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Immunology

Purification and characterization of a human tumor necrosis factor from the LuKII cell line

(lymphokine/antitumor factor/cancer therapy)

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ABSTRACT **A factor with tumor necrosis factor (TNF) activity produced by the LuKII human lymphoblastoid cell line [designated TNF(LuKII)] was purified sequentially by using controlled-pore glass, lentil lectin-Sepharose, and procion red agarose chromatography, yielding TNF with a specific activity of 1.5×10^7 units per mg of protein and an isoelectric point of ≈6.7. Purified TNF(LuKII) fractionated by NaDodSO$_4$/PAGE under reducing as well as nonreducing conditions was found to contain seven protein bands of M_r 80,000, 70,000, 43,000, 25,000, 23,000, 21,000, and 19,000. The proteins of M_r 80,000 and 70,000 could not be dissociated into lower molecular weight components. Peptide mapping analysis and immunoblotting analysis revealed that the seven protein bands in the purified TNF(LuKII) preparations are related. After fractionation of TNF(LuKII) by NaDodSO$_4$/PAGE under reducing conditions, TNF activity was recovered from the regions of M_r 70,000 and 19,000–25,000. Purified human TNF(LuKII) (*i*) produces hemorrhagic necrosis of Meth A mouse sarcoma in the standard *in vivo* mouse TNF assay; (*ii*) has the same pattern of reactivity as mouse TNF (cytotoxic/cytostatic/no effect) on a panel of human cancer cell lines; and (*iii*) has its anticellular effect potentiated by interferon, also a feature of mouse TNF.**

The presence of a tumor inhibitory factor in the sera of mice infected with bacillus Calmette–Guérin (BCG) and subsequently injected with endotoxin was reported by Carswell *et al.* (1). Sera from these mice cause necrosis and regression of certain tumors in mice and have a cytotoxic effect on tumor cells *in vitro* (1–5). By using similar methods, a factor with the same *in vivo* and *in vitro* properties can be induced in rats (1) and rabbits (1, 6, 7). The antitumor factor present in the sera of animals sensitized to BCG or other immunopotentiating agents, such as *Corynebacterium parvum* or Zymosan, and then challenged with endotoxin has been termed tumor necrosis factor (TNF). Biochemical studies have indicated that serum TNF activity is associated with both high molecular weight components (4, 8) and components in the M_r range of 40,000 to 70,000 (3–5, 9, 10).

We have recently reported that human cell lines of hematopoietic origin have the capacity to produce a factor with TNF activity (11). The product of one of the lines (LuKII) was chosen for detailed studies and, according to the following criteria, TNF(LuKII) and mouse TNF have identical properties: (*i*) mouse L cells made resistant to mouse TNF are resistant to TNF(LuKII), and L cells made resistant to TNF(LuKII) are resistant to mouse TNF; (*ii*) the anticellular response of a panel of human cell lines to TNF(LuKII) or mouse TNF is indistinguishable and can be potentiated in a synergistic fashion by interferon; and (*iii*) TNF(LuKII) causes hemorrhagic necrosis of Meth A sarcomas in the standard *in vivo* TNF assay (11).

In the present study, we report a sequential chromatographic procedure for the purification of TNF(LuKII) and describe biochemical, serological, and biological characteristics of purified TNF(LuKII).

MATERIALS AND METHODS

Production of TNF(LuKII). LuKII cells (8×10^5 cells per ml) in RPMI 1640 medium containing 8% fetal calf serum were incubated with 10 ng of mezerein per ml (L. C. Services, Woburn, MA) for 48 hr. The cells were separated by centrifugation, resuspended in fresh RPMI medium lacking any protein supplement, and incubated for an additional 48 hr. Cells were removed by centrifugation, and the culture media were used as the source of TNF(LuKII).

***In Vitro* TNF Assay.** TNF assays were performed in 96-well microtiter plates. Serially diluted fractions were sterilized by ultraviolet radiation and TNF-sensitive L cells were added to each well at a density of 2×10^4 cells per well in 100 μl. After incubation for 2 days at 37°C, the plates were examined microscopically and the percentage of dead cells was determined. The unitage of the sample was calculated as the reciprocal of the highest dilution that killed 50% of the cells. All TNF assays were run in parallel with a laboratory standard and titers are expressed in laboratory units.

***In Vivo* TNF Assay.** The standard Meth A sarcoma assay was performed as described (11).

Monoclonal Antibody to Human TNF. BALB/c mice were injected with 1600 units of TNF(LuKII), with a specific activity of 1.5×10^7 units/mg. For the initial injection, TNF(LuKII) was mixed with Freund's complete adjuvant (1:1) and was injected subcutaneously. Subsequent injections were given intraperitoneally in the absence of adjuvant. Serum antibody to TNF(LuKII) was determined by an ELISA with TNF(LuKII) bound to polystyrene plates. After nine immunizations over a period of 7 months, the spleen of one mouse with a high titer of TNF(LuKII) antibody was removed and fused with cells of the P_3U_1 mouse plasmacytoma cell line. Resulting clones were screened for their ability to bind TNF(LuKII) in ELISAs. A hybridoma (designated T1-18) producing antibody reactive with TNF(LuKII) was isolated and subcloned. Media from T1-18 hybridoma cultures served as a source of TNF(LuKII) antibody.

Affinity Chromatography. Affinity chromatography procedures were carried out at room temperature and column fractions were collected into polypropylene tubes or bottles. The column matrices used were controlled-pore glass 350 (Electro-Nucleonics, Fairfield, NJ), lentil lectin-Sepharose

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Abbreviation: TNF, tumor necrosis factor.

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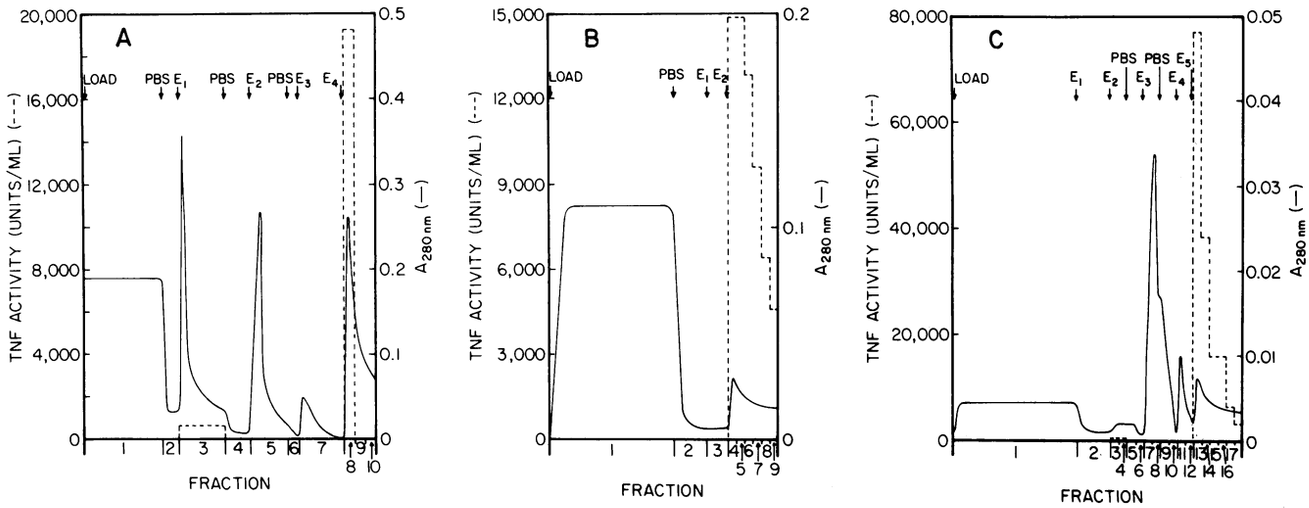

FIG. 1. (*A*) Controlled-pore glass column chromatography. LuKII culture medium (8 liters) containing 200 units of TNF activity per ml was applied to a controlled-pore glass column (50 ml) equilibrated with phosphate-buffered saline (20 mM sodium phosphate, pH 7.0/0.15 M NaCl) (PBS). The column was washed with the following buffers in sequence: PBS (75 ml); PBS containing 20% ethylene glycol (vol/vol) (E_1) (225 ml); PBS (120 ml)/20 mM sodium phosphate, pH 7.0/1.15 M NaCl (E_2) (175 ml); PBS (50 ml)/5 mM sodium phosphate, pH 6.8 (E_3) (225 ml); and 5 mM sodium phosphate, pH 6.8/5% triethylamine (vol/vol) (E_4) (150 ml). Eluted fractions were collected in polypropylene bottles. The material eluted with the E_4 buffer was collected in 50-ml aliquots. (*B*) Lentil lectin Sepharose column chromatography. Partially purified TNF(LuKII) (150 ml) eluted from the controlled-pore glass column was loaded onto a lentil lectin-Sepharose column (10 ml) equilibrated with PBS. The column was washed sequentially with PBS (40 ml), PBS/1 M NaCl (E_1) (24 ml), and PBS/1 M NaCl/0.2 M methyl-α-D-mannoside (E_2) (60 ml). The material eluted with the methyl-α-D-mannoside-containing buffer was collected in 10-ml aliquots. (*C*) Procion red agarose column chromatography. Partially purified TNF(LuKII) (60 ml) eluted from the lentil lectin-column was diluted 1:1 with PBS and loaded onto a procion red agarose column (4 ml) equilibrated with 20 mM sodium phosphate, pH 6.8/0.65 M NaCl (PBS/0.5 M NaCl). The column was washed with the following buffers in sequence: PBS/0.5 M NaCl (E_1) (30 ml); PBS/1 M NaCl (E_2) (8 ml); PBS (8 ml); PBS/50% ethylene glycol (vol/vol) (E_3) (8 ml); PBS (8 ml); 0.1 M Tris·HCl, pH 9.4/0.1 M NaCl (E_4) (8 ml); and 0.1 M Tris·HCl, pH 9.4/0.1 M arginine (E_5) (24 ml). The material eluted with the 0.1 M Tris·HCl, pH 9.4/0.1 M arginine buffer was collected in 4-ml aliquots.

(Pharmacia), and procion red agarose (Bethesda Research Laboratories).

Protein Determinations. Protein determinations were carried out with the Bio-Rad dye reagent (Bio-Rad) using bovine serum albumin as a standard.

Radioiodination of TNF(LuKII). TNF(LuKII) was labeled with ^{125}I using 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Iodo-Gen, Pierce). Polypropylene tubes were coated with 100 μg of Iodo-Gen (dissolved in chloroform) by evaporation of the solvent. A 2-ml sample of TNF(LuKII) (50,000 units/ml) with a specific activity of 1.5×10^7 units per mg of protein was incubated for 25 min at room temperature in an Iodo-Gen-coated tube containing 2 mCi of ^{125}I (1 Ci = 37 GBq). The labeled protein was then separated from the unbound ^{125}I by using a P-4 column (Bio-Rad) equilibrated with phosphate-buffered saline containing 50 μg of cytochrome *c* per ml. The iodinated material eluted in the void volume of the column was divided into aliquots and stored at −80°C.

NaDodSO$_4$/PAGE. NaDodSO$_4$/PAGE was carried out in 18-cm slab gels according to published methods (12).

Isoelectrofocusing. Isoelectrofocusing was performed by using Ampholine Pagplates (pH 3.5–9.5) (LKB). The gels were run at 30 W for 1.5 hr, at which time the pH gradient was measured and the gel was sliced into 18 equal pieces. The gel fractions were incubated for 18 hr in Eagle's minimum essential medium (ME medium) containing 10% fetal calf serum and fractions were assayed for TNF *in vitro*.

Peptide Mapping Analysis. A ^{125}I-labeled preparation of purified TNF(LuKII) was fractionated by NaDodSO$_4$/PAGE and individual bands localized by autoradiography were cut from the gel and treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin or *N*-α-tosyllysine chloromethyl ketone-treated chymotrypsin. Digested fractions were analyzed according to the methods of Elder *et al.* (13).

Immunoblotting Analysis. Immunoblotting was performed essentially as described (14). Briefly, preparations of purified TNF(LuKII) were transferred to nitrocellulose paper overnight at 100 mA. After incubation of the nitrocellulose paper in buffer containing bovine serum albumin, the paper was exposed for 2 hr to 40 ml of T1-18 antibody-containing culture medium. The nitrocellulose paper was then washed extensively and incubated overnight in 10 mM Tris·HCl, pH 7.4/0.9% NaCl/5% bovine serum albumin/^{125}I-labeled rabbit anti-mouse IgG. The nitrocellulose paper was further washed and exposed to x-ray film.

RESULTS

Purification of TNK(LuKII). Controlled-pore glass beads bound all TNF activity from LuKII culture fluids. After washing with several buffers in sequence, TNF activity was

Table 1. Purification of TNF(LuKII)

	Load		Recovery			
Column	Units	Specific activity, units/mg	Units	Specific activity, units/mg	% recovery	-Fold purification
Controlled-pore glass	1.6×10^6	5.3×10^3	9.6×10^5	3.8×10^5	60	72
Lentil lectin-Sepharose	9.6×10^5	3.8×10^5	6.3×10^5	1.3×10^6	39	245
Procion red agarose	6.3×10^5	1×10^6	6.3×10^5	1.5×10^7	39	2830

eluted with a 5 mM sodium phosphate buffer (pH 6.8) containing 5% triethylamine (Fig. 1*A*). The eluted TNF was then applied to a lentil lectin-Sepharose column, which was washed first with phosphate-buffered saline and then with 0.02 M sodium phosphate buffer (pH 6.8) containing 1.15 M NaCl (buffer A). TNF activity was eluted from this column with buffer A containing 0.2 M methyl-α-D-mannoside (Fig. 1*B*). All TNF activity bound to the lentil lectin-Sepharose column and 39% of the activity was recovered in the methyl-α-D-mannoside-containing buffer. (Further washing of the column with buffer containing 50% ethylene glycol elutes only a small amount of additional TNF activity.) TNF from the lentil lectin column was then diluted 1:1 with phosphate-buffered saline and loaded onto a procion red agarose column. The column was washed sequentially with several buffers that remove protein having no TNF activity. The column was then washed with 0.1 M Tris·HCl, pH 9.4/0.1 M arginine. TNF activity was eluted with this buffer, yielding TNF with a specific activity of 1.5×10^7 units per mg of protein. Table 1 summarizes the purification scheme for TNF(LuKII) with specific activities of the resulting fractions.

Biochemical Characterization of Purified TNF(LuKII). Isoelectric focusing of purified TNF(LuKII) indicates an isoelectric point of ≈6.7 (Fig. 2). ^{125}I-labeled TNF(LuKII) with a specific activity of 1.5×10^7 units per mg of protein was analyzed by NaDodSO$_4$/PAGE and found to contain seven protein bands with M_r values of 80,000, 70,000, 43,000, 25,000, 23,000, 21,000, and 19,000 (Fig. 3). The same seven protein bands were observed when nonlabeled purified TNF(LuKII) was fractionated by NaDodSO$_4$/PAGE and examined by silver staining. The proteins of M_r 80,000 and 70,000 were eluted from the gels and reanalyzed by NaDodSO$_4$/PAGE. They migrated once again to the M_r 70,000–80,000 region, and no lower molecular weight components were observed. In further experiments, purified TNF(LuKII) was boiled in NaDodSO$_4$, urea, and 2-mercaptoethanol, and the same characteristic seven bands were found.

To determine which bands in TNF(LuKII) showed TNF activity, parallel samples of purified TNF(LuKII), one ^{125}I-labeled and one unlabeled, were treated with 0.1% NaDodSO$_4$/0.1 M 2-mercaptoethanol and fractionated by NaDodSO$_4$/PAGE. After electrophoresis, the lane containing the unlabeled material was cut into 4.4-mm slices and the proteins were eluted from each slice by overnight incubation at 4°C in ME medium containing fetal calf serum. The parallel lane containing ^{125}I-labeled TNF(LuKII) was dried immediately after electrophoresis and protein bands were located by autoradiography. As seen in Fig. 4, TNF activity was recovered from the gel at M_r values of 70,000 and 19,000–25,000, corresponding to ^{125}I-labeled protein bands at these positions. TNF(LuKII) samples that were not exposed to 2-mercaptoethanol before NaDodSO$_4$/PAGE also showed TNF activity at M_r values of 70,000 and 19,000–25,000.

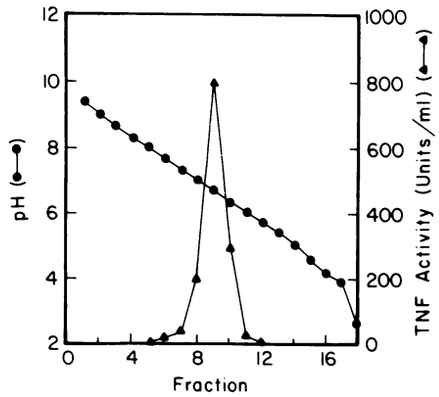

FIG. 2. Isoelectrofocusing of TNF(LuKII). A 60-μl sample of purified TNF(LuKII) containing 1500 units was applied to an ampholine gel (pH 3.5–9.5).

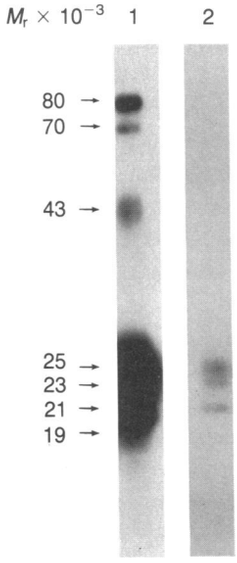

FIG. 3. NaDodSO$_4$/PAGE of purified ^{125}I-labeled TNF(LuKII). TNF(LuKII) was iodinated and fractionated by NaDodSO$_4$/PAGE. Autoradiographs were developed for 18 hr (lane 1) and 0.5 hr (lane 2). The following proteins provided M_r markers: myosin (M_r, 200,000), β-galactosidase (M_r, 130,000), phosphorylase b (M_r, 94,000), bovine serum albumin (M_r, 67,000), ovalbumin (M_r, 43,000), α-chymotrypsinogen (M_r, 25,700), β-lactoglobulin (M_r, 18,400), lysozyme (M_r, 14,300), and cytochrome *c* (M_r, 12,300).

To examine the relationships among the various protein bands in purified TNF(LuKII) preparations, two-dimensional chymotryptic and tryptic peptide mapping analyses were performed. As seen in Fig. 5*a*, the chymotryptic peptide maps demonstrate that the proteins of M_r 43,000, 25,000, 23,000, 21,000, and 19,000 are related and the proteins of M_r 80,000 and 70,000 are related. To examine the relationship of the larger molecular weight proteins to the smaller proteins, chymotryptic digests of the M_r 70,000 and 25,000 proteins containing equal amounts of radioactivity were mixed and analyzed. As seen in Fig. 5*b*, three of the fragments (termed A, B, and C) generated by digestion of the M_r 25,000 protein migrate to the same position as three fragments generated by digestion of the M_r 70,000 protein. A similar analysis was carried out using trypsin as the proteolytic enzyme. The results also indicate that the seven distinct forms are closely related.

Further evidence for the relationships among the various proteins in purified TNF(LuKII) comes from immunoblotting analysis with T1-18 monoclonal antibody to TNF(LuKII). Fig. 6 shows that the antibody reacts with the M_r 43,000 and the M_r 19,000–25,000 components.

Biological Characteristics of Purified TNF(LuKII). Limulus

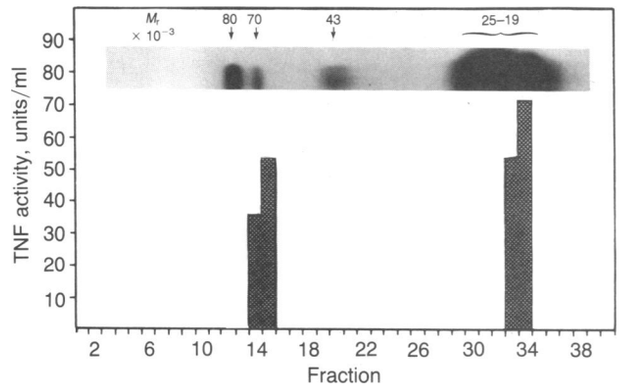

FIG. 4. Recovery of TNF activity after NaDodSO$_4$/PAGE fractionation of TNF(LuKII). A sample of TNF(LuKII) containing 6000 units adjusted to contain 0.1% NaDodSO$_4$ and 0.1 M 2-mercaptoethanol was applied to a 12% polyacrylamide gel. After electrophoresis, the gel was sliced and activity was eluted and assayed. In an adjacent lane, ^{125}I-labeled TNF(LuKII) was fractionated and autoradiographed to determine the molecular weight of the TNF(LuKII) active fractions.

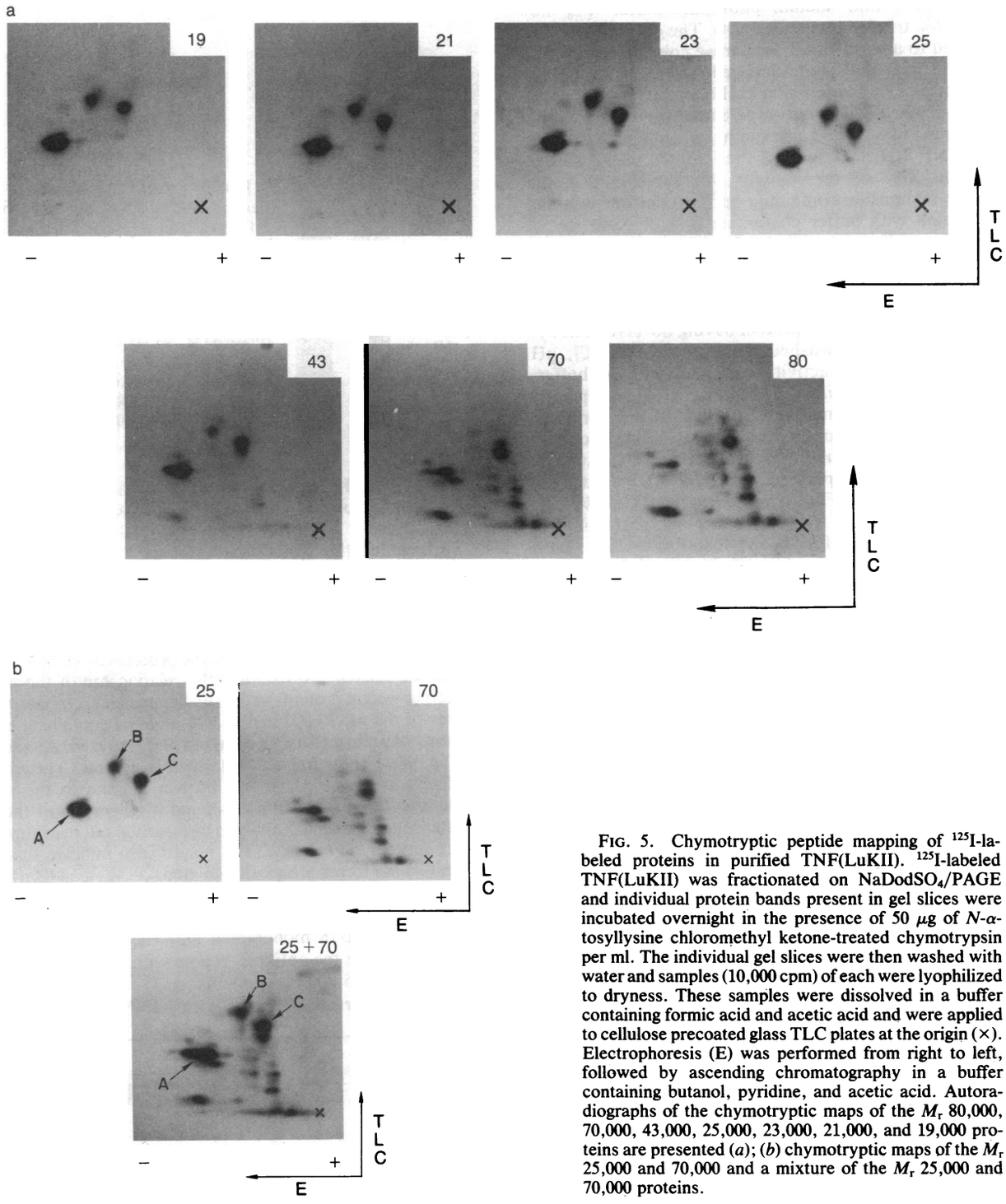

FIG. 5. Chymotryptic peptide mapping of ^{125}I-labeled proteins in purified TNF(LuKII). ^{125}I-labeled TNF(LuKII) was fractionated on NaDodSO$_4$/PAGE and individual protein bands present in gel slices were incubated overnight in the presence of 50 μg of *N*-α-tosyllysine chloromethyl ketone-treated chymotrypsin per ml. The individual gel slices were then washed with water and samples (10,000 cpm) of each were lyophilized to dryness. These samples were dissolved in a buffer containing formic acid and acetic acid and were applied to cellulose precoated glass TLC plates at the origin (×). Electrophoresis (E) was performed from right to left, followed by ascending chromatography in a buffer containing butanol, pyridine, and acetic acid. Autoradiographs of the chymotryptic maps of the M_r 80,000, 70,000, 43,000, 25,000, 23,000, 21,000, and 19,000 proteins are presented (*a*); (*b*) chymotryptic maps of the M_r 25,000 and 70,000 and a mixture of the M_r 25,000 and 70,000 proteins.

tests of purified TNF(LuKII) indicate 25 ng of endotoxin per ml. TNF(LuKII) causes hemorrhagic necrosis of Meth A sarcoma after intratumoral or intravenous injection and total tumor regression has been observed in some treated mice. L-cell lines made resistant to mouse TNF or to partially purified TNF(LuKII) are resistant to purified TNF(LuKII). With the panel of human cell lines studied by Williamson and coworkers (11), purified TNF(LuKII) showed the same pattern of reactivity (cytotoxic/cytostatic/no effect) as mouse TNF and partially purified TNF(LuKII). In addition, purified TNF(LuKII) and interferon showed synergistic cytotoxic activity for human tumor cells, similar to what has previously been reported for partially purified TNF(LuKII) and mouse TNF (12).

DISCUSSION

We have recently described the production, characterization, and biological properties of a human factor with TNF activity from the LuKII lymphoblastoid cell line (11). The further purification and characterization of this factor, designated TNF(LuKII), is the subject of this report. A protocol for the purification of TNF(LuKII) has been developed that yields both good recoveries of TNF and material with high specific activity. This purification protocol allows active fractions

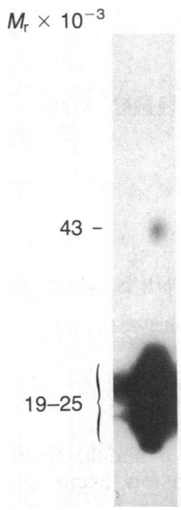

FIG. 6. Immunoblotting analysis of TNF-(LuKII) with T1-18 mouse monoclonal antibody. A sample of TNF(LuKII) containing 10,000 units was fractionated by NaDodSO$_4$/PAGE. Fractionated proteins were transferred to a nitrocellulose membrane and processed as described.

eluted from one column to be applied either directly or after dilution onto the next column, thereby eliminating any need for dialysis and thus avoiding the losses associated with dialysis. TNF(LuKII) has a M_r of 70,000 by gel filtration under nonreducing conditions and an isoelectric point of 6.7. Examination of purified TNF(LuKII) by NaDodSO$_4$/PAGE under reducing as well as nonreducing conditions revealed the presence of seven protein bands ranging from M_r 19,000 to 80,000. The M_r 80,000 and 70,000 proteins could not be dissociated into smaller molecular weight components even after boiling in NaDodSO$_4$/2-mercaptoethanol/urea. Fractions from NaDodSO$_4$/PAGE were assayed for TNF activity and proteins in the M_r 70,000 and 19,000–25,000 region had TNF activity. Using peptide mapping analysis, we found that the seven proteins present in purified TNF(LuKII) were related. Immunoblotting analysis with monoclonal antibody to TNF(LuKII) showed shared determinants on the M_r 43,000 and 19,000–25,000 proteins. Antibody did not react with the higher molecular weight forms, even though these have been shown to be related to the M_r 43,000 and lower molecular weight components. This could be due to the inaccessibility of the determinant on the M_r 70,000 and 80,000 species. Thus, our analysis indicates that there are a number of structurally related proteins in purified TNF(LuKII) and that TNF activity is associated with nondissociable high molecular weight and low molecular weight forms. We conclude that the seven proteins in our purified TNF(LuKII) are either the products of related genes or products DCof a single gene that undergoes extensive processing.

TNF(LuKII) has the full range of biological activities associated with mouse TNF. It produces hemorrhagic necrosis of Meth A sarcoma in the standard TNF assay and cannot be distinguished from mouse TNF in its pattern of reactivity on a large panel of human cancer cell lines. In addition, L cells made resistant to mouse TNF are resistant to TNF(LuKII), and cells made resistant to TNF(LuKII) are resistant to mouse TNF. Recent work has indicated that there are surface receptors for TNF on TNF-sensitive cells (unpublished data). Competitive binding studies showed that mouse TNF and TNF(LuKII) compete for the same receptor.

The relationship between TNF(LuKII) and lymphotoxin (15, 16) is unclear. While they share certain properties, such as their ability to kill mouse L cells, their affinity for lentil lectin, their multiple forms, and, in some cases, their cellular origin, they differ in certain biochemical properties. Aggarwal and coworkers (17, 18) have observed that lymphotoxin from the RPMI 1788 lymphoblastoid cell line has a M_r value of 25,000 and 20,000 under reducing conditions, whereas TNF(LuKII) exists in several molecular weight forms, some of which do not dissociate under reducing conditions. Granger *et al.* (15) have also reported that species of lymphotoxin exist in higher molecular weight forms. It seems likely that there is a family of cytotoxic factors with TNF activity and that lymphotoxin and TNF(LuKII) are members of this family. This would be comparable to the interferon system in which there are three major species, all having antiviral activities, but each being coded for by a different gene with varying degrees of homology. As in the interferon field, the cloning of factors with TNF activity will provide the basis for distinguishing various molecules of this family. The recent cloning of human TNF (19–21) and human lymphotoxin (22) has clarified the relationship between these two molecules. Recombinant TNF and lymphotoxin are distinct molecules of M_r 17,000 and 18,600 that share considerable sequence homology. Both kill L cells and have tumor necrosis activity. In light of the observation that TNF(LuKII) exists in higher molecular weight forms, it seems unlikely that all forms of TNF have been cloned.

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