CYTOPATHOGENIC ACTION OF L-FORMS OF CERTAIN PATHOGENIC SPECIES OF BACTERIA IN TISSUE CULTURE

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The existing method for studying the pathogenic properties of bacteria, based on the survival rate of sensitive animals after inoculation, is inadequate for the study of the pathogenicity of L-forms. The presence of filter-passing elements in the composition of L-forms of bacteria, with a diameter of 200-300 mμ, suggests that they may have a cytopathogenic action in tissue culture, thereby giving the L-forms pathogenic properties which are not revealed by the ordinary methods of infection of various species of laboratory animals.

There are only isolated reports in the literature [6,7] describing the discovery of 3 pathogenic strains of L-forms of the cholera vibrio possessing a cytopathogenic action on a tissue culture of epidermoid carcinoma of the cervix uteri (HeLa).

The object of the present study was to test the cytopathogenic properties of the available strains of stable L-forms of Salmonella typhosa and Streptococcus haemolyticus.

METHOD

Tests were carried out on a stable L-culture of S. typhosa No. 152L which we obtained in 1956 [1] from strain S. typhosa No. 5606; a stable L-culture of Str. haemolyticus No. 196L, obtained in 1957 from a β-hemolytic streptococcus of group A, No. 10-S [2]; two stable L-cultures of streptococci Nos. 406L and 409L isolated by us in 1959 from the blood of patients with rheumatic fever [3-5]. As a control we used the original strains of S. typhosa No. 5606 and of β-hemolytic streptococcus of group A, No. 10-S, and also revertant strains of streptococci from these L-forms (Nos. 196r, 406r, 409r) obtained by us at different times (1958 and 1960).

All the test strains were grown on synthetic medium No. 199: L-cultures for 7-10 days and bacterial for 24 h at 37°. The growing cultures were centrifuged, the supernatant fluid poured off, and a suspension with a density of 500 million/ml was used for inoculation of the corresponding tissue cultures.

Four monolayer primary trypsinized tissue cultures were used in the experiments, including cultures of fibroblasts of chick embryos (9-12 days), kidneys of monkeys (Macacus rhesus), kidneys of human embryos, and two transplantable cultures of Hep_2 and CMH.

The tissue cultures were prepared by the usual method. A suspension of cells in a concentration of 300,000-3,500,000/0.5 ml of medium was grown in a stationary position at 37° for 2-3 days. After formation of a monolayer on glass (after 2-3 days) the medium was removed. Fresh medium No. 199 was poured in a volume of 1 ml into test tubes, and inoculation was carried out simultaneously. In each experiment two controls were set up: a tissue culture control (K_1) and a control of the action of the medium in which the L-forms and bacterial forms were grown (K_2). To each tube with the tissue culture 0.1 ml of medium was added. Each strain of L-forms, and also of the bacterial forms, was tested in 10 samples of the corresponding tissue culture; 10 samples were also used for the control tests. The changes in the inoculated tissue cultures were inspected after 1,2,5, and 8 days under the low power of the microscope and microscopic examination of stained films was performed. The preparations were fixed in Bouin's fluid and stained with hematoxylin-eosin.

TABLE 1. Characteristics of Cytopathogenic Action of L-Forms on Different Tissue Cultures

Tissue used for growing culture	Test strains and inoculating dose																	
	S. typhosa No. 152 L ($2.5 \cdot 10^8$)			Str. haemolyticus No. 196 L ($5 \cdot 10^8$)			Str. haemolyticus No. 409 L ($2.5 \cdot 10^8$)			Str. haemolyticus No. 406 L ($2.5 \cdot 10^8$)			K_1			K_2		
	total no. of samples	no. of positive tests	intensity of action	total no. of samples	no. of positive tests	intensity of action	total no. of samples	no. of positive tests	intensity of action	total no. of samples	no. of positive tests	intensity of action	total no. of samples	no. of positive tests	intensity of action	total no. of samples	no. of positive tests	intensity of action
Fibroblasts of chick embryo.	10	10	++++	10	10	++	10	10	+++	10	10	+++	10	0	0	10	0	0
Monkeys' kidneys.....	10	10	+	10	10	+	10	10	+	10	10	+++	10	0	0	10	0	0
Cutaneo-muscular human fibroblasts.....	10	1	+	10	1	+	10	1	+	10	3	+	10	0	0	10	0	0
Human embryonic kidneys..	10	5	+	10	0	0	10	2	+	10	3	+	10	0	0	10	0	0
Hep_2.....	10	5	+	10	1	+	10	0	0	10	10	++	10	0	0	10	0	0
CMH.....	10	10	++	10	0	0	10	10	++	10	10	++	10	0	0	10	0	0

Legend: ++++ total slipping of layer in the form of a "stocking" during first 2 days; +++ severe rarefaction of layer, formation of large, round holes, deformation and pycnosis of nuclei of nearly all cells, early vacuolation of cytoplasm on 1st-2nd day; ++ rarefaction of layer, formation of round holes in layer, deformation and pycnosis of the majority of cells, vacuolation of cytoplasm on 1st-2nd days; + changes of the same character but less marked and observed in a minority of cells on 2nd-5th days; – continuous, firm layer, nuclei granular, normal, fine granular cytoplasm with basophilic inclusions, cells unchanged.

TABLE 2. Results of Titration of Minimal Cytopathogenic Dose of L-Culture Causing Marked Cytopathogenic Effect in 50% of Tests ($MCPD_{50}$)

L-cultures	$MCPD_{50}$
152	$8 \cdot 10^5 - 4 \cdot 10^5$
196	$25 \cdot 10^6 - 12.5 \cdot 10^6$
406	$6.25 \cdot 10^6 - 3.125 \cdot 10^6$
409	$6.25 \cdot 10^6 - 3.125 \cdot 10^6$

RESULTS

The results of the study of the pathogenic action of the L-forms of the bacteria on the different tissue cultures are shown in Table 1. These results demonstrate the difference in the degree of sensitivity of the various tissue cultures to the cytopathogenic action of the L-forms. Irrespective of the specific origin, the cytopathogenic effect of the L-culture on the cultures of the chick embryonic fibroblasts was high. The streptococcal L-cultures isolated from a patient had a more intensive action than the L-culture obtained in experimental conditions.

All the investigated strains of L-forms except one (a culture of L-forms of Str. haemolyticus No. 406, isolated from a patient) had a very slight cytopathogenic action on the tissue culture of monkeys' kidney. Strain No. 406L gave a higher cytopathogenic effect in this particular tissue culture.

The tested L-cultures had practically no cytopathogenic effect in human embryonic cutaneo-muscular and renal tissue; in the tissue culture of human embryonic kidney a cytopathogenic effect was found slightly more often (in 5 of 10 tests when L-forms of S. typhosa No. 152L were used and in 2 and 3 of 10 tests respectively when L-forms of Str. haemolyticus Nos. 406L and 409L were used).

In contrast to the other tested strains, culture No. 406L had a selective cytopathogenic action on the transplantable tissue culture of Hep_2. All the L-cultures except one strain of an experimentally obtained culture of L-forms of Str. haemolyticus No. 196L gave a marked cytopathogenic effect against the transplantable CMH tissue culture.

Having demonstrated the highest cytopathogenic effect in the culture of chick embryonic fibroblasts, we used this for the subsequent study of the degree of intensity and dynamics of the cytopathogenic action of the L-forms, and also in control experiments to study the character of the cytopathogenic action of the bacterial forms.

The results of these experiments demonstrated the differences in the character and intensity of the cytopathogenic effect of the L-forms of different origin, and also of the bacterial forms. For example, the L-form of S. typhosa caused sliding of the layer as a "stocking" and complete degeneration of the cells on the 2nd day. This culture did not alter the pH of the medium.

The L-forms of Str. haemolyticus caused a varying intensity of rarefaction of the layer; early and severe vacuolation of the cytoplasm and pycnosis of the nuclei were observed. Signs of degeneration appeared on the 1st-2nd day and reached their maximum on the 5th-8th day. These cultures altered the pH of the medium on the 2nd day. The L-forms of the streptococcus isolated in vivo (Nos. 409L and 406L) had a higher cytopathogenic action, for they caused more marked changes when inoculated in smaller doses than the L-culture obtained in an experiment in vitro. Quite different results were observed after inoculation of a tissue culture of chick fibroblasts with bacterial forms (S. typhosa No. 5606; Str. haemolyticus No. 10-S; revertants of L-forms of streptococcus Nos. 196r, 409r, and 406r). A uniform reaction was obtained in the form of complete degeneration of the layer on the 2nd-3rd day, marked turbidity of the medium on account of growth of the culture, and a change in pH towards the acid side, i.e., the picture usually observed after bacterial growth.

During titration of the minimal doses of L-cultures causing a marked cytopathogenic effect in 50% of test samples ($MCPD_{50}$), differences in the intensity of the cytopathogenic action were observed: it was highest in the L-culture of S. typhosa No. 152, slightly lower, but still quite high in the L-culture of Str. haemolyticus Nos. 406L and 409L isolated from patients with rheumatic fever, and finally, minimal in the L-culture of a streptococcus (No. 196L) obtained in experimental conditions in vitro (Table 2).

Hence, irrespective of the specific origin of the L-cultures, they possessed a high cytopathogenic action on a culture of chick embryonic fibroblasts and a moderate action on a culture of Macacus rhesus kidneys. In relation to the other tissue cultures studied, the cytopathogenic effect of the L-forms was selective in character depending on the specific origin.

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