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Evidence for Two Types of Cytotoxic Necrotizing Factor in Human and Animal Clinical Isolates of *Escherichia coli*

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We have characterized the in vitro and in vivo toxic properties of cell sonic extracts from 22 animal and human clinical isolates of *Escherichia coli* that caused both necrosis in the rabbit skin and multinucleation in tissue cultures, two toxic properties previously reported as being specific for *E. coli* cytotoxic necrotizing factor (CNF). Two distinct toxic phenotypes were observed. Type 1, which was displayed by originally described CNF strains, was characterized by extensive multinucleation and rounding of cells in HeLa cell culture assays, moderate necrosis in the rabbit skin test, and absence of necrosis in the mouse footpad test. Type 2, which has recently been shown to be associated with *E. coli* Vir plasmid, was characterized by moderate multinucleation, by polymorphism and elongation of HeLa cells, and by an intense necrotic response in both the rabbit skin test and the mouse footpad test. The distinction between the two cytotoxins accounting for these effects (CNF 1 and CNF 2), together with their partial relatedness, was confirmed by seroneutralization studies of both cytopathic effects and necrosis in the rabbit skin test. In addition, type 2 extracts were more lethal in the mouse intraperitoneal test and induced a moderate, although not totally repetitive, fluid accumulation in the ileal loop test. The original toxic properties of these recently recognized categories of *E. coli* strains, together with their association with enteritis and septicemia, suggest that these strains may play a significant role in pathology.

The existence of *Escherichia coli* toxic products causing cell multinucleation and enlargement in tissue cultures and necrosis in rabbit skin was first reported by Caprioli et al. (7) in strains from infant enteritis. These strains produced neither known enterotoxins nor verocytotoxins. Their toxic effects were attributed to a toxin, termed cytotoxic necrotizing factor (CNF), which was subsequently purified and identified as a protein of about 115 kilodaltons by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6) and immunoblotting (11). The production of CNF-like activity, as identified by the multinucleating effect on tissue cultures and necrosis in rabbit skin, was also detected in *E. coli* strains from enteritic piglets and calves (4, 9, 12, 14) and extraintestinal infections in humans (1, 8). Most CNF-producing strains also synthesized hemolysin, but CNF and hemolytic activities were dissociated, as shown by the fact that they did not copurify from cell lysates (7) and by the obtainment of hemolysin-negative laboratory mutants retaining the entire CNF activity of the parental strain (11). Extracts from CNF-producing strains induced no significant fluid accumulation by the rabbit ileal loop test (7), which suggested that CNF did not possess enterotoxic activity.

Another group of *E. coli* cytotoxic strains that causes multinucleation in HeLa cell cultures was detected by De Rycke et al. (9) in calves with enteritis. Immunologically, the new type of cytotoxin (type 2) was only partially related to CNF (type 1) and was totally unrelated to verocytotoxins, as shown by seroneutralization studies (9). Subsequently, Oswald et al. (16) demonstrated that type 2 cytotoxicity was coded by the transmissible Vir plasmid of the reference strain S5 (from H. W. Smith) (17) and, moreover, was produced by the formerly designated Vir strains of *E. coli*. The distinction between these two types of CNF-like toxicity was confirmed in vivo by a mouse footpad test (10). In that test, cell sonic extracts from both types of strains induced a strong and persistent local inflammatory response, resulting in necrosis with type 2 strains only. In the same test, extracts from verocytotoxin-producing strains produced a clearly different response, consisting of an intense but transient local infiltration with no apparent residual lesions. Blanco et al. (4), by using the Vero cell culture assay, did not observe noticeable differences in the cytopathic effect produced by CNF-like extracts, and they showed that both type 1 and type 2 extracts produced necrosis in the rabbit skin.

The collaborative investigation presented here was carried out to clarify the above separate observations and to define clearly the toxic phenotypes associated with *E. coli* strains with CNF-like activity, i.e., causing multinucleation in tissue cultures and necrosis in rabbit skin. For this purpose, we have tested a sample of 22 human and animal *E. coli* strains possessing the above properties isolated in different European countries, in most cases from enteritis.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* strains investigated are listed in Table 1. Except for the Vir control strain S5, which was isolated from a septicemic lamb (17), all isolates originated from cases of enteritis in human infants and neonatal calves or piglets. They were selected for the study because their sonic extracts were necrotizing in rabbit skin and produced a cytopathic effect of multinucleation in Vero or HeLa cell cultures. These strains were previously shown to be negative for heat-stable enterotoxin in the infant mouse test and for thermolabile enterotoxin and verocytotoxins in the assay with Vero cell cultures. CNF control strains EB21 and EB28 were kindly supplied by A. Caprioli (Istituto Superiore di Sanita, Rome, Italy), and Vir strain S5 by H. W. Smith

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TABLE 1. Evidence of two types of multinucleating cytotoxins (CNF 1 and CNF 2) in cell sonic extracts of *E. coli* strains

Strain	Origin[a]	Serotype	Hemolysin	Cytopathic effect in HeLa cell cultures			
				Type	Titer[b]	Seroneutralization titer[c]	
						CNF 1 antiserum	CNF 2 antiserum
EB21	Human (I)	O75:K95:H$^-$	+	CNF 1	128	1,024	64
EB28	Human (I)	O4:K12:H1	+	CNF 1	128	2,048	32
MR48	Human (S)	O75:K95	+	CNF 1	64	2,048	32
MR199	Human (S)	O6:K13	+	CNF 1	64	2,048	32
MR249	Human (S)	O6:K$^-$	+	CNF 1	4	2,048	64
BM2-1	Bovine (F)	O2:K53,93:H1	+	CNF 1	128	2,048	128
BM3-1	Bovine (F)	O8:K87:H$^-$	+	CNF 1	64	2,048	128
BM4-1	Bovine (F)	O8:K87:H$^-$	+	CNF 1	64	2,048	64
DM2-2	Bovine (F)	O8:K87:H$^-$	+	CNF 1	64	2,048	64
9AM1	Bovine (F)	O11:H$^-$	+	CNF 1	32	2,048	64
B17a	Bovine (F)	O8:K87	+	CNF 1	32	2,048	64
28C	Porcine (S)	O75:K95	+	CNF 1	64	2,048	64
13B	Porcine (S)	O2:K$^-$	+	CNF 1	32	2,048	64
7d	Porcine (S)	O75:K$^-$	+	CNF 1	16	2,048	64
S5	Lamb (UK)	O15:K?:H21	−	CNF 2	256	32	1,024
B24c	Bovine (S)	O76:K$^-$	+	CNF 2	256	32	2,048
B20a	Bovine (S)	O15:K14	−	CNF 2	256	32	2,048
B28b	Bovine (S)	O78:K80	−	CNF 2	64	32	1,024
B26a	Bovine (S)	O123:K$^-$	−	CNF 2	32	32	2,048
B9S2	Bovine (F)	O168:K?:H8	−	CNF 2	128	64	1,024
LM1-6	Bovine (F)	O?:H25	−	CNF 2	64	64	2,048
BM2-10	Bovine (F)	O88:H25	−	CNF 2	32	64	2,048

[a] Reference strain S5 was isolated from a septicemic lamb in the United Kingdom, while the remaining strains were fecal isolates from diarrheic humans and animals isolated in Italy (I), Spain (S), and France (F).

[b] Titer of multinucleating cytotoxic effect: highest dilution of sonic extracts (twofold dilutions) that produced at least 50% of cells with two or more nuclei after 72 h of incubation (9). Sonic extracts contained 2.5 to 3 mg of protein per ml.

[c] Titer of seroneutralization: highest dilution of antiserum (twofold dilutions) that neutralized at least 90% of the multinucleating effect produced by an amount of toxic material four times higher than the 50% cytotoxic dose.

(Institute for Animal Disease Research, Houghton, United Kingdom). In addition, enterotoxigenic *E. coli* m452-C1 (thermolabile enterotoxin and heat-stable enterotoxin producing) and nontoxigenic K12-185 were used as positive and negative controls, respectively, for enterotoxigenic activity.

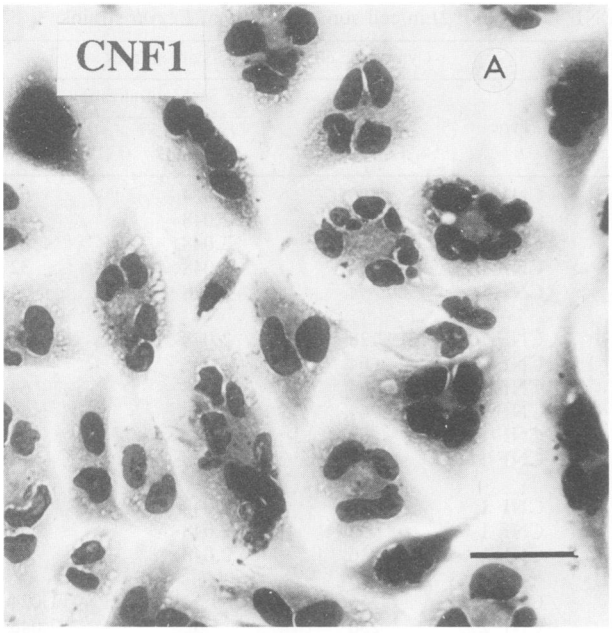
Toxicity assays. All assays were performed with cell sonic extracts, by the methods comprehensively described in previous papers. These were as follows: Vero cell culture assay (1, 3), HeLa cell culture assay in combination with seroneutralization (9), rabbit skin test (1, 3), mouse intraperitoneal test (1, 3), mouse footpad test (10), and rabbit intestinal loop test (13).

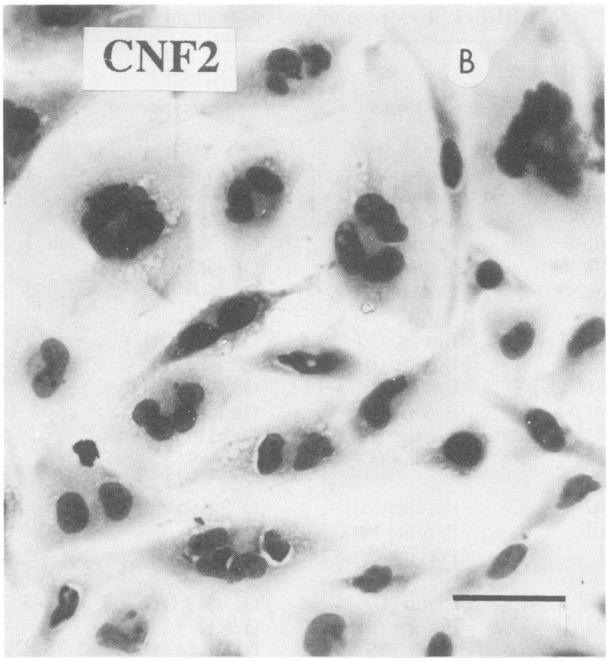
Briefly, sonic extracts were prepared in all cases from organisms in late-exponential aerated cultures in Trypticase (BBL Microbiology Systems) soy broth (pH 7.5). For assay of Vero cells, rabbit skin test, mouse intraperitoneal test, and rabbit intestinal loop test, whole cultures, corresponding to a bacterial density of about 7×10^9 CFU/ml, were ultrasonically disintegrated, and the resulting sonic extracts were clarified by centrifugation (6,000 × *g* for 15 min) and filtered (0.22-μm-pore-size filter) (1, 3). The range of the protein content of such extracts, as determined by the method of Bradford (5), was about 300 to 400 μg/ml. For the HeLa cell assay and mouse footpad test, organisms were first concentrated by centrifugation at 5,000 × *g* for 20 min, and the pellet was suspended in phosphate-buffered saline (pH 7.2). The bacterial suspension was disintegrated, clarified by centrifugation (10,000 × *g* for 2 h), and filtered (0.22-μm-pore-size filter), and each resultant extract was then assayed for total protein content by the method of Bradford (5).

The Vero cell culture assay was performed on cell monolayers grown nearly to confluence in plates with 24 wells, each well having a diameter of 16 mm. At the time of assay, the growth medium was changed (0.5 ml per well) and 0.05 ml of undiluted sonic extract was added. Specific morphological changes were observed after 24, 48, and 72 h of incubation by examining unfixed cell cultures under a phase-contrast inverted microscope (1, 3).

In the HeLa cell culture assay, the trypsinized cell suspension (4×10^4 cells per ml of growth medium) was distributed in 96-well microdilution plates, under a volume of 180 μl, immediately followed by the addition of 20 μl of twofold dilutions in phosphate-buffered saline of sonic extracts. After 72 h of incubation, cells were stained with Giemsa and the cytopathic effects were identified and titrated microscopically. The seroneutralization assays were carried out with antisera produced in rabbits against extracts from *E. coli* CNF-producing BM2-1 (type 1) and from strain S5, which produces the Vir cytotoxin (type 2), as described previously (9). Strains BM2-1 and S5 appear in Table 1.

For the titration of necrotizing activity in the rabbit skin test, cell sonic extracts were diluted to 1/10, 1/25, 1/50, 1/100, and 1/500 in sterile Trypticase (BBL Microbiology Systems) soy broth, and 0.1 ml of undiluted extracts and dilutions were each intradermally injected into three rabbits. Seroneutralization of necrotizing activity caused by type 1 extracts was performed by mixing a volume of undiluted sonic





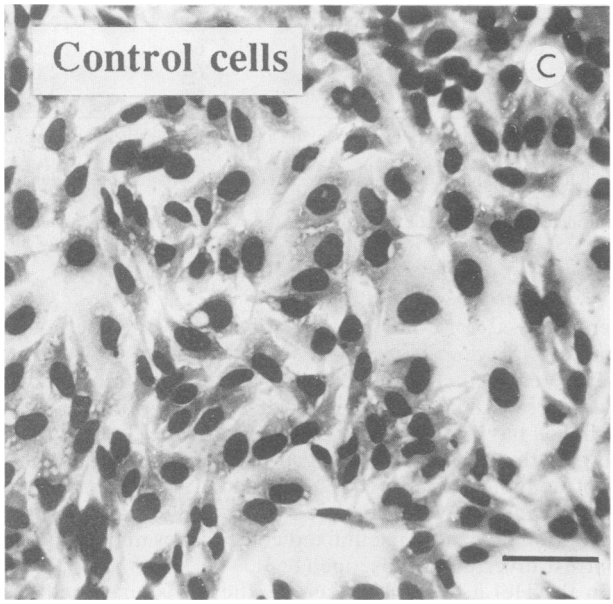


FIG. 1. Cytopathic effect exerted by CNF 1 and CNF 2 extracts in HeLa cell cultures assay after 72 h of incubation. Giemsa staining. Bar = 50 μm.

extract with the same volume of antiserum diluted in phosphate-buffered saline, whereas neutralization of necrotizing activity due to type 2 extracts was performed by mixing a volume of sonic extract diluted to 1/10 with the same volume of antiserum dilutions. The necrotizing effect of the mixture was assayed after incubation for 1 h at 37°C and then overnight at 4°C.

Lethality in the mouse intraperitoneal test (1, 3) was recorded during a period of 7 days following intraperitoneal administration of a 0.5-ml volume of undiluted sonic extract.

In the mouse footpad test (11), a dose of toxic extract containing about 150 μg of total protein was inoculated into the left hind footpad in a 50-μl volume and the occurrences of necrosis and of lethality were recorded over a period of 7 days.

Exterotoxic activity in the rabbit ileal loop test (13) was assayed after 6 and 2 h of incubation. For both incubation periods, 1.0 ml of undiluted sonic extract was inoculated into one loop of four rabbits. The ratio of accumulated fluid (in milliliters) per loop length (in centimeters) was calculated.

Hemolysin production was detected after 18 h of growth on a tryptose blood agar base (Difco Laboratories) containing 5% (vol/vol) washed sheep erythrocytes.

RESULTS

The sonic extracts of the 22 *E. coli* isolates produced both necrosis in rabbit skin and cell multinucleation-enlargement in tissue cultures. According to the cytopathic effect produced by these sonic extracts in HeLa cell cultures after 72 h of incubation, all of these strains belonged to one of two distinct toxicity groups (Table 1). Type 1 alteration was characterized by an extensive multinucleation of cells, together with enlargement and rounding, whereas type 2 alteration was characterized by a lower degree of multinucleation, elongation, polymorphism, and partial loss of viability (Fig. 1). The type 1 effect was caused by sonic extracts of the two reference CNF-producing strains (EB21 and EB28) obtained from Caprioli (7), and the type 2 effect was produced by extracts from Vir strain S5 from H. W. Smith (16, 17). This classification into two groups was confirmed by seroneutralization experiments (Table 1). However a significant cross-neutralization was observed between the two types, homologous serum being 16- to 64-fold more potent than heterologous serum. We have named CNF 1 and CNF 2, respectively, the postulated cytotoxins responsible for each type of cytopathic multinucleating effect.

In contrast to the HeLa cell culture assay, the Vero cell assay, applied in the conditions described above, did not reveal clear differences between strains. In this test, both types of extracts induced moderate cell multinucleation with increase of cell refringence and enhanced definition of cell outlines (photographs in references 4 and 12).

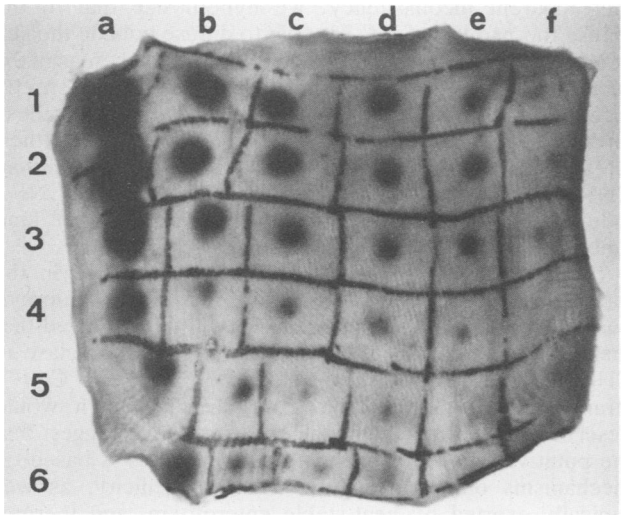


FIG. 2. Titration of necrotizing activity of sonic extracts from CNF 2 (1, 2, and 3)- and CNF 1 (4, 5, and 6)-producing strains in the rabbit skin test. Materials injected were as follows: undiluted sonic extracts (a) and sonic extracts diluted to 1/10 (b), 1/25 (c), 1/50 (d), 1/100 (e), and 1/500 (f).

Although both types of extracts caused necrosis in rabbit skin, CNF 2 extracts were up to 100-fold more potent than CNF 1 extracts, as shown by the titration assay (Fig. 2; Table 2). Seroneutralization of necrotizing activity in the rabbit skin test was done with sonic extracts from six CNF 1 and six CNF 2 extracts, with CNF 1 and CNF 2 antisera diluted to 1/10 and 1/100, respectively (Fig. 3). Considering that CNF 2 extracts were about 10-fold more necrotic than were CNF 1 extracts, the former were used at a 1/10 dilution and the latter were undiluted in the seroneutralization assay. In these conditions, a 1/100 dilution of CNF 2 antiserum totally neutralized the necrosis caused by the six CNF 2 extracts but not the necrosis caused by CNF 1 extracts, which was, however, partially neutralized by a 1/10 dilution of this antiserum. A 1/100 dilution of CNF 1 antiserum was unable to neutralize the necrosis caused by extracts of both types, whereas a 1/10 dilution of this serum partially neutralized the necrosis caused by CNF 1 extracts only.

Necrotizing activity in the mouse footpad test was produced only by CNF 2 extracts (Table 2). With an inoculum of about 150 μg of protein, all CNF 2 extracts indeed produced necrosis in 40 to 100% of the mice, whereas no sign of necrosis was ever observed with CNF 1 extracts. Both types nevertheless produced an intense and persistent inflammatory response, resulting in a 70 to 120% increase of footpad thickness 2 days after inoculation (data not shown). In addition, CNF 1 extracts from the highest producing strains were totally or partially lethal, whereas with CNF 2, only one extract, namely that from strain B24c, caused a partial lethality (Table 2).

In the mouse intraperitoneal test (Table 2), all CNF 2 extracts were 100% lethal, whereas CNF 1 extracts were most often only partially lethal. Death occurred within 48 h after inoculation and was not preceded by paralysis of the hind limbs.

The rabbit intestinal loop test was done with sonic extracts from five CNF 1 and four CNF 2 strains (Table 3). After 24 h of incubation, all type 2 extracts caused a significant fluid accumulation in at least two out of four loops tested, whereas CNF 1 extracts or the nonenterotoxigenic control gave none or only one positive control loop out of four. However, the fluid accumulation caused by CNF 2 extracts was much lower than that caused by the control enterotoxigenic strain.

Hemolysin was produced by all 14 CNF 1 strains but in only 1 of 7 CNF 2 strains (Table 1).

TABLE 2. Responses produced by the rabbit skin test, mouse intraperitoneal test, and mouse footpad test with cell sonic extracts of *E. coli* strains producing CNF 1 and CNF 2

Strains	Necrosis in rabbit skin test	Lethal activity in mouse intraperitoneal test	Mouse footpad test[a]	
			Lethality[b]	Foot necrosis[c]
CNF 1				
EB21	1/10[d]	5/5[b]	6/7	0/1
EB28	1	2/5	7/7	
MR48	1/10	1/5	7/7	
MR199	1	5/5	3/7	0/4
MR249	1	5/5	0/7	0/7
BM2-1	1	2/5	7/7	
BM3-1	1	3/5	1/7	0/6
BM4-1	1	1/5	0/7	0/7
DM2-2	1	1/5	0/7	0/7
9AM1	1	0/5	0/7	0/7
B17a	1	2/5	0/7	0/7
28c	1	3/5	6/7	0/1
13b	1	4/5	0/7	0/7
7d	1/10	5/5	0/7	0/7
CNF 2				
B24c	1/100	5/5	1/5	4/4
B20a	1/50	5/5	0/7	7/7
B28b	1/50	5/5	0/5	5/5
B26a	1/100	5/5	0/7	7/7
B9S2	1/100	5/5	0/7	4/7
LM1-6	1/50	5/5	0/7	7/7
BM2-10	1/100	5/5	0/7	3/7

[a] Inoculum of about 150 μg per mouse.
[b] Number of mice that died during a 7-day period of observation/number of mice inoculated.
[c] Number of mice with foot necrosis/number of surviving mice 7 days after inoculation.
[d] Highest dilution of sonic extract causing necrosis in three out of three rabbits tested.

DISCUSSION

We have established the existence of two categories of *E. coli* strains producing cell-associated factors causing both multinucleation in HeLa cell cultures and necrosis in rabbit skin. This study confirms and generalizes a preliminary observation, carried out on a restricted sample of *E. coli* strains from calf enteritis in France (9). In both studies, these toxic properties were not associated with the production of other recognized toxins, such as verocytotoxins or enterotoxins. In the HeLa cell assay, such as was described here, the two types of multinucleating cytotoxins caused distinct morphological traits. Such a distinction was not achievable with the Vero cell assay. The distinction between type 1 and type 2 toxicity was confirmed by the seroneutralization studies, which demonstrated, however, some degree of homology between the two toxic products. The type 1 effect was produced by the original CNF strains described by Caprioli et al (7). We have shown recently that these strains possessed the 115-kilodalton entity specific for CNF by

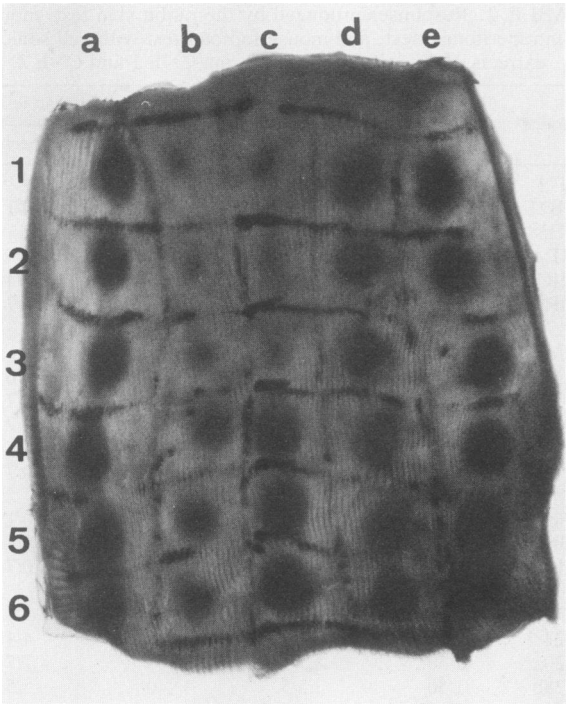


FIG. 3. Seroneutralization of necrotizing activity caused in the rabbit skin test by sonic extracts from CNF 2 (1, 2, and 3)- and CNF 1 (4, 5, and 6)-producing strains. Control sonic extracts without antiserum (a), with CNF 2 antiserum diluted to 1/10 (b) and 1/100 (c), and with CNF 1 antiserum diluted to 1/10 (d) and 1/100 (e).

sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (11). In the light of the present results, we propose to call CNF 1 the CNF originally described by Caprioli et al. (7). The second type of multinucleating effect was previously demonstrated to be associated with the Vir plasmid of *E. coli* and was present in presumed Vir strains cited in the literature (16). Although the postulated toxin responsible for this second type of toxicity has been neither identified immunochemically nor purified, we propose to call it provisionally CNF 2 because of its analogy with CNF 1. To detect and differentiate between CNF 1 and CNF 2 strains, we suggest the use of the HeLa cell culture assay with confirmation by seroneutralization studies.

The second objective of this study was to define the in vivo toxic properties associated with CNF 1 and CNF 2 extracts. The most important observation was that CNF 2 extracts were significantly more necrotic than were CNF 1 extracts. In the rabbit skin test, this difference could be expressed in a quantitative manner, CNF 2 extracts being up to 100-fold more potent than CNF 1 extracts. In the mouse footpad test, necrosis was induced exclusively by CNF 2 extracts. By our previous results, it was not possible to induce a necrotic response in this test with CNF 1 extract inoculated at higher doses (10). Increasing doses with such an extract indeed resulted in increased lethality with no occurrence of necrosis, whereas with a CNF 2 extract, the proportion of mice with necrosis was linearly related to dose, lethality occurring only with doses inducing at least 100% necrosis (10).

CNF 2 extracts were also more lethal than were CNF 1 extracts by the mouse intraperitoneal test. These results may appear contradictory to data from the mouse footpad test, in which only some CNF 1 extracts were lethal. To account for this apparent inconsistency, we hypothesize that in the mouse footpad test, CNF 1 is able to diffuse outside the site of inoculation and thus to exert a lethal effect, whereas in the same test, CNF 2 does not diffuse and produces a strictly local effect resulting in necrosis. Conversely, in the mouse intraperitoneal test, both toxins may fully express their lethal properties, CNF 2 being in that case more lethal than CNF 1. In contrast to verocytotoxins (4), CNF 1 and CNF 2 induced neither delayed mortality nor paralysis of hind limbs.

The absence of significant enterotoxic response in the intestinal loop test with CNF 1 extracts confirms the previous observation of Caprioli et al. (7), who also showed that their CNF strains failed to stimulate adenylate cyclase in CHO cell cultures. The slight effect observed with CNF 2 strains suggests some enterotoxic activity which would deserve further study. Nonetheless, our results suggest that the putative enteropathogenicity of the strains is based on mechanisms other than strict enterotoxigenicity, as was typically exerted by heat-stable enterotoxin- and thermolabile enterotoxin-producing strains. In this regard, it would be worthwhile to study the attaching-effacing ability of these strains, a mechanism of pathogenicity which has been demonstrated in human enteropathogenic *E. coli* synthesizing a Shiga-like toxin with cytotoxic activity on Vero cells (15).

All CNF 1 strains were hemolytic in the present study, versus only one CNF 2 strain. The association of hemolysin with CNF-producing strains has been reported repeatedly. In three epidemiological studies carried out in Italy, only one hemolysin-negative strain was detected in more than 60 human CNF-producing strains of various clinical origins (2, 7, 8). Studies carried out in Spain revealed the existence of three hemolysin-negative strains among 21 CNF-producing strains isolated from feces of children with or without diarrhea (3) and four among 24 CNF-producing strains isolated from human urinary tract infection (1). In the light of the present results, it would be interesting to examine if these former hemolysin-negative CNF-producing strains were not actually of the CNF 2 type.

TABLE 3. Enterotoxic activity of sonic extracts in the rabbit intestinal loop test after 6 and 24 h of incubation

Strains	RILT ratio[a] after incubation for:							
	6 h				24 h			
	R1	R2	R3	R4	R1	R2	R3	R4
CNF 1								
BM2-1	—	—	1.3	—	0.2	—	—	—
BM3-1	—	—	—	—	1.5	—	—	—
BM4-1	—	—	—	—	—	—	0.2	—
DM2-2	—	—	—	—	—	—	—	—
9AM-1	—	—	—	—	—	—	—	—
CNF 2								
S5	0.3	—	—	0.2	1.9	1.1	0.9	—
B9S2	0.9	—	—	0.4	—	—	1.0	0.6
LM1-6	0.8	—	0.4	0.2	—	1.2	1.3	—
BM2-10	0.7	—	—	0.4	—	—	1.2	0.5
E. coli control								
m452-C1 (LT^+ STa^+)[b]	1.3	1.0	1.5	0.9	2.9	2.7	2.4	1.9
K12-185 (nontoxigenic)	0.1	—	—	0.1	—	—	—	—

[a] RILT (rabbit intestinal loop test) ratios, accumulated fluid (in milliliters) per loop length (in centimeters). Each sonic extract was tested in four rabbits (R1, R2, R3, and R4) for both periods of incubation. —, No detectable fluid accumulation in intestinal loops.

[b] LT, Thermolabile enterotoxin; STa, heat-stable enterotoxin.

The necrotic and lethal properties of CNF 1 and CNF 2 suggest that they are true virulence factors. This hypothesis is strengthened by the association of producing strains with intestinal as well as extraintestinal infections. Although most of the strains examined in the present study were isolated from enteritis in humans and animals, CNF strains have been also isolated from septicemia and urinary tract infection in humans (1, 8). The production of the Vir toxin (CNF 2) was originally detected in invasive strains of *E. coli*, and the transfer of the Vir plasmid was shown to confer toxemic properties to transconjugant strains along with the CNF 2 toxicity (16, 17). We have also shown that both types of strains were highly lethal for mice inoculated intraperitoneally (9), which strongly suggests that both types of strains possess toxemic properties. Further study is obviously required to demonstrate the role of these new categories of toxic strains in both intestinal and extraintestinal pathology.

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