ZINC AND THE HEAT-LABILE ENTEROTOXIN OF *ESCHERICHIA COLI*

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SUMMARY. Enterotoxigenic *Escherichia coli* is a major cause of diarrhoea in man. When zinc in concentrations of 10^{-6}M or 10^{-5}M was added to the growth medium, there was a significant increase in heat-labile enterotoxin production by each of six toxigenic strains. Zinc in these concentrations did not alter bacterial growth or the activity of preformed toxin. Other heavy metals did not enhance toxin production and *o*-phenanthroline, a relatively specific zinc-chelating compound, blocked the enhancing effect. The significance of these findings is discussed in relation to the use of supplemental dietary zinc.

INTRODUCTION

Zinc is essential for all living organisms (Vallee, 1959; Riordan, 1976) where it functions, for example, in many metallo-enzymes, activation of biological compounds and membrane stabilisation (Reynolds and Schlesinger, 1969; Trotman and Greenwood, 1971; Chvapil, 1976; Failla, 1977). The alpha toxin of *Clostridium perfringens* is produced in an inactive form when the organism is grown in medium with low concentrations of zinc but it combines with zinc to form stable and active toxin (Sato and Murata, 1973; Sato *et al.*, 1978). Several other bacteria produce increased amounts of proteases, toxins or both when incubated with supplemental zinc (Feeney, Lightbody and Garibaldi, 1947; Jensen, Fecycz and Campbell, 1980; Riddle, Graham and Amborski, 1981; Kessler *et al.*, 1982), which probably reflects the presence of zinc in these proteins or in the nucleic-acid polymerases involved in their synthesis (Wegener and Romano, 1963; Williams and Loeb, 1973; Sugarman, 1983).

Toxigenic *Escherichia coli* is a major cause of diarrhoea in man (e.g., traveller's diarrhoea) and other mammals (DuPont *et al.*, 1980; Sack, 1980; Clements, Flint and Klipstein, 1982). Loss of time from work and economic losses in the raising of infected animals can be significant. The heat-labile enterotoxin (LT) closely resembles cholera toxin (DuPont *et al.*, 1980; Clements *et al.*, 1982), and produces a prolonged secretory response by the intestinal cells affected (Sack, 1980).

Because zinc is so often ingested as a nutritional supplement, alone or in

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proprietary vitamins-with-mineral preparations, an attempt was made to determine if zinc supplements may act as a virulence factor in the development or severity of disease caused by the LT of *E. coli*. This hypothesis is based upon aspects of zinc biochemistry mentioned above and on other data which suggest that zinc may promote some bacterial infections (Sugarman, 1983).

MATERIALS AND METHODS

Bacteria. Six LT-producing strains of *E. coli* (isolates 1, 2, and 5 also produced heat-stable toxin) were grown in a shaking apparatus for 18 h at 37°C in trypticase soy broth (TSB) (BBL, Cockeysville, MD, USA) or Eagle's minimal essential medium (MEM) (Gibco, Grand Island, NY, USA) with defined concentrations of zinc or other heavy metals. To prevent trace zinc contamination (Failla, 1977), only acid-washed glassware (Falchuck, 1977), disposable plastic-ware, reagent quality chemicals, and ultra-pure water of at least 18 megohm/cm resistivity and with no zinc detectable by atomic absorption spectrophotometry (AAS) (Perkin-Elmer, Norwalk, CT, USA) were used for these experiments. In certain experiments; polymyxin B 2000 units/ml of medium was added and incubated for a further 30 min in order to lyse the cells; this reduced the viable colony counts by > 99.99%. These cultures were centrifuged to remove the bacteria and supernates were filtered through 0.2- μ m membrane filters (Amicon, Danvers, MA, USA). Non-LT producing control strains were treated in the same way.

LT assay. LT in the supernates was assayed by the Y1 mouse adrenal cell assay (Donta, Moon and Whipp, 1974; Sack and Sack, 1975). Briefly, Y1 cells were grown at 37°C, in a humidified atmosphere of CO₂, 5% v/v and air 95%, in plastic multi-welled plates (Flow Laboratories, McLean, VA, USA) in Ham's nutrient F-10 mixture (Flow) supplemented with horse serum 15% (Flow), fetal bovine serum 2.5% v/v (Sterile Systems, Inc., Logan, UT, USA) and gentamicin 40 μ g/ml. When a monolayer was formed, the bacterial culture supernates were added in a 1 to 10 dilution to the medium bathing the Y1 cells and incubated in the same humidified atmosphere for up to 48 h. Rounding of the cells was assessed with a phase-contrast microscope and was graded, without knowledge of the contents, as 0, 1, 2, or 3 corresponding respectively to < 20% cells rounded, 20–49% rounded, 50–90% rounded, and > 90% rounded. Rounding of the Y1 cells as determined by light microscopy correlates with steroidogenesis by these cells and with the amount of LT present (Donta *et al.*, 1974). This technique was highly reproducible; duplicate plates were scored exactly the same after incubation for 24 h in 261 of 272 sets from the same days.

In other experiments 5×10^{-6} M *o*-phenanthroline was added to the bacterial growth medium with or without supplemental zinc. After incubation for 18 h, cultures were centrifuged and the supernates filtered through a 10 000-mol. wt ultrafiltration device (Amicon) to remove the *o*-phenanthroline. The retentates were diluted in isotonic buffer to their original volumes and assayed for LT after incubation with Y1 cells for 6 h.

Statistical method. The signed-ranks test of Wilcoxon was used to judge statistical significance, with $n \geq 5$.

RESULTS

Zinc effect

Supernates from non-LT producing strains of *E. coli* (controls) never produced any rounding, even when the bacteria were grown with up to 2×10^{-5} M supplemental zinc. Zinc alone added to Y1 cells in concentrations of up to 1×10^{-4} M never produced any rounding. In contrast, all six LT-producing strains of *E. coli* caused rounding of the Y1 cells when grown in either unsupplemented TSB or MEM. TSB contained 6.7×10^{-7} M zinc and MEM contained 1.8×10^{-7} M zinc as measured by AAS. The addition of supplemental zinc (1×10^{-6} M or 1×10^{-5} M) to the TSB growth medium did not affect

TABLE I
Assay of LT produced by *E. coli* strains after growth for 18 h in TSB with or without added zinc

<i>E. coli</i> strain no.	Mean rounding score ($\pm$ standard deviation of the mean) in the Y1 mouse adrenal cell assay after incubation for								
	6 h			24 h			48 h		
	None	with added zinc $1 \times 10^{-6}M$	$1 \times 10^{-5}M$	None	with added zinc $1 \times 10^{-6}M$	$1 \times 10^{-5}M$	None	with added zinc $1 \times 10^{-6}M$	$1 \times 10^{-5}M$
1	1.0 (± 0.4)	2.0 (± 0.4)*	2.0 (± 0.4)*	2.2 (± 0.5)	3.0 (± 0.0)*	3.0 (± 0.0)*	2.4 (± 0.5)	3.0 (± 0.0)*	3.0 (± 0.0)*
2	0 (± 0)	0 (± 0)	0.2 (± 0.4)	1.0 (± 0.7)	1.2 (± 0.7)	2.0 (± 0.9)*	1.2 (± 0.8)	1.5 (± 0.8)	2.0 (± 0.9)*
3	0 (± 0)	0 (± 0)	0 (± 0)	1.0 (± 0.5)	1.4 (± 0.6)	1.3 (± 0.5)	1.3 (± 0.8)	1.5 (± 0.9)	2.0 (± 1.0)*
4	0.3 (± 0.3)	0.6 (± 0.4)	0.3 (± 0.3)	1.6 (± 0.7)	2.0 (± 0.5)	2.5 (± 0.8)*	2.5 (± 0.5)	2.7 (± 0.6)	2.7 (± 0.6)
5	1.0 (± 0.8)	1.8 (± 0.4)*	1.3 (± 0.8)	2.3 (± 0.7)	2.7 (± 0.9)	2.3 (± 0.7)	3.0 (± 0.0)	3.0 (± 0.0)	3.0 (± 0.0)
6	1.2 (± 0.1)	1.7 (± 0.5)*	1.8 (± 0.5)*	3.0 (± 0.0)	3.0 (± 0.0)	3.0 (± 0.0)	3.0 (± 0.0)	3.0 (± 0.0)	3.0 (± 0.0)

* $p < 0.05$ compared to appropriate control without supplemental zinc.

bacterial growth as assayed by viable count, but significantly increased toxin production as manifested by quicker and more rounding of the Y1 cells (table I). Results with MEM were similar, though less rounding occurred in all groups. Addition of $1 \times 10^{-6}\text{M}$ or $1 \times 10^{-5}\text{M}$ zinc to each of the bacterial supernates immediately before incubation with the Y1 cells did not result in any differences compared with control supernates without supplemental zinc. Zinc chloride was used in all of the above experiments but zinc acetate also produced the same effects.

To determine the extent of toxin enhancement and whether increased release of preformed toxin was responsible, experiments were repeated with lysis of the bacteria by polymyxin B and serial dilution of the bacterial supernates. Lysis of the bacteria greatly increased the amount of toxin present in the supernates. Growth of the different isolates with zinc caused production of more toxin, approximately 140–220% of that produced by control cultures. Representative data are shown in table II.

O-phenanthroline, $5 \times 10^{-6}\text{M}$, a relatively specific zinc-binding compound, did not inhibit growth of the *E. coli* strains nor have any effect on LT production in unsupplemented medium. However, it did block the effect of supplemental zinc on elaboration of LT during co-incubation (table III).

Other heavy metals

Chloride salts of iron (ferrous and ferric), magnesium, manganese, zinc and

TABLE II
Assay of LT after lysis of E. coli strains with polymyxin B

<i>E. coli</i> strain no.	Mean rounding score ($\pm$ standard deviation) in Y1 mouse adrenal cell assay after incubation for 24 h with supernate					
	Undiluted		Diluted 1 in 10		Diluted 1 in 20	
	with added zinc None	$1 \times 10^{-5}\text{M}$	with added zinc None	$1 \times 10^{-5}\text{M}$	with added zinc None	$1 \times 10^{-5}\text{M}$
4	3.0 (± 0.0)	3.0 (± 0.0)	2.1 (± 0.4)	2.7 (± 0.5)*	1.1 (± 0.6)	1.5 (± 0.5)*
5	3.0 (± 0.0)	3.0 (± 0.0)	1.7 (± 0.8)	2.8 (± 0.6)*	1.0 (± 0.5)	1.9 (± 0.7)*

* $p < 0.05$ compared to appropriate control without supplemental zinc.

TABLE III
Effect of $5 \times 10^{-6}\text{M}$ o-phenanthroline on enhancement of LT production by zinc

<i>E. coli</i> strain	Mean rounding score ($\pm$ standard deviation) after growth for 18 h in TSB supplemented with			
	No supplement	Zinc‡	Zinc + $5 \times 10^{-6}\text{M}$ <i>o</i> -phenanthroline	$5 \times 10^{-6}\text{M}$ <i>o</i> -phenanthroline
<i>E. coli</i> strain no. 1	1.2 (± 0.2)	2.0 (± 0.2)*	1.0 (± 0.4)	1.2 (± 0.2)
<i>E. coli</i> strain no. 6	1.1 (± 0.1)	1.6 (± 0.4)*	1.1 (± 0.2)	1.2 (± 0.1)
Non LT-producing <i>E. coli</i> control	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)

‡ $1 \times 10^{-5}\text{M}$ for *E. coli* strain no. 1 and control; $1 \times 10^{-6}\text{M}$ for *E. coli* strain no. 6.

* $p < 0.05$ compared to control.

calcium in final concentrations of 1×10^{-6} M and 1×10^{-5} M were incubated with *E. coli* strain no. 1 and *E. coli* strain no. 6 in TSB. After 18 h, viable counts averaged $(1.8-2.1) \times 10^9$ cfu/ml for each strain grown without supplemental metal; results with the metals were not statistically different, averaging $\pm 20\%$ of the control values. Rounding of the Y1 cells after 6, 24 and 48 h was highly reproducible, with means $\pm 10\%$ of the controls for all metals except zinc.

DISCUSSION

These data show that significantly more LT was produced when *E. coli* isolates were grown with 10^{-6} – 10^{-5} M supplemental zinc. Zinc in these concentrations stabilises membranes (Sugarman, 1983) and lysis of the bacteria with polymyxin greatly increased the amount of toxin in the supernates, but the effect with zinc was still noted; therefore, it seems unlikely that the effect was due solely to an increased release of LT into the surrounding milieu. The lack of effect noted when zinc was added after bacterial growth suggests that this increased toxicity was not the result of activation or stabilisation of the toxin by zinc, unless the zinc present in unsupplemented growth media was adequate for maximal effect. Most media contain at least 10^{-7} M zinc; it is quite difficult to prepare media with less zinc, and this concentration is sufficient for optimal growth of most bacteria (Failla, 1977). The specificity of zinc to cause increased production of LT was determined by the inability of other heavy metals to cause this effect and by the inhibition of the response with a zinc chelator. The inability of *o*-phenanthroline alone to decrease LT production compared with bacteria grown without supplemental zinc suggests that only trace amounts of zinc are necessary for baseline LT production, that intracellular zinc is sufficient, or that the bacteria successfully compete with *o*-phenanthroline for zinc.

Normally, people ingest *c.* 10–15 mg of zinc daily and most of this is excreted in the faeces, the level excreted increasing with higher levels of ingestion (Sugarman, 1983). Zinc supplementation would increase the amount of zinc in the intestines and could promote the development of infection by increasing the attachment of bacteria to receptor cells (Sugarman, Epps & Stenback, 1982), elaboration of LT, or both. Zinc supplementation should not be considered harmless until in-vivo studies of associated infection risk are conducted.

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