Persistence in Mice of the L-Phase of Three Streptococcal Strains Adapted to Physiological Osmotic Conditions

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SUMMARY

Inability of the L-phase of Gram-positive bacteria to persist *in vivo* is explainable by its need for osmotic protection. Adaptation to physiological osmotic conditions did not enhance the ability of the L-phase to survive *in vivo* after intravenous or intraperitoneal injection in mice. One 'low-salt' L-strain survived in mice for 8 days *in vivo* when protected against phagocytosis by a filter box, whereas under the same conditions its 'high-salt' parent L-strain died in a few hours. The decrease of the number of surviving colony-forming units of this low-salt L-strain in the filter box contrasts sharply with the increase in number shown by the parent bacterial strain protected in the same way.

INTRODUCTION

The L-phase of group A streptococcus derives its importance from its being mentioned in considerations about the cause of rheumatic fever (Watson, Hirst & Lancefield, 1961; McCarty, 1964) and also of rheumatoid arthritis (Lack, 1968). Isolation from patients of the group A streptococcal L-phase has been claimed (Kagan & Mikhailova, 1963) as well as the persistence in mice (Schmitt-Slomska, Sacquet & Caravano, 1967; Schmitt-Slomska & Mauss, 1968) and in cell cultures (Schmitt-Slomska, Boué & Caravano, 1968) of an L-phase induced *in vitro*.

Gram-positive bacteria require strong osmotic protection when in the L-phase and even the growth of the L-phase derived from Gram-negative bacteria generally is better on media with a higher osmotic value than bacteriological media usually have. Therefore the generally observed loss of pathogenicity resulting from the induction of the L-phase is plausibly correlated with the osmotic lability of the L-phase (Hijmans, van Boven & Clasener, 1969).

To obviate the difficulties of working with strains requiring osmotic protection in an environment which does not offer this protection, several L-phase strains were adapted to grow in physiological conditions. Adaptation *in vitro* of the L-phase of three Gram-positive bacterial strains to physiological conditions did not make their capability to survive *in vivo* comparable to that of their bacterial phase.

METHODS

Strains. The L-strains were induced by the use of penicillin from the following three parent strains: group A haemolytic streptococcus strain GL-8, type 19; group A haemolytic streptococcus strain VB, type 6, obtained from Dr H. A. Valkenburg of this

Institute; and *Streptococcus faecalis* strain B9, obtained from Dr L. B. Guze of the Los Angeles School of Medicine, and originally used for producing pyelonephritis in rats (Guze & Kalmanson, 1964; Clasener & Ensering, 1970a).

Media. Trypticase soy broth dehydrated (Difco) 3 % (w/v) or brucella broth (Albimi) 2.7 % (w/v); ion agar no. 2 (Oxoid) or agar Noble (Difco) 1 % (w/v) when solid medium was required; bovine albumin (for bacteriological media, Poviet, Amsterdam) 0.3 % (w/v); NaCl 2.2 % (w/v); yeast extract (Oxoid) 1 % (w/v). Penicillin was routinely added (1000–1500 units/ml.).

Adaptation of L-strains to media not giving the usual osmotic protection

Recently induced L-strains were first adapted to liquid media by lowering the agar concentration of the successive transfer media. Then the NaCl concentration in the liquid medium was generally reduced from 3 % (w/v) to 1.2 % (w/v) in steps of 0.1 % (w/v) and thereafter in steps of 0.01 % (w/v) at each transfer until a final concentration in the medium of 0.9 % (w/v) NaCl was reached. On the basis of one sodium determination of the trypticase soy broth we assumed the NaCl concentration to be 0.78 % (w/v); therefore we considered the strains to be adapted to physiological osmotic conditions when not more than 0.2 % (w/v) of NaCl had to be added to the medium.

Liquid to liquid transfers were made with 10 % (v/v) inocula in media that had been warmed to 37°. Care was taken not to shake the freshly inoculated cultures. On solid media the adapted strains grew in the characteristic L-colonies. The yield of the adapted L-strains in low-salt liquid media was inferior to that in the usual L-media and to that of non-adapted strains in their usual media. The adapted strains will be called in this paper 'low-salt' L-strains, in contrast to the non-adapted high-salt L-strains. The low-salt VB L-strain turned out to be transferable to blood agar plates, on which the colonies were macroscopically similar to those of the bacterial phase. However, less haemolysis is produced by the L-strain, and microscopic observation was necessary to disclose the characteristic morphology of an L-phase colony. Haemolysin production of the low-salt GL-8 L-strain also was less than that of the bacterial phase, and even disappeared altogether with successive transfers of this strain.

We were not able to revert the group A streptococcus L-strains adapted to low-salt media. Neither could we demonstrate M-antigen production by the method of Freimer, Krause & McCarty (1959). The parentage of the most interesting L-strain, the low-salt VB L-strain that grows on blood agar, was confirmed for us by determination of the DNA base composition (Hill, 1968): Dr W. R. Maxted (Colindale) extracted the DNA from the L-strain and from the supposed parent strain after making labile the cell wall of the latter by means of streptomycin enzyme (McCarty, 1952); Mr L. R. Hill (Colindale) determined the denaturation temperatures. Both samples of DNA were identical, with a denaturation ('melting') temperature (T_m) of parent strain DNA = 85.3°: % GC = 39.02 and a (T_m) of adapted L-strain = 85.2°: % GC = 38.78.

Mice. Random-bred Swiss mice of both sexes, weighing 25 to 35 g., were used.

Homogenization of mouse organs

The numbers of L-elements contained in various mouse organs, as a result of intravenous or intraperitoneal injection, were counted after homogenization of the organs in 4.5 ml. L-medium by means of an all glass hand-operated Potter homogenizer. Pour plates containing 15 ml. L-medium and receiving 0.5 ml. homogenate were

incubated at 37° for 5 days. In a model experiment, homogenization of L-strain suspensions together with kidney tissue did not decrease the number of colony-forming units. Therefore the homogenization procedure was considered to be innocuous for L-phase elements.

Tissues have been shown to contain inhibitors of micro-organisms (Skarnes & Watson, 1957); when tissue cells are damaged, substances lethal for mycoplasma (Tully & Rask-Nielsen, 1967; Kaklamanis *et al.* 1969) and L-elements (Minck, 1954) seem to appear. In our experiments, tissue homogenates in the dilutions cultured did not decrease the number or viable elements more than L-medium. We draw this conclusion from 105 min. survival experiments in various dilutions of mouse tissue homogenates, using a GL-8 L-strain that has been kept in our laboratory during more than 900 transfers on L-medium containing 3% (w/v) NaCl. The high number of L-elements isolated from the mice in the first minute after injection (see Results) is the best indication that homogenizing mouse tissues does not seriously harm the L-phase strains.

After intraperitoneal injections, organs to be cultured were first thoroughly washed in distilled water to free them of adherent L-elements or bacteria.

Filter boxes. In some experiments the L-phase elements of group A haemolytic streptococcus strain VB were protected against phagocytosis by enclosure in boxes covered with a 220 nm. Millipore filter. Round cover-glasses were glued with a two-component epoxy cement on one side of perspex rings (inner diameter 10 mm., outer diameter 15 mm., thickness 3 mm.). After filling the box with L-elements in molten agar, Millipore filters were glued with Millipore cement on the other side of the ring. The filter boxes were implanted in the peritoneal cavity of mice, directly or after incubation in liquid L-medium and removed at various times. Removal of the opaque filters permitted high power microscopic observation of undisturbed contents.

RESULTS

Intraperitoneal and intravenous injections

Tables 1 to 3 indicate the survival of bacterial phase elements of the three strains (table sections A), of the elements of the derived L-phase strains requiring osmotic protection (table sections B), and of the elements of the derived L-strains adapted to physiological osmotic conditions (table sections C).

From table sections A it can be concluded that bacterial phase elements may persist for at least 1 day even when the injected dose is less than 1/1000 of the lethal dose. In contrast to this, the high-salt L-phase elements of the same strains were killed within a few hours, as is shown in table sections B. Adaptation of the L-strains to physiological osmotic conditions did not enhance their survival in the animal body because low-salt L-elements also did not survive for more than a few hours, as is shown in table sections C.

Survival of VB-L elements in filter boxes

When filter boxes, inoculated with the low-salt L-strain of group A streptococcus strain VB, were first incubated in liquid L-medium for 1 day and then implanted in the mouse peritoneal cavity, the 1-day-old L-colonies did not develop any further as did the control boxes that were kept in liquid medium. However, a few viable elements could be retrieved even after 8 days. The morphology of these small colonies was

Table 1. *Number of colony-forming units per organ after injection of streptococcal strain GL-8*

Time after injection	Peritoneal cavity	Intraperitoneal route				Time after injection	Intravenous route			
		Lungs	Liver	Spleen	Kidneys		Lungs	Liver	Spleen	Kidneys
A 1	2 min.	75,000	490	210	200	A 2	2 min.	7,500	360	90
	20 min.	39,000	560	390	940		15 min.	1,960	0	20
	1½ h.	—	960	1,350	250		1½ h.	150	30	0
	4 h.	0	3,770	1,270	2,950		4 h.	100	20	10
	6½ h.	500,000	1,680	4,540	6,160		6½ h.	0	10	0
B 1	30 h.	0	10	0	170		30 h.	4,500	910	10
	2 min.	243,000	550	10	10	B 2	2 min.	6,120	110	10
	15 min.	30,000	10	0	10		15 min.	690	50	0
	30 min.	50	0	0	0		30 min.	0	0	0
	1 h.	165	0	0	0		1 h.	10	0	0
C 1	2½ h.	0	0	0	0		2½ h.	0	0	0
	2 min.	45,000	720	240	190	C 2	2 min.	225,000	13,500	10
	15 min.	180,000	10	0	0		15 min.	30,000	0	10
	40 min.	90	0	0	0		30 min.	140	0	0
	1 h.	0	0	0	0		1 h.	50	0	0
							2 h.	0	0	0

A = Bacterial phase, after injection of 10^4 c.f.u. (A1), or 6×10^3 c.f.u. (A2).B = High-salt L-phase, after injection of 1.2×10^6 c.f.u. (B1), or 7×10^5 c.f.u. (B2).C = Low-salt L-phase, after injection of 8×10^6 c.f.u. (C1), or 2×10^6 c.f.u. (C2).

Table 2. Number of colony-forming units per organ after injection of streptococcal strain VB

Time after injection	Peritoneal cavity	Intraperitoneal route				Time after injection	Intravenous route			
		Lungs	Liver	Spleen	Kidneys		Lungs	Liver	Spleen	Kidneys
A1	2 min.	12,760	150	12	232	A2	2 min.	4,650	30,000	60
	30 min.	12,750	1,500	2,400	387		30 min.	730	15,000	60
	1½ h.	9,450	6,750	884	452		1½ h.	160	3,600	4,410
	6 h.	75	3	52	0		5 h.	390	810	10
	24 h.	9	27	0	21		24 h.	0	50	930
B1	1 min.	—	15,000	2,430	1,040	B2	1 min.	27,000	82,500	9,750
	30 min.	1,962	0	0	0		15 min.	770	5,100	260
	1 h.	567	0	0	0		1 h.	20	10	200
	2 h.	3	0	0	0		2½ h.	0	180	10
	6 h.	0	0	0	0		5 h.	0	10	0
C1	2 min.	900,000	122	139	186	C2	2 min.	400	1,110	200
	1 h.	744	9	11	43		15 min.	30	530	0
	2 h.	295	6	3	5		½ h.	40	490	0
	4 h.	2	0	0	0		1½ h.	10	0	0
	5 h.	0	0	0	0		2½ h.	0	0	0

A = Bacterial phase, after injection of 10^6 c.f.u. (A1), or 9×10^4 c.f.u. (A2).
 B = High-salt L-phase, after injection of 4×10^6 c.f.u. (B1), or 4.2×10^7 c.f.u. (B2).
 C = Low-salt L-phase, after injection of 4×10^6 c.f.u. (C1), or 3×10^6 c.f.u. (C2).

Table 3. *Number of colony-forming units per organ after injection of streptococcal strain B9*

Time after injection	Peritoneal cavity	Intraperitoneal route				Time after injection	Intravenous route			
		Lungs	Liver	Spleen	Kidneys		Lungs	Liver	Spleen	Kidneys
A1	2 min.	420,000	1,592	920	1,800	A2	2 min.	57,000	60,000	24,000
	20 min.	30,000	899	1,048	1,232		40 min.	4,170	32,400	28,650
	1½ h.	1,200	71	880	66		1½ h.	920	145,800	3,690
	3 h.	158	15	660	88		4 h.	3,530	10,520	3,490
	6 h.	208	10	184	31		6 h.	880	15,000	500
B1	24 h.	3	1	45	4		24 h.	230	2,710	960
	2 min.	450,000	300	19,500	1,610	B2	2 min.	4,500,000	900,000	96,000
	20 min.	156	0	60	0		30 min.	18,000	60,000	600
	40 min.	102	0	0	0		1 h.	290	13,500	76
	1 h.	57	0	0	0		3 h.	20	350	8
C1	2 h.	0	0	0	0		6 h.	60	216	0
							24 h.	0	0	0
	3 min.	450,000	7,500	22,500	1,230	C2	5 min.	360,000	31,000	42,000
	30 min.	450,000	930	4,180	1,150		45 min.	750	4,100	52
	1 h.	24	0	760	10		2 h.	20	44	14
	2 h.	0	0	0	10		4 h.	0	10	0
	4 h.	0	0	0	0		6½ h.	0	2	0
							24 h.	0	0	0

A = Bacterial phase, after injection of 3.2×10^6 c.f.u. (A1), or 3×10^6 c.f.u. (A2).B = High-salt L-phase, after injection of 4.4×10^7 c.f.u. (B1), or 8.3×10^7 c.f.u. (B2).C = Low-salt L-phase, after injection of 2.2×10^7 c.f.u. (C1), or 3.2×10^7 c.f.u. (C2).

preserved too. This is in contrast to the ghost-like appearance that resulted when high-salt L-colonies were implanted intraperitoneally in filter boxes. Furthermore, viable elements could be retrieved from implanted high-salt L-colonies of this strain for only a few hours.

When filter boxes were inoculated with low-salt vb L-elements in agar and implanted without pre-incubation in liquid L-medium, no colonies developed.

DISCUSSION

The cell wall-damaging capacity of serum factors has been studied more extensively (Muschel, 1968) than their action on the cell membrane, for example their lethal action on the L-phase (Kalmanson, Hubert, Montgomerie & Guze, 1968). The action of serum on the L-phase probably depends on the strain: while the GL-8 low-salt L-strain, protected in filter boxes, died in mice almost as fast as in distilled water (Clasener & Ensering, 1970*b*), suggesting a lethal action, the vb low-salt L-strain, in the filter box experiment described above, seemed to suffer only a bacteriostatic action from the serum as has been described for dermatophytes (Lorincz, Priestley & Jacobs, 1958).

In preliminary experiments, we found that 20% fresh rat serum in the medium did not sustain growth of the low-salt GL-8 L-strain in the same way as did 20% old rat serum (Seitz-filtered and stored at -20° for several weeks). Heating fresh rat serum for 20 min. at 56° did not abolish its inhibiting action, neither did addition of 12% fresh guinea-pig serum make the old rat serum inhibitory. These phenomena were observed not only on agar media that is anticomplementary, but also in liquid media. Therefore the growth-inhibiting action is not dependent on complement. Moreover, it is not a lethal action, since with longer incubation growth occurred.

We think that bactericidal action of fresh serum (Kalmanson *et al.* 1968) may play a role in killing some L-strains, but that serum contains bacteriostatic factors not identical to complement nor to lysozyme, which, in preliminary experiments, did not act on our L-strains.

Osmotic lability is not the only reason for the L-phase to perish in the mouse; as reported elsewhere (Clasener & Ensering, 1970*b*), the GL-8 low-salt L-strain, protected in filter boxes, died in mice faster than in physiological salt solution. Furthermore, osmotic stabilization does not abolish the lethal action of serum (Kalmanson *et al.* 1968).

In spite of these considerations, we think that the effect of phagocytosis surpasses all serum actions. This is indicated by the fact that, in the experiments described above, there is no difference in survival between the GL-8 low-salt L-strain and the vb low-salt L-strain upon intraperitoneal or intravenous injection. In this respect it is interesting that fluorescein labelled GL-8 membranes have been found to disappear within 72 h. after intramuscular injection of mice (Rickles *et al.* 1969).

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