DETERMINATION OF THE ACTIVITY OF CHOLERAGEN ON TADPOLES

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A method of titration of choleragen in tadpoles of Rana temporaria is suggested. Unlike in methods known hitherto, the animals are kept (10 at a time) in vessels with water containing serial dilutions of choleragen. The degree of activity of the choleragen is determined from the percentage of dying animals. The dilution of cholergen causing death of 50% of tadpoles after 24 h was taken as the unit of activity. The method is simple, accurate, easily reproducible, and economical in use.

KEY WORDS: intestine; choleragen; dehydration.

An important stage in the study of the pathogenetic mechanisms of cholera occurred in the 1960s, when the leading role of the exotoxin in this diseases was demonstrated. Several workers reproduced the typical clinical picture of diarrhea with the aid of highly purified exotoxin (choleragen) in animals [6, 7] and man [9]. This was the starting point for many attempts to obtain choleragen and to study its chemical nature and properties. However, no simple and accessible method yet exists in laboratory practice for determining its activity. At the present time two methods are used for this purpose: titration of the toxin on ligated loops of rabbit ileum [4] and Craig's intradermal test in rabbits and guinea pigs [5]. These methods are complicated and expensive. The accuracy of the results depends largely on the technique of the operation and the individual sensitivity of the animals to choleragen. The use of the second method is restricted also by the fact that it can determine only choleragens which contain permeability factor (PE). However, in recent years reports have been published of the production of choleragens in which the vascular permeability factor could not be found [2, 8].

The object of the present investigation was to develop a simpler, more accurate, and cheaper method of titrating choleragen than those mentioned above.

EXPERIMENTAL METHOD

Tadpoles of Rana temporaria were used as the model for determining choleragen activity. The tadpoles also were bred under laboratory conditions [3]. In these experiments the tadpoles were kept at 18-20°C in glass crystallizing tanks filled with dechlorinated tap water. The animals were fed on a mince of boiled nettle and burdock leaves, and the only protein which the tadpoles received was from eating individuals that died; the water was changed every 2 or 3 days. By keeping the tadpoles under these conditions the duration of the larval stage could be prolonged.

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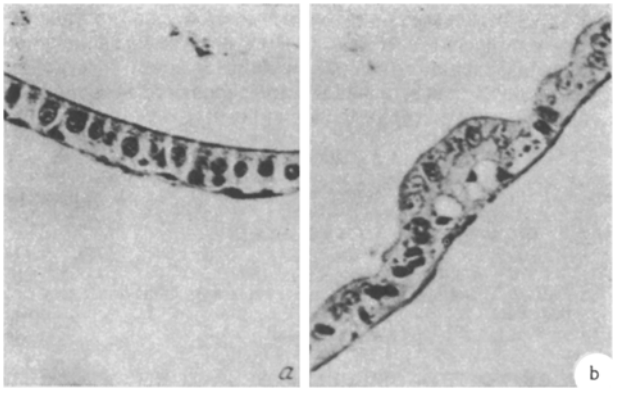

Fig. 1. Small intestine of tadpole: a) control; b) after treatment with choleragen. Hematoxylin–eosin, 500 ×.

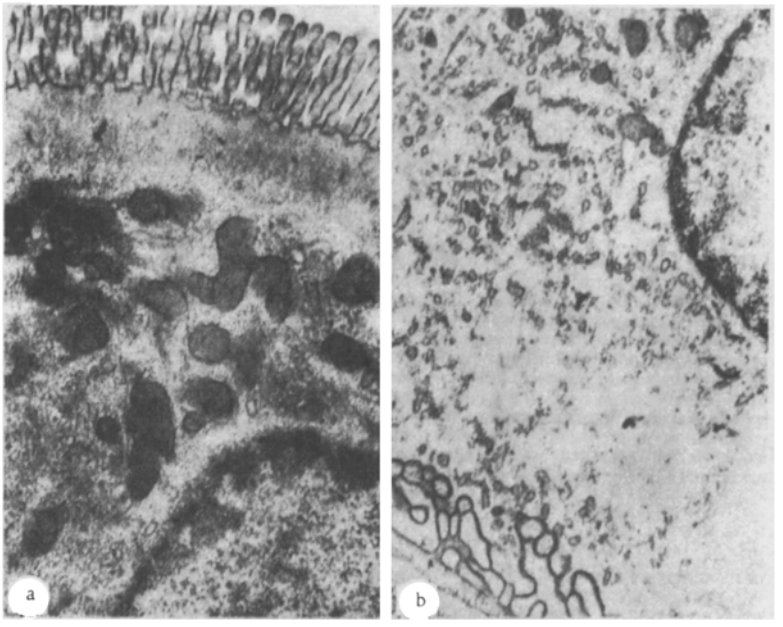

Fig. 2. Epithelial cell of small intestine of tadpole: a) control; b) after treatment with choleragen: edema of basal part of cytoplasm, 16,000 ×.

TABLE 1. Comparative Results of the Study of Activity of Choleragen Batches on Tadpoles of Rana temporaria and Ligated Loops of Rabbit Ileum

Batch of choleragen	Biological activity of choleragen	
	on tadpoles (LD_{50})	on rabbits (ED_{50})
1	1:336	1:320
P-10	1:240	1:220
19	1:270	1:280
20	1:300	1:310
41	1:144	1:160
49	1:200	1:210

To determine the activity of the choleragen, batches of the toxin obtained at different times from an international strain of Vibrio cholerae 569B, serotype Inaba of the Pakistan strain, were used.

The choleragen was titrated by the method of double serial dilution in a volume of 10 ml tap water. Each vessel contained 10 tadpoles. As a result, the animals swallowed the toxin in a dose corresponding to the dilution of the preparation. For the controls, the medium in which the choleragen was prepared and tap water were used. The experimental and control tadpoles were left at room temperature without food. The results of the test were read after 24 h as the number of dying animals compared with the total number taken for the test, in the absence of mortality in the controls.

The dilution of choleragen causing death of 50% of the tadpoles was taken as the unit of activity; calculation was done by interpolation (LD_{50}).

The dilution of choleragen causing death of 100% of tadpoles was 1:40, the next dilution (1:80) caused death of 80%, the dilution of 1:160 caused death of 30%, and finally, with a dilution of 1:320, no tadpoles died.

Altogether six batches of choleragen were tested by this method in 44 experiments on 6000 tadpoles. The results of at least four repetitions of the test were analyzed.

The results were compared with the results of titration of the same batches of choleragen on ligated loops of ileum of adult rabbits (4 rabbits for each batch).

EXPERIMENTAL RESULTS

The investigations showed (Table 1) that the titers of the same batches of choleragen obtained by the two methods agreed almost completely. This shows that tadpoles, like rabbits, are highly sensitive to the action of cholera toxin and that the method of titration of choleragen on tadpoles is just as accurate as the classical method [4].

A very important factor in the evaluation of the suggested model is proof of the specificity of action of choleragen on tadpoles. To obtain this proof a series of experiments was set up to abolish the toxic action of choleragen by means of neutralization tests with specific antiserum. Choleragen or choleragen with normal serum, when added to the nutrient medium of the tadpoles, caused death of 100% of the animals in a dilution of 1:80 (LD_{50}=1:200). Meanwhile a combination of choleragen with the specific antiserum gave complete protection of the tadpoles against death in all dilutions tested.

The results of the histological and electron-microscopic study of the mucous membrane of the small intestine of the experimental animals are of great interest. By the light-optical method swelling of the cytoplasm of the epithelial cells of the small intestine and displacement of their nuclei were found (Fig. 1); the RNA content in the damaged cells was reduced and the capillaries in the submucosa were widely dilated. Electron-microscopic investigation showed that under the influence of choleragen the intercellular spaces between the epithelial cells of the small intestine were wider than in the control and the nucleus and other organelles were displaced into the central and apical parts of the cells as the result of edema of the basal part of the cytoplasm (Fig. 2). Cisterns of the agranular endoplasmic reticulum formed vesicles of various sizes and large vacuoles. The mitochondria in these cells were round in shape, their matrix was branched, and their cristae were arranged radially.

Damage to the epithelial cells of the tadpoles' small intestine was basically similar to the lesion in the epithelial cells of the small intestine of young rabbits aged 10 days [1].

A method is thus suggested for determining the activity of choleragen on amphibians – tadpoles of Rana temporaria; the method is based on analysis of mortality among the animals from known concentrations of cholera toxin added to the aqueous medium in which the tadpoles are kept. The dilution of the preparation causing death of 50% of the tadpoles after 24 h is taken as the unit of activity of choleragen. The pathomorphological picture of the wall of the small intestine of tadpoles exposed to the action of a lethal dose of choleragen does not differ in principle from that observed in rabbits. This shows that the enteropathogenic action of the toxin has the same mechanism not only in different species, but also in different classes of animals. Besides its high sensitivity and accuracy, the suggested method is extremely simple, easily reproducible, economical in use, and it can be used to test the necessary number of dilutions of toxin with at least 10 tadpoles for each dilution, thus ruling out the possibility of errors connected with individual sensitivity of the animals.

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