Comparison of Recombinant Tumor Necrosis Factor and the Monocyte-derived Cytotoxic Factor Involved in Monocyte-mediated Cytotoxicity

Jon Nissen-Meyer, Rigmor Austgulen, and Terje Espevik

ABSTRACT

A direct comparison of recombinant tumor necrosis factor (rTNF) and the monocyte-derived cytotoxic factor (CF) which is involved in monocyte-mediated cytotoxicity revealed immunological, physicochemical, and biological similarities, indicating that TNF is an effector molecule in monocyte-mediated cytotoxicity. Neutralizing antiseraum raised against rTNF completely inhibited the ability of CF-containing monocyte supernatants to induce cytostasis and cell death of sensitive target cells and, conversely, antiseraum raised against purified CF completely inhibited the cytotoxic activity of rTNF. Both CF and rTNF have an apparent isoelectric point of 5.8-5.9 as determined by chromatofocusing, and a molecular weight of about 40,000 as determined by gel filtration. Moreover, when present in monocyte supernatants with a total protein concentration of about 1 mg/ml and 0.1% sodium dodecyl sulfate, both CF and rTNF migrated with a molecular weight of about 35,000 upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Pure rTNF, however, migrated with a molecular weight of 17,000, suggesting that the relative amount of sodium dodecyl sulfate to protein is critical for dissociating the apparent dimeric structure of TNF. CF and rTNF were also similar with respect to their ability to kill various types of target cells the sensitivity of which to TNF differ, and the dose-response curves of cytotoxicity obtained with CF-containing monocyte supernatants and rTNF were similar. As is the case with anti-CF serum, anti-rTNF serum inhibited drug-dependent cellular cytotoxicity and cytolysis mediated by both freshly isolated monocytes and in vitro cultured unactivated and lymphokine-like polysaccharide activated monocytes, indicating that TNF is an effector molecule in both drug-dependent cellular cytotoxicity and "classical" monocyte-mediated cytotoxicity.

INTRODUCTION

We have earlier reported and characterized a M, 40,000 CF which is released from human monocytes activated with interferons, lymphokines, and/or Escherichia coli endotoxin (LPS) (1-5). CF appears to be an important effector molecule involved in cytolysis mediated by monocytes (monocyte-mediated cytotoxicity) and in DDCC, which is monocyte-induced cytolysis of target cells treated with actinomycin D, a treatment which renders the target cells highly susceptible to killing by cells of the monocyte-macrophage lineage (6, 7). CF appears to be involved in all of these processes since neutralizing antiseraum against CF completely inhibited the monocyte-mediated cytotoxicity and antibody-dependent cytotoxicity (8, 9) and DDCC (10). Moreover, target cells which have become relatively resistant to CF-induced cytolysis upon long-time exposure to CF have also become relatively resistant to monocyte-mediated cytolysis (11) and DDCC (12).

Monocytes also produce and release TNF, a protein which induces necrosis of tumors in mice (13-17). TNF was first found in sera from endotoxin-treated mice and rabbits previously infected with Mycobacterium bovis Bacillus Calmette Guérin (13, 17). More recently, human TNF has been extensively characterized (18), and the gene coding for this protein has been cloned and expressed in Escherichia coli (19-22). In this study we show that TNF and the effector molecule involved in monocyte-mediated cytotoxicity, CF, are similar as judged by comparing the biological, physicochemical, and immunological characteristics of human recombinant TNF and CF.

MATERIALS AND METHODS

Monocytes and Preparation of Monocyte-derived CF. Human monocytes were separated from defibrinated venous blood as previously described (23) and cultured in RPMI 1640 medium (Gibco Laboratories), containing 25% pooled human serum, 0.1 mM L-glutamine, and 30 μg/ml gentamicin (HS-M). For production of CF, monocyte monolayers cultured for 3 days were incubated for 24 h at 37°C with a 1:2 dilution of lymphokines prepared from human lymphocytes stimulated with Bacillus Calmette Guérin as described previously (23). The lymphokine-containing medium was then removed, and serum-free RPMI 1640-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid medium containing 0.1 μg/ml LPS from E. coli 026:B6 (Sigma Chemical Co.) was added. After further incubation of the monocyte culture for 7 h, the culture medium (monocyte supernatant) containing monocyte-released CF was rendered cell free by low speed centrifugation (400 x g) and stored at -20°C.

Target Cells. WEHI 164 murine fibrosarcoma cells (24) (in this communication termed WEHI 164 parental cells or P-WEHI 164 cells) were obtained from Dr. H. W. Loms Ziegler-Heitbrock (Institute of Immunology, University of Munich, Federal Republic of Germany). WEHI 164 clone 13 cells were obtained by cloning P-WEHI 164 cells by limiting dilution in microplates (Costar 3596). The WEHI 164 clone 13 cells were selected on the basis of their extreme sensitivity to CF-induced cytolysis (10-fold more sensitive than P-WEHI) (25). P-WEHI 164, WEHI 164 clone 13, murine fibroblast L929, and nonadherent human leukemia K-562 cells (26) were all maintained as stationary cultures in RPMI 1640 containing 10% fetal calf serum (Gibco), 0.1 mM L-glutamine, and 30 μg/ml gentamicin (FCS-M).

Incubation of Target Cells with Actinomycin D. In experiments where monocyte-mediated cytolysis of actinomycin D-treated target cells was studied, P-WEHI 164 cells were incubated for 3 h before cytolysis assay with 1 μg/ml actinomycin D in FCS-M as described in (27).

Cr Release Assay for Measuring rTNF and CF-induced Cytolysis. Monocyte supernatants containing CF, supernatants containing rTNF (kindly provided by Biogen, S.A. and BASF; specific activity: 1.5 x 10^7 units/mg in a 24-h bioassay with actinomycin D-treated L929 cells; data from Biogen/BASF), or RPMI 1640 alone were added to 6-mm microculture wells (Costar 3799) together with 1 x 10^5 WEHI 164 clone 13 cells which had been labeled with 51Cr as described previously (27). Each well contained a final volume of 200 μl FCS-M. After culturing 8 h at 37°C, 50 μl HBSS (Gibco) were added to each well. To some wells 50 μl of 2.5% SDS were added instead of 50 μl HBSS in order to determine total amount of releasable 51Cr. The plates were then centrifuged (400 x g; 10 min), and the radioactivity was deter-
mined in 100-µl samples of cell-free supernatants from cultures containing monocytes, CF, or rTNF (experimental release, E), cultures with RPMI alone (spontaneous release, S), and cultures to which SDS had been added (total release, T).

The percentage of specific lysis was calculated as

\[ \frac{E - S}{T - S} \times 100 \]

Under our labeling conditions the total release T was between 2000 and 3000 cpm, and the spontaneous release S after 8 h was 20–25% of the total release.

Colorimetric MTT Assay for Measuring CF- and rTNF-Induced Target Cell Death. The MTT cytotoxicity assay (28) was used to measure the percentage of dead cells. Target cells (2 × 10^4 cells in 100 µl FCS-M) were added to microculture wells (Costar 3596) together with various dilutions of CF or rTNF, giving a final volume of 200 µl FCS-M. After 20 h of incubation at 37°C, 10 µl MTT (Sigma) at a concentration of 5 mg/ml in phosphate-buffered saline were added. The cultures were then incubated for 4 h at 37°C, after which 100 µl supernatant were removed from each well and replaced with 100 µl 0.04 N HCl in isopropanol. After dissolving the dark blue formazan, the absorbance of each well was measured with a Dynatech MR 600 Microplate reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm. The percentage of dead target cells was calculated as

\[ \frac{100 - \text{Absorbance in wells with CF/TNF}}{\text{Absorbance in control wells without CF/TNF}} \times 100 \]

One cytotoxic unit was defined as the amount of CF or rTNF which induced 50% target cell death using the MTT assay as described above and WEHI 164 clone 13 cells as target cells.

Monocyte-mediated Cytolysis. To determine cytolysis mediated by freshly isolated monocytes, monocytes separated from defibrinated venous blood were washed twice in HBSS, suspended to a concentration of 4 × 10^6 cells/ml in HS-M, and 1.5 ml of the cell suspension was added to 35-mm tissue culture wells (Costar 3506). Nonadherent cells were then removed after 90 min incubation at 37°C, and the adherent cells were washed three times with HBSS and detached by a 5-min treatment with 0.02% EDTA in Ca^2+/-Mg^2+-free HBSS. The cells were then washed twice in HBSS and suspended in FCS-M at appropriate dilutions (see Figs.). One hundred µl of the monocyte suspension were added to 6-mm microwells (Costar 3596) together with 100 µl target cells (5 × 10^4 cells/ml FCS-M) which had been 51Cr-labeled as described previously (27). After culturing 6 h at 37°C the microculture plates were centrifuged (400 × g; 10 min), and the amount of radioactivity released from target cells was determined by measuring radioactivity in 100 µl of cell-free supernatants.

To determine cytolysis mediated by in vitro-cultured monocytes activated with lymphokines and LPS, monocytes separated from defibrinated venous blood were washed twice in HBSS, suspended to a concentration of 4 × 10^6 cells/ml in HS-M, and 0.2 ml of the cell suspension was added to 6-mm microwells (Costar 3596). After 90 min incubation at 37°C the nonadherent cells were removed, the monolayers washed three times with HBSS, and HS-M was added. After culturing the monocyte monolayers for 3 days at 37°C, the monocytes were activated with lymphokines and LPS by culturing the monocytes 24 h with a 1:2 dilution of lymphokines prepared from human lymphocytes as previously described (23). On the fourth day of culture the lymphokine-containing medium was removed, and 200 µl of 51Cr-labeled target cells (2 × 10^4 cells/ml) in FCS-M containing 0.1 µCi/ml LPS were added. At this time each well contained about 8 × 10^4 monocytes as judged by visual counting. After culturing 6 h at 37°C the amount of radioactivity released from target cells was determined as described above. Cytolysis of in vitro-cultured unactivated monocytes was determined in an identical manner, except that media containing lymphokines and LPS were replaced with HS-M and FCS-M, respectively.

The percentage of specific lysis was calculated as

\[ \frac{E - S}{T - S} \times 100 \]

where S is the spontaneous release of radioactivity (measured in supernatants from target cells cultured without monocytes), T is the total radioactivity (measured in supernatants from target cell cultures which received SDS to a final concentration of 0.5%), and E is the experimental release (measured in supernatants from monocyte-target cell cocultures).

Chromatofocusing rTNF and CF Using the Fast Protein Liquid Chromatography System. Chromatography was performed as described in Ref. 4. Briefly, CF in monocyte culture medium or rTNF was transferred to 20 mm imidazole hydrochloride, pH 7.3, by gel filtration using Sephadex G-25 PD-10 columns (Pharmacia Fine Chemicals) and applied to a Mono P HR5/20 prepacked column (Pharmacia) equilibrated with imidazole buffer. Proteins were eluted with Polybuffer 74 (Pharmacia), pH 4.0, and the absorbance of the eluent was monitored at 280 nm. Fractions of 2.5 ml were collected and the pH and cytotoxicity of each fraction measured. The optical monitor, pumps, recorder, fraction collector, and gradient programmer were all part of the fast protein liquid chromatography system (Pharmacia). The chromatography was carried out at room temperature.

Sephacryl S-200 Gel Filtration. The column was calibrated with blue dextran, bovine serum albumin (M, 67,000), ovalbumin, (M, 43,000), chymotrypsinogen (M, 25,000), and RNase A (M, 13,700). CF or rTNF was transferred to 20 mm phosphate buffer, pH 6.9, containing 0.3 M NaCl by gel filtration as described above and applied to a Sephacryl S-200 column (1.6 x 80 cm) equilibrated with the same buffer. Elution was carried out with this buffer and fractions of 3 ml were collected and assayed for cytotoxic activity. The absorbance of each fraction was measured at 280 nm. The chromatography was carried out at 2°C.

SDS-Polyacrylamide Gel Electrophoresis. CF and rTNF were analyzed by SDS-PAGE (10% acrylamide gels with 0.8% bis-acrylamide in 0.1 M phosphate buffer, pH 7.2, and 0.1% SDS) and relative molecular weights were estimated, all according to the method of Weber and Osborn (29). Monocyte supernatants containing CF or rTNF in RPMI 1640 or in monocyte supernatants received SDS to a final concentration of 0.1% before electrophoresis. The electrophoresis was run for 3 h with 10 mA/gel in tubes with an inner diameter of 5 mm. The gels were sliced (2 mm) with a Bio-Rad Model 190 gel slicer, and the proteins in each slice were eluted by incubating each slice in RPMI 1640 at 20°C for 15 h. The cytotoxic activity eluted from each slice was then determined. Standard proteins [low-molecular-weight protein electrophoresis calibration kit (Pharmacia) treated with 0.1% SDS] were phosphorylase b (M, 94,000), bovine serum albumin (M, 67,000), ovalbumin (M, 43,000), carbonic anhydrase (M, 30,000), soybean trypsin inhibitor (M, 20,100), and L-lactalbumin (M, 14,400). Gels with standard proteins were stained with Coomassie blue as described in (29).

Antiserum against CF and rTNF. Purification of CF for immunization was performed by ion exchange chromatography, chromatofocusing, and gel filtration as described previously (3), and resulted in an approximately 1000-fold reduction of contaminating proteins in the CF preparation (3). A rabbit was given multiple intraarterial injections of purified CF, and after the fourth injection a neutralizing antiserum of sufficient potency was obtained (5).

Antiserum against rTNF was made by giving a chinchilla rabbit injections three times intradermally at 14-day intervals with 12, 80, and 70 µg rTNF (99% pure; kindly provided by Biogen, S. A. and BASF), respectively. The first two injections were given in the presence of 50% Freund's complete adjuvant and the third injection with 50% Freund's incomplete adjuvant.

Statistics. Results are given as mean ± SD of quadruplicate determinations in single experiments.

RESULTS

Comparison of the Cytotoxic Activity of CF and rTNF. Using the MTT assay for measuring cell death, identical dose-response curves were obtained with CF-containing monocyte supernatants and rTNF (Fig. 1A). The dose-response curves of CF- and rTNF-induced cytolysis measured using the Cr release...
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Fig. 1. Cytotoxic activity of rTNF and CF-containing monocyte supernatants. Various amounts of rTNF- or CF-containing monocyte supernatants (C) were added to WEHI 164 clone 13 target cells, and (A) cell death was measured using the MTT-assay, and (B) cytolysis was measured using the Cr release assay. Bars, SD.

A. MTT-assay

<table>
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<tr>
<th>ng/ml rTNF</th>
<th>CF dilution</th>
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<td>5 x 10^{-7}</td>
<td>5 x 10^{-9}</td>
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<td>5 x 10^{-8}</td>
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B. Cr-release assay

Assay were also identical (Fig. 1B). CF-containing monocyte supernatants and rTNF were also similar with respect to their ability to kill different cell types. Using the MTT assay for measuring cell death, WEHI 164 clone 13 cells were much more sensitive for both CF and rTNF than were L929 cells (Figs. 1 and 2), which in turn were more sensitive than P-WEHI 164 cells (Fig. 2). Moreover, K-562 cells (human chronic myelogenous leukemia cells) (26), HL-60 cells (human promyelocytic leukemia cells) (30), NHIK 3025 cells (human epithelial carcinoma cells) (31), and PC-3 (human prostatic adenocarcinoma cells) (32) were all relatively resistant to both CF-containing monocyte supernatants and rTNF at the concentrations of cytotoxins given in Fig. 2 (Fig. 2 for K-562 cells only; data not presented for HL-60, NHIK 3025, and PC-3 cells). These results are as one would expect if CF and rTNF were similar or identical cytotoxins.

Physicochemical Comparison of CF and rTNF. We have earlier characterized CF and have shown that its isoelectric point is between 6.0 and 5.0, and that its molecular weight as determined by gel filtration is in the range of 45,000–35,000 (3, 4). In order to further compare CF and rTNF we analyzed rTNF by chromatofocusing and gel filtration. CF and rTNF behaved similarly upon chromatofocusing, because rTNF and CF eluted in the pH range of 6.0–5.5, the activity peaks eluting at pH 5.9–5.8 (Fig. 3). Thus, CF and rTNF appear to have similar isoelectric points. CF and rTNF also behaved similarly upon gel filtration, because rTNF eluted with a molecular weight between 45,000 and 35,000 (Fig. 4).

A comparison of CF and rTNF with respect to their molecular weights as determined by SDS-PAGE, was also made. When CF in monocyte supernatants was treated with 0.1% SDS and analyzed by SDS-PAGE, CF migrated with a molecular weight of about 1 mg/ml, whereas the total protein concentration in monocyte supernatants is about 1 mg/ml, the contaminating proteins being largely serum proteins which were originally present in the monocyte culture media (3). In order to obtain similar electrophoresis conditions for CF and rTNF with respect to the relative amount of protein and SDS in the samples which were analyzed, rTNF was added to the same monocyte supernatants containing 0.1% SDS, and the rTNF in the supernatants was then analyzed by SDS-PAGE. The rTNF was added to the monocyte supernatants in a 100-fold excess compared to the amount of CF present in the supernatants, and thus the cytotoxic activity due to rTNF masked that due to CF. At the same time, rTNF did not contribute significantly to the overall protein concentration in the monocyte supernatants, because contaminating proteins were in a 100-fold excess compared to the amount of rTNF added. Under these conditions, rTNF migrated in a manner similar to that of CF, with a molecular weight of about 35,000 (Fig. 5B). When the SDS concentration was raised to 1% in the monocyte supernatants to which rTNF was added, cytotoxic activity was detected at both molecular weights of 35,000 and 17,000 (data not shown), and when pure rTNF in the presence of 0.1% SDS was analyzed directly by SDS-PAGE, cytotoxic activity was detected only at a molecular weight of about 17,000 (Fig. 5C), in agreement with what others have found for the molecular weight of pure TNF as determined by SDS-PAGE (18).

Immunological Comparison of CF and rTNF. CF has previously been purified by ion exchange chromatography, chromatofocusing, and gel filtration, which resulted in approximately a 10^4-fold reduction in the protein content of the CF preparation (3). A rabbit antiserum which neutralizes all the cytotoxic activity in monocyte supernatants has been produced by immunization with the purified CF preparation (5). This anti-CF serum inhibited the cytotoxic activity of rTNF as well as the
cytotoxic activity of CF (Fig. 6). In addition, fusing plasmacytoma cells (NSO) with spleen cells from mice immunized with partially purified CF yielded a hybridoma which secreted an IgM k light chain antibody which bound to rTNF. These results indicate that CF and rTNF are immunologically related and that the cytotoxin, CF, which we previously have characterized, may in fact be natural TNF. This conclusion is further supported by the fact that anti-rTNF serum, which completely inhibited both rTNF-induced cell death (measured with the MTT assay) and cytolysis (measured with the Cr release assay), also completely inhibited the ability of CF-containing monocyte supernatants to induce both cell death and cytolysis (Fig. 7). This indicates that all cytotoxic activity, measured with either the MTT or Cr release assay and WEHI 164 clone 13 cells as target cells, in the crude monocyte supernatants is due to a cytotoxin immunologically related to rTNF.

TNF as an Effector Molecule in Monocyte-mediated Cytotoxicity. CF appears to be an effector molecule in monocyte-mediated cytotoxicity and DDCC, since anti-CF serum inhibits both of these processes (8–10), and target cells which are relatively resistant or susceptible to CF-induced cytolysis are also relatively resistant or susceptible to monocyte-mediated cytotoxicity and DDCC (11, 12). One would consequently expect if CF and natural TNF are identical that anti-rTNF serum would also inhibit monocyte-mediated cytotoxicity and DDCC. This was found to be the case. Anti-rTNF serum inhibited DDCC (Fig. 8) and cytolysis mediated by both freshly isolated monocytes and in vitro-cultured unactivated and lymphokine/LPS-activated monocytes (Fig. 9). This indicates that TNF is an effector molecule in both DDCC and monocyte-mediated cytotoxicity, and that the molecular mechanism in these two processes may be basically similar.

DISCUSSION
Monocyte-mediated cytotoxicity appears to involve a monocyte-derived cytotoxic protein factor, CF, because neutralizing antiserum raised against CF inhibits both monocyte-induced cytolysis and cytoknosis of various target cells (8, 9). We have earlier purified and characterized CF and have shown that its apparent isoelectric point as determined by chromatofocusing is between 6.0 and 5.0 and that its molecular weight is about 40,000, as determined by gel filtration (3, 4).

Monocytes also produce TNF, a protein which induces necrosis of tumors in mice (17). A direct comparison of CF and rTNF revealed immunological, physicochemical, and biological similarities, which thus suggest that TNF is an effector molecule in monocyte-mediated cytotoxicity. Neutralizing antiserum

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*Fig. 3. Comparison of CF and rTNF by chromatofocusing. CF-containing monocyte supernatant (5 ml) (A) and rTNF (10 µg) (B) were transferred to 20 mM imidazole buffer, pH 7.3, and chromatographed on a Mono P chromatofocusing column. C.U., cytotoxic units.*

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*T. Espevik, unpublished results.*
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raised against rTNF completely inhibited the ability of CF-containing monocyte supernatants to induce cytolysis and cell death of sensitive target cells and, conversely, antiserum raised against purified CF completely inhibited the cytotoxic activity of rTNF. Both CF and rTNF have an apparent isoelectric point between pH 6.0 and 5.5, as determined by chromatofocusing and a molecular weight of about 40,000, as determined by gel filtration. Moreover, when present in monocyte supernatants the total protein concentration of which is about 1 mg/ml and to which SDS had been added to a concentration of 0.1%, both CF and rTNF migrated with a molecular weight of about 35,000 using SDS-PAGE. Pure rTNF, however, migrated with a molecular weight of 17,000, in agreement with what others have found (18). This result suggests that in the presence of relatively large amounts of protein (1 mg/ml), 0.1% SDS is not enough to dissociate what appears to be TNF’s dimeric structure, whereas one may obtain a partial dissociation by increasing the SDS concentration to 1% (data not presented) and a full dissociation with 0.1% SDS if the protein content is low, as was the case when pure rTNF was analyzed.

CF and rTNF were also similar with respect to their ability to kill different types of target cells, and the dose-response curves of cytotoxicity obtained with CF-containing monocyte supernatants and rTNF were identical. If CF and rTNF have the same specific activity, one may estimate from the dose-response curves (Fig. 1) that the concentration of CF in monocyte supernatants was about 0.1 µg/ml, which is in agreement with earlier estimates based on the absorbance at 280 nm of a purified CF preparation and on the amount of 125I incorporated into the proteins of this preparation upon iodination (3). A TNF/CF concentration of 0.1 µg/ml was also obtained when TNF/CF in monocyte supernatants was quantitated with anti-

Fig. 6. Effect of increasing amounts of anti-CF and normal rabbit sera on cell death induced by (A) CF-containing monocyte supernatants (0.01 µl/culture well) and (B) rTNF (1 pg/culture well). Cell death was measured using the MTT assay and WEHI 164 clone 13 target cells. The amount of serum given indicates the number of µl of anti-CF or normal serum added per culture well. Bars, SD.
rTNF antibodies and an enzyme-linked immunosorbent assay procedure using rTNF as standard (data not presented).

As has previously been shown to be the case with anti-CF serum (8, 9), anti-rTNF serum inhibited both monocyte-mediated cytotoxic activity and DDCC, again indicating that CF is similar or identical to TNF and that TNF is an effector molecule involved in monocyte-mediated cytotoxicity and DDCC. Moreover, the results suggest that the molecular mechanism in monocyte-mediated cytotoxicity and DDCC may be basically the same. This notion, however, is somewhat contrary to results reported by Colotta et al. (33), which indicate that "classical" monocyte-mediated cytotoxicity and DDCC are separate and distinct effector functions, since macrophages from mice strains with defective classical cytotoxicity may be competent in DDCC. This apparent discrepancy may possibly be due to the fact that Colotta et al. (33) used a long-time (48 h) [3H]-thymidine release assay and TU5 cells as target cells when measuring classical cytotoxicity (33), and that the TU5 cells may possibly be resistant to TNF but sensitive to other monocyte products upon a long-time exposure.

Human monocyte/macrophage cytotoxic factor(s) have also been reported by other groups (34–43). Although more characterization data on these factors are needed to determine definitely whether or not they are related to TNF, we suspect that many of these factors are similar or identical to this cytotoxin. Although IL-1 is a monocyte-produced protein which has many characteristics in common with TNF (44–45), including cytotoxic activity towards some cell types (46), IL-1 does not appear to contribute to the cytotoxic activity in our monocyte supernatants. The production of IL-1 is largely restricted to the first 2 days of in vitro-cultured monocytes (47), whereas our cytotoxic activity may be produced throughout an 8-day in vitro culture period, and our monocyte supernatants produced on day 4 of in vitro culture appeared to contain little IL-1 activity (5). Neither did lymphotoxin contribute to the cytotoxic activity in our supernatants, because the cytotoxic activity measured using the MTT assay and WEHI 164 cells was not inhibited by neutralizing antiserum raised against recombinant lymphotoxin. The fact that anti-rTNF serum inhibited all cytotoxic activity (measured using either the MTT or Cr release assay) in our supernatants suggests that TNF is the major cytotoxin produced by monocytes and the one which is most easily detected when such commonly used TNF-sensitive target cells as WEHI 164 and L929 cells are used for assaying cytotoxic activity. Our results showing that TNF is an effector molecule in monocyte-mediated killing of WEHI 164 cells support studies published while this article was under preparation; those other published studies also show that antiserum against rTNF inhibits mouse natural cytotoxic activity (48) and the killing of tumor cells by activated murine macrophages (49) and human monocytes (50).

We used CF-induced cytostasis of K-562 cells to assay for CF when CF originally was characterized and purified for the production of anti-CF serum (3, 5). The fact that this anti-CF serum inhibited all cytostatic activity in our crude monocyte supernatants (51) and all rTNF activity (Fig. 6B) suggested that all cytostatic activity in the crude monocyte supernatant was due to CF/TNF. We were therefore surprised to find that although rTNF is cytostatic towards K-562 cells, the monocyte supernatant is relatively much more potent in this respect, and that anti-rTNF serum only partially inhibited the cytostatic activity in monocyte supernatants under conditions where it inhibited all the cytotoxic activity measured using the MTT or Cr release assay (Fig. 7). These findings suggest that either (a) due to some structural difference between natural TNF (CF) and rTNF, natural TNF is relatively more cytostatic towards
K-562 cells and antiserum raised against rTNF does not completely neutralize this activity, or (b) there is another monocyte-derived protein in addition to TNF which also has cytostatic activity towards K-562 cells but no detectable cytotoxic activity (measured using MTT or Cr release assay) towards WEHI 164 cells, and this protein copurifies with TNF upon ion exchange chromatography, chromatofocusing, and gel filtration. We are presently attempting to determine which of these two alternatives is correct.

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