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Concentrations of Immunoreactive Human Tumor Necrosis Factor Alpha Produced by Human Mononuclear Cells In Vitro

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The concentrations of tumor necrosis factor (TNF) produced by human peripheral blood mononuclear cells (MNC) were measured using a radioimmunoassay (RIA) for human TNF. This was developed using a rabbit antiserum against human recombinant TNF (Hu rTNF), and Hu rTNF labeled with Na125I by a modification of the chloramine T method. This RIA does not detect human lymphotoxin, interleukin-1 alpha or beta, interleukin 2, interleukin 6, interferon alpha or gamma, granulocyte-macrophage-colony stimulating factor, and C5a des arg. A good correlation (r = 0.89) was found between the RIA and the cytolytic bioassay for TNF. The sensitivity of the RIA is between 3 and 78 pg/ml (median 11 pg/ml).

The mean concentration of TNF in 24-h culture supernatants of human MNC exposed to different concentrations of lipopolysaccharide (LPS) was found to increase in dose-dependent fashion and then level off between 50 and 100 ng/ml. The concentrations of IL-1 beta and alpha detected by specific RIAs in these supernatants were between 0.2 and 19 ng/ml and 0.04 and 1 ng/ml, respectively. The amount of TNF produced by human MNC in vitro was determined in a cohort of 50 normal volunteers. Without exogenous stimuli, TNF concentrations were almost always below the detection limit; with 0.5 ng/ml LPS, the median concentration of TNF was 2 ng/ml, and with PHA the median was 3.8 ng/ml. In cultures performed in the presence of indomethacin significantly (p<0.005) more TNF was produced.

Using this RIA, we could detect TNF in the circulation of mice injected with Hu rTNF. When plasma samples of patients with febrile illnesses were added directly to the RIA, TNF was not detectable, with the exception of patients with malaria.

These studies demonstrate the range and sensitivity of LPS-induced and mitogen-induced production of immunoreactive TNF by human MNC in vitro without interference of similar cytokines in bioassays.

Key words: tumor necrosis factor, interleukin-1, monocyte, lymphocyte, radioimmunoassay

INTRODUCTION

Tumor necrosis factor-alpha (TNF, synonym cachectin) is a cytokine with a wide variety of actions [1]. A number of activities of the molecule are shared with another cytokine, interleukin-1 (IL) (α and β). For instance the pyrogenic effect; the ability to induce an acute-phase response; and the stimulatory effects on fibroblasts, osteoclasts, endothelial, and synovial cells are common to both molecules [2-4]. Recent studies also indicate that there is a strong synergism between TNF and IL-1 β [5], and it has become apparent that TNF stimulates the production of IL-1 both in vivo and in vitro [2].

Measurement of TNF in culture supernatant has focused on the cytotoxicity assay. However, IL-1 and other substances that are often present with TNF in cell supernatants affect the TNF cytotoxicity assay, since IL-1 also exhibits cytotoxic properties [6]. In the present paper we have employed a radioimmunoassay (RIA) for TNF secreted by human mononuclear cells in vitro under a variety of conditions. Furthermore, we compare the concentrations of TNF to IL-1 β and IL-1 α using endotoxin (lipopolysaccharide, LPS) as a stimulus.

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MATERIALS AND METHODS
Preparation of the Antiserum

Female New Zealand white rabbits (Pine Acres, Burlington, VT) were immunized with 50 μg recombinant human tumor necrosis factor (Hu rTNF), obtained from three sources: Genentech Inc. (South San Francisco, CA), Cetus Corp. (Emeryville, CA), and Biogen (Cambridge, MA). The initial immunization took place in complete Freund’s adjuvant, using 12 intradermal injections. After 8 weeks the animals were boosted monthly with 50 μg TNF in incomplete Freund’s adjuvant intramuscularly. Unfractionated serum was used as anti-TNF antiserum. Cross-reactivity of this antiserum with human recombinant interleukin-1 β (Cistron Technology Inc., Pine Brook, NJ), human recombinant interleukin-1 α (kindly provided by Dr Alan Shaw, Biogen, Geneva, Switzerland), human recombinant interleukin-2 (Cetus Corp.), human recombinant interleukin-6 (Genetic Institute, Cambridge, MA), human recombinant interferon alpha (Schering Corp., Kenilworth, NJ), human recombinant interferon gamma (Schering Corp.), human recombinant granulocyte-macrophage-colony stimulating factor (Genetics Institute, Cambridge, MA), human C5a des arg (kindly provided by Dr KB Yancey, Uniformed Services University of the Health Sciences, Bethesda, MD), human recombinant lymphotoxin, and murine recombinant TNF (both kindly provided by Dr MA Palladino Jr, Genentech, Inc.) was investigated in various concentrations in the radioimmunoassay as described below.

Radiolabeling Hu rTNF

Hu rTNF was labeled using a modification of the chloramine T method [7]; 5 μg Hu rTNF (500 μg/ml) and 0.5 mCi Na[125]I (100 mCi/ml, New England Nuclear, Boston, MA) were added to 10 μl of 0.5 M sodium phosphate buffer (pH 7.4) and mixed. Next, 10 μg chloramine T (Sigma Chemical Co, St Louis, MO; 2.5 mg/ml in 0.25 M sodium phosphate buffer, pH 7.4) were added and mixed by gently pipetting for exactly 10 s. The reaction was terminated by adding 100 μg of the reducing agent sodium metabisulfite (Sigma; 5 mg/ml in 0.25 M sodium phosphate buffer, pH 7.4). Finally, 300 μl of BSA-buffer containing 0.01 M phosphate buffered saline, 0.25% bovine serum albumin (BSA, Sigma), and 0.05% sodium azide) was added. The radiolabeled material was chromatographed on a Sephadex G50 (fine) (Pharmacia, Piscataway, NJ) column (0.8 × 30 cm) to separate the radiolabeled protein from the free [125]I-BSA-buffer was used to equilibrate and to run the column. Twenty-five fractions of 0.7 ml were collected. The radioactivity of aliquots of these fractions was determined in a gamma counter. Next, small aliquots from each fraction were incubated with a 1:100 dilution of the anti-TNF antiserum for 18 h at room temperature, and then precipitated with 6% (w/v) polyethylene glycol 8000 (Fisher Scientific Company, Fair Lawn, NJ) and 1% sheep antirabbit IgG (Sigma).

The homogeneity of the radiolabeled TNF was determined with SDS-polyacrylamide gel electrophoresis (17%) [8] and autoradiography. The biological activity of the radiolabeled TNF was measured in the bioassay for TNF (see below). The specific activity of the labeled TNF was calculated assuming 75% recovery and using the known efficiency of the gamma counter.

Titration of Anti-TNF Antiserum

Aliquots of the radiolabeled TNF containing 15,000 cpm were diluted in 100 μl BSA-buffer and incubated with serial twofold dilutions of the anti-TNF antiserum for 18 h at room temperature, and then precipitated with an equal volume of 6% polyethylene glycol and 1% sheep antirabbit IgG.

Radioimmunoassay for TNF

Both standards and samples were assayed in duplicate or in some experiments in triplicate in 10 × 75-mm polystyrene tubes (Stockwell Scientific, Walnut, CA). In every assay, 12 standards of Hu rTNF (5,000, 2,500, 1,250, 625, 313, 157, 79, 40, 20, 10, 5, and 0 pg/ml) were employed. Depending on the nature of the samples, the standards were diluted in either human serum, urine, or BSA-buffer. On the first day of the assay, 100 μl of the anti-TNF antiserum (diluted in order to precipitate 40% of the radiolabeled TNF) was added to 100 μl of the standards or to 100 μl of the samples. To determine the nonspecific binding (NSB), 100 μl BSA-buffer was added to a standard containing no TNF. On the same day, 300 μl of BSA-buffer containing 0.3% heat-inactivated normal rabbit serum was added to each tube. After vortexing, the tubes were incubated at room temperature. The next day, 100 μl of BSA-buffer containing 10,000 cpm of radiolabeled TNF were added to each tube, and after vortexing, again incubated at room temperature. On the third day, 700 μl of BSA-buffer containing 9% of polyethylene glycol 8000 and 1.4% sheep antirabbit IgG were added. The tubes were vortexed and then centrifuged at 1,500g for 15 min. Thereafter, the supernatants were decanted, and the tubes were kept inverted and allowed to dry for at least 15 min before being counted. The mean cpm of duplicate or triplicate standards were calculated and the counts of the NSB subtracted. All standards were then expressed as a percentage of the standard containing no TNF (zero standard). The values obtained were converted using the logit transformation, according to the formula logit x = ln(x/100 – x). The logit values were plotted on the x-axis against the concentrations on a logarithmic y-axis. An exponential curve fit was performed, using Cricket graph (Cricket Software, Philadelphia, PA) on an Apple Macintosh Plus computer. The formula for the curve fit obtained was used to calculate
the “backfit” of the standard curve and to determine the concentration of TNF in samples. Values <95% of the zero standard (logit x < 2.94) were accepted.

Radioimmunoassays for IL-1α and β

Radioimmunoassays for human IL-1β [9] and α [10] were performed as described.

Bioassay for TNF

To measure the TNF activity in a bioassay, standards and samples were incubated with L929 cells (ATCC CCL1) as described by Flick and Gifford [11]. In short, the L929 cells were cultured in RPMI 1640 containing 2 mM L-glutamine and 10% heat-inactivated fetal calf serum (all from microbiological Associates, Walkersville, MD) in 75-cm² polystyrene tissue culture flasks (Falcon, Oxnard, CA) and split every 3 days. One day after subculture, the nonadherent cells were decanted after vigorous shaking of flask. After adding fresh medium the adherent cells were scraped with a policeman (Costar, Cambridge, MA), and after washing (450g, 10 min), 100-µl aliquots of the cell suspension (5 × 10⁵/ml) were pipetted in 94 wells of a 96-well flat-bottom microtiterplate (A/S Nunc, Roskilde, Denmark). After incubation overnight (37°C, 5% CO₂), the culture supernatants were decanted, and 100 µl of 15 µg/ml actinomycin D (Sigma) in saline, and 100 µl of RPMI 1640 with 10% fetal calf serum was added to each well. After adding 100 µl of either medium (control), TNF standards or samples to at least quadruplicate wells, the plates were reincubated. The next day, the supernatants were decanted and the wells were rinsed with saline. After staining of the cells with 0.1% (w/v) crystal violet (Sigma) in 100% methanol for 20 min, the plates were rinsed with tap water and dried. Absorbance (A) was measured in an ELISA reader at 595 nm and the percentage of cytotoxicity determined as

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\frac{[(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100}{}
\]

Isolation and Culture of Human Mononuclear Cells

Heparinized blood (10 U heparin/ml blood) from human volunteers was diluted threefold in 0.15 M saline and centrifuged on pyrogen-free Ficoll Hypaque. The mononuclear cells (MNC) of the interphase were washed three times in ultrafiltered [12] RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml benzylpenicillin, 100 µg/ml streptomycin, 10 mM HEPES (all from Microbiological Ass., Walkersville, MD), and 1% heat-inactivated AB serum. A volume of 100 µl of cell suspension (final concentration 2.5 × 10⁶ cells/ml) was added to flat-bottom microtiter plates (A/S Nunc), to which either 100 µl of medium or medium containing Escherichia coli LPS (Sigma) in various concentrations, or 0.5 µg/ml phytohemagglutinin (PHA) (Burroughs Wellcome, Research Triangle Park, NC) was added. Cultures were performed either with or without 0.5 µg/ml indomethacin (Sigma). After 24 h of culture at 37°C in a water-saturated atmosphere with 5% CO₂, either the culture supernatants or the contents of each well after three freeze-thaw cycles were harvested, centrifuged at 10,000g (Micro centrifuge, Fisher Scientific), for 1 min and frozen at −70°C until assay.

Hu rTNF in Mice

TNF concentrations were measured in EDTA-treated plasma obtained by cardiac puncture at various time points from 25g, female Swiss Webster mice (Charles River Laboratories, Wilmington, MA) that received an intraperitoneal injection 2 µg of Hu rTNF.

Measurement of TNF Concentration in Human Plasma

Plasma samples prepared from either citrated or heparinized blood from patients with febrile illnesses were tested for TNF in the RIA.

RESULTS

Validation and Specificity of the RIA for Human TNF

As shown in Figure 1, fractions of radiolabeled Hu rTNF which had the highest binding to anti-TNF were found in the first peak of eluting radioactivity, whereas the second peak contained the nonprecipitable free iodine. The fractions with greater than 85% of precipitable radiolabel were pooled and used as radiolabeled TNF in the RIA. The specific activity of this pool was approxi-

![Fig. 1. Gel filtration chromatography of the radiolabeled TNF on Sephadex G50. The square symbols represent the counts in 5 µl of each fraction. The closed circles represent the counts obtained in each fraction after incubation with anti-TNF antisem and precipitation with polyethylene glycol/antirabbit IgG.](image-url)
Fig. 2. Functional and structural integrity of the radiolabeled TNF. Biological activity of the radiolabeled TNF (□) is compared with that of unlabeled Hu rTNF (■) on L929 cells. The insert shows autoradiography of SDS-polyacrylamide gel electrophoresis of radiolabeled Hu rTNF. A single band is obtained at approximately 17 kD.

Fig. 3. Titration of anti-TNF antiserum with radiolabeled TNF. The antigen antibody complexes were precipitated with polyethylene glycol/antirabbit IgG. The results are expressed as percent of the total counts per min.

Fig. 4. Standard curves for the TNF RIA in BSA-buffer (■ — ■) and serum (○ — ○). The cpm of the standards are converted into logit. The curve fit for these curves can be described as $y = 64.97 \times 10^{-0.35x}$ for the curve in buffer (regression coefficient 0.98) and as $y = 56.80 \times 10^{-0.50x}$ for the curve in serum (regression coefficient 0.96). The sensitivity of the assays is less than 10 pg/ml (logit < 2.94).

Titration of the antiserum with the pooled radiolabeled ligand is shown in Figure 3. A 41% precipitation with polyethylene glycol/anti rabbit IgG at an anti-TNF dilution of 1:20,000 was detected and this dilution was chosen for use in the subsequent RIAs.

Representative standard curves for TNF in BSA-buffer and frozen-thawed human serum are shown in Figure 4. The standard curves in these fluids do not coincide, which implies that standard curves should be generated not only in BSA-buffer, but also in relevant biological fluids. From these standard curves it can be seen that the sensitivity of the assay is < 10 pg/ml (95% confidence limit, 2.9 logit). In more than 20 radioimmunoassays using radiolabeled TNF that was iodinated at different occasions, the sensitivity in BSA-buffer was in the same order of magnitude (between 3 and 78 pg/ml, median 11 pg/ml).

The RIA for Hu TNF did not show cross-reactivity with either 100 or 10 ng/ml human recombinant IL-1 β; 100 or 10 ng/ml human recombinant IL-1 α; 1,000 or 100 ng/ml human recombinant IL-2; 100 ng/ml human recombinant IL-6; 1,000 or 100 U/ml human recombinant interferon α; 1,000 or 100 U/ml human recombinant interferon γ; 10 or 1 ng/ml human recombinant GM-CSF; 50, 5, 0.5, or 0.05 ng/ml lymphotoxin; 100 or 50 ng/ml human C5a des arg; or 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, or 0.020 ng/ml murine recombinant TNF.

Measurement of TNF Concentrations in Human MNC Lysates and Supernatants

When supernatants from 24-h cultures of peripheral blood MNC, either unstimulated or stimulated, were tested in both the RIA and bioassay, a good correlation was found (Fig. 5). Because of the different detection ranges of both assays (bioassay, 500-15 pg/ml; RIA, 2,000–10 pg/ml) these samples were tested in the sensitive portion of either assay at various dilutions, and the data shown were calculated from the dilutions. Repeat-
was found: 2 ng/ml TNF with 1 ng/ml LPS up to 100 ng/ml TNF with 50 ng/ml LPS, and thereafter leveling off. The IL-1 β concentration in these supernatants measured in an RIA showed an increase from 0.2 ng/ml to 19 ng/ml with the same concentrations of LPS. Concentrations of IL-1 α increased from 0.04 to 1 ng/ml (Fig. 6).

To investigate the intraindividual consistency of TNF production, human MNC of three donors isolated and cultured on three different days were incubated with medium alone, medium with 1 ng/ml LPS, and medium with 30 µg/ml PHA. These experiments show good consistency for unstimulated and LPS stimulated cells but less so far PHA stimulation (Fig. 7).

**Distribution of TNF Produced by MNC of a Cohort of 50 Healthy Humans**

The amount of TNF produced by human MNC in vitro was determined in a cohort of 50 normal volunteers (laboratory personnel). We studied total TNF production by measuring the combined supernatant and cell lysate material present after 24 h of incubation. The data are depicted in Figure 8. With few exceptions, TNF levels were below the lower detection limit without exogenous stimuli. The unstimulated MNC show a narrow concentration range of TNF. LPS-stimulation led to TNF concentrations up to nearly 6 ng/ml (median 2.0 ng/ml). In 4 individuals, the TNF concentrations were below detection level. With PHA as a stimulus, a wider distribution of TNF concentrations was observed (> 14 ng/ml). Only one TNF determination was below detection level. Cultures performed in the presence of indomethacin showed
edly freezing and thawing of the samples (up to three times) was found not to affect the results of the RIA.

**Comparison of Amounts of TNF, IL-1 β and α Secreted by MNC**

Next, the dose-effect relationship for LPS stimulation of peripheral blood MNC was assessed in supernatants from cells of three normal donors, cultured for 24 h (Fig. 6). A dose dependent increase of the TNF production...
higher TNF concentrations for approximately 75% of the donors (Fig. 8). The differences in TNF production in the presence of indomethacin were not significant for the unstimulated cells; however, for both LPS- and PHA-stimulated cells, indomethacin significantly increased the amount of TNF ($p < 0.005$, Wilcoxon matched-pairs signed-ranks, two-tailed).

The MNC preparations after Ficoll Hypaque separation contained on average 19.1% (SD ± 10.7) monocytes. No correlation was found between the number of monocytes and the amount of TNF produced (data not shown).

Use of the RIA With Plasma Samples

To investigate whether we could recover TNF from the circulation, we injected mice with Hu rTNF intraperitoneally, and detected the appearance and clearance of Hu rTNF in the plasma. An injection of 2 $\mu$g of human TNF, which is a nonlethal dose, produced a peak concentration of 266 ng/ml after 12 min. After 15 min, the concentration had fallen to 127 ng/ml.

When plasma samples (with heparin or citrate) from patients with a variety of febrile illnesses (burns, sepsis, Still’s disease, Hodgkin’s disease) were added directly to the RIA, TNF was not detected. We observed that in the presence of these acute-phase samples, precipitable radioactivity was often greater than 100% of the zero standard, suggesting enhanced binding of the radiolabel, even if the standard curves for these assays were performed in normal plasma. However, in heparinized plasma samples of four patients with malaria, we were able to detect TNF concentrations of 274 ± SEM 45 pg/ml.

DISCUSSION

Employing the modified chloramine T method of radiolabeling and selecting the highest binding fractions to the specific polyclonal anti-TNF, we report the development of a sensitive and useful RIA. The process of radiolabeling of the TNF molecule does not degrade the molecule as demonstrated by SDS-polyacrylamide gel electrophoresis, nor does it affect the bioactivity. Although we have found a good correlation between the RIA and the bioassay for TNF, the RIA is clearly superior to the bioassay. The latter is more laborious and less specific than the RIA. Substances inhibiting or promoting growth or adherence of the L929 cells may lead to false-positive or false-negative results. Also factors, such as IL-1β, may synergize with TNF and lead to increased cytotoxicity (Ikejima et al, unpublished results).

The RIA is particularly useful for the detection of TNF produced by cultured human monocytes and natural killer cells [13–15]. Using a solid-phase RIA for TNF, Cuturi et al recently reported that T lymphocytes exposed to phorbol esters or calcium ionophore also produce TNF [15]. For studies like ours, in which lymphocytes are not separated from monocytes, it is important to know that the RIA does not detect the comparable lymphocyte product, lymphotoxin (TNF $\beta$), despite its structural homology with TNF (TNF $\alpha$) [16, 17]. In the experiments using PHA as a stimulant, production of lymphotoxin could be expected to have occurred [15, 18].

The concentrations of TNF produced in the cultures of the unstimulated human cells in our experiments show a narrow range, owing to strict measures to eliminate contaminating bacterial products (such as LPS) from reagents and culture media [12]. Incubation with indomethacin showed a slight but significant increase in the amount of TNF produced by LPS- or PHA-stimulated
cells, pointing to an inhibitory effect of products of the cyclooxygenase pathway. Using the RIA's to TNF, IL-1α and β, we measured the concentrations of these three major cytokines secreted by human MNC stimulated with various doses of LPS. In a highly consistent fashion, the cells of the three donors secreted large amounts of TNF and a tenfold lesser amount of IL-1β. The amount of IL-1α secreted was considerably lower. If, however, total (i.e., secreted and cell-associated) IL-1β is compared with total TNF, the amount of IL-1β greater than the amount TNF (Endres et al, unpublished data). The explanation should be sought in differences in the molecular structure of TNF and IL-1. TNF has a prominent signal peptide for cleavage and transport [16], and thus the amount of TNF secreted approximates the total amount of TNF produced. IL-1 is lacking a clear signal peptide [19], and this results in a variable amount of extracellular IL-1β and cell-associated IL-1α [20]. Since LPS only stimulates TNF production from monocytes and not from T cells [15], it can be calculated from the numbers of monocyte that stimulation with LPS results in the secretion of up to 0.2 pg of TNF per monocyte in 24 h.

The experiments using the cells of 50 different healthy donors demonstrate the normal distribution pattern of TNF production in vitro. With LPS as a stimulant, the cells of 10% of the donors produce less than 0.5 ng/ml TNF, and the cells of most donors produce more than 1 ng/ml. With PHA, even higher concentrations of TNF are found. It is conceivable that the greater amount of TNF produced upon stimulation with PHA is mediated via an effect on the lymphocyte. We have not been able to correlate the amount of TNF produced to obvious characteristics such as sex, race, and age. Since these studies were performed over a period of 6 weeks, seasonal influences can also be ruled out. The experiments performed to investigate the intra-individual consistency of TNF production suggest that high and low responders with regard to TNF production exist. Since the gene for TNF is closely located to the genes for HLA [21], the linkage of HLA haplotypes to TNF production should be investigated.

The results of the experiments in which Hu rTNF was injected into mice suggest a slightly shorter half-life of human TNF than the 10-min half-life found by Flick and Gifford with murine TNF [22]. Detection of TNF in the circulation of patients is still a problem when plasma or serum samples are put directly in the RIA. Similar problems have been reported by Scuderi et al [23], who were also unable to detect TNF in the majority of patients with infectious or neoplastic disease. Like them, we detected circulating TNF in malaria. The detectability of TNF in mice injected with human recombinant IL-1 and the observation that acute-phase plasma samples bind more than 100% of the radiolabeled TNF points to substances in the circulation of humans with various diseases that compete with anti-TNF for binding of the labeled TNF. Studies are under way to separate TNF from such substances. However, the RIA is highly useful to measure TNF in stimulated MNC from humans, e.g., in disease states, on drugs, and during cytokine treatment. At a more basic level, detection of immunoreactive TNF in studies on cytokine interaction, and studies of how various agents affect TNF synthesis and gene expression in vitro will help to clarify our understanding of the control of TNF production.

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