Radioimmunoassay of Tumor Necrosis Factor in Serum
Anna-Maija Teppo and C. P. J. Maury

We present a double-antibody radioimmunoassay for determination of the concentration of tumor necrosis factor (TNF) in serum. TNF in serum competes with a fixed amount of labeled TNF for the binding sites of specific rabbit antibodies. The bound TNF is precipitated with Sepharose-bound anti-rabbit IgG, then centrifuged, and the radioactivity of the pellets is counted. The detection limit of the assay is 7 ng/L (B0 = 3 SD). Bound radioactivity in the range of 10% to 90% of the B0 counts corresponds to TNF concentrations of 26 to 10,000 ng/L. Of 40 sera from healthy subjects, 21 (53%) contained TNF concentrations >7 ng/L (range 8–40 ng/L). Some patients with parasitic or neoplastic disease and patients with septic shock had highly increased TNF values. Three of the 14 sera (21%) from patients with rheumatoid arthritis had TNF concentrations >40 ng/L.

Additional Keyphrases: cachectin - neoplastic disease - parasitic disease - septic shock - rheumatoid arthritis - reference interval

Tumor necrosis factor (TNF) is a monocyte/macrophage-derived protein with cytotoxic or cytoplastic activity against certain tumor cells (1, 2), and antimalarial activity (3). Recent studies suggest that TNF is identical with cachectin (4, 5), a macrophage product that specifically suppresses lipoprotein lipase activity of adipocytes (5, 6). Cachectin/TNF has been implicated in the pathogenesis of the endotoxin-induced shock syndrome (7).

Although TNF may be an important mediator of various host responses to neoplasia and infection, its role in vivo has not yet been defined.

Two studies of the concentrations of TNF in diseased persons have been so far reported (8, 9). In one, the enzyme immunoassay was used to demonstrate increased TNF concentrations in parasitic infections, and in some patients with neoplastic disease (8). In the other study the concentrations of TNF in meningococcal infections were determined by a bioassay (9). To gain additional insight into the role of TNF in various disease processes, we have developed a radioimmunoassay for quantifying the concentrations of TNF in human serum. The method may also be useful for monitoring the concentrations of TNF in connection with clinical trials with recombinant TNF.

Materials and Methods

Tumor necrosis factor. Escherichia coli-derived recombinant TNF, obtained from Dr. G. R. Adolf, Ernst-Boehringer-Institut für Arzneimittelforschung, Wien, Austria, had a specific activity of 6 × 10^10 arb. units per gram of protein, as measured by bioassay with mouse L 929 cells (10). The preparation contained endotoxin, <125 int. units/L (11), and protein, 0.5 μg/L. TNF was dissolved in phosphate-saline buffer containing, per liter, 10 mmol of sodium phosphate and 200 mmol of sodium chloride (pH 7.0), then stored at 0 °C. To prepare standards, we diluted various amounts of this in assay buffer (see below).

Antiserum to TNF. Rabbit antiserum to recombinant human TNF was also obtained from Dr. Adolf. One milliliter of antiserum bound 9 × 10^5 units of TNF assayed in a mouse L 929 cell cytolyis bioassay (10).

Reagents. 125I was obtained from Amersham International, Amersham, Bucks., U.K.; Chloramine T, sodium bisulfite, bovine serum albumin, and KI were from Sigma Chemical Co., St. Louis, MO 63178; Sephadex G-25 and "Decanting Suspension 3" (Micro Sepharose-anti-rabbit IgG) from Pharmacia, Uppsala, Sweden. Assay buffer was phosphate-buffered saline, pH 7.3, containing, per liter, 5 g of bovine albumin, 50 mmol of phosphate, and 150 mmol of NaCl.

Radioimmunoassay. Mix 0.10 mL of standards or sera and 50 μL of antiserum to TNF, diluted 80,000-fold in assay buffer, and incubate at room temperature overnight. Add 50 μL of 1000-fold-diluted tracer (about 5000 ng/L, 8000 counts/min for 50 μL) and incubate for 5 to 6 h at room temperature. Separate bound and free TNF by adding 0.5 mL of Decanting Suspension 3, incubating at room temperature for 30 min, then centrifuging (1500 × g, room temperature, 15 min) and decanting. Simultaneously assay samples with phosphate-buffered saline added instead of antiserum, to measure the nonspecific binding of the labeled TNF. Then measure the radioactivity of the pellets and use logit-log linear transformation curve for the calculations. Report TNF concentrations as the mean ± SD of triplicate estimations.

Iodination procedure. Label TNF with 125I by the Chlora- mine T method (12), separating it from unbound iodine by gel filtration through a Sephadex G-25 column (2 × 20 cm) that has been presaturated with bovine albumin. Store the labeled TNF in the presence of 10 g of bovine albumin and 1 g of KI per liter.

Sera. We assayed 40 sera from healthy blood donors (Finnish Red Cross, Helsinki, Finland), 85 sera from patients with parasitic disease (most of the patients had malaria), 28 sera from patients with bacterial infections, 43 sera from patients with malignant neoplastic disease, and 14 sera from patients with rheumatoid arthritis.

Results

Assay Conditions

Tracer diluted 1000-fold gave a radioactive activity measurement of about 8000 counts/min in 50 μL; we used this dilution throughout the study. To determine the optimum dilution of the antiserum, we incubated 50-μL aliquots of 1000-fold diluted 125I-labeled TNF (about 10 000 ng/L, 8000 counts/min for 50 μL) with 50 μL of antiserum diluted 10 000-, 20 000-, 40 000-, 80 000-, and 160 000-fold, and then separated the bound 125I-labeled TNF by adding 0.5 mL of Decanting Suspension 3. The 80 000-fold-diluted antiserum bound 68% of the diluted tracer (Figure 1), so we used that antiserum dilution in further studies.
The suspension was incubated at room temperature before addition of the tracer. Closed circles: tracer was added simultaneously with the antiserum. In both cases the procedure was continued as described in Methods.

doses of 26, 480, and 10 000 ng/L correspond to 90%, 50%, and 10% tracer binding relative to the zero dose (100% = \( B_0 \)). \( B_0 \) values ranged between 55% and 69% of the tracer. Nonspecific binding remained <5.5%. The minimum concentration of TNF clearly distinguishable from zero (i.e., \( B_0 - 3 \ SD \) was 7 ng/L.

Analytical recovery of TNF, and conditions for blood sampling and storage. Different amounts of recombinant TNF (50 and 200 ng/L) were added to aliquots (16 each) of fresh serum and EDTA-treated plasma. One batch of aliquots (eight each) were analyzed directly, another (eight each) after freezing at −20 °C. Analytical recoveries ranged from 86 to 103% (Table 2). We noticed no difference between results for serum and plasma or for fresh and frozen samples. Evidently, either serum or plasma can be used for determining TNF concentrations, and the samples can be stored frozen. No corrections for recoveries are needed.

Precision of the assay. To calculate within-run and between-run precision (CV), we analyzed during 26 days aliquots of three sera (TNF from 15 to 270 ng/L) that had been stored frozen (−20 °C), in three parallel determinations, each in nine separate assays, for the between-run assessment, and 10 to 15 parallel determinations, each in one run, for assessing within-run precision. Within- and between-assay CVs were respectively 8.2% and 9.4% for a TNF concentration of 15 ng/L, 6.3% and 7.0% for 92 ng/L, and 5.1% and 5.7% for 272 ng/L.

Clinical Results

Concentration of TNF in normal sera. Sera from 40 healthy blood donors (26 men, 14 women, ages 26 to 56 years) were analyzed for TNF. In 19 (48%) the concentration was below the detection limit for our method (Figure 4); the median was 9 ng/L; the range was <7–40 ng/L. We noticed no correlation with age.

Concentrations of TNF in diseased patients. TNF was detectable in 53 sera from 65 patients with parasitic disease

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**Table 1. Effect of Duration of Pre-incubation**

<table>
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<tr>
<th>TNF concn, ng/L</th>
<th>Pre-incubation time, h</th>
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<th>16</th>
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Standards were incubated with the antiserum at room temperature for 0 to 16 h before addition of tracer, then processed as described in Methods. Results are expressed as the ratio of \( B/B_0 \).
After 500 mg/L we much in injury, infection, and neoplasia, with monocyte/macrophage factors, monokines, being important mediators of the host response. Recent interest has focused on TNF, which has a relative molecular mass of 17 000, an isoelectric point of 5.3, and contains a disulfide bridge (10). The complementary DNA for TNF has been cloned and expressed in Escherichia coli (11, 13). Recombinant TNF has similar anti-tumor activity and the same molecular mass as the natural TNF (11). TNF displays about 30% amino acid sequence homology with lymphokinin, a lymphocyte product functionally related to TNF (11, 14).

To gain insight into the role of TNF in human diseases, we developed this radioimmunoassay for determining TNF in serum. As little TNF as 7 ng/L can be detected and as much as 10 000 ng/L can be measured. We detected TNF in 21 serum samples (52%) from 40 healthy control subjects (81%), 25 sera from 28 patients with bacterial infections (89%), 28 sera from 43 patients with neoplastic disease (65%), and six sera from 14 patients with rheumatoid arthritis (43%). The mean concentration of TNF for each group was 305, 84, and 20 ng/L, respectively (Figure 4). In our calculations those values <7 ng/L were regarded as 0 ng/L.

**Discussion**

Numerous metabolic changes occur in response to tissue injury, infection, and neoplasia, with monocyte/macrophage factors, monokines, being important mediators of the host response. Recent interest has focused on TNF, which has a relative molecular mass of 17 000, an isoelectric point of 5.3, and contains a disulfide bridge (10). The complementary DNA for TNF has been cloned and expressed in Escherichia coli (11, 13). Recombinant TNF has similar anti-tumor activity and the same molecular mass as the natural TNF (11). TNF displays about 30% amino acid sequence homology with lymphokinin, a lymphocyte product functionally related to TNF (11, 14).

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and found that TNF concentrations were increased in patients with parasitic disease, with bacterial infections, and with neoplastic disease. In two earlier studies involving either a bioassay (9) or an enzyme-linked immunoabsorbent assay (8), increased concentrations of TNF—values comparable to those of this study—were found in patients with meningococcal meningitis (9) and with parasitic disease (8). Also in agreement with our results TNF concentrations remained <40 ng/L in 92% of healthy subjects (8). On the other hand, an enzyme-linked immunoabsorbent assay (8) TNF values >40 ng/L (the detection limit of that assay) were found only in 8% of patients with neoplastic disease, whereas we found the concentrations >40 ng/L in 43% of cancer patients. However, because synthesis of TNF is increased in response to tumor cells (1), increased concentrations of TNF could be expected in patients with advanced cancer. Also, perhaps the increased amounts of TNF could be involved in the pathogenesis of cancer-related cachexia (16).

The cause of slightly increased TNF concentrations in the serum of some rheumatic patients is not known. Observations that TNF stimulates collagenase and prostaglandin E₂ production by synovial cells and by fibroblasts (16) and causes osteoclastic bone resorption (17) are interesting in this connection.

We thank Dr. G. R. Adolf (Ernst-Boehringer Institut für Arzneimittelforschung, Wien, Austria), not only for sending us the recombinant human tumor necrosis factor and antiserum but also for giving valuable advice and constructive criticism. This work was financially supported by the Sigrid Juselius Foundation.

References