Turk J Med Sci
35 (2005) 385-393

EXPERIMENTAL / LABORATORY STUDIES

Development of ELISA Systems for Measurement of Human Tumor Necrosis Factor-Alpha (TNF-α)

Ayşegül YÜCEL, Cemalettin AYBAY
Department of Immunology, Faculty of Medicine, Gazi University, Ankara - Turkey

Received: June 30 2005

Abstract: The aim of this study was to develop a human tumor necrosis factor alpha (hTNF-α) ELISA system because specific and sensitive measurement of low levels of circulating TNF-α is very important for enlightening the immunopathologial mechanisms associated with TNF-α. Monoclonal antibodies 6A4c and 8A6 were produced against hTNF-α and were used as the capture antibody and the tracer antibody respectively for the first hTNF-α ELISA system (6A4c/biotin-8A6). Murine polyclonal IgG was used as the tracer antibody in the second hTNF-α ELISA system (6A4c/biotin-polyclonal IgG). Both systems could detect both recombinant hTNF-α and native hTNF-α. The detection limits defined as minimal concentration of hTNF-α were less than 4 pg/ml for the first and less than 12 pg/ml for the second ELISA systems. 6A4c/biotin-8A6 system resulted in some non-specific reactions to some extent with human sera; however, 6A4c/biotin-polyclonal IgG system produced acceptable background levels with human sera. A prominent inhibitory effect of TNF receptors-I and -II did not occur in any of the ELISA systems at physiological concentrations. Two different types of ELISA systems with high sensitivity and specificity were developed to measure hTNF-α level both in human serum and cell culture supernatant.

Key Words: TNF-α, ELISA

Introduction

Tumor necrosis factor-alpha (TNF-α) is a very potent cytokine which is an endogenous mediator of immune and inflammatory functions (1, 2). In response to various stimuli such as bacterial toxins and inflammation, it is predominantly produced by activated macrophages, but also by many other normal cells including astrocytes, fibroblasts, basophiles, mast cells, NK cells, Kupffer cells, smooth muscle cells and epidermal cells as well as by various malignant cell types in some instances (1, 2). Although it is generally thought that TNF-α is not produced by normal cells constitutively and its physiological role has not been completely understood yet, it is believed to be effective in diseases due to bacterial, viral and parasitic infections as well as autoimmunity (3). Therefore, TNF-α is one of the key cytokines in immunology-based studies. Measurement of TNF-α by ELISA method has been widely used in clinical investigations and research. There are quite a number of commercially available TNF-α ELISA kits. However, considering the economical status of Turkey, the cost is very high when the commercial kits are used. Besides, it is very important for each academic unit to produce the necessary biotechnological products for its use.

In view of all these factors, we aimed to develop a highly sensitive but of much lower cost TNF-α ELISA kit with all its components in our department.

Material and Methods

Anti-hTNF-α monoclonal antibodies:

Nine different hybridoma cell lines secreting monoclonal antibodies (mAb) against human TNF-α (hTNF-α) were produced in our preliminary research studies (data not presented here). It was found that the 6A4c mAb was suitable to be used as capture antibody. 8A6 mAb was used as the tracer antibody (conjugate)

after being labeled with biotin. Consequently, these two mAb's were used to develop the hTNF-α ELISA kit.

Purification of monoclonal antibodies:

Monoclonal antibodies were purified from the culture supernatants using the protein G affinity column as described previously (4).

Biotinylation of 8A6 mAb and polyclonal murine IgG for conjugate formation:

Biotinylation of tracer antibody was performed using EZ-Link Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Inc., Rockford, IL 61105) according to the manufacturer's instructions. Free biotin was eliminated chromotographically by using Sephadex G-25 Fine-packed 1x30 cm column.

ELISA systems

High binding capacity, 96-well flat-bottomed microplates (Costar, Corning Incorporated, Corning, NY) were coated with 100 µl/well of 6A4c mAb at 5 µg/ml in 0.05 M carbonate-bicarbonate buffer (CBB) pH 9.6 and were incubated overnight at +4°C. For blocking purpose, 200 µl of phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) was added to each well and then incubated for 2 hrs at room temperature (RT). After washing the plates five times with washing buffer consisting of PBS plus 0.05% Tween 20 (PBS-T), 50 ml of sample diluent (PBS containing 1% BSA, 0.5% foetal bovine serum (FBS) and 0.5% inactivated murine serum) was added to each well. A 100 µl of the standards or the samples was added into each well. The plates were incubated for 2 hrs at RT. After washing three times with PBS-T, 100 µl of conjugate was added to each well. The conjugate used was 1/800 diluted biotin-8A6 mAb or 1/100 diluted biotin-polyclonal murine IgG in 6A4c/biotin-8A6 and 6A4c/biotin-polyclonal murine IgG TNF-α ELISA systems respectively. The plates were incubated for 1 hr at RT, and then washed three times with PBS-T. 100 µl of Streptavidin-HRP in %1BSA-PBS was added into each well and incubated for 30 min at RT. After washing three times, 100 µl/well TMB was added and the plates were reincubated for 30 min at RT; then the reaction was stopped with 50 µl/well of 1 M H_2SO_4. The optical density of each well was measured with an ELISA reader (Sunrise Remote/Touch Screen, Tecan Austria GmbH, Grödig, Austria) at OD450 nm or OD450/620 nm.

Statistical Analysis

Regression-correlation analysis was performed. Calculations were done using SSPS for the Windows version 10.0 and Microsta computerized statistics programme.

Results

Standard curve for 6A4c/biotin-8A6 hTNF-α ELISA system

Figure 1A shows the standard curve obtained from developed 6A4c/biotin-8A6 hTNF-α ELISA system. Standard recombinant human TNF-α (rhTNF-α) was prepared at various concentrations in RPMI-1640 containing 10% FBS and added into microplates as 100 µl/well. Data given here consisted of mean OD450 nm values of a study performed in quadruplicate wells The analytical sensitivity of developed 6A4c/biotin-8A6 hTNF-α ELISA system was investigated. The detection limit defined as minimal concentration of hTNF-α that produces a signal equal to non-specific background signal + 2SD, of this in-house ELISA, was measured to be less than 4 pg/ml.

Measurement of native human TNF-α using the 6A4c/biotin-8A6 hTNF-α ELISA system

A supernatant of human peripheral blood mononuclear cells (1 x 10^6 cells/ml) stimulated with LPS of *S.typhimurium* at 1 µg/ml for 48 hrs produced high amounts of hTNF-α. For both standard rhTNF-α and cell culture supernatant (native hTNF-α), absorbance values at OD450 nm increased in a concentration dependent manner (Figure 1B). Data given here consisted of mean of OD450 nm values of a study performed with peripheral blood mononuclear cells of two different persons in triplicate wells. This data suggested that the developed 6A4c/biotin-8A6 hTNF-α ELISA system could recognize native antigen as effectively as rhTNF-α so that this system could be used to measure both recombinant and native human TNF-α.

Standard curve for 6A4c/biotin-polyclonal IgG TNF-α ELISA system

Although TNF-α measurement could be performed in culture supernatants by using the 6A4c/biotin-8A6 hTNF-α ELISA system, non-specific reactions were seen with

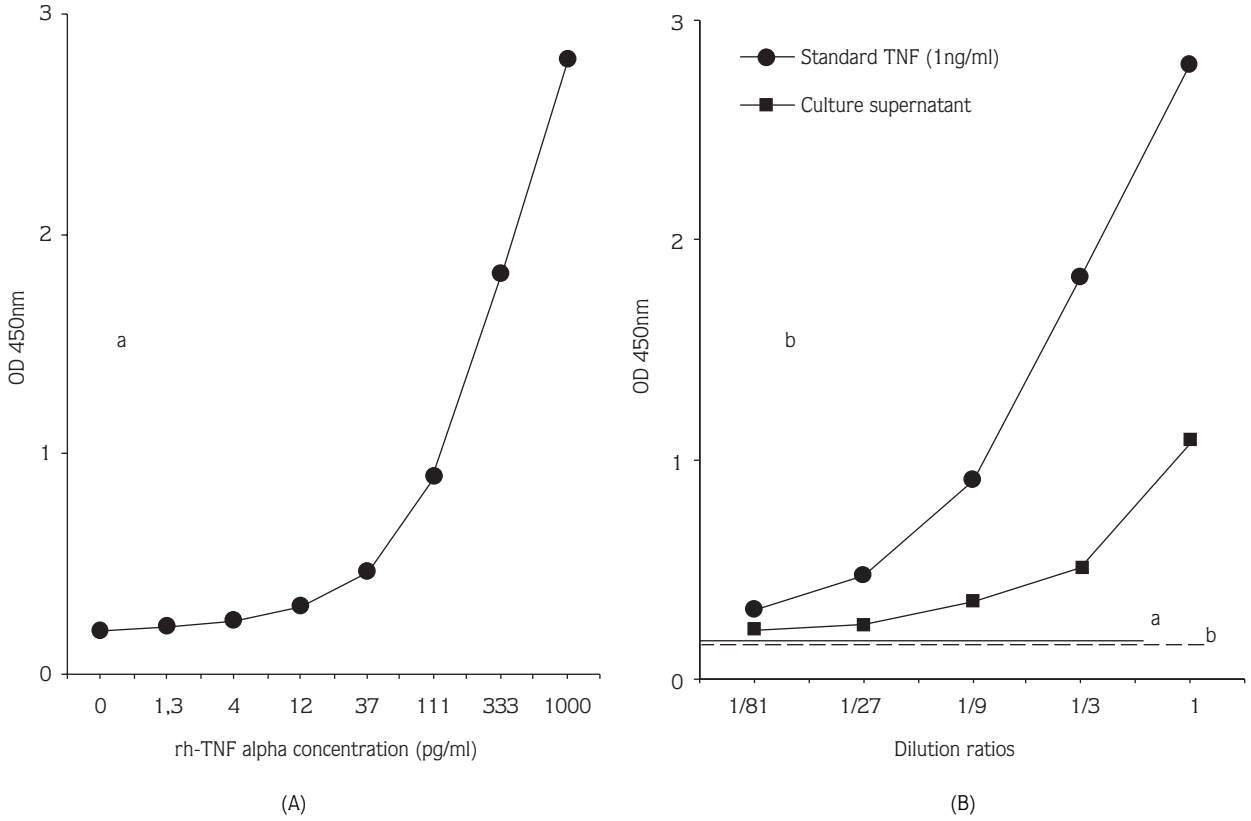

Figure 1. Analytical sensitivity of 6A4c/biotin-6A6 hTNF-α ELISA system. (A). Different concentrations of rhTNF-α, diluted in RPMI-1640 containing 10%FBS, were measured using the developed system. The ELISA system gave a significant standard curve (r^2=0.9208, p=0.00016) and an analytical sensitivity less than 4 pg/ml. Native hTNF-α in the cell culture supernatant of LPS-stimulated human peripheral blood mononuclear cells (PBMNC) was also measured by using the same ELISA system (B). Different concentrations of standard rhTNF-α as well as non-stimulated (a) and (1 µg/ml) LPS-stimulated (b) PBMNC culture supernatant were also measured simultaneously with the same system. It was shown that 6A4c/biotin-8A6 hTNF-α ELISA system also recognized native hTNF-α.

human sera (data not shown here); therefore, it was decided to develop another ELISA system for hTNF-α measurement in human serum. For this purpose, biotinylated polyclonal murine IgG at 1/100 diluted form was used as the conjugate while the solid phase was still coated by 6A4c mAb (6A4c/biotin-polyclonal IgG). The assay procedure was performed as described above. Figure 2A shows the standard curve obtained from the 6A4c/biotin-polyclonal IgG hTNF-α ELISA system. Data given here consisted of mean OD450 nm values of a sample study performed in quadruplicate wells. The analytical sensitivity of this hTNF-α ELISA system was also investigated. The detection limit defined as minimal concentration of hTNF-α that produces a signal equal to non-specific background signal + 2SD, of this in-house ELISA, was measured to be less than 12 pg/ml.

Measurement of native human TNF-α using 6A4c/biotin-polyclonal IgG TNF-α ELISA system

Supernatant of human peripheral blood mononuclear cells (1 x 10^6 cells/ml) stimulated with LPS of *S.typhimurium* at 1 µg/ml for 48 hrs produced high amounts of hTNF-α. For both standard rhTNF-α and cell culture supernatant (native hTNF-α), absorbance values at OD450 nm increased in a concentration dependent manner with lower background (Figure 2B). Data given here consisted of mean OD450 nm of a sample study performed with peripheral mononuclear cells of two different persons in triplicate wells. This data suggested that the 6A4c/biotin-polyclonal IgG TNF-α ELISA system could recognize native antigen as effectively as rhTNF-α so this system could also be used to measure both recombinant and native human TNF-α.

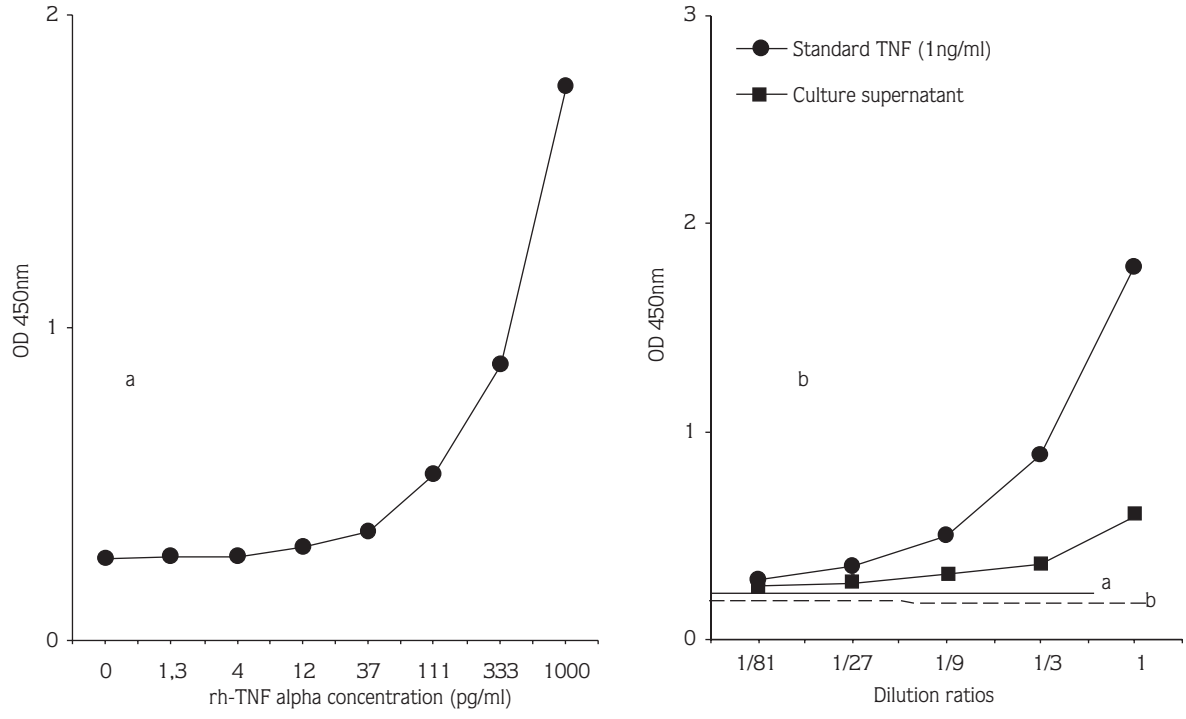

Figure 2. Analytical sensitivity of 6A4c/biotin-polyclonal IgG hTNF-α ELISA system (A). Different concentrations of rhTNF-α, diluted in RPMI-1640 containing 10%FBS, were measured using the developed system. 6A4c/biotin-polyclonal IgG hTNF-α ELISA system gave a significant standard curve (r^2=0.9917, p<0.00001) and an analytical sensitivity less than 12 pg/ml. Native hTNF-α in the cell culture supernatant of LPS-stimulated human PBMNC was also measured using the same ELISA system (B). Different concentrations of standard rhTNF-α as well as non-stimulated (a) and (1 μg/ml) LPS-stimulated (b) PBMNC culture supernatants were also measured simultaneously with the system. It was shown that 6A4c/biotin-polyclonal IgG hTNF-α ELISA system also recognized native hTNF-α.

Serum dilution study

When recombinant hTNF-α was added into pooled human serum at 1ng/ml and then threefold serially diluted within the same serum, it was observed that the dilution curve was very similar to those produced by the same antigen added to RPMI-1640 containing 10% FBS using the 6A4c/polyclonal murine IgG hTNF-α ELISA system (Figure 3). Data given here consisted of mean OD450 nm values of a sample study performed in triplicate wells. These results showed that the 6A4c/biotin-polyclonal IgG hTNF-α ELISA system was useful for measuring hTNF-α, not only in the culture medium but also in human serum samples.

Effect of TNF-RI and/or TNF-RII on TNF-α measurement in both developed ELISA systems

The microplates of both of the hTNF-α ELISA systems developed were coated with 6A4c mAb; however, the conjugate was biotin-8A6 mAb in one and biotin-polyclonal IgG in the other. It was investigated whether the addition of various concentrations of recombinant TNF receptor (TNFR)-I and/or TNFR-II to the medium had inhibitory effects on hTNF-α measurement or not because one of the aims of this project was to show whether the developed hTNF-α ELISA systems measured active free TNF or total TNF. Recombinant TNFR-I and recombinant TNFR-II at 0, 4.5, 13.5, 4.0, 122, 1100 and 3300 ng/ml concentrations were added alone or together to rhTNF-α prepared at a constant concentration (110 pg/ml). Data given here consisted of mean OD450 nm values of a prepresentative study performed in triplicate wells. The physiological concentrations of TNFR-I and TNFR-II are 4-8 ng/ml (12). It was seen that addition of TNFR-I and/or TNFR-II at physiological concentrations did not cause a significant decrease in OD values, while higher doses caused a

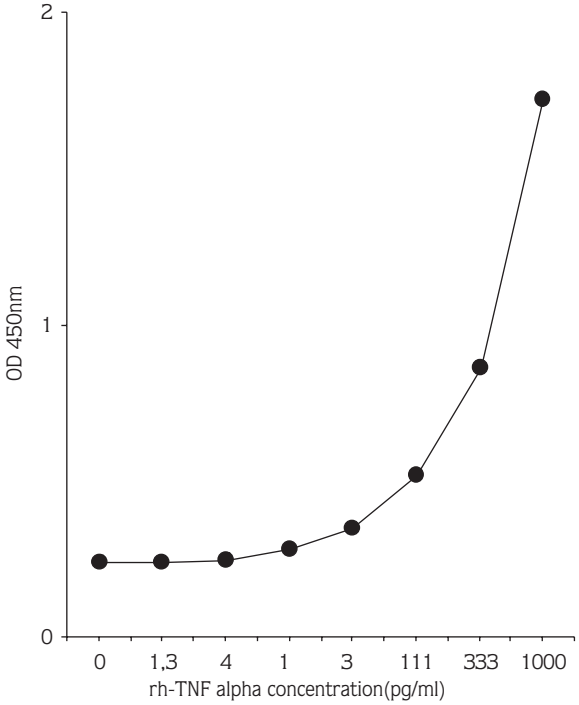

Figure 3. Analytical sensitivity of 6A4c/biotin-polyclonal IgG hTNF-α ELISA system in the measurement of level of rhTNF-α, diluted in human serum. The ELISA system gave a significant standard curve (r^2=0.9889, p<0.00001) and an analytical sensitivity less than 12 pg/ml for human serum.

decrease to some extent. The decrease was more obvious with addition of TNFR-I or TNFR-I+TNFR-II compared to addition of TNFR-II alone (Figure 4). However, since there was no significant decrease at physiological concentrations of TNFR-I and/or TNFR-II, it was thought that most of the measured hTNF-α was total TNF-α.

Discussion

TNF-α is a pleiotrophic cytokine with many different effects; for example, it triggers the production and secretion of proinflammatory mediators, it is cytotoxic against various tumor cells and induces apoptosis of various cells (1, 3). TNF-α expresses its biological activities by binding to its two receptors (TNFR-I and TNFR-II) (5). Today, it is accepted that TNF-α plays role in the pathogenesis or progression of many diseases (6-9). Therefore, some monoclonal antibodies blocking TNF-α or recombinant TNFR's have been used in the treatment of some malignant and autoimmune diseases (1, 2, 10-14).

Because of all these factors, it is very important to measure the very low amounts of circulating TNF-α very accurately and precisely for elucidation and investigation of many TNF-α-dependent pathological conditions as well as for clinical research and follow up of such conditions. The most widely used TNF-α measurement methods depend on the bioactivity (bioassays) or immunoreactivity (immunoassays) of TNF-α (15-17), and these methods are ELISA, RIA and bioassays (15). The bioassays provide sensitive measurement of bioactive forms of TNF-α; however, they have low reproducibility and specificity but high cost; therefore, they are not suitable for routine use (15, 17). The basic disadvantage of the RIA methods is use of radioisotopes (15, 16). The main non-isotopic immunoassays for measurement of TNF-α are ELISA's which have good reproducibility and high sensitivity (16, 17). The sensitivity of TNF-α obtained by the ELISA method is better with chemiluminescent material compared to colorimetric ones but there can still be need for long incubation times (17).

The WHO study group investigated test validity and reproducibility of TNF-α measurements in 11 different laboratories (18). Serum samples were tested with immunoassays (ELISA and RIA) and bioassays. The variation coefficient among the used methods was evaluated. Very large differences were found in the measured values and sensitivities among tests of both commercial kits and laboratory-developed ones. RIA's gave the highest results in the same samples when compared to ELISA's and bioassays. The researchers concluded that using international standards was very important for correct interpretation of biological and medical data in the case of cytokine tests (18). In the study presented here we investigated the performance characteristics of the hTNF-α ELISA systems developed in our laboratory.

In the first clinical studies, TNF-α was measured by cellular cytotoxicity tests (19), but ELISA was accepted as the conventional method for TNF-α quantitation after 1987 (20). Most frequently two different mAb or polyclonal antibodies against two different epitopes of TNF-α have been used in the ELISA development. Therefore, they are classified as heterologous ELISA's. These ELISA systems potentially recognize both monomeric and oligomeric forms of TNF, but cannot differentiate between biologically active oligomers from inactive monomers (21). The amounts of active oligomers

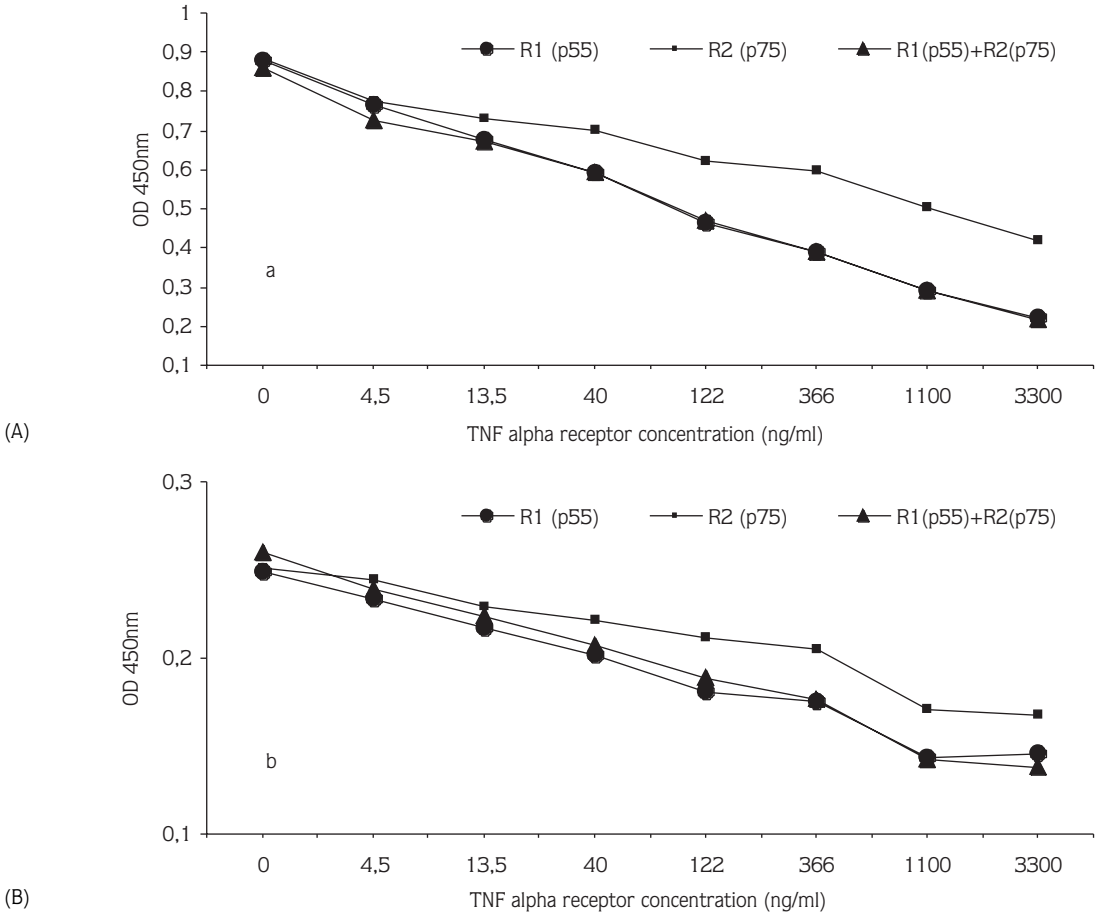

Figure 4. Inhibitory effects of TNF Receptor-I (TNFR-I) and/or TNF Receptor-II (TNFR-II) on the measurement of antigen at 110 pg/ml concentration in 6A4c/biotin-8A6 system (A), and in 6A4c/biotin-polyclonal IgG system (B). It was shown that a prominent inhibitory effect of TNF receptors occurred in both ELISA systems only at supraphysiological concentrations.

in body fluids may be very important. The stabilization of a TNF oligomer with soluble TNFR in vivo can be very important during the progress of the disease. Petyovka et al. (22) used the same mAb both for coating of solid phase and as the conjugate after labeling in the homologous ELISA system they developed to measure TNF. Homologous ELISA systems can only recognize the oligomeric forms of TNF but not monomeric forms. When the previously developed homologous TNF ELISA systems were compared to heterologous ELISA systems, they were not found usually suitable for the clinical studies since they have lower sensitivities (23). Both of the hTNF-α ELISA systems presented in this study (6A4c/biotin-8A6 and 6A4c/biotin-polyclonal IgG) are heterologous ELISA systems using different antibodies for coating (capture antibody) and labeling (conjugate).

The monoclonal antibody-based immunoassays are still a new state of art technique for both clinical and research applications. However, different monoclonal antibodies used in different tests may have differences for antigen recognition. Therefore, Madersbacher et al. (24) concluded that high specificity, cross-reactivity, epitope placement and competitions of monoclonal antibodies and overlap and overhang specificities of monoclonal antibody pairs should be known while preparing sandwich ELISA's. They suggested that narrow measurement spectrum and restricted sensitivity of immunoenzymometric (IEMA) techniques could be corrected in different ways. Addition of 3, 3', 5, 5' tetramethybenzidine (TMB) without affecting the features of the technique would widen the measurement spectrum, while labeling of highly purified monoclonal antibodies with horseradish peroxidase

(HRPO) would increase the sensitivity. Today the most important amplification method known in increasing the sensitivity of ELISA systems is biotin-streptavidin amplification system (25). Kittigul et al. (26) developed a sensitive ELISA for measurement of TNF-α in serum where biotin-streptavidin labeled anti-hen Ig conjugate was used in anti-TNF-α mouse mAb covered plate. Hedeyati et al. (27) used biotin-streptavidin-HRPO amplification systems in the labeling of conjugate to develop a highly sensitive TNF-α ELISA system.

In the study presented here, two different hTNF-α ELISA systems have been developed. Both systems used a non-neutralizing anti-TNF-α mAb 6A4c as capture antibody for coating the plates. The conjugate was anti-TNF-α mAb 8A6 in one system, and polyclonal mouse IgG in the other. The used mAb's were purified using a protein G column and contaminant solutes other than antibodies were removed by passing through Sephadex-G25 column. In order to widen the measurement spectrum, TMB was used as substrate while biotin and Streptavidin-HRPO were added to amplify the system.

Ameloot et al. (28) studied the interaction of murine TNF and the mAb they developed against it and its effects on the ELISA results. They showed that the neutralizing mAb they developed could change the monomeric structure of TNF; however, the reversible dissociation of heterodimeric TNF did not affect the measurement in their ELISA systems (29).

In the hTNF-α ELISA systems we developed, the coating (capture) mAb 6A4c was not a neutralizing antibody, and the use of 8A6 or polyclonal murine IgG as recognition antibody did not significantly change the level of measured hTNF-α. Both recombinant hTNF-α (Figure 1A) and the culture medium of peripheral blood mononuclear cells stimulated with LPS (Figure 1B) gave higher hTNF-α values in the ELISA system with 8A6 mAb conjugate compared to polyclonal murine IgG conjugate (Figure 2A and 2B). Various dilutions of rhTNF-α were measured in the 6A4c/biotin-8A6 and 6A4c/biotin-polyclonal IgG ELISA systems and the analytical sensitivities were found to be less than 4 pg/ml and 12 pg/ml respectively.

The performance of TNF-α immunoassays can be affected by making TNF-α complexes with different plasma elements like circulating TNFR fragments (29) because TNF-α displays its biological activities by binding to its receptors TNFR-I (p55) and TNFR-II (p75). The number of TNFR's in serum is normally higher than the number of TNF-α molecules (30). Hwu et al. (31) tried to produce TNF-α from lymphocytes, and they observed that the amount of measured TNF-α decreased when they mixed with lymphocytes in their ELISA studies. They also showed that the bioactivity of TNF-α in the solution decreased when it was kept for a long time at 37°C although it was very stable in lyophilized form and they decided to add recombinant TNFR's to the ELISA system for more precise measurement of TNF-α in the presence of lymphocytes in a time dependent manner (2, 32).

Ledur et al. (33) measured levels of TNF-α in plasma, synovial fluid and human monocyte cell culture activated by LPS by various commercial ELISA kits and found very high variations in the measured values. They obtained calibration curves for all ELISA kits by using intenationally accepted standards from NIBSC (U.K.) but they could not correct variation coefficients for TNF-α kits. They concluded that the reason for this variation was due to the inability of each kit to detect cytokine levels in the samples in a comparable manner, and they suggested one of the reasons of this result could be binding of cytokine to ligands such as TNFR (33)

In the study presented here, when soluble recombinant TNFR's were added to the ELISA systems, we showed that addition of TNFR-I or TNFR-II at physiological levels, 4-8 ng/ml (13), did not have a significant effect on the system; however, very high levels of TNFR's (especially TNFR-I and TNFR-I+TNFR-II) had inhibitory effects on the measurement (Figure 4). Therefore, it was suggested that the developed hTNF-α ELISA system was not affected by TNFR's present in the serum during hTNF-α measurement in serum and that the measured TNF-α was mainly total TNF-α.

The measurement of TNF-α by the ELISA method has been very widely used in immunology-based research and clinical studies. However, a commercial TNF-α ELISA kit is sufficient only to measure at most 88 samples (except for standards and when the kit works perfectly) at a high cost. It may be necessary to use dozens of ELISA kits to test a scientific hypothesis and to investigate the effects of different factors. Considering the economical status of our country, almost all academic units are urged to work to develop biotechnological products for their scientific studies.

Due to these factors, the need to produce our own hTNF-α ELISA system became important. By the partial support of Tubitak (project number: SBAG-AYD 427), we completed the R&D studies and developed two different ELISA systems for the measurement of human TNF-α. The systems we developed have very high sensitivity, specificity, and good reproducibility with very low cost. We believe that these systems will be very useful as a model for many different research studies concerning antigen-antibody binding, hybridoma technology, antibody purification, in vitro cell cultures, evaluation of immune response in vitro, etc. The newly developed hTNF-α ELISA systems will be used in our department as well as in collaborative studies with other departments and faculties.

Acknowledgement

This work was partly supported by Tubitak with Project number of SBAG-AYD 427.

Corresponding author:

Ayşegül YÜCEL

Gazi University,

Faculty of Medicine,

Department of Immunology,

06510 Beşevler, Ankara - Turkey

E-mail: aatakyucel@yahoo.com

References

1. Bharat B, Aggarwal B, Samanta A et al. TNF-α. The TNF Family. Cytokine Reference, Volume 1, (Eds-in-chief: Oppenheim JJ, Feldman M. Eds: Durum SK, Hirano T, Vilcek J, Nicola NA), Academic Press-A Hartcout Science and Technology Company, London, 2001, pp 413-434.
2. Aggarwal BB, Reddy S. Tumor necrosis factor (TNF). Guidebook to Cytokines and Their Receptors. (Ed. Nicola NA), Sambrook & Tooze Publication-Oxford University Press, Oxfrod, 1994, pp 103-104.
3. Beutler B. Tumor necrosis factors: the molecules and their emerging roles in medicine, Raven Publishing, New York, 1992, pp 1-590.
4. Aybay C, Imir T. Development of a rapid, single-step procedure using protein G affinity chromatography to deplete fetal calf serum of its IgG and to isolate murine IgG1 monoclonal antibodies from supernatants of hybridoma cells. J Immunol Methods 233: 77-81, 2000.
5. Wallach D. TNF Ligand and TNF/NGF Receptor Families. The TNF Family. Cytokine Reference, Volume 1, (Eds-in-chief: Oppenheim JJ, Feldman M. Eds: Durum SK, Hirano T, Vilcek J, Nicola NA), Academic Press-A Hartcout Science and Technology Company, London, 2001, pp 377-412.
6. Aggarwal BB, Natarajan K. Tumor necrosis factors. Developments during last decade. Eur Cytokine Network 7: 93-124, 1996.
7. Tracey KJ, Ceramy A. Tumor necrosis factor, other cytokines and disease. Ann. Rev Cell Biol 9: 317-343, 1993.
8. Gardlung B, Sjolin J, Nilson A et al. Plasma levels of cytokines in primary septic shock in humans: Correlation with disease severity. J Infect Dis 172: 296-301, 1995.
9. Mackay F, Kalled SL. TNF Ligands and receptors in autoimmunity: an update. Curr Opin Immunol. 14: 783-790, 2002.
10. Klausner JD, Freedman VH, Kaplan G. Thalidomide as an anti-TNFa inhibitor: implications for clinical use (review). Clin Immunol Immunopathol 81: 219-223, 1996.
11. Walsh CJ, Sugerman HJ, Mullen PG et al. Monoclonal antibody to tumor necrosis factor alpha attenuates cardiopulmonary dysfunction in porcine gram-negative sepsis. Arch Surg 127: 138-145, 1992.
12. Williams RO, Feldmann M, Maini RN. Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. Proc Natl Acad Sci USA 89: 9784-9788, 1992.
13. Neutrah M, Fuss I, Pasparakis M, et al. Predominant pathogenic role of tumor necrosis factor in experimental colitis in mice. Eur J Immunol 27: 1743-1750, 1997.
14. Baker D, Butler D, Scallon BJ et al. Control of established experimental allergic encephalomyelitis by inhibition of tumor necrosis factor activity within the central nervous system using monoclonal antibodies and TNF receptor-immunoglobulin fusion proteins. Eur J Immunol 24: 2040-2048, 1994.
15. Remick DG, Whiteside TL. Cytokine Assays. Cytokine Reference, Volume 1, (Eds-in-chief: Oppenheim JJ, Feldman M. Eds: Durum SK, Hirano T, Vilcek J, Nicola NA), Academic Press-A Hartcout Science and Technology Company, London, 2001, pp 53-81.
16. Mire-Sluis AR, Gaines-Das R, Thorpe R. Immunoassays for detecting cytokines: What are they really measuring? J Immunol Methods 186: 161-164, 1995.
17. Petrovas C, Daskas SM, Lianidou ES. Determination of TNF-α in serum by a highly sensitive enzyme amplified Lanthanide luminescence immunoassay. Clin Biochem 32: 241-247, 1999.
18. De Kossodo S, Houba V, Grau GE. WHO Collaborative study group. Assaying tumor necrosis factor concentrations in human serum. A WHO international collaborative study. J Immunol Methods 182: 107-114, 1995.

19. Meager A, Leung H, Wooley J. Assays for tumour necrosis factor and related cytokines. J Immunol Methods 116: 1-17, 1989.

20. Meager A, Parti S, Leung H et al. Preparation and characterization of monoclonal antibodies directed against antigenic determinants of recombinant human tumour necrosis factor (rTNF). Hybridoma 6: 305-311, 1987.

21. Corti A, Fassina G, Marcucci F et al. Oligomeric tumour necrosis factor alpha slowly converts into inactive forms at bioactive levels. Biochem J 284: 905-910, 1992.

22. Petyovka N, Laych L, Voitenok NN. Homologous ELISA for detection of oligomeric human TNF: properties of the assay. J Immunol Methods 186: 161-170, 1995.

23. De Groote D, Grau G, Dehart I et al. Stabilization of functional tumor necrosis factor-alpha by its soluble TNF receptors. Eur Cytokine Netw 4: 359-62, 1993.

24. Madersbacher S, Berger P. Antibodies and immunoassays. Methods 21: 41-50, 2000.

25. Diamandis EP, Christopoulos TK. The biotin-(strept)avidin system: principles and applications in biotechnology. Clin Chem, 1991 625-636, 1991.

26. Kittigul L, Temprom W, Sujirarat D et al. Determinantion of tumor necrosis factor-alpha in dengue virus infected patients by sensitive biotin-streptavidin enzyme-linked immunosorbent assay. J Virol Methods 90: 51-57, 2000.

27. Hedeyati M, Yazdanparast R, Azizi F. Determination of human tumor necrosis factor-a by a highly sensitive enzyme immunoassay. Biochem Biophys Res Com. 289: 295-298, 2001.

28. Ameloot P, Fiers W, Burman WA et al. Characterization of the interaction between murine tumor necrosis factor and monoclonal antibodies. Cytokine 18: 158-163, 2002.

29. Thorpe R. Development of immunological standardization. J Immunol Methods 216: 93-101, 1998.

30. Rose-John S, Heinrich PC. Soluble receptors for cytokines and growth factors: Generation and biological functions. Biochem J 300: 281-290, 1994.

31. Hwu P, Schwarz S, Custer M, et al. Use of soluble recombinant TNF receptor to improve detection of TNF secretion in cultures of tumor infiltrating lymphocytes. J Immunol Methods 151: 139-147, 1992.

32. Geigert J, Panschar BM, Taforo C et al. Parameters for the evaluation of long-term stability of tumor necrosis factor preparations. Dev Biol Standard 69: 129-138, 1988.

33. Ledur A, Fitting C, David B et al. Variable estimates of cytokine levels produced by commercial ELISA kits: Results using international cytokine standards. J Immunol Methods, 1995; 182: 171-179.