OSMOTIC FRAGILITY AND MOUSE VIRULENCE IN ENTEROCOCCAL L-FORMS

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THE stable L-forms of pathogenic bacteria are much less virulent than the parent organism.

Penicillin-induced L-forms of *Staphylococcus aureus* do not infect mice, rabbits or guinea-pigs even when doses of 1.2×10^{10} organisms are injected intraperitoneally (Prozorovskii, 1959; Kagan *et al.*, 1963; Pratt, 1965; Godzeski, Brier and Farran, 1967). These L-forms require high salt concentrations for growth *in vitro*, and Young and Dahlquist (1967) suggest that osmotic fragility accounts for the rapid disappearance of staphylococcal L-forms injected into rabbits. However, Schmitt-Slomska, Sacquet and Caravano (1967) have shown that osmotically sensitive L-forms of group-A streptococci can be recovered from the peritoneal cavity of mice as long as 25 days after challenge.

One suggestion is that spermine, present in mouse blood at a concentration of 25–100 μg per ml (Rosenthal and Tabor, 1956) might stabilise the L-form membrane *in vivo*, because Gooder (1964) has reported that up to 20 per cent. of enterococcal protoplasts survived suspension in hypotonic media if pretreated with 5 μg per ml spermine. Another possibility is that L-forms in the kidney resist osmotic shock under acid conditions; Gnärpe and Edebo (1967) have shown that osmotically fragile protoplasts of *Escherichia coli* and *Proteus vulgaris* remain viable in urine at pH 5.0–5.5. Osmotic fragility cannot be the only factor responsible for the avirulence of L-forms because the L-forms of *Streptobacillus moniliformis* and *Salmonella typhimurium* grow on standard media approximately isotonic with serum although they are non-pathogenic for mice (Klieneberger, 1938; Silberstein, 1953). Perhaps these L-forms have lost surface antigens responsible for the virulence of the parent organism.

The aim of the present work was to determine the effect of spermine and increased hydrogen-ion concentration on the minimal osmotic requirements for the induction and growth of penicillin-induced L-forms of *Streptococcus faecalis* and to study the ability of the L-form to survive in mouse serum and urine and initiate infection in mice challenged by various routes (see also Watt, 1969).

MATERIALS AND METHODS

Culture media. The basal medium ("L-form agar") used for the culture of L-forms consisted of Difco Brain Heart Infusion broth solidified with 1 per cent. (w/v) Oxoid Ionagar no. 2 and containing 10 per cent. (v/v) sterile horse serum. For routine use the osmotic strength was increased by adding salt to a final concentration of 0.5M-NaCl. NaCl or KCl was added to the basal medium to give a series of L-form media with the osmotic protection increasing in steps of 50 m.osmoles per kg from 400 to 1000 m.osmoles per kg. The osmolality was observed with a Fiske osmometer. Urea-based medium was prepared by mixing equal volumes of double-strength basal medium with 3M urea previously sterilised by membrane filtration. This full-strength urea medium was diluted with basal medium to give a series of urea media with osmolality increasing in steps of 50 up to 1500 m.osmoles per kg. For "L-form broth" the agar was omitted.

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Culture of L-forms. Strain B of *Streptococcus faecalis* was isolated from a patient with endocarditis and identified by the presence of the group-D antigen and its ability to ferment sorbitol but not arabinose. To enhance their virulence for mice the enterococci were injected intravenously into mice and recovered from the kidneys 72 hr after challenge. After a second passage the kidneys were removed aseptically, emulsified in 16 per cent. (v/v) glycerol broth and stored at -20°C . L-forms were induced in liquid culture by the method described by Hamburger and Carleton (1966). One drop of an overnight culture of enterococci was inoculated into 100 ml of "L-form broth" containing 500 μg penicillin per ml. The effective antibiotic concentration was maintained by adding 0.1 ml of a 500 mg per ml solution of penicillin on alternate days. Cultures were incubated at 37°C and kept for 2 mth or until growth became apparent. The growth was readily sub-cultured on "L-form agar" without penicillin and did not revert to the parent organism even on the gelatin-based medium described by King and Gooder (1965). Stock cultures of L-forms in broth without added penicillin were kept at 37°C and remained viable for some 4-6 wk. To prepare the L-form cultures used in subsequent experiments one drop of the stock culture was added to 100 ml of "L-form broth" with an osmolality of 750 m.osmoles per kg. The broth, in screw-capped bottles, was incubated at 37°C without shaking. The concentration of L-forms reached 10^7 per ml of broth after 4 days' incubation. The L-form preparations used in each experiment consisted exclusively of L-forms, for they gave typical L-form colonies on serum agar containing 0.5M-NaCl without added penicillin, but no colonies at all on unprotected, isotonic serum agar.

To determine the minimal osmotic requirements for growth, L-forms were induced on solid media containing varying concentrations of osmotic stabiliser. The plates were inoculated with 0.1 ml of an overnight culture of the enterococcus and a paper disk soaked in 10 per cent. (w/v) benzylpenicillin was placed in the centre of the plate. Plates were incubated in plastic bags to reduce evaporation.

Viable counts. L-form broth was delivered aseptically in 0.9-ml volumes with an automatic syringe and serial ten-fold dilutions of the L-forms were prepared with 0.1-ml pipettes. Sterile bent glass rods were used to spread 0.1-ml samples of the neat culture and successive dilutions of it over the surface of "L-form agar" plates. Plates were incubated for 3 days and viable counts were made with an electronic colony counter. Counts are expressed as logarithms to base 10. The average of six counts on a single culture was 3.82, with a range of 3.77-3.88. After homogenisation by repeated insertion of a teflon grinder (Pierce, Dubos and Schaeffer, 1953) revolving at about 5000 r.p.m., the average viable count increased to 4.27, with a range of 3.95-4.56. This finding suggests that a viable unit is composed of several L-form cells.

Mice. Male albino mice weighing 30-35 g were anaesthetised with pentobarbitone sodium and the anterior abdominal wall was shaved. In animals with an easily palpable bladder a 25-gauge needle was inserted and the urine aspirated. A volume of 0.1 ml of L-form culture was then injected into the bladder, the peritoneum or a vein. The mouse serum used in the experiments was collected from mice with enterococcal pyelonephritis and had an osmolality of 340 m-osmoles per kg. Pooled specimens of mouse urine collected by the suprapubic pressure technique of Silverstein (1961) were sterilised by membrane filtration. The urine had a sodium concentration of 152 m.equiv. per litre and a potassium concentration of 132 m.equiv. per litre. About 3 ml of urine was filtered in 1 cm dialysis tubing (Visking Cellophane) under 600 mm pressure for 2 hr and yielded 0.5 ml of ultrafiltrate.

RESULTS

Minimal osmotic requirements for L-form growth

On media containing NaCl or KCl occasional L-form colonies grew on plates with an osmotic protection of 650 m.osmoles per kg, but salt concentrations giving at least 750 m.osmoles per kg were required for optimal growth. This osmotic requirement was not reduced by the incorporation of 10 or 100 μg

spermine per ml into the medium. With medium prepared at pH 5.0, 5.5, 6.0 or 7.0 the degree of acidity did not influence the osmotic requirement for the induction of L-form growth, but the growth was partially inhibited at pH 5.0. L-forms could not be induced on urea-based media containing up to the growth-inhibiting concentration of 1.5M urea.

In liquid media typical viscid L-form growth appeared after some 20–40 days' incubation in all bottles where the osmotic protection exceeded

FIG. 1.—The survival of penicillin-induced L-forms of *Streptococcus faecalis* incubated *in vitro* at 37°C in samples of pooled serum from mice with enterococcal pyelonephritis that were: (1) protected osmotically by the addition of 0.5M sucrose, (2) unprotected osmotically but heated at 56°C for 30 min. to inactivate complement, and (3) unprotected and unheated.

750 m.osmoles per kg. This osmotic requirement was unaffected by the presence of spermine in concentrations of 10, 50 and 100 µg per ml.

Survival of L-forms in serum and urine

The survival of L-forms in “immune serum” obtained from mice with enterococcal pyelonephritis is shown in fig. 1. To reduce the possibility of agglutination of L-forms by antibodies present in the mouse serum the L-form cultures were diluted in the serum to a concentration of between 10^3 and 10^4 viable units per ml. All the L-forms survived in serum containing 0.5M sucrose, but in unprotected serum the rate of killing was not affected by inactivating the complement, suggesting that the rapid destruction of L-forms in unprotected serum was due to osmotic rupture and not to the action of complement. Since

FIG. 2.—The effect of the presence of different concentrations of spermine on the survival of enterococcal L-forms incubated in mouse serum at 37°C *in vitro*. The lines for 100 and 200 µg per ml of spermine were coincident with that for 50 µg per ml.

FIG. 3.—The survival of enterococcal L-forms incubated in pooled mouse urine at 37°C *in vitro* with and without the addition of M sucrose for osmotic protection.

the presence of sucrose did not prevent the natural antibodies present in rat serum from killing *Streptococcus faecalis* L-forms (Kalmanson *et al.*, 1966), it is unlikely that the effect of sucrose was to inhibit the action of antibodies on the L-forms. Spermine in concentrations of 50, 100 and 200 μg per ml had no osmotic stabilising action on L-forms suspended in mouse serum and at higher concentrations the polyamine was bactericidal (fig. 2).

The survival of enterococcal L-forms in urine was increased by the addition of molar sucrose (fig. 3) suggesting that L-forms were not completely stabilised in mouse urine. The gradual destruction of L-forms in osmotically protected

FIG. 4.—The effect of ultrafiltration on the survival of enterococcal L-forms incubated at 37°C in pooled mouse urine protected osmotically by the addition of M sucrose.

urine was not mediated by antibody because the lethal factor remained in ultra-filtered urine (fig. 4).

Virulence of enterococcal L-forms for mice

Intravesicular injection. Twenty-four mice were given intravesicular injections of 2×10^6 L-forms and killed in groups of four at 1, 2, 4, 8, 24 and 72 hr afterwards. Up to 8 hr after the injection, decreasing numbers of L-forms were recovered from bladder urine plated directly on L-form agar (table I). L-forms were isolated from broth cultures of the bladders of 15 out of 16 mice killed within 8 hr of challenge, but the bladders of animals killed at 1 or 3 days proved sterile.

Intraperitoneal injection. Twenty mice were given intraperitoneal injections of 2×10^6 enterococcal L-forms and killed in groups of four at 2, 4, 8, 24 and 72 hr afterwards. Samples of peritoneal membrane and heart blood were inoculated into "L-form broth" and the peritoneal exudate was plated on "L-form agar". The results (table II) show that the L-forms were rapidly cleared from the peritoneal cavity and did not invade the bloodstream.

The hypertonic L-form medium provoked a rapid migration of phagocytes into the peritoneal cavity and an average of 5.3×10^6 cells per ml was present in the exudate at 4 hr after injection. In mice receiving cortisone 1 mg twice daily by subcutaneous injection this inflammatory exudate was suppressed, but the clearance of L-forms from the peritoneal cavity was unaffected (table II), suggesting that the destruction of L-forms was not due to phagocytosis.

Intravenous injection. Twenty mice were given intravenous injections of 2×10^6 L-forms and killed in groups of four at 1, 2, 4, 8 and 24 hr thereafter. L-forms could not be recovered from the blood, kidney, spleen or liver of any animal.

TABLE I

Clearance of the L-forms of Streptococcus faecalis from the urinary bladder of mice: 24 mice were given intravesicular inoculations of 2×10^6 viable L-forms and killed in groups of four at intervals up to 72 hr

Mouse no. (in group of four)	Log ₁₀ no. viable L-forms per ml bladder urine from mouse at time (hr) after inoculation					
	1	2	4	8	24	72
1	4.95	4.63	2.82	2.3	<1.0	<1.0
2	2.66	3.72	3.43	<1.0	<1.0	<1.0
3	3.15	1.85	<1.0	<1.0	<1.0	<1.0
4	3.52	3.97	<1.0	<1.0	<1.0	<1.0
Mean for four mice*	3.57	3.55	1.56	0.56	0	0

* To calculate mean values the urines that appeared sterile were given an arbitrary value of 0.00 log units.

DISCUSSION

The *Streptococcus faecalis* L-forms required a minimum osmotic protection of 750 m.osmoles per kg for adequate growth, but mouse serum has an osmolality of only about 340 m-osmoles per kg and the destruction of L-forms in the serum can therefore be explained by osmotic lysis. Presumably this mechanism accounts for the rapid disappearance of L-forms injected into mice, because no antibodies capable of killing enterococcal L-forms could be detected in the mouse serum and treatment with cortisone, which inhibits phagocytosis (Cohn, 1962), did not affect the clearance of L-forms from the peritoneal cavity. These findings suggest that mouse tissues do not contain any system capable of osmotically stabilising L-forms, and the fact that spermine did not reduce the osmotic requirement for L-form induction or the survival of L-forms in mouse serum would support this conclusion. Harold (1964) found that enterococcal proto-plasts protected with spermine, though apparently morphologically intact, did in fact leak small-molecular-weight substances, such as alanine labelled with ¹⁴C and acid-soluble substances labelled with ³²P. He suggested that the loss of these substances from the cell might be an important element in the osmotic

stabilisation of the protoplast. Such a mechanism might explain how enterococcal protoplasts protected with spermine could resist and remain viable throughout a brief exposure to hypotonic conditions (Gooder, 1964), yet fail to multiply under these conditions.

TABLE II

Survival of L-forms injected into the peritoneal cavity and the results of culture of the peritoneal membrane and blood for L-forms in normal mice and mice receiving 1 mg cortisone twice daily

Time after injection of L-forms (hr)	Results in immunologically normal mice			Results in cortisone-treated mice		
	Log ₁₀ no. of viable L-forms per ml peritoneal exudate	Culture of peritoneal membrane	Blood culture	Log ₁₀ no. of viable L-forms per ml peritoneal exudate	Culture of peritoneal membrane	Blood culture
2	4.72	+	—	4.59	+	—
	4.53	+	—	4.93	+	—
	3.62	+	—	3.88	+	—
	3.75	+	—	3.60	+	—
4	3.47	+	—	2.76	+	—
	2.36	+	—	3.32	+	—
	3.00	+	—	2.51	+	—
	1.30	+	—	2.22	+	—
8	1.67	+	—	2.31	+	—
	<1.0	+	—	1.21	+	—
	<1.0	+	—	<1.0	+	—
	<1.0	—	—	<1.0	+	—
24	<1.0	—	—	<1.0	—	—
	<1.0	—	—	<1.0	—	—
	<1.0	—	—	<1.0	—	—
	<1.0	—	—	<1.0	—	—
72	<1.0	—	—	<1.0	—	—
	<1.0	—	—	<1.0	—	—
	<1.0	—	—	<1.0	—	—
	<1.0	—	—	<1.0	—	—

Since mice excrete a highly concentrated urine with an average osmolality of 2.6 osmoles per kg (Silverstein, 1961), it might be thought that L-forms would be osmotically stable in the urinary tract. However, urea cannot stabilise protoplasts (Mitchell and Moyle, 1956) and L-forms cannot be induced on urea-based media, so that total urine osmolality cannot be equated with osmotic protection. The concentration of sodium and potassium ions in the urine together with that of their corresponding anions, would provide an osmotic protection of some 600 m.osmoles per kg and although other substances such as ammonium ions (Gooder, 1968) stabilise protoplasts, enterococcal L-forms are

not completely protected in mouse urine as is shown by the fact that they survived longer in urine containing M sucrose. However, osmotic rupture cannot explain the rapid disappearance of L-forms injected into the mouse bladder; presumably many are voided in the urine.

Like enterococcal L-forms, the osmotically fragile L-forms of staphylococci and listeria are avirulent for mice (Prozorovskii, 1959; Louria *et al.*, 1967), whilst the L-forms of group-A streptococci do not survive in diffusion chambers implanted in rabbits (Haller and Lynn, 1968). In contrast, Schmitt-Slomska *et al.* (1967) recovered osmotically fragile group-A streptococcal L-forms from the peritoneal cavity of mice 25 days after challenge. However, bacterial cocci in pairs and chains were observed in direct smears of the peritoneum, suggesting that the L-forms might have reverted to osmotically stable forms in the mouse, but were recovered as L-forms on the penicillin-containing media used. Nevertheless, the coccal forms were not typical streptococci because they failed to grow on standard media.

SUMMARY

Osmotic protection by the addition of sodium chloride to the level of 750 m.osmoles per kg was required to support an adequate growth of penicillin-induced L-forms of *Streptococcus faecalis* on a serum agar medium. This requirement was not reduced by the addition of spermine or by the acidification of the medium.

L-form preparations that gave typical L-form colonies on serum agar containing 0.5M-NaCl without added penicillin, but no colonies on isotonic serum agar, were tested for the ability to survive in the serum and tissues of mice. The L-forms were rapidly killed when incubated *in vitro* in mouse serum unprotected by the addition of NaCl, and disappeared rapidly after being injected into the bloodstream or peritoneal cavity. Osmotic lysis was probably the cause of their death and disappearance, since the mouse serum did not contain antibodies capable of killing the L-forms, and treatment with cortisone, which inhibits phagocytosis, did not affect the rate of their clearance from the peritoneum. Although the L-forms slowly underwent lysis in mouse urine, ones that were injected into the urinary bladder were probably also removed to a large extent by the voiding of the urine.

The findings show that L-forms were not virulent in mice.

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