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Accelerated cell death produced by a cholera cytotoxin on isolated epithelial cells from rabbit ileum

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Villus and crypt cells were removed from rabbit ileum by a modification of a method previously described. Villus cells were isolated by vibration of everted bowel segments in citrate buffer, and crypt cells were removed by the expansion of these segments with air and simultaneous vibration in citrate buffer. Several culture media were tested for the maintenance and/or culture of these cells. Two defined media, Eagle's minimum and medium 199, were unsatisfactory and led to rapid cell death. The presence of whole serum was beneficial and both cell types could be maintained for several hours when homologous rabbit serum was employed. Initially 70–80 % of villus and crypt cells were viable in homologous serum, and the effect of cholera toxin on cell viability was studied by incubating the cells with peptone dialysate supernatant (PSUP) toxin from Vibrio cholerae *for 4 h. PSUP toxin reduced the viability of both villus and crypt cells compared with control preparations, as measured by the uptake of trypan blue ; cell death was accelerated with time. A purified, diarrhoea-inducing cholera toxin also reduced the viability of these cells and the results were comparable to those with PSUP toxin. The effect was usually immediate, and a significant reduction in viability occurred within 1 hour of the incubation of cells with toxin. The toxin was heat-labile (60°C/30 min) and nondialysable, and its cytotoxic activity could be completely neutralized with cholera antitoxin. Dose–response studies indicated that as little as 0.0275 equivalent units of ligated ileal loop toxin were active in this system.*

In neither human nor experimental animal cholera is the integrity of the ileal epithelium affected by contact with the exotoxins formed by the cholera vibrio in the lumen of the bowel (16). Detailed studies of experimental canine infection during the first 20 h of the disease have also indicated that the epithelium remains intact. Other changes in the bowel tissue are consistent with the inference that hypersecretion, resulting from the enterotoxin-induced movement of water and ions from the tissues into the lumen of the bowel, occurs mainly in the crypt cells, so that the cholera stool is produced primarily in the crypts (6).

In vitro studies on the cytotoxicity of cell-free preparations with enterotoxic activity have, in contrast, shown that a cytopathic effect may be produced. Specifically, Read (15) found that crude preparations of intracellular enterotoxin-containing substances from vibrios produced a rounding-up and detachment of L cells in cell monolayer cultures and inhibited the uptake of tritiated thymidine by cells in spinner cultures. Similarly, Basu Mallik et al. (1) found that cholera stools contained a factor that was toxic to monkey kidney cells. Felsenfeld et al. (7) utilized this cytotoxic effect of cell-free preparations for the titration of the toxicity and of its neutralizing antibody in tissue cultures. It is, however, difficult to interpret the results of these studies in terms of a possible cytotoxic effect of the enterotoxin because of the impure nature of the toxin-containing preparations. Inwood & Tyrell (11) used purified preparations with enterotoxic activity and found a cytopathic effect on HeLa cells in culture. In addition, Greenough et al. (8) described a lipase-activating effect on rat fat cells produced by purified enterotoxin-containing material. In the latter instance, the toxin preparations contained the vascular permeability factor (PF) described by Craig (3).

Recently Sultzer & Craig (17) reported that puri-

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fied cholera toxin inhibited the synthesis of DNA, RNA, and protein in isolated splenic lymphocytes. These workers postulated that the level of cyclic AMP in their mouse spleen cell system might not have been a critical factor in explaining the activity of the cholera toxin. In other tissue culture studies, Donta et al. (5) reported that purified cholera toxin caused rounding and steroidogenesis in monolayer cultures of Y1 adrenal cells. The system was very sensitive and it was found that only picogram amounts of purified cholera toxin were required to induce the morphologic and steroidogenic changes that occurred in these tissue cultured cells.

The method of separation of villus and crypt cells from rat small intestine, reported by Harrer et al. (9) and Webster & Harrison (19), was adapted for the separation of these cells in rabbit ileum in the present experiments. This method has been described elsewhere (14) and the maintenance of these epithelial cells in various media has also been studied. The present report is concerned with the effect of purified cholera enterotoxin on the viability of epithelial cells from the lower ileum of the rabbit, as determined by trypan blue exclusion.

MATERIALS AND METHODS

Experimental animals

New Zealand white rabbits, aged 10–12 weeks, were used throughout these experiments. The animals were restricted from food for 24 h prior to sacrifice in order to reduce the luminal contents of the small bowel. After exsanguination at the time of sacrifice, the serum was separated by centrifugation and sterilized for immediate use in the tissue culture experiments.

Removal of epithelial cells

Villus cells were dissociated from everted segments of rabbit lower ileum with a Vibro-Mixer (Chemap AG, Zurich), as previously described (14). Briefly, each everted ileal tissue segment was vibrated in sterile citrate buffer for 7 min. In the earlier experiments, crypt cells were removed by vibrating a segment, distended with buffer, at maximum amplitude for 20 min in fresh buffer. However, further experiments indicated that expansion of the segment with air and concomitant vibration for 3 additional minutes in fresh buffer gave greater yields of crypt cells. Gross contamination of each cell fraction with the other cell type appeared to be negligible, as verified by haematoxylin and eosin stained preparations (14). The preparation of cell suspensions was complete within 45–60 min of sacrificing the animal. The cells were dispersed by progressive passage through 22- to 26-gauge needles before dilution, and their numbers were determined by counting in a haemocytometer. Initially, approximately 75% of the villus cells and 75–80% of the crypt cells were viable, as determined by trypan blue exclusion. It was found that 1 ml of packed villus or crypt cells contained 2 to 3×10^8 cells. In all short-term tissue culture experiments, the packed cells were diluted 10 : 1 or greater in order to give a final cell concentration of 2×10^7 cells/ml.

Tissue culture media

Initial experiments were undertaken to determine the most suitable medium for maximum viability of epithelial cells. The viability of the cells was poor in Eagle's minimum essential medium (MEM) and in medium 199 with HEPES [a] buffer, both with and without 10% calf or fetal calf serum, but was enhanced by the addition of whole calf serum. However, homologous rabbit serum was more effective in maintaining the viability of these cells than heterologous rabbit, calf, or fetal calf serum. All the culture media employed contained penicillin (100 units/ml) and streptomycin (100 μg/ml).

Viability measurements

After dissociation and sedimentation, the cells were resuspended in the appropriate medium at a concentration of 10^7 cells/ml, with or without toxin, and incubated in sterile plastic tubes (16 × 150 mm) with continuous rocking at 37°C. At hourly intervals, a tube was removed from the incubator, 2 drops of the cell suspension were immediately mixed with 1 drop of 0.5% trypan blue and then spread with a coverglass on a microscope slide to give approximately 50–100 cells per high power (×430) field. At least 6 different fields were counted at each hourly interval tested to determine the proportions of viable cells.

Preparation of cholera toxin(s)

Crude cholera toxin was produced by a modification of the method described by Coleman et al. (2). The culture was cleared of bacteria by centrifugation and filtration of the supernatant through 0.45 μm Millipore filter, and then concentrated 10-fold on an Amicon UM-2 membrane. The concentrate was dialysed with deionized water (dH_2O), which also

[a] 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

served to remove the dialysable constituents of the unaltered culture medium and the products of bacterial growth. The concentrate was used in preliminary experiments, and fractionated on A25 DEAE Sephadex by elution in dH_2O to give a first peak described as enterotoxic by Kaur et al. (13). After thorough washing to eliminate UV-absorbing substances, the column was eluted with NaCl (0.5 mol/l) to give the material designated as nonenterotoxic fraction II by Coleman et al. (2). Enterotoxicity was determined by bioassay in the rabbit ileal loop (12), and PF activity by intradermal inoculation in the rabbit according to the method of Craig (3). More purified toxin was kindly provided by Dr C. Miller from the National Institutes of Health.

Toxicity of fractions

The PSUP (peptone supernatant) preparation contained both enterotoxic (750 units/ml) and PF (500 blueing doses/ml) activities as determined by bioassay of the dialysed concentrate. Assay of the first peak eluted from A25 DEAE Sephadex showed essentially all of the enterotoxic activity of the starting material, i.e., 180–850 units of enterotoxicity per ml, and amounts of PF activity ranging from a trace, i.e., <10 units/ml, up to 1 000–1 500 units/ml. The PF activity of the fractions was titrated in a single rabbit to avoid differences in sensitivity between individual animals, which Craig (4) showed could be as great as 16-fold. Eighteen hours after the intradermal injection of toxin, 3 ml of a 5% solution of Pontamine Sky Blue dye was injected intravenously and blueing diameters were recorded 1 h later.

RESULTS

Cell suspensions

The method used for separating the ileal epithelium produced villus cells in strips of 3–4 cells and occasionally more, and crypts that were often removed intact. The villus cells were morphologically distinguishable even after 4 h of *in vitro* culture; specifically a brush border, bulging cytoplasm, and a distinct nucleus could be observed. Within these 4 hours, however, the crypt cells tended to become rounded and had less distinct nuclei and cytoplasmic components.

Although such cell preparations showed synthesizing activity, as indicated by the incorporation of isotope-labelled amino acids and bases into protein and nucleic acid respectively, mitotic figures were not observed and the isolated epithelial cells failed to replicate (unpublished observations). No significant differences were noted between villus and crypt cells other than a tendency for the crypt cells to become rounded. At the end of 1 h, only 25–30% of the cells suspended in medium 199 or MEM were viable. The addition of whole serum increased the proportion of viable cells to about 50%, heterologous rabbit serum being more effective than calf serum. Maximum viability (70–80%) was obtained with homologous rabbit serum, i.e., that obtained at sacrifice from individual animals as described above. There appeared to be no significant differences in the death rates of the untreated cells between preparations from individual animals although there were differences, probably technical in origin, in the initial proportions of viable cells. It was evident from our results that the low death rate of the cells suspended in homologous serum allowed a period of 4 h for the study of any acceleration in the death rate.

Cytotoxic effects

In the first series of experiments, PSUP toxin from *Vibrio cholerae* 569B was tested at a concentration of 5 and 50 ileal loop units per 10^7 cells and the results are presented in Table 1. In these experiments, the number of viable untreated cells at 0 time was adjusted to 100% and other values were expressed as the percentage ratio of viable cells to the number of viable untreated cells at 0 time. The PSUP toxin was effective in reducing the viabil-

Table 1. Loss of viability of villus and crypt cells after treatment with peptone supernatant (PSUP) toxin

Time (hours)	Villus			Crypt		
	units of toxin [a]		control	units of toxin [a]		control
	5	50		5	50	
0	32 [b]	51 [b]	100 [c]	48 [b]	66 [b]	100 [c]
1	28	34	64	33	39	84
2	20	19	54	21	25	69
3	7	7	37	16	12	61
4	5	1	11	10	3	36

[a] As determined by the rabbit ligated loop technique.

[b] Each toxin concentration represents a separate experiment using toxin and cells (from a single rabbit) prepared at different times; the control values are an average of these two experiments.

[c] The control viability at 0 time was adjusted to 100% and the other values for toxin-treated and control cells were compared with this.

ity of both villus and crypt cells. The toxic effect was usually rapid and clearly apparent during the first hour of incubation.

As it was possible that several cholera products might have a toxic effect on these cells, a more purified toxin (the kind gift of Dr C. Miller at the N.I.H.) was also employed at 2 concentrations (5 and 50 ligated ileal loop units) with isolated villus and crypt cells (Table 2). It was readily apparent that this toxin preparation was equally effective in reducing the viability of both villus and crypt cells. In these experiments, the initial percentage viabilities of both types of cells were higher than those reported in the previous experiment (Table 1). Other experiments (not tabulated) showed that the toxin fraction obtained by elution with dH_2O from A25 DEAE Sephadex gave results similar to those reported in Table 2.

Since more than one toxic manifestation of cholera supernatants has been reported (3), the 0.5 mol/l fraction from DEAE Sephadex (fraction II) was tested with these isolated epithelial cells. This fraction was purposely selected, because it exhibited little diarrhoea-inducing toxin activity but had 1 000 blueing dose (BD) units/ml. As can be seen in Table 3, there was no effect on isolated, rabbit ileal mucosal cells with a single exception of villus cells treated for 3 h. This apparent effect at 3 h was probably due to a technical error, since it is unlikely that toxic activity would have been noted at 3 h and not at any of the other intervals tested. This would suggest that the cytopathic effect on these cells is perhaps not associated with the PF toxin; further studies, however, are necessary to clarify this point.

Table 2. Loss of viability of villus and crypt cells after treatment with purified cholera toxin

Time (hours)	Villus			Crypt		
	units of toxin [a]		control	units of toxin [a]		control
	5	50		5	50	
0	76 [b]	79 [b]	100 [c]	74 [b]	93 [b]	100 [c]
1	50	58	84	66	59	89
2	35	33	64	39	28	63
3	14	16	41	13	16	44
4	8	9	22	7	4	24

[a] As determined by the rabbit ligated loop technique.

[b] Each toxin concentration represents a separate experiment using toxin and cells prepared at different times; the control values are an average of these two experiments.

[c] The control viability at 0 time was adjusted to 100 % and the other values for toxin-treated and control cells were compared with this.

Nature of the cytotoxin

In order to establish more firmly the nature of this cytopathic effect, the following experiment was performed. A purified toxin (13), containing 850 units of ileal loop toxin and 50 PF units per ml, was tested (as previously described) with crypt epithelial cells. As a control, a portion was heated at 60°C for 30 min. The results of this experiment are expressed in Table 4. Toxin-treated cells again showed a marked decrease in viability, which was significantly different from that for control cells. The heated fraction was without effect on the cells during the first 3 intervals tested; comparable results were

Table 3. Loss of viability of villus and crypt cells after treatment with fraction II [a]

Time (hours)	Villus		Crypt	
	fraction II	control	fraction II	control
0	98 (92–105)	100 [b] (92–108)	99 (97–107)	100 [b] (97–102)
1	97 (88–101)	102 (96–107)	96 (93–101)	95 (91–99)
2	76 (67–86)	75 (70–79)	79 (75–85)	79 (74–83)
3	75 (69–79)	48 (41–52)	62 (55–70)	67 (64–74)
4	42 (34–46)	42 (37–47)	53 (49–55)	51 (48–53)

[a] This fraction contained a PF toxin titre in excess of 1000 units/ml.

[b] The control viability at 0 time was adjusted to 100 % and the other values for fraction II-treated and control cells were compared with this. The percentage range of viability from six determinations is expressed in parenthesis.

obtained when no toxin was employed. A slight toxic effect was noted at 3 and 4 h with heated toxin, but this was much lower than the effect observed with unaltered toxin. Thus, the component responsible for the cytopathic effect was, as with the diarrhoea-inducing toxin, heat labile.

In addition, a purified toxin was tested with a hyperimmunized antiserum which exhibited a high antitoxin titre. In this experiment, 600 units of antitoxin (12) were mixed with 100 units of ligated loop toxin, incubated for 30 min at 37°C, and tested for cytotoxic activity. The results are expressed in Fig. 1. Complete neutralization of the cytotoxin could be effected by the concentration of antitoxin employed, while toxin plus normal serum accelerated the death of crypt cells.

Since 50% of the isolated epithelial cells were still viable after incubation for 2 h *in vitro*, it was possible to utilize this epithelial cell system for the development of a cytotoxin assay. In an attempt to study the dose–response relationship of cytotoxicity in this system, purified toxin was added in increasing doses to freshly isolated crypt epithelial cells and the percentage loss of viability was recorded. From these experiments, it was apparent that the degree of toxin-induced cytotoxicity was dose-dependent when the experiment was performed for 1, 2, or 3 h. A characteristic sigmoid-shaped curve was obtained at each interval studied when the percentage loss of crypt cell viability was plotted against the logarithm of the toxin dose. The results of these experiments are summarized in Table 5. The dose–response curve obtained after 1 h of incubation of cells with toxin had an upper asymptote of 34%, and the sensitive,

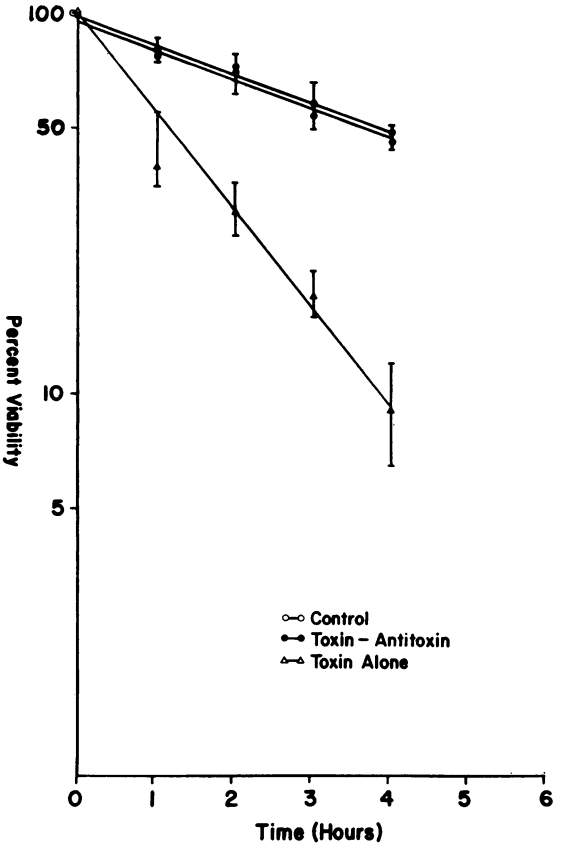

Fig. 1. The neutralization of cytotoxic activity with antitoxin: 100 units of ileal loop toxin were mixed with 600 units of antitoxin, incubated at 37°C for 30 min., and tested with isolated crypt cells.

Table 4. Effect of heated and unheated cholera toxin on the loss of viability of isolated rabbit ileal crypt cells

Time (hours)	Toxin [a]		Control
	heated	not heated	
0	102	101	100 [b]
1	83	48	84
2	47	16	49
3	34	12	43
4	19	6	31

[a] At a concentration of 5 units per ml.

[b] The control viability at 0 time was adjusted to 100% and the other values in toxin-treated and control cells were compared with this.

Table 5. Dose–response of different concentrations of cytotoxin which induced loss of viability of 10^7 crypt epithelial cells

Time (hours)	Upper asymptote [a]	Fifty % effective dose (ED_{50}) [b]	Average ED_{50} [b]
1	34%	0.015	
2	62%	0.0375	0.0275
3	74%	0.030	

[a] Percentage loss of cell viability.

[b] Equivalent units of ligated ileal loop toxicity.

linear portion of the curve allowed the interpolation of a 50% effective dose (ED_{50}). At 1 h, the ED_{50} was 0.015 equivalent units of ligated loop toxicity; and 0.0375 and 0.0300 equivalent units were obtained from the 2 and 3 h dose–response curves, respectively. The average ED_{50} of 0.0275 equivalent units suggested that this system was suitable for the titration of cholera cytotoxin. The steeper curves after 2 and 3 h of incubation became asymptotic at 62% and 75% of cytotoxicity and reflected the toxin-induced, accelerated rate of cell death. Since approximately one-half or more of the initially viable cells were killed by toxin treatment for 2 hours, this would be the optimum and recommended time for titration of this cytotoxin.

DISCUSSION

In recent years attention has been focused on the dissociation of living epithelial cells from the small intestine. Harrer et al. (9) first reported the successful removal of these cells in the rat, and others have since been able to dissociate and separate villus and crypt cells in this species (10). We have previously reported a modification of Harrison & Webster's technique for the separation of rabbit ileal epithelium (14), by which it was possible to remove homogeneous, living villus or crypt cells.

Attempts to establish a primary culture of these isolated cells have been unsuccessful. Among the media investigated were Eagle's minimum and medium 199, with and without 10% calf or fetal calf serum. In addition, whole calf, fetal calf, and rabbit sera were also tested in our attempts to establish primary cultures. Although the viability of the dissociated epithelial cells in whole, homologous serum was high, no cells in the act of division could be detected. Morphologically, the cell membrane was intact, and villus cells exhibited a distinct brush border even after 4 h *in vitro*. Usually whole crypts were isolated by the dissociation procedure, and incubation for 4 h in homologous serum did not result in a noticeable swelling or lysis of the cells. Some free nuclei were observed in both villus and crypt cell preparations immediately after their separation, indicating that cell damage probably resulted from the dissociation procedure.

Since these cells could be maintained *in vitro* for 4 h with a constant and reproducible death rate, and since they would be the first cells to come in contact with the cholera vibrio and its products *in vivo*, it was of interest to study the effects of cholera toxin on them. It was immediately apparent that peptone supernatants of cultures of *Vibrio cholerae* did contain a substance or substances, which reduced the viability of both villus and crypt cells in tissue culture. It is clear from Fig. 1 that the death rate of cells was accelerated logarithmically indicating that these dissociated cells became more susceptible to toxin with increased exposure. After the incubation of cells with toxin for 2 h, the viability of villus and crypt cells was approximately halved. Since both water and ions began to accumulate in the rabbit ileal loop at this time, it would be of interest to discover the subcellular changes in these isolated, toxin-treated, epithelial cells.

The use of purified cholera toxin led to the suggestion that the cytotoxic material was present together with the diarrhoea-inducing toxin. Indeed, a single molecule could perhaps be responsible for both toxic manifestations. The reduction in the viability of epithelial cells by PSUP and purified toxin was equally effective at both 5 and 50 units of ileal loop toxicity, suggesting further that the cytotoxic effect might be associated with all or a part of the enterotoxin. In this regard, it was of interest that fraction II (which contained no detectable ligated loop toxicity) did not significantly reduce cell viability (Table 3); this fraction nevertheless had permeability factor (PF) toxin activity and trace amounts of endotoxin activity. Perhaps subtle changes occurred in the cells with such treatment, but more sensitive techniques will be needed to detect them. It is also possible that PF toxin could be active at other intestinal sites, such as the capillary beds, without having any effect on the intestinal epithelial cells. The cytopathic effect was specific because heated toxin had no apparent effect on either villus or crypt cells. In addition, the cytotoxic effect could be neutralized completely with an excess of antitoxin.

From this study, it was clear that the death rate of epithelial cells after treatment with increasing concentrations of toxin followed a typical dose–response curve. From this curve one could estimate an upper asymptote of 34% of cell deaths after toxin treatment for 1 h, and approximately 60–75% after 2–3 h. The cytotoxin was found to be present in crude and purified enterotoxin preparations, was heat labile and nondialysable, and gave a typical dose–response curve when expressed as gut enterotoxin activity. This would suggest that the *in vitro* death of toxin-treated epithelial cells might be another effect of the diarrhoea-inducing, cholera enterotoxin.

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RÉSUMÉ

MORT CELLULAIRE ACCÉLÉRÉE PROVOQUÉE PAR UNE CYTOTOXINE CHOLÉRIQUE SUR DES CELLULES ÉPITHÉLIALES ISOLÉES DE L'ILÉON DE LAPIN

On a réussi à isoler des cellules de villosités et des cellules glandulaires de l'iléon de lapin grâce à une modification de la méthode permettant l'isolement de cellules intestinales du rat. A l'origine, 70 à 80% de ces cellules sont viables, comme l'atteste l'épreuve du Bleu trypan, mais leur viabilité disparaît progressivement en l'espace de 4 heures. On a mis à l'essai différents milieux pour tenter de prolonger la vie des cellules. Le milieu d'Eagle et le milieu 199, additionnés ou non de sérum de veau, ne se prêtent pas à leur culture. Les sérums complets atténuent dans une certaine mesure la perte de viabilité et le meilleur à cet égard est le sérum homologue de lapin. Ce système (cellules épithéliales isolées et sérum homologue) permet de mesurer l'activité cytotoxique de substances entraînant la mort des cellules dans un intervalle de 4 heures.

Lorsque des cellules intestinales de lapin isolées sont mises en présence de préparations de toxine cholérique, on note une réduction immédiate de la viabilité des deux types de cellules. Une entérotoxine cholérique purifiée provoquant de la diarrhée a une action du même ordre. Il semble donc que la cytotoxine soit identique à la toxine provoquant la diarrhée ou corresponde à une de ses fractions. Cette conclusion est étayée par le fait qu'une fraction dépourvue d'activité choléragène décelable, mais renfermant une certaine quantité de facteur de perméabilité vasculaire, n'a aucune action sur la viabilité des cellules épithéliales isolées.

L'activité cytotoxique disparaît après dialyse et est complètement détruite par chauffage à 60° pendant 30 min, ce qui semble indiquer que la cytotoxine est de nature protéique.

La cytotoxine est en outre totalement neutralisée par l'antitoxine cholérique: 600 unités d'antitoxine neutralisent 100 unités de toxine et suppriment complètement l'effet cytotoxique sur les cellules épithéliales glandulaires isolées. Les résultats de ces expériences donnant à penser que l'activité cytotoxique s'exerce de façon spécifique sur les cellules épithéliales isolées, on a établi une courbe dose-réponse afin de déterminer la sensibilité et l'utilité d'un tel système cellulaire pour le titrage de l'activité de la toxine cholérique. Il en ressort qu'une dose très faible, 0,0275 unité de toxine, suffit à tuer plus de la moitié des cellules initialement viables en 2 heures, durée qui semble optimale pour le titrage de l'activité de la cytotoxine cholérique.

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