Purification and physicochemical characterization of a human cytotoxic factor produced by a human haemic cell line

Hava NEUMANN and Abraham KARPAS

Department of Haematological Medicine, University of Cambridge Clinical School, Hills Road, Cambridge CB2 2QL, U.K.

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A cytotoxic factor, produced by a human lymphoblastoid cell line [Karpas (1977) Br. J. Cancer 35, 152–160; Karpas (1977) Br. J. Cancer 36, 437–445], was purified both from the cell extracts and from the culture medium containing the cell lysate, by using ammonium sulphate precipitation, DEAE-cellulose chromatography, gel filtration and affinity chromatography on concanavalin A–Sepharose and on [3H]aminoethanol–glass beads. Two factors, Factor I and Factor II, were separated by DEAE-cellulose chromatography. Factor I was eluted from this column at 30 mM-aminoethanol/HCl buffer, pH 8.0, whereas Factor II was bound strongly to DEAE-cellulose and was eluted only at 325 mM-aminoethanol/HCl buffer, pH 8.0. The purified Factor I migrated as a single band on polyacrylamide-gel electrophoresis. Its isoelectric point, pI, was 8.0 ± 0.3. Its sedimentation coefficient, $s_{20,w}$, was 3.5 ± 0.1 S and its apparent molecular weight, $M_r$, was 65000 ± 1000 as determined by sedimentation-velocity and sedimentation-equilibrium measurements. A linear relationship between molecular weight and concentration was found in equilibrium runs, suggesting a non-spherical shape of the molecule. Factor I is not a glycoprotein, inasmuch as it does not bind to concanavalin A–Sepharose. It consists of two subunits ($M_r$, 32000 ± 4000), migrating on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as a single band. Factor II had pI 6.0 ± 0.4 and $M_r$, 75000 ± 3000. Factors I and II are thus different proteins.

The properties of a unique human haemic cell line, established from a patient with non-Hodgkin's lymphoma who became leukaemic a week before his death, have been described previously (Karpas, 1977a). The cell line (Karpas 160) was established from the leukaemic (105 × 10⁹/litre) white blood cells obtained from the peripheral blood 1 day before the patient's death. Although the leukaemic cells were thymus-derived (T-cells), had C3 receptors and were negative for Epstein–Barr virus, the cultured cells were Epstein–Barr-virus-positive, had surface-membrane immunoglobulin and also synthesized and secreted immunoglobulin M ($\lambda$-chains). It was therefore assumed that this cell line did not represent a proliferation of the patient's malignant cells, but rather represented a proliferation of a subpopulation of lymphoid B-cells that were 'immortalized' by Epstein–Barr virus. The most striking property of this cell line was its ability to kill, in mixed culture, a wide range of human and animal cell lines even at a ratio of 1:1. Preliminary studies suggested that malignant human cells were more susceptible than normal human cells. It was also established that the cell killing was mediated by a humoral cytotoxic factor that was synthesized by the cells, and that the factor could be found intra- and extra-cellularly and was released from the cells by an apocrine form of secretion. This factor could be precipitated and concentrated by 30–60% (w/w) (NH₄)₂SO₄. When the cytotoxic factor was injected into fibrosarcoma-bearing mice, approx. 50% survived, whereas all control animals died (Karpas, 1977b). This cell line might represent a proliferation in vitro of a population of cells that are involved in cell-mediated anti-tumour immunity in vivo. Therefore we have undertaken the purification and characterization of this soluble cytotoxic factor.

Materials and methods

Materials

DEAE-cellulose (DE-52) and CM-cellulose (CMC-32) were purchased from Whatman (Maid-
stone, Kent, U.K.); Sephadex G-25, G-100 and G-200, Sepharose 4B, DEAE-Sepharose and CM-Sepharose were from Pharmacia Fine Chemicals (Uppsala, Sweden); concanavalin A-Sepharose was from Miles-Yeda, Rehovot, Israel; acrylamide (gel-electrophoresis grade), ethanolamine, \((\text{NH}_4)_2\text{SO}_4\), EDTA (disodium salt), ammonium persulphate, acrylamide (specially purified for electrophoresis), \(\text{NN}'\text{-methylenebisacrylamide}\) and 2-aminooethanol were from BDH Chemicals (Poole, Dorset, U.K.); dithiothreitol (Cleland’s reagent), guanidinium chloride (crystalline), urea and Tris were from Bethesda Research Laboratories, Bethesda, MD, U.S.A.; \(\text{NNN}'\text{N}'\text{-tetramethylethylenediamine} \) with 5-brominated groups was purchased from Koch-Light Laboratories (Colnbrook, Bucks., U.K.); Trizma base was from Sigma Chemical Co. (Poole, Dorset, U.K.); \([^{14}\text{C}]\)methylated protein mixture, \([^{125}\text{I}]\)iodide and \([^{3}\text{H}]\)aminoethanol were from The Radiochemical Centre (Amersham, Bucks., U.K.), and Aquacide III was from Calbiochem (San Diego, CA, U.S.A.). Other materials used were of analytical grade purchased elsewhere.

**DEAE-cellulose chromatography**

DEAE-cellulose was treated as described by Peterson & Chiazze (1962) and adjusted to pH 7.2 with dilute HCl. The absorbent was then equilibrated with 5 mM-aminoethanol/HCl buffer, pH 8.0. The sample, dialysed against the starting elution buffer, was applied to the column (3 cm x 80 cm). Elution with a stepwise concentration gradient was then performed with equal volumes of starting buffer and the next chosen concentration (50–100 ml of each concentration; the concentration steps were 5–50, 50–100, 100–180, 180–250, 250–500 and 500–800 mM). Recycling was performed in a small column of DEAE-cellulose, usually 10% of the initial column size. A linear concentration gradient of buffer was used to elute the purified protein in a conveniently small volume from the second column.

**Preparations of aminoethanol–glass affinity-chromatography materials**

\([^{3}\text{H}]\)Aminoethanol was coupled to activated glass beads to monitor the amounts of covalently bound \([^{3}\text{H}]\)aminoethanol. Activated glass beads were prepared by the procedure of Weetall (1969).

**Iodination of the partially purified killer Factor I**

The fraction possessing the biological activity after partial purification was labelled with \(^{125}\text{I}\) (10 mCi of carrier-free iodine) in 0.10 M-sodium phosphate buffer, pH 7.2, at 4°C. The unreacted iodine was removed by gel filtration on Sephadex G-25. The labelled proteins, containing Factor I, had a specific radioactivity of approx. \(6 \times 10^6\) c.p.m./mg of protein. The fraction was frozen at –15°C and used as a marker in purification procedures.

**Molecular-weight determinations**

(a) **Sedimentation analysis.** Boundary-sedimentation, including sedimentation-equilibrium, studies were performed in a Beckman model E ultracentrifuge equipped with u.v.-absorption optics, photoelectrical scanning system and schlieren optical system as described by Chervenka (1969) and Yphantis (1960). Protein solutions for ultracentrifugal analysis were dialysed at 4°C against 0.1 M-sodium phosphate buffer, pH 7.2, or against the same buffer containing 4 M-guanidinium chloride for 24 h. All samples were sedimented at 56 000 rev./min for 18 h. Equilibrium sedimentation was performed at 18 000 rev./min or, at concentrations below 0.15 mg of protein/ml, at 24 000 rev./min for 18 h. All runs were performed at 20°C.

(b) **Sephadex G-200 and Sepharose-4B gel filtration.** Two sets of marker proteins were used: (1) \([^{14}\text{C}]\)methylated protein mixture; (2) calibration mixture composed of Blue Dextran (M, 20 000 000), fructose bisphosphate aldolase (M, 125 000), alkaline phosphatase (Escherichia coli) (M, 80 000), bovine serum albumin (M, 67 000), egg albumin (M, 46 000), pepsinogen (M, 40 800), pepsin (M, 35 000), soya-bean trypsin inhibitor (M, 21 500) and cytochrome c (M, 11 500). The eluates were monitored at 650 nm for Blue Dextran, at 435 nm for cytochrome c and at 278 nm for the other proteins. Enzymic activities of fructose bisphosphate aldolase, alkaline phosphatase and activated pepsinogen were also measured.

**Isoelectric focusing**

Isoelectric focusing of the purified Factor I was performed in an LKB 8101 isoelectric-focusing column (28 ml) with 2% Ampholines of pH 3.5–10.0 at 800 V for 16 h. Fractions of volume 1.0 ml were collected. Protein content and pH were measured in each fraction.

**Polyacrylamide-gel electrophoresis**

Electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulphate and absence of reducing agent was performed in column and in slab gels (7.5%, pH 9.0) as described by Laemmli (1970). The gels were stained with Coomassie Blue R-250.

**Radioactivity measurements**

The radioactivity of \(^3\text{H}\)-labelled compounds was measured in toluene/Triton scintillation liquid by using a Beckman model LS200 liquid-scintillation counter, and \(^{125}\text{I}\) radioactivity by using a Wilson γ-radiation counter.
Protein concentration

Protein concentrations were measured routinely at 278 nm, the absorption coefficients $A_{1%}^{1cm}$ being taken to be 10 ± 0.5. Calibration values were determined for the most purified material by the method of Lowry et al. (1951), with bovine serum albumin as standard.

Reduction and carboxymethylation of Factor I

A 2 mg sample of the material obtained after several steps of purification was reduced and carboxymethylated by the procedure of Craven et al. (1965). The carboxymethylated derivative was dialysed against 0.1 M-sodium phosphate buffer, pH 7.2, containing 4.0 M-guanidinium chloride.

Enzymic assays

Partially purified fractions and the purified Factor I were assayed for proteolytic activity with $[^3H]$acetyl-casein (200 Ci/mol) or $[^3H]$acetyl-haemoglobin as substrate (Hatcher et al., 1978), and for alkaline phosphatase activity by the method of Neumann et al. (1967).

Assay of the cytotoxic factor

The concentration of the cytotoxic factor was assayed by the method described in detail previously (Karpas, 1977b). Samples, with known protein concentrations, were dialysed at 4°C for at least 20 h against several changes of 20 ml or larger volume of fresh growth medium (Dulbecco's modified Eagle's medium; Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.), filtered through Millipore filters (0.45 μm pore size). A serial 2-fold dilution was done in fresh growth medium containing 5% (v/v) foetal bovine serum. A 1 ml volume of diluted material was added to each well (Linbro tray FB1G-24TC) containing $10^4$ target cells (human rhabdomyosarcoma line A-204). Samples were assayed in duplicate. The wells with the highest dilution, which contained after 5 days of incubation at 37°C only 50% of the original number of cells, were deemed to contain 1 killer unit, which, when multiplied by the dilution factor, gave the concentration of killer units/ml.

Extraction of the killer factor from the growth medium

The cells were separated from the growth medium by low-speed centrifugation (4000g for 5 min at 4°C). Several extraction methods were tried. The best results were obtained when the cells were resuspended in 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.1% Emulphogene, a non-ionic detergent, and homogenized at 4°C. The homogenates were ultracentrifuged at 30000 rev./min for 30 min at 4°C in an MSE Superspeed 65 ultracentrifuge. The supernatant was used for purification of the killer factor.

Results

Purification of the killer factor from the cells and from the medium containing the cell lysate

Separation and purification of the killer factor were conducted with as starting materials fraction A, the homogenates of the isolated killer cells (10$^{10}$ cells), and fraction B, the medium containing also the cell lysate. The protein was precipitated with (NH$_4$)$_2$SO$_4$ (60%, w/w) at room temperature; the precipitate was separated by ultracentrifugation (30000 rev./min for 30 min at 20°C), dissolved in 5 mM-aminoethanol/HCl buffer, pH 8.0, and dialysed against the same buffer for 48 h at 4°C. In the sample the protein content and killer activity were measured and the specific activity was calculated (killer units/mg of protein) (see Table 1). Preliminary experiments have shown that the protein isolated from the medium alone, which had not been in contact with the cells, with the same method did not express any cytotoxic activity even at a protein concentration of 140 mg/ml.

The material obtained from either fraction A or fraction B, with known amounts of killer activity, was applied on a DEAE-cellulose column (3 cm x 50 cm), and the protein was eluted with stepwise gradient concentrations of buffer. Typical chromatograms of fractions A and B are illustrated in Figs. 1 and 2 respectively. By the above chromatography ten protein peaks could be separated, but killer activity appeared in only two peaks, namely peaks P2 and P9. Peak P2 material was eluted at 15 mM-aminoethanol/HCl and peak P9 material at 325 mM-aminoethanol/HCl buffer concentrations.

The material eluted in peaks P2 and P9 was designated Factor I and Factor II respectively. Killer activities, specific activities and recoveries of the biological activity are given in Table 1 for typical DEAE-cellulose chromatography of material from fractions A and B.

Additional columns

The preparations of Factor I and Factor II, obtained both from the isolated cell homogenate (fraction A) and from the medium containing the cell lysate (fraction B) after DEAE-cellulose column chromatography, were further purified by Sepharose-4B gel filtration, concanavalin A-Sepharose affinity chromatography, recycling on a small DEAE-cellulose column, Sepharose-4B gel filtration and $[^3H]$aminoethanol–glass-bead affinity chromatography. We describe in detail the purification of Factor I obtained after DEAE-cellulose chromatography of the medium containing the cell lysate (fraction B).
Table 1. Purification of Factor I and Factor II

The DEAE-cellulose chromatography, Sepharose-4B gel filtration, concanavalin A-Sepharose affinity-chromatography and aminoethanol-glass-bead affinity-chromatography steps in the purification of the twice-(NH₄)₂SO₄-precipitated killer factor of the cell homogenates (A) and of the medium containing also the cell lysate (B) were performed as described in the text. Biological activities and specific activities of the starting material and of the material recovered for each column separation are given. These data were calculated as described in the Materials and methods section.

<table>
<thead>
<tr>
<th>Type of column</th>
<th>Material</th>
<th>Activity (killer units)</th>
<th>Sp. activity (killer units/mg)</th>
<th>Values for starting material</th>
<th>Values for recovered material</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. DEAE-cellulose</td>
<td>A</td>
<td>1600</td>
<td>7.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. DEAE-cellulose</td>
<td>B</td>
<td>5000</td>
<td>0.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Sepharose 4B</td>
<td>B (Factor I)</td>
<td>1500</td>
<td>46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Concanavalin A--</td>
<td>B (Factor I)</td>
<td>600</td>
<td>153</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. *DEAE-cellulose</td>
<td>B + MR1 prepn.</td>
<td>10000</td>
<td>0.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. *Sepharose 4B</td>
<td>B (Factor I)</td>
<td>4000</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. *Concanavalin A--</td>
<td>B (Factor I)</td>
<td>1500</td>
<td>246</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. *Aminoethanol--glass beads</td>
<td>B (Factor I)</td>
<td>800</td>
<td>750</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. Sepharose 4B</td>
<td>B (Factor II)</td>
<td>1500</td>
<td>20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* A new batch of killer cells including the medium containing also the ¹²⁵I-labelled marker, MR1 preparation.

Fig. 1. DEAE-cellulose chromatography of the isolated killer-cell extract

Twice-(NH₄)₂SO₄-precipitated fraction A, after dialysis against 5 mM-aminoethanol/HCl buffer, pH 8.0, was subjected to chromatography on a DEAE-cellulose column (3 cm x 50 cm) equilibrated with 5 mM-aminoethanol/HCl buffer, pH 8.0. The sample of fraction A contained 500 mg. The column was eluted by the following stepwise gradient concentrations of aminoethanol/HCl buffer, pH 8.0: 5–50 mM (60 ml and 60 ml); 50–250 mM (90 ml and 90 ml); 250–500 mM (40 ml and 40 ml); 500–1000 mM (50 ml and 50 ml). A volume of 3.0 ml was collected every 20 min. The vertical broken lines indicate the fractions pooled. In the pooled fractions the killer activity and protein content (A₂₇₈) were assayed. Only peaks P2 and P9 had killer activity. Peak P2, eluted at 30 mM buffer concentration, contained Factor I, whereas peak P9, eluted at approx. 325 mM buffer concentration, contained Factor II.
Cytotoxic factor from a human haemic cell line

Fig. 2. DEAE-cellulose chromatography of the twice-(NH₄)₂SO₄-precipitated medium containing the cells

A 5 ml volume of the dialysed concentrated substance was applied to a DEAE-cellulose column (3 cm x 50 cm) and eluted from the column; fractions were pooled as indicated by the arrowheads and assayed for biological activity. Only peak P3 had only biological activity. For molecular-weight determinations the column was calibrated before the application of the sample with the designated molecular-weight marker proteins (see the Materials and methods section). The biologically active substance was found to have a molecular weight 63000 ± 3000.

Sepharose-4B gel filtration of the Factor I preparation obtained after DEAE-cellulose column chromatography

Concentrated Factor I preparation (2.0 ml) was applied on a Sepharose-4B column (1.5 cm x 10 cm), which was eluted with 10 mM-aminoethanol/HCl buffer, pH 8.0, at 4°C, 2.0 ml of liquid being collected per 20 min. Four distinct peaks were obtained (see Fig. 3a), of which only fraction P3 had killer activity. Since peak P3 was a broad peak, it was divided into eight subfractions (Fig. 3b), and each subfraction was assayed for biological activity. Subfractions 4, 5 and 6 had such activity, with a symmetrical distribution. About 50% of the initial activity was recovered with activity 153 killer units/mg, a 425-fold purification compared with the starting material [twice-(NH₄)₂SO₄-precipitated medium containing the cells, specific activity 0.36 killer unit/mg].

Concanavalin A-Sepharose affinity chromatography of the Factor I preparation

Concentrated preparation of Factor I obtained from the previous columns (0.3 ml, 10 mg of pro-
tein) was dialysed against 50 mM-sodium phosphate buffer, pH 7.2, at 4°C for 24 h, and then applied on a concanavalin A-Sepharose column (10 ml volume), elution being with the same buffer, 1.0 ml of liquid being collected per 20 min. After all the proteins that did not bind to concanavalin A-Sepharose were eluted, the elution was continued with the same buffer containing 0.5 M-D-glucose. Five peaks were separated (see Fig. 4), of which only peak P2 (eluent containing no D-glucose) had killer activity (160 killer units; \( A_{270} \) 0.358; specific activity 447 killer units/mg, representing a 1270-fold purification compared with the starting material). This fraction was iodinated with \(^{125}\)I (see the Materials and methods section) and used as marker (MR1 preparation) in further purification procedures.

**Sepharpse-4B gel filtration of the Factor I preparation after DEAE-cellulose column chromatography and labelling with MR1 preparation**

A new batch with a large amount of killer cells was prepared, and the medium containing also the cells was fractionated with \((\text{NH}_4)_2\text{SO}_4\) and purified by DEAE-cellulose column chromatography. The starting material contained 10000 killer units. After the DEAE-cellulose chromatography the recovery of Factor I in peak P2 was 4000 killer units (40%). To this material was added 0.1 ml of MR1 preparation, and the mixture was applied on a Sepharose-4B column (1.2 cm \(\times\) 95.0 cm), which was eluted with 10 mM-aminoethanol/HCl buffer, pH 8.0, at 4°C, 1.8 ml of liquid being collected per 20 min (see Fig. 5). Five protein peaks were obtained, but only peak P5 contained killer activity and radioactivity. The protein peaks and radioactivity peak did not coincide. Tubes in peak P5 were pooled according to the radioactivity content (see Fig. 5); tubes 40–46 contained 160 killer units, with a specific activity of 246 killer units/mg, representing a 680-fold purification. This material was concentrated by dialysis against 50 mM-sodium phosphate buffer, pH 7.2, and recycled on a concanavalin A-Sepharose column.
similar to that previously used. Again the killer activity was eluted in peak P2 (specific activity 750 killer units/mg, representing a 2000-fold purification).

\[ ^3H \text{Aminoethanol-glass-bead affinity chromatography of the Factor I preparation} \]

The Factor I preparation (specific activity 750 killer units/mg) was dialysed first against 100 mM-sodium phosphate buffer, pH 7.2, containing 50 mM-EDTA for 24 h at 4°C, and consecutively against 5 mM-aminoethanol/HCl buffer, pH 8.0. The sample was applied to a \[^3H\]aminoethanol–glass beads column (10 ml) that had been equilibrated with the same buffer. Elution was performed with a 5–100 mM linear gradient of the same buffer (50 ml and 50 ml), 2.9 ml being collected per 20 min (see Fig. 6). The killer activity was found in peak P3. Material in this sample migrated on polyacrylamide-gel electrophoresis as a single protein band (see Fig. 7), suggesting that the preparation of Factor I is relatively pure.

**Sedimentation coefficient, molecular weight and isoelectric point of Factor I**

The sedimentation coefficient, \( s_{20,w} \), was determined at protein concentrations of 0.1, 1.0 and 5.0 mg/ml in 0.1 mM-sodium phosphate buffer, pH 7.2. The values obtained are given in Table 2. The molecular weight, \( M_r \), was determined by the conventional sedimentation-equilibrium method (18000 and 24000 rev./min) and by the Yphantis (1960) schlieren method, and was found to be 63 000 ± 1000. The plot of \( M_r \) versus protein concentration gave a linear relationship (Fig. 8), indicating the purity of the preparation. The slope of this plot is greater than 1, thus suggesting a non-spherical shape of the molecules. Factor I was eluted from an isoelectric-focusing column with a single but broad peak, in the pH range 7.7–8.3. The estimated \( pI \) value of Factor I is 8.3 ± 0.3.

**Subunit composition of Factor I**

The molecular weight of reduced and carboxymethylated Factor I, determined by ultracentrifuge and gel-filtration methods, was found to be 32 000 ± 4000. This value represents half the value of the molecular weight of the untreated Factor I. Thus it could be concluded that Factor I is composed of two polypeptide chains covalently linked by disulphide bond(s). The reduced and carboxymethylated Factor I migrated on polyacrylamide gel as a single band, indicating the identity of the subunits (Fig. 7, column E).

**Purification of the Factor II preparation obtained from DEAE-cellulose chromatography**

Factor II obtained from a former DEAE-cellulose chromatography (see Fig. 2) in peak P9 was recycled on a smaller-size DEAE-cellulose column (0.4 cm x 20 cm), which was eluted with a 250–600 mM linear gradient of aminoethanol/HCl buffer, pH 8.0 (100 ml and 100 ml), 2.0 ml being collected per 20 min (see Fig. 9). Only peak P1 had biological activity [32 killer units, specific activity 20 killer units/mg, representing a 55-fold purification compared with the starting material (specific activity
The material was eluted from the column (1.5 cm x 100 cm) with 10 mM-aminoethanol/HCl buffer, pH 8.0. A volume of 1.8 ml was collected every 20 min. The arrows indicated the fractions pooled. In the pooled fractions the killer activity, protein (●, $A_{278}$) and radioactivity (□, c.p.m.) were assayed. Only peak P5 had killer activity.

The pattern of a typical $^3$H]aminoethanol–glass-beads affinity chromatography of the Factor I preparation obtained after DEAE-cellulose, Sepharose-4B, concanavalin A–Sepharose and Sepharose-4B chromatography is shown. The Factor I preparation, after dialysis against 5 mM-aminoethanol/HCl buffer, pH 8.0, was applied to the column, and eluted with a linear buffer concentration gradient (5–100 mM) of the buffer (—). A 0.29 ml volume was collected every 20 min. In the fractions the killer activity and protein content (●, $A_{278}$) were assayed. Four distinct peaks were obtained, of which only peak P3 had killer activity.

For experimental details see the text. Samples A to D were applied under non-reducing conditions. A, Concentrated peak-P2 material from DEAE-cellulose chromatography; B, peak-P2 material from concanavalin A–Sepharose chromatography; C, subfraction 5 material from Sephadex-4B gel filtration; D, peak-P2 material from $^3$H]aminoethanol–glass-beads chromatography; E, reduced and carboxymethylated peak-P3 material obtained after $^3$H]aminoethanol–glass-beads chromatography. For details of the electrophoresis see the Materials and methods section.
Table 2. Sedimentation coefficients and apparent molecular weights of various preparations of Factor I

The \( s_{20w} \) and \( M_r \) values were calculated from the measurements of sedimentation velocity and sedimentation equilibrium in the analytical ultracentrifuge (for experimental conditions see the text). The samples obtained after several purification steps were designated \( A \), \( B \), \( C \) etc. \( A \), Factor I obtained after two DEAE-cellulose and Sepharose-4B columns; \( B \), \( A \) subjected to and additional concanavalin A–Sepharose column; \( C \), \( B \) subjected to an additional Sepharose-4B gel-filtration column; \( D \), \( C \) after an aminoethanol–glass-beads column; \( E \), the same as \( D \) after dialysis against buffer containing 4 M guanidinium chloride.

<table>
<thead>
<tr>
<th>Designation of the sample</th>
<th>Concentration of protein (mg/ml)</th>
<th>( s_{20w}^0 ) (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( A )</td>
<td>0.6</td>
<td>3.5</td>
</tr>
<tr>
<td>( B )</td>
<td>0.4</td>
<td>3.5</td>
</tr>
<tr>
<td>( C )</td>
<td>0.2</td>
<td>3.3</td>
</tr>
<tr>
<td>( D )</td>
<td>0.6</td>
<td>3.6</td>
</tr>
<tr>
<td>( D )</td>
<td>0.2</td>
<td>3.3</td>
</tr>
<tr>
<td>( D )</td>
<td>0.4</td>
<td>3.6</td>
</tr>
<tr>
<td>( E )</td>
<td>0.5</td>
<td>3.0</td>
</tr>
</tbody>
</table>

The molecular weight of Factor I was measured at several protein concentrations in sedimentation-equilibrium runs and plotted versus protein concentration. For details see the text.

Fig. 8. Molecular weight of Factor I at different protein concentrations

The molecular weight of Factor I was measured at several protein concentrations in sedimentation-equilibrium runs and plotted versus protein concentration. For details see the text.

Fig. 9. Purification of Factor II obtained from a previous DEAE-cellulose column in peak P9, and recycled on a smaller-size DEAE-cellulose column

Substance obtained previously on a DEAE-cellulose column in peak P9 (see Fig. 2) was concentrated and re-applied to a smaller-size column (0.4 cm x 20 cm). Elution was performed with a linear concentration gradient of buffer (100 ml of 250 mM and 100 ml of 600 mM) (—). A 2.0 ml volume was collected every 20 min. In the fractions the killer activity and protein content (\( \bullet \), \( A_{278} \)) were assayed. Only peak P1 had killer activity [32 killer units, specific activity 17.8 killer units/mg, representing a 50-fold purification compared with the starting material (specific activity 0.36 killer unit/mg)]. Factor II was eluted from the column at 290–350 mM buffer concentration.

0.36 (killer unit/mg). Factor II was eluted at 290–350 mM buffer concentration.

Sepharose-4B gel filtration of the Factor II preparation

The Factor II preparation, after being concentrated, was further purified on a Sepharose-4B gel column. Four peaks were separated. Only peak P2 had killer activity. The Factor II preparation thus obtained had a specific activity of 60 killer units/mg, representing a 170-fold purification.

Vol. 194
Discussion

By a series of steps involving column chromatography, gel filtration and affinity chromatography, two cytotoxic factors, Factor I and Factor II, were separated both from the homogenates of the isolated cells of the haemical cell line (Karpas, 1977a,b) and from the medium containing the cell lysate. The purification procedure was repeated several times for various preparations and for large quantities. In every case the same pattern was obtained with each separation step. No significant improvement of the purification was found when CM-cellulose, DEAE-Sepharose and CM-Sepharose chromatography was also included in the purification process.

Factor I is a basic protein (pI 8.0) and is eluted from DEAE-cellulose at 20 mM-aminoethanol/HCl buffer, pH 8.0, whereas Factor II is an acidic protein (pI 6.0) and is bound strongly to DEAE-cellulose, being eluted only at 350 mM concentrations of the same buffer. Factor I was found to be a homogeneous protein both in polyacrylamide-gel electrophoresis, migrating as a single protein band, and in ultracentrifuge measurements. The plot of $M_r$ versus protein concentrations gave a slope, $x/y$, greater than 1, indicating a non-spherical shape of the molecule (Fig. 8). After reduction and carboxymethylation Factor I is dissociated to two identical subunits. The subunit migrated on gel electrophoresis as a single protein band. Its $M_r$, measured by equilibrium sedimentation and Sepharose-4B gel filtration, is $32000 \pm 4000$. The purified Factor I has no proteolytic activity. It does not catalyse the hydrolysis of denatured proteins with known sequence or of low-molecular-weight synthetic peptides.

Factor II was only partially purified. Its $M_r$ is 75000, as estimated by Sepharose-4B gel filtration. It has a slight proteolytic activity, but at this stage of purification it is hard to say that this activity is not due at least in part to contamination by other proteins.

The physicochemical and catalytic properties of Factor I and Factor II are different from those of lymphotoxins described by Granger et al. (1975), those of arginase reported by Currie (1978) and those of the cytotoxic proteinase reported by Hatcher et al. (1978).

The method of purification of the cytotoxic factors described should enable their production in large quantities and the preparation of specific antibodies. This in turn may simplify the quantification assay, and could also lead to a rapid selection of clones for higher production of the cytotoxic factor with or without exogenous stimulation (e.g. hormonal). Specific antibodies could also help determine whether this cytotoxic factor is present in human serum and whether certain cell populations have a higher or lower concentration of this factor. With the aid of the pure factor it should also be possible to study the mode of action of the cytotoxic factor and to investigate its preferential binding to malignant cells.

The availability of large quantities of the pure cytotoxic factor should allow its potential beneficial effect as an anti-tumour agent in vivo to be evaluated.

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