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### Research Article

Tumor necrosis factor (TNF) is a pleiotropic mediator of inflammatory responses. A cysteine-rich, highly glycosylated 30-kD TNF-binding protein (TNF-BP) purified from urine may have a role in regulation because it protects in vitro against the biological effects of TNF. The cytotoxic effect of TNF on the fibrosarcoma cell line WEHI 164 was inhibited by 50% at a 10-fold excess of TNF-BP. The binding of TNF to the receptor was partially reversed after the addition of TNF-BP. Results from biosynthetic labeling of cells with <sup>35</sup>S-cysteine followed by immunoprecipitation with anti-TNF-BP indicated that TNF-BP is formed and released at the cell surface by cleavage because no corresponding cellular polypeptide was observed. A cellular 60-kD polypeptide, which was immunoprecipitated with anti-TNF-BP, may correspond to the transmembrane TNF-receptor molecule and be the precursor of TNF-BP. Thus, TNF-BP appears to be a soluble form of a transmembrane TNF-receptor. Moreover our results demonstrate that the production of TNF-BP is increased when the TNF receptor is downregulated in cells by treatment with TNF or by activation of protein kinase C with phorbol esters. TNF-BP may be an important agent that blocks harmful effects of TNF, and, therefore, useful in clinical applications.

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# Characterization In Vitro of a Human Tumor Necrosis Factor-binding Protein

## A Soluble Form of a Tumor Necrosis Factor Receptor

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### Abstract

Tumor necrosis factor (TNF) is a pleiotropic mediator of inflammatory responses. A cysteine-rich, highly glycosylated 30-kD TNF-binding protein (TNF-BP) purified from urine may have a role in regulation because it protects in vitro against the biological effects of TNF. The cytotoxic effect of TNF on the fibrosarcoma cell line WEHI 164 was inhibited by 50% at a 10-fold excess of TNF-BP. The binding of TNF to the receptor was partially reversed after the addition of TNF-BP. Results from biosynthetic labeling of cells with  $^{35}\text{S}$ -cysteine followed by immunoprecipitation with anti-TNF-BP indicated that TNF-BP is formed and released at the cell surface by cleavage because no corresponding cellular polypeptide was observed. A cellular 60-kD polypeptide, which was immunoprecipitated with anti-TNF-BP, may correspond to the transmembrane TNF-receptor molecule and be the precursor of TNF-BP. Thus, TNF-BP appears to be a soluble form of a transmembrane TNF-receptor. Moreover our results demonstrate that the production of TNF-BP is increased when the TNF receptor is downregulated in cells by treatment with TNF or by activation of protein kinase C with phorbol esters. TNF-BP may be an important agent that blocks harmful effects of TNF, and, therefore, useful in clinical applications. (*J. Clin. Invest.* 1990. 86:1396-1402.) Key words: inflammation • protein kinase C • biosynthesis • lymphotoxin • endotoxin

### Introduction

Tumor necrosis factor (TNF)<sup>1</sup> produced primarily by macrophages is a pleiotropic mediator of many inflammatory responses (1). TNF was initially recognized because of its cytotoxic and antitumor effects (2). It was found to be identical with a factor called cachectin (3, 4), which suppresses lipoprotein lipase and gives rise to hypertriglyceridemia of chronic inflammatory disease and malignancy (5). TNF/cachectin is presumed to possess beneficial effects for the host by stimulation of inflammatory cells and by inhibition of viral replica-

tion and may be involved in the regulation of normal tissue homeostasis (1). On the other hand, when the production of this cytokine is inappropriately controlled harmful manifestations occur (3). Thus, TNF/cachectin plays a major role in the pathogenesis of gram-negative endotoxic shock (6, 7), which can lead to cardiovascular collapse, organ failure, and death. These effects are reversed by passive immunization with anti-TNF monoclonal antibodies (6, 7). Many, perhaps all, effects of lipopolysaccharide (LPS) are mediated by TNF/cachectin (3). An association has been reported between the concentration of TNF in serum and fatal outcome in meningococcal disease (8). TNF/cachectin may also be an effector of skin and gut lesions of the acute phase of graft-versus-host disease (9).

Attempts to control the adverse effects of TNF/cachectin would be of clinical importance. We have identified a TNF-binding protein (TNF-BP) of serum and urine from patients with chronic renal failure (10), which interferes with the binding of TNF to its receptor and inhibits the biological activity of TNF (10). A TNF-inhibitory activity was also described by Seckinger et al. (11). We have purified TNF-BP from urine of patients with renal failure (12). TNF-BP was found to be a cysteine-rich glycoprotein with mol wt of 30 kD, without significant homologies with previously described protein sequences. Recently others have also reported the purification of a TNF inhibitor (13, 14), which seems to be identical with TNF-BP. Because TNF-BP acts as a regulator of TNF/cachectin bioactivities it has a potential clinical application to counteract harmful manifestations such as septic shock and cachexia. In this report we describe biological and biochemical characteristics of TNF-BP and its production. The data presented suggest that TNF-BP is a soluble form of a TNF receptor released by proteolytic cleavage of the receptor at the cell surface.

### Methods

**Tumor necrosis factor (TNF) and lymphotoxin (LT).** Recombinant human TNF (rTNF) and lymphotoxin (Genentech Inc., San Francisco, CA) were supplied by Boehringer Ingelheim (Vienna, Austria). The rTNF contained  $38 \times 10^6$  U/mg (646 U/pmol) and the recombinant lymphotoxin  $230 \times 10^6$  U/mg (3,960 U/pmol). These activities were assayed as cytotoxic activities on mouse L 929 fibroblasts in the presence of 1  $\mu\text{g/ml}$  actinomycin D. Radioiodination of TNF and TNF-BP was carried out accordingly to the two-phase method of Tedjedor and Ballesta (15).

**Purification of TNF-BP and production of antibodies.** All experiments were performed with TNF-BP purified to homogeneity from urine of patients with renal failure by ion exchange chromatography, affinity chromatography on TNF-Sepharose and reverse-phase chromatography as previously described (12). The purified TNF-BP contained only one 30-kD chain both under reducing and nonreducing conditions as judged by SDS-PAGE. The aminoterminal sequence was previously reported (12). An antiserum against TNF-BP was produced by immunization of rabbits subcutaneously with 0.2 mg purified

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1. Abbreviations used in this paper: LPS, lipopolysaccharide; RIPA, radioimmunoprecipitation assay; rTNF, recombinant tumor necrosis factor; TNF, tumor necrosis factor; TNF-BP, tumor necrosis factor-binding protein.

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TNF-BP in complete Freund's adjuvant. Booster injections of 0.2 mg TNF-BP in incomplete Freund's adjuvant were given after 4 wk and the rabbits were bled 2 wk later. The antiserum could be diluted 1:8,000 and still detect TNF-BP in the enzyme-linked immunosorbent assay (ELISA) described below. TNF and lymphotoxin did not interfere when the antiserum was used in the ELISA for detection of TNF-BP.

**Cell lines.** The HL-60 (16) and the HeLa cell line were maintained in suspension culture in RPMI 1640 medium containing 10% fetal calf serum (FCS). Exponentially growing cells were used for assays.

**Assays of TNF-BP.** TNF-BP was assayed by competition with the binding of  $^{125}\text{I}$ -rTNF to cells, by inhibition of the cytotoxic effect of rTNF on the fibrosarcoma cell line WEHI 164 clone 13 cells (17) and by a specific enzyme-linked immunosorbent assay.

In the competition assay, the inhibition by TNF-BP of the  $^{125}\text{I}$ -rTNF-binding to HL-60 cells was measured. Cells ( $7.5 \times 10^6$ ) were washed with binding buffer consisting of RPMI-1640, 10% FCS, 20 mmol/liter Hepes (pH 7.4) and incubated with 150 pmol/liter of  $^{125}\text{I}$ -rTNF for 2 h at 4°C by rotation in 1.5 ml Eppendorf tubes in a total volume of 300  $\mu\text{l}$ . After centrifugation for 10 s at 8,000  $g$  the cell pellet was resuspended and washed twice in ice-cold binding buffer to separate unbound from membrane-bound  $^{125}\text{I}$ -rTNF. Specific binding was defined as the difference between total binding and the nonspecific binding that occurred in the presence of a 1,000-fold excess of unlabeled rTNF.

The neutralizing effect of TNF-BP on the cytotoxicity of rTNF was assayed by use of WEHI 164 clone 13 cells (17). These cells were seeded in microplates at a concentration of  $2 \times 10^4$  cells per well in 50  $\mu\text{l}$  of RPMI-1640 medium with 10% FCS. Different dilutions of TNF-BP with or without various concentrations of rTNF were made up, incubated for 30 min at 37°C and added to the target cells. After 20 h of incubation at 37°C the percentage of dead cells was determined as described in (17).

TNF-BP was also measured by an enzyme-linked immunosorbent assay (ELISA) based on inhibition of binding of anti-TNF-BP to immobilized TNF-BP. Samples of 50  $\mu\text{l}$  containing TNF-BP to be measured were loaded in triplicate in 96-well round-bottom incubation plates (Nunc 2-62170). A 50- $\mu\text{l}$  aliquot of the anti-TNF-BP antibody diluted 1:4,000 in incubation buffer (0.1 mol/liter NaCl, 0.05 mol/liter  $\text{NaH}_2\text{PO}_4$ , 0.05% Tween-20, adjusted to pH 7.5 with NaOH) was added to each well followed by overnight incubation at 4°C to allow equilibration between TNF-BP and anti-TNF-BP. In parallel, 96-well flat-bottom immunoplates (Nunc 4-39445) were coated with TNF-BP, 100  $\mu\text{l}$ /well diluted to 7.5 ng/ml in 0.1 mol/liter  $\text{NaHCO}_3$ , and incubated overnight at room temperature followed by three washes in washing solution (0.15 mol/liter NaCl, 0.05% Tween-20). The content of the sample plate was transferred to the plate coated with TNF-BP which was incubated for 3 h at 4°C to allow binding of free anti-TNF-BP antibodies to immobilized TNF-BP and washed three times. For detection of bound anti-TNF-BP antibodies, an aliquot of 100  $\mu\text{l}$  secondary antibody, diluted 1:200 in incubation buffer supplemented with 1 mg/ml bovine serum albumin was added to each well. The secondary antibody was goat anti-rabbit antibody conjugated to biotin (Vectastain ABC kit, AK-5001, Vector Laboratories, Inc., Burlingame, CA). The plates were incubated for 1 h at room temperature followed by three washes. To increase the absorbance and thereby the sensitivity, 100  $\mu\text{l}$  of a solution containing avidin-biotin (Vectastain ABC kit, AK-5001) was added to each well followed by incubation of the plates for 1 h, washing, and addition of phosphatase substrate (Sigma 104) dissolved in 1 mol/liter diethanolamine buffer, pH 9.8, supplemented with 0.5  $\mu\text{mol}$ /liter  $\text{MgCl}_2$  in a final volume of 100  $\mu\text{l}$ . The absorbance was measured at 405 nm in an automatic Titertek multiscan ELISA plate reader. Values were calculated from a standard curve based on freshly prepared dilutions of TNF-BP (0.3–30 ng/ml) made up in the same solution used to test the samples.

Gel chromatography was applied to visualize the complex formation between TNF-BP and rTNF. Superose 12 and Sephacryl S-200 HR columns (1  $\times$  30 cm) (Pharmacia Fine Chemicals, Piscataway, NJ)

were equilibrated with 0.15 mol/liter NaCl, 0.01 mol/liter Hepes, pH 7.4, and 0.1% BSA and eluted using the fast-protein liquid chromatography (FPLC) system of Pharmacia Fine Chemicals. The Superose 12 column was also used to investigate the effect of pH on complex formation; the column was equilibrated and eluted with 0.15 mol/liter NaCl, 0.05 mol/liter citrate-phosphate buffer, pH 6.0, pH 5.0, or pH 4.0. The Superose and Sephacryl columns were eluted at a flow rate of 0.5 ml/min and 0.25 ml/min, respectively.

**Biosynthetic labeling of TNF-BP.** HL-60 or HeLa cells,  $2 \times 10^6$ /ml, were first suspended in cysteine-free medium for 30 min, then  $4 \times 10^7$  cells were suspended in 10 ml of cysteine-free Eagle's minimal essential medium (Flow Laboratories, Inc., McLean, VA) containing 10% dialyzed FCS and 165  $\mu\text{Ci}$ /ml  $^{35}\text{S}$ -cysteine (sp act > 1,000 Ci/mmol) and incubated 30–60 min at 37°C. The label of the cells was chased for various periods of time in complete medium with 10% FCS at a cell concentration of  $2 \times 10^6$ /ml. In some experiments the cells were incubated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) for 24 h before labeling with  $^{35}\text{S}$ -cysteine.

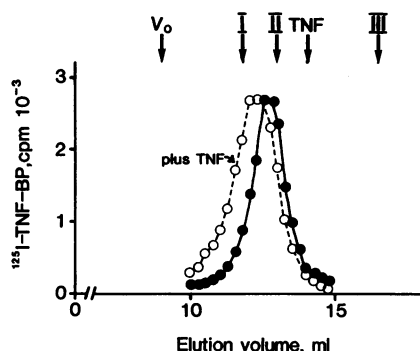
Cell lysates were prepared in cold radioimmunoprecipitation assay (RIPA) buffer consisting of 0.15 mol/liter NaCl, 30 mmol/liter Hepes (pH 7.3), 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and freshly added phenylmethylsulfonyl fluoride (PMSF) at 1 mmol/liter  $2 \times 10^7$  cells were lysed with 1 ml of RIPA buffer. After 1 h, cell lysates were clarified by centrifugation at 32,000  $g$  for 30 min at 4°C. RIPA buffer extracts, 0.5–1.5 ml, of whole cells or cell supernatant were mixed with 2  $\mu\text{l}$  anti-TNF-BP, 50  $\mu\text{l}$  protein A–Sephacryl (200 mg/ml) suspension, and incubated overnight at 4°C with continuous rotation. SDS-PAGE was then performed on 16% acrylamide slab gels as described (18). After electrophoresis the gels were stained, destained, treated with Amplify (Amersham International, Amersham, UK), and dried on filter paper. Dried gels were exposed to x-ray film (Hyperfilm MP, Amersham International) at  $-80^\circ\text{C}$  for 1 wk. Apparent molecular weight values were determined by use of molecular weight standards (Pharmacia Fine Chemicals), which included phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; and lactalbumin, 14,400.

**Digestion with N-glycanase.**  $^{125}\text{I}$ -TNF-BP was deglycosylated with N-glycanase (Genzyme Corp., Boston, MA) according to the manufacturer's description. TNF-BP was denatured by boiling 4 min in 0.1 M  $\beta$ -mercaptoethanol and 0.5% SDS. The sample was diluted to a final concentration of 0.2 mol/liter sodium phosphate pH 8.6, 10 mmol/liter 1, 10-phenanthroline hydrate and 1.25% NP-40. N-Glycanase was added (0.08–10 U/ml) followed by incubation at 37°C for 4 h. Controls were incubated without N-glycanase.

## Results

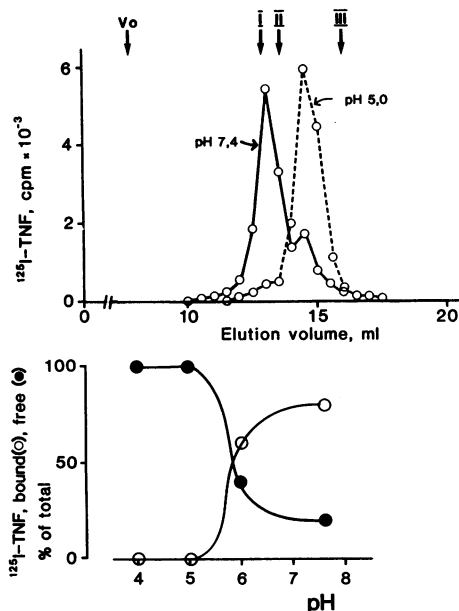
**Stoichiometry of the binding between TNF-BP and rTNF.** The complex formation between TNF-BP and rTNF was analyzed by gel chromatography. TNF-BP and rTNF were eluted from a Sephacryl S-200 column corresponding to molecular weights of 50 and 35 kD, respectively (Fig. 1). In its active form TNF is thought to be trimeric (19) and it might have been retarded on the column because of adsorption. Mixtures of equimolar amounts of  $^{125}\text{I}$ -TNF-BP and rTNF were chromatographed and all radioactivity was found to be associated with a complex that had an apparent molecular weight of 65 kD (Fig. 1). It is not possible to conclude from these results whether TNF bound to TNF-BP as a trimer, dimer, or monomer.

The binding between TNF-BP and TNF was highly susceptible to low pH. No binding was observed at pH 5.0 or below (Fig. 2). The binding was also susceptible to reduction because it was abolished in the presence of 1.25% 2-mercaptoethanol (data not shown).

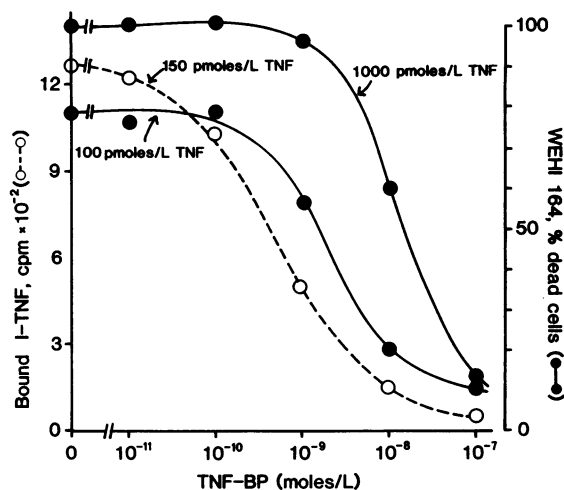


**Figure 1.** Gel chromatography of TNF-BP on a Sephacryl S-200 column (1 × 30 cm). 1.8 pmol of  $^{125}\text{I}$ -TNF-BP (●) or a mixture of 1.8 pmol of  $^{125}\text{I}$ -TNF-BP and 1.8 pmol of rTNF (○) were chromatographed. The column was eluted with 0.15 mol/liter NaCl, 0.01 mol/liter Hepes, pH 7.4, and 0.1% BSA and 0.25 ml fractions were collected to determine radioactivity. Arrows indicate void volume ( $V_0$ ) and elution volume for BSA,  $M_r$  67,000 (I), ovalbumin,  $M_r$  43,000 (II), and ribonuclease,  $M_r$  13,700 (III). The elution volume of rTNF is also indicated.

**Inhibition of TNF effects by TNF-BP.** The inhibitory effects of TNF-BP on the binding of rTNF to the cell surface receptor of HL-60 cells and the rTNF-mediated cytotoxicity on WEHI 164 cells were determined. The results with various concentrations of TNF-BP are shown in Fig. 3. In both cases a



**Figure 2.** Effect of pH on complex formation between TNF-BP and  $^{125}\text{I}$ -rTNF. Mixtures of 1.8 pmol of TNF-BP and 1.8 pmol of  $^{125}\text{I}$ -rTNF were chromatographed on a Superose 12 column equilibrated with 0.15 mol/liter NaCl, 0.01 mol/liter Hepes, pH 7.4, and with 0.15 mol/liter NaCl, 0.05 mol/liter citrate-phosphate buffer pH 6.0, pH 5.0, or pH 4.0. Fractions of 0.25 ml were collected and assayed for radioactivity. The upper part of the figure shows the elution profiles at pH 5.0 and pH 7.4. The lower part of the figure shows the fractions of bound (○) and free (●)  $^{125}\text{I}$ -rTNF and different pH values. Arrows indicate void volume ( $V_0$ ) and elution volumes for BSA,  $M_r$  67,000 (I), ovalbumin,  $M_r$  43,000 (II), ribonuclease,  $M_r$  13,700 (III), and for  $^{125}\text{I}$ -rTNF (TNF).

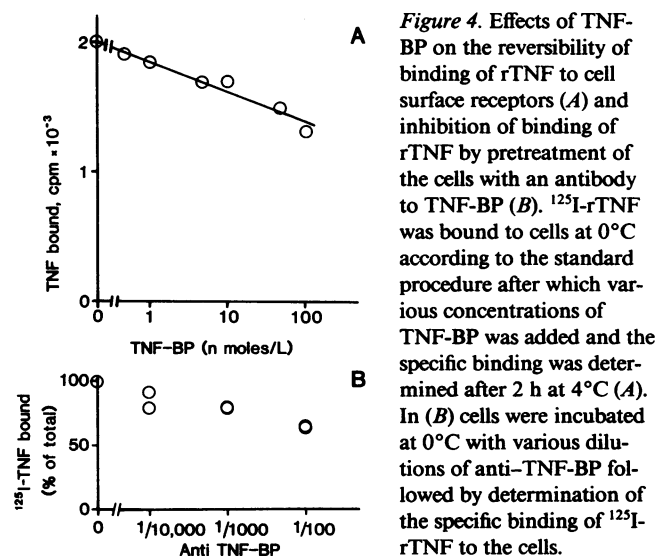


**Figure 3.** The inhibitory effect of TNF-BP on the binding of  $^{125}\text{I}$ -rTNF to HL-60 cells and the inhibitory effect of TNF-BP on TNF-mediated cytotoxicity for WEHI 164 cells. In the binding assay the concentration of rTNF was 150 pmol/liter and the concentration of TNF-BP was varied between 10 pmol/liter and 100 nmol/liter (○). In the cytotoxicity assay the concentration of rTNF was 100 pmol/liter or 1,000 pmol/liter and the concentration of TNF-BP was varied between 10 pmol/liter and 100 nmol/liter (●).

50% reduction of the TNF effects is observed at approximately a 10-fold molar excess of TNF-BP.

The specific binding of  $^{125}\text{I}$ -rTNF to the surface of HL-60 cells was partially reversed by TNF-BP. Cells were incubated with  $^{125}\text{I}$ -rTNF for 2 h after which TNF-BP was added. A dose-dependent decrease of specific rTNF binding was observed (Fig. 4). Experiments were also performed to investigate if antibodies to TNF-BP bound to cells and interfered with TNF binding to cell surface receptor. Actually preincubation of HL-60 cells with anti-TNF-BP inhibited to ~40% the specific binding of  $^{125}\text{I}$ -rTNF (Fig. 4).

**Deglycosylation of TNF-BP.** When TNF-BP was treated with *N*-glycanase, its molecular weight was reduced from 31 to



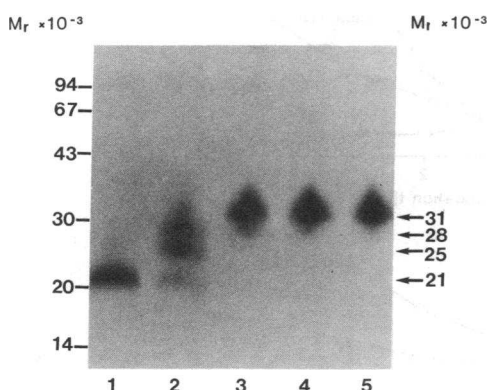
**Figure 4.** Effects of TNF-BP on the reversibility of binding of rTNF to cell surface receptors (A) and inhibition of binding of rTNF by pretreatment of the cells with an antibody to TNF-BP (B).  $^{125}\text{I}$ -rTNF was bound to cells at 0°C according to the standard procedure after which various concentrations of TNF-BP was added and the specific binding was determined after 2 h at 4°C (A). In (B) cells were incubated at 0°C with various dilutions of anti-TNF-BP followed by determination of the specific binding of  $^{125}\text{I}$ -rTNF to the cells.

21 kD (Fig. 5). *N*-Glycanase cleaves *N*-linked oligosaccharides independent of type. These results demonstrate the presence of a high carbohydrate content. In addition, partial digestion with *N*-glycanase shows three separate cleavage products consistent with the presence of at least three glycosylation sites (Fig. 5).

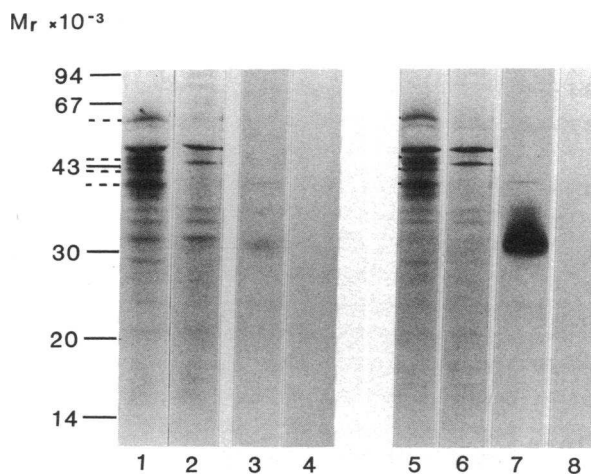
**Biosynthesis of TNF-BP.** The finding of inhibition of  $^{125}\text{I}$ -rTNF binding to cells by antibodies to TNF-BP suggested an homology between TNF-BP and TNF receptor. Therefore, experiments were performed to immunoprecipitate TNF-BP produced by biosynthetically-labeled cells.

TNF-BP was biosynthetically labeled with  $^{35}\text{S}$ -cysteine because of its high cysteine content (12). Several polypeptides were immunoprecipitated with anti-TNF-BP from HL-60 cell lysates and had molecular weights of  $\sim 60$ , 45, 42, and 40 kD (Fig. 6). Apparently none of these corresponded to the intact TNF-BP molecule. However, when the label was chased and then followed by immunoprecipitation of the cell supernatant with anti-TNF-BP, a labeled polypeptide presumably mature TNF-BP appeared (Fig. 6). It is likely that the cellular components specifically precipitated with anti-TNF-BP represent precursor forms for TNF-BP. The 60-kD polypeptide may correspond to the transmembrane TNF-receptor molecule, whereas the other components as well as mature TNF-BP may represent cleavage products of the receptor. Fig. 6 also demonstrates that incubation of cells with PMA results in a large increase in the production of extracellular TNF-BP. This effect was most probably mediated by activation of protein kinase C because 4  $\alpha$ -PMA, which does not activate protein kinase C, did not increase production of TNF-BP (data not shown). No mature TNF-BP polypeptides could be detected in extracts of PMA-treated HL-60 cells (Fig. 6). Thus, it is likely that the mature TNF-BP molecule is formed at the cell surface followed by its immediate release. The time course for production of TNF-BP in  $^{35}\text{S}$ -labeled HL-60 cells treated with PMA is shown in Fig. 7. Further, it was observed that TNF itself induced production of TNF-BP (Fig. 7).

Also results from biosynthetic labeling of other cell lines supported the above notions. Immunoprecipitation with anti-TNF-BP of lysates of HeLa cells incubated with  $^{35}\text{S}$ -cysteine



**Figure 5.** Deglycosylation of  $^{125}\text{I}$ -TNF-BP with *N*-glycanase.  $^{125}\text{I}$ -TNF-BP was incubated with 10 U/ml (lane 1), 2 U/ml (lane 2), 0.4 U/ml (lane 3), and 0.08 U/ml (lane 4) of *N*-glycanase or in the absence of *N*-glycanase (lane 5) for 4 h. The samples were analyzed by SDS-PAGE and fluorography. The molecular weights of fully glycosylated (lane 5), partially deglycosylated (lane 2), and completely deglycosylated TNF-BP (lane 1) are indicated to the right. Molecular weight markers are indicated on the left of the fluorograph.

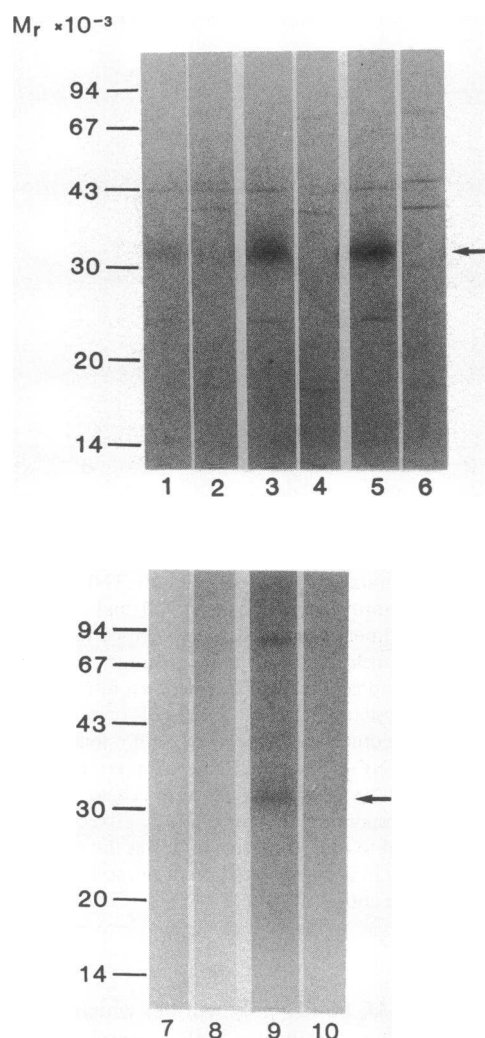


**Figure 6.** Immunoprecipitation with anti-TNF-BP of biosynthetically labeled HL-60 cells. The cells were labeled with  $^{35}\text{S}$ -cysteine for 60 min. Cell extracts (lanes 1, 2, 5, and 6) and cell supernatants (lanes 3, 4, 7, and 8) were immunoprecipitated with anti-TNF-BP (lanes 1, 3, 5, and 7) or preimmune serum (lanes 2, 4, 6, and 8). Lanes 5–8 represent an experiment where cells were exposed to 10 ng/ml PMA for 24 h before labeling with  $^{35}\text{S}$ -cysteine in the presence of PMA. Extracts for immunoprecipitation were prepared immediately after labeling with  $^{35}\text{S}$ -cysteine from half of the cells (lanes 1, 2, 5, and 6). The  $^{35}\text{S}$ -label of the other half was chased for 6 h followed by immunoprecipitation of TNF-BP from cell supernatants (lanes 3, 4, 7, and 8). PMA was present in the cell culture during chase of the label in the experiments corresponding to lanes 7 and 8. Molecular weight standards are indicated to the left. Arrow indicates the position of TNF-BP. Broken lines indicate the position of intracellular components precipitated with anti-TNF-BP.

revealed in particular an  $M_r$  60-kD polypeptide, which may correspond to the intact transmembrane TNF receptor molecule (Fig. 8). Additional polypeptides were immunoprecipitated from cell lysates with molecular weights of  $\sim 40$  and 37 kD, which may represent cleavage products of the receptor. As for HL-60 cells none of these cellular polypeptides corresponded to TNF-BP, but chase of the label in the presence of PMA resulted in the occurrence of labeled TNF-BP in the cell supernatant (Fig. 8). In addition to TNF-BP, an extracellular  $M_r$  34-kD polypeptide that was absent from cell lysates was also released and reacted with anti-TNF-BP. The latter may represent another cleavage product of the transmembrane TNF receptor, which is released extracellularly.

Production of TNF-BP by HL-60 cells was also determined by an ELISA. Both rTNF and recombinant lymphotoxin induced TNF-BP production during incubation for 4 d (Fig. 9). An increased production of TNF-BP was visible after 2 d. It is possible that the production of TNF-BP correlates with the TNF- and lymphotoxin-induced differentiation of HL-60 cells. However, biosynthetically labeled TNF-BP was detected to be released from cells within 20 h of incubation with TNF. On a molar basis, lymphotoxin seemed to be more efficient than TNF. The optimal concentration was  $\sim 1,000$  pmol/liter for both lymphotoxin and TNF.

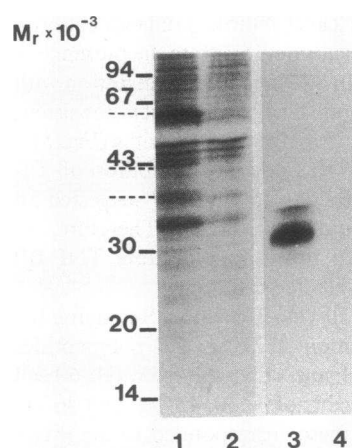
TNF-BP was detected in the supernatant of a large number of cell lines, mononuclear blood cells, neutrophil granulocytes, and fibroblasts (data not shown). The production of TNF-BP was generally increased by incubation with PMA.



**Figure 7.** Radioimmunoprecipitation of extracellular TNF-BP produced by PMA- and TNF-treated cells. HL-60 cells were labeled with  $^{35}\text{S}$ -cysteine for 30 min and the label was chased for 60 min followed by the addition of 10 ng/ml PMA (lanes 1–6) or 1,000 pmol/liter TNF (lanes 7–10). Cell supernatants were recovered after 30 min (lane 1), 60 min (lane 3), and 90 min (lane 5) of incubation with PMA and immunoprecipitated with anti-TNF-BP and preimmune serum (lanes 2, 4, and 6). Cell supernatant was also recovered after chase of the label for 60 min without any addition to the cell culture (lane 7) and after incubation with TNF for 20 h (lane 9) followed by immunoprecipitation and anti-TNF-BP and preimmune serum (lanes 8 and 10). Molecular weight markers are indicated to the left. Arrow indicates the position of TNF-BP.

## Discussion

In the present study, we report characteristics of a TNF binding protein purified to homogeneity from urine of patients with renal failure (12). The amino-terminal amino acid sequence has not revealed significant homologies with previously described protein sequences. Analysis by SDS-PAGE has shown that TNF-BP is composed of a single polypeptide chain with an apparent  $M_r$  of 30 kD (12), but it was eluted from a gel filtration column corresponding to a molecular weight of  $\sim 50$  kD. Perhaps TNF-BP forms a dimer at non-denaturing conditions or has a high excluded volume because of its high carbohydrate content. Analysis of immunoprecipitation

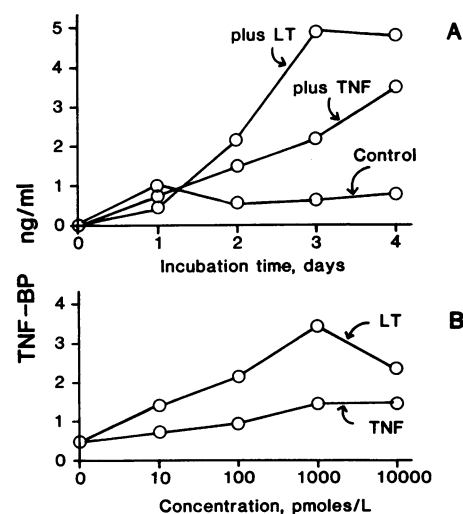


**Figure 8.** Immunoprecipitation with anti-TNF-BP of biosynthetically labeled HeLa cells. The cells were labeled with  $^{35}\text{S}$ -cysteine for 30 min. The cell extract was immunoprecipitated with anti-TNF-BP (lane 1) or preimmune serum (lane 2). In another experiment cells were labeled with  $^{35}\text{S}$ -cysteine for 30 min followed by chase of the label for 60 min in the presence of 10 ng/ml PMA after which the

cell supernatant was immunoprecipitated with anti-TNF-BP (lane 3) or preimmune serum (lane 4). Molecular weight standards are indicated to the left. Arrow indicates the position of TNF-BP. Broken lines indicate the position of intracellular components precipitated with anti-TNF-BP.

tated TNF-BP by SDS-PAGE showed that the molecular weight of TNF-BP was reduced from 30 to 21 kD by treatment with *N*-glycanase, whereas results from partial digestion with *N*-glycanase suggested that TNF-BP had at least three *N*-linked oligosaccharide side chains. We could not establish whether TNF-BP binds to the trimeric form of TNF, which is the biologically active form (19).

Purified TNF-BP competed with the TNF receptor for binding of rTNF. Excess TNF-BP was required to reduce the binding of rTNF. Similarly the cytotoxic effect of TNF on fibrosarcoma WEHI 164 cells was inhibited by TNF-BP. The binding of TNF to the receptor was partially reversed after the addition of TNF-BP provided that internalization was prevented by low temperature. Similarly the binding of TNF to



**Figure 9.** Production of TNF-BP from HL-60 cells. Cells ( $10^6$ /ml at the start point) were incubated for 4 d (A) with 1,000 pmol/liter lymphotoxin (LT), 1,000 pmol/liter TNF or without added cytokine. Incubations were also performed in the presence of various concentrations of lymphotoxin and TNF (B). TNF-BP of cell supernatants was determined with an ELISA.

TNF-BP can be partially reversed, which may explain the molar excess of TNF-BP needed for inhibition of receptor binding and biological activities of TNF.

Several observations suggest that TNF-BP is a soluble fragment of a TNF receptor. Pretreatment of cells with anti-TNF-BP resulted in decreased receptor binding of rTNF, which may indicate an antigenic relationship between the TNF receptor and TNF-BP. Work by Engelmann et al. (20) also demonstrated that antibodies to TNF-binding proteins from urine had an inhibitory effect on the binding of TNF to cells suggesting that these proteins are structurally related to the TNF receptors. The results from their work are consistent with the notion that urinary TNF-binding proteins constitute soluble forms of two molecular species of the cell surface TNF receptors (20). High-affinity TNF receptors with a  $K_D$  of 0.1–1.0 nmol/liters have been identified on a variety of cells (21–23). Results from cross-linking experiments of  $^{125}\text{I}$ -rTNF to its receptor on HL-60 cells (23, 24) suggested oligomer binding of TNF to a single receptor molecule with an apparent  $M_r$  of 70 kD. The TNF receptor has been purified 165,000-fold by immunoaffinity chromatography and partially characterized (25). It showed an  $M_r$  of  $\sim 65$  kD when gel filtration was carried out in the absence of ligand. Our results from biosynthetic labeling with  $^{35}\text{S}$ -cysteine followed by immunoprecipitation with anti-TNF-BP revealed extracellular release of newly synthesized TNF-BP. The absence of a corresponding intracellular polypeptide indicates that mature TNF-BP is formed at the cell surface presumably by cleavage of a TNF receptor and rapidly released. The cellular  $M_r$  60-kD polypeptide precipitated by anti-TNF-BP may represent an intact transmembrane TNF receptor. The antibodies reacted stronger with this polypeptide in lysates of HeLa cells as compared to HL-60 cells. Even if we assume that TNF-BP is a soluble form of a receptor, antibodies raised against TNF-BP do not necessarily have a high affinity for an intact receptor because TNF-BP purified from urine might have been antigenically modified even if it retains full binding capacity for TNF. The additional cellular polypeptides of cell lysates precipitated with anti-TNF-BP most likely represent degradation products of a TNF receptor. Results from sequencing of complementary DNA clones encoding a TNF receptor chain (26, 27, Himmler, A., I. Maurer-Fogy, M. Krönke, P. Scheurich, K. Pfizenmaier, M. Lantz, I. Olsson, R. Hauptmann, C. Stratawa, and G. R. Adolf, submitted for publication) have indeed shown that the extracellular ligand binding domain encodes the soluble TNF-BP. TNF-BP is most likely proteolytically derived because there was no indication that alternative splicing of mRNA is responsible for its generation. Three potential *N*-glycosylation sites were present in the sequence corresponding to TNF-BP consistent with our results from partial digestion with *N*-glycanase.

The findings of TNF-BP in the supernatant of a large number of cell lines as well as in the supernatants from mononuclear blood cells, neutrophils, and fibroblasts are consistent with the notion that TNF-BP is a cleavage form of the transmembrane TNF receptor because this receptor seems to be present on most cells. It is known that interleukin-2 receptor is released in a soluble form from activated lymphocytes (28). Also, other cytokine receptors may be released into body fluids including soluble receptors for both interleukin-6 and interferon- $\gamma$  detected in urine (29). In addition, activation of protein kinase C by phorbol esters has been shown to induce

proteolytic cleavage of the CSF-1 receptor in its extracellular domain at a site near the membrane-spanning sequence (30). This form of transmodulation, possibly due to activation of a protease, may occur with several different cell surface receptors and serve to regulate the inflammatory response.

TNF and LT are internalized through receptor-mediated endocytosis and the ligand is degraded in lysosomes (31, 32). We assume that binding of TNF to TNF-BP imitates receptor binding of TNF. Many receptor-ligand complexes are dissociated after internalization into receptosomes. The complex formed between TNF and TNF-BP was highly sensitive to low-pH environment and completely dissociated at pH 5.0 or lower. If this result is relevant to binding of TNF to a receptor, then dissociation of the ligand-receptor complex in receptosomes may be due to acidity.

In this study we also demonstrate an increased production of TNF-BP from cells under conditions which favor downregulation of the TNF receptor. A spontaneous turnover of the TNF receptors has been reported with a half life of  $\sim 2$  h (24, 33, 34). Agents which activate protein kinase C such as PMA and diacylglycerol 1-oleoyl-2-acetyl-glycerol, downregulate the TNF receptor (32, 33). Also, TNF itself has been shown to downregulate its receptor (24, 35). A receptor occupancy above a certain level resulted in downregulation that persisted for a prolonged time period (24). The production of TNF-BP from HL-60 cells was activated when TNF receptor was downregulated by PMA or rTNF. If TNF-BP is a soluble cleavage fragment of a TNF receptor the results imply that agents like PMA and TNF induce cleavage of the transmembrane receptor with release of TNF-BP by activation of a protease. Alternatively, PMA and TNF may induce *de novo* synthesis of an active protease. However, the production of TNF-BP by the cells was prolonged and would imply that downregulation of a receptor for TNF also initiated new synthesis of receptor with continuous formation of TNF-BP. There is evidence for development of tolerance to the toxic effect of TNF after exposure to TNF itself (36, 37), indicating that effects of TNF can be modulated by antagonistic mechanisms. One explanation could be the production of TNF-BP. Our finding of a release of TNF-BP during conditions when the receptor is downregulated (e.g., by TNF itself) may provide an explanation for the protection against deleterious effects of TNF induced by TNF itself. It still remains to show whether TNF-BP can function as an antagonist to TNF *in vivo*. If this occurs, it might contribute to the elimination of TNF.

In conclusion we have characterized a TNF binding protein purified to homogeneity from urine. The evidence available suggests that TNF-BP is a soluble form of a TNF receptor. Our results also show that the production of TNF-BP is increased when TNF receptor is downregulated with TNF itself or with phorbol ester. Responses to endotoxemia can be explained by the synthesis of TNF, which is a central mediator of host response to bacterial infection and inflammation. Therefore, TNF-BP could have clinical importance in neutralizing harmful manifestations of TNF/cachectin.

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