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Vero Response to a Cytotoxin of *Escherichia coli*

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A cytotoxin was found in culture filtrates of a number of *Escherichia coli* strains that differed from the known heat-stable and heat-labile enterotoxins of *E. coli*. It was cytotoxic for Vero but not for Y-1 or CHO cells, and its effect on Vero was distinctly different from that of heat-labile enterotoxin. It was labile to heat and antigenically different from heat-labile enterotoxin, and membrane filtration indicated a molecular weight of 10,000 to 30,000.

Recently we described the effect of *Escherichia coli* heat-labile enterotoxin (LT) on the continuous Vero cell line (6). The response of these cells compared favorably with that of Y-1 (mouse adrenal) and CHO (Chinese hamster ovary) cells and of rabbit ileal loops. Nontoxigenic *E. coli* strains showed no response when culture filtrates were tested in any of the above systems.

Some strains of *E. coli* induced a distinctive cytotoxic effect on Vero that was different and easily distinguished from that produced by LT. This report describes the production and properties of this toxin referred to here as VT (toxic to Vero cells).

MATERIALS AND METHODS

Toxin production. *E. coli* enterotoxigenic human or porcine strains, H10401, H10407, B7A, and B2C were obtained from H. L. DuPont (Houston, Tex.); P155, P307, 339, E57, P16, and 711(P307) were from C. Gyles (Canada); SSU 3496, SSU 3515, and M80 31 M1 came from I. K. Wachsmuth (Atlanta, Ga.); and O124, an invasive strain (4), was obtained from C. Park (this laboratory). Strains isolated from infants with diarrhea, H19, H30, and H.I. 1 to H.I. 10, were obtained from C. Gyles, and HW 1 to HW 7 and HSC 1 to HSC 15 came from H. Farkas-Himsley (Canada). Nontoxigenic strains 711 and K-12 were obtained from C. Gyles and V. N. Iyer (Canada), respectively. Eighty-six *E. coli* food isolates were supplied by C. Park and E. Todd (this laboratory). Table 1 lists further details of the strains used.

Erlenmeyer flasks, 250 ml, containing 20 ml of Trypticase soy broth were inoculated and mechanically shaken at 37°C. After 20 to 24 h, the cultures were centrifuged at 17,000 × *g* for 30 min. The supernatants were filtered through 0.45-μm membrane filters (Millipore Corp., Bedford, Mass.) and stored at 4°C until assayed. Filtrate dilutions were made in phosphate-buffered saline (PBS), pH 7.0.

Cell culture assay. Stocks of Y-1, CHO, and Vero cells, purchased from the American Type Culture Collection, Rockville, Md., were passaged by trypsinization and grown as monolayers at 36°C in a 5% CO_2 atmosphere. Y-1 cells were grown in Ham nutrient mixture F10 (Connaught Laboratories Ltd., Toronto), CHO in Ham nutrient mixture F12 (Grand Island Biological Co., Grand Island, N.Y.), and Vero in medium 199 with Earle salts (Connaught); all media were supplemented with 10% fetal calf serum (GIBCO). Maintenance of the cell cultures was described elsewhere (6).

Toxin activity was assayed in plastic dishes (Falcon 3008) containing 24 16-mm diameter wells with 0.5 ml of cell culture in each well. Y-1 monolayers were obtained by seeding 10^5 cells per well 3 to 4 days before use; the growth medium was changed at the time of assay. CHO cells, 10^4 in medium F12 without serum, were seeded in each well at the time of assay. For Vero monolayers, 10^5 cells in growth medium were seeded in each well 1 to 2 days before use; the medium was not changed at the time of assay. (The LT effect on Vero monolayers was more defined when growth medium was replaced with PBS just before addition of bacterial filtrates (6). Although the VT effect was more advanced in the presence of PBS than in growth medium after 24 h, Vero cells could not be maintained in PBS for the 4 days necessary to quantitate the toxin by end-point determination.)

To 0.5 ml of cell culture, 0.05 ml of bacterial filtrate was added. For controls, cultures received PBS or Tripticase soy broth. Cultures were incubated at 36°C; the incubation periods are indicated below. Morphological effects were recorded as 1, 2, 3, and 4, these ratings corresponding to roughly ≤25, 50, 75, or ≥90% of cells affected.

Rabbit ileal loop assay. The ileal loop assay was performed in duplicate or triplicate in 9-week-old rabbits by the method of Kasai and Burrows (2). Two milliliters of filtrate or sterile medium was used for each 10-cm loop; after 18 h, the fluid accumulation was measured and expressed as milliliters of fluid per centimeter of gut. A ratio of 1.0 or more was considered positive for LT.

Infant mouse test. Culture filtrates (0.1 ml) mixed with Evans blue dye were injected orally into 1- to 3-day-old mice and incubated for 4 h at 22°C (7). A strain was considered positive for heat-stable toxin (ST) if the ratio of combined weight of the intestines of four mice to the combined weight of the bodies was over 0.09 (1).

TABLE 1. *Characterization of the E. coli isolates examined*

Strain	Serotype	Source	Disease	Enteropathogenic status[a]
H10401 and H10407	O78:K2	Humans[b]	Diarrhea	LT and ST
B7A	O148:H28			
B2C	O6:H16			
339	O15:H11			
SSU 3496 and 3515	O6:K15:H16			
M80 31 M1	—[c]			ST, no LT
O124	O124:K27(B17)	Cheese	Diarrhea in humans	Invasive
P155	O149:K91, 88a, c	Young pigs	Diarrhea	LT and ST
P307	O8:K87, 88a, b			
P16	O9:K9			ST, no LT
E57	O138:K81			
711(P307)	O18:K?[d]	Laboratory derived		LT and ST
711	O18ab:K?:H14	Fowl	Bacteremia	No LT, no ST
K-12	Not typeable	—	—	
H19 and H30	O26	Human infants	Diarrhea	Little or no response in rabbit leal loops
H.I. 1, 8, and 9	O128:B12			
H.I. 2, 3, and 10	O55:B5			
H.I. 4 and 5	O111:B4			
H.I. 6	O18:B21			
H.I. 7	O125:B15			
HW 1	O26:B6			—
HW 2, 3, and 5	O126:B16			
HW 4	O125:B15			
HW 6	O126:B16?			
HW 7	O125:B15?			
HSC 1, 2, 3, 7, 8, 11, 12, and 14	—			
HSC 4 and 5	O55:B5			
HSC 6	O111:B4			
HSC 9	O125:B15			
HSC 10	O126:B16			
HSC 13	O127:B8			
HSC 15	O128:B12			
64 isolates	—	Cheese, one meat	None	
183	O68:H12	Cheese	None	
185	O26:K60(36):H32			
20 isolates	—	Various foods	Diarrhea in humans	

[a] Prior to present study.
[b] Not infants.
[c] —, No information.
[d] Unknown.

Fractionation of bacterial filtrates. A 200-ml pool of bacterial filtrate of strain H30 was fractionated by filtration, in series, through 76-mm diameter Diaflo membranes, XM300, XM100A, PM30, UM10, UM2, and UM05 (Amicon Corp., Lexington, Mass.), which restrict macromolecules in excess of molecular weight 300,000, 100,000, 30,000, 10,000, 1,000, and 500, respectively. Ten milliliters of filtrate from each membrane was retained for assay; the remaining fluid was filtered through the next grade of membrane. The 5- to 10-ml retentate of each membrane was made up to the starting volume with PBS before assay.

Preparation and testing of antisera. Adult albino rabbits were pre-bled and then given, at 7-day intervals, seven sequential intramuscular injections of 1 ml of concentrated culture filtrates from freeze-dried preparations of *E. coli* H10407 or H30. The first injection consisted of a seven times concentration of filtrate emulsified with an equal volume of incomplete Freund adjuvant. The remaining doses were given as a 7 or 10 times concentration without adjuvant. For neutralization studies, culture filtrates or dilutions in PBS were mixed with equal volumes of serial fourfold dilutions of rabbit sera in PBS. Mixtures were incubated for 1 h at ambient temperature (about 22°C) before assaying on Y-1 cells for LT or on Vero cells for VT.

RESULTS

Culture filtrates of 10 out of 136 *E. coli* strains induced a cytotoxic response in Vero cells. Eight of these were associated with diarrhea, seven in human infants and one in a weanling pig; two were isolates from cheese not implicated in disease. Microscopically, the VT-affected cells ap-

peared round but shriveled; many floated free in the medium (Fig. 1). Undiluted culture filtrates induced the above effect in at least 50% of the monolayer within 24 h, but the cytopathic effect advanced with time; maximum titers were obtained in 4 days (Table 2) and varied, with the strain, from 16 to 1,024 (Table 3).

The VT response was readily distinguished from that of LT in Vero cells. With LT, the cells were enlarged, thick-walled, refractile, and possessed several filamentous tendrils (Fig. 2) (6). If the assay of LT was performed in medium 199 with 10% serum, the effect on the cells faded with time; after 3 days, the monolayers appeared

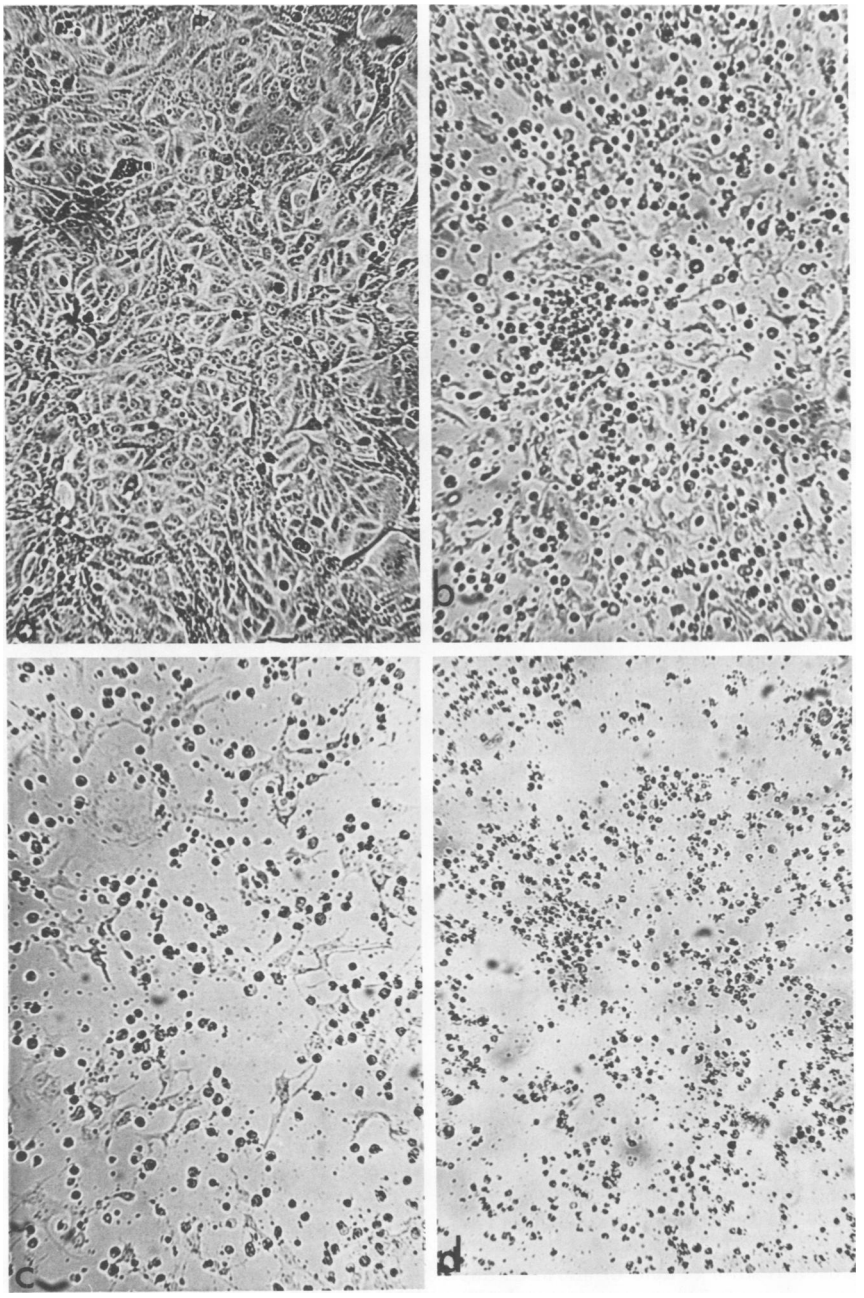

FIG. 1. *VT effect on Vero monolayers: medium control, 1 day (a); with H30 filtrate, 1 day (b), 2 days (c), and 5 days (d). Control monolayers appeared unchanged over 8 days.* ×237.

TABLE 2. *Time course of VT effect of E. coli H19 filtrate on Vero monolayers*

Dilution in PBS of culture filtrate	Day postinoculation					
	1	2	3	4	5	8
Undiluted	4[a]	4	4	4	4	4
1/4	2	3	3	4	4	4
1/16	1	2	3	4	4	4
1/64	—	1	2	3	3	3
1/256	—	—	1	2	2	2
1/1,024	—	—	—	1	1	1
1/4,096	—	—	—	—	—	—

[a] Code: —, no response; 1, ≤25%; 2, about 50%; 3, about 75%; and 4, ≥90% of cells affected.

TABLE 3. *Titer of VT-positive E. coli strains, in Vero monolayers, 4 days postinoculation*

Strain	Source	Disease	Reciprocal of highest dilution inducing morphological change
H19	Human infants	Diarrhea	1,024
H30			1,024
H.I. 5			1,024
H.I. 6			16
H.I. 8			64
HW 1			256
HSC 10			256
E57	Weanling pig	Diarrhea	64
183	Cheese	None	64
185			64

normal. In contrast, cells affected by VT did not recover.

The LT- and ST-positive strains, H10401, H10407, B7A, B2C, 339, 711(P307), P155, P307, SSU 3496, and SSU 3515 were negative for VT as were the ST (only)-positive strains, P16 and M80 31 M1 and the invasive strain 0124. E57, a weanling-pig strain was positive for ST and VT but negative for LT. Of 34 strains isolated from infants with diarrhea, 7 were positive for VT, all were negative for ST, and only HSC10 was positive for LT (as well as VT) (Table 4). Nonenteropathogenic strains 711 and K-12 were negative for VT, ST, and LT. The 86 food isolates were all negative for LT and ST, but two (from cheese) were positive for VT.

VT in filtrates of the positive strains was destroyed by heating at 98°C for 15 min. The titer of H30 filtrate was reduced 50% after heating at 65°C for 15 min.

Membrane filtration of VT in H30 filtrate indicated a size between 10,000 and 30,000 (Table 5).

Neutralization results (Table 6) showed that antiserum against H30 filtrate neutralized VT of all strains except H.I. 8 and E57. Antiserum against H10407 filtrate did not neutralize LT of HSC10. There was no cross-neutralization between LT of H10407 and VT of H30 when different culture filtrates and antisera from three rabbits were used.

DISCUSSION

In general, *E. coli* strains that produced LT did not produce VT. An exception was strain HSC10, but its LT was antigenically different from that of the classical strain H10407. In addition, VT of strains H.I. 8 and E57 were anti-

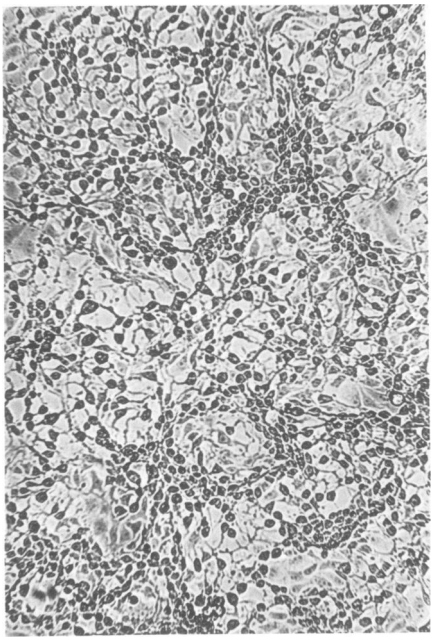

FIG. 2. *LT effect on Vero monolayer with H10407 filtrate, 1 day. For control see Fig. 1a. ×237.*

TABLE 4. *Assays of enterotoxic activity (LT and ST) in VT-positive E. coli strains*

Strain	Enterotoxic activity			
	LT response in:			ST response in infant mouse
	Y-1	CHO	Rabbit ileal loop	
H19	−	−	−	−
H30				
H.I. 5				
H.I. 6				
H.I. 8				
HW 1				
183				
185				
HSC 10	+	+	+	−
E57	−	−	−	+

TABLE 5. *Fractionation of VT of H30 culture filtrate by membrane filtration*

Membrane size	Reciprocal of highest dilution inducing morphological change in Vero cells	
	Filtrate	Retentate
Control (not filtered)	1,024	
XM300	1,024	0
XM100A	1,024	4
PM30	1,024	64
UM10	64	256
UM2	4	16
UM05	1	0

TABLE 6. *Neutralization of cell response to E. coli toxins by rabbit antibodies*

Strain	Reciprocal of culture filtrate dilution[a]	Cell type for test	Reciprocal of highest dilution to neutralize with antiserum to:	
			H30	H10407
H19	100	Vero	64	0
H30	100	Vero	64	0
H.I. 5	100	Vero	64	0
H.I. 6	1	Vero	64	0
H.I. 8	4	Vero	0	0
HW 1	16	Vero	64	0
183	4	Vero	64	0
185	4	Vero	64	0
HSC 10	1	Y-1	0	0
HSC 10	16	Vero	64	0
E57	4	Vero	0	0
H10407	1	Y-1	0	256

[a] Eight to 16 times the amount that causes toxic effect in cell culture.

genically different from VT of the other eight strains.

The VT titers were generally higher than those of LT in Y-1 or Vero cells (6). As indicated by membrane separation, VT of H30 was a smaller molecule than LT of H10407, i.e., 10,000 to 30,000 as compared to 30,000 to 300,000 (6). Both toxins were destroyed by heating at 98°C for 15 min.

Since the Vero cells failed to recover, the effect of VT was apparently cytotoxic; in totally involved monolayers, many of the cells lifted from the surface. This is in contrast to the cytotonic effect of LT (3). Preliminary studies (not reported here) indicated that in contrast to LT, VT did not stimulate cyclic adenosine 5'-monophosphate production in Vero cells.

It is apparent that several different *E. coli* exotoxins are produced, and they can be measured in cell cultures. The newest of these, VT, may contribute to diarrheal disease in human infants and possibly young pigs. It would be expected that fluid accumulation would result as a cytotoxic response by intestinal cells rather than by the stimulation of cyclic adenosine 5'-monophosphate as with LT. Recently, we noted that the human amnion cell line, FL, was also sensitive to VT (unpublished data). In our studies, the rabbit ileum showed little response with culture filtrates of VT-producing strains. However, fluid accumulation, in this host, has been demonstrated by C. Gyles with concentrated lysates of H19 and H30 (personal communication) and by our laboratory with concentrated purified VT of H30 (unpublished data). The presence of VT in 2 out of 66 culture filtrates from food isolates (not implicated in disease) need not detract from the possibility that VT may be involved in disease. In a similar survey of food, Sack et al. (5) found that 8% of the isolates were enterotoxigenic. Further investigation is obviously required to demonstrate the specific role of VT in enteropathogenic disease.

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