Proc. Nat. Acad. Sci. USA
Vol. 73, No. 2, pp. 381–385, February 1976
Biochemistry

Partial purification of a serum factor that causes necrosis of tumors

(anti-tumor factor/bacillus Calmette-Guérin/endotoxin/glycoprotein)

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Communicated by Lewis Thomas, November 14, 1975

ABSTRACT **Tumor necrosis can be induced in transplanted mouse methylcholanthrene-induced sarcoma by a tumor necrosis factor in the serum of mice infected with bacillus Calmette-Guérin and given bacterial endotoxin. Sera from normal mice, endotoxin-treated mice, and mice infected with bacillus Calmette-Guérin do not contain this factor. A 20- to 30-fold purification of the serum factor has been achieved by $(NH_4)_2SO_4$ fractionation, Sephadex G-100 and G-200 gel filtration, and preparative polyacrylamide electrophoresis. Tumor necrosis factor is not bacterial endotoxin. It migrates with α-globulins, is made up of at least four subunits, and has a molecular weight of about 150,000. The active factor is a glycoprotein that contains sialic acid and galactosamine.**

Carswell *et al.* (1) have described a tumor necrosis factor (TNF) that is found in the serum of endotoxin-treated mice that had been infected with bacillus Calmette-Guérin (BCG). TNF is not interferon and is not pyrogenic for rabbits. Serum containing TNF induces necrosis in a variety of transplanted mouse tumors, with no toxic effect upon the tumor-bearing recipient. In the standard assay for TNF (1), virtually all tumors become grossly necrotic within 24 hr of TNF administration, and about 20% of these regress completely. Two transformed cell lines in culture were also inhibited by TNF whereas cultured mouse embryo cells were not (1).

This report concerns the partial purification and preliminary characterization of TNF from mouse serum.

MATERIALS AND METHODS

Preparation of Mouse Serum Containing TNF. CD-1 female Swiss mice weighing 35–40 g were injected intravenously with 2×10^7 viable BCG organisms (Tice strain) obtained from the Institute for Tuberculosis Research (University of Illinois Medical Center, Chicago, Ill.). After 14 days, 25 μg of endotoxin (lipopolysaccharide W from *Escherichia coli*, Difco, Detroit, Mich.) was injected intravenously, and the mice were exsanguinated from the axilla 2 hr later. Control mice received either viable BCG organisms only, 25 μg of endotoxin only, or no treatment. Serum was pooled and stored in sealed vials at −78°.

TNF Bioassay. The TNF activity of all fractions was bioassayed in (BALB/c × C57BL/6)F_1 hybrid mice bearing 7-day subcutaneous transplants of methylcholanthrene-induced fibrosarcoma (Meth A) averaging 7–8 mm in diameter. The degree of tumor necrosis developing at 24 hr after intravenous injection of 0.5 ml of the test sample was graded as previously detailed (1). Only samples inducing central necrosis extending over half the tumor surface (++) or massive necrosis, leaving a small viable rim along the tumor periphery (+++), were considered to contain TNF. Three mice bearing Meth A were scored for assay of each sample. One unit of TNF activity is defined as the lowest concentration of protein in the test sample that will produce necrosis (+++) in 100% of the Meth A tumors.

Assay Procedures. Alkaline (EC 3.1.3.1) and acid phosphatase (EC 3.1.3.2) (2, 3) β-glucuronidase (EC 3.2.1.31) (4), β-glucosidase (EC 3.2.1.21) (5), α-galactosidase (EC 3.2.1.22) (6), lactate dehydrogenase (EC 1.1.1.27) (7), aspartate aminotransferase (EC 2.6.1.1) (8), lysozyme (EC 3.2.1.17) (9), neuraminidase (EC 3.2.1.18) (10), NAD^+ glycohydrolase and (NADase; EC 3.2.2.5) (11, 12) were assayed as described. Total protein was determined by the method of Lowry *et al.* (13); DNA and RNA by the method of Hotchkiss (14); endotoxin, by the *Limulus* lysate assay of Levin *et al.* (15); sialic acid, by the resorcinal method of Warren (10); polyacrylamide gel electrophoresis, by the method of Ornstein (16); cellulose acetate electrophoresis as described in the Beckman Manual RM-1 M3 (17).

RESULTS

Electrophoretic Pattern of Serum Proteins. The profiles of serum proteins from mice treated with endotoxin or BCG, or with both are shown in Fig. 1. No significant difference was found between normal and endotoxin-treated mice. Sera from the mice infected with BCG or infected with BCG followed by endotoxin showed increases in the α-globulin region and in the region between the β and γ globulins.

Changes in Serum Enzymes. Table 1 shows that enzyme levels in endotoxin-treated mice were essentially similar to those of normal mice, with the exception of a small rise in lysozyme. The serum of BCG mice displayed marked elevation in all the enzymes measured; four of these—acid phosphatase, β-glucuronidase, β-glucosidase, and α-galactosidase—are lysosomal. In the BCG-endotoxin-treated group these elevations (with the exception of alkaline phosphatase) were even more marked. NADase, which is normally bound in microsomes (12), was present only in the serum of the BCG-endotoxin-treated mice. As we have shown previously (1), TNF was demonstrable only in serum of BCG-endotoxin-treated mice. Similar trace amounts of endotoxin (*Limulus* assay) were found in all four pools, indicating that TNF is not endotoxin.

Partial Purification of TNF Obtained from Serum. All steps were carried out at 4°. Glassware was steam-sterilized at 250°F (121°C) for 20 min, and solutions were autoclaved. Sephadex gels were equilibrated and rinsed with 10 volumes of sterile 0.01 M potassium phosphate buffer, pH 7.0 containing 0.15 M NaCl (phosphate-buffered saline).

A typical purification of TNF was as follows: 80 ml of TNF-rich serum was diluted with an equal volume of sterile 0.15 M NaCl, and 36.8 g of $(NH_4)_2SO_4$ was added with con-

Abbreviations: Meth A, methylcholanthrene-induced fibrosarcoma; TNF, tumor necrosis factor; BCG, bacillus Calmette-Guérin; NADase, NAD^+ glycohydrolase (EC 3.2.2.5); M_r, molecular weight.

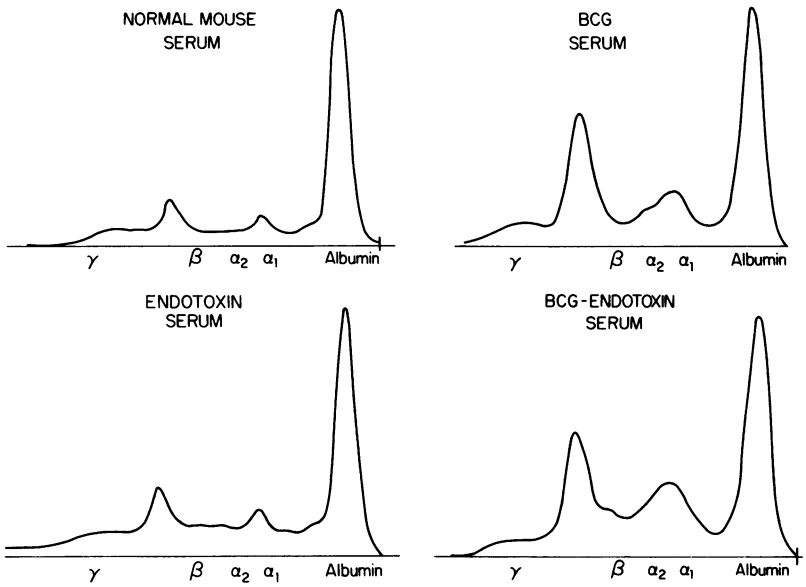

FIG. 1. Cellulose acetate.electrophoresis of serum from untreated, endotoxin-treated, BCG-treated, and BCG- and endotoxin-treated mice.

stant stirring (35% saturation). The pH was maintained at 7.0 by dropwise addition of concentrated NH_4OH. After 1 hr the mixture was centrifuged for 15 min at 105,000 × *g* and 36.8 g of $(NH_4)_2SO_4$ was added to the supernatant solution with constant stirring. After 1 hr the precipitate was suspended in 15 ml of distilled water and dialyzed against eight successive 500-ml volumes of sterile phosphate-buffered saline until free of sulfate. The solution was brought to 50–75 mg of protein per ml, and 10-ml aliquots were filtered through a Sephadex G-100 gel column. Five-milliliter fractions were collected (Fig. 2). TNF activity was found in the fractions under peak 1. These fractions (called G-100-1) were pooled, concentrated by lyophilization, and filtered through a G-200 Sephadex gel (Fig. 3). Three peaks of protein were eluted from this column, and TNF activity was found in the sample collected under peak 2 (called G-200-II).

The proteins in G-200-II were separated by preparative polyacrylamide gel electrophoresis in a modified Canalco model 1200 disc electrophoresis apparatus (16). Separating gels with a volume of 15 ml each were prepared from a 7% acrylamide solution and were polymerized in 16 × 150 mm glass tubes. After polymerization a 2-ml stacking gel was placed on each, the gel tubes were immersed in 2.6 liters of ice-cold 0.025 M Tris–0.2 M glycine buffer, pH 8.3, and the upper electrode chamber was filled with the same buffer. A 1-hr pre-electrophoresis was carried out at 5° and at 3 mA per tube. One-milliliter aliquots containing 3.0–3.5 mg of G-200-II protein, Tris-glycine buffer, and 0.6 M sucrose were layered over the stacking gel. Electrophoresis was carried out at 3.0 mA per tube until the tracking dye (0.05% bromphenol blue), reached the separating gel. The amperage was increased to 5 mA per tube and electrophoresis continued for 16.5 hr.

For the first run, 0.05 ml of TNF-rich whole serum was applied to one gel and aliquots of the G-200-II containing

Table 1. Analysis of sera from treated and untreated mice

	Treatment of serum donor			
	None	BCG	Endotoxin	BCG and endotoxin
Alkaline phosphatase[a]	71.0	320	67	331
Acid phosphatase[b]	2.7	28	3.1	280
β-Glucuronidase	0.6	3.2	0.2	58
β-Glucosidase	1.6	4.7	2.2	10.7
α-Galactosidase	4.6	146	6.3	1,394
Lactate dehydrogenase	976	2,472	1,162	>25,000
Aspartate aminotransferase[c]	236	735	270	4,886
Lysozyme[d]	40	60	70	160
NADase	0	0	0	5.0
Endotoxin, μg/ml	0—1.0	0—1.0	0.5—1.0	0.5—1.0
TNF[e]	0	0	0	+++

Procedures and assays are described in *Materials and Methods*. Values are the average of duplicate analyses on pools of serum from 10 mice.
[a] Where noted, enzyme activities are in μmol of substrate converted per min/ml of serum.
[b] Bodansky units (one unit is 1.0 mg of substrate converted per hr/ml).
[c] One unit is the change in A of 0.001 per min/ml at 340 nm.
[d] One unit is the change in A of 0.001 per min/ml at 450 nm.
[e] See *Materials and Methods*.

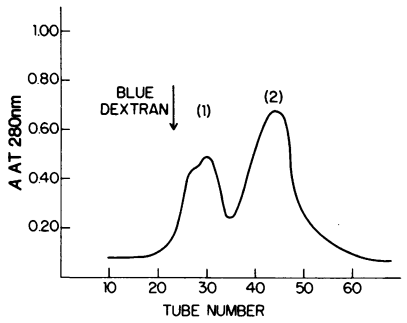

FIG. 2. Gel filtration of the proteins present in the 35–70% $(NH_4)_2SO_4$ fraction of BCG-endotoxin-treated mouse serum, through a column (2.5 × 100 cm) of Sephadex G-100 in phosphate-buffered saline (pH 7.0). TNF activity was recovered in the first major peak.

3.0–4.0 mg of protein were placed on the remaining 11 gels. After electrophoresis the gel containing the whole serum sample (Fig. 4A) and one of the gels containing G-200-II were stained for protein with amido Schwarz. A second gel containing G-200-II was stained for glycoprotein (18). Remaining gels were cut into three 3.3-cm segments (A, cathodic; B, center; C, anodic), and protein was extracted into 0.9% NaCl by homogenization in a Polytron homogenizer. Recovery of the protein in pooled extracts was 94–99%. TNF activity was located in the B (center) segment, which contained four major protein bands (Fig. 4B). Periodic acid-Shiff stain (Fig. 4C), showed that this area contained carbohydrate. Extracts from gel segments in the B region were re-electrophoresed. One gel was stained for protein (Fig. 4D), and the B segments of the remaining gels were sliced into four 1-cm segments. TNF assay showed activity in the top two segments (G-200-II, B_1 and B_2). When the extracts from the B_1, B_2, B_3, and B_4 segments were re-electrophoresed, the patterns shown in Fig. 4 (gels E, F, G, and H) were obtained. The most cathodic protein (B_1) separated into four bands, B_2 into three bands, B_3 into two bands, and B_4 migrated as one band.

To determine whether the protein fractions, generated when G-200-II-B was electrophoresed, were separating on the basis of charge or of their molecular weight, aliquots of G-200-II, G-200-II-B_1, G-200-II-B_2, and G-200-II-B_3 were electrophoresed on cellulose acetate strips. Fig. 5 shows that B_1, B_2, and B_3 migrated as the α-globulin of the whole G-200-II fraction. Thus, these fractions may be separating on the basis of their molecular weight when electrophoresed on the polyacrylamide gels.

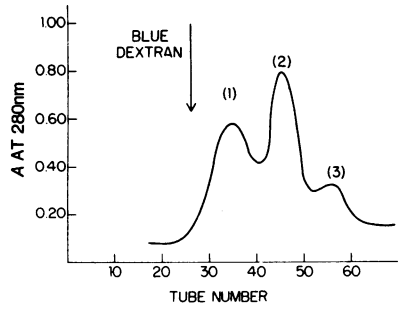

FIG. 3. Gel filtration of the proteins present in the first major peak from the Sephadex G-100 filtration through a column (2.5 × 100 cm) of Sephadex G-200 in phosphate-buffered saline (pH 7.0). The TNF activity was recovered in the second major peak (G-200-II).

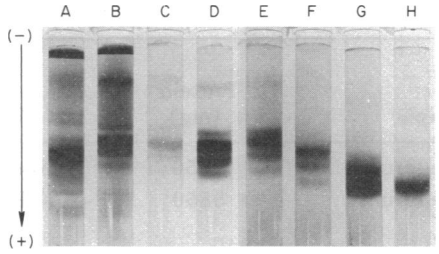

FIG. 4. Electrophoresis for 16.5 hr at 4° and at 5.0 mA per tube of fraction G-200-II on polyacrylamide gels (1.4 × 10.0 cm) at pH 8.9. Migration was toward the anode (top to bottom), and protein was stained with amido Schwarz. (A) TNF serum; (B) G-200-II; (C) G-200-II, stained for glycoprotein with periodic acid-Shiff reagent; TNF activity was in the central third of the gel (B segment); (D) re-electrophoresis of protein extracted from the B segment; (E-H) re-electrophoresis of each of the four proteins in the B segment, i.e., (E) first band, (F) second band, (G) third band, and (H) fourth band.

Characterization of G-200-II-B_1 and B_2. The molecular weights of protein in a mixture of G-200-II-B_1 and B_2 were determined on Sephadex G-200 gel column by the method of Andrews (19). The column was calibrated with purified glyceraldehyde 3-phosphate dehydrogenase [molecular weight 140,000 (M_r)]; bovine serum albumin (M_r 67,500); pepsin (M_r 35,000); soybean trypsin inhibitor (M_r 21,000); and cytochrome *c* (M_r 12,000). The mean elution volume and average deviation of this volume of each standard protein was determined, and the best straight line between each of these points was determined by the method of least squares. The results with G-200-II-B_1 and B_2 showed two components, one of M_r about 170,000 and one of M_r about 100,000. Both had TNF activity, when a sample of the mixture in 0.9% NaCl was centrifuged at 50,000 rpm in a no. 50 Ti Spinco rotor for 16.5 hr. All the TNF activity was found in the pellet. The *g*-force produced by this speed sediments proteins of M_r 150,000 or more. These data are summarized in Table 2.

Preliminary analysis of some chemical and physical characteristics of the G-200-II, B_1–B_2 mixture have been carried out. TNF activity was not lost after 1 hr at 56° but was completely lost after 1 hr at 70°C. Repeated freezing and thawing did not inactivate TNF. All preparations contained be-

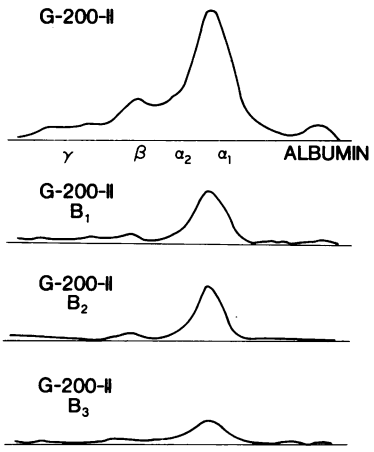

FIG. 5. Cellulose acetate electrophoresis of fraction G-200-II and of each of the proteins extracted from the B segment of polyacrylamide gels: fraction B_1, fraction B_2, and fraction B_3. Reading left (cathode) to right (anode), the major peak occurs in the α-globulin region.

Table 2. Isolation of TNF from BCG-endotoxin-treated mice

Sample	Protein (mg)	Treatment[a]	Fraction	Protein (%)	TNF activity[b] (units/mg protein)
(*1*) Whole serum	5850	None	—	100	0.033
(*2*) Whole serum	5850	$(NH_4)_2SO_4$ fractionation	0—35% ppt.[c]	11	0
			35—70% ppt.	56	0.061
			70—100% ppt.	26	0
			Supernatant	7	0
(*3*) Protein ppted. by 35—70% $(NH_4)_2SO_4$	3276	G-100 Sephadex filtration	Peak 1	35	0.105
			Peak 2	60	0
(*4*) Pooled protein under peak 1	1147	G-200 Sephadex filtration	Peak 1	30	0
			Peak 2	53	0.154
			Peak 3	10	0
(*5*) Pooled protein under Peak 2	604	Acrylamide gel electrophoresis	(A) Cathodic segment (3.5 cm)	20	0
			(B) Center segment (3.5 cm)	33	0.250
			(C) Anodic segment (3.5 cm)	35	0
(*6*) (B) Center segment	200	Extraction, lyophylization, dialysis, and electrophoresis	Band (1)	14	0.526
			Band (2)	68	0.301
			Band (3)	10	0
			Band (4)	6	0

[a] See *text* for details.
[b] One unit of TNF activity is the lowest concentration of protein which produces necrosis in 100% of the Meth A tumors under the conditions specified.
[c] ppt., precipitate.

tween 0.4 and 4.0 μg of endotoxin-like material per ml (*Limulus* assay). In these amounts, *E. coli* endotoxin does not produce necrosis in sarcoma Meth A. No Gram-negative or Gram-positive organisms, or fungi, were demonstrable after 24–48 hr of culture. The mixture did not react with antibody to human C-reactive protein (20) nor did it precipitate with purified pneumococcal C-polysaccharide (21).

Biochemical analysis of the G-200-II-B_1-B_2 containing 3.0 mg of protein per ml showed: Enzymes—no measurable acid phosphatase, alkaline phosphatase, β-glucosidase, β-glucuronidase, α-galactosidase, NADase, neuraminidase, or lysozyme; carbohydrates—sialic acid (98 μg), galactosamine (90 μg), glucose (15 μg), and fucose (5 μg); fatty acids—myristic (3 μg), palmitic (16 μg), oleic (3 μg), and stearic (5. μg); nucleic acids—no measurable DNA or RNA.

DISCUSSION

The scheme used to isolate TNF is summarized in Table 2. Our data show TNF is present in a glycoprotein fraction with the electrophoretic mobility of α-globulins and a molecular weight of approximately 150,000. Acrylamide gel electrophoresis yields evidence that the molecule is a polymer, composed of at least four subunits with different molecular weights. Fig. 4 shows the TNF activity is located in two bands in the central segment of the gel. Re-electrophoresis of band 1 on polyacrylamide yields four bands which migrate as α-globulins during cellulose acetate electrophoresis. This suggests that the protein in band 1 dissociates into subunits having the same electrical charge but different molecular weights.

The evidence presented here indicates TNF is not endotoxin. Thus, TNF gives only trace reactions in the *Limulus* assay; TNF lacks the sugar 2-keto-3-deoxyoctonate (22) and the fatty acid 3-D-myristo-myristic (23) which are characteristic of the lipid A moiety of bacterial endotoxin (23).

What is the cellular source of TNF? As reported here, BCG-infected mice show a general rise in serum levels of enzymes, particularly those that are abundant in lysosomes of activated macrophages. The levels of these enzymes are increased still further in the serum of BCG-infected mice in shock after injection with endotoxin. There are now many indications that macrophages, activated by BCG or endotoxin, acquire discriminating toxicity *in vitro* for transformed cells as compared with normal cells (24, 25, 29). Because TNF also has shown such selective toxicity (1) we have proposed that TNF may mediate this cytotoxic property of activated macrophages.

How is TNF released into the serum? Mice infected with BCG become inordinately sensitive to the toxic effects of endotoxin (26). Endotoxin damages hepatocytes and liver macrophages of normal mice (27), and we have observed pyknosis and disruption of macrophages in the greatly enlarged spleens of mice treated with BCG followed by endotoxin (S. S. Sternberg and L. J. Old, unpublished results). The outpouring of enzymes that accompanies endotoxin shock in BCG-infected mice is another indication of tissue disruption. Especially striking is our finding that NADase, an enzyme normally bound to membranes (12) and absent from serum, is found in the serum of BCG mice after treatment with endotoxin (28).

Thus, we favor the view that endotoxin causes the disruption of cellular components, i.e., activated macrophages, *in vivo* and that as a consequence a cellular glycoprotein, identified as TNF, is solubilized and released. If this is true, it should be feasible to prepare TNF directly from the tissues of mice treated with BCG or *Corynebacterium parvum*.

We thank Dr. Lewis Thomas and Miss Dorothy McGregor (Memorial Sloan-Kettering Cancer Center, New York, N.Y.) for the endotoxin assays with *Limulus* lysate; Drs. Marion Barclay and Francis Archibald (Memorial Sloan-Kettering Cancer Center, New York, N.Y.) for the carbohydrate analysis; Dr. Robert Rosenfeld (Montefiore Hospital, New York, N.Y.) for the fatty acid determinations. Supported in part by grants from: NCI CA-09748; New York Cancer Research Institute, Inc.; The Fuller Foundation, Inc.; The Martin Elk League for Cancer Research.

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