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RESEARCH ARTICLE

Molecular Cloning of the Complementary DNA for Human Tumor Necrosis Factor

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A factor that became known as tumor necrosis factor (TNF) was first reported by Carswell and colleagues in the mid-1970's (*1*). Sera from endotoxin-treated mice, rabbits, or rats that had been previously sensitized with an immunopotentiator such as bacillus Calmette-Guérin (BCG) were found to contain a substance that, when injected into mice harboring transplanted tumors, caused extensive hemorrhaging of the tumors without undesirable side effects on the recipient. The sera were thus presumed to contain a substance that caused necrosis of tumor cells but had no effect on normal tissue; hence its designation TNF. The ability to cause selective tumor destruction when injected into whole animals became a standard assay for indicating the presence of TNF in vivo.

Several investigators (*2–4*) have attempted to isolate and purify native TNF from rabbit and mouse sera. The factor isolated from rabbit serum is a protein with a molecular weight of 39,000 (39K) to 55K on gel filtration and an isoelectric point of *p*H 5.1 to 5.2 (*2*). The factor from mouse serum had low (50K to 60K) (*4, 5*) and high (100K to 225K) (*4*) molecular weight forms and an isoelectric point of *p*H 4.8 (*3*). Purified preparations of murine TNF were tested against murine and human cell lines in vitro (*6*). In contrast to normal cells, tumor cell lines from both species were susceptible to the cytotoxic activity of the mouse TNF. Furthermore, the murine TNF was active against tumors transplanted from both humans and mice to nude mice (*7*).

Tumor necrosis factor is produced in the medium of mononuclear phagocytes from BCG-infected rabbits and macrophage-enriched peritoneal exudate cells from BCG-infected mice after induction with endotoxin (*5, 8, 9*). In addition, Williamson *et al.* (*10*) have reported the production of TNF by B-lymphoblastoid cells. This partially purified protein exhibited cytostatic and cytotoxic activity against human tumor cell lines in vitro and had tumor necrotic activity in animals.

We now describe the production and purification of human TNF from the human promyelocytic leukemia cell line HL-60 and the determination of its biologic characteristics and amino terminal amino acid sequence. We also describe the molecular cloning of the factor and its production in *Escherichia coli* and in mammalian cells.

Purification and biologic characterization. A number of human cell lines were compared as sources of TNF protein and messenger RNA (mRNA). These included several B-lymphoblastoid lines: Daudi and line 8866; monocyte line U937; a myelogenous leukemia cell line, ML2; and a promyelocytic leukemia line, HL-60. The HL-60 cell line was chosen because it can be induced to differentiate into monocytes upon treatment with phorbol myristate acetate (PMA) (*11*) and produces substantial amounts of TNF, which is thought to be generated by monocytes (*5, 8*) after treatment with endotoxin (lipopolysaccharide; LPS).

Previous attempts to purify TNF from cell culture supernatants have been unsuccessful because the protein is produced in minute amounts by human monocytes (*8*). Our success in purifying the protein was largely due to our ability to induce the HL-60 cell line to produce large amounts of TNF (*12*) and by our ability to determine amino acid sequences from minute quantities of protein samples.

About 4 to 8 liters of culture supernatant from induced HL-60 cells were concentrated by hollow-fiber ultrafiltration (1-square-foot cartridge with a 10K molecular weight cutoff; Amicon). The concentrated, conditioned medium was purified by DEAE ion-exchange chromatography, gel filtration on Sephadex G-75 (Superfine), preparative sodium dode-

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Abstract. *Tumor necrosis factor (TNF) is a soluble protein that causes damage to tumor cells but has no effect on normal cells. Human TNF was purified to apparent homogeneity as a 17.3-kilodalton protein from HL-60 leukemia cells and showed cytotoxic and cytostatic activities against various human tumor cell lines. The amino acid sequence was determined for the amino terminal end of the purified protein, and oligodeoxyribonucleotide probes were synthesized on the basis of this sequence. Complementary DNA (cDNA) encoding human TNF was cloned from induced HL-60 messenger RNA and was confirmed by hybrid-selection assay, direct expression in COS-7 cells, and nucleotide sequence analysis. The human TNF cDNA is 1585 base pairs in length and encodes a protein of 233 amino acids. The mature protein begins at residue 77, leaving a long leader sequence of 76 amino acids. Expression of high levels of human TNF in* Escherichia coli *was accomplished under control of the bacteriophage* λP_L *promoter and gene N ribosome binding site.*

cyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and reversed-phase high-performance liquid chromatography (HPLC) (Fig. 1, A and B) (*13*). The yield from a typical purification was about 10 to 20 μg of TNF protein at greater than 90 percent purity (Fig. 1B, lane 2). The resulting preparation of TNF was sufficiently pure to be used for amino acid sequence analysis (*14*) by means of a gas-phase Sequenator (Applied Biosystems). Typical recoveries of biologic activity throughout the purification scheme were as follows: 80 percent after the ultrafiltration-concentration step, 30 percent after ion-exchange chromatography, and 10 percent overall after gel-permeation chromatography. After exposure to SDS and low *p*H, the biologic activity of TNF was partially destroyed; this effect rendered the values for recovered activity meaningless after SDS-PAGE and reversed-phase HPLC. However, partial recovery in vitro of TNF cytotoxicity by removing the SDS and neutralizing the TNF protein solution after these two purification steps enabled us to ensure that the 17-kilodalton (kD) TNF molecule was being analyzed. The sequence of the first 22 amino acids was determined (Fig. 1C) and was confirmed by analysis of two additional preparations of purified TNF protein.

Partially purified TNF protein was tested for its biologic activity against 11 human tumor cell lines, three normal human fibroblast and epithelial cell lines, and one primary breast epithelial cell culture. Native TNF at less than 60 units (U) per milliliter was cytotoxic against five tumor cell lines, was cytostatic against three others, and had no effect on the remaining three cell lines. In addition, TNF had no effect on three normal cell lines; however, it had a cytostatic effect on primary breast epithelial cells at high concentrations ($\sim 10^3$ U/ml) (Table 1). These results suggest that the TNF protein shows selective activity against cell lines from different tumor pathologies, has no effect on normal cell lines, and has a growth-inhibitory effect on a primary breast epithelial cell culture. Like native TNF, recombinant TNF had a cytotoxic effect on breast carcinoma cells (MCF-7) and no effect on cervical carcinoma cells (HeLa) and a normal foreskin fibroblast cell line (Hs27F).

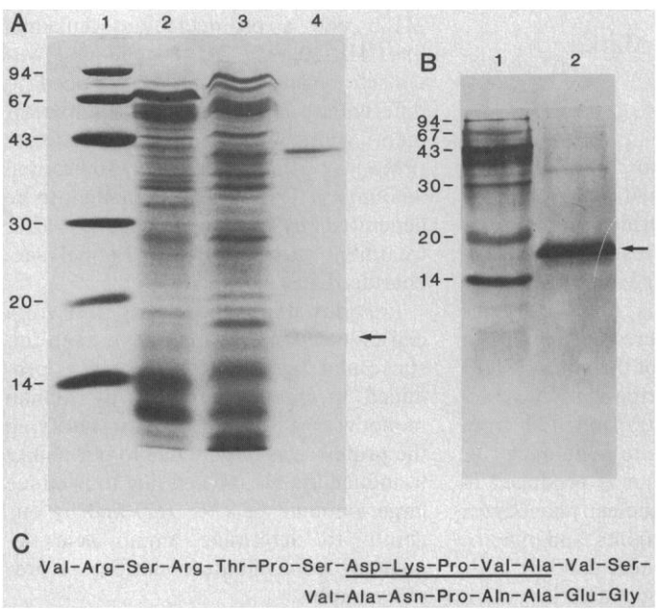
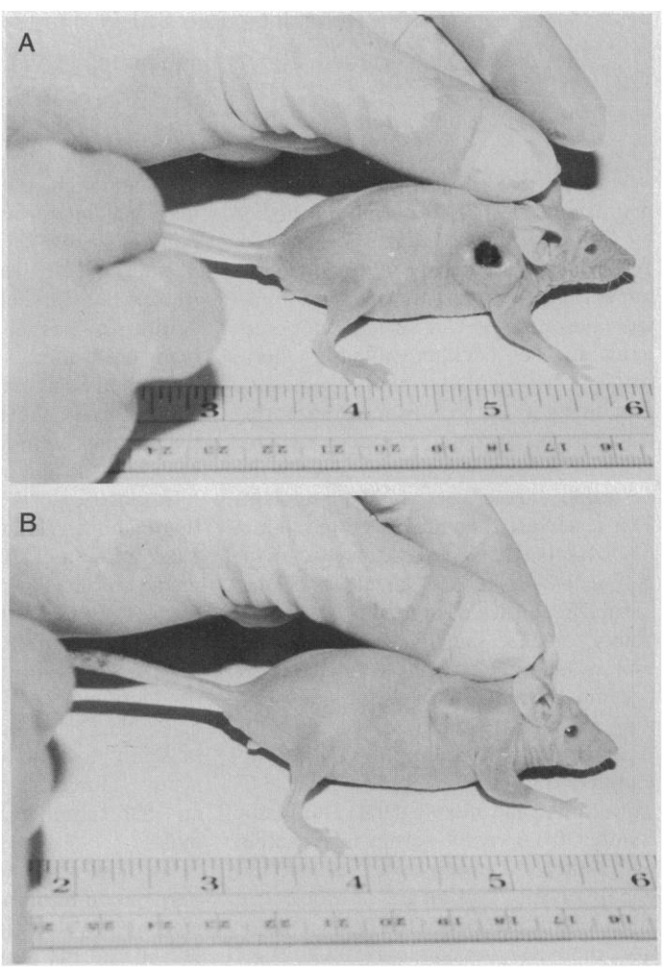


Fig. 1 (left). Purification of TNF (*13*). (A) SDS-PAGE with the Laemmli gel system (*26*). (Lane 1) Molecular weight standards (×1000) (Pharmacia), (lane 2) culture supernatant, (lane 3) DEAE unbound fraction, and (lane 4) gel-permeation peak fraction. The gel was stained with Coomassie brillant blue. (B) (Lane 1) Molecular weight standards (×1000) and (lane 2) SDS-PAGE (*26*) of HPLC peak fraction for amino acid sequence determination. The gel was silver stained. (C) Amino terminal sequence of purified TNF protein. Fig. 2 (right). Tumor necrotic activity of purified TNF on transplanted human tumors in nude mice. (A) Nude mouse bearing a 14-day-old human breast carcinoma explant (MX-1) was treated intravenously with a single dose (~1 μg) of partially purified TNF (G-75 fraction; Fig. 1). (B) Control mouse treated similarly with buffer. Necrosis was evaluated macroscopically 48 hours after administration of TNF.



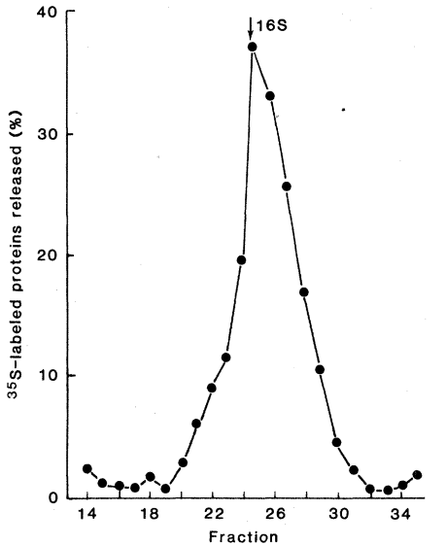


Fig. 3. Sucrose-gradient fractionation of TNF mRNA from HL-60 cells induced as described (*12*). Messenger RNA was prepared as described (*27*). The polyadenylated mRNA's were purified on an oligo(dT)-cellulose column; after precipitation with ethanol, they were fractionated on a 5 to 25 percent sucrose gradient in a Beckman SW40 rotor at 38,000 rev/min for 17 hours. The mRNA from gradient fractions was injected into *X. laevis* oocytes at a concentration of 2 μg/μl according to standard methods. After incubation for 24 to 48 hours, the oocytes were homogenized in Barth's medium, and the homogenate was assayed for cytotoxicity by the ^{35}S-labeled protein release assay (*15*).

To show that highly purified native TNF has tumor necrosis activity, we injected approximately 1 μg of TNF protein (from the gel-filtration pool) into the tail veins of nude mice carrying 14-day-old transplanted human breast carcinoma explants (MX-1). A single dose of TNF caused necrosis of an established tumor 48 hours after administration (Fig. 2).

Molecular cloning of human tumor necrosis factor. Four hours after induction, mRNA was isolated from the HL-60 cell lines and fractionated on a 5 to 25 percent sucrose gradient. The fractionated mRNA was analyzed by injection into *Xenopus* oocytes, and the oocyte extracts were assayed for TNF cytotoxic activity against mouse L929 cells (*15*). The mRNA coding for TNF biologic activity migrated as a 16*S* peak in the sucrose gradient (Fig. 3). The fractions containing the most TNF mRNA activity were pooled, and 5 μg was used for constructing a complementary DNA (cDNA) library as described (*16*) containing 325,000 individual transformants (*17*). Restriction enzyme digestion of plasmids isolated from a number of randomly selected clones suggested that 200,000 individual transformants (60 percent) carry inserts larger than 1000 base pairs (bp).

Oligodeoxyribonucleotide probes were synthesized on the basis of the amino acid sequence of TNF (Fig. 1C, underlined region). Even though the sequence selected contained amino acids with the least number of degenerate codons, we synthesized 64 oligodeoxyribonucleotides, each 14 nucleotides in length, in 4 pools of 16, each to complement all possible coding sequences for these five amino acids.

Because of the degeneracy of the oligodeoxyribonucleotide probes, we used a hybrid-arrest translation technique (*18*) to identify the pool containing the sequence complementary to the TNF mRNA. Briefly, the four pools of probes were hybridized to TNF mRNA, and the mixtures were injected into *Xenopus* oocytes. The pool that contains a sequence complementary to the TNF mRNA will inhibit its translation into biologically active TNF protein. Therefore, by assaying the oocyte extracts, the pool that inhibits TNF synthesis can be identified. Using this technique, we identified one pool of oligodeoxyribonucleotide sequences [GCNACNGGCTTGTC, where N is A, G, T, or C (G, guanine; C, cytosine; A, adenine; T, thymine)] that was complementary to the TNF mRNA and inhibited the synthesis of TNF activity by a factor of about 5.

On the basis of the nucleotide sequences in this pool of oligodeoxyribonucleotides, eight pairs of probes, each 14 nucleotides in length, were synthesized and subjected to the same hybrid-arrest analysis; one mixed probe (number 11; GCYACAGGCTTGTC, where Y is T or C) was identified. Further analysis of this pair identified a probe (number 14; GCTACAGGCTTGTC) as the sequence complementary to TNF mRNA. Probe 11 was characterized further in Northern blot hybridizations to induced and uninduced HL-60 mRNA to confirm that it hybridizes specifically to a 1.6-kb mRNA present in induced HL-60 cells but absent in uninduced cells.

Probe 11 was then used to screen the TNF cDNA library, and 25 clones were identified; of these, 18 hybridized to probe 14. To show that the cDNA clones encoded TNF cytotoxic protein, we subjected selected cDNA clones to a hybrid-selection assay and to direct expression in COS-7 cells. Plasmid DNA's from TNF clones were immobilized on a nitrocellulose filter and hybridized to TNF mRNA, and the bound mRNA was eluted from the filter and assayed in the oocyte translation assay. Several cDNA clones, including B8, B11, B18, and E4, hybridized to TNF mRNA in this assay; this confirms that the clones encoded the same mRNA sequence as that in the HL-60 cell line.

The TNF cDNA library was con-

Table 1. Relative sensitivity of human cell lines to purified human TNF (primary breast epithelial cells were from M. Stamper; all cell lines were obtained from the Naval Biosciences Laboratory or the American Type Culture Collection). Sensitivity was determined by exposing cells to biologically active fractions of TNF protein (from G-75 gel-permeation chromatography). Briefly, fractions were serially diluted (twofold) into Dulbecco minimum essential medium (DMEM; Gibco) containing 10 percent fetal bovine serum. Diluted samples (100 μl each) were applied to the cells, which had been seeded overnight into 96-well microtiter plates at a density of 2×10^4 cells per 100 μl of medium per well. The plates were incubated at 37°C for 5 days, after which the number of viable cells per well was counted with a hemacytometer. Abbreviations: N.T., not tested; N.E., no effect.

Cell line		TNF (U/ml)*	
Origin	Designation	Native	Recombinant
	Tumor		
Melanoma	Hs939T	4	N.T.
Melanoma	Hs294T	N.E.†	N.T.
Breast carcinoma	MCF-7	2	5
Breast carcinoma	BT-20	5	N.T.
Breast carcinoma	SKBR-3	900‡	N.T.
Lung carcinoma	A427	20	N.T.
Bladder carcinoma	5637	1600‡	N.T.
Cervical carcinoma	HeLa	N.E.	N.E.
Colon carcinoma	HT-29	60	N.T.
Fibrosarcoma	HT-1080	600‡	N.T.
Erythroleukemia	K562	N.E.	N.T.
	Normal		
Skin diploid fibroblast	Hs939sk	N.E.	N.T.
Foreskin fibroblast	Hs27F	N.E.	N.E.
Fetal intestinal epithelial	FHs74Int	N.E.	N.T.
Primary breast epithelial	184	1000‡	N.T.

*Amount of TNF causing 50 percent cytotoxicity or cytostasis. †No effect at 1600 U/ml. ‡Cell line in which TNF caused cytostasis.

structed with the vector that contains the simian virus 40 origin of replication and has the transcriptional promoter in the correct orientation for expression of the cDNA insert in mammalian cells (*16*); therefore, plasmid DNA from several clones was transfected into COS-7 cells, and the culture medium was assayed for TNF biologic activity at 24, 48, and 72 hours after transfection. Clones B11 and E4 expressed TNF activity in the culture medium by 24 hours after transfection; the activity increased by a factor of 2 to 3 by 48 hours (Table 2). By 72 hours after transfection, E4 produced more than 512 U of TNF activity per milliliter of culture medium.

When these clones were analyzed by restriction enzyme digestion, they formed two size classes of 1.6 and 1.3 kb, but both classes had the same internal restriction sites. Therefore, the shorter clones may be incomplete because of a preferential termination site for reverse transcriptase. This was confirmed by nucleic acid sequence analysis of several clones (Fig. 4). The nucleotide sequence of the entire clone is 1585 bp in length and has an open reading frame that encodes a protein of 233 amino acids. The amino terminal amino acid for the mature TNF protein begins at amino acid 77, leaving an unusually long apparent leader sequence of 76 amino acids. Comparison of the 22 amino terminal amino acid residues of the purified protein with the amino acid sequence of the protein predicted from the nucleotide sequence revealed that there are two minor differences. Amino acid residues 80 and 81 of the predicted protein sequence are two serine residues that are not present at the corresponding positions in the purified protein. Amino acid residues 91 and 92 of the predicted protein sequence are histidine and valine, respectively; in the purified protein, the corresponding amino acids are valine and serine.

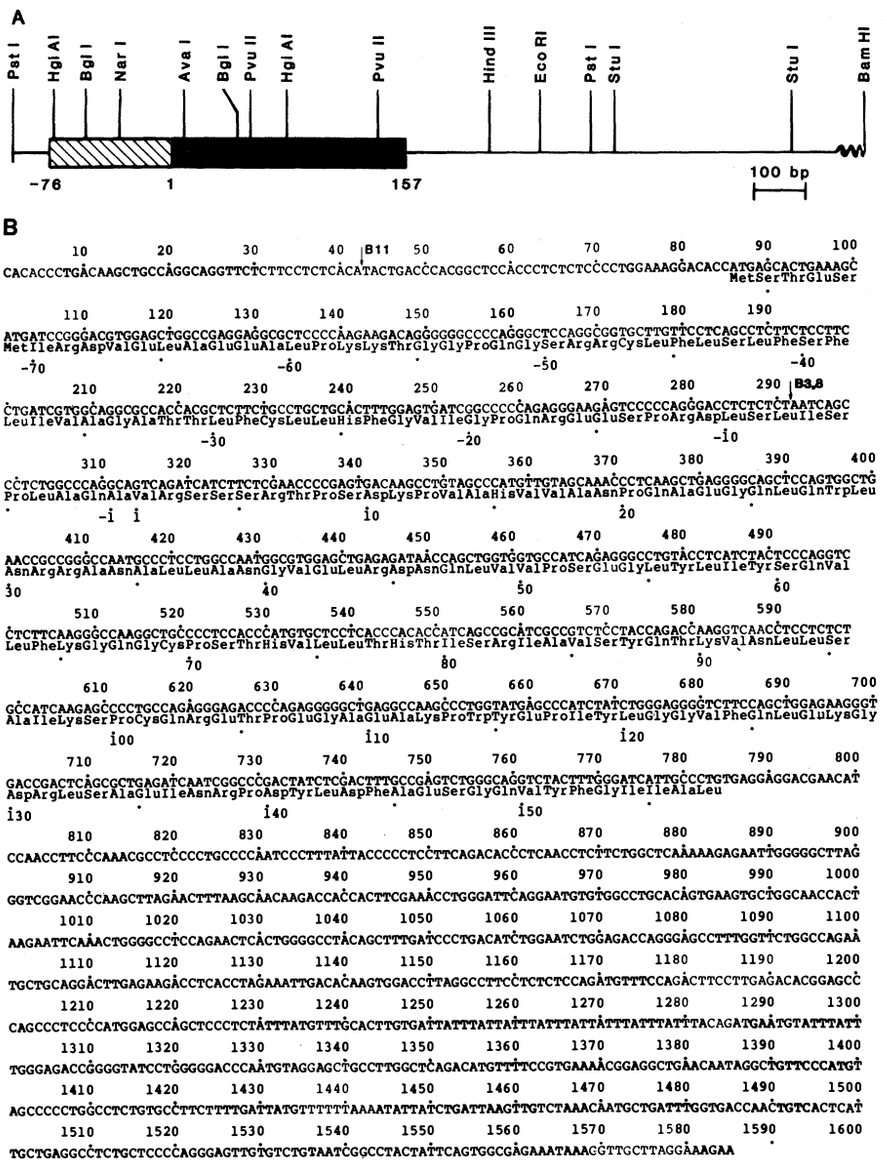


Fig. 4. (A) Restriction map of TNF cDNA inserts of pE4 and pB11. The cDNA insert of clone pE4 was subcloned into bacteriophage M13, in both orientations, as Pst I and Pst I–Bam HI fragments. The complete nucleotide sequence was determined by a combination of methods (*28*). (B) Nucleotide sequence and protein sequence of clone pE4. The amino terminal amino acid (valine) of mature TNF is labeled as amino acid residue 1. The arrows point to the 5′ end of the shorter cDNA clones (B3 and B8) and of another full-length clone, B11.

Expression of TNF protein in E. coli. Expression of the mature TNF protein in *E. coli* required the deletion of the nucleotide sequence encoding the apparent leader sequence of TNF (Fig. 4). This was accomplished by subcloning the Pst I fragment of the cDNA clone E4 (which is the entire coding region; see Fig. 4) into bacteriophage M13mp18 and by using an oligodeoxyribonucleotide (GAAGATGATCTGACCATAAGCTTT-GCCTGGGCC) to introduce a Hind III restriction enzyme site and an ATG initiation codon before the GTC codon encoding the first amino acid (valine) of the mature TNF protein by site-specific mutagenesis (*19*). The resulting clone, M13-pAW701, was used to provide the coding sequence for TNF in an expression scheme (Fig. 5). The plasmid constructed for TNF expression, pAW711, contained the coding sequence for the mature TNF protein under the regulatory control of the bacteriophage λ P_L promoter and the λ gene N ribosome binding site. The *Bacillus thuringiensis* positive retroregulating element (*20*) was downstream of the TNF cDNA (Fig. 5). In addition, the vector origin derived from ColE1 contained mutations conferring a temperature-sensitive Cop^- phenotype (*21*). The *E. coli* strain DG95 ($\lambda N_7 N_{53}$ cI_{857} *sus* P_{80}) harboring plasmid pAW711 was cultured at 30°C to an optical density at 640 nm of 0.5, and the P_L promoter and increased plasmid replication were induced by shifting the temperature to 42°C. Extracts of induced cultures containing pAW711 had 225,000 U of TNF activity per milliliter, with TNF protein representing about 8 percent of the total *E. coli* cellular protein.

Discussion. The demonstration that the factor we purified is cytotoxic to tumor cells in vitro and causes tumor necrosis in some transplanted tumors in animals is suggestive of the isolation and identification of the TNF originally described by Carswell (*1*). Of 11 human tumor lines, eight showed various degrees of responsiveness to this factor, yet none of the normal continuous cell lines was affected by it and only a primary breast

epithelial culture had growth inhibited by 10^3 U of TNF per milliliter. These normal breast cells are fastidious so that their growth pattern is perturbed by minimal changes in the growth medium. Further experimentation with TNF and primary cultures from breast tissue and organs would substantiate the significance of this observation. These findings suggest that the TNF molecule may have potential use in abrogating some types of cancers in vivo with minimal cytotoxicity to the normal cellular counterparts.

Tumor necrosis factor and lymphotoxin (*22*) have the same characteristic in the assay for lysis of mouse L929 cells. However, lymphotoxin is produced and secreted mainly by cells of T- and B-cell lineage, whereas TNF is produced by cells of macrophage lineage. Furthermore, oligodeoxyribonucleotide probes based on the amino acid sequence of TNF did not inhibit the translation of lymphotoxin mRNA (from the B-cell line 8866) in the *Xenopus* oocyte translation assay. This implies that there is little homology in the nucleotide sequences of TNF and lymphotoxin. In addition, the amino acid composition of TNF as deduced from the cloned DNA sequence is different from the composition of lymphotoxin described (*22*). The most notable difference was the absence of any methionine in the mature TNF protein (lymphotoxin has three methionine residues per molecule).

Sequence analysis of the cloned TNF cDNA has shown that it codes for a polypeptide of 157 amino acids and has a calculated molecular size of 17.3 kD. The leader sequence contains roughly 76 amino acids, beginning with the first available Met start codon. We have purified mature human TNF to homogeneity from the same HL-60 cell line used as the source of the mRNA in these studies. The amino acid sequence for human TNF, as predicted from the nucleotide sequence, varies slightly from the actual amino acid sequence for the mature protein. Specifically, the two serine amino acids, at positions 4 and 5 in the predicted amino acid sequence, are not found in the protein sequence, and the His-Val sequence at positions 15 and 16 of the predicted sequence are Val-Ser in the corresponding positions of the protein sequence. The reason for the absence of the two additional serine residues in the protein sequence and the substitution of His-Val by Val-Ser in the purified native protein is not clear. It is possible that there are two TNF genes encoding similar proteins, with the protein sequence being the product of one allele and the cDNA clone being the other. It is also possible that these differences are due to a technical error in the sequence analysis of the purified protein. This, however, is less likely because the same amino acid sequence was obtained from three different preparations of purified TNF protein.

When the cDNA library was screened again with nick-translated probes isolated from the pE4 cDNA insert, an additional 22 partial cDNA clones were isolated. In addition to the 18 clones identified with probe 11, this cDNA library of 200,000 individual clones contained a total of 40 TNF cDNA clones. Assuming that there was a tenfold enrichment of the mRNA by sucrose-gradient fractionation, the concentration of TNF mRNA in induced HL-60 cells was probably less than one copy in 50,000 mRNA molecules.

Initial experiments with human breast carcinoma explants (MX-1) transplanted into nude mice have shown that the purified native protein does have tumor necrotic activity in mice. In addition, the mature protein is toxic to several tumor cell lines in culture and only cytostatic to one primary epithelial cell culture. Thus,

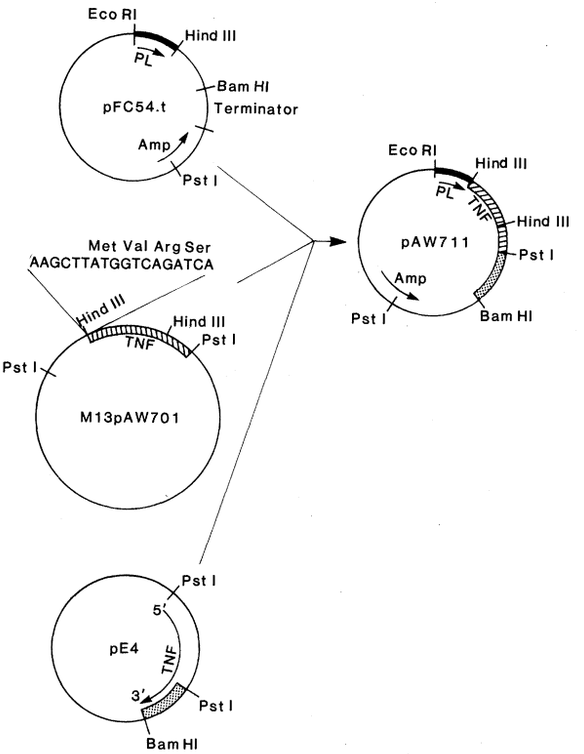


Fig. 5. Expression of TNF in *E. coli*. The clone M13-pAW701 was digested with Pst I and then digested partially with Hind III to obtain the Hind III–Pst I TNF coding sequence. The Pst I–Bam HI fragment containing the 3′ noncoding sequence of the TNF gene was purified from pE4 after digestion of the DNA with Pst I and Bam HI. Together, the two fragments make up the coding sequence plus a 600-bp 3′ untranslated portion of DNA. The temperature-sensitive Cop$^-$ plasmid, pFC54.t (*20, 21*), was digested with Hind III and Bam HI, and the vector fragment was purified on an agarose gel. The isolated fragment was then ligated with the above Hind III–Pst I and Pst I–Bam HI segments in a three-fragment ligation, and the mixture was used to transform *E. coli* K12 strain DG95 to the ampicillin-resistant phenotype, resulting in the plasmid pAW711.

Table 2. Transfection of COS-7 cells. Cells were seeded into T-25 flasks 1 day before transfection and were fed again 3 hours before the addition of DNA with DMEM–high glucose without pyruvate. The calcium phosphate–DNA preparation was carried out by adding 5 μg of plasmid DNA in 250 m*M* $CaCl_2$ to an equal volume of 2× Hepes-buffered saline [HBS; 280 m*M* NaCl, 50 m*M* Hepes, and 2.8 m*M* Na_2HPO_4 (*p*H 7.1)] and allowing the solution to stand at room temperature for 30 minutes before transferring by pipette into 2 ml of medium covering the cells. After a 5-hour incubation, the cells were washed and glycerol-shocked by adding 1.5 ml of 15 percent glycerol-HBS and incubating for 3 minutes at 37°C. The cells were then washed and fed with DMEM–high glucose without pyruvate and 10 percent fetal bovine serum. At 24, 48, and 72 hours after transfection, the medium was removed from the cells by aspiration and was cleared of cellular debris by centrifugation (5 minutes at 4500*g*). The medium was assayed for TNF activity at dilutions of from 1:4 to 1:512. Abbreviation: N.D., not determined.

Plasmid	Hybridization with probe 14*	TNF activity (U/ml)				
		Experiment 1		Experiment 2		
		24 hours	48 hours	24 hours	48 hours	72 hours
B8	+	<1	<1	<1	<1	<1
B11	+	<4	32	<1	32	64
B18	+	<1	<1	<1	<1	<1
E4	+	N.D.	N.D.	8 to 16	512	>512
B16	−	<1	<1	<1	<1	<1
E9	−	<1	<1	N.D.	N.D.	N.D.
Without DNA†		<1	<1	<1	<1	<1

*COS-7 cell culture supernatant was subjected to the ^{35}S-labeled protein release assay (*15*). †Mock infection.

although the amino acid sequence of the mature protein deviated from that predicted from the cloned cDNA nucleotide sequence, the purified protein is biologically active. Direct comparison of TNF activity on the same cell line in vivo and in vitro has not been possible with the human breast carcinoma explant (MX-1) because it has not been established as a cell line in culture. However, direct comparison of responsiveness to TNF in vivo and in vitro could be accomplished with the MCF-7 tumor model (*23*). Results of initial experiments with recombinant TNF show that the factor is biologically active and that it has a biologic profile, including cytotoxicity to MCF-7 in vitro, similar to its native counterpart (*24*).

The isolation of a cDNA clone encoding human TNF has also made possible the expression of the gene in eukaryotic (COS-7) cells, whose growth is unaffected by large doses (1600 U/ml) of TNF. The protein secreted from those cells is cytotoxic to mouse L929 cells in vitro. In addition, we have accomplished expression of the gene in *E. coli* and have isolated sustantial amounts of pure, active protein. The availability of large amounts of recombinant TNF should facilitate the study of the biology of TNF, its mechanism of action on tumor cells, and its potential application in clinical medicine against solid and metastatic tumors that are hard to combat with conventional therapies. There is also evidence suggesting a possible application for TNF in the treatment of parasitic diseases (*25*).

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12. HL-60 cells were grown to a density of 2×10^6 cells per milliliter in suspension cultures in RPMI 1640 medium supplemented with 20 percent fetal bovine serum. The cells were collected by centrifugation, washed with serum-free medium, and resuspended in serum-free medium containing PMA (100 ng/ml) at a density of 10^7 cells per milliliter. The cells were incubated for 30 minutes at 37°C, collected by centrifugation, and resuspended at a density of 10^7 cells per milliliter in serum-free medium containing 10 μg of bacterial endotoxin (LPS) and 10 μg of calcium ionophore A23187 per milliliter. The cells were incubated at 37°C with constant agitation for 4 hours. The culture supernatant was harvested, assayed, and then used for the purification of TNF.
13. Approximately 300 ml of the concentrated culture fluid was centrifuged to remove cell debris, and the supernatant was adjusted with 30 m*M* ammonium bicarbonate buffer (*p*H 8.2) to a conductance of 6.2 mS. The solution was concentrated further by filtration through a PM10 membrane (Amicon) and clarified by centrifugation (20,000*g* for 10 minutes). The supernatant (160 mg) was then applied to a 30-ml DEAE ion-exchange column that was equilibrated with 30 m*M* ammonium bicarbonate and 1 m*M* NaCl (*p*H 8.2) and washed with the same buffer. Fractions were collected, and the protein was monitored at 280 nm. The unbound fractions were assayed by means of the L929 cytotoxicity assay, and those fractions having TNF activity were pooled and concentrated again by ultrafiltration. The concentrated solution (15 mg) was applied to a Sephadex G-75 Superfine column (2.6 by 75 cm; Pharmacia) equilibrated in 30 m*M* ammonium bicarbonate buffer (*p*H 7.4). Fractions obtained by eluting with the same buffer were monitored at 280 nm and assayed for TNF. Fractions containing maximum TNF activity were lyophilized. The lyophilized protein was resuspended in Laemmli SDS sample buffer and subjected to SDS-PAGE (*26*). The gel was sliced into 2-mm sections, and the protein from each section was eluted (*29*) by immersion in 1 ml of 30 m*M* ammonium bicarbonate buffer (*p*H 7.4) and overnight shaking at room temperature. The sections of the gel containing TNF activity were applied to a C-4 reversed-phase HPLC column (Vydac) equilibrated in 0.1 percent trifluoroacetic acid (TFA), and the activity was eluted by means of a linear gradient (0 to 60 percent acetonitrile in 0.1 percent TFA). Protein was monitored at 280 and 214 nm, and the fractions were assayed for biological activity after lyophilization and suspension in 30 m*M* ammonium bicarbonate buffer (*p*H 7.4). Fractions containing TNF activity were again lyophilized.
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15. Briefly, L929 cells were seeded (2×10^5 cells per milliliter) overnight to form monolayers in microtiter plates. The test samples were diluted twofold across the plate, exposed to ultraviolet radiation, and then added to the prepared cell monolayers. Actinomycin D (1 μg/ml) was then added to the culture medium, and the plates were incubated for 18 hours at 37°C and then scored visually under a microscope. Each well was given a 25, 50, 75, or 100 percent mark, signifying the extent of cell death in the well. One unit of TNF was defined as the reciprocal of the dilution at which 50 percent cytotoxicity occurred. Another version of this assay was developed that measures the release of ^{35}S-labeled peptides from labeled cells when they are treated with the test sample and actinomycin D. This assay is a measure of cytotoxicity and can be used to measure potency (for example, the relative specific toxicity of oocyte-translated material). Briefly, actively growing L929 cultures were labeled with [^{35}S]methionine (200 μCi/ml) for 3 hours in methionine-free medium supplemented with 2 percent dialyzed fetal bovine serum. The cells were then washed and plated into 96-well plates, incubated overnight, and treated the next day with twofold dilutions of test samples and actinomycin D (1 μg/ml). The cultures were then incubated at 37°C for 18 hours. Portions (100 μl) of the supernatant from each well were then transferred onto another 96-well plate, and the cells were precipitated with trichloroacetic acid and harvested onto glass fiber filters. The filters were washed with 95 percent ethanol and dried, and the radioactivity was counted. A Nonidet P-40 detergent control was included in every assay to measure maximum release of label from the cells. The percent of ^{35}S released was then calculated by the ratio $[(N_t - N_c)/(N_d - N_c)] \times 100$, where N_t is the label released from cells treated with test sample, N_d is the label released from cells treated with detergent, and N_c is the label released from control cells. Higher potency of TNF results in higher values of this ratio. Routinely, samples assayed by this version of the L929 assay are also evaluated microscopically as described (*10*).
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