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A CELL DIVISION-ACTIVE PROTEIN FROM E. COLI

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A purification procedure for a protein obtained from an pathogenic strain of E. coli is described. The protein - called CNF - is active in inhibiting the duplication of cultured mammalian cells. Since nuclei division is apparently normal, treatment of cultured cells with CNF leads to the formation of gigantic, polynucleated cells. The purified protein is chromatographically and electrophoretically homogeneous. A partial characterization of CNF protein is also given.

We recently described a new toxic factor - termed CNF - produced by several E. coli strains isolated from enteritis-affected children, and we suggested its role as a virulence factor (1).

The examination of mammalian cultured cells used for enterotoxin detection (2) eventually led us to discover that their exposure to soluble lysates obtained from these strains causes striking morphological alterations in the treated cell lines. Such effects are apparently due to the suppression of the separation of the daughter cell, which follows an otherwise normal nuclear division (unpublished results). After approximately 48 hours in the presence of bacterial lysates, a great majority of the treated cells (about 98%) becomes polynucleated and considerably larger (an average of 4 or 5 times in mean diameter) than the controls.

The raw material was shown to exhibit the same kind of activity in different lines of cultured cells. Indeed we found the same activity in each of the six cell lines tested (HeLa, Vero, CHO, MA-104, Mc Coy, EHEp-2).

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Subsequently, evidence obtained indicated that CNF activity was due to a protein (1). Thus, we undertook a research program aimed at purifying the substance active in cell division and also to clarify its mode of action. The following results show the purification procedure such as it was developed in recent months and reports preliminary data on the characterization of the material thus purified.

MATERIAL AND METHODS

Bacterial growth. E. coli strain ISS 51 (1) was maintained in Dorset egg medium (Oxoid Ltd, Basingstoke, Hampshire, UK) at room temperature. For bacterial production, 500 ml of trypticase soy broth (BBL Microbiological Systems, Cockeysville, MD, USA) in a 2000 ml Erlenmeyer flask were inoculated with approximately 10^5 cells from exponentially growing cultures, and then incubated 18 hours at 37°C in a rotatory shaker.

Harvesting and ammonium sulphate fractioning. Bacterial cells from 500 ml of culture were harvested by centrifugation (15 minutes at 15,000 x g), washed twice, resuspended in 20 ml of 25 mM tris-HCl pH 7.2 and disrupted by sonication in ice bath. Cell extracts were centrifuged at 20,000 x g for 60 minutes and the supernatant was filtered through a 0.45 µ MF membrane (Millipore Co. Bedford, MA, USA). The soluble lysate was then fractioned with $(NH_4)_2SO_4$ between 0 and 45% of saturation. After 30 minutes of stirring in an ice bath, the ammonium sulphate suspension was centrifuged for 30 minutes at 20,000 x g, the pellet was resuspended at 0.5 g/ml in 25 mM tris-HCl pH 7.2, centrifuged again (30 minutes at 45,000 x g) and finally filtered through a 0.22 µ MF membrane.

Cell assays. Hela cells, obtained from the American Type Culture Collection (Rockville, MD, USA), were cultured as described elsewhere (1). Assays were carried out in microtiter plates (Falcon Plastics, Oxnard, CA, USA) seeded with approximately $5x10^3$ HeLa (3) cells per well. Each well was inoculated with 20 µl of serial dilutions of the sample under test. After 48 hours of incubation (37°C at 5% CO_2), monolayers were washed with saline, stained with 10% Giemsa, washed twice with water and read at 200X with an inverted microscope. Data were expressed as activity units/ml. These were determined as the reciprocal of the last dilution causing multinucleation in at least 50% of the cells.

Ultrafiltration. The many steps necessary for concentration were performed by using Pellicon PCAC and PTGC membranes (Millipore Co., Bedford, MA, USA) in Millipore stirred cells.

Chromatography. Steric exclusion chromatography was performed with a 42.0x600 or a 7.6x600 mm TSK G3000SW and a 7.6x500 mm TSK G4000 column (Toyo Soda Ldt., Tokyo, Japan) eluted at room temperature at a flow rate of 2.2 ml/cm^2/minute. Steric exclusion in denaturing conditions was performed at 25°C on the G4000 column eluted at 0.5 ml/cm^2/minute with 6 M guanidine-HCl in 50 mM tris-HCl pH 7.0. The column was calibrated (4,5) by using an LKB standard (LKB Instruments, Bromma, Sweden) plus the following proteins: bovine serum albumine monomer and dymer; egg albumin; chymotrypsinogen; horse heart myoglobin; lysozyme. The standard curve was interpolated with a linear least

squares procedure. Ion exchange chromatography was performed at room temperature using a 7.6x150 mm DEAE-TSK silica-based ion exchange column (Toyo-Soda) developed at pH 6.8 with a tris-HCl concentration gradient.

Polyacrylamide electrophoresis was performed by means of a commercially available apparatus with minor modifications of the method of Laemmly (6) in the presence of sodium dodecylsulphate (SDS) and with or without 8 M urea.

Amino acid analyses were performed in triplicate on acid-hydrolyzed material (24 hours at 110°C in 6 N HCl under low pressure) using a Carlo Erba 3A29 amino acid analyzer (Carlo Erba Strumentazione, Corsico, Italy). Tryptophane was determined according to Edelhoch (7), cystine was converted into cysteic acid (8) and determined by amino acid analysis.

Material. HCl for hydrolyses was Merck Suprapur grade (E. Merck, Darmstadt, DBR). All other chemicals were of reagent grade and used without further purification.

RESULTS

1. Chromatography. The resuspended ammonium sulphate pellet was first applied to a 42.0x600 mm TSK G3000 column equilibrated in 150 mM tris-HCl pH 7.2 and eluted at 0.6 ml/cm^2/minute. The resulting chromatogram is shown in figure 1.

The following step in the purification process was ion exchange on a DEAE-TSK column. The fractions indicated by the shaded area in figure 2 were pooled, concentrated, dialyzed against 150 mM tris-HCl pH 6.8 and applied to the DEAE column developed at 1 ml min^{-1} with the tris-HCl gradient indicated in figure 2, panel a. Under these conditions, active material was eluted at a

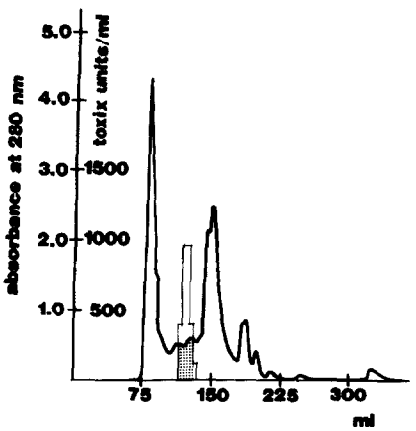

FIGURE 1: Steric exclusion chromatography of the ammonium sulphate-fractionned material. 3.8 ml of the resuspended pellet were applied to a 42.0 x 600 mm TSK G3000 column eluted as described in the text. Solid line represents absorbance at 280 nm. Histogram bars represent activity on HeLa cells; shaded area represents pool made.

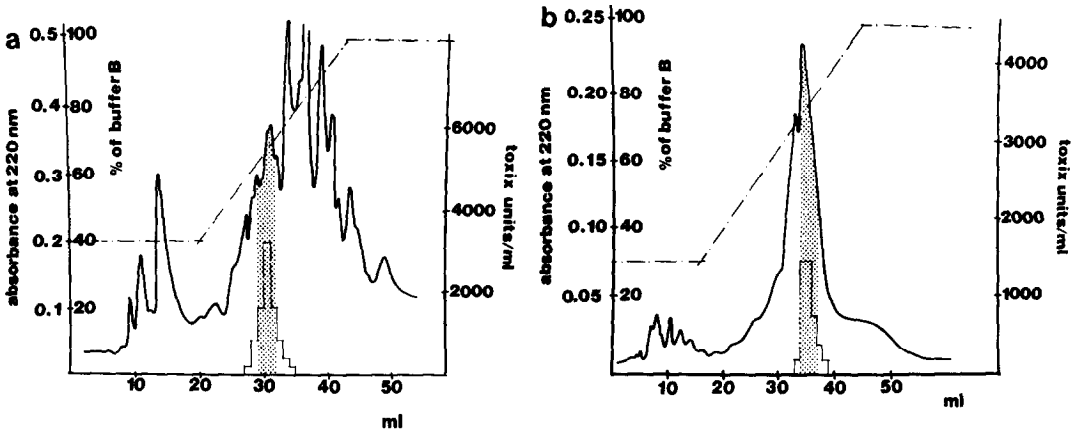

FIGURE 2: Panel a: ion exchange chromatography of active fractions from figure 1. Active material was pooled, concentrated, applied to a 7.6x150 DEAE-TSK column and eluted with the gradient indicated. Panel b: ion exchange chromatography of active fractions from the column in panel a. Active fractions were pooled, concentrated and rechromatographed on the same ion-exchange column with a slightly different gradient. Both panels: solid line represents absorbance at 280 nm; dash-dotted line represents tris-HCl concentration; histogram bars represent activity on HeLa cells; shaded area indicates pools made.

nominal 0.25 molar tris concentration. The active material was then pooled, dialyzed against 50 volumes of 100 mM tris-HCl pH 6.8, concentrated and rechromatographed on the same column with a slightly different gradient, as shown in figure 2, panel b.

At this stage the active material - revealed by SDS electrophoresis - was not yet homogeneous. It was therefore concentrated again and applied to two TSK columns connected in series (a 7.6x500 mm G4000 followed by a 7.6x600 mm G3000) and eluted with 25 mM tris-HCl pH 7.2. Under these conditions the chromatogram shown in figure 3 was obtained.

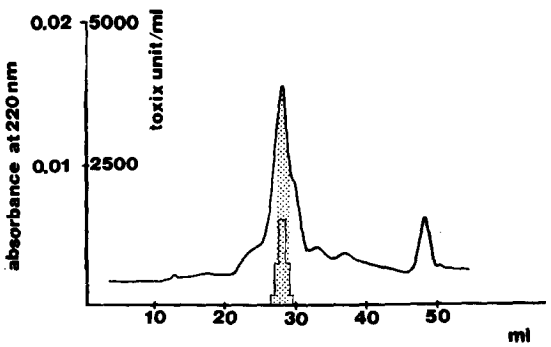

FIGURE 3. Steric exclusion chromatography of material from figure 2b. Active fractions were pooled, concentrated and applied to a 7.6 x 500 mm TSK G4000 and a 7.6x600 mm TSK G3000 connected in series. Solid line represents absorbance at 280 nm; shaded area indicates pool analyzed by electrophoresys.

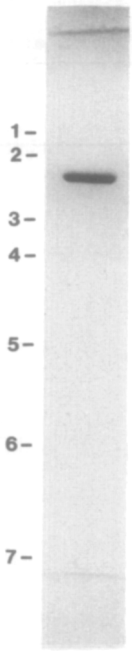

FIGURE 4. Polyacrylamide gel electrophoresys of purified CNF. Gels were made 10% in acrylamide and run in the presence of 1% SDS. The following proteins were used as standard: 1. RNA polymerase; 2. Bovine serum albumin dimer; 3. RNA polymerase; 4. Bovine serum albumin monomer; 5: Ovalbumin; 6. RNA polmymerase; 7. Soy bean trypsin inhibitor.

2. Purity assessment. The active material, as obtained from the final purification step, was analyzed by polyacrylamide gel electrophoresis in the presence of SDS, and found to migrate as a single coomassie blue-stained band (figure 4) with faint traces of lower and higher molecular weight material. Upon densitometric scanning, the main band was estimated to account for more than 90% of the total material. Similar results were obtained on acrylamide gel electrophoresis performed in the presence of 8 M urea (not shown).

3. Amino acid composition. Amino acid analyses of purified material were performed as described under "Methods". The amino acid composition of CNF protein is reported in table I.

4. Molecular weight. Because of the very limited quantity of purified material available, molecular weight was estimated by SDS electrophoresis and on the basis of its hydrodynamic properties in steric exclusion chromatography under denaturing conditions.

SDS electrophoresis was standardized with proteins of known molecular weight and the standard curve interpolated with a least squares procedure ($r=0.991$). On the basis of its electrophoretic motility, an apparent molecular weight of 115,000 Daltons was calculated for the CNF band.

Table I. CNF amino acid composition

Amino acid	Moles %
Cystine½[a]	3.0
Aspartic acid	9.0
Threonine	4.3
Serine	7.1
Glutamic acid	12.2
Proline	2.1
Glycine	13.3
Alanine	9.2
Valine	5.4
Methionine	n.d.[c]
Isoleucine	4.7
Leucine	7.7
Tyrosine	3.0
Phenylalanine	4.0
Histidine	4.4
Lysine	6.2
Arginine	4.2
Tryptophan[b]	0.2

[a]Determined as performic acid (8)
[b]Determined spectrophotometrically(7)
[c]n.d., not detectable

High pressure steric exclusion chromatography in 6 M guanidine hydrochloride was carried out as described in the experimental section. From the standard curve (r=0.998), an apparent molecular weight of 109,000 Daltons was calculated for the elution volume of the active material. Such a value tallies very well with the estimate based on electrophoretic data.

DISCUSSION

The purification procedure outlined in the previous sections allows one to obtain the material active in cell division in a state of considerable purity. However, the purification procedure - such as it has been developed - is not devoid of limits since it is made rather cumbersome by the high number of chomatographic steps required. Yet, the complexity of the procedure seems to be due - at least partially - to the considerable heterogeneity of the

original material. Finally, the absolute amount of protein obtainable by the reported procedure is quite low, in the order of 1×10^{-4} g per preparation. We are now working to improve the purification procedure in order to circumvent these difficulties.

Summing up, the reported evidence shows that a protein, contained within the cell bodies of several E. coli strains, interferes with the process of mammalian cell duplication. Although several other substances - mainly active on spindle assembly - are already known to interfere with the processes leading to cell duplication, e.g. cytochalasine B (9), colchicine (10), nocodazole (11), caffeine (12), none is of a proteinaceous nature. Indeed, no protein known so far is active in cell duplication.

Although our knowledge of CNF activity is still very preliminary, the role of this protein in the bacterial organism will hopefully be ascertained in due time. It is also possible that CNF may become instrumental in studyng the even more fascinating subject of the physiological mechanisms of eukariotic cell division.

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