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Partial Purification and Characterization of an *Escherichia coli* Toxic Factor That Induces Morphological Cell Alterations

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A factor produced by several strains of *Escherichia coli* isolated from enteritis-affected children has been shown to produce both a necrotizing effect on rabbit skin and striking morphological alterations on CHO, Vero, and HeLa cells. The same strains were found to have hemolytic activity on sheep erythrocytes. The toxic, cell-altering factor was demonstrated to be different from both heat-labile and heat-stable enterotoxins and from Vero toxin. The main effect induced by the isolated factor on cultured cells was the formation of large multinucleated cells. The partial purification achieved suggests that the same factor (most likely a protein with a molecular weight of 70,000 to 80,000) is responsible for toxic and cell-altering activities, whereas a different molecular species is responsible for hemolytic activity.

A number of *Escherichia coli* strains are known to produce heat-labile enterotoxin (LT) and heat-stable enterotoxin, whose properties and role in diarrheal disease have been widely investigated (1–4, 9, 11, 18, 19). A cytotoxin active on Vero cells has also been described by Konowalchuk et al. (13, 14), and its role in human infection by enteropathogenic *E. coli* has subsequently been discussed (17). In addition, several *E. coli* strains involved in sepsis or urinary tract infections have been reported to be hemolysin producers (12).

In this paper we report the finding of a new *E. coli* factor, designated CNF, obtained from strains of clinical relevance and showing an activity which is cytotoxic for HeLa cells and necrotizing on rabbit skin. We also describe the main biological properties and chemical characteristics of CNF.

MATERIALS AND METHODS

Bacterial strains. A total of 213 *E. coli* strains tested for toxic activity were isolated from stool specimens of 110 children (age, 2 years and under) hospitalized for acute diarrheal disease. Strains were identified by the API 20E technique (La Balme, Les Grottes, France). The following *E. coli* strains were used as controls: E9638 (LT^+) and E1106/0 (positive for Vero toxin), kindly supplied by B. Rowe (Public Health Laboratory Service, London, United Kingdom); and ISS132 (positive for heat-stable enterotoxin) and K-12 J62 carring the hemolysin plasmid p212, from our laboratory collection. *E. coli* K-12 J53 (*pro met*) was used as negative control in all experiments.

Culture conditions. Cultures were maintained on nutrient agar slants at 25°C. Erlenmeyer flasks (250 ml) containing 20 ml of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) were inoculated with 10^4 bacterial cells from exponentially growing cultures of control and test strains. After incubation in a rotary shaker at 37°C for 18 h, the cultures were centrifuged at 10,000 × *g* for 30 min at 4°C, and the supernatants were filtered through 0.45-μm MF membranes (Millipore Corp., Bedford, Mass.). If required, mitomycin C was added (0.5 μg/ml) 3 h after the inoculation of the cultures. To obtain higher amounts of LT (2), cultures were sonicated in an ice bath at an amplitude of 14 μm peak to peak (three strokes, 20 s each) in an MSE apparatus (MSE Scientific Instruments, Crawley, United Kingdom). The cell extracts thus obtained were centrifuged at 20,000 × *g* at 4°C for 20 min, and supernatants were filtered as described above.

Cell culture assays. Chinese hamster ovary (CHO), Vero, and HeLa cells, obtained from the American Type Culture Collection (Rockville, Md.), were passaged by trypsinization and grown as monolayers at 36°C in Eagle minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) with Earle salts. The medium was supplemented with 5% fetal calf serum and 1% nonessential amino acids (GIBCO). LT assays were carried out in microtiter plates (Falcon Plastics, Oxnard, Calif.) as described by Guerrant et al. (10). Bacterial extracts were tested in Vero and HeLa cultures by the same procedure. To obtain monolayers in eight-chamber tissue culture slides (Lab-Tek 4808; Lab-Tek Products, Naperville, Ill.) each chamber was seeded with about 10^4 cells in 0.36 ml of minimal essential medium containing 1% fetal calf serum and then was inoculated with 0.04 ml of bacterial sample. After incubation at 36°C, monolayers were fixed in methanol and stained with May Grunwald-Giemsa (E. Merck, Darmstaadt, Federal Repub-

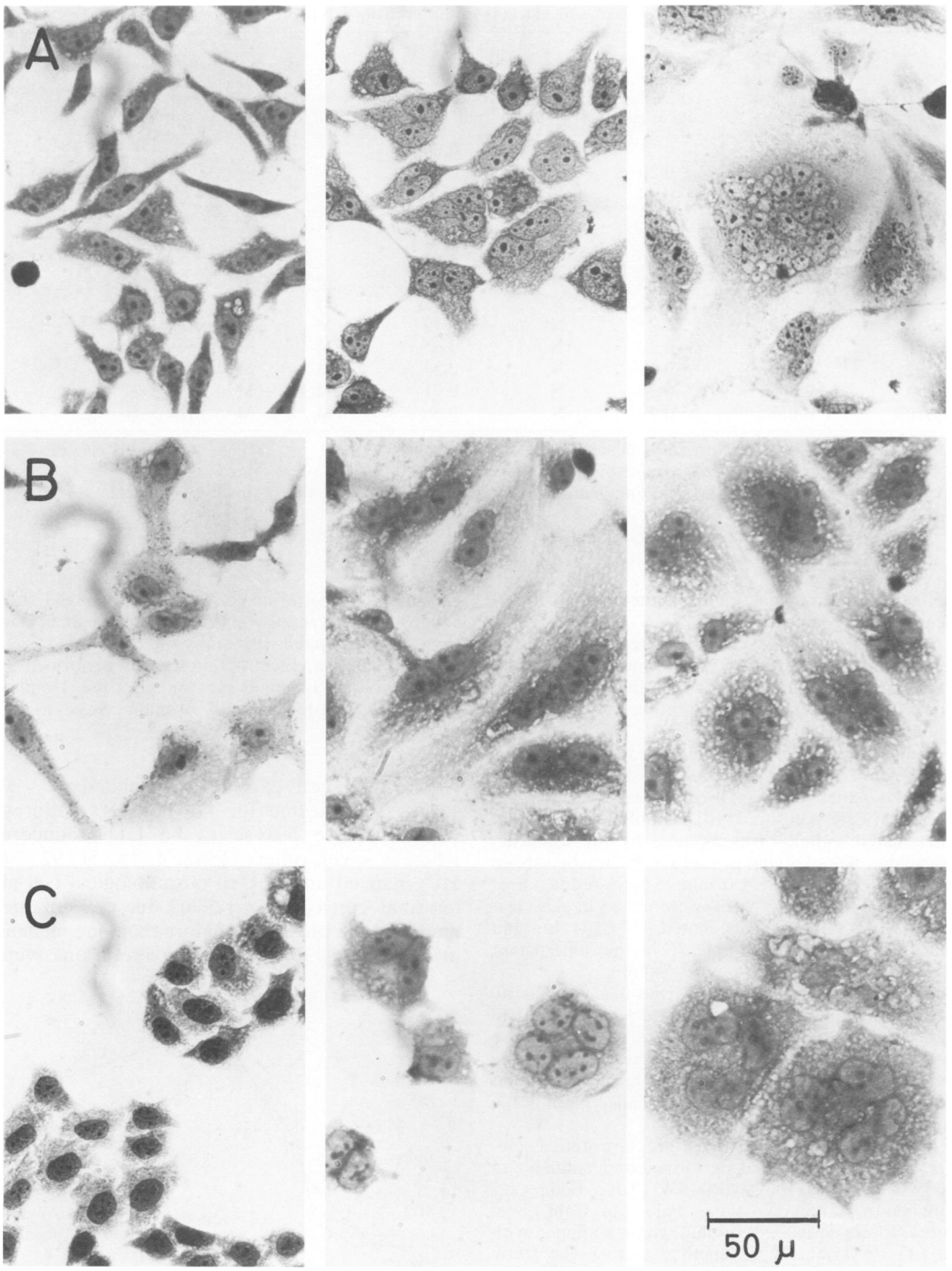

FIG. 1. Toxin effect on monolayers of CHO (A), Vero (B), and HeLa (C) cells. From left to right, cells treated with: K-12 J53 cell extract, 18 h; ISS51 cell extract, 18 h; ISS51 cell extract, 3 days.

lic of Germany). To quantify toxin effects in HeLa cultures, 20 microscopic fields (×400) for each sample were examined; viable cells (by trypan blue exclusion) and cells containing one, two, or more nuclei were counted. Data were expressed as cells per square millimeter. Adenylate cyclase stimulation in CHO cell cultures was evaluated as described by Guerrant et al. (10); cAMP was determined by the protein binding

TABLE 1. Biological effects of cell extracts obtained from CNF-producing *E. coli* strains[a]

Strain	Test results							
	Infant mouse (intestine wt/ body wt)	Ileal loop (ml of fluid/ cm of gut)	Skin test[b]	cAMP increase (mean level/ control level)	Cell culture[c]			Hemolysin production
					CHO	Vero	HeLa	
Control[d]	0.061	0.15	–	0.90	–	–	–	–
K-12 J53	0.060	0.20	–	0.48	–	–	–	–
ISS132	0.140	0.40	–	1.08	–	–	–	–
E9638	0.058	1.43	PF	6.08	E	E	–	–
E1106/0	0.061	1.11	–	0.82	–	K	–	–
K-12 J62	0.064	0.16	–	0.66	–	–	–	+
ISS2	0.064	0.19	N	0.71	M	M	M + K	+
ISS4	0.060	0.22	N	0.48	M	M	M + K	+
ISS18	0.058	0.25	N	0.39	M	M	M + K	+
ISS51	0.065	0.22	N	0.65	M	M	M + K	+
ISS92	0.070	0.19	N	0.54	M	M	M + K	+
ISS94	0.061	0.28	N	0.91	M	M	M + K	+
ISS107	0.057	0.19	N	0.86	M	M	M + K	+

[a] Experiments were performed as described in the text.
[b] –, No effect; PF, vascular response; N, necrosis.
[c] –, No effect; K, killing; M, multinucleation; E, typical morphological alterations by LT (elongation in CHO cells, enlargement in Vero cells).
[d] Uninoculated Trypticase soy broth.

method with a reagent kit from the Radiochemical Centre (Amersham Corp.).

Serial dilutions of each sample were tested on HeLa cell cultures to follow every step of purification. Data were expressed as toxic units per milliliter, determined from the reciprocal of the last dilution showing detectable morphological alteration.

Hemolysis test. Two different procedures were used. (i) *E. coli* strains were tested on 5% sheep blood agar plates, and after overnight incubation (37°C), hemolysis halos were measured. (ii) Hemolytic activity on fractionated samples was assayed by the following procedure. Samples (100 μl each) of 5% washed sheep erythrocytes and of the test sample (adjusted to a final concentration of 0.9% NaCl) were mixed in glass test tubes (3 by 30 mm), incubated (37°C, 4 h), and centrifuged. Hemoglobin release in the supernatant was evaluated by absorbance at 540 nm.

Animal assays. To reveal LT, the ileal loop assay and skin test in rabbits were carried out as described by Evans and co-workers (7, 8). The suckling mouse test for heat-stable enterotoxin was performed by the method of Dean et al. (5). Necrotic reaction in rabbit skin was evaluated by inspecting the ulceration in the injection site.

Fractionation procedure. High-pressure steric exclusion chromatography was performed on a column (7.5 by 500 mm) of TSK G4000 SW (Toyo Soda Co., supplied by Varian Associates, Palo Alto, Calif.) on a Perkin Elmer series 2/1 chromatograph equipped with an LC 75 variable wavelength detector. The steric exclusion column was calibrated with the following proteins as standards: bovine serum albumin (monomer and dimer), egg albumin, chymotrypsinogen, myoglobin (from horse hearts). A standard curve was fitted with a least-squares computer program.

Detection methods. Protein content was followed by absorbance at 280 nm and by a microbiuret method (16). Amino acid analyses of hydrolyzed samples (24 h in constant boiling 6 N HCl at 110°C under reduced pressure) were performed on a Carlo Erba model 3A29 amino acid analyzer (Carlo Erba Strumentazione, Corsico, Italy) equipped with ninhydrin detection.

Electrophoresis. Electrophoresis was performed by the method of Laemmli (15) on an LKB Uniphor apparatus (LKB Instruments, Bromma, Sweden).

RESULTS

Sonicated cell extracts of isolated *E. coli* strains were tested for LT in CHO cultures. Four of these proved to be LT producers, whereas seven others (ISS2, 4, 18, 51, 92, 94, 107) induced unexpected morphological cell alterations consisting of remarkable cell enlargement and the presence of more than one nucleus in each cell (Fig. 1). Similar alterations were

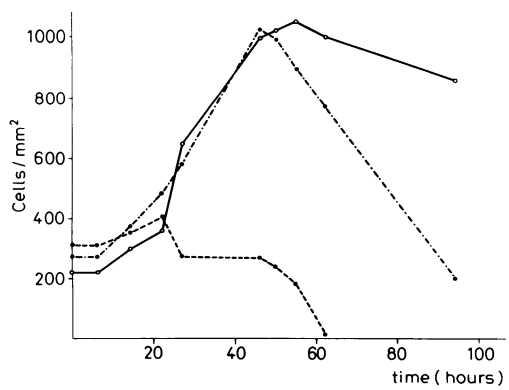

FIG. 2. Survival of HeLa cells with time. Cells treated with ISS51 cell extract (– – –), K-12 J53 cell extract (– · – · –), and medium control (——). Experiment was performed as described in the text.

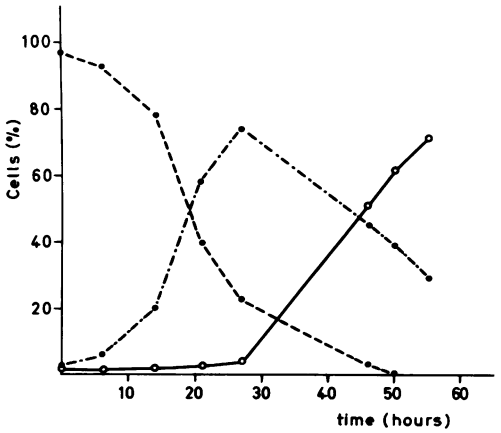

FIG. 3. Relative frequencies of HeLa cells with one (– – –), two (– · – · –), and more (——) nuclei, by time of exposure with ISS51 cell extract. Percentages of mononucleated cells in control monolayers treated with K-12 J53 cell extract were constantly about 97%.

observed when cultures of the same strains were treated with mitomycin C, thus inducing release of the inner cell contents. The occurrence of cell lysing was confirmed by a 50% decrease in turbidity, as determined by Klett readings, and by a drop of >90% in viability. Conversely, no effect was detected when CHO monolayers were inoculated with the supernatants of untreated cultures of the toxic strains. All 7 toxic strains were found to be positive when tested for hemolytic activity, whereas only 20 hemolytic strains were found among the remaining 206.

To characterize the seven toxic strains, their cell extracts were compared with extracts from control strains in a set of biological assays (Table 1). Cell extracts did not elicit fluid accumulation in rabbit ileal loops (the same result was obtained by inoculating about 10^{10} living bacteria per loop), nor did they stimulate adenylate cyclase in CHO cultures. In addition, anticholera hyperimmune serum failed to neutralize the cytotoxic activity. These results as a whole suggest the presence of a specific factor other than LT. Furthermore, all seven *E. coli* strains were negative in the infant mouse assay for heat-stable enterotoxin.

Cell extracts were also able to produce morphological alterations in Vero and HeLa cell cultures within 18 h, and the formation of giant multinucleated cells was observed after a few days (Fig. 1). HeLa cells died within 60 h of incubation unless the medium was changed daily. CHO and Vero cells appeared to be more resistant, their viability being unaffected over 5 days. Medium substitution 6 h after inoculation did not influence the outcome of morphological changes in any of the cell lines tested.

Multinucleation was observed at low cell densities (less than 5,000 cells per cm^2) as well as in confluent monolayers.

Because the *E. coli* strains tested were more toxic for HeLa cells, this cell line was used for the quantitative evaluation of the alterations induced by the cytotoxic factor. Monolayers were treated with bacterial extracts and stained at different times as described above. Determinations were made of cell viability (Fig. 2) and the relative frequencies of cells with one, two, or more nuclei (Fig. 3).

Intradermal injection of cell extracts in rabbits caused an induration area with a necrotic center up to a dilution of 1:20 (Fig. 4). Subcutaneous inoculation in the abdomens of guinea pigs elicited a large necrotic reaction; slightly more than 50% of the animals died within 48 h and showed a diffuse hemorrhagic pattern during autopsy.

Heating at 75°C for 15 min or at 60°C for 1 h destroyed the toxic activity of the sample, which was found to be stable at pH values ranging from 6 to 10.5. Since the above-mentioned observations led us to suppose that toxic activity was carried by proteinaceous material, further experiments were carried out to obtain evidence on this point.

Samples of bacterial culture (20 ml) were centrifuged (20 min at 4°C, 1,500 × g), suspended in 5 ml of 25 mM Tris-hydrochloride buffer (pH 7.2), spun down again, suspended in 1.5 ml

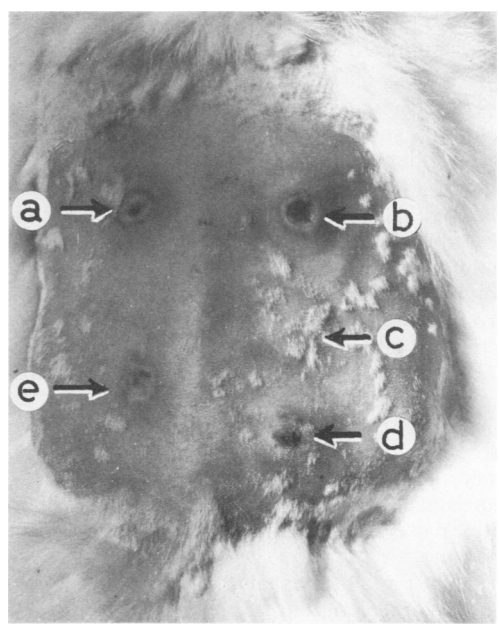

FIG. 4. Local reactions in rabbit 40 h after intradermal injection (0.1 ml) of cell extracts obtained from ISS51 (a), ISS4 (b), K-12 J53 (c), ISS18 (d), and ISS2 (e).

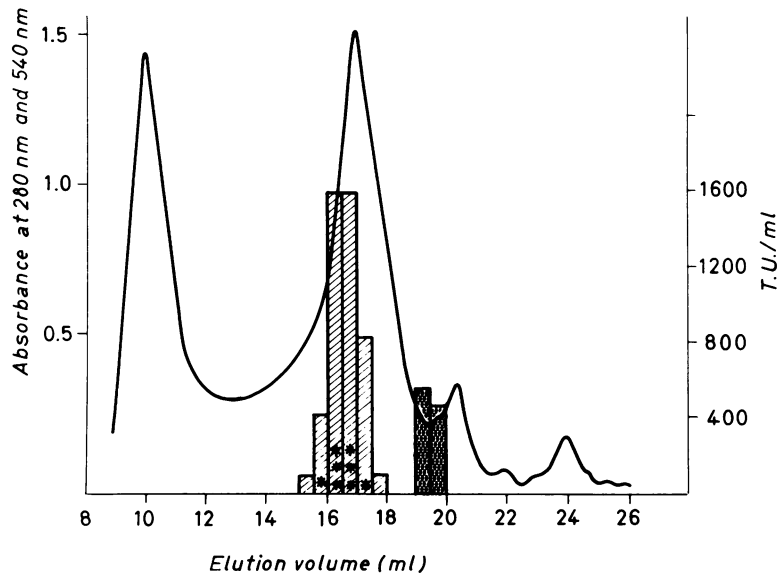

FIG. 5. Chromatogram from TSK G4000 SW column (7.5 by 500 mm). The column, equilibrated in 25 mM Tris-hydrochloride (pH 7.2) and loaded with 175 μl of filtered supernatant (45,000 × *g*), was eluted at 1 ml min^{-1}. The solid line represents absorbance at 280 nm; hatched bars represent toxic activity on HeLa cells; dark bars represent hemolytic activity measured as absorbance at 540 nm; the number of asterisks is proportional to necrotic activity in the skin test assay. TU, Toxic units.

of Tris buffer, and sonicated as described above. Sonicated cells were finally centrifuged (20 min at 4°C, 45,000 × *g*), and the supernatant, filtered through a 45-μm Millipore MF filter, was immediately applied to a TSK G4000 SW column (7.5 by 500 mm). The column, equilibrated in the same buffer, was eluted at 1 ml/min, and the effluent was monitored at 280 nm.

The chromatogram obtained from the G4000 column is shown in Fig. 5 together with the toxic activity determined by HeLa cell and skin test assays. Furthermore, the hemolytic activity of the TSK G4000 fractions was also determined as described above. Figure 6 shows the same chromatogram as Fig. 5 together with the carbohydrate and protein contents of each fraction. From the results shown, it is evident that toxic activity is eluted by the G4000 column from 15 to 17 ml, approximately 75% of the total toxic activity being localized at 16 ml, whereas according to the biuret assay, only 22% of the total protein is localized in fraction 16. On the contrary, hemolytic activity (Fig. 5, dark bars) is eluted at 19 ml. Figure 7 shows the electrophoretic pattern of the fractions from the TSK column.

From Fig. 6, it is also clear that the total carbohydrate content of the original sample is quite low, and no more than 25 μg of equivalent hexose is associated with each fraction, whereas the protein content is considerable. Indeed, the maximum protein concentration is associated with the second main peak in Fig. 5 and 6. Toxic activity is also associated with the same peak. This datum makes it very likely that the toxic activity is due to material of a proteinaceous nature. To confirm this hypothesis, amino acid analyses of fractions 14 through 18 were run as described above. It is clear from these analyses that amino acids are associated with all of the

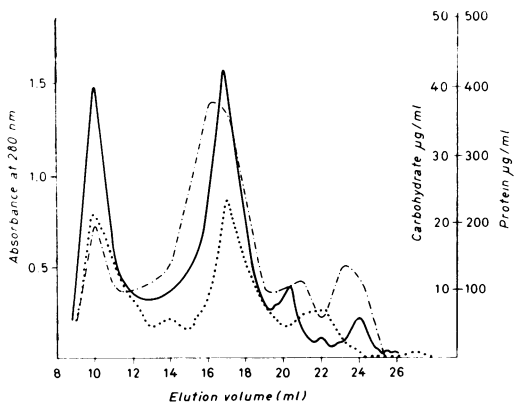

FIG. 6. Chromatogram from the TSK G4000 column. Chromatographic conditions were the same as for the experiments shown in Fig. 5. Symbols: ———, absorbance at 280 nm; – · – · – · – protein as determined by biuret assay; · · · · · ·, carbohydrate (as hexose) in micrograms per milliliter determined by the method of Dubois et al. (6).

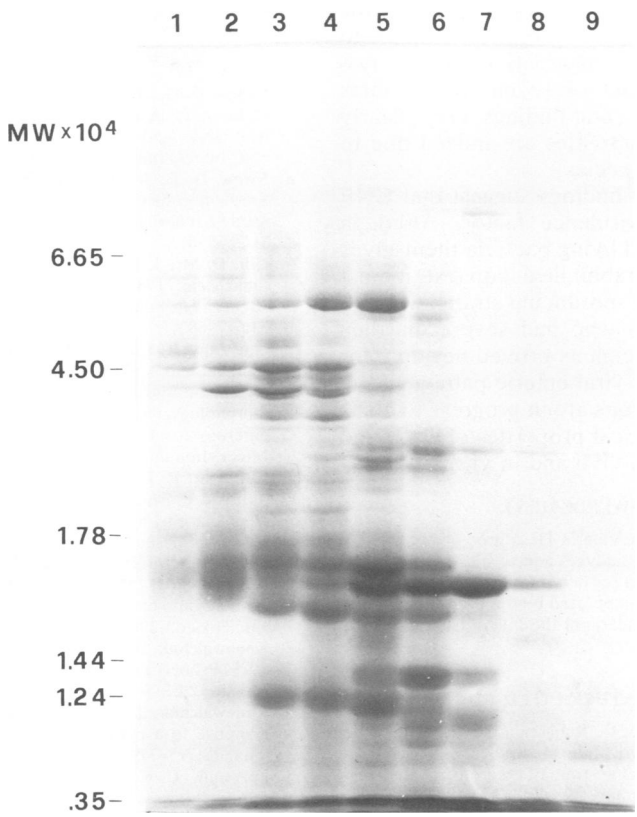

FIG. 7. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, performed on 0.6-ml fractions from the TSK G4000 SW column. Fractions corresponding to effluent at 15 to 17 ml (wells 1 through 4) are cytotoxic for HeLa cells; fractions corresponding to 19 ml (wells 7 and 8) are hemolytic. Inactive fractions, corresponding to 18 and 20 ml, are in wells 5, 6, and 9.

assayed fractions, and 4.5 nM amino acid is present in fraction 16, confirming again the proteinaceous nature of the material to which toxic activity is due.

The TSK G4000 column was calibrated as described above. The resulting calibration curve regression coefficient was 0.98, and in the calibration curve (data not shown) the elution volume of the main toxic fraction (fraction 16) corresponds to the elution volume of a globular protein with a molecular weight of approximately 76,000.

DISCUSSION

The most relevant biological properties of this *E. coli* toxin, termed CNF, can be summarized as follows. (i) It appears to be cytotoxic for HeLa cells. (ii) Its action on CHO and Vero cells distinguishes it from Vero toxin and LT. (iii) It causes the formation of large multinucleated cells when tested in cell cultures; this effect is more remarkable in HeLa and CHO cells than in Vero cells. Vero and CHO cells remained viable just as long as untreated controls did, whereas HeLa cultures died within 60 h after inoculation. (iv) There is good evidence that the toxin is of a proteinaceous nature.

The outcome of morphological alterations is not influenced by the density of the toxin-treated monolayers; such a result makes the occurrence of a cell fusion process unlikely, thus supporting the hypothesis of an inhibition of cell division.

In vivo experiments show that CNF causes not only a local necrotic reaction but also systemic damage consisting of widespread hemorrhage. No target organ was evidenced, and the observed alterations appear rather to be the result of a diffuse vascular involvement.

The data also tend strongly to support the hypothesis that toxic activity is associated with material of a proteinaceous nature, and specifically with a protein with a molecular weight of about 70,000 to 80,000. Furthermore, hemolytic activity is consistently separated from toxic activity and is related to material with a much lower apparent molecular weight. The low frequency of hemolytic strains among *E. coli* isolat-

ed from stool specimens (12), confirmed in the present study, makes it extremely difficult to hypothesize a casual association of the two activities in seven out of seven toxic strains examined. However, our findings very clearly show that the two activities are indeed due to different molecular species.

In conclusion, our findings suggest that CNF plays the role of virulence factor. Although bacterial extracts and living bacteria themselves were negative in the rabbit ileal loop test, it must be stressed that CNF-producing strains were all derived from infants who had severe diarrhea and whose stool specimens proved negative for all other bacterial or viral enteric pathogens.

Further investigations are in progress to characterize the biochemical properties of CNF and its mode of action in vivo and in vitro.

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