RESPONSE OF MAMMALIAN CELL LINES TO THE TOXINS OF
ESCHERICHIA COLI

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SUMMARY. The effects of the thermolabile (LT) and thermostable (ST) enterotoxins and the Vero-cell (VT) toxin of *Escherichia coli* were studied in continuous cell lines in tissue culture. The LT enterotoxin induced morphological changes in mouse adrenal (Y-1), Chinese hamster ovary (CHO-K1) and African green-monkey kidney (Vero) cells. VT toxin produced cytotoxic effects in adult Rhesus-monkey kidney (LLC-MK2), human embryonic foreskin (HFS) and Vero cells. The ST enterotoxin had no effect on any of the cell lines.

INTRODUCTION

Enterotoxigenic strains of *Escherichia coli* are now recognised as important causes of diarrhoeal disease in man in several parts of the world (Sack *et al.*, 1971; Woodward *et al.*, 1974; Giugliano, Meneguetti and Trabulsi, 1978a; Goldhar *et al.*, 1980). These bacteria produce at least two enterotoxins—thermolabile (LT) and thermostable (ST). The identification of enterotoxigenic strains depends upon the demonstration of the production of LT or ST because the strains do not necessarily have any other bacteriological or biochemical characteristic to distinguish them from non-toxigenic strains.

Classically, enterotoxins have been detected in whole-animal models such as suckling mice (Dean *et al.*, 1972) or the rabbit ileal loop (Drucker, Yeivin and Sacks, 1967; Moon, Whipp and Baetz, 1971). However, tissue cultures of mouse adrenal cells (Y-1; Donta, Moon and Whipp, 1974) and Chinese hamster ovary cells (CHO-K1; Guerrant *et al.*, 1974) have been used to study the LT enterotoxin (Giugliano *et al.*, 1978b; Kunkel and Robertson, 1979; Reis *et al.*, 1980). Culture filtrates of some strains of *E. coli* also contain Vero-cell toxin (VT) that is cytotoxic for African green-monkey kidney (Vero) cells (Konowalchuk, Speirs and Stavric, 1977). This cytotoxin differs from LT and ST enterotoxins but its role in diarrhoeal disease is not established.

Tissue-culture systems have proved useful for studying the epidemiology of LT-producing strains. The assays are performed easily and they avoid costly and variable animal models. Furthermore, the action of toxins on cells in culture has allowed successful investigation of the molecular basis of pathogenicity of the enterotoxins of *Vibrio cholerae* and *E. coli* (Guerrant *et al.*, 1974).

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It has been suggested that the use of cell cultures for the assay of the enterotoxins of *E. coli* has been superseded by the development of ELISA (Yolken et al., 1977; Sack et al., 1980) and RIA (Frantz and Robertson, 1981; Gianella, Drake and Luttrell, 1981) tests. However, although such assays may detect specific molecules of toxin, they lack the functional amplification of a specific biological assay. The amplification associated with the enzymic activity in ELISA or the radioactive decay in RIA does not depend on the activity of the toxin.

The aim of this study was to determine the effects of the *E. coli* toxins on several mammalian cell lines in tissue culture. Only three of these cell lines had been studied previously.

**Materials and Methods**

*Bacterial strains.* Nine enterotoxin-producing stains of *Escherichia coli* were studied. Strains P307, H296, H19 and P3 were donated by Dr H. W. Smith (Houghton Poultry Research Station, Huntingdon, Cambridgeshire). The strain P307 was isolated from a pig with diarrhoea and produces LT and ST; strain H19 was from a human infant and produces LT and VT; strain P3 was from a pig and produces ST and VT; and strain H296 was from an adult with diarrhoea and produces LT; strains H43B, H31B, H76B and H105B were isolated from cases of infantile diarrhoea in Manaus, Brazil, and produce LT and ST (Giugliano et al., 1978b). Strain E14350 produces only ST enterotoxin; it was donated by Dr B. Rowe of the Division of Enteric Pathogens, Central Public Health Laboratory, Colindale, London NW9 5HT. *E. coli* K12 was used as a non-enterotoxigenic control.

*Conditions for growth and toxin production.* Cultures were grown in 250-ml conical flasks containing 20 ml of Evans Medium (Evans, Evans and Gorbach, 1973) aerobically at 37°C in a rotary shaking water bath (Grant Instruments, Barrington, Cambridge CB2 5QZ). After 18–20 h, the cultures were centrifuged at 3000 g for 20 min and the supernates were filtered through a membrane filter of pore size 0.22 μm (Millipore). The filtrates were stored in 0.5 ml amounts at -70°C.

*Cell culture.* Ten established mammalian cell lines were used: Chinese-hamster ovary (CHO-K1), mouse adrenal-cortex tumour (Y-1), mouse areolar and adipose tissue (L-929), African green-monkey kidney (Vero), adult Rhesus-monkey kidney (LLC-MK₂), rabbit kidney (RK-13), dog kidney epithelial cells (MDCK), embryonic human intestine (INT-407), human embryonic foreskin (HFS) and human embryonic lung (MRC-5).

Y-1 and CHO-K1 cells were grown in Ham Nutrient Mixture F-12 (Flow Laboratories Ltd, Irvine, Ayrshire KA12 8NB, Scotland); Vero and LLC-MK₂ cells in medium 199 with Earle’s salts (Flow Laboratories); MRC-5, HFS, RK-13, MDCK, L-929 and INT-407 cells in Minimum Essential Medium with Earle’s salts (Gibco Europe Ltd, Paisley, Strathclyde PA3 4EP, Scotland). All media were supplemented with fetal-calf serum (Gibco) 10% v/v, penicillin G 100 units/ml and streptomycin 100 μg/ml (Glaxo Laboratories Ltd, Greenford, Middlesex).

*Toxin assays* were performed in microtitration plates containing 96 6-mm-diameter wells (Falcon Microtest II; Becton, Dickinson, Cockneysville, USA). All assays were conducted with confluent monolayers and freshly seeded cells of each cell line. Confluent monolayers were prepared by seeding 2 × 10⁴ cells in 0.1 ml of medium into each well and incubating for 1–2 days at 37°C in a humidified incubator with an atmosphere of air + CO₂ 5%. Growth medium was then removed by inversion of the plate and replaced by an equal volume of maintenance medium that was the same basic formula as the growth medium but contained fetal-calf serum 1% v/v and HEPES buffer pH 7.2 (N-2 hydroxyethyl)piperazine N-2-ethane-sulfonic acid) (Sigma London Chemical Co Ltd, Poole, Dorset BH17 7HF) 2.5% v/v. For the assays with freshly seeded cells, 2 × 10⁴ cells in 0.1 ml of the maintenance medium were put in the wells immediately before the toxin preparation. The inoculum of each well was 25 μl of serial twofold dilutions of the sterile bacterial filtrate. The plates were sealed with plastic film and incubated at 37°C.

Cells were examined microscopically 18–20, 48 and 72 h after addition of the filtrates to detect any effects on cell growth and morphology. The effects observed were scored as 0, 1, 2, 3 or 4, corresponding to c. 25, 50, 75 or 90% of the cells having an abnormal appearance.
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Characterisation of the responses of the cell types to purified toxin. The responses of Vero, LLC-MK₂, HFS, MRC-5 and CHO-K1 cells to N°, O°-dibutrylcytidosine 3':5'-cyclic monophosphoric acid (cAMP; Sigma 0627), N°, O°-dibutrylguanosine-3':5'-cyclic monophosphoric acid (cGMP; Sigma D3510) and cholera toxin (Sigma C3012) were examined. Solutions of cAMP 5 mg/ml, cGMP 100 mg/ml and cholera toxin 1 mg/ml in phosphate-buffered saline (Oxoid) were prepared and 25 µl of twofold serial dilutions of each solution were added to freshly seeded cells. The cells were examined microscopically after c. 3, 20 and 48 h.

RESULTS

Effect of the filtrates on the ten cell lines

None of the filtrates of the E. coli cultures had any effect on the monolayers or the freshly seeded cultures of MRC-5, L-929, RK-13 or INT-407 cells at the highest concentration tested. CHO-K1 and Y-1 showed characteristic cytopathology after incubation with the filtrates of strains P307, H296, H43B, H31B and H76B, that were known to produce LT. Vero cells were affected by the filtrates from LT-producing strains and by filtrates from strains H19 (LT and VT) and P3 (ST and VT). The filtrate of strain E14350 had no effect on any of the cell lines studied. Comparison of the results obtained with filtrates of the LT-producing strains on confluent monolayers showed that Y-1 cells were more sensitive than Vero and CHO-K1 cells in the same conditions.

The 50% endpoint of the characteristic effect was taken as the titre of toxin in the filtrate and was calculated by interpolation when necessary. The LT titre measured in Vero and Y-1 cells varied and depended upon the strain (table I). The highest titres were obtained with filtrates of strains H296 and P307, which gave absolute end points of 256 in Y-1 and Vero cells, and 16 in CHO cells. Vero, LLC-MK₂ and HFS cells gave a clear response to the filtrates of strains H19 and P3 but the titres were different

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Filtrate from strain no.</th>
<th>Effect on the cell line of filtrate dilutions of toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 4 8 16 32 64 128 256</td>
</tr>
<tr>
<td>Y-1</td>
<td>H296</td>
<td>4 4 4 4 4 4 3 1 192</td>
</tr>
<tr>
<td></td>
<td>P307</td>
<td>4 4 4 4 4 3 2 1 128</td>
</tr>
<tr>
<td></td>
<td>H43B</td>
<td>4 4 4 4 4 2 1 0 64</td>
</tr>
<tr>
<td></td>
<td>H31B</td>
<td>4 4 4 4 3 2 1 0 64</td>
</tr>
<tr>
<td></td>
<td>H76B</td>
<td>4 4 4 3 1 0 0 0 24</td>
</tr>
<tr>
<td>Vero</td>
<td>H296</td>
<td>4 4 4 4 4 4 3 1 192</td>
</tr>
<tr>
<td></td>
<td>P307</td>
<td>4 4 4 4 4 4 3 1 192</td>
</tr>
<tr>
<td></td>
<td>H43B</td>
<td>4 4 4 4 4 2 2 0 96</td>
</tr>
<tr>
<td></td>
<td>H31B</td>
<td>4 4 4 4 4 2 1 0 64</td>
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<tr>
<td></td>
<td>H76B</td>
<td>4 4 4 2 1 0 0 0 16</td>
</tr>
<tr>
<td>CHO-K1</td>
<td>H296</td>
<td>2 2 1 1 0 0 0 0 3</td>
</tr>
<tr>
<td></td>
<td>P307</td>
<td>2 2 1 1 0 0 0 0 3</td>
</tr>
<tr>
<td></td>
<td>H43B</td>
<td>2 2 1 1 0 0 0 0 3</td>
</tr>
<tr>
<td></td>
<td>H31B</td>
<td>2 2 1 0 0 0 0 0 3</td>
</tr>
</tbody>
</table>

0 = no response; 1 = ≤ 25% of cells affected; 2 = c. 50%; 3 = c. 75% and 4 = ≥ 90%.
* See Materials and methods.
Effect of filtrates of VT-producing strains of E. coli on freshly-seeded cultures of Vero, LLC-MK₂ and HFS cells after incubation for 48 h

**Table II**

Effect of filtrates of VT-producing strains of E. coli on freshly-seeded cultures of Vero, LLC-MK₂ and HFS cells after incubation for 48 h

<table>
<thead>
<tr>
<th>Cell line*</th>
<th>Filtrate from strain no.</th>
<th>Effect on the cell line of filtrate dilutions of</th>
<th>Titre of toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero</td>
<td>H19</td>
<td>4 4 4 4 4 4 4 3 &gt;256</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>4 4 3 2 1 1 0 0</td>
<td>16</td>
</tr>
<tr>
<td>LLC-MK₂</td>
<td>H19</td>
<td>4 3 3 2 2 2 1 0</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>3 2 1 0 0 0 0 0</td>
<td>4</td>
</tr>
<tr>
<td>HFS</td>
<td>H19</td>
<td>4 4 3 3 2 2 1 1</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>3 3 2 1 1 0 0 0</td>
<td>8</td>
</tr>
</tbody>
</table>

Footnotes as in Table I.

with each cell line (table II). Filtrates of strain H19 were more active than those of strain P3.

MDCK cells were not affected by the filtrates from LT- or ST-producing strains but gave an equivocal response to the filtrates of strain P3; a cytotoxic response was observed with only some cell cultures.

![Figure 1](image-url)

**Fig. 1.**—African green-monkey kidney (Vero) cells after incubation for 24 h with a twofold dilution of E. coli K-12 filtrate (A) and with a 32-fold dilution of E. coli strain P307 filtrate (B).

Chinese-hamster ovary cells after incubation for 24 h with a twofold dilution of E. coli K-12 filtrate (C) and with a fourfold dilution of E. coli strain P307 filtrate (D). (All × 100).
Specificity of the cytotoxic effects

In the presence of LT, CHO-K1 cells became elongated and Y-1 cells became round; Vero cells became refractile with several filamentous tendrils extending from the cells (fig. 1). The most clearly defined effects with the three sensitive cell lines were observed after 18–20 h.

The cytopathic effect caused by filtrates of strains P3 and H19 on Vero, LLC-MK₂ and HFS cells was similar; the cells became round and the monolayer was disrupted.

Fig. 2.—Vero cells after incubation for 72 h with a twofold dilution of *E. coli* K-12 filtrate (A) and with a 64-fold dilution of *E. coli* strain H19 filtrate (B). Rhescus-monkey kidney (LLC-MK₂) cells after incubation for 48 h with a twofold dilution of *E. coli* K-12 filtrate (C) and with a 64-fold dilution of *E. coli* strain H19 filtrate (D). Human embryonic foreskin (HFS) cells after incubation for 48 h with a twofold dilution of *E. coli* K-12 filtrate (E) and with a 64-fold dilution of *E. coli* strain H19 filtrate (F). (All × 100).
This effect appeared after 24 h and increased with further incubation. However, the time required to obtain the most characteristic effect varied with the cell type. LLC-MK2 and HFS cells showed the most characteristic response after 48 h and Vero cells after 72 h.

**Effect of cholera toxin, cAMP and cGMP on the cell lines**

The addition of cholera toxin and cAMP to cultures of Vero and CHO cells produced a characteristic morphological effect similar to that produced by LT-producing *E. coli* strains. MRC-5, HFS and LLC-MK2 cells were not affected by cAMP or cholera toxin. The lowest concentrations of cholera toxin that produced morphological changes in c. 25% of Vero and CHO cells were $3 \times 10^{-3}$ ng/ml and 2 ng/ml respectively. The lowest concentrations of cAMP that produced the characteristic effect in c. 25% of Vero and CHO cells were 60 µg/ml and 120 µg/ml respectively.

Cyclic GMP caused rounding of Vero cells at concentrations of 6–25 mg/ml. Concentrations below 6 mg/ml reduced the growth rate of this cell line and the morphology of the cells was similar to that produced by cAMP or cholera toxin. LLC-MK2, MRC-5, HFS and CHO cells were also affected by cGMP. The cells appeared round and more refractile; c. 25% of cells were affected by 12 mg/ml of cGMP.

**Comparison of freshly seeded cells and confluent monolayers**

The titres of the toxic activity in the filtrates that produced cytopathic effects on the sensitive cell lines are shown in table III. The results were recorded at the time when the characteristic response was most clearly seen. In general, the freshly seeded cells were more sensitive to the toxins and gave higher titres than the confluent monolayers.

**TABLE III**

*Comparison of freshly seeded cells and confluent monolayers for the detection of *E. coli* enterotoxins*

<table>
<thead>
<tr>
<th>Cell line*</th>
<th>Type of culture</th>
<th>Titre (50% endpoint) of toxic activity in filtrate of strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero</td>
<td>FS</td>
<td>P307 192 H296 192 H19 256 16 96 64 16 M</td>
</tr>
<tr>
<td>CHO</td>
<td>FS</td>
<td>3 3 &lt;2 &lt;2 &lt;2 3 3 ... M</td>
</tr>
<tr>
<td>Y-1</td>
<td>FS</td>
<td>128 192 &lt;2 &lt;2 &lt;2 64 64 24 M</td>
</tr>
<tr>
<td>LLC-MK2</td>
<td>FS</td>
<td>&lt;2 &lt;2 &lt;2 &lt;2 24 4 &lt;2 &lt;2 &lt;2 &lt;2 M</td>
</tr>
<tr>
<td>HFS</td>
<td>FS</td>
<td>&lt;2 &lt;2 &lt;2 &lt;2 48 8 &lt;2 &lt;2 &lt;2 &lt;2 M</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>&lt;2 &lt;2 &lt;2 &lt;2 16 4 &lt;2 &lt;2 &lt;2 &lt;2</td>
</tr>
</tbody>
</table>

FS = freshly seeded cells; M = confluent monolayer.
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DISCUSSION

Guerrant et al. (1974), Donta et al. (1974) and Speirs, Stavric and Konowalchuk (1977) described the characteristic morphological changes caused by the LT enterotoxin of E. coli in CHO-K1, Y-1 and Vero cells respectively. In our studies, sterile filtrates of enterotoxigenic E. coli strains produced morphological changes in \( \geq 90\% \) of the Y-1 and Vero cells and in c. 50\% of the CHO-K1 cells. Guerrant and Brunton (1977) found that filtrates of E. coli strain 334 caused elongation of c. 37\% of CHO-K1 cells in tissue culture and purified E. coli toxin caused elongation of c. 33\% of cells. Guerrant et al. (1974) found that c. 50\% of cells were affected by purified cholera toxin. Diluted supernates of enterotoxigenic E. coli strains cause morphological changes in \( \geq 90\% \) of Y-1 and Vero cells (Donta and Smith, 1974; Isaacson and Moon, 1975; Speirs et al., 1977). There are two possible explanations for these results: CHO-K1 cells may be affected only at a particular phase of growth, or the population of CHO-K1 cells may not be homogeneous and the cells may vary in sensitivity to the enterotoxin. McDonel and McClane (1979) demonstrated two distinct populations of Vero cells with different sensitivity to Clostridium perfringens enterotoxin.

Isaacson and Moon (1975) and Donta and Smith (1974) found LT titres of eight and 160 respectively in filtrates tested in Y-1 cells. The highest dilution of an enterotoxin filtrate that affected Y-1 cells in our studies was 256. The yield of enterotoxin from E. coli strains varies from strain to strain and is influenced by the culture medium (Gilligan and Robertson, 1979). Furthermore, several factors in the cell culture system can influence the cell's response to the enterotoxin, e.g., the concentration of fetal-calf serum in the growth medium (Guerrant et al., 1974), the concentration of cells (Speirs et al., 1977), and the phase of growth in which the toxin is added (table III).

We have demonstrated that two more cell lines, LLC-MK2 and HFS, are sensitive to the cytotoxin of E. coli in tissue culture; unlike Vero cells, these are not affected by LT. We could not detect any morphological changes in CHO-K1 or Y-1 cells treated with filtrates of the E. coli strain H19 that produces VT. This strain was isolated from the faeces of a baby with diarrhoea by Smith and Linggood (1971) who found that it caused dilatation of segments of rabbit intestine; this effect was neutralised by homologous antiserum and by an antiserum prepared against strain H30, which also produced VT. However, Konowalchuk et al. (1977) failed to detect fluid accumulation in rabbit ileal loops or morphological changes in Y-1 or CHO-K1 cells with filtrates of strain H19. Furthermore, the effect of the filtrates on Vero cells was neutralised by antiserum raised against strain H30 but not by antiserum against the LT-producing strain H10407. This evidence indicates that strain H19 produced only VT cytotoxin under the conditions used by Konowalchuk et al. (1977) and in the present study.

Puck, Waldren and Hsie (1972) showed that cAMP caused morphological changes in CHO-K1 cells. Cholera toxin stimulates cAMP production and Guerrant et al. (1974) established a CHO-cell assay for cholera toxin. The results of our study showed that cholera toxin or cAMP cause the same morphological changes in Vero cells which suggests that the accumulation of cAMP is responsible for the morphologic effects observed in Vero cells.

None of the 10 cell lines studied in our experiments was affected by the ST
producing strains of *E. coli* and the infant-mouse assay is still required for the identification of this toxin. ST enterotoxin stimulates cGMP production in sensitive cells (Field et al., 1978; Guerrant et al., 1980) and fairly high concentrations of cGMP affected five of our cell lines but there was no response with the filtrates of the ST producing strains of *E. coli*. These results suggest that the cells lack appropriate receptors for ST enterotoxin or that the stimulation produced by ST was insufficient to induce the accumulation of enough cGMP to produce a cytopathic effect. The development of RIA (Frantz and Robertson, 1981; Giannella et al., 1981) and ELISA (World Health Organization, 1981) for ST have been reported recently and these assays may reduce the problems of screening strains for ST production. However, the need for a cell-culture test system remains. The presence of ST receptors on cells in culture remains to be demonstrated but the response of the cell lines to cGMP suggests that the development of such an assay would be aided by the selection of a cell line that is exceptionally sensitive to cGMP.

The present study has shown that several continuous cell lines are sensitive to some of the toxins produced by *E. coli* and that there is good reason to suggest that such assays can be developed for other bacterial toxins.

REFERENCES


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