EFFECTS OF CHOLERAGENOID AND GLUCOSE ON THE RESPONSE OF DOG INTESTINE TO *ESCHERICHIA COLI* ENTEROTOXINS

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THE small intestine of the dog responds differently to the thermostable (ST) and the thermolabile (LT) enterotoxins of *Escherichia coli* (Nalin, Bhattacharjee and Richardson, 1974). In ligated jejunal loops, ST acts within minutes of contacting the mucosa and its effect ends when it is rinsed out of the loops. The effect of LT is detectable only after a lag of 3–4 h and is unchanged by rinsing the loops after exposure to the toxin. The differences in onset and duration of these ST and LT effects in dog intestinal loops permit the study of *E. coli* culture supernates containing ST, LT or both.

Culture supernates containing LT and concentrated 60- to 100-fold invariably cause net fluid secretion in the dog intestinal loop. As the response to ST is more variable, ST from a series of strains was tested to establish quantitative criteria for its effect on absorption in the dog loop. As the characteristics of dog-loop responses to ST or LT produced by different strains remained constant, culture supernates derived from a single strain producing both ST and LT (ST⁺/LT⁺) were used to study the effect of glucose on absorption in toxin-treated loops, and to determine whether the two enterotoxins are blocked by natural cholera toxoid (choleragenoid). The glucose effect has a clinical application in the oral glucose-saline therapy of non-cholera diarrhoeas (Nalin and Cash, 1970) whilst a possible choleragenoid effect is relevant to studies on the identity, or otherwise, of mucosal toxin-binding sites for enterotoxins of *E. coli* and *Vibrio cholerae* (Pierce, 1973).

MATERIALS AND METHODS

Strains of E. coli isolated from the faeces of 40 Bangalee patients with acute idiopathic diarrhoea were tested for LT by the Chinese hamster-ovary (CHO) cell assay (Guerrant *et al.*, 1974). The effects on absorption of ST and LT produced by these strains were then tested in dog intestinal loops, to define the criteria for a positive ST response. Then the effects of glucose and choleragenoid on the ST and LT responses were determined.

Preparation of E. coli culture supernates. Pools of 10 colonies of *E. coli* were chosen from cultures on MacConkey's agar of rectal swabs obtained from patients before antibiotic therapy was begun. Clinical details of the patients have been reported (Nalin *et al.*, 1975). Additional strains of *E. coli* from patients with diarrhoea were supplied by courtesy of Dr Doyle Evans. Isolates were stored at room temperature in the dark on blood-agar base slopes. The identification of *E. coli* was confirmed by standard biochemical tests. To

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prepare culture supernates, broth was inoculated with pooled isolates, except in a few cases noted in the text when sucrose-fermenting (S+) or sucrose-nonfermenting (S-) colonies from the pooled isolates were tested individually. Culture supernates for CHO assay were prepared as described previously (Nalin *et al.*, 1975).

As net fluid loss in dog intestine occurs at high concentrations of ST (Pierce and Wallace, 1972), concentrated cell-free supernates were prepared from broth cultures of pooled isolates by the method of Evans, Evans and Gorbach (1973) at pH 8. Supernates were concentrated approximately 60- to 100-fold (mean 73-fold ± 0.6 ; all figures for means in the text are accompanied by the standard error of the mean). Concentrates were hypotonic (4-50 m-osmole per litre), had low ammonia content (30-100 mg per litre) and when stored at 5°C retained their ST and LT activity for more than 1 year. Heated supernates of three strains were produced by heating at 100°C for 20 min. in a boiling water bath and were tested after cooling to room temperature.

As the ST responses in dog intestine were similar, whether induced by supernates containing ST alone or both ST and LT, culture supernates of a single ST+/LT+ strain (CRL10 400) were used to test for the effects of glucose and choleraenoid on the loop responses to both enterotoxins. Concentrated supernates derived from this strain had a mean protein concentration of 8.29 ± 0.35 g per litre.

As CRL10 400 was also used as the positive control, Dr I. Craig and Dr A. Andreumont kindly compared its toxigenicity with that of a typical ST+/LT- strain (CRL35 647) in the rabbit-skin and suckling-mouse tests (Dean *et al.*, 1972; Evans *et al.*, 1973). The tests were carried out on supernates from unheated and shaken aerobic cultures grown in casamino acid-yeast extract medium. ST was expressed in "effective doses" (ED per ml) and LT in "4-mm blueing" doses (BD4 per ml). One ED is defined as the amount yielding a ratio of gut-weight to remaining body weight of 0.1 in the suckling-mouse assay. Dose-response titrations were performed to obtain ED per ml and BD4 per ml endpoints.

Reference numbers of CRL (Cholera Research Laboratory) stock cultures of *E. coli* isolates are given below to facilitate comparison in other laboratories. ST+/LT+: 10 400, 20 476(S+), 35 639, 35 671, 35 678, 35 687, 35 760, 35 875, 35 880, 36 004(S+); ST+/LT-: 20 178(S+), 20 546(S+), 20 580(S+), 20 590, 35 631, 35 642, 35 647, 36 000, 41 591, 41 736; ST-/LT-: 19 487(S-), 19 521, 19 587, 19 632, 20 483(S-), 20 546(S-), 20 902, 20 913, 22 141, 22 205, 35 622, 35 733, 35 766, 35 797, 35 981, 36 013, 40 835, 41 251, 41 575, 41 788. The toxigenic stock strains have all retained their toxigenicity over the period of 3 to 7 years during which they have been used to make supernates for testing in dogs.

Net water and sodium flux measurements in dog intestinal loops. Dogs were fasted overnight and kept lightly anaesthetised with intravenous sodium pentobarbital. At laparotomy, four or six ligated cannulated-jejunal or ileal loops were constructed in each dog, as previously described (Nalin *et al.*, 1974). The mean loop length was 17 ± 0.3 cm and basal loop absorption was measured with a test solution of NaCl 150 m.moles per litre (300 m.osmoles per litre; pH 6.9) containing phenolsulphonphthalein (PSP) 50 mg per litre or polyethylene glycol (PEG) 10 g per litre as a non-absorbable marker. After rapid intraluminal injection and mixing, a zero time sample was collected and a second sample was obtained on emptying the loops at the end of each 10-min. test period. Changes in water or sodium absorption or both were calculated from changes in marker and sodium concentrations over successive 10-min. periods. Sodium concentrations were measured with a flame photometer, PSP was measured spectrophotometrically after alkalisation and PEG was measured by the turbidimetric method (Hydén, 1956).

In each instance basal absorption was measured first and the effect of ST was measured by repeating the test procedure after mixing supernates with the marker-test solution and injecting the mixture intraluminally through the cannula. Total volumes injected in the control and toxin periods were equal. After a 10-min. exposure to supernate-test solution mixtures, the loops were drained and absorption was measured again. After this, the loops were rinsed with physiological saline at 37°C. The effect of LT was detected by measuring changes in absorption over a 4-h period after exposure to supernates. Toxic and non-toxic supernates were randomly distributed in adjacent loops.

Studies with glucose. The effects of glucose on ST and LT responses were studied separately. In respect of ST, the effect of glucose on sodium absorption was examined by comparing control and experimental loops. In control loops, net absorption was measured for the saline test solution without glucose before, during and after the exposure to ST. The action of ST was stopped by rinsing the loops out 20 min. after injection of the supernates. In experimental loops the procedure was the same, except that the test solution contained 110 m.moles glucose per litre. This glucose concentration was chosen because it is optimal for enhancement of intestinal absorption in patients with diarrhoea (Nalin and Cash, 1973).

In respect of LT, absorption was monitored after exposure to supernates until net secretion appeared some 3–4 h after the ST effect had ended. Then the rate of net loop secretion was measured in successive 10-min. periods for a total of 30 min., by means of the NaCl test solution without glucose. The same procedure was repeated, first with added glucose and finally without glucose. The effect of glucose on the response to LT was determined by comparing absorption in the presence of glucose with the mean absorption before and after the periods when glucose was present.

Choleraegenoid studies. The effect of pre-treatment with choleraegenoid on the response to ST and LT was tested after rinsing the loops with normal saline at 37°C. Then a mean of 149 µg (range 125–190 µg) of choleraegenoid in 10 ml of normal saline (three loops) or distilled water (three loops) was injected intraluminally. The choleraegenoid (Finkelstein, Fujita and Lospalluto, 1971), kindly supplied by Dr R. Finkelstein, was freshly reconstituted from lyophilised material. Control loops received 10 ml physiological saline or distilled water to correspond with the diluent of the choleraegenoid given to adjacent test loops.

After 3 h, both control and choleraegenoid solutions had been absorbed and baseline net-water fluxes were measured as described above. In choleraegenoid studies, 4 ml of a culture supernate of strain CRL10 400 concentrated 100-fold were mixed with 8 ml NaCl-PEG test solution and injected intraluminally into all loops. After mixing, a zero-time specimen was taken and 10 min. later the residual loop fluid was withdrawn for analysis. Net water fluxes were determined from changes in marker concentrations. A second 10-min. net-water flux measurement was made to detect any ST effect and the loops were again rinsed to remove any unbound toxin. Serial net fluxes were then measured in the same way by means of the test solution alone at intervals up to 6 h, to determine the effect of choleraegenoid on LT.

RESULTS

In tests with culture supernates of 40 strains of *E. coli*, 10 strains induced both ST and LT responses, 10 induced only ST responses, and the rest induced neither response (see fig. 1 and strain reference list above). Supernates containing ST showed a rapid, significant decrease in absorption within 20 min. of contact of mucosa with the supernate, whereas with LT net secretion began after 3–4 h. The CHO cell-assay results for LT were in agreement with the LT responses in dog ileal loops. No strain tested produced LT alone.

ST response

Supernates classified as ST⁺ decreased absorption by at least 50% of basal loop-absorption rate, within 20 min. of intraluminal injection of heated or unheated concentrated supernates. As loops varied in baseline absorption rate, it was convenient to express the ST effect as a percentage decrease in absorption relative to baseline ($\pm$ SEM), as well as to record absolute values for decrease in absorption (table).

TABLE

Comparison of the effects of ST-containing culture supernates with the effects of control supernates on dog ileal-loop absorption

Test supernate	Number of		Absorption (ml H ₂ O per 10 min. per loop)		Significance (P)
	strains	ileal loops	Mean (and range)	Percentage change (and range)	
ST+/LT—	9	21	-1.6 ± 0.2 (–0.4 to –4.0)	-123 ± 6 (–57 to –218)	≤ 0.001
ST+/LT—	1	11	-1.5 ± 0.24 (–0.4 to –2.4)	-122 ± 10 (–80 to –114)	≤ 0.001
ST–/LT—	21	24	$+0.23 \pm 0.05$ (–0.2 to +0.8)	$+27 \pm 7$ (–17 to +125)	≤ 0.001
None	0	7	$+0.4 \pm 0.09$ (+0.2 to +0.7)	$+53 \pm 23$ (+8 to +64)	≤ 0.01

Net secretion can clearly occur only in loops with a decrease in absorption greater than 100%. It could be detected after aspiration without measuring marker concentration changes in loops with decreases in absorption greater than –110% of baseline. In 18 of 38 loops showing the ST response, net secretion did not occur despite decreases in net absorption averaging -0.85 ± 0.13 ml per 10 min. per loop ($-69 \pm 5\%$ of baseline; $P \leq 0.001$).

Three of the ST+/LT— supernates were tested in dogs before and after heating and gave the same results (fig. 1). Supernates from a given strain also gave similar results in different dogs (table).

There was almost no overlap between the effects of ST+/LT— and control ST–/LT— strains; insignificant decreases of absorption of –12% and –17% of baseline occurred in only two of 24 loops receiving non-toxic supernates. The response of the other 22 loops was similar to that of control loops in seven dogs given no supernates; in these absorption *increased* above the baseline whereas adjacent loops treated with ST showed decreases in absorption. The increased absorption after exposure to ST–/LT— supernates was expected because of their hypotonicity. Control loops given no supernates as well as loops given ST–/LT— supernates, continued to absorb during the remainder of the experimental period (fig. 1). Thus, under the conditions of this study, the ST response may be defined as a decrease in absorption of $\geq 50\%$, taking place within 20 min. of exposing the loops to concentrated *E. coli* culture supernates and ending within 20 min. of rinsing out the loop.

Suckling-mouse and rabbit-skin assays showed that the ST content of unheated shaken-culture filtrates of strain CRL35 647 was 912 ED per ml whereas the LT content of these filtrates was 10 BD4 per ml or less. By comparison, filtrates of strain CRL10 400 had an ST content of 800 ED per ml and an LT content of 400–500 BD4 per ml. The ST content of filtrates from these strains was similar and was consistent with the quantitatively-similar ST responses in loops exposed to their culture supernates.

The effect of glucose on absorption

As ST production and the intestinal response to ST from ST+/LT— and ST+/LT+ strains grown under identical conditions were similar, supernates

from a single ST+LT+ strain (CRL10 400) were used to compare the effect of glucose on ST and LT responses. Glucose enhanced sodium absorption and reduced the net luminal-sodium accumulation brought about by both ST and LT (fig. 2). Glucose increased absorption by 49 ± 7 μ Eq per 10 min. per loop during the ST effect and by 72 ± 3 μ Eq per loop during the LT effect. The glucose effect in toxin-treated loops was slightly less than in control loops without toxin (77 ± 20 μ Eq per 10 min. per loop), but this difference was not statistically significant.

The choleraenoid blockade of LT

Pre-treatment of loops with natural choleraenoid had no effect on a subsequent ST response but completely blocked the LT response (fig. 3). In both control and choleraenoid-treated loops the ST response began within 20 min. and was not affected by choleraenoid. In choleraenoid-treated loops

FIG. 1.—Effects of *E. coli* culture supernates on absorption. Bars above line = decrease in loop water (absorption from loop). Bars below line = increase in loop water (net secretion into loop). All loops absorbed normally before exposure to supernates (–20 to 0 min.) and absorption continued in untreated loops and in loops treated with non-toxic supernates. Absorption fell within 20 min. of exposure to ST+ supernates, and recovered after rinsing. Supernates that contained LT produced net secretion 3–4 h later. Numbers in parentheses = number of loops/number of dogs.

FIG. 2.—Addition of glucose to test fluid (black bars) increased net sodium absorption (or reduced net sodium secretion) during both ST and LT effects. n = Number of loops represented by each bar.

absorption returned to normal within 20–40 min. of rinsing and remained normal during the 6-h observation period. The LT response, however, developed only in control loops. In two loops given 225 μ g and 300 μ g of glutaraldehyde-treated cholera toxoid (Wyeth) no blocking effect was seen and baseline absorption changed to secretion after giving toxin. The mean secretion was -0.4 ml per 10 min. per loop for both ST and LT responses.

DISCUSSION

Recent work has suggested that the effects of glucose and cholera toxin on the responses to *E. coli* and cholera enterotoxins are different (Pierce, 1973; Holmgren, 1973a; Sherr *et al.*, 1973). However, in these reports the effects of *E. coli* ST and LT toxins were not usually distinguished. In the present study the different responses of dog small bowel to ST and LT were used to re-examine the glucose and cholera toxin effects. The results indicate that glucose enhances absorption during both ST and LT responses, and that cholera toxin blocks the LT but not the ST response. These data constitute additional evidence for the similarity of LT, but not of ST, to cholera toxin, as first suggested by work with enterotoxins from porcine strains of *E. coli* (Gyles and Barnum, 1969; Smith and Gyles, 1970).

FIG. 3.—The blocking action of choleraenoid on LT but not on ST. ST produced fluid accumulation (bars below line) within 20 min. in both control and choleraenoid-treated loops. Absorption (bars above line) returned to normal 20–40 min. after rinsing loops. In controls, but not in choleraenoid-treated loops, the LT effect became evident 3–4 h later. n = Number of loops represented by each bar.

Our findings extend to LT-treated loops earlier observations on the efficacy of glucose in ST-treated loops (Pierce and Wallace, 1972), and show that, as in the case of cholera toxin (Carpenter, Sack and Feeley, 1968), the glucose effect is independent of the effects of the enterotoxins. Whipp and Moon (1973) reported that glucose increased absorption in loops infected with enteropathogenic *E. coli* serotypes but the type of toxin produced was not specified. The effect of glucose in the present study is consistent with the efficacy of oral glucose-electrolytes solutions in tropical diarrhoeas (Nalin and Cash, 1970; Sack *et al.*, 1970) which are known to be associated with enterotoxigenic *E. coli* Sack *et al.*, 1971; Pierce and Wallace, 1972; Nalin *et al.*, 1974; Nalin *et al.*, 1975; Sack *et al.*, 1975).

As choleraenoid, like the cholera toxin molecule, binds to cell receptors (Holmgren, 1973*b*), the blocking of *E. coli* LT by choleraenoid suggests a common binding site and common toxin-binding sub-unit for cholera toxin and for *E. coli* LT. Conversely failure of choleraenoid to block the ST

response implies a separate binding site for ST. This indicates different mechanisms of action for ST and LT, a suggestion that is supported not only by their differing pathophysiological effects but also by a recent report showing that ST, unlike LT or cholera toxin, does not stimulate adenylate cyclase-related phenomena such as platelet aggregation (Fumarola, 1976).

The difference between the present results and previous reports of a lack of choleraenoid effect on *E. coli* enterotoxin (Holmgren, 1973a; Pierce, 1973) may be explained by the lack of differentiation of LT and ST effects in the earlier work. It is possible that, in addition, other factors may have played a role, such as the lower doses of choleraenoid used and species differences between the dog and the rabbit. The absence of an effect with glutaraldehyde-treated cholera toxoid is attributable to structural differences between it and choleraenoid, and is consistent with the ability of choleraenoid, but not glutaraldehyde-treated toxoid, to block cholera toxin (Pierce, 1972).

It is worthy of note that strains of enterotoxigenic *E. coli* producing only LT, such as those reported elsewhere (Morris *et al.*, 1975), were not detected in our series nor in another series reported from the same area (Ryder *et al.*, 1976). Only four such strains have been reported from Bangladesh, in a study of *E. coli* from 65 patients (Sack *et al.*, 1977), based on Y-1 adrenal-cell assay for LT and a negative suckling-mouse test for ST. This suggests that geographical differences may exist in the prevalence of strains producing particular enterotoxins, and that strains producing LT alone are relatively uncommon in Bangladesh. Furthermore, it has been shown recently that under certain storage conditions, stock strains that are LT+/ST+ revert to LT+/ST-, possibly by loss of a plasmid (Evans *et al.*, 1977). Therefore, the true nature of LT+/ST- strains remains to be defined by comparative studies of their culture supernates in different in-vivo and in-vitro models.

The fact that 47% of ST+/LT- supernates reduced absorption by more than 57% without causing net secretion also suggests that use of net secretion as the only criterion for enterotoxicity may result in failure to detect significant absorptive decreases, particularly those caused by ST. As the suckling mouse test for ST depends only on net fluid accumulation, the question arises as to whether in the mouse, as in the dog, some strains that produce ST may go unrecognised when absorptive decreases are not measured. Preliminary evidence (Bergquist, Levine and Nalin, 1977) suggests that this is so.

SUMMARY

Culture supernates of *Escherichia coli* from diarrhoea patients were tested in dog jejunal loops for thermostable (ST) and thermolabile (LT) enterotoxins. Pre-treatment with choleraenoid blocked LT but not ST. Glucose was found to reduce fluid accumulation induced by both types of enterotoxin. The findings confirm and extend previous evidence of similarities between cholera toxin and *E. coli* LT and differences between it and *E. coli* ST.

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