Purification of rabbit tumor necrosis factor

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Rabbit tumor necrosis factor (TNF) was purified and shown by SDS–PAGE to be a single protein of 18 kDa. TNF in 355 ml rabbit serum was precipitated with ammonium sulfate, and purified by repeated DEAE-Sephadex and Sephacryl S-200 chromatographies, and the final fractionation on Blue-Sepharose 6B. By this procedure its yield was 22% and its specific activity was 2.4×10^7 U/mg protein. The sequence of the N-terminal 20 amino acids was determined.

Tumor necrosis factor *Purification* *Amino acid sequence*

1. INTRODUCTION

The tumor necrosis factor (TNF) was first discovered by Carswell et al. [1] in a murine system. They found that it was induced in the serum of animal-infected Bacillus Calmette-Guérin (BCG) and subsequently injected with endotoxin. Serum-containing TNF induces necrosis in a variety of transplanted tumors in vivo and has cytotoxic effects on various tumors in vitro. Ruff and Gifford [2] partially purified the TNF from rabbits. But their purification has, so far, not been sufficient for the determination of its partial amino acid sequence.

This paper reports the purification of rabbit TNF and its identification as a protein of 18 kDa by SDS–PAGE. The sequence of its N-terminal 20 amino acids was also determined;

2. MATERIALS AND METHODS

2.1. *Induction of rabbit TNF*

Serum-containing TNF was prepared by the method of Carswell et al. [1]. Briefly, Japanese albino rabbits were infected intravenously (i.v.) with 18 mg lyophilized viable Bacillus Calmette-Guerin (Japan BCG Ind., Tokyo). Two weeks later the animals were challenged i.v. with 80 μg lipopolysaccharide from *E. coli* 0127:B8 (Difco Lab. Detroit, MI) and bled 1.5 h later to obtain serum-containing TNF.

Abbreviations: TNF, tumor necrosis factor

2.2. *Cytotoxicity assay*

TNF activity was determined by assay of killing of L 929 cells, as described by Ruff and Gifford [2]. Briefly, L 929 cells (8×10^4/well), dilutions of TNF and actinomycin D (0.2 μg/well) were incubated for 18 h in 96 well trays (Nunc, OK4000, Denmark) containing 200 μl medium (Eagle's MEM supplemented with 5% fetal calf serum). Then the cells were stained with 0.2% crystal violet for 15 min, washed with PBS(−) and treated with 100 μl of 0.5% sodiumdodecylsulfate. The absorbance at 590 nm of the solubilized material was measured. The cell survival ratio was calculated as S/S_o, where S_o is the the A_{590} of the control tray without TNF and S is that of the experimental tray. One unit of TNF activity was arbitrarily defined as the amount giving a survival ratio of 50% under these conditions.

2.3. *Purification procedure*

Ammonium sulfate fractionation and DEAE-Sephadex and Sephacryl S-200 chromatographies

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were performed essentially as described by Ruff and Gifford [2]. Saturated ammonium sulfate was added to serum-containing TNF to a final concentration of 60% at pH 7.0. The precipitate was dissolved in 50 mM sodium phosphate buffer and loaded onto a column (2.5×25 cm) of DEAE-Sephadex (Pharmacia, NJ) previously equilibrated with the starting buffer (50 mM phosphate, 120 mM NaCl, pH 7.0). The column was washed with the starting buffer and then the material was eluted with a linear NaCl gradient (120–400 mM). Fractions with activity were concentrated to 15 ml on an ultrafiltration membrane (Toyo Kagaku KK., Tokyo) and applied to a column (2.5×120 cm) of Sephacryl S-200 (Pharmacia, NJ) with phosphate buffer described above. Fractions with TNF activity were rechromatographed on Sephacryl S-200 and then on a DEAE-Sephadex column (1.0×10 cm) under similar conditions to those for the first column. The fraction containing TNF was recovered and loaded onto a column (1.0×10 cm) of Blue-Sepharose 6B (Pharmacia, NJ), previously equilibrated with the starting buffer (50 mM Tris-HCl, 100 mM Kcl, pH 7.0).

The purified TNF (12 μg) recovered from the Blue-Sepharose was subjected to reverse-phase high-pressure liquid chromatography (HPLC, Spectra Physics, SP8000, Calf) in a C_4 column (25 cm×4.6 mm; Bio-Rad, CA). The material was eluted with a linear gradient of 20–90% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. Fractions with absorption (A_{210} nm) were collected and lyophilized.

For determination of the amino acid sequence, 6 μg TNF was introduced into a protein sequencer (Applied Biosystem Co., CA, Model 470 A). Protein was determined by the method of Lowry et al. [3]. Polyacrylamide gel electrophoresis was carried out as described by Laemmli [4]. The protein on the gel was located by silver staining [5].

3. RESULTS

Typical results in the purification are summarized in table 1. The first step of purification is based on the results of Ruff and Gifford [2]. We confirmed their results on a large scale up to the first Sephacryl S-200 column chromatography. The TNF-enriched fraction obtained by this step caused necrosis of MH134 hepatoma transplanted intradermally 7 days before.

The active fraction was rechromatographed on Sephacryl S-200 and DEAE-Sephadex. In these steps, the TNF activity was eluted in fractions corresponding to a molecular mass of 30–40 kDa and with 250–300 mM NaCl, respectively. Although about a 10000-fold purification of TNF from crude serum was achieved at this stage, the preparation gave two major protein bands of 18 and 65 kDa on SDS-PAGE. Since the latter band corresponded to serum albumin, the preparation was finally subjected to Blue-Sepharose 6B chromatography. The unadsorbed fraction from Blue-Sepharose contained only TNF activity and gave a single band of 18 kDa on SDS-PAGE, as shown in fig.1. The final TNF preparation was purified 11000-fold from the serum in a 22% yield.

To confirm that the protein band of 18 kDa was associated with TNF activity, we sliced the gel and assayed TNF activity as described by Aggarwal et al. [6]. Fig.2 shows that TNF activity was associated with the 18-kDa band and recovery of TNF

Table 1

Summary of purification

Sample	Protein (mg)	Total TNF activity ($\times 10^3$ units)	Specific activity ($\times 10^3$ unit/mg protein)	Purification factor	Recovery (%)
Crude Serum	22000	48000	2.2	1.0	100
1st DEAE-Sephadex	96	45000	470	220	94
1st Sephacryl S-200	12	43500	3600	1700	91
2nd Sephacryl S-200	2.5	14000	5500	2600	30
2nd DEAE-Sephadex	0.51	12000	23000	10000	25
Blue-Sepharose 6B	0.43	10500	24000	11000	22

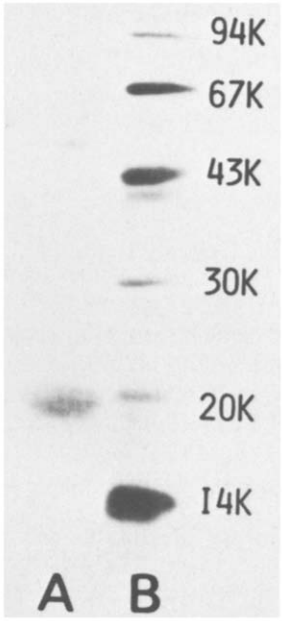

Fig.1. Polyacrylamide gel electrophoresis of rabbit TNF. (A) Purified TNF (4 μg). (B) Molecular markers in a Pharmacia Marker Kit. SDS-gel electrophoresis was carried out at pH 8.3, and then the gels were stained with a silver staining kit (Bio-Rad).

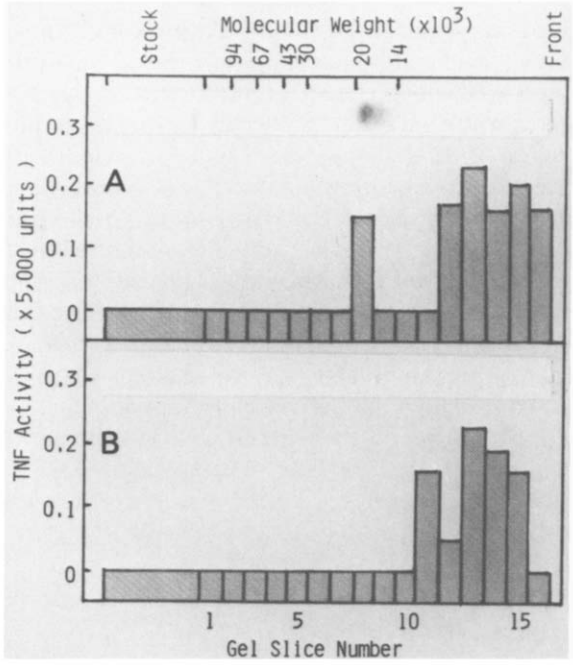

Fig.2. Profile of purified TNF after SDS-polyacrylamide gel electrophoresis. Samples of 8 μg purified TNF (A), or sample buffer (0.1% SDS and 0.1 M β-mercaptoethanol) (B) were subjected to electrophoresis on slab gel (0.1 mm thick, 15% polyacrylamide). The gels were cut into 6 mm wide slices, which were eluted with 0.5 ml of 50 mM NH_4HCO_3 and assayed for activity.

activity was 0.1% of the original TNF value before SDS treatment. There was also some activity at the dye front of the lanes applied with the TNF sample as well as with control buffer, which was reported [6] to be due to SDS concentrated at the dye front. To exclude the possibility that SDS may be concentrated to the 18-kDa band non-specifically, we confirmed that slices containing bovine serum albumin and ovalbumin in gels after SDS–PAGE had no cytotoxic activity.

The molecular mass of native TNF was estimated to be 34 kDa by gel filtration (Sephacryl S-200) of a mixture of TNF and marker proteins (not shown). These results indicate that purified TNF had a molecular mass of 18 kDa on SDS–PAGE but of about 34 kDa on gel filtration.

Reverse-phase HPLC of the purified TNF gave one major peak (75%, retention time = 11.79 min) and a minor peak (25%, retention time = 10.6 min) which was eluted with 45–50% acetonitrile. The solvent used for HPLC completely inactivated TNF.

The N-terminal amino acid sequences of the purified TNF and material in the major peak obtained by HPLC were examined in a protein sequencer. The sequence deduced from the two samples was: (His) - Ser - His - Val - Gly - Gln - Pro - Pro - Pro - Leu - Glu - Pro - (X) - Val - Ser - Glu - Arg - Gly - Arg - His - Try - Gln -. The amino acids in parentheses were not confirmed on analysis of the purified TNF.

4. DISCUSSION

Here we purified rabbit TNF 11 000-fold (table 1) to essentially single homogeneous species of 18 kDa as judged by SDS–PAGE. Ruff and Gifford [2] also reported the purification of rabbit TNF and estimated its molecular mass as 68 kDa by SDS–PAGE of a ^{125}I-labeled preparation [2]. Our value is supported by the following findings: (i) TNF activity was recovered from gel slices in a

position corresponding to 18 kDa; (ii) The TNF activity was finally separated from the 65-kDa protein by Blue-Sepharose chromatography; (iii) The purification factor (11 000-fold) by our procedure was larger than that reported by Ruff and Gifford (2200-fold). These results imply that native rabbit TNF has a molecular mass of approx. 34 kDa-, which is nearly equal to 39 kDa reported in a previous work by Matthew et al. [7] and is dissociated into 18 kDa species by SDS. We believe that the latter is a functioning monomer. These observations on rabbit TNF may be analogous to very similar findings on human lymphotoxins [6].

Information on the partial primary structure of rabbit TNF will be helpful for use of TNF in genetic engineering.

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