RESISTANCE OF BACTERIAL L-FORMS TO HEATING AND FREEZING

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The significance of bacterial L-forms in the etiology and pathogenesis of chronic and subacute infections [5, 8, 12] which often respond poorly to conventional therapy, demands a many-sided investigation of the characteristics of these forms of microorganism. From the epidemiological point of view it is important to elucidate their resistance to the action of unfavorable external factors including physical ones. There have been few such studies [11, 13, 14], these examining the resistance of only single species of L-forms to the action of separate physical factors.

In this study we have investigated the resistance of L-forms of a number of bacterial species—Str. hemolyticus, Pr. vulgaris, S. typhosa, S. typhimurium—original bacteria and form reversion to the action of heating and freezing.

METHODS

We used five strains of stabilized bacterial L-forms: two strains of the L-form of the hemolytic streptococcus (L196 and L409), one strain of Proteus vulgaris (LP), one strain of the enteric fever bacillus (L152) and one strain of murine typhus (L5710). Four of these cultures—L196, L409, L152 and LP—were provided for us by the department of general medical microbiology of the N. F. Gamaleya Institute of epidemiology and microbiology, and one strain—L5710—by the microbiology department of the N. I. Pirogov Second Moscow Medical Institute. The majority of L-cultures were artificially transformed by the authors at different times using penicillin [2, 4, 7, 10]. Strain L409 was isolated as an L-form from the blood of a patient with rheumatism and then reverted to the α-hemolytic streptococcus 409p [8]. The initial "parent" cultures were strains No. 10-S (β-hemolytic streptococcus), No. 3156 (Proteus vulgaris), No. 5606 (S. typhosa) and No. 5710 (S. typhimurium). In addition we used strain No. 4 β-hemolytic streptococcus, the L-forms of which died, during the experiments, but the reversion culture 190r remained. Four revertant cultures were used: 190r, 196r, 409r, RP, obtained from, respectively, L190, L196, L409, LP. The revertants of 190r, 196r and RP were described previously [9].

Then-day broth culture of the L-forms were centrifuged for 15 min at 3000 rpm, the supernatant liquid was decanted and the dense precipitate was carefully homogenized physiological saline added. The resultant suspension was brought to a concentration of 1 milliard viscosity (according to an optical bacterial standard), poured into test tubes and subjected to subsequent freezing or heating. Suspensions of twenty-four hour broth cultures of the bacteria (original and revertants) were prepared in exactly the same manner.

The thermoresistance of the culture was determined in the following manner: two ml of a one-milliard suspension of the original bacteria, L-form or reversion form in physiological saline were heated in a water bath at 55-56° for two to 60 min and at 60° for 30-60 min, then were quickly cooled to room temperature.

The resistance of these bacterial forms to freezing was investigated by cooling: brief freezing (1-2 hours) at -76° or prolonged freezing (up to $7\frac{1}{2}$ months) at -10°. In the former instance, suspensions of cultures in tubes of molybdenum glass rapidly (within two minutes) froze in Dewar flasks containing a mixture of dry ice and acetone, and then after 1-2 h were heated rapidly (within two minutes) by submerging in a water bath at 37°. In the second instance the suspension of the cultures in tubes were placed in a refrigerated chamber where they were slowly subjected (over several hours) to freezing at a temperature higher than eutectic (for sodium chloride) and after varying periods were removed and slowly thawed (5-7 minutes) at room temperature. After these treatments the culture suspensions were inoculated by 0.5 ml aliquots onto penicillin-containing or penicillin-free medium containing

TABLE 1. Comparative Resistance of L-forms, Original Bacteria and Reversion Forms to Heating

Species of bacteria	Type of culture	Strain	Temperature and duration of treatment (in min.)									
			55—56°								60°	
			2	5	10	15	20	30	45	60	30	60
Str. hem. β	Orig.	10—S	+	+	+	+	+	+	+	+	+	—
	L-cult.	L196	+	+	+	—	—	—	—	—	—	—
	Rev.	196 r	+	+	+	+	+	+	+	+	+	—
Str. hem. β	Orig.	4	+	+	+	+	+	+	+	+	+	—
	L-cult.		No culture									
	Rev.	190r	+	+	+	+	+	+	+	+	—	—
Str. hem. α	Orig.		No culture									
	L-cult.	L409	+	+	—	—	—	—	—	—	—	—
	Rev.	409 r	+	+	+	+	+	+	+	+	—	—
Proteus vulgaris	Orig.	3156	+	+	+	+	—	—	—	—	—	—
	L-cult.	LP	+	+	+	—	—	—	—	—	—	—
	Rev.	RP	+	+	—	—	—	—	—	—	—	—
S. typhosa	Orig.	5606	+	+	+	+	+	+	—	—	—	—
	L-cut.	L152	+	+	—	—	—	—	—	—	—	—
	Rev.		No culture									
S. typhimurium	Orig.	5710	+	+	+	+	+	+	—	—	—	—
	L-cut.	L5710	+	—	—	—	—	—	—	—	—	—
	Rev.		No culture									

0.3% or 1.3% agar and 10% serum albumin. For L-forms of streptococci 0.1% $MgSO_4$ and 15% sucrose were added to supplement the medium. In addition the bacterial suspensions in 0.05 ml aliquots were inoculated into liquid nutrient medium. For controls, a suspension of each culture which had not undergone the thermal experiment in the same volume was plated on similar media. Calculation of growth (colony counts, phase contrast microscopy, etc.) for L-forms was made after seven days of incubation, and for bacterial forms, after 24 h of incubation at 37°.

RESULTS

The results of these experiments are presented in Tables 1 and 2.

As seen from Table 1, the L-forms possess low thermostability and withstand heating to 55-56° only for two to ten min. The thermostability of L-forms partially depends on species characteristics and was considerably lower than for the original bacteria.

Inoculation of heated suspensions of L-cultures was usually accompanied by growth of a small number of L-colonies. As a rule, the diameter of the colonies appeared two to four times smaller than the control, not exceeding 0.2-0.5 mm. The L-colonies which formed from the heated culture consisted mainly of granular masses, tiny spheres, a small number of vacuoles and detritus. Only the culture of Proteus L-forms appeared comparatively resistant to heating. Plating of a suspension heated for ten min. Resulted in an abundant, deep growth of L-forms (tens of colonies) however, the typical superficial L-growth observed in the control, disappeared.

As seen from Table 2, in experiments with freezing the cultures the results obtained depended on the degree of cooling. With brief freezing at extremely low temperatures (-76°), the L-forms, original bacteria and revertants, independent of species affiliation, possessed the same resistance in absolute numbers. Only one strain of the L-form of the α-hemolytic streptococcus—L409—died after two hours at -76°. In the L-form of the S. typhimurium strain L5710, frozen for two hours at -76° and subsequently inoculated on semi-solid penicillin-free medium, one of the L-colonies then formed reverted to the original form. It was discovered, using phase contrast microscopy, at the central part of this colony were small, moderately and grossly homogeneous or vacuolated spheres and light refractile bodies,

TABLE 2. Comparative Resistance of L-forms, Original Bacteria and Reversion Forms to Freezing

Species of bacteria	Type of culture	Strain	Temperature and duration of treatment (in min.)									
			−76°		−10°							
			Hours		Days							
			1	2	1	3	6	12	30	100	140	225
Str. hem. β	Orig.	10–S	+	+	+	+	+	+	+	+	+	+
	L-cult	L196	+	+	+	—	—	—	—	—	—	—
	Rev.	196r	+	+	+	+	+	+	+	+	+	—
Str. hem. β	Orig.	4	+	+	+	+	+	+	+	+	+	—
	L-cult		No culture									
	Rev.	190r	+	+	+	+	+	+	+	+	+	+
Str. hem. α	Orig.		No culture									
	L-cult	L409	+	—	—	—	—	—	—	—	—	—
	Rev.	409r	+	+	+	+	+	+	+	+	+	—
Proteus vulgaris	Orig.	3156	+	+	+	+	+	+	+	+	—	—
	L-cult	LP	+	+	+	+	+	—	—	—	—	—
	Rev.	RP	+	+	+	+	+	+	+	+	—	—
S. typhosa	Orig.	5606	+	+	+	+	+	+	+	+	—	—
	L-cult	L152	+	+	+	+	+	—	—	—	—	—
	Rev.		No culture									
S. typhimurium	Orig.	5710	+	+	+	+	+	+	+	+	—	—
	L-cult	L 5710	+	+*	+	+	+	—	—	—	—	—
	Rev.		No culture									

Note: Orig) original culture; L-cult) L-culture; Rev) reverted (from L-form) culture; + presence of growth in culture which has undergone thermal treatment; (−) lack of growth.

*Under given conditions reversion was observed in culture L5710.

whereas at the periphery were a mass of non-motile rods, morphologically unchanged (we did not succeed in isolated pure culture of the revertants).

After prolonged freezing at moderately low temperatures (-10°) the L-forms appeared ten times less resistant (up to 1-6 days) that the corresponding bacterial forms (up to 100-225 days). The resistance depended on the species affiliation of the L-culture. Among the L-forms the most resistant were the salmonella and proteus strains (L152, L5710, LP), which could be preserved at -10° for six days, and the least resistant were the streptococcus (up to one day).

The original streptococcus (10-S) and revertant (190r) withstood -10° for 225 days, and the original salmonellae, proteus and its revertant, up to 100 days.

Discovery of the lower resistance of the L-forms in the last experiment was explicable, evidently, by the greater sensitivity of these cell-wall lacking forms to a long period of freezing and thawing, and also to the denaturing effect of liquid eutectic concentrations of sodium chloride [1], reaching up to 14 g% at -10°. The latter circumstance in the first place affects the L-form streptococcus, which is particularly sensitive to the qualitative and quantitative composition of the osmotic salt stabilizers in the medium [2].

In the original bacteria and the revertant cultures there was no principal difference in resistance at preservation at -10°. The data concerning resistance in salmonella preserved at -10° are similar to the results of other investigator [6].

It must be noted that upon comparing the L-forms with the original bacteria, the L-forms have a lower resistance to the effect of thermal factors, which, evidently results from the loss of the rigid cell wall, which functions

as defense in L-transformation. Certain differences in degree of resistance of separate L-cultures, probably, are determined by their various species origins and basic metabolic characteristics, preserved from the original "parent" cultures. In this connection, L-forms of bacteria with developed enzyme systems (P. vulgaris) exhibit greater vital capacity and resistance to thermal treatments. This upholds the rule of relatively unchanging forms of bacteria mentioned in the literature [3]. The lack of important differences in resistance of the cultures reverting from L-forms and of the original bacteria to heating and freezing, undoubtedly, is related to the restoration in the former of the rigid cell wall and other characteristics found in the original bacterial species.

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