possibility which is indeed not unlikely in conventional breeding. That is why we duplicated a part of this study with germ-free animals.

A careful preliminary examination of 14 of these mice failed to reveal the existence of any bacteria, L form, or *Mycoplasma*. The results of ip inoculation are shown in Table 2 and are comparable to those for conventional mice. One of the L forms isolated from peritoneal cultures reversed

TABLE 2. *Isolation of L forms from germ-free mice with ip injection of strain A 392*[a]

Sacrificed after days	Controls	L forms isolated from peritoneum and blood	Inoculated mice	L forms isolated		Bacterial forms		Identified as group A
				Peritoneum	Blood	Peritoneum	Blood	
5	2	0	5	5	3	2	5	2
10			5	4[b]	3	0	4	3
15	2	0	5	4	1	1	5	
Total	4	0	15	13	7	3	14	

[a] Figures give the number of animals.
[b] One isolate reverted to group A *Streptococcus*.

to the bacterial form and was identified as group A *Streptococcus*. Five atypical streptococci isolated from blood were able to grow in egg yolk-enriched TSB, and were identified as group A streptococci; the other nine gave weak cultures and were finally lost. We were not able to make yolk sac cultures in these series.

DISCUSSION

These data suggest that group A streptococcal L forms, when experimentally inoculated in mice, may persist for at least 25 days. (Our testing did not exceed this period.) Two facts prove indirectly that the isolated L forms are really identical to the inoculum. We never found them in control animals, and we isolated them from inoculated germ-free mice, otherwise free from any other bacterial contact.

The results differ as to the inoculation route. L forms injected iv disappear very quickly from the blood stream. We must point out, however, that in our series these animals were observed only 3 days. It may be that, after a temporary disappearance, they could be found again, if observations were prolonged as long as those for ip injections. In the latter case, as a matter of fact, continuous isolation of L forms was possible from peritoneum and blood stream. Precise evaluation of the density of isolated L forms is difficult, but a rough examination does not reveal any apparent decrease throughout the experiment. This leads us to suspect that this prolonged isolation does not result solely from survival of the inoculum. It appears that some degree of multiplication takes place, since a continuous inoculation of blood stream apparently occurs.

It seems also that isolated L forms differ somewhat in their cultural and metabolic characteristics from those of the inoculum, as if the contact with living cells induced some modifications. This has also been observed in present experiments in progress with tissue culture cells, but further investigations are needed for conclusive results.

Our observations also suggest the possibility of spontaneous in vivo reversion of L forms to original bacteria. We noticed, in a significant number of cases, *Streptococcus*-like bacteria on direct slides or in the first culture. These organisms were sensitive, very delicate, and difficult to grow. During the first subcultures, they were resistant to the high concentration of penicillin used in the medium. Adjustment on ordinary media and identification as group A streptococci could be achieved only by culture in yolk sac or in egg yolk extract broth. They then had the same characteristics as the original strains, including those of virulence, sensitivity to penicillin, and serological reactions, which were lacking in the early stage of these very peculiar bacterial forms. Because of these features, we cannot consider these organisms as common contaminants such as those one may occasionally find in conventionally bred animals. Spontaneous streptococci found in mice grow easily on ordinary media and almost never belong to group A. Besides, we wish to stress that our atypical bacteria were never found in control animals, whereas they were frequently encountered in inoculated germ-free mice.

There is no indisputable evidence that this partial reversion does actually occur in vivo. Since we observed these organisms more frequently in the first culture, one may object that the greatest part of this reversion occurred, in fact, in vitro. Against this interpretation, we point out that spontaneous reversion of well-induced streptococcal L forms never occurs in a single subculture on penicillin media. Mortimer showed recently that living streptococci of low virulence may spontaneously produce L forms in vivo (3). Facts reported by Wittler suggest that, in mice, L forms of *Haemophilus pertussis* may spontaneously revert to the bacterial form (6). Needless to say, if this evolution in both senses is firmly established by further experiments, this would be a major new trend in the understanding of the mechanisms of bacterial infection.

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