INFECTION AND IMMUNITY, Aug. 1975, p. 240-244

Vol. 12, No. 2
Printed in U.S.A.

Heat-Stable *Escherichia coli* Enterotoxin Production In Vivo

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Received for publication 17 March 1975

Hysterectomy-derived, colostrum-deprived piglets were infected with enterotoxigenic *Escherichia coli* on day 4 of life. Samples of feces and intestinal contents were collected and tested in infant mice for enterotoxic activity. Positive enterotoxic responses were observed in mice given filtrates of feces and intestinal contents from piglets infected with enterotoxigenic *E. coli* known to produce heat-stable enterotoxin but not heat-labile enterotoxin in vitro. It is concluded that heat-stable enterotoxigenic *E. coli* induce diarrhea by production of heat-stable enterotoxin in vivo.

Enterotoxigenic *Escherichia coli* (TEC) are known to induce cholera-like diarrheal disease in man and several species of domestic animals (6, 14). It is thought that TEC induce diarrheal disease through release of enterotoxins which stimulate copious secretion by mucosa of the small intestine (10). TEC produce two types of enterotoxins in vitro, a heat-stable toxin (ST) and a heat-labile toxin (LT) (13). Some TEC strains produce only ST (ST TEC) and some produce both ST and LT (ST-LT TEC). ST has been characterized as a nonantigenic small-molecular-weight substance, whereas LT is antigenic and has a large molecular weight. Furthermore, the host responses evoked by these toxins differ. Although both induce copious intestinal secretion, ST has a very rapid onset and a short duration of action, whereas LT has a delayed onset and prolonged duration of action. In consideration of the pathogenesis of colibacillosis in any species, it is pertinent to ask whether the toxins produced by these organisms in vitro are also produced in vivo and in fact induce a diarrheal disease.

Miniats and Gyles (9) presented evidence of LT in intestinal contents of gnotobiotic swine exposed to ST-LT TEC. More recently (4), an adrenal tissue culture assay was used to demonstrate LT in feces and intestinal contents of piglets infected with ST-LT TEC. In contrast, feces and intestinal contents from piglets with diarrhea caused by ST TEC gave negative results when tested in the adrenal tissue culture assay. Since the adrenal tissue culture responds only to LT and not to ST (3), it was inferred that the diarrhea observed in the latter piglets was caused by in vivo production of ST (4). This report confirms that inference in that ST is demonstrated in intestinal contents and fecal fluids collected from piglets with diarrhea caused by ST TEC.

MATERIALS AND METHODS

Organisms. All strains of TEC used in these experiments were originally isolated from swine. The serotypes of these organisms and the enterotoxins which they produce in vitro are listed in Table 1. These organisms were grown overnight in Trypticase soy broth (BBL) at 37 C, and 0.25 ml of these cultures was transferred to a tube containing 7 ml of fresh Trypticase soy broth and grown as above for an additional 3 h. The entire contents of this tube was suspended in 50 ml of milk and fed immediately to a single pig. The inoculum for each pig contained 10^9 to 10^{10} colony-forming units of *E. coli*.

Animals. Newborn hysterectomy-derived, colostrum-deprived piglets were housed in isolation units (J. R. Songer, R. G. Mathis, and S. M. Skartvedt, submitted for publication) with two piglets per unit. Within the isolation unit, each piglet was in a separate metabolism cage which permitted total collection of urine and feces as a mixture. Samples of this mixture, hereafter referred to as feces, were collected at various intervals before and after TEC exposure. All piglets were exposed to TEC on day 4 of life. They were killed at varying times after exposure, and the luminal contents of the large and small intestine were harvested. Fluid intake was limited to 100 ml of SPF-Lac (Borden Chemical Co., Norfolk, Va.) fed twice daily.

Experimental procedure. Samples of feces were collected from 55 pigs of which 14 were uninoculated controls and the remainder were exposed to TEC as follows: 6 to strain Troyer, 12 to strain 2176E8, 8 to strain 431, and 15 to strain 72-2502. Fecal samples were collected daily and represented the combined urinary and fecal excretion for 24-h intervals. The pigs were killed between 1 and 10 days postinoculation, and the contents of the small and large intestines were collected. Fecal and intestinal samples were centrifuged at 10,000 × *g* for 20 min. The supernate was

TABLE 1. *Total daily excreta collected from pigs infected with TEC*

TEC strain	TEC serotype	Enterotoxin produced in vitro	Feces[a] (ml/24 h)	
			Range	Mean
None			28-117	72
Troyer	09:K35:NM	ST only	205-380	292
431	0101:K(A):NM	ST only	175-340	270
2176E8	0138:K81	ST only	37-155	88
72-2502	0149:K91,88	ST and LT	27-225	101

[a] Combined urinary and fecal loss.

harvested, filtered through 0.45-μm filters or sterilized by addition of beta-propiolactone to 0.03%, and stored frozen. All samples were tested in the infant mouse bioassay for enterotoxic activity.

Fecal samples and postmortem samples of intestinal contents were tested for the presence of ST using the infant mouse bioassay as described (2), except that the inoculum was administered through a stomach tube (polyvinyl tube: inner diameter, 0.020 inches [ca. 0.05 cm]; outer diameter, 0.036 inches [ca. 0.09 cm]) attached to a 23-gauge needle. Each mouse received 0.1 ml of inoculum. Each sample was tested in three groups of mice, each group containing four mice. Each day the assay was performed, one group of mice was inoculated with preformed (in vitro) ST produced by strain 2176E8 (2176E8 BA) as a positive control. Similarly, most, but not all, assays included one group of mice which received preformed (in vitro) LT from strain 263 (263 BA) and one group which was not inoculated as negative controls. The preformed enterotoxins which were used were prepared as described previously (11).

To demonstrate that the toxin activity being detected was ST as opposed to LT, some of the fecal samples which had given positive responses were retested after heating for 15 min at 60 or 100 C. No loss of enterotoxic activity was observed in samples heated to 60 C, but three of four samples heated to 100 C gave negative results when retested in infant mice. Consequently, the following experiment was performed to detect fecal destruction and to validate the observed effects of heat.

Samples of fecal fluids which came from TEC-infected pigs with diarrhea but which were negative in the infant mouse test were mixed with an equal quantity of preformed ST enterotoxin. These samples were tested in infant mice after heating for 60 min at 37 C, for 15 min at 100 C, and for 30 min at 100 C. Each group of mice inoculated with a sample of feces-toxin mixture was accompanied by a group of mice receiving a sample of toxin-saline (1:1).

RESULTS

Limits of enterotoxin assay. Observations were made on 50 groups of uninoculated control mice with at least four mice per group. The ratio of the weight of intestinal tract to carcass weight (weight ratio) for these groups of mice ranged from 0.045 to 0.089 with a mean of 0.059. The weight ratios of 94 groups of mice inoculated with preformed LT (263 BA) ranged from 0.053 to 0.077 with a mean of 0.060. A 99% confidence interval was calculated for these respective means based on a random sample of any three observations since all unknown determinations were the mean of three observations. These included weight ratios of 0.047 to 0.072 for uninoculated controls and from 0.051 to 0.070 for the 94 groups of mice inoculated with 263 BA. There were 114 groups of mice inoculated with a standard dose of preformed ST (2176E8 BA). The weight ratios ranged from 0.062 to 0.160 with a mean ratio of 0.113. A confidence interval set to include 95% of the values derived from the mean of any three determinations includes weight ratios from 0.085 to 0.137. Consequently, 0.085 was selected as the minimum mean ratio for any fecal or intestinal sample to be called positive after three determinations. This value was selected with the knowledge that 5% of the samples containing the same quantity of enterotoxin as the 2176E8 BA standard would be called negative. It also follows that samples containing less enterotoxin than the 2176E8 BA standard will be called negative more than 5% of the time. However, this value was selected to insure against a "false positive" interpretation. It was noted that only 5 of 144 negative control samples (3.5%) fell in the area between 0.075 and 0.085.

Incidence and severity of diarrhea. None of the control piglets developed diarrhea, whereas all of those infected with ST strains of TEC did. TEC strains 431 and Troyer induced severe diarrhea. Piglets infected with these strains experienced mean fluid losses of 292 ml (Troyer) and 270 ml (431) within the first 24 h postinoculation (Table 1). These losses represent 22.1 and 20.5% of the original body weight of these piglets. This severe fluid loss and dehydration necessitated killing these piglets 1 day postexposure since they were judged unable to survive another 24 h. However, TEC strain 2176E8 induced a mild diarrhea compared to the other ST strains of TEC. The maximum fluid loss observed from any piglet infected with this strain was only 155 ml in 24 h, which represented 13.5% of the original body weight. Diarrhea induced by the ST-LT strain of TEC (72-2502) varied from mild to severe, and the fluid loss which was observed varied from close to normal up to 18% of the original body weight.

ST in feces and intestinal contents. When fluids derived from samples of feces and intestinal contents collected from these piglets were tested for enterotoxin content, none of the

samples from control piglets gave a positive response (Table 2). Only 2 of the 53 samples tested from these piglets gave responses resulting in weight ratios between 0.075 and 0.085. However, positive responses (weight ratios greater than 0.085) were observed in feces and in samples of intestinal contents from both the large and small intestines of piglets infected with either strain 431 or Troyer. In only one of eight piglets was a negative response observed from a fecal sample when the corresponding sample of intestinal contents gave a positive response. The proportion of positive responses obtained with fecal samples was 7 of 14 samples tested and 12 of 28 with samples of intestinal contents from pigs exposed to these two strains. The incidence of positive responses obtained with fecal fluids from piglets infected with strain 2176E8 was 16 of 58 samples tested. At no time was a positive response obtained with samples of intestinal contents from piglets infected with this strain. However, a relatively high percentage (15.4%) of the 90 samples of feces and intestinal contents from piglets infected with the ST TEC strains used resulted in weight ratios between 0.075 and 0.085, compared to 3.8% of the samples from uninfected piglets. Samples from only 1 of 20 piglets infected with the ST-LT strain (72-2502) gave positive enterotoxin responses, and both feces and large intestinal contents from this pig gave positive responses. Furthermore, none of the responses given by these negative samples resulted in weight ratios in the intermediate range (0.075 to 0.085).

Enterotoxin responses after heating. When preformed ST was mixed with fecal fluids or saline and subjected to heat treatment, there was no loss of enterotoxin in samples incubated at 37 C for 60 min (Table 3), but a consistent loss was observed with samples heated at 100 C for 15 min or at 100 C for 30 min. When these data were analyzed with an analysis of variance, heat treatment was the only factor that was statistically significant ($P < 0.05$). However, there was a significant interaction ($P < 0.05$) between treatment and mixture (saline or feces). From the mean values contained in Table 3, it was concluded that this interaction is the result of the low ratio of the saline toxin control and does not indicate a difference due to the presence of feces. Thus no degradation due to the presence of feces was demonstrated.

Using the least significant difference method at $P < 0.01$, both treatment 1 (unheated) and treatment 2 (37 C for 60 min) were significantly different than either treatment 3 (100 C for 15 min) or treatment 4 (100 C for 30 min) for either feces mixture or saline mixture. However, treatment 1 was not significantly different from treatment 2, nor was treatment 3 different from treatment 4 for either mixture. Thus severe inactivation of the enterotoxic activity detected by the infant mouse bioassay was observed after heating at 100 C for either 15 or 30 min.

TABLE 2. *Infant mouse bioassay: response to samples of fecal fluids and intestinal contents from pigs infected with TEC*

No. of pigs	TEC strain	Mouse response[a]		
		Feces	Intestinal contents	
			Large	Small
9	None	0/33	0/12	0/8
6	Troyer	4/6	4/6	2/6
8	431	3/8	3/8	3/8
12	2176E8	16/58	0/12	0/12
15	72-2502	1/20	1/6	0/6

[a] Number of positive responses/number of samples tested.

TABLE 3. *Effects of heat and swine fecal fluids on enterotoxic activity of preformed ST TEC*

Sample	No. of samples	Heat treatment			
		None	37 C, 60 min	100 C, 15 min	100 C, 30 min
Feces	6	0.066[a]			
Feces + toxin	6	0.117	0.106	0.081	0.075
Saline + toxin	6	0.097	0.112	0.078	0.068

[a] Gut-to-body weight ratio of infant mice 4 h after oral inoculation of 0.1 ml of sample; each value represents the mean of six samples each tested in three separate groups of mice.

DISCUSSION

Positive infant mouse enterotoxin assays were repeatedly obtained with fecal and intestinal fluids collected from piglets exposed to TEC known to produce only ST in vitro. That these positive responses were due to TEC enterotoxin and that the enterotoxin was ST is indicated by the following facts: (i) no positive responses were elicited by samples of fecal or intestinal fluids from control pigs; (ii) the infant mouse did not respond to preformed LT (263 BA); (iii) no loss in enterotoxic activity was demonstrable in positive fecal samples heated at 60 C for 15 min; and (iv) in a previous study (4), no LT activity was demonstrable in fecal or intestinal contents from pigs with diarrhea caused by ST TEC. It was recognized that some of the posi-

tive responses obtained with fecal samples could represent enterotoxin produced in the collection bag and does not necessarily represent ST production in vivo. However, the fact that 12 of the 52 samples from large and small intestine of piglets infected with ST TEC were positive clearly represents in vivo ST production. Furthermore, the fact that a positive sample of intestinal contents was usually accompanied by a positive fecal sample suggests that both responses are due to toxin produced in vivo. The TEC strain with which the piglets were inoculated was demonstrated in fecal samples which were negative, indicating that conditions were not conducive to ST production in fecal samples held in collection bags.

It was assumed that the more severe diarrheas would be associated with greater in vivo toxin production and a higher incidence of positive responses in infant mice. This assumption was validated to the extent that intestinal contents from piglets infected with strains causing more diarrheas (strains 431 and Troyer) evoked a markedly higher incidence of positive responses (12 of 28) than those from piglets infected with strain 2176E8 (0 of 24). The incidence of positive responses (7 of 14) elicited by fecal samples collected from the former group of piglets was also greater than that observed (16 of 58) from fecal samples collected from piglets infected with strain 2176E8. However, negative responses were obtained with fecal samples obtained from some piglets with very severe diarrheas, and positive responses were elicited by fecal samples collected from piglets which were excreting fecal volumes similar to preinfection excretory volumes. However, the severity of the clinical syndrome induced by enterotoxic TEC is dependent not only upon in vivo enterotoxin production but also upon the degree to which these organisms can colonize the anterior small intestine (10).

Of the samples from piglets infected with ST-LT TEC (strain 72-2502), positive responses were observed in samples from only one piglet. Samples of feces and large intestinal contents were both positive from this piglet. The ST activity in feces and intestinal contents from piglets infected by this strain is at a very low level and is less than in samples from piglets infected with ST-only strains. This is consistent with the relationship observed with ST-LT strains in vitro in that ST is present in higher concentrations in broth supernatant (1) from ST-only strains than from ST-LT strains. Production of LT in vivo has previously been demonstrated in piglets infected with ST-LT TEC (4). It is reasonable to conclude that the diarrhea observed in piglets exposed to strain 72-2502 is primarily due to in vivo LT production.

Since the infant mouse bioassay detects ST in fecal fluids, it has the potential to be a useful adjunct to diagnostic laboratory procedures. The fact that over half of the fecal samples tested from infected piglets were negative is, however, disappointing. Since these pigs had diarrhea and since the infecting organism was recovered from these same feces, it is reasonable to assume that ST either was or had been present in the intestinal tract of these pigs also. This concept is supported by the fact that samples which were from pigs infected with ST TEC but which gave negative responses in the infant mouse test induced a much higher incidence of responses characteristic of low levels of toxin (weight ratios between 0.075 and 0.085) than did samples from noninfected pigs (15.4 versus 3.7%). Thus, it must be concluded that ST was either present in concentrations which were below the sensitivity of the infant mouse bioassay or was present but was inactivated during processing and storage by some component of fecal and intestinal fluids; however, we were unable to demonstrate such inactivation. That the concentrations of toxin which were present were low is indicated by the fact that at no time was a maximum response observed in infant mice injected with samples from infected pigs.

Fecal samples which had given positive enterotoxin responses in infant mice were heated to 100 C for 30 min and retested to demonstrate that the enterotoxic activity which had been observed was heat stable. This temperature was selected because it has been used for previous work in this laboratory (1, 11) to distinguish between ST and LT and since other workers (5, 12, 15) had considered ST relatively stable at this temperature. It was disconcerting to observe that samples treated in this manner lost most of their enterotoxic activity. It was initially thought that this loss could have been facilitated by some component of fecal fluids. However, it was subsequently demonstrated that this was an effect of heating to 100 C and was not influenced by fecal fluids. Thus, this observation corroborates that made by Jacks and Wu (8) in that heat inactivation of ST from a human strain of TEC could be demonstrated after heating to 100 C using the infant mouse bioassay. This contrasts with results obtained in previous studies in which enterotoxic activity was measured in ligated intestinal segments of rabbit and pig small intestine (1, 5, 12, 15). Using pig loops, enterotoxic activity was only

slightly impaired by heating at 100 C (1, 15) and was still demonstrable but diminished by heating to 121 C for 30 min (15). Similarly, using rabbit loops, enterotoxic activity was present after 15 min at 100 C and was only slightly impaired by 121 C for 15 min (5, 12). Therefore, it is concluded that this inactivation by heating to 100 C was demonstrable by the infant mouse bioassay because of its greater sensitivity or that the specificity of the receptors in the infant mouse intestinal mucosal cells are such that heating at 100 C alters the toxin in a manner which interferes with toxin receptor interaction in the infant mouse more than in other species.

It is concluded that: (i) the infant mouse bioassay is sufficiently sensitive to detect ST in feces and intestinal contents of TEC-infected piglets; (ii) TEC which produce only ST in vitro also produce ST in vivo and thereby induce diarrhea; and (iii) this along with the previous demonstration of in vivo production of LT indicates that both LT and ST must be considered in the diagnosis and pathogenesis of diarreas caused by enterotoxigenic *E. coli*.

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