Examination of enteropathogenic Escherichia coli strains for an adenylcyclase stimulating factor

D. LAW, K. M. WILKIE*, and R. FREEMAN

Microbiology Department, Freeman Hospital, Freeman Road, High Heaton, Newcastle upon Tyne NE7 7DN and *Department of Chemical and Life Sciences, Elison Building, Newcastle Polytechnic, Elison Place, Newcastle upon Tyne

Summary. Enteropathogenic Escherichia coli strains are a common cause of infantile diarrhoea but do not produce recognised enterotoxins. Three strains of proven virulence were examined for toxins which may be missed in conventional tests. Cell lysates and concentrated culture supernates of organisms grown in five different media gave negative results when examined for adenylcyclase stimulating activity. The additions of zinc ions or lincomycin to these media or the use of iron-depleted media also gave negative results. The significance of these findings and the possible role of other toxins in diarrhoea due to enteropathogenic E. coli are discussed.

Introduction

Certain strains of Escherichia coli are associated with diarrhoeal disease in children. These strains belong to a number of distinct O serotypes and are designated enteropathogenic E. coli (EPEC) (Edelman and Levine, 1983). Despite numerous investigations, the mechanism by which these strains produce diarrhoea is still uncertain. Most strains give negative results when examined in assays for the heat-labile (LT) and heat-stable (ST) enterotoxins of enterotoxigenic E. coli (ETEC) and the invasive properties of enteroinvasive E. coli strains (Levine et al., 1978; Robins-Browne et al., 1982).

The clinical and histopathological features of infantile diarrhoea caused by EPEC strains suggest that enterotoxins may be involved in the pathogenesis of this disease (Edelman and Levine, 1983). It has been proposed that EPEC strains may elaborate enterotoxins which are not detected in conventional enterotoxin assays; this could be because the toxin is not reactive in such systems, or the yields of toxins are low, or the culture conditions are not appropriate for optimal toxin production (Robins-Browne et al., 1982).

Klipstein et al. (1978) demonstrated enterotoxic products in some EPEC strains by a rat perfused jejunum assay, and a factor that stimulated adenylcyclase in a similar manner to cholera toxin and LT has been described in some EPEC strains (Kantor et al., 1974; Scheftel et al., 1980). A later study failed to verify these findings (Long-Krug et al., 1984) although media factors, toxin preparation methods and different sources of adenylcyclase (rabbit intestine, rat heart or pigeon erythrocytes) could account for this.

The purpose of this investigation was to examine various preparations of EPEC strains, grown in different cultural conditions, for the presence of an adenylcyclase stimulating factor that may represent an LT-like toxin that is unreactive in conventional enterotoxin assays.

Materials and methods

Bacterial strains

Three enteropathogenic E. coli strains, E2348/69 (O127H6), E851/71 (O142H6) and E12801 (O114H2) were kindly provided by Dr M. Levine. These strains had been shown to produce diarrhoea in human volunteers but did not produce LT or ST (Levine et al., 1978, 1985). An enterotoxigenic E. coli strain TD 327-C1 was supplied by Dr K. Wachsmuth. Strains were stored lyophilised, and fresh cultures were used for each batch of assays.

Media

The following media were used to cultivate the organisms before enterotoxin testing: (1) modified Casamino acid Yeast Extract Broth (CAYE) (Mundell et al., 1976); (2) Brain Heart Infusion Broth (BHI) (Lab M); (3) Syncase Broth (SB) (O'Brien et al., 1982); (4) AKI Medium (AKI) (Iwanaga and Yamamoto, 1985); (5) Tryptone Soya Broth (TSB) (Oxoid).

The media were also prepared containing either

Received 6 June 1986; accepted 9 Sep. 1986.
lincomycin 90 mg/L or 10⁻⁵ M zinc chloride; both treatments enhance LT production by ETEC strains (Levner et al., 1977; Sugarman and Epps, 1984). Media were dispensed in 50 ml volumes in 250 ml Erlenmeyer flasks and seeded with 200 µl of an overnight culture of the E. coli strain grown in Nutrient Broth (Oxoid). Incubation was for 24 h at 37°C except for lincomycin-containing media for supernate preparation which were incubated for 48 h which is known to enhance LT yields from ETEC strains (Levner et al., 1977).

Iron depleted media

Iron depleted (Fe-) TSB and SB were prepared with Chelex 100 (Bio Rad), by the method of O’Brien et al. (1982); 0.2 ml of a cation supplement containing CuSO₄·5H₂O 0.0115 g/L, MgSO₄·7H₂O 2.38 g/L, CaCl₂ 2.8 g/L and ZnSO₄·7H₂O 0.68 g/L was added to 200 ml of Chelex-treated medium to restore cations to pretreatment levels.

Fe- media were dispensed as previously described into flasks cleaned by soaking in Decon 90 (BDH), once in HCl 1% v/v, again in double distilled water (DDW), once in HCl 1% v/v, again in DDW and soaked in EDTA 0.01% before rinsing well with DDW. Fe- media were incubated as described by O’Brien et al. (1982). The inoculum was 100 µl of an 18-h culture of an EPEC strain inoculated for 48 h which is known to enhance LT yields from ETEC strains (Levner et al., 1977).

Enterotoxin preparations

Concentrated culture supernates were prepared by the method of Scheftel et al. (1980) and dialysed against physiological saline for 48 h at 4°C.

Cell lysates were prepared by the method of Donohue-Rolle and Keusch (1983), with polymyxin at a concentration of 300 µg/ml. Each supernate and cell lysate was divided into two aliquots; one was activated by treatment with trypsin and dithiothreitol (Coulson et al., 1984).

Preparation of adenylcyclase

Crude rat liver membranes from one rat were used as the adenylcyclase source. These were prepared by the procedure of Neville (1968), up to step 5. The pellet from the final centrifugation was resuspended in 20 mM morpholinopropanesulfonic acid-50 mM sucrose buffer, pH 7.5, diluted to 50 ml in the same buffer and stored frozen at -20°C in 1 ml volumes.

Preparation of chromatography columns

Dowex and alumina chromatography columns were prepared and the optimal elution volumes for adenosine triphosphate (ATP) and cyclic adenosine 3',5' monophosphate (cAMP) were determined by the method of Salomon (1979). Solutions (50 mM) of cAMP and ATP replaced the radiolabelled compounds, and the E₂₅₉ of the eluant was measured. Fresh alumina columns were used for each batch of assays.

**Adenylcyclase assay**

The adenylcyclase (AC) assay was performed by the method of Coulson et al. (1984) with modifications; 40 µl of each toxin preparation was examined in the assay. The assay cocktail contained 1 µCi of [2-³H] ATP (Amersham International) replacing P³² ATP. The reaction was stopped with 1 ml of sodium dodecyl sulphate 0-2% containing 9 µl of 100 mM cAMP.

Of the 4-ml final eluant, 2 ml was used for liquid scintillation counting (Salomon, 1979). To determine the recovery efficiency of the chromatography procedure for each tube, 1 ml of eluant was diluted and the E₂₅₉ determined and compared with a standard containing 10 µl of 100 mM cAMP suitably diluted.

Blanks, basal enzyme activity and total counts were calculated as described by Salomon (1979). Sodium fluoride (final concentration 5 mM) or 1 µg of cholera toxin (Sigma) were used as positive controls. Polymyxin and toxin activating solution were also tested to determine their effect upon adenylcyclase activity. Each test was performed in duplicate and results were determined from the means of paired samples.

The amount of cAMP formed in tube A was determined by the following formula:

\[
\left( \frac{E_{259} \text{ cAMP std}/E_{259} \text{ tube A}}{\text{total count} - \text{background count}} \right) \times 2 \times \left( \text{cpm tube A} - \text{background count} \right) \times 20 \times 10^{-4} \\
\text{AC activity in tube A (pmoles cAMP formed per min)} \\
\frac{\text{(pmoles cAMP formed in tube A)}}{\text{10}} - \frac{\text{pmoles cAMP formed in blank}}{\text{Basal AC activity}} \\
\]

Degree of adenylcyclase stimulation

\[
\text{AC activity in tube A} \\
\text{Basal AC activity} \\
\]

**Results**

The assay was found to give reproducible results and stimulation of the enzyme was demonstrated by cholera toxin (eight-fold stimulation over basal levels) and sodium fluoride (five-fold stimulation). Results obtained with controls are shown in table I. Results of the enterotoxin preparations are given in table II.

None of the concentrated supernates or cell lysates of the three strains grown in any of the five basal media showed stimulation of adenylcyclase. All values were similar to basal enzyme values showing that neither stimulatory or inhibitory
### Table I. Adenylcyclase activity of controls

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Ac activity/basal activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal activity</td>
<td>1.0</td>
</tr>
<tr>
<td>Toxin activating reagents</td>
<td></td>
</tr>
<tr>
<td>Polymyxin</td>
<td>1.01</td>
</tr>
<tr>
<td>Cholera toxin (1 µg)</td>
<td>8.1</td>
</tr>
<tr>
<td>Sodium fluoride (5 mM)</td>
<td>5.2</td>
</tr>
<tr>
<td>ETEC supernate U</td>
<td>1.9</td>
</tr>
<tr>
<td>ETEC supernate A</td>
<td>6.4</td>
</tr>
<tr>
<td>ETEC cell lysate U</td>
<td>2.1</td>
</tr>
<tr>
<td>ETEC cell lysate A</td>
<td>6.8</td>
</tr>
</tbody>
</table>

* A = activated with trypsin and dithiothreitol; U = unactivated.

activity was present in the preparations. The addition of lincomycin or zinc ions to each of the five media did not alter the basal adenylcyclase activity. When strains were grown in iron-depleted media, again no adenylcyclase stimulatory activity was detected in any of the preparations tested.

### Discussion

We have failed to demonstrate the presence of an LT-like toxin that activates adenylcyclase in concentrated culture supernates or cell lysates of three EPEC strains. These results support and extend the findings of Long-Krug *et al.* (1984) by showing that the use of various culture media or iron depleted media and the addition of zinc ions or lincomycin, factors which affect toxin production by other intestinal pathogens (Levner *et al.*, 1977; Mundell *et al.*, 1976; O'Brien *et al.*, 1982; Sugarman and Epps, 1984; Iwanaga and Yamamoto, 1984), do not stimulate enterotoxin production by EPEC strains.

It may be argued that these strains have lost their pathogenic properties; however, the only method of demonstrating virulence is to perform feeding studies with human volunteers. The strains have been shown by others to be pathogenic many years after their original isolation (Levine *et al.*, 1978, 1985), suggesting that the pathogenic properties are stable over long periods. There is now evidence, however, that other toxins are produced that may be relevant to the production of diarrhoea. A toxin very similar to *Shigella dysenteriae* type-1 toxin (SDT) is produced by EPEC strains grown in iron-depleted media (O'Brien *et al.*, 1982). SDT production is much more common amongst EPEC strains than amongst non-EPEC strains and the quantities of toxin produced are higher (Cleary *et al.*, 1985). However, when compared with the quantity of toxin produced by *S. dysenteriae*, toxin yields from EPEC strains are very low. The close attachment between EPEC and the small intestinal mucosa

### Table II. Adenylcyclase stimulatory activity of enterotoxin preparations of EPEC strains

<table>
<thead>
<tr>
<th>Medium</th>
<th>E2348 supernate</th>
<th>E2348 cell lysate</th>
<th>E851 supernate</th>
<th>E851 cell lysate</th>
<th>E12801 supernate</th>
<th>E12801 cell lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAYE</td>
<td>0.83</td>
<td>0.8</td>
<td>0.9</td>
<td>1.0</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>CAYE + linc</td>
<td>0.91</td>
<td>1.01</td>
<td>0.95</td>
<td>0.84</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>CAYE + Zn</td>
<td>1.21</td>
<td>1.02</td>
<td>1.08</td>
<td>1.07</td>
<td>0.87</td>
<td>1.0</td>
</tr>
<tr>
<td>BHI</td>
<td>1.22</td>
<td>0.94</td>
<td>1.0</td>
<td>0.98</td>
<td>0.95</td>
<td>0.81</td>
</tr>
<tr>
<td>BHI + linc</td>
<td>1.12</td>
<td>0.91</td>
<td>1.02</td>
<td>0.96</td>
<td>1.2</td>
<td>0.8</td>
</tr>
<tr>
<td>BHI + Zn</td>
<td>0.98</td>
<td>1.1</td>
<td>1.08</td>
<td>1.11</td>
<td>0.87</td>
<td>0.86</td>
</tr>
<tr>
<td>AKI</td>
<td>1.2</td>
<td>0.9</td>
<td>1.16</td>
<td>1.14</td>
<td>1.19</td>
<td>1.1</td>
</tr>
<tr>
<td>AKI + linc</td>
<td>0.8</td>
<td>0.91</td>
<td>0.87</td>
<td>1.1</td>
<td>0.89</td>
<td>1.2</td>
</tr>
<tr>
<td>AKI + Zn</td>
<td>1.09</td>
<td>1.23</td>
<td>1.11</td>
<td>0.91</td>
<td>1.12</td>
<td>0.98</td>
</tr>
<tr>
<td>TSB</td>
<td>0.9</td>
<td>1.0</td>
<td>0.91</td>
<td>1.08</td>
<td>1.19</td>
<td>1.1</td>
</tr>
<tr>
<td>TSB + linc</td>
<td>0.89</td>
<td>0.91</td>
<td>0.75</td>
<td>1.15</td>
<td>0.89</td>
<td>1.2</td>
</tr>
<tr>
<td>TSB + Zn</td>
<td>1.05</td>
<td>1.23</td>
<td>1.07</td>
<td>1.2</td>
<td>1.05</td>
<td>1.23</td>
</tr>
<tr>
<td>Fe - TSB</td>
<td>1.1</td>
<td>1.05</td>
<td>1.0</td>
<td>0.95</td>
<td>0.9</td>
<td>1.05</td>
</tr>
<tr>
<td>SB</td>
<td>1.2</td>
<td>1.16</td>
<td>1.16</td>
<td>1.2</td>
<td>1.17</td>
<td>0.96</td>
</tr>
<tr>
<td>SB + linc</td>
<td>0.79</td>
<td>0.91</td>
<td>0.7</td>
<td>0.89</td>
<td>0.89</td>
<td>1.15</td>
</tr>
<tr>
<td>SB + Zn</td>
<td>1.05</td>
<td>1.2</td>
<td>0.9</td>
<td>1.21</td>
<td>1.12</td>
<td>0.98</td>
</tr>
<tr>
<td>Fe - SB</td>
<td>0.9</td>
<td>0.87</td>
<td>0.95</td>
<td>1.1</td>
<td>1.0</td>
<td>0.9</td>
</tr>
</tbody>
</table>

For abbreviations see table I and Materials and methods.
(Edelman and Levine, 1983) may facilitate delivery of small quantities of SDT to the mucosal cells where it can exert its enterotoxic effect. The mechanism(s) by which SDT brings about intestinal secretion is not yet known. There is disagreement about its ability to activate adenylcyclase because of differences in assay conditions (Cantey, 1985).

SDT activation of adenylcyclase may, however, account for some reports of an LT-like toxin in EPEC strains.

This study was supported by a grant from Newcastle Health Authority. We thank staff in the Pathology and Medical Physics Departments at Freeman Hospital and at Newcastle Polytechnic for invaluable assistance and advice.

REFERENCES


Levine M M *et al.* 1985 The diarrheal response of humans to some classic serotypes of enteropathogenic *Escherichia coli* is dependent on a plasmid encoding an enteroadhesiveness factor. *Journal of Infectious Diseases* **152**: 550–559.


