

Purification and characterization of an inhibitor (soluble tumor necrosis factor receptor) for tumor necrosis factor and lymphotoxin obtained from the serum ultrafiltrates of human cancer patients

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ABSTRACT Serum ultrafiltrates (SUF) from human patients with different types of cancer contain a blocking factor (BF) that inhibits the cytolytic activity of human tumor necrosis factor α (TNF- α) *in vitro*. BF is a protein with a molecular mass of 28 kDa on reducing sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE). The active material was purified to homogeneity by a combination of affinity chromatography, PAGE, and high-pressure liquid chromatography. Amino acid sequence analysis revealed that BF is derived from the membrane TNF receptor. Purified BF blocks the lytic activity of recombinant human and mouse TNF- α and recombinant human lymphotoxin on murine L929 cells *in vitro*. However, BF inhibits the lytic activity of TNF- α more effectively than it does that of lymphotoxin. The BF also inhibits the necrotizing activity of recombinant human TNF- α when coinjected into established cutaneous Meth A tumors in BALB/c mice. The BF may have an important role in (i) the regulation and control of TNF- α and lymphotoxin activity in cancer patients, (ii) interaction between the tumor and the host antitumor mechanisms, and (iii) use of systemically administered TNF- α in clinical trials with human cancer patients.

Tumor necrosis factor α (TNF- α) and lymphotoxin (LT or TNF- β) are two interrelated proteins that, in native form, are homotrimers of 17- and 17.5-kDa peptides, respectively (1, 2). Their genes are located in tandem within the major histocompatibility complex of mammals (3). While lymphotoxin is predominantly produced by lymphocytes (4), TNF- α is produced by macrophages, lymphocytes, and other cells in selected situations (5). These cytokines have many effects *in vitro* when tested on different kinds of cells (6) such as (i) growth inhibition or lysis of transformed cells, (ii) activation of phagocytic cells, (iii) upregulation of various cell surface proteins, and (iv) control of the development and expression of cell-mediated antitumor responses. They also can cause a spectrum of reactions *in vivo* (7–9), some of which include (i) necrosis of tumors, (ii) leukocytosis and inflammation, and (iii) wasting and shock. The activities attributed to these cytokines have evoked interest in the potential use of these molecules as anticancer agents (10). However, clinical trials to date indicate that systemically administered TNF- α has, at best, limited clinical efficacy (11).

We recently demonstrated that serum ultrafiltrates (SUF) obtained from patients with various types of cancer contained factor(s) that could inhibit the cytolytic activity of both recombinant human TNF- α and lymphotoxin on L929 cells *in vitro* (12, 13).

In the present study, the active material in the SUF was purified and shown to be a 28-kDa protein and to express the biologic activity measured in SUF. This material termed "blocking factor" (BF) has N-terminal amino acid sequences similar to a soluble TNF-binding factor identified previously in the urine of postmenopausal women and patients with chronic renal failure (14–17). We have demonstrated (18) that BF is the extracellular N-terminal domain of a protein derived from the membrane TNF receptor.

MATERIALS AND METHODS

SUF. All SUF were obtained from Rigdon Lentz at the John Kennedy Hospital (Indio, CA). The ultrafiltration procedure has been described (12). Patients used in these studies had advanced stages of cancers of prostate, breast, brain, and bowel. They had not received chemotherapy for 1–2 months prior to ultrafiltration.

Assays for Activity of TNF, Lymphotoxin, and BF. The recombinant human TNF- α and lymphotoxin were donated by Genentech, and recombinant murine TNF was purchased from Amgen Biologicals. Activity was assayed on L929 mouse fibroblasts as described (19).

To assay the activity of the BF, monolayers of nondividing L929 cells (8×10^4 cells per well) were incubated in a final volume of 200 μ l with 1 μ g of actinomycin D (Sigma) per ml. Various dilutions of TNF- α or lymphotoxin and 25 μ l of sample solution containing either BF or control medium were added to the L929 cells in 96-well microplates. After 18 hr in 5% CO₂/95% air at 37°C, the media were aspirated and cells were stained with 1% crystal violet for 5 min, washed with water, and solubilized with 100 μ l of 100 mM HCl in methanol. Adherent cell number is determined by the OD at 600 nm measured in an EAR 400 AT ELIZA plate reader (SLT-Labinstruments, Salzburg, Austria). Neutralization of TNF *in vitro* activity with rabbit antibody was performed by the method of Kashiwa *et al.* (20). One unit of inhibition is calculated by the following formula: 1 unit = [LD₅₀ (TNF or lymphotoxin + sample) – LD₅₀ (TNF or lymphotoxin alone)]/LD₅₀ (TNF or lymphotoxin alone).

TNF-Affinity Column. SUF from patients with the highest levels of BF activity as measured in the L929 cytolytic assay (13) were pooled. A total of three separate purification runs were performed on a total of 52 liters of SUF. Proteins in the SUF were precipitated by the addition of crystalline, ultra-pure ammonium sulfate (Fisher) to reach 80% saturation and

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Abbreviations: TNF- α , tumor necrosis factor α ; BF, blocking factor; SUF, serum ultrafiltrates; i.t., intratumor(ally).
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then were incubated overnight at 4°C. The precipitate was collected by centrifugation and then solubilized in 2 liters of phosphate-buffered saline (PBS; 0.01 M phosphate/0.15 M NaCl, pH 7.2). The solution was dialyzed against three changes of 10 liters each of 10 mM Tris-HCl/50 mM NaCl, pH 7.4, overnight at 4°C.

A TNF-affinity column was prepared by coupling 4 mg of TNF- α to 1.0 g of CNBr-activated Sepharose 4B (Pharmacia) in coupling buffer (0.1 M NaHCO₃/1 M NaCl, pH 9.0). The TNF-Sepharose was poured into a 5-ml column and was washed three times in 100 ml of 1 M NaCl/0.1 M sodium acetate, pH 8.0, and 1 M NaCl/0.1 M boric acid, pH 4.0, alternately before use. The 2 liters of dialyzed sample was applied to the TNF-affinity column at a flow rate of 10 ml/hr. The column was then washed with 50 mM Tris-HCl (pH 8.0) until the absorbance at 280 nm of the output solution was negative. The column was then eluted with three consecutive 3-ml aliquots of 0.2 M glycine-HCl (pH 2.5), and the total eluate was dialyzed against PBS at 4°C for 16 hr. Protein concentration in all samples was determined by absorbance at 260/280 nm in a Beckman spectrophotometer.

Reducing and Nonreducing Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis (SDS/PAGE). A total of 1–10 μ g of protein from the affinity column eluate was subjected to both reducing and nonreducing SDS/PAGE as described by Laemmli (21). Protein was visualized by silver staining (22). The eluate was also subjected to nonreducing SDS/PAGE in which the individual lanes were cut into 4-mm slices (16, 23). Proteins in each slice were eluted by overnight incubation in 1 ml of 10 mM Tris-HCl/1 mM EDTA, pH 7.4. The eluates from gel slices were dialyzed overnight against PBS to remove SDS and then were assayed for BF activity as described in the preceding section. The molecular mass of BF was calculated by comparison with coelectrophoresed samples from an electrophoresis calibration kit (Pharmacia) with the following markers: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α -lactalbumin.

Amino Acid Sequence Analysis. Samples of eluate from the TNF-affinity column were next separated by HPLC. Samples were loaded on small (1.5 mm \times 50 mm) glass columns packed with 15- μ m C₁₈ reversed-phase material (J. T. Baker).

The column was eluted on an HP 1090 high-pressure liquid chromatograph with a linear gradient of 1–60% *n*-propyl alcohol in 0.1% CF₃COOH (Applied Biosystems) in water at a rate of 0.2 ml/min. Each protein peak was collected and sequenced. Sequence analysis was conducted by Edman degradation on a gas/liquid-phase prototype sequencer (patent no. EP0257735). The liquid-phase buffer was 0.1 M Quadrol (pH 9.0; Pierce), the Edman reagent was phenyl isothiocyanate (Pierce), and the cleavage reagent was CF₃COOH. Analysis of the phenyl isothiocyanate derivatives of the Edman chemistry was done on an HP 1090 high-pressure liquid chromatograph.

Biological Activity of BF *in Vivo*. A 0.05-ml aliquot of Meth A tumor (4×10^6 cells per ml; obtained from L. J. Old, Memorial Sloan-Kettering Cancer Center, New York) was transplanted intradermally into the abdominal wall of BALB/c mice (24). Seven days later, the animals were separated into three groups that received the following intratumor (i.t.) injections: (i) 100 μ l of PBS, (ii) 100 ng of TNF- α in PBS, or (iii) 100 ng of TNF- α followed in a few minutes by 200 units of BF. The injection volume was kept constant for a total of 100 μ l at each site. The animals were examined daily and the level of tumor necrosis was recorded over a 3-day period.

RESULTS

Purification of TNF/Lymphotoxin BF. Three individual lots of SUF were purified as described in *Materials and Methods*. The equivalent at 20 liters (160–300 g of protein) of the SUF was passed through the TNF-affinity column, and about 25,000 units of the BF (200–500 μ g of protein) were routinely eluted from the TNF-affinity column. The eluate from the TNF-affinity column derived from each of the lots contained multiple bands when subjected to nonreducing SDS/PAGE as shown in Fig. 1A. The proteins were eluted from slices of the other half of this gel and tested in the TNF/lymphotoxin BF assay. As shown in Fig. 1B, the activity against both TNF- α and lymphotoxin was localized to a single peak (between slice no. 8 and 10) (Fig. 1B *Inset*). The blocking activity against lymphotoxin was about 1 order

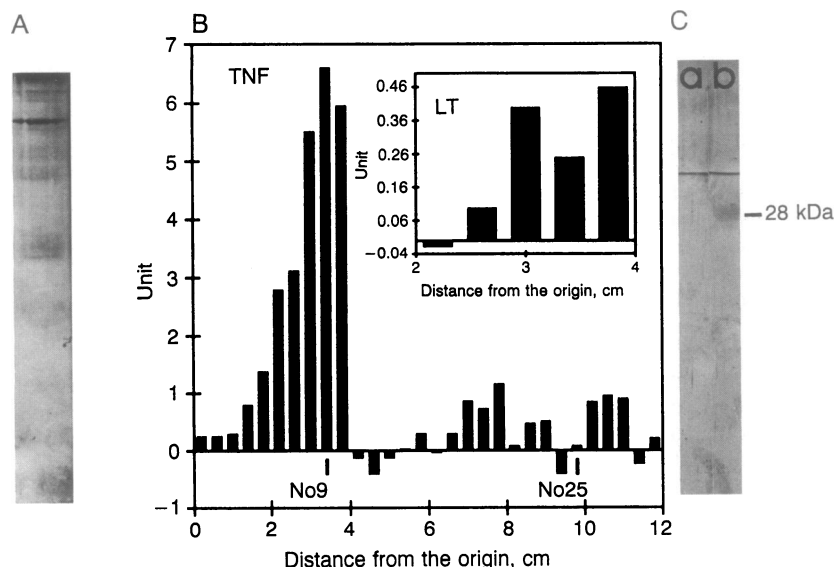


FIG. 1. Isolation of TNF/lymphotoxin BF from the SUFs of cancer patients. The SUF was passed over and eluted from a TNF-affinity column. A sample of the eluate was then subjected to horizontal electrophoresis in nonreducing SDS/PAGE. The gel was sliced into two sections, and one section was stained by the silver method (A). The other half was cut into 4-mm slices, and the protein was eluted and tested for its ability to block TNF- α - and lymphotoxin (LT)-induced lysis of L929 cells (B). The inhibition in both B and in B *Inset* represents the level of blocking activity in individual gel slices. Material eluted from nonactive slice no. 25 and the peak of blocking activity in slice no. 9 were reelectrophoresed in reducing SDS/PAGE. Silver-stained gels of these fractions are shown in C. Lanes: left, no. 25; right, no. 9.

| | | | | | | | | | | |
|----|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| a) | Asp | Ser | Val | Cys | Pro | Gln | Gly | Lys | Tyr | Ile |
| b) | Asp | Ser | Val | Cys | Pro | ----- | ----- | ----- | ----- | ----- |
| c) | Asp | Ser | Val | X | Pro | Gln | Gly | Lys | Tyr | Ile |
| | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| a) | His | Pro | Gln | Asn | Asn | Ser | Ile | Cys | Cys | Thr |
| b) | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| c) | His | Pro | Gln | Val | Asn | Ser | Ile | X | Lys | Thr |

FIG. 2. Comparison of N-terminal amino acid sequences of TNF BF and TNF-binding factor. Lines: a, TNF BF from cancer patient SUF; b, TNF-binding factor from urine of healthy postmenopausal women (17); c, TNF BF from urine of patients with chronic renal failure (15).

of magnitude weaker than against TNF- α . An active and an inactive fraction from the nonreducing SDS/PAGE were subjected to further electrophoresis in a reducing SDS/PAGE gel, and a single band of 28 kDa in the active fraction was detected by silver staining (Fig. 1C).

Amino Acid Sequencing. Approximately half of the eluate from the TNF affinity column was subjected to further separation on HPLC. A total of three protein peaks were sequentially eluted from the HPLC column. Each peak protein was collected and sequenced on a prototype sequencer (patent no. EP0257735). The amino acid sequences obtained were then compared with the protein sequences in the data base by using the DFASTP program. The sequence from the first peak was the only unknown sequence. All remaining peaks are known serum proteins (immunoglobulins and transferrin). The sequencing data for the BF was obtained from two separate purification lots (Fig. 2, line a). Reducing SDS/PAGE of this unknown peak showed a single band of 28 kDa (data not shown). Fig. 2

compares the three N-terminal amino acid sequences reported for these factors by the different groups. The partial N-terminal amino acid sequence is identical to the five N-terminal amino acids reported by Engelmann *et al.* (17) (Fig. 2, line b) and differs at positions 4, 14, 18, and 19 from the 20-residue N-terminal amino acid sequence published by Olsson *et al.* (15) (Fig. 2, line c).

In Vitro Activity of BF Against Human and Murine TNF. The activity of BF derived from the TNF-affinity column fraction was tested against recombinant murine and human TNF- α . The results in Fig. 3A show that BF inhibited the cytolytic activity of both recombinant human and murine TNFs. In contrast, rabbit anti-human and anti-mouse TNF antibodies neutralized only the specific TNF- α (Fig. 3A).

BF Effects on TNF-Induced Necrosis of Meth A Tumors in BALB/c Mice. Cutaneous Meth A tumors were produced on the abdominal wall of BALB/c mice by the intradermal injection of 2×10^5 Meth A tumor cells. After 7 days, tumor-bearing animals were divided into three groups, each containing four animals: (i) one group was injected i.t. with PBS, (ii) a second group was injected i.t. with TNF- α in PBS, and (iii) the last group was injected i.t. with TNF- α and BF derived from the TNF-affinity column fraction. All injections were made in the same final 100- μ l volume. TNF caused central necrosis of the transplanted tumors. Tumor necrosis was measured over a 72-hr period. The photographs in Fig. 3 illustrate the result 36 hr after injection. Necrosis was clearly evident at 36 hr in TNF- α treated tumors (Fig. 3Bb). In contrast, tumors treated with TNF and BF did not exhibit the TNF-induced necrosis (Fig. 3Bc). Control untreated tumors are shown in Fig. 3Ba. Although the data are not shown, BF alone had no effect.

DISCUSSION

The serum and the SUF obtained from human patients with various forms of cancer contain a protein that can block the

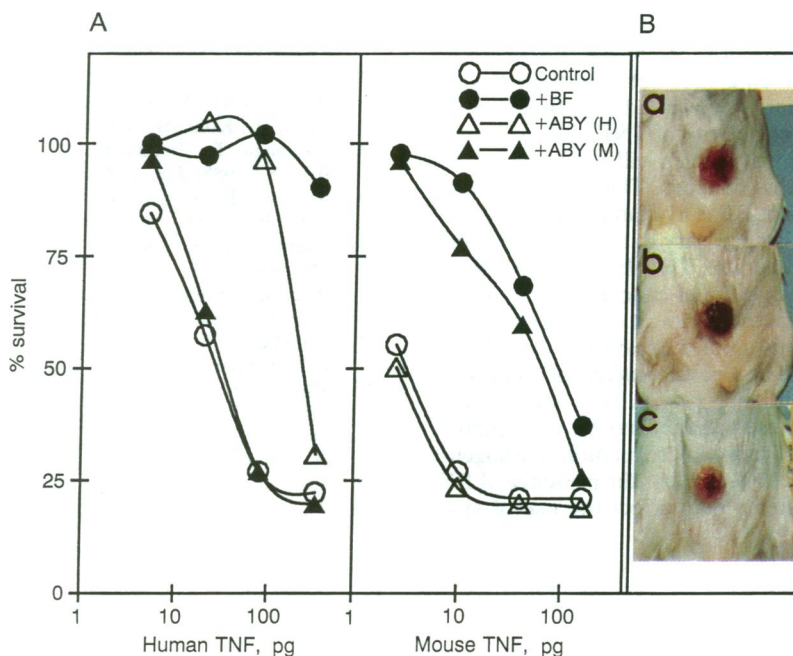


FIG. 3. Biological activity *in vitro* and *in vivo*. (A) Comparison of blocking activity against human and murine TNF-induced cytotoxicity of murine L929 cells *in vitro*. The cytotoxic assay was the same as Fig. 1. One hundred units of BF derived from the TNF-affinity column fraction were added with TNF to each assay well. In one set of assays, 25 neutralizing units of polyclonal rabbit anti-murine [ABY-(M)] TNF obtained from Genzyme and 10 neutralizing units of polyclonal rabbit anti-human [ABY-(H)] TNF produced in our laboratory were added. (B) Effect of BF on the necrotizing activity of human TNF- α *in vivo*. Sixteen BALB/c mice bearing 7-day cutaneous Meth A tumors were separated into three groups. Each group received injections i.t. of 100 μ l of PBS (a), 100 ng of TNF- α and PBS (b), and 100 ng of TNF- α and 200 units of BF derived from the TNF-affinity column fraction (c). The photographs show tumor necrosis at 36 hr; necrosis is clearly evident in TNF-treated tumors (b) and absent in BF/TNF- α -treated tumors (c) and PBS-treated tumors (a).

cytolytic activity of recombinant human TNF- α and, to a lesser extent, recombinant human lymphotoxin *in vitro* (13). In this study, we purified this material to homogeneity, determined its molecular weight in SDS/PAGE, and obtained a partial N-terminal amino acid sequence. The apparent molecular mass of the BF in SDS/PAGE is 28 kDa. It appears to be slightly smaller than the molecular mass reported (30 kDa) for other TNF-binding factors (15, 17). The partial N-terminal amino acid sequence is identical to the 5-residue N-terminal amino acid sequence reported by Engelmann *et al.* (17) and differs at positions 4, 14, 18, and 19 from the 20-residue N-terminal amino acid sequence published by Olsson *et al.* (15).

Engelmann has postulated that the TNF BF in urine from postmenopausal women is the membrane TNF receptor (17). Recently, we finished sequencing a TNF receptor cDNA clone obtained from human placental cDNA libraries, using degenerative probes deduced from the amino acid sequences of TNF BF derived from SUF (18). The nucleotide sequence has an open reading frame that can be translated to a peptide of 455 amino acids with a calculated molecular mass of 55 kDa. In the same journal Lesslaure and coworkers (25) reported an identical sequence of a cDNA for TNF membrane receptor. Collectively, these data suggest that all of the soluble BF isolated to date are apparently derived from the 55-kDa TNF membrane receptor (18). Thus, the BF in the serum from cancer patients appears to be a fragment derived from the TNF membrane receptor. A second possibility is that BF is produced by alternative mRNA splicing. However, our preliminary data derived from Northern blotting analysis of mRNA prepared from different human cell lines does not support this possibility.

It has been proposed by several authors (26, 27) that regulation of specific membrane receptors could serve as a mechanism of cytokine regulation. The SUF BF might be an example of this kind of regulation. The inhibitory activity of BF may be exerted by binding to the TNF and the lymphotoxin so that these cytokines can no longer interact with the relevant cell and tissue receptor(s). The finding that the BF can block the cytolytic activity of recombinant mouse TNF suggested that the active binding sites between the BF and the TNF has been evolutionarily conserved. Our finding that BF can inhibit the necrotizing activity of TNF *in vivo* is very significant; however, additional studies will have to be conducted to further support this concept. These studies may have to wait until recombinant BF is available to provide the quantity of purified materials necessary to test this question.

The mechanism(s) that generates TNF BF and the significance of this material in the serum of cancer patients is not yet clear. If it is derived from the tumor, it could represent a means of inhibitory host anti-tumor mechanisms, and if it is from normal tissues, it could be a natural means of control of these cytokines. Only additional studies will resolve this question; however, BF in these patients may be very important to our understanding of cytokine regulation, pathogenesis, and tumor-host interaction. Finally, the presence of BF in the serum of cancer patients may affect the use of sys-

temically administered TNF in clinical trials with cancer patients.

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