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Physicochemical Characterization of Cytostatic Factors Released from Human Monocytes

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Cultured human monocytes released cytostatic activity upon in vitro activation with lymphokines and lipopolysaccharide. This activity was mainly due to the presence of two different cytostatic factors, termed CstF I and II, which were separated by ion-exchange chromatography. At neutral pH, CstF I bound to the weak anion exchanger DEAE-Sephacel but not to the weak cation exchanger CM-Sepharose, whereas CstF II bound to CM-Sepharose but not to DEAE-Sephacel. The molecular weights of CstF I and II as determined by gel filtration were 55,000 and 40,000, respectively. Upon chromatofocusing, CstF I behaved as if it had an isoelectric point of 5.3. Neither CstF I nor CstF II bound specifically to concanavalin A-Sepharose, indicating the absence of carbohydrate residues containing α-D-mannopyranosyl, α-D-glucopyranosyl, or sterically related components. Both factors were susceptible to inactivation by proteinase K, demonstrating their protein nature. CstF II was purified more than 3,000-fold upon chromatography on CM-Sepharose and Sephacryl S-200. Ion-exchange chromatography and chromatofocusing of CstF I removed 97% of the proteins in the monocyte supernatant, but only 15% of the activity was recovered, resulting in a fivefold purification of CstF I.

Activated macrophages are selectively cytotoxic to neoplastic cells in vitro (5, 14, 24) and may be involved in host resistance against neoplasia (4, 5). Considerable evidence suggests that extracellular components secreted by activated macrophages play a central role in the tumoricidal activity of these cells. Thus, activated macrophages probably release the 55,000-dalton tumor necrosis factor (TNF) which has been found in sera of endotoxin-treated mice and rabbits previously infected with *Mycobacterium bovis* BCG (16, 18, 19). TNF induces necrosis of tumors in mice and shows cytolytic activity against transformed cells in vitro (7, 17, 22, 25, 26). Recent results implicate this factor as an effector molecule in the cytotoxic activity of activated in vitro cultured macrophages (16). Activated macrophages from mice also release a 40,000-dalton neutral serine protease (1–3) and, when appropriately triggered, large amounts of H_2O_2 (23). In vitro, these two components have been shown to act synergistically to promote lysis of neoplastic cells (2).

Human monocytes, upon in vitro differentiation into large macrophage-like cells, develop cytostatic activity toward various types of transformed target cells (9). Along with monocyte-mediated cytolysis, this cytostatic activity is stimulated by in vitro activation of the monocytes with lymphokines from human lymphocytes treated with *M. bovis* BCG (10, 13). Further enhancement of cytostasis and cytolysis is observed after subsequent addition of *Escherichia coli* endotoxin (lipopolysaccharide [LPS]) (11). Monocytes at an intermediate stage of in vitro differentiation (after 3 to 5 days of in vitro culture) show the greatest increase in cytostatic and cytolytic activities after treatment with lymphokines and LPS (10, 11). The involvement of extracellular factors in monocyte-mediated cytostasis or cytolysis or both is suggested by the appearance of cytostatic activity in the monocyte culture media after in vitro activation of the monocytes (11). Cytostatic activity was greatest in media from monocyte cultures activated at an intermediate stage of in vitro differentiation (12). In this paper we report that the cytostatic activity in media from activated human monocyte cultures is primarily due to the presence of two different protein factors released from the activated monocytes. These two factors were partially purified and characterized.

MATERIALS AND METHODS

Monocytes and lymphocytes. Human monocytes and lymphocytes were separated from defibrinated venous blood of healthy tuberculin-positive volunteers as previously described (10). All cells were cultured in RPMI

1640 medium (Bio-Cult; GIBCO Laboratories) supplemented with 25% pooled human AB Rh^+ serum, 0.1 mM L-glutamine, and 40 μg of gentamicin per ml (HS-M). Adherent cells were more than 97% monocytes, as judged by nonspecific esterase staining (10).

Preparation of lymphokines. Lymphokines were prepared from human lymphocytes as described previously (10).

Preparation of monocyte-derived CstF. Cytostatic factors (CstF) were prepared essentially as described previously (12). Monocyte monolayers cultured for 3 days were incubated for 24 h at 37°C with a 1:2 dilution of lymphokines. The lymphokine-containing medium was then removed, and RPMI 1640 medium containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and 0.1 μg of LPS from *E. coli* O26:B6 (Sigma Chemical Co.) per ml was added. After further incubation of the monocyte cultures for 7 h, the culture medium (monocyte supernatant) containing monocyte-released CstF was rendered cell-free by low-speed centrifugation (1,500 × *g*) and stored at −20°C.

Assay for cytostatic activity. Column fractions were transferred to RPMI 1640 medium before they were assayed for cytostatic activity. Human nonadherent leukemia K-562 cells (15) were used as target cells. Varying amounts of CstF and 10^4 K-562 cells were incubated in 6-mm tissue culture wells (type 3596; Costar) containing RPMI 1640 medium supplemented with 25 mM HEPES, 20% AB serum, 0.1 mM L-glutamine, and 40 μg of gentamicin per ml. After incubation for 48 h, 1 μCi of [*methyl*-^{3}H]thymidine (specific activity, 5 Ci/mmol) was added, and the cultures were harvested after 4 h of [*methyl*-^{3}H]thymidine incorporation (12). The results were calculated as percentages of inhibition of target cell [*methyl*-^{3}H]thymidine incorporation (%TI), as follows: %TI = 100 − [(counts per minute in CstF-exposed cells)/(counts per minute in HS-M control) ×100].

One CstF unit (CstFU) was defined as the amount of CstF which inhibited target cell DNA synthesis by 30% (%TI = 30) under the assay conditions described above.

Buffer exchange. Transfers of CstF from cell culture media to the appropriate starting buffer for subsequent chromatography, from one chromatography buffer to another, and from chromatography buffer to RPMI 1640 medium for subsequent assay for cytostatic activity were performed by filtration using Sephadex G-25 PD-10 columns (Pharmacia Fine Chemicals, Inc.) at 2°C.

Ion-exchange chromatography. Monocyte culture medium containing CstF was transferred to 20 mM sodium phosphate (pH 6.9) (phosphate buffer) by filtration as described above and applied to a CM-Sepharose (Pharmacia Fine Chemicals) column (diameter, 1 cm; 30 to 70 mg of protein per column; about 10 mg of protein per ml of bed volume) equilibrated in phosphate buffer. The column was washed with 1 volume of phosphate buffer. The proteins binding to the column were then eluted with a linear gradient (12 column volumes) of 0 to 0.6 M NaCl in phosphate buffer. Fractions equivalent to 8% of the gradient volume were collected and assayed for cytostatic activity as described above.

The cytostatic activity which did not bind to the CM-Sepharose column (flow-through fraction from the CM-Sepharose column) was applied to a DEAE-Sephacel (Pharmacia Fine Chemicals) column (diameter, 1 cm; 20 to 50 mg of protein per column; about 10 mg of protein per ml of bed volume) equilibrated in phosphate buffer. The proteins which bound to the column were eluted in a manner identical to that described above for the elution of proteins from the CM-Sepharose column. Ion-exchange chromatography was carried out at 2°C.

Sephacryl S-200 gel filtration. The NaCl concentration of a 2.5-ml sample from the ion-exchange fractions containing cytostatic activity was adjusted to 0.3 M by adding 5 M NaCl, and then this preparation was applied to a Sephacryl S-200 (Pharmacia Fine Chemicals) column (1.6 by 80 cm) equilibrated with 0.3 M NaCl in phosphate buffer. The cytostatic activity was eluted from the column with the same buffer at a flow rate of 30 ml/h, and 3.6-ml fractions were collected. The column was calibrated with blue dextran, bovine serum albumin (molecular weight, 67,000), ovalbumin (molecular weight, 43,000), chymotrypsinogen (molecular weight, 25,000), and RNase A (molecular weight, 13,700). Gel filtration was carried out at 2°C.

Chromatofocusing. The CstF was transferred to either 25 mM imidazole hydrochloride (pH 7.4) or 25 mM Tris-hydrochloride (pH 9.0) by filtration as described above, and the resulting preparation was applied to a Polybuffer exchange 94 (Pharmacia Fine Chemicals) column (bed volume, 6 ml) equilibrated with buffer identical to that in which the CstF was applied to the column. The proteins binding to the column were eluted with Polybuffer 96 (pH 7.0; Pharmacia Fine Chemicals) when the sample had been applied to the column in 25 mM Tris-hydrochloride (pH 9.0). Polybuffer 74 (pH 4.5; Pharmacia Fine Chemicals) was used as the elution buffer when the sample had been applied to the column in 25 mM imidazole-hydrochloride (pH 7.4). The elution rate was 30 ml/h, 4-ml fractions were collected, and the cytostatic activity and pH of each fraction were determined. Chromatofocusing was carried out at 2°C.

ConA-Sepharose chromatography. A concanavalin A (ConA)-Sepharose (Pharmacia Fine Chemicals) column (bed volume, 1 ml) was equilibrated with phosphate buffer containing 0.5 M NaCl. CstF in the same buffer (2.5 ml) was applied to the column. The column was subsequently washed with 5 ml of 0.5 M NaCl in phosphate buffer, and the proteins binding to the column were then eluted with 3 ml of 0.5 M α-D-methylmannoside–0.5 M NaCl in phosphate buffer.

Digestion of CstF. CstF obtained after ion-exchange chromatography was incubated for 1 h at 37°C in RPMI 1640 medium containing 20 μg of proteinase K (Sigma) per ml. The CstF preparation was then diluted with 3 volumes of RPMI 1640 medium and assayed for cytostatic activity.

Protein determination. Protein concentrations were determined by the method of Bradford (6), using the Bio-Rad Laboratories Protein Assay Kit and lyophilized bovine gamma globulin as the standard protein.

RESULTS

Quantitation of CstF. Optimal conditions for CstF release by human monocytes (12) were used for the production of CstF in all experiments. Dose-response curves of CstF activity in

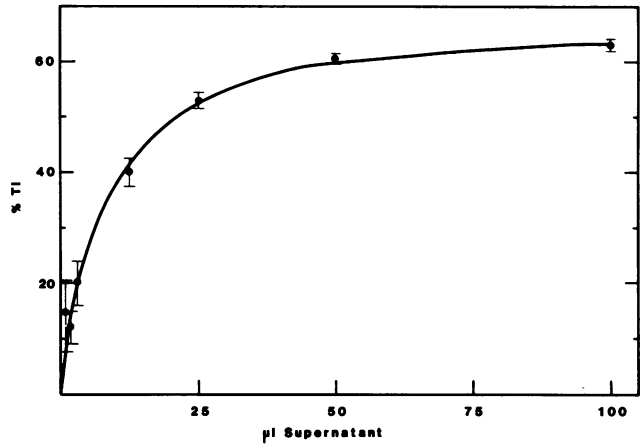

FIG. 1. Cytostatic activities in monocyte supernatants. Different amounts of CstF-containing monocyte supernatants were added to target cell cultures, and CstF-induced %TI was measured as described in the text.

monocyte supernatants revealed poor proportionality between the amount of CstF present and %TI (Fig. 1). Moreover, large changes in CstF dilution resulted in relatively small changes in %TI (Fig. 1). Consequently, it was somewhat misleading to quantitate CstF in terms of %TI measured at a single concentration of CstF without any reference to a dose-response curve. Hence, in this work amounts of CstF were quantitated in terms of CstFU, where 1 CstFU was defined as the amount of factor which gave a %TI of 30 under the assay conditions described above.

Presence of two cytostatic components in monocyte supernatants. Ion-exchange chromatography of CstF revealed the presence of at least two cytostatic components (Fig. 2A). One of the components (termed CstF I) did not bind to the weak cation exchanger CM-Sepharose in the presence of 20 mM sodium phosphate (pH 6.9) and consequently was obtained in the flow-through fraction upon chromatography on a CM-

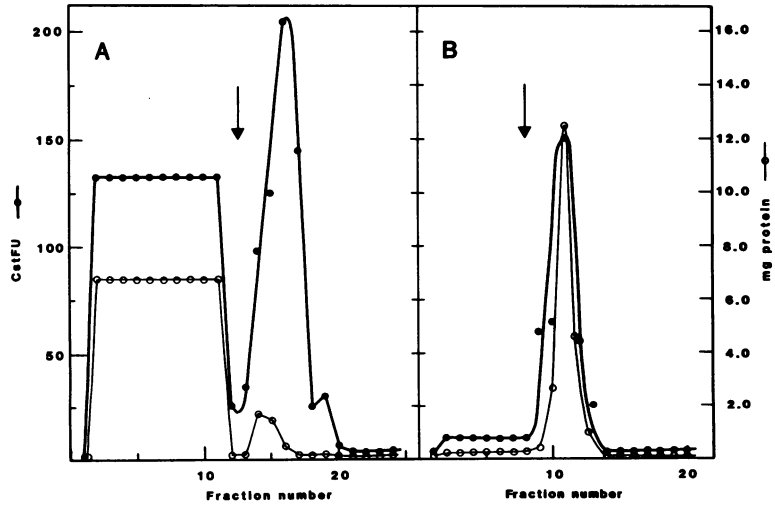

FIG. 2. Ion-exchange chromatography of cytostatic activity released from activated monocytes. (A) The cytostatic activity (approximately 2,000 CstFU) in the supernatant (30 ml) from lymphokine–LPS-activated monocyte cultures was transferred to phosphate buffer and chromatographed on a CM-Sepharose column as described in the text. (B) The cytostatic activity (approximately 600 CstFU) which did not bind to the CM-Sepharose column (fractions 2 through 11, Fig. 2A) was chromatographed on a DEAE-Sephacel column. The proteins which bound to the columns were eluted with a 0 to 0.6 M linear NaCl gradient in phosphate buffer. The arrows indicate the starting points of the gradients.

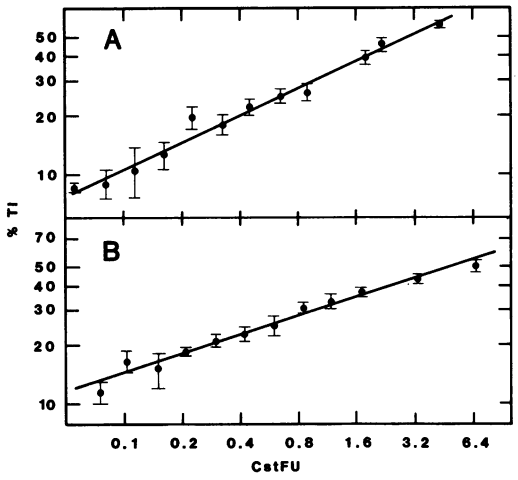

FIG. 3. Inhibition of target cell DNA synthesis measured as a function of the amount of CstF I (A) and CstF II (B) added to the target cell culture. CstF I was purified by chromatography on CM-Sepharose and DEAE-Sephacel, whereas CstF II was purified by CM-Sepharose chromatography, as described in the text.

Sepharose column (Fig. 2A). The other component (termed CstF II) bound to the CM-Sepharose column and could be eluted by raising the NaCl concentration to approximately 0.15 M (Fig. 2A). Subsequent chromatography of CstF I and II on the weak anion exchanger DEAE-Sephacel showed that less than 15% of CstF II was retained on a DEAE-Sephacel column in the presence of 20 mM sodium phosphate (pH 6.9) (data not shown). However, CstF I bound to the column under these conditions and could subsequently be eluted by raising the NaCl concentration to about 0.15 M (Fig. 2B).

The dose-response curves of CstF I and II were nearly linear when logarithmic scales were used on both abscissas and ordinates (Fig. 3). The cytostatic activity of CstF I appeared to be somewhat more sensitive to dilution than the activity of CstF II (Fig. 3). The dose-response curves allowed us to quantitate the amounts of CstF in CstFU by measuring CstF-induced inhibition of DNA synthesis at a single concentration of CstF.

Physicochemical characteristics of CstF I and II. Both CstF I and CstF II were analyzed by gel filtration, using Sephacryl S-200 (Fig. 4). When applied to a Sephacryl column in the presence of 0.3 M NaCl, CstF I eluted with a molecular weight of approximately 55,000 (Fig. 4A), whereas CstF II eluted with a molecular weight of about 40,000 (Fig. 4B).

CstF I was also analyzed by chromatofocusing, which is a chromatographic method for separating proteins according to their isoelectric points (27, 28). CstF I eluted as a major peak of cytostatic activity at a pH of 5.3, suggesting that the isoelectric point of CstF I is about 5.3 (Fig. 5). A minor peak of cytostatic activity eluted at a pH of about 4.9 together with most of the proteins (Fig. 5). The cytostatic activity of CstF II was lost when attempts were made to purify it by chromatofocusing.

Affinity chromatography on ConA-Sepharose revealed that neither CstF I nor CstF II bound specifically to ConA (data not shown). However, whereas CstF I eluted from the ConA-Sepha-

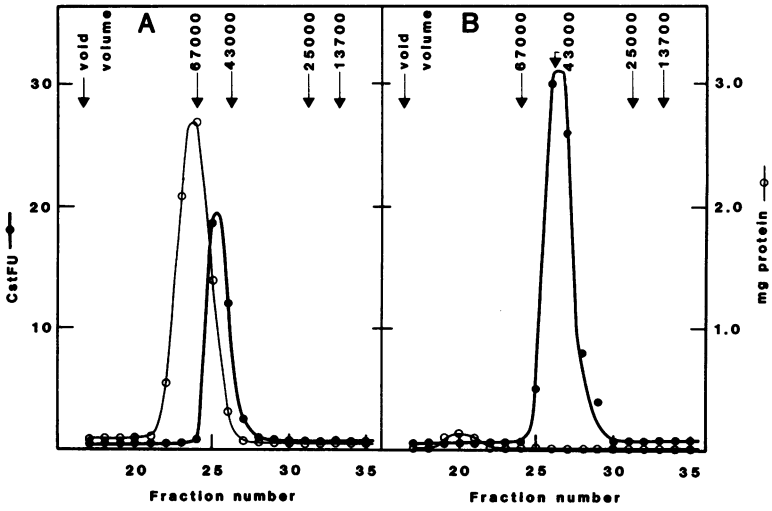

FIG. 4. Gel filtration analysis of CstF I and II. About 60 CstFU of CstF I (A) or CstF II (B) was chromatographed on a Sephacryl S-200 column as described in the text. CstF I and II were purified by ion-exchange chromatography as described in the legend to Fig. 3.

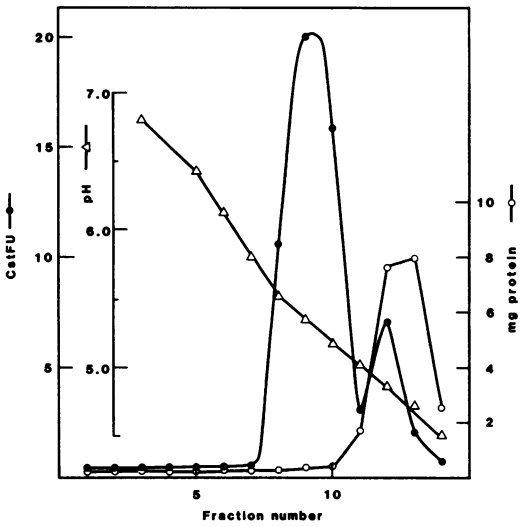

FIG. 5. Analysis of CstF I by chromatofocusing. CstF I (approximately 150 CstFU) which had been purified by ion-exchange chromatography on CM-Sepharose and DEAE-Sephacel was applied to a Polybuffer exchange 94 column and eluted from the column by using Polybuffer 74 (pH 4.5), as described in the text.

rose column largely in the flow-through fraction, CstF II was partially retained on the column and eluted mainly in the wash (data not shown). Consequently, CstF II appeared to have a slight, although probably nonspecific affinity for ConA-Sepharose.

Both factors are proteins, since more than 95% of the cytostatic activity was lost when CstF I and II were incubated for 1 h at 37°C in RPMI 1640 medium containing 20 μg of proteinase K per ml. Incubating the factors in the absence of proteinase K had no effect on the cytostatic activity. Control experiments showed that the presence of proteinase K at the concentrations used in these experiments had no significant effect on the growth of the target cells. The factors could be stored at −20°C for at least 2 months, although significant loss (20 to 50%) of cytostatic activity was detected upon repeated (three to five times) freezing and thawing of either factor.

Purification scheme for CstF I and II. The extents of purification of CstF I and II by ion-exchange chromatography, gel filtration, and chromatofocusing were evaluated (Table 1). Ion-exchange chromatography of monocyte supernatants on CM-Sepharose appeared to result in very little loss of cytostatic activity (Fig. 2A and Table 1). Consequently, we estimated that about 70 and 30% of the cytostatic activity in the monocyte supernatants were contributed by CstF I and II, respectively (Fig. 2A). Of the proteins applied to the CM-Sepharose column, about 95% were recovered in the flow-through fraction together with CstF I, and approximately 3% eluted together with CstF II (Fig. 2A and Table 1). Determining the increases in the specific activities (CstFU per milligram of protein) of CstF I and II revealed that CstF II was purified about 30-fold upon CM-Sepharose chromatography, whereas CstF I was not purified to any significant extent (Table 1).

Nearly all of the proteins in the CstF II preparation obtained after CM-Sepharose chromatography eluted ahead of CstF II upon gel filtration on Sephacryl S-200, and 70 to 90% of the cytostatic activity was generally recovered from the Sephacryl column (Fig. 4B and Table 1). Consequently, CstF II was purified more than 3,000-fold after chromatography on CM-

TABLE 1. Purification summary

Sample	CstF I			CstF II		
	Protein (%)	Recovery (% of CstFU)	Purification (-fold)	Protein (%)	Recovery (% of CstFU)	Purification (-fold)
Monocyte supernatant	100[a]	100[b]	1	100[a]	100[c]	1
CM-Sepharose	95	90–100	1	3	90–100	30
DEAE-Sephacel	95	50	0.5			
Sephacryl S-200[d]	20	30	1.5	<0.025[e]	70–90	>3,000
Chromatofocusing[d]	3	15	5			

[a] A 30-mg amount of protein in a monocyte supernatant before purification of CstF I and II equaled 100% protein.

[b] Seventy percent of the total cytostatic activity in the monocyte supernatant equaled 100% of CstF I activity (see text for details).

[c] Thirty percent of the total cytostatic activity in the monocyte supernatant equaled 100% of CstF II activity (see text for details).

[d] CstF obtained from ion-exchange chromatography was either applied to a Sephacryl S-200 column or chromatofocused.

[e] Protein not detected (concentration less than 0.5 μg/ml or 0.025% of the proteins present in the monocyte supernatant).

Sepharose and Sephacryl (Table 1).

The CstF I in the flow-through fraction from the CM-Sepharose column was concentrated on a DEAE-Sephacel column. Only about 50% of the cytostatic activity was recovered from the DEAE-Sephacel column, and nearly all of the protein applied to the column eluted together with CstF I (Fig. 2B and Table 1). As a result, the specific activity was reduced 0.5-fold (Table 1). After subsequent gel filtration of CstF I, about 30% of the activity was recovered together with approximately 20% of the proteins in the monocyte supernatant, resulting in only a 1.5-fold purification (Table 1). Chromatofocusing of CstF I separated most of the proteins from CstF I, but much of the cytostatic activity was lost (Fig. 5). Thus, only about 15% of the CstF I activity in the monocyte supernatant was recovered together with about 3% of the proteins present in the supernatant, resulting in a five-fold purification of CstF I (Table 1).

DISCUSSION

Cultured human monocytes release cytostatic activity upon in vitro activation with lymphokines and LPS (12). The release of cytostatic activity is greatest at an intermediate stage of monocyte in vitro differentiation (around day 4 of culture) (12), which corresponds to the period during which monocytes are most responsive to lymphokine activation for cytolytic activity (10). The cytostatic activity is mainly due to the presence of two different protein factors, CstF I and II. Ion-exchange chromatography revealed that CstF I has a dominant negative charge at neutral pH, whereas CstF II has a dominant positive charge. CstF I behaved as if it had an isoelectric point of about 5.3 upon chromatofocusing. The molecular weights of CstF I and II were determined by gel filtration to be 55,000 and 40,000, respectively. Neither factor appeared to contain carbohydrate residues with α-D-mannopyranosyl, α-D-glucopyranosyl, or sterically related components, as judged by the inability of the factors to bind specifically to ConA-Sepharose. Although CstF I and II clearly behave differently upon chromatography, we cannot exclude the possibility that they are derived from basically the same molecule, which has been cleaved by proteases present in the monocyte culture media. Such cleavage, which may be of no biological significance, could account for the differences in molecular weight and elution from ion-exchange columns. An immunological comparison of CstF I and II should reveal whether these factors are basically different proteins.

Upon chromatographic analysis, both CstF I and CstF II behaved relatively homogeneously, and they were separated from most of the proteins in the monocyte supernatant, suggesting that the cytostatic activity was not merely a nonspecific effect due to the bulk of proteins present in the supernatant nor the result of a multitude of various hydrolytic enzymes which might be released from damaged cells. CstF II was purified more than 3,000-fold by the simple two-step procedure of ion-exchange chromatography on CM-Sepharose and gel filtration on Sephacryl. Purification of CstF I by ion-exchange chromatography and chromatofocusing resulted in only a five-fold increase in the specific activity, due to loss of 85% of the cytostatic activity. Better purification might be obtained if gel filtration and chromatofocusing of CstF I were run in series.

Two cytostatic components were identified. However, more sensitive assays and refined separation techniques with higher resolution may resolve additional components. The minor CstF activity peak eluting from the chromatofocusing column at pH 4.9 may represent such an additional component, or this peak could be due to affinity between CstF I and the proteins eluting at pH 4.9.

CstF I or CstF II or both may be identical to the human monocyte-derived cytotoxin recently reported by Matthews (20), although the culture conditions inducing cytotoxin release differ. As far as the physical properties of CstF I and TNF (18, 21, 25) have been determined, they are very similar. CstF I and TNF behave similarly upon ion-exchange chromatography (18, 21, 25), bind poorly to ConA-Sepharose (21, 25), have similar molecular weights as determined by gel filtration (18, 25), and appear to have isoelectric points somewhere between 5.4 and 5.0 (21, 25). Mouse TNF may induce lysis of target cells (26), whereas no cytolytic activity was detected in our CstF preparations (unpublished data). However, the possibility that CstF I or CstF II or both have cytolytic activity at higher concentrations or that they may act cytolytically in the microenvironment of monocyte-target cell contact cannot be excluded.

The mechanism whereby CstF I and II induce cytostasis has yet to be determined. The possibility that the action of CstF is due to neutral protease activity as described by Adams et al. (1, 3) seems unlikely, since cytostasis was demonstrated in the presence of 25% serum. Arginase activity (8) does not appear to be involved, as the presence of an excess of arginine (1 mg/ml) in the assay medium did not influence the CstF activity (unpublished data). More knowledge of the mechanism of CstF-induced cytostasis is likely to clarify the function of CstF I and II in the in vitro cytotoxic activity of monocytes against tumor cells. The pertinent question of

the in vivo role of CstF must await the development of a sensitive assay for CstF in body fluids.

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