ASSAY OF THE HEAT-LABILE ENTEROTOXIN OF *ESCHERICHIA COLI* IN INFANT RABBITS

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STRAINS of *Escherichia coli* enteropathogenic for swine have been shown to secrete enterotoxins *in vitro* (Smith and Halls, 1967*b*; Gyles and Barnum, 1969). These enterotoxins cause accumulation of fluid when they are introduced into ligated intestinal loops prepared in rabbits or pigs (Smith and Halls, 1967*a*). The strains of *E. coli* involved are believed to produce two different types of enterotoxin, namely a heat-labile non-dialysable immunogenic toxin (LT) and a heat-stable dialysable toxin (ST).

The use of infant rabbits for the detection of LT has been reported by Kutas, Vetési and Semjén (1974). The present paper described the response of infant rabbits to LT in greater detail, and the evaluation of them as small-animal models for the detection of LT.

The quantity of LT required for assessment of the infant-rabbit model necessitated a large-scale fermentation, and during the course of this work, it was reported that fermentation of strains of *E. coli* in the presence of lincomycin (Levner, Wiener and Rubin, 1977) or mitomycin C (Isaacson and Moon, 1975) increased LT synthesis. Hence, it was decided to investigate the effect of these agents on the yield of LT from *E. coli* strain P307, and these results are also reported here. The preliminary assessment of increase in yield of LT was determined in rabbit ligated-intestinal loops.

MATERIALS AND METHODS

Organisms. *E. coli* strain P307 (08: K87: K88a, b) was obtained from Dr H. Williams Smith (Houghton Poultry Research Station, Houghton, Huntingdonshire). It produced LT and ST. Five substrains resistant to lincomycin 300 µg/ml were obtained by sequential transfer of liquid cultures into media containing increasing concentrations of lincomycin, followed by purification of resistant clones as reported by Levner *et al.* (1977). The Lin^r phenotype was stable in the absence of the drug.

Culture media. Large-scale growth of strain P307 was produced on syncase medium (Finkelstein, Norris and Dutta, 1964) modified by the substitution of glucose for sucrose and by the addition of yeast extract 0.6% (w/v).

The lincomycin-resistant strains were grown on the modified casamino acids and yeast extract medium described by Evans, Evans and Gorbach (1973*b*) in the presence and absence of lincomycin 250 µg/ml.

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Fermentation details. Five freeze-dried cultures of strain P307 were resuspended in five 200-ml volumes of modified syncase medium in 1-litre Erlenmeyer flasks. After incubation for 18 h at 37°C with shaking (200 r.p.m.) in a Gallenkamp Rotary Incubator (Gallenkamp, London), a 5% (v/v) inoculum was used to seed 20 litres of modified syncase medium in a 20-litre bottle. After incubation for 24 h at 37°C with aeration (6 litres/min.) and stirring (250 r.p.m.), this culture was used to inoculate 400 litres of medium in a fermenter as described by Melling and Scott (1972). After incubation for 24 h at 37°C with aeration (120 litres/min.) and stirring (250 r.p.m.), phenol was added to a final concentration of 0.5% (w/v) and the culture held at 37°C for 30 min. The culture was then centrifuged in a De Laval centrifuge at maximum speed of approximately 8000 r.p.m. (Melling and Phillips, 1975). Twenty litres of the supernatant fluid were harvested for future processing whilst the cell paste was washed with 100 litres of sterile phosphate-buffered saline (PBS; Oxoid, Basingstoke, Hampshire) to remove any residual ST, and was re-centrifuged. The cell paste was stored at -20°C.

The effects of lincomycin and of mitomycin C (MTC) were investigated by means of small-scale fermentations as follows. A 10-ml volume of peptone water was inoculated with strain P307 from a Dorset's Egg Slope (Oxoid) and incubated for 18 h at 37°C; 1 ml of this culture was added to 100 ml of medium in a 500-ml Erlenmeyer flask and incubated for 48 h at 37°C with shaking (200 r.p.m.). Phenol was then added to a final concentration of 0.5% (w/v) and the culture left for 30 min. at 37°C. After centrifugation (28 000 g) for 30 min. at 4°C, the cell pellet was washed with 50 ml of sterile PBS to remove any residual ST, and re-centrifuged. The cell paste was stored at -20°C. The five lincomycin-resistant substrains and the parent strain P307 were grown in the absence and presence of lincomycin. The effect of MTC was investigated by the addition of MTC (final concentration 0.5 µg/ml) to a syncase-glucose yeast-extract culture seeded 2 h earlier. Processing was then as above.

Cell-lysate production. Unless otherwise stated, cell lysates were prepared by ultrasonic disintegration of a 5% (w/v) wet weight (approximately a 1.2% w/v dry weight) suspension of cells in sterile PBS. The cell suspensions were disintegrated for 15 min. in 100-ml volumes with an MSE Ultrasonicator (MSE, Crawley, Sussex) at maximal power (7 microns peak to peak). An ice bath was used to cool the cell suspension during disintegration. The cellular debris was then removed by centrifugation (28 000 g) for 30 min. at 4°C. The supernatant fluid was decanted, sterilised by filtration through 0.45-µm Millipore filters (Millipore, London), and stored at -20°C.

Extracellular-LT production. The 20 litres of cell-free P307 culture supernate were concentrated to 50 000; this was achieved by means of an Amicon CH3 Concentrator (Amicon, High Wycombe, Bucks.). The ultrafiltrate was stored whilst the retentate was washed with a 10-fold excess of deionised water, freeze dried and held at -20°C. The ultrafiltrate and washing were processed as above by means of a hollow fibre membrane with a m.w. exclusion limit of 10 000; the resulting freeze-dried retentate was reconstituted in 200 ml of PBS, sterilised by filtration through a 0.22-µm Millipore filter, and held at -20°C. The resulting ultrafiltrate was processed once again, this time by means of a hollow fibre membrane with a m.w. exclusion limit of 5000; the ultrafiltrate produced, containing ST, was discarded.

Chemicals. Crude cholera enterotoxin was obtained from Wyeth Laboratories, lincomycin was obtained from Upjohn Ltd, Crawley, Sussex, and mitomycin C and other chemicals were obtained from Sigma Ltd, London.

Statistics. Analysis of variance and Student's *t* test were used.

Assays of enterotoxic activity by the infant-rabbit method. The method was similar to that of Kutas *et al.* (1974). Infant rabbits (New Zealand White) were separated from their mothers shortly before use. Each animal was dosed orally by means of a polyethylene catheter, with the sample under test at the rate of 50 ml/kg body weight. The addition of 1 ml of 5% Evans blue dye to each 100-ml volume of test sample enabled the presence of the material in the stomach to be confirmed at necropsy. Unless otherwise stated, animals were killed 5 h after inoculation and their entire intestines removed. The ratio of gut weight to remaining body weight was calculated for each rabbit. Ratios below 0.070 were considered negative. In our hands rabbits inoculated with heated toxin preparations (65°C for 15 min.) invariably gave ratios of 0.050-0.065, with a mean coefficient of variance of 8%.

Assay of enterotoxigenic activity by the rabbit ligated-loop method. The method used was that of Evans, Evans and Pierce (1973a) with an 18-h incubation period. Adult New Zealand White rabbits were used with a maximum of 12 loops per rabbit. The inoculum volume was 2 ml per loop and each sample was tested in at least four loops.

RESULTS

Fermentation

Various parameters monitored during the large-scale fermentation of *E. coli* strain P307 are shown in table I. The culture had reached the stationary phase after 12 hours' incubation, although there was a subsequent rise in the pH of the culture. There was no need to add antifoam during growth. The modified syncase medium was found to give consistently good growth and a high yield of LT.

TABLE I
Large-scale growth of E. coli strain P307 in modified syncase medium

Age of culture (hours)	pH	Optical density at 620 nm, 1 cm pathlength	% CO ₂	Viable count (10 ⁹ organisms per ml)	Concentration of glucose (g/litre)
0	7.5	0.12	0.00	0.14	4.3
4	6.7	1.08	2.80	ND	ND
12	7.9	1.84	0.15	8.82	< 0.005
18	8.4	1.84	0.10	5.28	ND
24	8.4	1.84	0.10	5.23	< 0.005

ND = Not determined.

Study of the infant-rabbit model

Numerous preliminary tests showed that rabbits aged 7–9 days were responsive to oral inoculation with cell lysate (50 ml/kg) produced from strain P307. Fluid accumulation was noted in the intestine, and gut weight to body weight (GW/BW) ratios in excess of 0.090 were routinely obtained. Heated cell lysates (65°C for 15 min.) never produced GW/BW ratios above 0.070 when inoculated into infant rabbits.

Time course of response to LT

Groups of rabbits aged 7–9 days were killed at various intervals after oral inoculation with P307 cell lysate (50 ml/kg). The time course of fluid accumulation is shown in fig. 1.

Fluid accumulation was evident 3 h after inoculation; maximum GW/BW ratios occurred 5–7 h after inoculation. A 5-h incubation time was subsequently used as a routine.

FIG. 1.—Time course of fluid accumulation in infant rabbits dosed orally with cell lysate of *E. coli* strain P307. Numbers next to symbols indicate the number of animals used to determine each point. Each point represents the mean ratio; the bars indicate the standard error of the mean (SEM). GW/BW ratio = ratio of gut weight to remaining body weight.

Dose response

A dose-related response was obtained with P307 cell lysate and is shown in fig. 2. Doses in excess of 30 ml/kg gave good positive responses.

Effect of cell concentration on lysate activity

Cell suspensions of 2.5, 5.0 and 25% wet weight/volume were used to prepare cell lysates, which were then assayed in infant rabbits at a dose of 50 ml/kg. The results are shown in table II; the 5% cell suspension lysate was as active, if not more so, than the 25% cell suspension lysate.

Age response

Animals at 7, 10, 14 and 21 days of age were dosed at 50 ml/kg with P307 cell lysate and heated (65°C for 15 min.) cell lysate. The results can be seen in fig. 3. The gastric pH of the animals dosed with unheated cell lysate was measured at necropsy and is also shown in fig. 3.

FIG. 2.—Dose response of infant rabbits to cell lysate of strain P307. ● = Cell lysate; ○ = heated cell lysate (65°C for 15 min.). Numbers next to symbols indicate the number of animals used to determine each point. Each point represents the mean ratio; the bars indicate the SEM.

TABLE II

The toxicity for infant rabbits of lysates prepared from suspensions containing different concentrations of E. coli cells

Concentration of cell suspension (% wet weight/volume)	Number of animals	Gut weight to body weight ratio (GW/BW) of rabbits 5 h after dosing (Mean $\pm$ standard error of mean [SEM])
2.5	6	0.097 $\pm$ 0.006
5	5	0.133 $\pm$ 0.013
25	4	0.117 $\pm$ 0.011

The animals remained responsive at 14 days of age, but became unresponsive by 21 days: the high control level at 21 days appeared to be due to increased faecal content. The gastric pH was 3.0 at 21 days of age; pH values of below 5.0 have previously been found to inactivate LT (Evans *et al.*, 1973a).

Effect of withholding food before dosing

Rabbits were deprived of food for 18 h before they were dosed with P307

FIG. 3.—Effect of age on response of infant rabbits to cell lysate of strain P307, and gastric pH at necropsy. ● = Cell lysate; ○ = heated cell lysate. Numbers next to symbols indicate the number of animals used to determine each point. Each point represents the mean ratio; the bars indicate the SEM.

TABLE III
Effect of withholding food before dosing with cell lysate

Treatment of infant rabbits before dosing	Observations made on six infant rabbits 5 h after dosing (means $\pm$ SEM)	
	GW/BW ratio	Gastric pH
Food withheld for 18 h	0.071 $\pm$ 0.009*	3.6 $\pm$ 0.1†
Left with dam	0.104 $\pm$ 0.005	5.5 $\pm$ 0.1

* Significantly lower GW/BW ratio ($p < 0.025$) than in untreated animals (Student's *t* test).

† Significantly lower gastric pH ($p < 0.001$) than in untreated animals (Student's *t* test).

cell lysate. The gastric pH of the animals was measured at necropsy (table III). Fasting significantly reduced the response to LT and the final gastric pH.

Stability of cell lysates during storage at -20°C

Two cell-lysate preparations were assayed weekly during storage at -20°C for 7 weeks; the combined results are shown in fig. 4.

FIG. 4.—Stability of cell lysate of strain P307 at -20°C . Toxicity was tested in infant rabbits and numbers next to symbols indicate the number of animals used to determine each point. Each point represents the mean ratio; the bars indicate the SEM.

The line of best fit was then calculated by linear regression and is shown in fig. 4; the data gave a significant linear correlation ($p < 0.001$) indicating that crude LT was not stable when stored at -20°C . However, cells stored at -20°C maintained their ability to produce active cell lysates for more than one year.

Extracellular LT and cholera toxin

Extracellular LT (m.w. $> 50\,000$) and cholera enterotoxin were administered to infant rabbits (table IV). Crude cholera toxin was active at a dose of 25 mg/kg and the extracellular LT had comparable activity at a dose of 100 mg/kg. Crude cholera toxin and extracellular LT were totally inactivated by heating at 65°C for 15 min.

Effect of lincomycin on LT yield

Cell lysates from the five lincomycin-resistant substrains of *E. coli* pro-

TABLE IV
Response of infant rabbits to extracellular LT and crude cholera toxin

Treatment	Dose (mg/kg)	Mean GW/BW ratio ($\pm$ SEM) 5 h after dosing*	p (Student's <i>t</i>)
Cholera toxin	25	0.106 $\pm$ 0.013 (6)	<0.001
Inactivated cholera toxin	200	0.055 $\pm$ 0.002 (4)	
Extracellular LT	100	0.107 $\pm$ 0.007 (7)	<0.001
Inactivated extracellular LT	100	0.056 $\pm$ 0.003 (6)	

* Numbers of animals in parenthesis

duced from strain P307 were assayed, together with the parent strain, in adult-rabbit ligated loops (table V). All five lincomycin-resistant strains gave higher titres of LT when grown in the presence of lincomycin. When compared with the parent strain, no significantly increased levels of LT were observed from any of the lincomycin-resistant strains, and three (B, C and E) gave significantly lower levels of LT than did the parent strain P307.

The two strains (A and D) that gave levels of LT comparable with those of the parent strain were assayed, together with the parent strain, at a 1 in 5 dilution. The results confirmed that elevation of LT titre had not occurred.

The biological activity of the cell lysates was invariably destroyed by heating at 65°C for 15 min., confirming the heat-lability of the toxin.

TABLE V
Effect of lincomycin on the LT yield as assayed in ligated intestinal loops of adult rabbits

Strain or sub-strain	Concentration (μ g/ml) of lincomycin in growth medium	Mean volume/length ratio ($\pm$ SEM) of four loops	Significance† (p)
P307	0	1.49 $\pm$ 0.13	...
A*	0	0.73 $\pm$ 0.29	<0.01
	250	1.46 $\pm$ 0.11	NS
B*	0	0.05 $\pm$ 0.03	<0.001
	250	0.72 $\pm$ 0.32	<0.02
C*	0	0.19 $\pm$ 0.13	<0.001
	250	0.50 $\pm$ 0.30	<0.01
D*	0	0.44 $\pm$ 0.22	<0.001
	250	1.58 $\pm$ 0.19	NS
E*	0	0	<0.001
	250	0.33 $\pm$ 0.21	<0.01

* Lin^r isolate of strain P307

† Significance (by analysis of variance) of result as compared with that of the parent strain P307.

NS = Not significant.

Effect of MTC on LT toxin yield

Cell lysates produced from strain P307 grown in the presence and absence of MTC 0.5 µg/ml were assayed in rabbit ligated loops (table VI). The biological activity of the samples was inactivated by heating at 65°C for 15 min., confirming the heat-lability of the toxin.

The presence of MTC resulted in no increased toxin levels.

TABLE VI
Effect of mitomycin C (MTC) on LT yield as assayed in ligated intestinal loops of adult rabbits

Strain	Concentration (µg/ml) of MTC in growth medium	Mean volume/length ratio ($\pm$ SEM) of four loops
P307	None	1.74 $\pm$ 0.14
	0.5	1.48 $\pm$ 0.15

Extracellular LT

The extracellular LT preparations were assayed in rabbit ligated loops and compared with a 5% cell-lysate sample (table VII). All the samples were inactivated by heating at 65°C for 15 min., confirming the heat-lability of the toxin. Extracellular LT appeared to be heterogeneous, with a wide range of molecular weights. A dose-response curve for the material with m.w. of > 50 000 was obtained by the ligated-loop method and is shown in fig. 5. A dose of 20 mg/loop had activity comparable with that of the 5% cell lysate.

TABLE VII
Assay of extracellular LT samples in ligated intestinal loops of adult rabbits

Sample	Range of molecular weights (10^3)	Mean volume/length ratio ($\pm$ SEM) of four loops
Cell lysate	...	1.81 $\pm$ 0.22
Extracellular LT	5-10	1.38 $\pm$ 0.16
Extracellular LT	10-50	1.96 $\pm$ 0.32
Extracellular LT	> 50	1.84 $\pm$ 0.23

DISCUSSION

Several sensitive tissue-culture techniques have been developed for the detection and quantitative assay of *E. coli* LT activity (Donta, Moon and Whipp, 1974; Guerrant *et al.*, 1974; Kantor, Tao and Wisdom, 1974). However, we have been interested in studying the pathophysiological effect of LT and have therefore preferred to use the intact animal. To this end, we have

FIG. 5.—Dose response of ligated intestinal loops of adult rabbits to extracellular LT. ● = Extracellular LT (m.w. > 50 000); ○ = heated extracellular LT (65°C for 15 min.). Each point represents the mean ratio; the bars indicate the SEM of four loops.

studied the effect of oral administration of LT to infant rabbits, to avoid the of the standard rabbit-ileal loop system.

The dosing of infant rabbits with LT was first described by Smith (1972) and further investigated by Kutas *et al.* (1974). We have examined the response in more detail and have attempted to measure it by determining the ratios of gut weight to remaining body weight (GW/BW) 5 h after the oral administration of LT. We hoped that this system might then be used to detect and quantify LT in a manner similar to that by which *E. coli* heat-stable enterotoxin (ST) can be detected and quantified in the infant mouse (Dean *et al.*, 1972; Mullan, Burgess and Newsome, 1978).

The results show that GW/BW ratios obtained from infant rabbits dosed with LT were invariably above 0.090, whereas heat-inactivated LT always gave ratios below 0.070. These ratios correspond to those found by means of the infant mouse assay for ST (Dean *et al.*, 1972). In the infant mouse a near-maximal response occurs in 2 h (Mullan *et al.*, 1978), but the response of infant rabbits to LT was much less rapid. Whilst fluid secretion was evident 3 h after inoculation, maximal GW/BW ratios were not obtained until the 5th hour. The infant rabbits were still responsive to LT at 14 days of age, but became unresponsive at 21 days. These findings correspond closely to those obtained in a study of mouse responsiveness to ST (Mullan *et al.*, 1978). The failure of older rabbits to respond to LT may be associated with their gastric pH, which is low enough to inactivate LT (Evans *et al.*, 1973a).

LT is unstable when stored at -20°C, losing approximately half of its activity within 4 weeks. Similar findings have recently been reported by Mundell, Anselmo and Wishnow (1976). Both lincomycin (Levner *et al.*, 1977) and mitomycin C (Isaacson and Moon, 1975) have previously been reported to

increase the yield of LT from strains of *E. coli*. However, we were unable to confirm either claim from experiments with strain P307 in which the LT levels were measured by means of rabbit ligated-intestinal loops. Wadström, Möllby and Söderlind (1977), referring to a manuscript in preparation, noted that LT synthesis was apparently not increased by mitomycin C if intestinal loops were used for assay, but was dramatically increased if tissue culture was used for assay.

A possible explanation for this difference may be found in the different m.w. values reported for LT preparations; the values vary from 20 000 (Evans *et al.*, 1976) to > 100 000 (Dorner, Jaksche and Stöckl, 1976) and this suggests a pronounced degree of heterogeneity. With our preparation of extracellular LT from *E. coli* P307, molecular weights ranging from < 10 000 to > 50 000 were noted.

Heating destroyed the activity of intracellular and extracellular LT and confirmed the absence of ST in the preparations used in this study.

SUMMARY

Infant rabbits were shown to respond to *Escherichia coli* heat-labile enterotoxin by a consistent increase in intestinal fluid content, which was maximal 5 h after oral dosing. Infant rabbits could be used in a simple quantitative assay for heat-labile *E. coli* enterotoxin based on the ratios of gut weight to remaining body weight 5 h after oral dosing. Infant rabbits remained responsive to heat-labile enterotoxin up to 14 days of age, after which their gastric pH became low enough to destroy the enterotoxin. Rabbits that had been deprived of food before being dosed had a reduced gastric pH and a reduced response to the enterotoxin.

Lincomycin and mitomycin C were found not to increase the yield of heat-labile enterotoxin from *E. coli* strain P307.

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