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A CYTOTOXIC FACTOR IN CHOLERA TOXIN

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SUMMARY.—Preparations of cholera toxin prevented HeLa cells from spreading on a plastic surface. The toxic factor was heat labile. The toxin was filtered through Sephadex G25 and the cytotoxic component appeared to enter the resin, and cytotoxic fractions sensitized sheep cells to antitoxic serum. These fractions were then run on a G50 column and amino acid analysis suggested the presence in active fractions of a peptide containing 11 amino acids.

IN order to exploit the potential advantages of studying the action of toxins *in vitro* we have tried to develop a technique for detecting a toxic effect of *Vibrio cholerae* material on mammalian cells.

In preliminary experiments it was found that a preparation of cholera toxin, when used in high concentrations, caused the cells of monolayer tissue cultures to become rounded. The effect was observed in mouse and chick embryo fibroblast cells, but seemed to be most clear-cut with HeLa cells. We then used a modification of a method developed by Sanderson to study the toxic effect of an adenovirus antigen (Sanderson, unpublished) and found that an effect could be detected at high dilution of toxin. The toxic factor has been purified and seems to be related to enterotoxin, and this paper summarizes the results obtained.

MATERIALS AND METHODS

Cell toxin assay.—A strain of HeLa cells obtained from the Wellcome Laboratories, Beckenham, Kent, was maintained by serial passage in 10 per cent foetal calf serum and 10 per cent tryptose phosphate broth in Eagle's medium. The medium was poured off a bottle and replaced with a 0·2 per cent EDTA in saline, which was poured off about 5 min. later. After 2 hr incubation at 37° the cells were resuspended at a concentration of 8000 cells/ml. in a mixture of 10 per cent tryptose phosphate broth in Eagle's MEM medium with antibiotics. Cells suspended after trypsin dispersal were unsatisfactory, as were versene dispersed cells suspended in a medium containing serum. Similar results were obtained with another strain of HeLa cells. Culture chambers were made by cementing sections of stainless steel tubing (10 mm. long × 16 mm. internal diameter) to the bottom of plastic petri dishes (Esco AA). Cell suspension (2·5 ml.) was mixed with 0·5 ml. of buffer or a dilution of test material in a test tube, and then 0·9 ml. of mixture was put into a chamber. The chambers were incubated at 37° in a CO_2 incubator for about 18 hr. The medium and ring were then removed and the dish stained by flooding it with 3·3 per cent carbol fuchsin.

Gel filtration.—Sephadex columns 200 × 2·5 cm. were prepared and run in the cold room.

RESULTS

The majority of cells in cultures set up with buffer are flattened (Fig. 1a) and the number in this state were counted with a × 10 objective by a standard procedure in about 10 non-overlapping fields across a diameter of the circular area of stained cells. In the presence of a dilution of cholera toxin cells stuck to the

plastic but did not flatten out (Fig. 1b). Again the number of flattened cells was counted. As shown in Fig. 2 the number of flattened cells increased with increasing dilution of toxic material. However, the rounded cells were not permanently injured—if the culture was continued for a further 24 hr they spread out and apparently multiplied. It was also possible to see structural changes in monolayer cultures of flattened cells treated with large doses of toxin—these consisted largely of cytoplasmic vacuolation and retraction of cell from contact with each other.

We wondered whether the toxic activity detected in tissue culture was due to the same substances as those which caused the toxic effects described by the authors. The following observations were important in this regard.

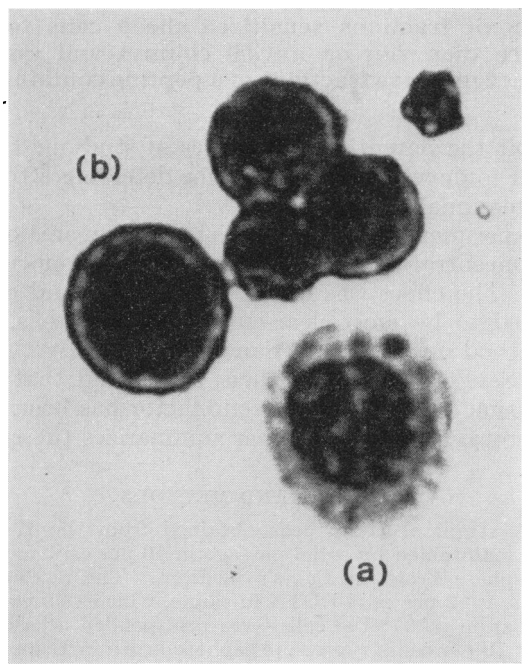

FIG. 1.—Typical appearance of (a) spread and (b) unspread cells fixed after 18 hr of culture. Stained carbol-fuchsin.

(a) Cell toxicity was regularly found in preparations of cholera toxin, including NIH-001 kindly supplied by John R. Seal, Chairman of the NIH Cholera Advisory Committee, National Institutes of Health, Bethesda, Md. 20014—this material has been shown to be active in enhancing skin capillary permeability and in promoting fluid accumulation in the dog and rabbit intestine. A sample of cholera filtrate prepared here for virological work had low activity and the culture medium from which it was made had none. Purified cholera toxin (MW 60,000), prepared and kindly supplied by the Wellcome Research Laboratories, showed an effect at high dilution.

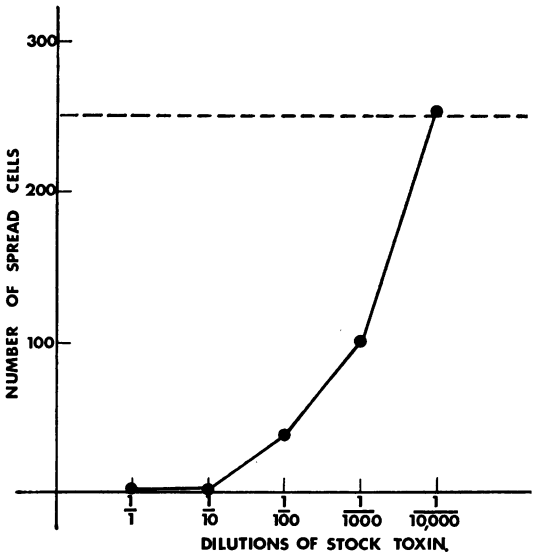

FIG. 2.—Number of spread cells observed after incubation with varying dilutions of toxin. Stock toxin contained 0·3 g. per ml. of dried toxin. The final concentration of toxin in contact with cells was about 1/20 of that shown on the abscissa. The number of cells in cultures receiving no toxin is indicated by the interrupted line.

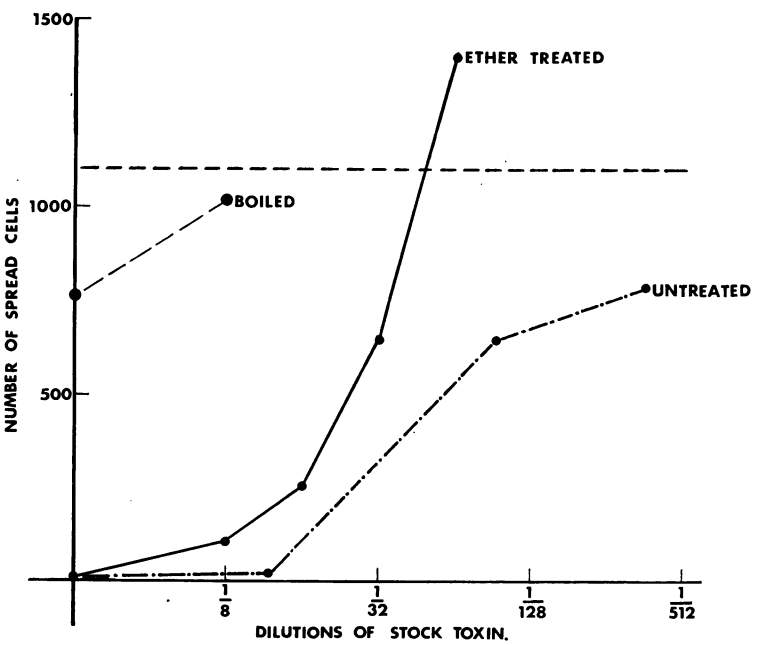

FIG. 3.—Inactivation of toxin. Toxin was heated in a boiling water bath for 10 min. before dilution. Another sample was shaken with ether at intervals at room temperature before dilution. The shift of the dose response curve to the left indicates at least 100-fold reduction of activity on heating and 2- or 3-fold by ether treatment.

(b) The properties of the cell toxin were studied by treating cholera toxin preparations in various ways and then assessing their activity. As shown in Fig. 3, activity was partly destroyed by ether treatment and completely lost on boiling and heating at 57° for 30 min. It was also partly inactivated in some experiments by incubation with a final concentration of 2·5 μg./ml. trypsin at 37° for 1 hr, but not by DNAse. Attempts to show an effect with papain were unsuccessful because the enzyme alone had a marked effect upon the cells.

Gel filtration.—The activity of the contents of the bag declined when toxin was dialysed overnight at 4° against saline—the fluid against which the filtrate was dialysed was freeze dried and reconstituted and activity was detected again.

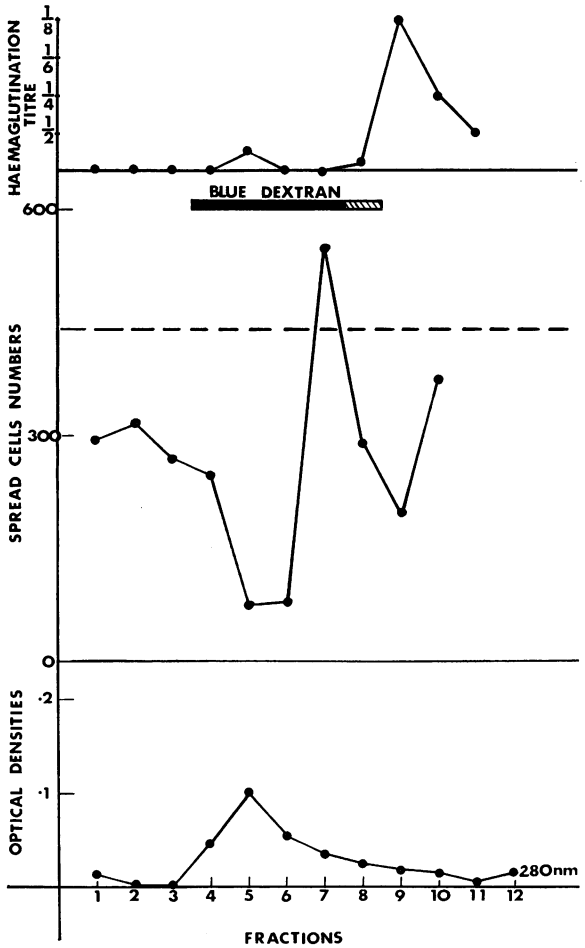

FIG. 4.—Passage of toxin through G25 column 210 × 2·5 cm. equilibrated with 0·2 M sodium phosphate buffer pH 7·2. The blue dextran had a cytotoxic effect, but in parallel experiments without blue dextran toxic activity was detected in both the excluded volume and also in later fractions, as shown here. Fractions up to 45 were tested but showed no toxic effect except for some containing high concentrations of amino acids and salts. The results of red cell agglutination tests in a parallel run are also shown in the upper part of the graph.

It therefore seemed likely that an active peptide was present and we therefore tried to separate it by gel filtration on a Sephadex G25 column. The results of preliminary experiments indicated that, although some biological activity was excluded, some active material just entered the gel. The active fractions seemed to be substantially purified, but lost activity on storage at 4° and when frozen at −20°; active fractions were resalted into 0·05 M ammonium bicarbonate buffer pH 8 on a short G25 column and freeze dried, more of the activity was lost. Ammonium bicarbonate buffer was used in further separations (Fig. 4). The cytotoxic fractions which appeared after the blue dextran were combined and reduced to about 4 ml. on a rotary evaporator at about 0°. The concentrate was

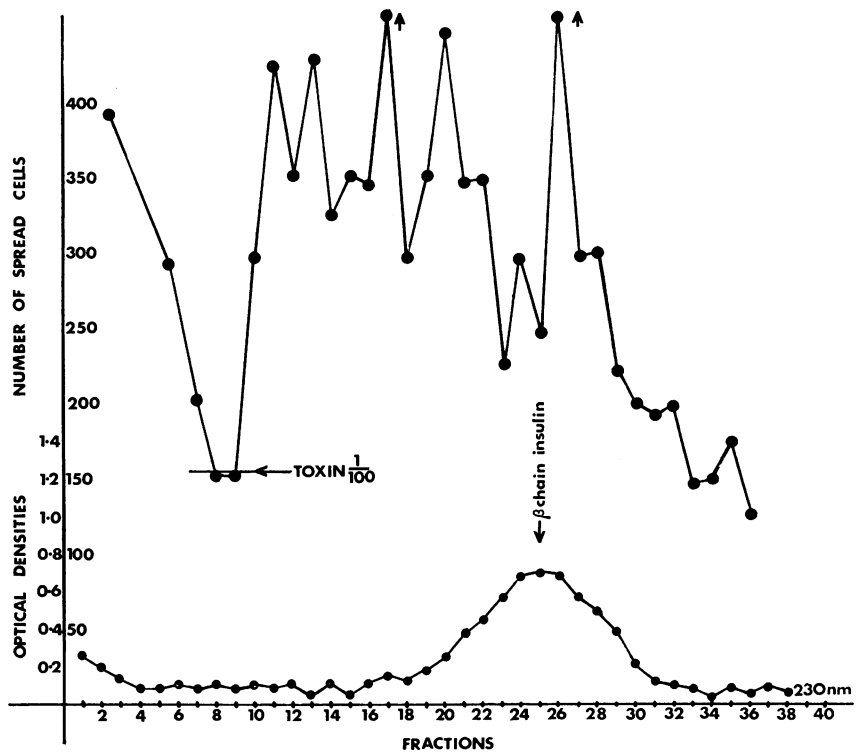

FIG. 5.—Passage of concentrated active fractions 8–11 from G25 column (see Fig. 4) through G50 column. The cell count using crude toxin diluted 1/100 is shown. The toxic effect in late fractions is again presumably due to salts or other low molecular weight materials. In a parallel run pure β-chain of insulin was found at the point indicated by the arrow. Fractions 7–9 were used for amino acid analysis.

tested in the skin capillary permeability test in guinea-pigs as described by Craig (1965). It was found to increase skin capillary permeability in 4 hr to the same extent as a 1/500 dilution of crude filtrate, and its cell toxicity was roughly one hundredth that of the crude material.

The concentrate was then run on a G50 column, in ammonium bicarbonate buffer and, as shown in Fig. 5, the biological activity travelled well ahead of 230 nm absorbing components. The active fractions were concentrated by evaporation

but lost their biological activity; however, after hydrolysis they contained a limited number of amino acids, suggesting the presence of a peptide with the composition shown in the Table. The indicated number of amino acid residue is consistent with the fact that β-chain of insulin (30 residues) appeared in the column at the point shown in Fig. 5. Similar results were obtained on amino acid analysis of another run from which rather less peptide was available for analysis.

TABLE.—*Amino Acid Composition of Fractions from G50 Column which Contained Biological Activity* (Fig. 5)

Amino acids	μ moles	Possible no. of residues
Lysine	0·007	1
Aspartic acid	0·08	3
Threonine	0·015	1
Serine	0·064	3
Glutamic acid	0·075	3
Proline	0·066	3
Glycine	0·29	11
Alanine	0·058	2
Valine	0·036	2
*Iso*leucine	0·008	1
Leucine	0·019	1

No other amino acid was detected.

Serological activity.—A constituent of crude cholera toxin adsorbs to red cells which are then agglutinated by specific antitoxic sera (Hochstein, Feeley and Richardson, 1970). We found that crude toxin could be titrated by making serial dilutions in phosphate buffered saline, adding an equal volume (0·2 ml.) of 1 per cent sheep red cells and allowing the cells to stand, with occasional shaking, for 1 hr. The addition of 2–4 agglutinating doses of antitoxic serum produced agglutination in cells treated with dilutions of toxin up to about 1/160. This test was also applied to the fractions from the column and it can be seen that serologically reactive material was found in fractions from the G25 column which showed cytotoxic activity—mainly in those representing material of low molecular weight; in addition there was weak activity in the most cytotoxic fractions from the G50 column.

DISCUSSION

These results suggest to us that we have isolated a toxic peptide, which may be associated with lipid because biological activity is somewhat ether labile, and could represent a fragment of a larger toxic molecule. Attempts to determine the amino acid sequence will now be made. However, other problems remain to be solved. For instance, it seems that choleragen is derived from the cholera vibrio by being shed from the surface under appropriate growth conditions (Kennedy and Richardson, 1969)—it remains to be discovered how the large and small molecular weight toxins are interrelated and whether they are related chemically and serologically to any component of the cell surface.

It seems that the cytotoxic factor is similar to that which increases vascular permeability; if biological activity could be better preserved during purification we could find out whether it also has an enterotoxic effect or not, although the results of comparative tests of antitoxic activity in the rabbit skin and the ileal

loop strongly suggest that these respond to the same component in crude toxin (Mosley, Aziz and Ahmed, 1970). Other workers using tissue cultures have observed vacuolation in intact cell sheets due to a heat labile component in cholera toxin (Read, 1965), and similar changes in cultures inoculated with filtrates of stools from some cholera patients (Mallik, Bannerjee, Mallik, Ghosh and Monol, 1969); it remains to be discovered whether these effects were due to the factor we have been studying. Our fraction is clearly distinct from therm toxic " choleragenoid " (Finkelstein and Lospalluto, 1969), for it is toxic and of smaller molecular weight; however, our fraction might well lose biological activity if it were recovered by a lengthy fractionation procedure, in which case it would possess only serological activity.

The type of change seen in HeLa cells suggests a reversible effect on the cell structure, perhaps membranes or microtubules, and this can now be studied in isolated cells *in vitro*, using purified toxic peptide. This may well shed light on the disordered physiology observed in the disease, or, on the other hand, indicate whether the cytotoxic factor is likely to produce the effects known to occur in cholera.

We would like to thank Dr. R. A. Phillips and Professor G. M. Bull for obtaining samples of cholera toxin for us. We are very grateful to Dr. D. Smythe who gave us much helpful advice in the use of columns and Mr. Arthu Ko who operated the amino acid analyser. Dr. W. E. van Heyningen kindly supplied antitoxic serum.

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