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Determination of Tumor Necrosis Factor-α (TNF-α) in Serum by a Highly Sensitive Enzyme Amplified Lanthanide Luminescence Immunoassay

CONSTANTINOS PETROVAS, SPIROS M. DASKAS, and EVRIKLIA S. LIANIDOU

Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, 15771 Athens, Greece

Objectives: To develop a highly sensitive enzyme amplified lanthanide luminescence (EALL) immunoassay for tumor necrosis factor-α (TNF-α).

Methods: The method is based on the use of two monoclonal antibodies against TNF-α, one "capture" antibody and one labeled with biotin, in a "sandwich type" assay format. Alkaline phosphatase (ALP) conjugated to an antibiotin-polyclonal antibody is used as the enzyme label. ALP cleaves phosphate from diflunisal phosphate (DIFP) to produce diflunisal (DIF). The detection system is based on the combination of enzymatic amplification introduced by ALP and the formation of a highly fluorescent terbium complex that can be monitored by time resolved or conventional fluorimetry.

Results: By using 50 μL of sample, the dynamic range of the assay extends up to 2000 ng/L of TNF-α, with a detection limit of 1 ng/L, within-run CVs ranging from 3 to 15% and recoveries of 97 ± 2%. By using 100 μL of sample the dynamic range of the assay extends up to 1000 ng/L of TNF-α with a detection limit of 0.2 ng/L, recoveries of 94 ± 13%, within-run CVs ranging from 2 to 6.5% and between-run CVs ranging from 5 to 15%, in a total incubation time of 3h. No interference by the presence of other cytokines (IL-1β, IL-2, IL-4, IL-6, IFN-γ) or by rheumatoid factors has been observed. The results obtained by the proposed method and by a commercially available kit (Medgenix TNF-α EASIA) correlated well ($n = 26$, $r = 0.934$).

Conclusion: The proposed method is highly sensitive, simple and rapid and can reliably measure TNF-α in the ng range in biological specimens. Copyright © 1999 The Canadian Society of Clinical Chemists

KEY WORDS: TNF-α; cytokines; enzyme amplified lanthanide luminescence immunoassay; terbium.

Introduction

Tumor necrosis factor-α (TNF-α) is a potent preinflammatory and immunoregulatory cytokine, which is secreted as an unglycosylated 157-amino-acid polypeptide of relative molecular mass (M_r) of 17 KDa under denaturing and 45-50 KDa under native conditions (1,2). TNF-α is produced under appropriate conditions by a large number of different cell types mainly by activated macrophages, lymphocytes and polymorphonuclear leukocytes in response to bacterial toxins, inflammatory and other invasive stimuli (3,4). TNF-α is able to induce a great diversity of cellural responses, the most important being the triggering of synthesis and secretion of most preinflammatory mediators (3,4), cytotoxicity to certain types of tumor cells and apoptosis of mature T-cells (5). TNF-α exerts its biological effects through binding to two distinct receptors, TNF-R_1 and TNF-R_2 (6,7) and its response heterogeneity can be explained by a diversification of post-receptor signal-transducing pathway, including activation of protein kinases and transcription factors (8,9).

TNF-α is rapidly gaining recognition as a critical mediator of the inflammatory response and has been shown to play an important role in hematopoiesis, protection against infection, in the regulation of insulin activity and is a key mediator of obesity related insulin resistance (4). TNF-α is important in the regulation and pathogenesis of several disease states like cachexia, autoimmune disorders, septic shock, insulin dependent diabetes melitus (IDMM), inflammatory myocarditis, ischemia, cancer and AIDS (4,10–14). High concentrations of TNF-α are detected in plasma from patients with septic shock (11) and in serum and synovial fluid from patients with rheumatoid arthritis (RA) (12). High levels of circulating TNF-α seem to be well correlated with a poor prognosis in acute graft vs. host disease (13) and with the severity and progression of multiple sclerosis (14).

The ability to measure accurately and precisely very low amounts of circulating TNF-α is very important for the elucidation and clinical investigation of many TNF-α dependent pathological conditions. Commonly used TNF-α assays are based on TNF-α bioactivity (bioassays) or TNF-α immunoreactivity (immunoassays). The most commonly used methods to determine TNF-α are ELISA, RIA, and bioassays.

Correspondence: Dr. E. S. Lianidou, Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, 15771, Greece. E-mail: lianidou @chem.uoa.gr

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Bioassays offer sensitive measurements of bioactive forms of TNF-α but they are not suitable for routine clinical use, since they have the disadvantages of poor reproducibility, low specificity, high costs and a low capacity of processing samples (15), while RIAs main disadvantage is the use of radioisotopes (16). Non-isotopic immunoassays for TNF-α, mainly ELISA (17–21) offer the advantages of good reproducibility and high specificity. Sensitivity for TNF-α obtained by ELISA methods is generally better when chemiluminescent substrates are used in respect to colorimetric substrates but still very long incubation times are needed (18).

However, the performance of TNF-α immunoassays is affected by the complex interaction of TNF-α with various plasma components like TNF-α binding proteins, circulating receptor fragments, α_2-macroglobulin and rheumatoid factors (22,23), by the nature of monoclonal antibodies, TNF-α standards used and various preanalytical factors (24–26). Moreover, a wide variation in TNF-α values was obtained when TNF-α was measured in the same samples using ELISA kits from different companies (27), so an internationally accepted standard preparation for TNF-α was established, to facilitate the development of TNF-α diagnostic and therapeutic potential (28).

The most sensitive and reliable immunoassay methods for TNF-α reported so far (19,20), with detection limits in the order of less than 1 ng/L, use europium labeled antibodies and are based on a time-resolved fluoroimmunoassay detection approach. However, these methods require long incubation times and a large amount of sample. Enzyme amplified lanthanide luminescence (EALL) , firstly introduced by Evangelista and coworkers (29), is one of the most sensitive non-isotopic immunoassay methodologies available today, because it combines amplification provided by using an enzyme as label in combination with the unique fluorescent properties of the lanthanides, a fact that enables the use of time-resolved fluorescence detection. The basic principle of this detection approach is that under the action of an enzyme used as a label, a substrate which under the conditions of the measurement does not form a highly luminescent chelate with a lanthanide ion, such as terbium, is converted to a product which does form such a chelate. This results in exceptionally low detection limits for the analyte (30). This method is well established as an ultrasensitive non-isotopic detection system and has been succesfully applied in immunoassays (30–32), and DNA hybridization assays (33).

In this work, we describe a highly sensitive enzyme immunoassay for TNF-α in serum and plasma, based on the EALL detection approach (29). In this assay, exceptional sensitivity is achieved in a short incubation time by using a relatively small amount of sample. This is due to the combination of enzymatic amplification by using alkaline phosphatase (ALP) as the enzyme label and fluorescence detection by the formation of a highly fluorescent terbium complex, which can be monitored by time-resolved or conventional fluorometry. The proposed method is particularly suitable to reliably measure TNF-α in the ng range in biological specimens.

Methods

Reagents

Human recombinant TNF-α (hrTNF-α) international WHO standard 87/650, was obtained from the National Institut of Biological Standards and Controls, (NIBSC, Pottersbar, UK). Two monoclonal antibodies against TNF-α, TNF-α/7 and TNF-α/5 were kindly donated by Prof. L. Aarden (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, Netherlands) and were used for the TNF-α immunoassay. The other cytokines (IL-2β, IL-2, IL-4 and IFN-γ) were commercial kit components while IL-6 was obtained from NIBSC (WHO standard, 88/514). The antibiotin-ALP conjugate was obtained from Abbott Laboratories (IMx, HBsAg assay). The ELISA microplates used for the assay were NUNCLON (Nunc, Denmark). The phosphate ester of diflunisal (5-[2,4-Difluorophenyl]salicylic acid), DIFP, was synthesized and purified as previously described (32). Terbium chloride was obtained from Aldrich (Steinheim, Germany). Unless otherwise stated the chemicals used were of analytical grade and purchased from Sigma (St. Louis, MO, USA).

Solutions

All solutions were prepared in deionized, distilled water. Coating solution, blocking solution, the substrate buffer for ALP and developing solution were prepared as described previously (34,35). The TNF-α stock solution (1 mg/L) was prepared by dissolving the lyophilized hr-TNF-α international standard preparation (NIBSC 87/650) in 1000 μL PBS buffer. TNF-α working stock solutions (100 μg/L) were prepared by appropriate dilutions of the stock solution in a 50 mmol/L Tris buffer, pH 7.40 containing 60 g/L bovine serum albumin (BSA), 0.5 g/L of NaN_3 and 9 g/L of NaCl and were kept in aliquots at −20° C. Under these conditions TNF-α in standard preparations and samples is stable for at least 3 months, provided that the samples are not subjected to repeated freeze-thaw cycles (26,28,36). TNF-α calibrators at a concentration range of 2–2000 ng/L were prepared by appropriate dilutions of the TNF-α stock solution both in assay buffer and in TNF-α free serum. The assay buffer was a 60 g/L BSA solution in a 50 mmol/L Tris buffer, pH 7.40, containing 0.5 g of NaN_3, 9 g of NaCl, 50 mL of mouse serum and 0.5 g Tween-20 per liter. This solution was used also for dilution of biotinylated anti-TNF-α monoclonal antibody. The DIFP stock solution (10 mmol/L) was prepared in 0.1 mol/L NaOH and kept at 4° C, whereas DIFP working solution (500 μmol/L) was

prepared just before use by 20-fold dilution of the stock solution in the substrate buffer of ALP (32).

Plasma and serum samples

For serum, blood samples were collected in clean glass tubes, left at room temperature for 1 h and centrifuged at 4° C. For plasma, blood samples were collected in EDTA-containing Vacutainer tubes (Beckton Dickinson) and centrifuged at 4° C. Serum and plasma samples were stored at −20° C until assayed.

Procedure

A typical "sandwich type" immunoassay was used. Assay conditions involving all steps, such as reagent concentrations, sample volume and time of incubation periods, were optimized in preliminary experiments in respect to the best signal-to-noise ratios, sensitivity and rapidity. The final optimized assay protocols were the following: (a) immobilization of the anti-TNF-α monoclonal antibody (stock solution 2.5 g/L), diluted 1000-fold (100 μL/well) in coating solution, for a minimum of 12 h, wash; (b) blocking with blocking solution (200 μL/well) for 2 h, wash; (c) addition of the TNF-α standard or sample (100 μL/well/Protocol A or 50 μL/well/Protocol B) diluted with assay buffer (50 μL/well/Protocol A or 100 μL/well/Protocol B), incubation for 90 min, wash four times; (d) addition of the biotinylated anti-TNF-α monoclonal antibody (stock solution 1g/L), diluted 3000-fold in assay buffer (100 μL/well), incubation for 30 min, wash four times; (e) addition of antibiotin-ALP conjugate, (100 μL/well), incubation for 30 min, wash six times; (f) addition of the DIFP working solution (100 μL/well), incubation for 30 min. The enzymatic reaction was stopped by adding developing solution (100 μL/well). All incubation steps were performed at room temperature. Aliquots of 180 μL of the DIF-Tb^{3+}-EDTA ternary complex, developed finally in each well and 400 μL of developing solution, were brought into a 1.00 mL cuvette and mixed thoroughly. Fluorescence measurements were performed in a Perkin Elmer M 512-A fluorescence spectrophotometer (λexc = 284 nm, λem = 546 nm) as previously described (32).

Results

Standard curve and detection limit

A set of TNF-α calibrators in the concentration range of 2 to 2000 ng/L was prepared daily by appropriate dilutions of the TNF-α international standard (NIBSC 87/650) stock solution (100 μg/L) both in assay buffer and in a pool of normal sera that did not contain any detectable immunoreactive TNF-α.

In Protocol A, where 100 μL of serum or standard are used as a sample volume, the assay's dynamic range extended up to 1000 ng/L. When the TNF-α standards were diluted in serum the obtained fluorescence signal was ~30% lower than that obtained by dilutions of standards in assay buffer. The detection limit defined as the minimal concentration of TNF-α that produces a fluorescent signal equal to the nonspecific background signal ±2SD was as low as 0.2 ng/L in serum (n = 12).

In Protocol B, where 50 μL of serum or standard are used as a sample volume, the assay's dynamic range extended up to 2000 ng/L. When the TNF-α standards were diluted in serum the obtained fluorescence signal was ~12 % lower than that obtained by dilutions of standards in assay buffer. In this case, the detection limit was as low as 1 ng/L (n = 12).

Table 1
Analytical Recovery of TNF-α Added to Serum Samples

Protocol	TNF-α, ng/L added	TNF-α, ng/L found[a]	Recovery, %
A	10	8.8 ± 1.9	88
	50	54 ± 6	109
	100	85 ± 11	85
Mean ± SD			94 ± 13
B	10	9.5 ± 2.3	95
	100	99 ± 10	99
	500	482 ± 13	96
Mean ± SD			97 ± 2

[a]Mean ± SD value of four different serum samples (three replicates per specimen).

Analytical recovery and precision

Analytical recovery was assessed by analyzing four serum samples supplemented with hrTNF-α at three different concentrations. When hrTNF-α calibrators were prepared in assay buffer, analytical recoveries were low for protocol A (66 ± 13%, n = 36) and satisfactory for protocol B (89 ± 10%, n = 36). When hrTNF-α calibrators were prepared in a pool of TNF-α free serum, satisfactory analytical recoveries were obtained for both protocols A (94 ± 14%, n = 36) and B (97 ± 7%, n = 36) (Table 1).

To determine within-run precision of the assay, serum samples containing different concentrations of TNF-α were assayed in eight parallel determinations for both Protocols A and B. To determine between-run precision of the assay, aliquots of three serum samples containing different concentrations of TNF-α were stored frozen (−20° C) and analyzed over a period of 2 weeks, by following Protocol A, in three parallel determinations, each in six separate assays (Table 2).

Serum dilution study

Serum dilution experiments showed that both recombinant and native TNF-α are equally recognized by the assay. When two serum samples from patients found to contain high concentrations of

TABLE 2
Precision of the TNF-α Immunoassay

Serum	Within-run[a] Protocol A TNF-α, ng/L	CV, %	Between-run[b] Protocol A TNF-α, ng/L	CV, %	Within-run[a] Protocol B TNF-α, ng/L	CV, %
1	1.9 ± 0.1	6.5	—	—	—	—
2	10.0 ± 0.5	4.6	10 ± 1.4	14	10 ± 1.5	15
3	100 ± 2	2.3	96 ± 14	15	87 ± 5	6.1
4	500 ± 11	2.2	336 ± 17	4.6	500 ± 13	2.7

[a]Mean value of eight replicates per specimen (same run).
[b]Mean value of three replicates per specimen (six runs).

TNF-α and one serum sample spiked with hrTNF-α at a final concentration of 1000 ng/L were serially diluted in a pool of TNF-α negative control serum and analyzed by Protocol A, the dilution curves were parallel in all cases.

SPECIFICITY OF THE ASSAY

Both monoclonal antibodies used for the formation of the TNF-α "sandwich" were previously selected by Prof. Aarden among a variety of different antibodies (personal oral communication). The specificity of the TNF-α assay was assessed by checking cross reactions with other cytokines. No interference by the presence of IL-1β, IL-2, IL-4, IL-6 and IFN-γ, at concentrations up to 10000 ng/L, has been observed (Figure 1).

To avoid any possibly falsely increased results for TNF-α, due to the presence of anti-mouse immunoglobulins and rheumatoid factors (RF) present in human sera, we added 50 mL of normal mouse serum per liter (5% NMS) of assay buffer (37,38). To check the effectiveness of this addition, 18 serum samples with known high concentrations of RFs, between 160 and 2540 U/mL (RapiTex RF, Behring), were analyzed in two separate TNF-α assays. The assays were run both with and without the addition of 5% NMS in assay buffer. In the assay containing 5% NMS in assay buffer all the sera were found negative for TNF-α, while in the assay without 5% NMS in assay buffer, five of the sera were found to be positive, with TNF-α values ranging from 5 to higher than 500 ng/L.

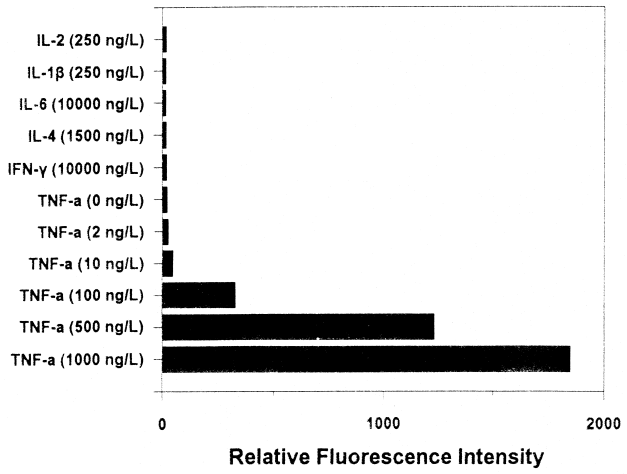

Figure 1 — Specificity of the TNF-α EALL immunoassay. No significant elevation of the relative fluorescence intensity (RFI) was observed with any of the five cytokines assessed by the TNF-α EALL immunoassay.

COMPARISON WITH ELISA

TNF-α concentrations in 26 serum samples from apparently healthy individuals and patients with various diseases were compared with values obtained by a commercially available ELISA immunoassay (Medgenix, TNF-α EASIA). The results obtained by our method (y) and by ELISA (x), correlated firmly well, $y = 0.77x - 25.7$ ng/L, $r = 0.934$. Samples with high TNF-α concentrations gave similar results in both assays, whereas generally the values obtained by the proposed EALL immunoassay were slightly lower than those obtained by the ELISA kit. However, it has been recently stated (27) that even when the same international standards (provided by the NIBSC) were used, a wide variation in TNF-α values was obtained when the same serum samples were analyzed by several commercial ELISA kits.

Discussion

The ability to measure accurately and precisely very low amounts of circulating TNF-α is very important for the elucidation and clinical investigation of many TNF-α dependent pathological conditions. Usually the concentrations of circulating TNF-α in healthy states are very low, although a wide variation has been reported that is mainly due to differences in assay methods and sensitivity (27,39). The most commonly used methods to determine TNF-α are ELISA, RIA, and bioassays. Non-isotopic immunoassays for TNF-α, mainly ELISA (17–21) offer the advantages of good reproducibility and high specificity.

A time-resolved fluoroimmunoassay with a europium labeled antibody for TNF-α was described by

Ogata *et al.* (19). They report a large measuring range and a very low detection limit (0.1 ng/L), however their total incubation time is longer than 24 h at 0° C, a fact that limits the practical use of this method. A modified time-resolved fluoroimmunoassay for TNF-α was also described later by Turpeinen *et al.* (20). In this case, a detection limit of 6 ng/L (in a total incubation time of more than 6 h) and low recoveries (in serum 83% and in plasma 76%) have been reported. Moreover, both of these methods are vulnerable to contamination of trace amounts of europium and special precautions must be taken during all incubation steps.

For this reason we believed that there was still a need to develop a reliable, sensitive and rapid method for the determination of TNF-α in biological samples. In the present work, we describe a highly sensitive enzyme immunoassay for the determination of TNF-α in serum and plasma, based on the enzyme amplified lanthanide luminescence (EALL) detection approach (29). Ultrasensitivity is obtained by the powerful detection system that is based on the combination of enzymatic amplification introduced by the enzyme ALP and the formation of a highly fluorescent terbium complex (31,32), which can be monitored by time-resolved or conventional fluorometry. The resulting fluorescence signal is quite stable, so that many plates can be run simultaneously, therefore, making the method suitable for routine clinical use. This detection approach has been successfully applied for the ultra-sensitive determination of AFP (30,32,34) and IL-6 (35) in serum and can be also used for the determination of other cytokines.

The data presented here show that a reliable and sensitive immunoassay for TNF-α has been developed. During the development of the assay, a systematic analytical work was undertaken so that all assay conditions were optimized in respect to the best signal-to-noise ratios and analytical characteristics. The optimized assay demonstrates exceptional sensitivity (detection limit 0.2 ng/L in serum) and good recoveries in a relatively short incubation time (3 h).

By using 100 μL as sample volume (protocol A) the detection limit is as low as 0.2 ng/L in serum while the assay's dynamic range extends from 2 to 1000 ng/L of TNF-α, a concentration range which is usually required for several disease states. By using 50 μL as sample volume (protocol B) the assay's dynamic range extends up to 2000 ng/L of TNF-α, the detection limit is 1 ng/L, while even lower sample volumes (*e.g.,* 10 μL) or even shorter incubation times can be also used, provided that exceptional sensitivity is not required.

The standard curves obtained by both protocols gave lower slopes when hrTNF-α calibrators were prepared in TNF-α free pool serum, (~30% for protocol A, 12% for protocol B) in respect to those obtained by preparation of hrTNF-α calibrators in assay buffer. This can be explained by the presence of various TNF-α binding proteins in serum and plasma, which can affect the accuracy of TNF-α quantification by competing with the assay antibodies for binding to TNF-α. The main binding proteins reported are the soluble forms of the TNF-α receptors, sTNFR1 and sTNFR2 (6,7) and α_2-macroglobulin (23). According to a WHO study on TNF-α (28), the diluent or matrix should be identical for the TNF-α standards and for the samples, so in our study TNF-α standards were prepared in serum, shown previously to give only background levels. In this case, satisfactory analytical recoveries for both protocols were obtained. This can be explained by the fact that normal serum contains soluble forms of the TNF-α receptors in a great molecular excess to TNF-α (7) and in this way the sTNFR effect on the TNF-α determination is compensated. However, in protocol B, the TNF-α calibrators can also be prepared in assay buffer, because it was shown that satisfactory recoveries are obtained, possibly due to the fact that a lower amount of sample is diluted in a larger amount of assay buffer, therefore, minimizing the effect of sample matrix in this case. The within-run CVs ranged from 2 to 6.5% while between-run CVs ranged from 5 to 15%. Serum dilution experiments showed that both recombinant and native TNF-α are equally recognized by the assay. The specificity of the assay was optimized by addition of normal mouse serum in assay buffer, which was shown to prevent false-positive interferences caused by rheumatoid factors and heterophilic antibodies. No interference by the presence of other cytokines (IL-1β, IL-2, IL-4, IL-6, IFN-γ) has been observed. Comparison of our EALL method and a commercially available ELISA (Medgenix TNF EASIA) using the same serum samples showed acceptable correlation ($r = 0.934$), although values obtained by EALL were lower. When the TNF-α standards of the ELISA kit were measured by EALL, the concentrations obtained were also lower than their expecting values, suggesting that the discrepancies in sample values between the two methods were due to differences in standardization.

The proposed method is highly sensitive, simple and rapid and can reliably measure TNF-α in the ng range in human serum. The method can be also applied for the determination of TNF-α in cell culture supernatants, rheumatoid synovial fluids and other biological samples. Moreover, in case that time resolved fluorescence detection is used, the total incubation time for the assay can be much shorter (35).

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