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Immunology

Purification, characterization, and antitumor activity of nonrecombinant mouse tumor necrosis factor

(cytotoxic factor/monokine/tumor regression)

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ABSTRACT **Mouse tumor necrosis factor (TNF) was purified from serum through a series of steps, and each step was monitored for L-cell cytotoxicity *in vitro* and tumor-necrotizing activity *in vivo*. The two activities copurified and could not be dissociated. Purified mouse TNF has a specific activity of 2.2 × 10⁷ (L-cell assay in the absence of actinomycin D) and 1 μg causes necrosis of the standard TNF-sensitive sarcoma Meth A. TNF has a M_r of 39,000 ± 2000 by gel filtration and a M_r of 16,000–18,000 by $NaDodSO_4$/PAGE. Both molecular weight forms display cytotoxic and necrotizing activities. TNF has a pI of 3.9 and is destroyed by trypsin, protease, elastase, and α-chymotrypsin but not by neuraminidase or papain. These characteristics of nonrecombinant mouse TNF clearly resemble those of recombinant human and mouse TNF.**

Tumor necrosis factor (TNF) was initially recognized as a constituent in the blood of bacillus Calmette–Guerin (BCG)-infected mice, rats, or rabbits injected with endotoxin (1). Other agents, such as *Corynebacterium parvum*, can substitute for BCG in priming mice for TNF production, but endotoxin appears to be uniquely effective in eliciting TNF release (1–3). Serum containing TNF causes hemorrhagic necrosis of mouse and human tumors *in vivo* (1, 4) and has cytotoxic and cytostatic activity for mouse and human tumor cells *in vitro* (1, 5–7). Early studies indicated that the hemorrhagic necrosis effect of TNF-containing serum was not due to residual endotoxin (1, 8) and that the necrotizing and cytotoxic activities were not separable (9, 10). Considerable indirect evidence pointed to the macrophage as the source of serum TNF (1, 11), and subsequent studies demonstrated that peritoneal macrophages and cloned lines of histiocytoma produce a factor with TNF-like activity after stimulation with endotoxin (12–16).

Recently, several groups have reported the cloning of human TNF (17–21) and mouse TNF (22, 23). Recombinant TNF has a M_r of 45,000, which dissociates into M_r 17,000–18,000 monomer subunits on $NaDodSO_4$/PAGE. Recombinant TNF displays *in vivo* necrotizing activity and *in vitro* cytotoxic activity, establishing that both activities are the property of a single molecule.

As work on TNF progresses, it is important to have sufficient amounts of nonrecombinant TNF as a basis for comparison with recombinant TNF, particularly with regard to antitumor activity and toxicity. In the present study, we describe (*i*) methods for the large-scale production of partially purified mouse serum TNF with good recovery, (*ii*) the relation between *in vivo* and *in vitro* activities during sequential steps of TNF purification, and (*iii*) the characteristics of purified mouse serum TNF.

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MATERIALS AND METHODS

TNF Serum Production. Young adult CD-1 (Charles River Breeding Laboratories) and DDY (Shizudokyo, Shizuoka, Japan) female mice were injected intraperitoneally with 1 mg of *C. parvum* (Burroughs Wellcome, Research Triangle Park, NC) or *Propionibacterium acnes* IID912 (The University of Tokyo, Japan). After 9 days, mice received an intravenous injection of 10 μg of *Escherichia coli* endotoxin (0127:B8; 0111:B4W) (Difco) and were bled 1.5–2 hr later.

***In Vitro* Assay.** Serially diluted samples were incubated with 2 × 10⁴ TNF-sensitive L-M cells (American Type Culture Collection). At 48 hr, cells were fixed with methanol and stained with methylene blue. The dye was extracted with 3% HCl and the absorbance was measured by a Titertek multiscan spectrophotometer. Fifty percent cytotoxicity (titer) was assessed and expressed as the dilution factor (DF) (24). TNF specificity was assessed by tests with TNF-resistant L-cell lines (25).

***In Vivo* Assay.** (BALB/c × C57BL/6)F_1 or BALB/c female mice bearing 7-day intradermal transplants of Meth A sarcoma measuring 7–8 mm received a single intravenous injection of the TNF preparation. Tumor hemorrhagic necrosis was graded after 24 hr (see ref. 1) and relative tumor weight was estimated from linear measurements of the tumors (4).

Protein Concentration and Determination. Fractions were concentrated in Amicon stirred cells using PM10 ultrafiltration membranes. Protein content was determined by Coomassie brilliant blue dye-binding assays.

***Limulus* Amebocyte Lysate Assay.** Endotoxin activity of purified TNF was determined with reagents (Pyrodick) from Teikoku Hormone, Tokyo (26).

RESULTS

Production of Mouse Serum TNF. Several variables influence TNF production. Mouse strains differ in their capacity to produce TNF (1, 24). C3H/HeJ and A/J are low producers, and DDY, CD-1, SJL/J, and DBA/2 are high producers. DDY and CD-1 were employed as the source of TNF in the present study. Although BCG and zymosan are capable of priming mice for TNF production, *C. parvum* and *P. acnes* provide stable material that is consistently effective (1–3). The optimal time for eliciting TNF is day 9 after *C. parvum* or *P. acnes*, prior to the development of maximal hepatosplenomegaly. Endotoxin preparations from various sources and of varying purity have been tested, but none has proved superior to the Difco preparations. A broad range of endotoxin doses can be used to elicit TNF; 2.5 μg is the

Abbreviations: TNF, tumor necrosis factor; DF, dilution factor; BCG, bacillus Calmette–Guerin; FPLC, fast protein liquid chromatography.

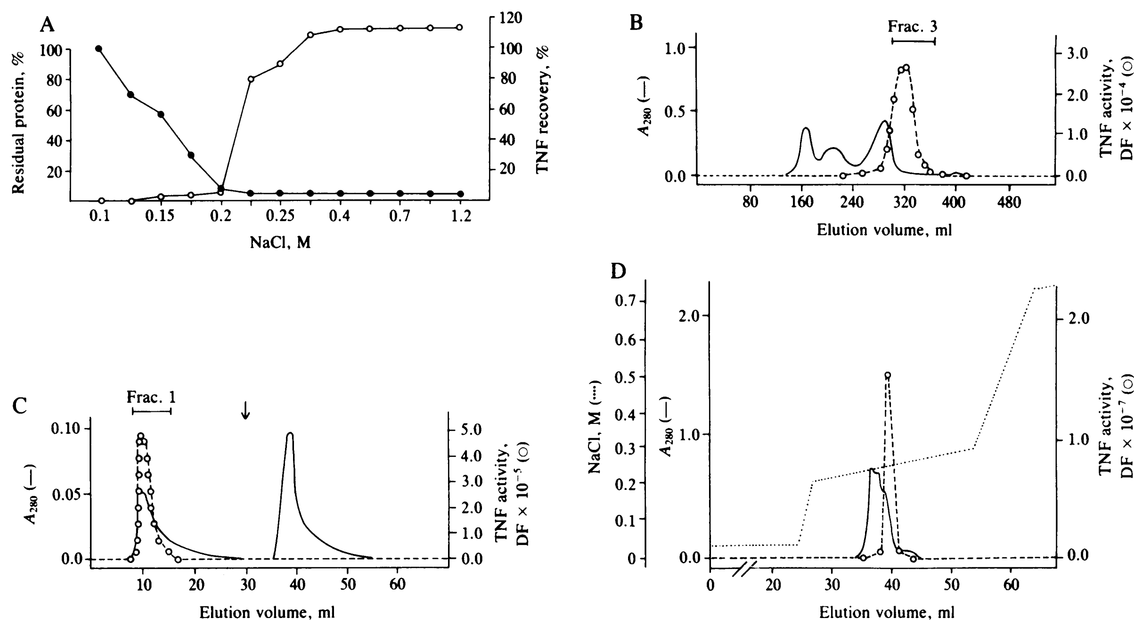

FIG. 1. Purification of mouse serum TNF by sequential chromatography. (*A*) DEAE Sephadex A-50 gel chromatography. Protein elution and TNF recovery following elution with buffers of different ionic strength. ●, Percentage of applied protein remaining on the gel after elution with buffer; ○, percentage of TNF activity recovered after elution with buffer. Gel, swollen and equilibrated in 0.04 M Tris·HCl buffer (pH 7.8) containing 0.1 M or 0.15 M NaCl, was poured into a Buchner funnel covered with a Whatman no. 1 paper filter. After excess buffer was drained, TNF serum (delipoproteinated by centrifugation at 110,000 × *g* for 12 hr) (ratio of 1 g of serum protein to 20 g of dry gel) was pipetted uniformly onto the gel surface. The gel was covered with a second filter, and the elution buffer, equal to 3 gel bed volumes, was poured into the funnel. Eluted protein was concentrated and tested *in vitro* for TNF activity. Optimal conditions used for large-scale TNF purification (see Table 1): gel was equilibrated with Tris buffer containing 0.15 M NaCl. After serum was applied, >95% of the protein (TNF-inactive) could be removed with 3 volumes of Tris/0.195 M NaCl buffer. TNF was then eluted with 3 volumes of Tris/0.4 M NaCl buffer. (*B*) Sephadex G-200 column chromatography. Five-milliliter samples of DEAE A-50 TNF were applied to a 2.6 × 100 cm Sephadex G-200 (Pharmacia) column equilibrated with 0.04 M Tris·HCl/0.7 M NaCl buffer, pH 7.8. Protein was eluted at a flow rate of 20 ml/hr. Frac. 3, fraction 3. (*C*) Affi-Gel blue affinity chromatography. Concentrated samples of Sephadex G-200 TNF were applied to a 0.7 × 10 cm column of Affi-Gel blue (Bio-Rad) (ratio of 1 ml of gel to 10 mg of sample) equilibrated with 0.04 Tris·HCl buffer (pH 7.2). The TNF-active fraction was eluted at a flow rate of 2.5 ml/hr. The albumin fraction was eluted (↓) with Tris/1.4 M $MgCl_2$ buffer. Frac. 1, fraction 1. (*D*) Fast protein liquid chromatography (FPLC) ion-exchange chromatography. Five-milligram samples of Affi-Gel blue TNF were applied to a Mono Q HR 5/5 column (Pharmacia) equilibrated with 0.04 M Tris·HCl buffer (pH 7.8). Protein was eluted with a linear NaCl gradient at a flow rate of 2 ml/min. TNF-active fractions were rechromatographed two times; the final elution pattern is shown.

lowest dose that consistently elicits peak TNF levels. Since *C. parvum*- or *P. acnes*-primed mice develop signs of shock after endotoxin injection, it is important to bleed mice before vascular collapse has occurred. To increase the recovery of TNF-containing blood, mice are perfused with 1 ml of warm saline before exsanguination. Using these methods, 1–2 ml of

Table 1. Purification of TNF from mouse serum

Step	Fraction	Volume, ml	DF × 10^{-3}	Protein, mg/ml	Specific activity, (DF per mg) × 10^{-3}	Total DF × 10^3	Recovery, %	Purification factor	Necrosis of Meth A *in vivo*,* mg
TNF serum		12,500	6.4	56.7	0.1	80,000	100		20.0
DEAE A-50 Sephadex batch chromatography	0.15 M NaCl†	21,250	—	17.8	—	—	—	—	—
	0.195 M NaCl†	22,750	—	13.1	—	—	—	—	—
	0.4 M NaCl	1,600	56.4	8.0	7.1	90,240	113	64	8.0
Sephadex G-200 column chromatography	Frac. 1†	550	—	19.2	—	—	—	—	—
	Frac. 2†	250	12.4	5.3	2.3	3,100	4	21	2.0
	Frac. 3	250	236.0	2.1	111.1	59,000	74	1,010	0.25
Affi-Gel blue affinity chromatography	Frac. 1	20	2,840.0	8.0	355.0	56,800	71	3,227	0.03
	Frac. 2†	30	—	9.0	—	—	—	—	—
FPLC Mono Q column chromatography	First‡	60	907.7	0.7	1,295.3	54,400	68	11,776	0.013
	Second	15	2,580.0	0.3	7,854.1	38,700	48	71,401	0.003
	Third	2	14,750.0	0.9	16,573.0	29,500	37	150,664	0.002
PAGE without $NaDodSO_4$		1	983.0	0.05	21,594.5	9,830	1.2	196,314	0.001

*The lowest concentration of protein administered intravenously that produced grade (++) necrosis of Meth A tumor.
†Discarded materials.
‡Sequential applications to Mono Q columns.

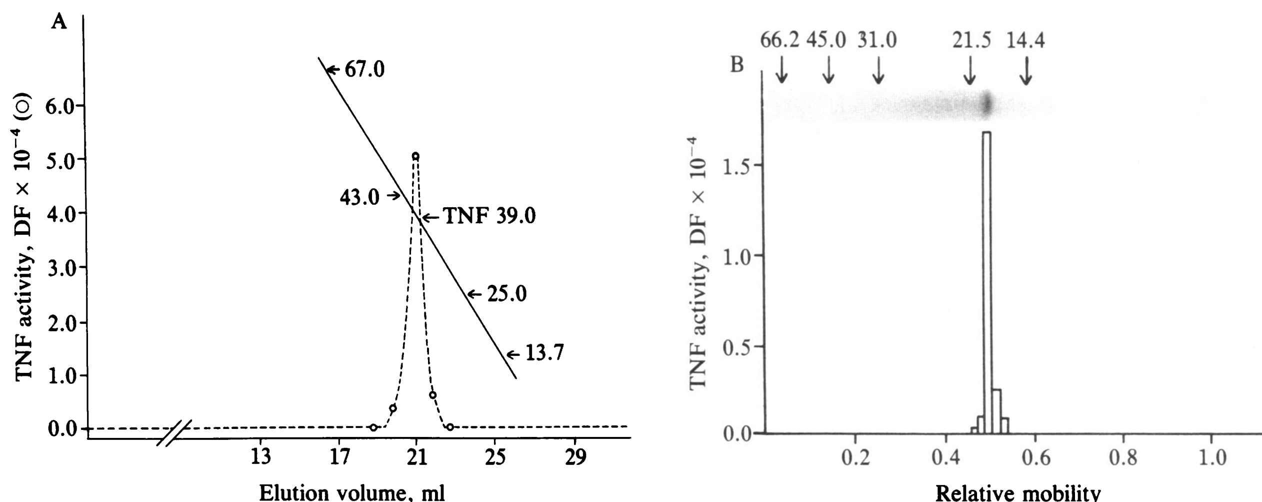

FIG. 2. Molecular weight determination of TNF. (A) Molecular weight of nondenatured TNF determined by Sephadex G-200 column chromatography. Concentrated FPLC Mono Q TNF was applied to a 0.9 × 120 cm G-200 column equilibrated with 0.04 Tris·HCl/0.7 M NaCl buffer, pH 7.8. Fractions of 0.3 ml were collected at a flow rate of 3.5 ml/hr and were tested *in vitro* for TNF activity. As estimated by comparison with molecular weight standards (shown as $M_r \times 10^{-3}$), TNF eluted at a M_r of 39,000 ± 2000. (B) Molecular weight of denatured TNF determined by $NaDodSO_4$/PAGE. Concentrated FPLC Mono Q TNF was electrophoresed on an 8% slab gel without $NaDodSO_4$. The gel was cut into 2-mm strips and samples were eluted with Tris/1.0 M NaCl buffer for 24 hr. Each sample was applied to several tube gels (10% $NaDodSO_4$/polyacrylamide). After electrophoresis, one tube from each set was stained with Coomassie brilliant blue, and the others were cut into 1-mm sections and tested *in vitro* for TNF activity. The TNF-active samples were concentrated, reduced with 2-mercaptoethanol at 100°C for 2 min, and applied to a tube gel (10% $NaDodSO_4$/polyacrylamide). A single band was demonstrated by Coomassie brilliant blue staining (shown here). A single band was also demonstrated by silver staining after applying the same reduced samples to a 12% $NaDodSO_4$/polyacrylamide slab gel.

blood is obtained per mouse, with an L-cell titer of 0.5–6 × 10^{-4} and Meth A necrotizing activity of ++ to +++ after injection of 0.25–0.5 ml of serum.

Purification of Mouse Serum TNF. *DEAE A-50 Sephadex chromatography.* Fig. 1A illustrates the elution pattern of TNF activity after DEAE A-50 ion-exchange chromatography and exposure to different ionic strength buffers. Using 0.195 M NaCl, >95% of the protein could be eluted from the gel without any release of TNF. Ionic strengths >0.225 M NaCl resulted in virtually complete recovery of TNF activity. With this information, a simple DEAE A-50 batch method was devised. Large amounts of material could be processed in a short period of time with excellent recovery of TNF. This resulted in a 64-fold purification of TNF L-cell activity (Table 1).

Sephadex G-200 chromatography. Previous studies (9) indicated that TNF activity was found in a high molecular weight fraction (M_r 150,000) after G-200 chromatography with low ionic strength buffers (0.15 M NaCl). Using high ionic strengths (0.7 M NaCl), TNF activity is detected shortly after the albumin peak, with an estimated M_r of 39,000 (Fig. 1B). Whether high ionic strength buffers prevent aggregation or dissociate TNF from a carrier molecule is not yet clear.

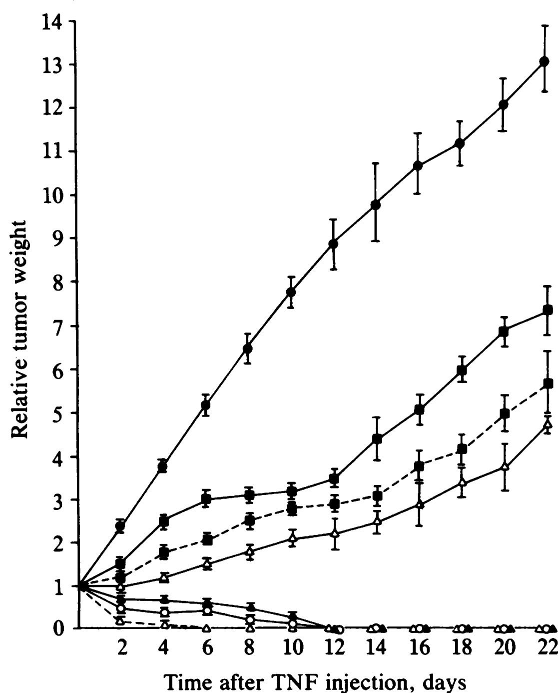

FIG. 3. Growth of Meth A sarcoma in BALB/c female mice injected with purified M_r 39,000 or M_r 16,000–18,000 TNF. Seven days following intradermal inoculation of Meth A cells, mice received a single intravenous injection of TNF. Lines represent changes in relative tumor weight in groups of three mice. Estimations of tumor weight were made from linear measurements; to standardize viability, relative tumor weights were calculated according to ref. 4. ●–●, Control; M_r 16,000–18,000 (■–■), 7000 DF; △–△, 10,000 DF; ▲–▲, 12,000 DF; ○–○, 14,000 DF; M_r 39,000 (■--■), 5000 DF; △--△, 10,000 DF.

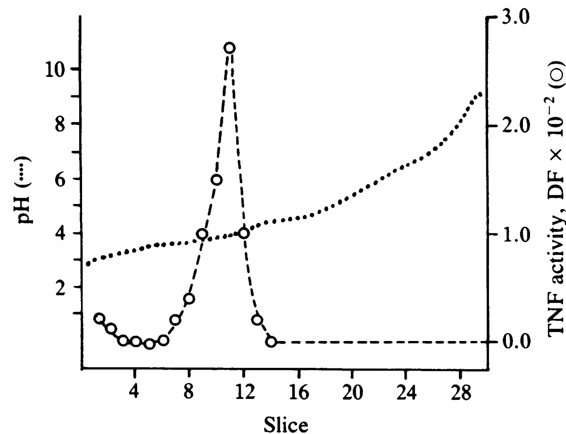

FIG. 4. Isoelectric focusing of mouse TNF. Preparative flat-bed isoelectric focusing (granulated Ampholine carrier system).

Employing the higher strength buffer in the sequential purification scheme outlined in Table 1, the G-200 fraction of DEAE-purified TNF resulted in 74% recovery of L-cell activity and a 1000-fold purification over TNF serum.

Affi-Gel blue affinity chromatography. Contaminating albumin from the Sephadex G-200 fraction could be removed by chromatography on Affi-Gel blue (Fig. 1C). This step resulted in a 3-fold purification and no loss of TNF (Table 1).

FPLC Mono Q chromatography. The TNF-containing fraction from Affi-Gel blue was subjected to three rounds of FPLC Mono Q chromatography. Fig. 1D illustrates the elution pattern from the third application. TNF derived from this step was ≈50-fold purified over the Affi-Gel blue fraction and 150,000-fold purified over TNF serum, with a recovery of ≈50% of the applied material (Table 1).

PAGE. In the final step, the fraction from FPLC Mono Q chromatography was separated by PAGE in the absence of $NaDodSO_4$. Gels were cut into 2-mm strips, eluted, and assayed for L-cell activity. Fractions with highest activity had a specific activity of 2.2×10^7, representing a 200,000-fold purification over TNF serum (Table 1). Fig. 2A shows a molecular weight determination of purified TNF. By Sephadex G-200 column chromatography, TNF had a M_r of 39,000 ± 2000. On $NaDodSO_4$/PAGE, under reducing conditions, purified TNF showed a single band by Coomassie blue or silver staining with a M_r of 16,000–18,000 (Fig. 2B).

In vivo TNF assay. Each of the purification steps was monitored for *in vivo* TNF activity (Table 1). This assay requires far more TNF (≈30,000 times more) and is less quantitative than the *in vitro* assay. Nevertheless, both activities copurified through the sequential fraction steps. There was a 2.5-fold purification of necrotizing activity after DEAE-Sephadex, 80-fold after Sephadex G-200, 667-fold after Affi-Gel blue, 10,000-fold after FPLC Mono Q, and 20,000-fold after PAGE. *Limulus* assays of purified TNF showed an endotoxin activity of <0.2 ng/ml. Whereas 15–20 mg of protein is required for ++ necrosis of Meth A using TNF serum, 1 μg of purified TNF induced a comparable reaction (Table 1). The M_r 16,000–18,000 components separated by $NaDodSO_4$/PAGE also exhibited tumor-necrotizing and tumor-inhibitory activity, but higher amounts (9 μg) were required to induce ++ tumor necrosis. Fig. 3 illustrates a comparative study of the tumor-inhibitory activity of the purified M_r 39,000 and M_r 16,000–18,000 components.

Characteristics of Purified Mouse Serum TNF. *pI.* The pI of mouse TNF as estimated by preparative flat-bed isoelectric focusing was pH 3.9 (Fig. 4). Chromatofocusing assays using a FPLC system also showed that the pI for TNF was <4.3 and that TNF activity was eluted by high ionic strength buffers.

Con A affinity chromatography. Affinity chromatography on Con A-Sepharose indicated heterogeneity in TNF binding (Fig. 5). Approximately 60% of applied TNF did not bind to Con A, whereas the residual activity bound and could be eluted with α-methyl-D-mannoside.

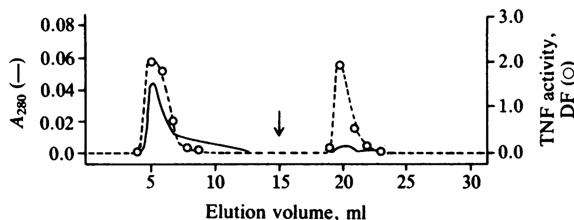

FIG. 5. Con A-Sepharose affinity chromatography of mouse TNF. After application of TNF, the Con A-Sepharose column (0.3 × 10 cm) was washed with 20 mM phosphate-buffered saline (pH 7.2). Bound TNF was eluted (↓) with α-methyl-D-mannoside at a flow rate of 2.5 ml/hr.

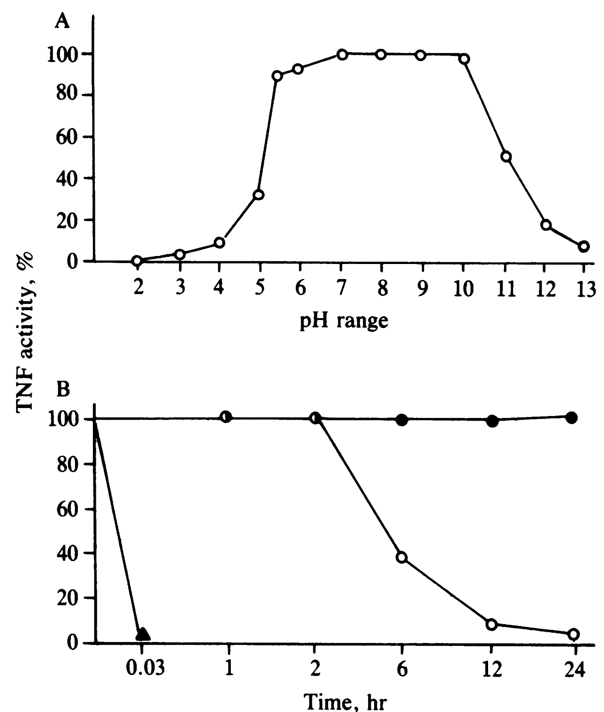

FIG. 6. pH stability and thermostability of mouse TNF. (A) TNF dialyzed against different pH buffers for 24 hr. (B) TNF stability at 37°C (●), 56°C (○), and 100°C (▲). TNF activity was determined by *in vitro* assay.

pH stability and thermostability. TNF activity was stable in the pH range of 5.5–10.0 but was rapidly destroyed at a higher or lower pH. Incubation of TNF at 37°C for 40 hr caused no loss of activity. However, activity was decreased at 56°C for 6 hr and destroyed at 100°C for 2 min (Fig. 6).

Sensitivity of mouse TNF to various enzymes. TNF was incubated with various enzymes and residual TNF activity was assayed (Table 2). Trypsin, protease, elastase, and α-chymotrypsin destroyed TNF activity. Papain, neuraminidase, and pepsin exerted little or no effect.

DISCUSSION

Several sources of nonrecombinant TNF have now been characterized. The properties of mouse TNF (as defined here), rabbit TNF (27), and human TNF (28) are quite similar.

Table 2. Stability of mouse TNF to digestion by various enzymes

Enzyme	Final concentration, units	TNF activity remaining	
		DF × 10^{-3}	% of control
Control*		167.4	100
Trypsin	60 BAEE	1.4	0.8
Protease	0.24	5.6	3.3
Elastase	0.34	3.6	2.2
α-Chymotrypsin	180 ATEE	11.2	6.7
Papain	0.04	155.8	93.1
Control†		112.4	100
Neuraminidase	0.01	99.8	88.8
	0.04	85.6	76.2
Pepsin	16	101.4	90.2
	40	65.6	58.4

BAEE, N^{α}-benzoyl-L-arginine ethyl ester; ATEE, N-acetyl-L-tyrosine ethyl ester.
*Tris·HCl, pH 7.8.
†Acetate buffer, pH 5.1.

The natural forms have a M_r of ≈40,000 and are composed of M_r 16,000–18,000 monomers associated through noncovalent bonds. The pI of mouse TNF is 3.9, rabbit TNF is 4.0, and human TNF is 5.3. Similar characteristics have been reported for recombinant human TNF (28) and recombinant mouse TNF (23). There appears to be species variation in the extent of TNF glycosylation. As described here, a fraction of mouse serum TNF binds to Con A, and there are several glycosylation signals in the coding sequence for mouse TNF (22, 23). Rabbit TNF (27) and human TNF (28) do not bind to Con A, and there are no N-linked glycosylation signals in the coding sequence for human TNF (17–19). In contrast, a molecule from the LuKII human lymphoblastoid cell line (29), which has all of the *in vivo* and *in vitro* characteristics of TNF and which binds to the same surface receptors as mouse and human TNF (30), is known to be heavily glycosylated. It is not yet clear whether this factor, designated TNF(LuKII), is related to lymphotoxin (31), a cytotoxic factor with TNF-like activity that has been cloned recently (32). Nonrecombinant lymphotoxin binds to Con A (31, 33) and glycosylation signals are found in the coding sequences for lymphotoxin (32). In future studies, it will be important to relate these differences in glycosylation to biological activities, such as antitumor activity and toxicity, and to compare the *in vivo* and *in vitro* activities of glycosylated (nonrecombinant) and nonglycosylated (recombinant) forms of mouse TNF.

It is now established that the *in vivo* necrotizing and *in vitro* cytotoxic activities of TNF are properties of one and the same molecule. In the present study, we observed no dissociation between these two activities throughout the purification steps, and recombinant human TNF displays both activities (refs. 17–19; unpublished observations). Past suggestions that the necrotizing and cytotoxic activities of TNF were dissociable (34, 35) point out the difficulties of comparing two assays with very different quantitative end points (tumor necrosis requires 30,000 times more TNF protein than required in the L-cell assay) and different specificity requirements (tumor necrosis can be induced by endotoxin-contaminated materials and L cells can be killed by a variety of unrelated factors). For this reason, cytotoxic assays should include the TNF-resistant L-cell line as a specificity control (25), particularly when actinomycin D or mitomycin C is used to enhance the sensitivity of the L-cell assay, and materials tested for necrotizing activity should be assayed for endotoxin by *Limulus* assays. Antibodies to TNF, as they become available, provide additional specificity controls.

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