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Stimulation of Adenyl Cyclase by *Escherichia coli* Enterotoxin

THE acute diarrhoea of cholera is caused by an enterotoxin produced by *Vibrio cholerae*[1]. Experimentally, the activity of this enterotoxin can be demonstrated in the ligated ileal loop of rabbits and dogs[2–4]. In response to intraluminal application of cholera enterotoxin, these loops demonstrate net secretion of fluid and electrolytes[5]. *V. cholerae*, however, is not the only enteropathogen associated with acute diarrhoea. Certain strains of *Escherichia coli*, an organism usually considered to be normal bowel flora, have been associated with epidemics of acute diarrhoea in infants[6], adults[7], and a variety of animal species[8]. Like cholera, *E. coli* diarrhoea is the result of accidental introduction and colonization of the small intestine by an offending organism capable of producing an enterotoxin[7].

Recently, it has become clear that the action of cholera enterotoxin is mediated by increase of intracellular concentrations of cyclic 3′,5′-adenosine monophosphate (cyclic AMP) secondary to stimulation of adenyl cyclase[9–11] As *E. coli* and *V. cholerae* enterotoxins possess similar biological and biochemical properties[12,13], fluid accumulation produced by *E. coli* enterotoxin might also be associated with increased adenyl cyclase activity.

The *E. coli* strains that we used were isolated by rectal swab from adult "non-vibrio cholera" patients[14] admitted to our hospital with acute diarrhoea, copious rice-water stool, marked dehydration and circulatory collapse, but bacteriologically negative for *V. cholerae*. Viable cultures of these *E. coli* strains produced gross fluid accumulation when administered to ligated rabbit ileal loops. *E. coli* cultures were incubated with vigorous shaking for 18 h at 37° C in TRY medium[15] (50 ml./500 ml. Erlenmeyer flasks). TRY medium consisted of the following: 1.0% casamino-acids; 0.05% glycerol; 0.005 M Tris-maleate, *p*H 7.5; 0.25% NaCl; 0.25% KCl; 0.02% Na_2HPO_4; 0.005% yeast extract; 1.0 ml./l. salts (5% $MgCl_2$, 0.5% $FeCl_2$, 0.5% $MnCl_2$ in 0.001 N H_2SO_4). Sterile cell-free culture supernatants were obtained by centrifugation and passage through a 'Millipore' filter. These supernatants also consistently caused gross fluid accumulation in rabbit

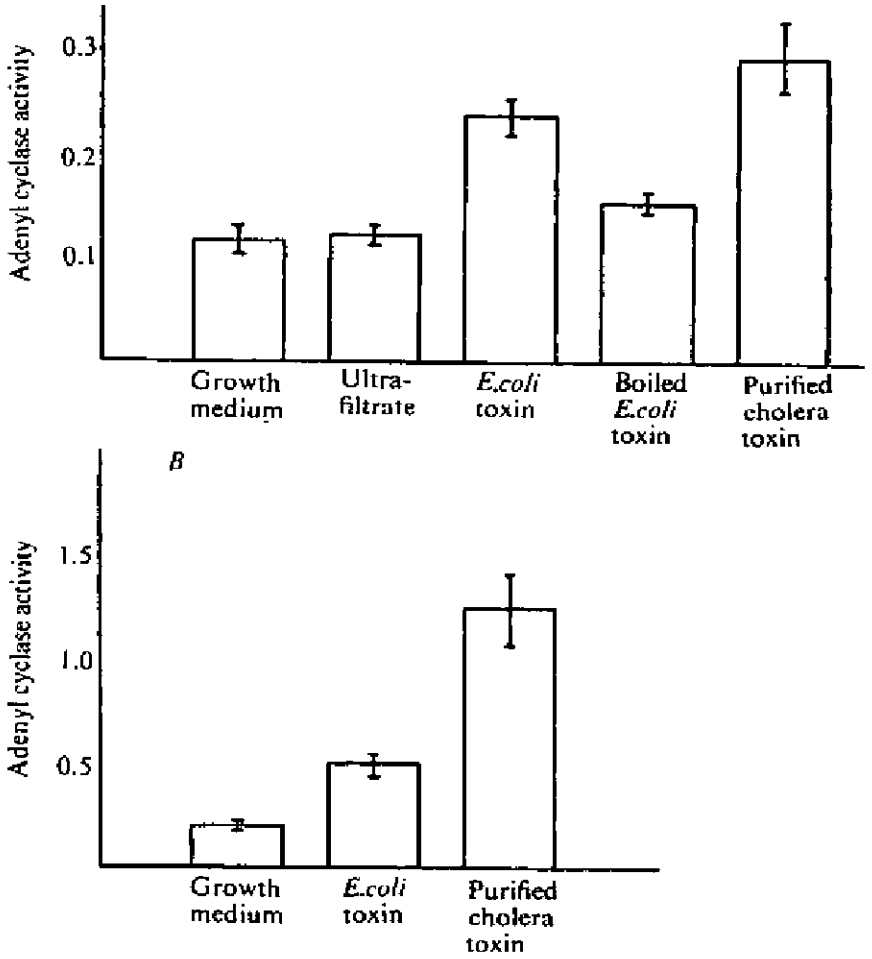

Fig. 1 *A*, The effect of *E. coli* enterotoxin (ten-fold concentrated culture supernatant) and purified *V. cholerae* enterotoxin on adenyl cyclase activity of rabbit ileal mucosa. Controls include TRY medium, Diaflo ultrafiltrate and ten-fold concentrate boiled for 20 min. Adenyl cyclase activity is expressed in nmol cyclic AMP formed/mg protein/20 min incubation. Cross bars represent ± 1 s.e. ($n=8$). *E. coli* and *V. cholerae* enterotoxin stimulated activities are significantly elevated from controls ($P<0.01$). *B*, The effect of TRY medium (control), *E. coli* enterotoxin (ten-fold concentrated culture supernatant), and purified *V. cholerae* enterotoxin on adenyl cyclase activity of isolated rat adipocytes. Adenyl cyclase activity is expressed in nmol cyclic AMP formed/mg protein/20 min incubation. Cross bars represent ± 1 s.e. ($n=6$). *E. coli* and *V. cholerae* enterotoxin stimulated activities are significantly different from control ($P<0.01$).

ileal loops. The enterotoxic component was found to be heat-labile (destroyed at 100° C for 20 min), non-dialysable, and precipitable by ammonium sulphate. This component was concentrated by passing culture supernatants through a 'Diaflow' apparatus fitted with a UM-10 membrane filter

followed by 'Millipore' filtration. The effect of two such *E. coli* enterotoxin preparations on intestinal adenyl cyclase activity was studied by inoculating adjacent 10 cm ligated rabbit ileal loops with ten-fold concentrate, sterile TRY medium (control), heat-inactivated concentrate, and 5 μg of purified cholera enterotoxin[15], respectively. Eight 2 kg rabbits were employed in which the sequence of test and control loops was varied, but *E. coli* and cholera enterotoxin-treated loops were never immediately adjacent to each other.

After 4 h, the animals were killed and mucosal adenyl cyclase activity was determined by the method of Krishna *et al.*[16]. Mucosal cells were isolated by scraping with glass slides and homogenization was completed with Ten Broeck hand homogenizers. To minimize breakdown of labelled cyclic AMP by phosphodiesterase, 0.1 mM cyclic AMP was included in the reaction mixture[17]. To begin the incubation, 20 μl. of tissue homogenate (equivalent to 30–50 μg of protein) was added to 30 μl. of incubation mixture containing 1 μCi-ATP-α^{32}P and incubated at 37° C for 20 min. The final composition of the assay mixture consisted of 30 mM Tris-HCl buffer (*p*H 7.5), 5 mM $MgCl_2$, 0.1 mM cyclic AMP, 0.1 mM ATP, 5 mM phosphoenolpyruvate, 40 μg/ml. phosphoenolpyruvate kinase, and 20 μg/ml. myokinase. After incubation, a known amount of ^{3}H-cyclic AMP was added to determine the efficiency of recovery of cyclic AMP after cation exchange and precipitation.

Both *E. coli* and *V. cholerae* enterotoxins markedly stimulated adenyl cyclase activity as compared with control and heat-inactivated concentrate (Fig. 1*A*). All loops with enhanced enzymatic activity exhibited gross fluid accumulation but control and heat-inactivated enterotoxin-treated loops displayed no fluid accumulation. In similar experiments, viable cultures of *E. coli* strains obtained by rectal swab from four non-diarrhoeal normal adults in East Bengal failed to produce fluid accumulation. In addition, culture supernatants and ten-fold concentrates of these strains failed to elicit fluid accumulation or enhanced adenyl cyclase activity.

Six experiments to determine the effect of *E. coli* enterotoxin on adenyl cyclase activity and lipolysis of rat adipocytes (fat cells) were performed by incubating isolated intact adipocytes with enterotoxin for 3 h. *E. coli* ten-fold concentrate, 0.1 μg of purified cholera enterotoxin, or TRY medium control was added to each incubation. Adipocytes were prepared and lipolysis was determined by the method of Vaughn *et al.*[18]. Adenyl cyclase activity was determined on a membrane-nuclear fraction which was prepared by homogenization and centrifugation (1,000*g* for 20 min). *E. coli* enterotoxin, like cholera enterotoxin, clearly stimulated adenyl cyclase activity

of rat adipocytes (Fig. 1*B*). Similarly, both enterotoxin preparations were found to stimulate lipolysis, a known consequence of increased intracellular levels of cyclic AMP[19].

The suggestion that the adenyl cyclase-cyclic AMP system may be the intracellular mediator of choleraic diarrhoea has considerable supporting evidence. Added cyclic AMP and agents which increase the intracellular level of cyclic AMP such as theophylline stimulate short circuit current and net secretory ion flux in isolated rabbit[9] and human ileal mucosa[20], changes which are qualitatively similar to those produced by cholera enterotoxin. Enhanced adenyl cyclase activity and elevated levels of cyclic AMP have been demonstrated in rabbit[10,21], guinea-pig[11], and dog[22,23] intestinal mucosa after exposure to cholera enterotoxin. Increased adenyl cyclase activity has been recently demonstrated in jejunal mucosa of patients with naturally acquired cholera[24]. In addition, cholera enterotoxin stimulates cyclic AMP mediated responses in a variety of non-intestinal tissues such as lipolysis in adipocytes[18] and glycogenolysis in liver homogenates and platelet lysates[25].

Our study demonstrates that *E. coli* enterotoxin, like cholera enterotoxin, stimulates adenyl cyclase activity in intestinal mucosa and in isolated rat adipocytes. The enterotoxins are not identical as our *E. coli* concentrated enterotoxin preparations failed to elicit the skin response[26], characteristic of cholera enterotoxin. But because of the similarity of their properties with respect to production *in vitro*, temperature sensitivity, high molecular weight, lipolytic activity in rat adipocytes, and fluid accumulating and adenyl cyclase stimulating activities on intestinal mucosa, both enterotoxins may possess similar toxic moieties. These findings strongly support the hypothesis that both enterotoxins exert their effect through the same intracellular mediation system. We therefore suggest that therapy directed at inhibiting the common intracellular enzyme system affected by enterotoxin may prove to be effective for both *V. cholerae* and *E. coli* diarrhoea.

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