Novel Method for Detection of Ex Vivo Tumor Necrosis Factor α Production by Monocytes

Kikumi Suzuki,¹ Takatoshi Koyama,² Shizuko Kobayashi,¹ Koji Kobayashi,³ Koji Inagaki,³ Yoshiko Abe,³ Kiyoshi Kuriyama,³ and Kiyoko Shiba^{2*}

¹Department of Molecular Physiology, Kyoritsu College of Pharmacy Tokyo, Japan

²Graduate School of Allied Health Sciences, Tokyo Medical and Dental University, Tokyo, Japan

³Medical Research Laboratory, Sekisui Chemical Co., Ltd., Osaka, Japan

Tumor necrosis factor α (TNF- α) has come into recent focus as a proinflammatory cytokine derived from monocytes/macrophages. We developed a novel system (the SEK-5001 system) for measurement of ex vivo production of TNF- α stimulated by lipopolysaccharide (LPS) as a marker of immune function. In the present study, we evaluated the performance of this system as a diagnostic tool. Furthermore, we compared TNF- α levels with data from other immune function tests, including lymphocyte blast formation test and differential leukocyte counts. Incubation of whole blood with a stimulation of low-dose LPS (100 EU/mL blood) for 4 hr at 37°C gave

acceptable results. After incubation, plasma TNF- α levels were determined by enzyme-linked immunosorbent assay (ELISA). The specificity, reproducibility, and recovery of the SEK-5001 system were excellent. No correlation between TNF- α levels and total leukocyte counts was found. Lymphocyte blast formation test and monocyte counts, however, were correlated with TNF- α levels in blood from patients with hematological malignancy and aplastic anemia before treatment. This assay system may potentially be clinically applicable to assess in vivo immune function. *J. Clin. Lab. Anal.* 16:273–278, 2002. © 2002 Wiley-Liss, Inc.

Key words: TNF- α production; low-dose LPS stimulation; whole-blood culture; monocyte/macrophage; lymphocyte blast formation test

INTRODUCTION

Immune function tests are critical to assess changes in activity of the immune system, and to understand the pathology of certain diseases. Current immune function tests can be classified roughly into two types. The first type detects quantitative changes in immunocompetent cells, such as differential leukocyte counts, T or B cell percentage, and CD4/CD8 subset ratio. The other type, which includes the lymphocyte blast formation test, detects qualitative changes, such as the ability to produce cytokines. Differential leukocyte counts are determined primarily to assess declines in immune function due to medications such as antitumor drugs and steroids. In certain cases, however, decreased immune function is not always reflected as a decrease in leukocyte counts, and an infection has often been supervened by a decline of immune function. Currently, detection of qualitative changes has little clinical use because of inherent time constraints and assay limitations.

We focused on cytokine production by monocytes/macrophages. The monocyte/macrophage is the cell that initiates inflammatory reaction. It is reported that changes in cytokine levels are more sensitive than leukocyte counts for assessing the effects of chemotherapy on leukemia (1,2). TNF- α is one of the most important proinflammatory cytokines. It has been shown to play major roles in the pathogenesis of both systemic diseases, such as sepsis, and diseases with significant, localized tissue destruction, such as rheumatoid arthritis. However, TNF- α has protective effects on hematopoiesis in mammals receiving cycle-specific chemotherapy. TNF- α also possesses antiviral activity and enhances neutrophil

*Correspondence to: Kiyoko Shiba, Department of Moleculo-Genetic Sciences, Graduate School of Allied Health Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. E-mail: k.shiba.mtec@tmd.ac.jp

Received 18 December 2001; Accepted 4 June 2002

Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jcla.10050

function, and thus may be beneficial in controlling infections. Since TNF- α production by monocytes/macrophages may become a marker of changes in immune function caused by medications or diseases in which immune function is associated with the pathology, we developed a TNF- α production measurement kit, called the SEK-5001 system, that measures the ability of TNF- α production by monocytes/macrophages.

Our goal was to be able to measure TNF- α levels accurately and easily without concern for lipopolysaccharide (LPS) contamination when using a closed system with LPS-free containers. We also tried to reduce incubation time from 24 hr to 4 hr with low-dose LPS (physiological stimulus concentration (3)) to make rapid testing possible.

After establishing the kit system, we examined the relations between TNF- α levels and various immune function tests, including the lymphocyte blast formation test, leukocyte counts, and monocyte counts.

MATERIALS AND METHODS

Blood Samples

We obtained blood samples from 85 healthy volunteers (14 men and 71 women, ages 20–39 years) and 11 outpatients (seven men and four women, ages 58–77 years) at the First Department of Internal Medicine, Tokyo Medical and Dental University. The patients all had a form of hematological malignancy before treatment, such as malignant lymphoma ($n=5$), acute myelocytic leukemia ($n=3$), and multiple myeloma ($n=1$), and aplastic anemia ($n=2$). Informed consent was obtained from all patients.

Measurement of TNF- α Levels With the SEK-5001 System

Blood collection

Blood samples (2 mL) were drawn directly into heparinized control tubes (C tube, 15 U/mL final). One mL of blood from each control tube was transferred into an LPS tube (L tube) containing 100 EU LPS per tube. The LPS was isolated from *Escherichia coli* 055B5.

Induction of TNF- α

C tubes and L tubes were incubated for 4 hr at 37°C in a programmable incubator (IN-61; Yamato Science Co., Ltd., Tokyo, Japan) on a rocking platform. Tubes were centrifuged for 10 min at 4°C, 2,600 g. The plasma supernatants were then collected and stored at –40°C until use.

Measurement of Levels of TNF- α After LPS Induction

TNF- α levels in plasma samples were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, a microtiter plate coated with monoclonal mouse anti-human-TNF- α -antibody (anti-TNF- α 1 μ L/mL) was washed five times with Dulbecco's buffer containing 0.05% Tween-20 (washing buffer, pH 7.2). Standard samples (20 μ L) for calibration and plasma samples (20 μ L each) were added to each well of the microtiter plate. Bovine serum albumin (BSA, 5%, 80 μ L) was then added to each well. After the samples were incubated for 2 hr at 37°C and washed three times with washing buffer, 100 μ L of biotin-labeled polyclonal anti-human-TNF- α -antibody was added to each well. After the samples were incubated for 1 hr at 37°C and washed three times with washing buffer, 100 μ L of horseradish peroxidase-conjugated streptavidin was added to each well. After incubation for 15 min at 37°C and three washes with washing buffer, 100 μ L of substrate solution (tetramethylbenzidine, hydrogen peroxide) was added to each well. After incubation for 7 min at 37°C, the reaction was stopped with 1 N sulfuric acid, and the indicator color changed to yellow. The color intensity was determined at 450 nm with a spectrophotometer (Multiskan[®] Bicromatic; Dainippon Pharmaceutical Co., Ltd., Tokyo, Japan). A calibration curve was developed with Micro Plate Manager III software (Bio-Rad Laboratories, Richmond, CA). TNF- α production was calculated with the following equation:

$$(\text{TNF-}\alpha \text{ production}) = (\text{TNF-}\alpha \text{ production in L tube}) - (\text{TNF-}\alpha \text{ production in C tube}).$$

PHA-Induced Lymphocyte Blast Formation Test

Lymphocyte blast formation was examined by the ethidium bromide fluorescence assay (fluorometric blast formation test (Jimro FBT); Jimro Co., Ltd., Gunma, Japan) as described previously (4). [³H]-thymidine was replaced with 7.5 μ g/mL of ethidium bromide (EB). The fluorescence intensity of the EB was determined with a fluorescence detector (E_x 525 nm, E_m 600 nm; Hitachi, Tokyo, Japan). Lymphocyte reactivity stimulated by PHA was expressed as the stimulation index (SI). SI was calculated with the following equation:

$$\text{SI} = (I_2 - I_0 / I_1 - I_0) \times 100$$

where I_0 is the fluorescence intensity of the background, I_1 is the fluorescence intensity of the control, and I_2 is the fluorescence intensity of the PHA group (sample).

Fig. 1. Mean dose response of TNF- α production in whole blood stimulated with LPS. One milliliter of heparinized peripheral blood was stimulated with LPS for 4 hr at 37°C. Mean $\pm$ SD values are shown ($n = 5$).

Fig. 2. Mean LPS-stimulated production of TNF- α over time. One milliliter of heparinized peripheral blood was incubated for the indicated times with 100 EU of LPS at 37°C. Mean $\pm$ SD values are shown ($n = 5$).

Blood Cell Count and Leukocyte Classification

Blood cell counts and differential leukocyte counts were determined on an automatic analyzer (SE-9000; Sysmex, Kobe, Japan).

Influence of Substances

Assessments of the influences of hemoglobin, bilirubin, chyle, and rheumatoid factor (RF) were examined with the reagents Interference Check A Plus and Interference Check RF Plus (Kokusai Shiyaku Co., Ltd., Kobe, Japan). The reagents consisted of five substances: hemolysis hemoglobin (4,800 mg/dL), free bilirubin (181 mg/dL), conjugated bilirubin (216 mg/dL), chyle (24,730 turbidity unit), and RF (550 IU/mL).

Statistical Analyses

All data are expressed as mean $\pm$ SD. Paired t -tests were used for statistical analyses. Linear regression was used to analyze the relation between TNF- α levels and results of lymphocyte blast formation tests, leukocyte counts, and monocyte counts. Correlation was evaluated by Pearson's correlation analysis. A P -value of <0.05 was accepted as statistically significant.

RESULTS

Conditions of TNF- α Production

To examine the effect of various LPS concentrations on TNF- α production, heparinized whole-blood samples were incubated with LPS at 5, 24, 53, 123, 339, 675, 6,112, and 53,936 EU, and TNF- α levels were then measured. LPS stimulated TNF- α production in a dose-dependent manner (Fig. 1). We then used a low dose of

LPS (100 EU = 15.3 ng/mL blood) for experiments on TNF- α production by monocytes under physiological conditions.

Whole-blood samples were incubated with 100 EU LPS at three temperatures (35°C, 37°C, and 39°C) to determine the optimal temperature for TNF- α production. TNF- α production at 35°C (9.77 ± 3.96 ng/mL) was significantly lower than that at 37°C (16.47 ± 7.79 ng/mL, $P = 0.05$). At 39°C, TNF- α production (18.22 ± 6.83 ng/mL) was increased slightly over that at 37°C, although the difference was not statistically significant. Accordingly, we used 37°C as the incubation temperature for further experiments.

We next studied the time course of LPS-stimulated TNF- α production (Fig. 2). One mL of whole blood was incubated with 100 EU LPS at 37°C and followed for 6 hr. TNF- α production increased rapidly between 1 and 4 hr of incubation, and the slope flattened and reached a plateau after 4 hr. We selected 4 hr as the optimal incubation time for a rapid test.

Next, the effects of anticoagulants on TNF- α production were examined. Blood samples were treated with one of three anticoagulants (sodium heparin, 10% citric acid, or K₂EDTA), and LPS-induced TNF- α production (4 hr, 37°C) was analyzed. The mean $\pm$ SD values of samples from five healthy donors were 10.84 ± 3.41 ng/mL for sodium heparin, 4.83 ± 2.06 ng/mL for 10% citric acid, and 1.09 ± 1.25 ng/mL for K₂EDTA. TNF- α production in samples treated with 10% citric acid and K₂EDTA was 44.5% and 10.0%, respectively, compared with that of samples treated with sodium heparin. Therefore, we selected sodium heparin as the anticoagulant.

Ideally, blood should be analyzed immediately after collection, but there may be cases in which samples

Fig. 3. Standard curve for TNF- α ELISA. Standard purified human TNF- α was diluted with human plasma to obtain various concentrations of TNF- α . Mean $\pm$ 2 SD values are shown ($n=5$).

should be stored prior to use. Therefore, we examined the effect of storage on the assay. After collection, blood samples were stored for different time periods (24, 48, 72, and 96 hr) at 4°C. Samples were preincubated for 45 min at 37°C, and then incubated with LPS for 4 hr at 37°C. Controls were incubated immediately after collection for 4 hr at 37°C. In comparison to controls (6.71 ± 1.45 ng/mL), storage for 24 hr (8.48 ± 1.74 ng/mL) and 48 hr (7.80 ± 1.60 ng/mL) at 4°C did not affect TNF- α production. However, TNF- α production by samples stored at 4°C for 72 hr and 96 hr was decreased significantly (5.12 ± 1.54 ng/mL, $P<0.1$; and 4.64 ± 1.43 ng/mL, $P<0.05$, respectively).

Assessment of Performance of the SEK-5001 System

A standard curve for the TNF- α ELISA was generated to determine the lower limit of sensitivity of the assay (Fig. 3). We took the lower limit of detection as the value of TNF- α within the mean -2 SD value at which absorbance was just above 0, and the lower limit of detection for ELISA of the SEK-5001 system was 0.24 ng TNF- α /mL. Specificity was evaluated with control plasma at seven concentrations. The mean specificity was 99.8%, and the average coefficient of variation (CV) was 4.8%.

We then examined the reproducibility of the ELISA measurement of TNF- α production. Three different types of samples were used. Samples comprised of plasma collected at a single time were used to determine “within-run” variations. Samples collected at two different times (1100 and 1400 hr) in a single day were used for analysis of “same-day” variations. “Day-to-

TABLE 1. Recovery tests for ELISA measurements

TNF- α solution	Control plasma (6.76 ng/mL)		
	Theoretical value	Measured value	Recovery (%)
Control	6.08	6.29	—
25 ng/mL	8.58	9.06	106.6
50 ng/mL	11.08	11.40	104.9
100 ng/mL	16.08	13.62	90.7
		Mean	100.7

Control plasma: TNF- α solution = 9:1.

Fig. 4. Dilution linearity test of ELISA measurements. Plasma from five healthy donors was mixed with human pooled plasma at each dilution rate. Mean $\pm$ SD values are shown ($n=5$).

day” variations were examined with blood collected on 3 different days within a 2-week period. The reproducibility of the ELISA measurements was assessed with various concentrations of control plasma. The mean CV for within-run precision was 4.8%. The reproducibility of TNF- α production was obtained using blood from five healthy donors. The mean CVs of the within-run, same-day, and day-to-day precisions were 10.4%, 7.2%, and 13.4%, respectively.

Finally, a recovery test for ELISA measurements was performed. Control plasma (6.76 ng TNF- α /mL) was mixed with different concentrations of TNF- α at a 9:1 ratio, and the mixtures were measured. The recoveries for the three different concentrations were obtained (Table 1), and the mean recovery was 100.7%. A dilution linearity test of ELISA measurements yielded a dilution-dependent straight line for TNF- α (Fig. 4).

The presence of substances such as hemolysis hemoglobin, free bilirubin, conjugated bilirubin, and chyle had no effect on the ELISA assay. However, RF decreased measurements in a dose-dependent manner. An RF of more than 330 IU/mL decreased the measurement value by approximately 30%. Thus, attention must be paid to RF contamination in the ELISA reaction.

TABLE 2. Correlations between TNF- α levels and various immune function tests

Immune function test	Healthy donors	Patients ^a
	(n = 27)	(n = 11)
Blast formation test	$y = 0.0026x + 9.04$ $r = 0.014$	$y = 0.1443x - 33.13$ $r = 0.473$
Leukocyte count	$y = 0.0006x + 5.88$ $r = 0.368$	$y = -0.002x + 21.18$ $r = -0.466$
Monocyte count	$y = 0.0132x + 5.14$ $r = 0.458, p < 0.01$	$y = 0.0221x + 5.52$ $r = 0.610, p < 0.05$

^aPatients had hematological malignancy and aplastic anemia before treatment as described in the Materials and Methods section.

Comparison of TNF- α Levels and Lymphocyte Blast Formation Test Data in Patients and Healthy Donors

To determine whether ex vivo measurement of TNF- α production reflects in vivo immune function, we investigated the relation between TNF- α production and data from the blast formation test (Table 2).

A positive correlation was found between TNF- α levels and blast formation test results in blood from patients with hematological malignancy and aplastic anemia before treatment, but no relation between these items was observed in blood from healthy donors.

Comparison of TNF- α Levels and Differential Leukocyte Counts in Patients and Healthy Donors

No correlation between TNF- α levels and total leukocyte counts was found in blood from patients, whereas a positive correlation was found in blood from healthy donors. In blood from both patients and healthy donors, positive significant correlations were observed between TNF- α levels and monocyte counts (patients: $r = 0.610$, $n = 11$, $P < 0.05$; healthy donors: $r = 0.458$, $n = 27$, $P < 0.01$; Table 2).

DISCUSSION

The present findings enabled us to establish the optimal protocol for assessment of TNF- α production by monocytes. Blood is collected into heparinized tubes and cultured for 4 hr at 37°C with a low dose of LPS (100 EU/mL blood). Previous methods were impractical, because blood culture in microplates is complicated and time-consuming. With our method, blood is collected directly into a vacuum syringe that contains less than 0.1 EU LPS, and the blood is then transferred to another tube for the reaction to induce TNF- α production. Because these processes occur in a closed system, LPS contamination is eliminated, and the time from collection to incubation is reduced. Rapid testing is

possible because the culture time is also shortened. Furthermore, the present method is less sensitive to effects of sample storage than previous methods (5), which may be important in clinical settings.

With respect to performance of the present method, the precision for each measure—specificity, reproducibility, recovery, and dilution linearity—was excellent. The lower limit of the ELISA was 0.24 ng TNF- α /mL, which is adequate for clinical assessments, because the normal level of TNF- α in plasma is ≥ 1.00 ng/mL.

There are two pathways by which LPS stimulates TNF- α production via activated macrophages (6–8): 1) interaction of the LPS-LPS binding protein (LBP) complex with the CD14 receptor on macrophages, and 2) CD14 receptor-independent stimulation. Low doses of LPS act through the CD14 receptor-dependent pathway, which is considered a protective mechanism to increase sensitivity against Gram-negative bacteria. In the present study, LPS induced release of TNF- α in a dose-dependent manner. This release occurred in a stepwise, rather than linear, manner, suggesting that release was mediated via the CD14 receptor pathway until a critical amount of LPS (123 EU/mL blood) triggered activation of the CD14 receptor-independent pathway. At high doses of LPS where CD14 receptor binding was saturated, TNF- α was produced by monocytes/macrophages and neutrophils, mast cells, and lymphocytes. LPS concentrations in blood from patients infected with Gram-negative bacteria range from 5–500 pg/mL (3). In previous studies, TNF- α production was stimulated by doses of LPS in the μ g/mL range (5,9,10). Therefore, our method is a more sensitive test of TNF- α production because low doses of LPS (100 EU = 15.3 ng/mL), which are closer to physiological conditions, yield measurable TNF- α . Additionally, the optimal culture time for previous methods was 24 hr after addition of LPS (11,12), whereas TNF- α production reached a plateau between 4 and 6 hr of incubation and decreased after 24 hr of incubation in the present method (data not shown). It is possible that these differences may be due, at least in part, to differences in blood samples, LPS concentrations, and culture conditions.

We compared TNF- α levels and data from several immune function tests of blood from patients and healthy donors. TNF- α levels in blood from patients with aplastic anemia and acute myelocytic leukemia (AML) were nearly 0, and the levels in blood from other patients were slightly higher than those in healthy donors. The lymphocyte blast formation test assesses immune response, including antigen-presenting function and accessory function, and is represented as an index of lymphocyte proliferation. The normal level is ≥ 290 . Since both the ex vivo TNF- α production test and the

lymphocyte blast formation test detect qualitative changes in the immune system, and since the latter is also associated with production of several cytokines from monocytes/macrophages, we chose to examine the relation of TNF- α levels with the lymphocyte blast formation test. Lymphocyte blast formation levels from healthy donors were mostly >290, and a correlation with TNF- α level was not observed—possibly because many of the levels were around 310. A positive correlation was found in blood from patients; however, this was not statistically significant because of the small number of patients.

When TNF- α levels and total leukocyte counts were compared, a difference between samples from patients and healthy donors was observed. No correlation was found in the patient group, whereas a positive correlation was found in the healthy donor group. On the other hand, significant positive correlations between monocyte counts and TNF- α levels were observed in blood samples from both patients and healthy donors.

In the present study, we have established a novel method to measure TNF- α production by monocytes. Changes in TNF- α levels are suggested to be a more sensitive marker of the cytotoxic effect of chemotherapy than leukocyte counts (1). While determining the clinical significance of ex vivo TNF- α production by monocytes will require further studies, the assay system described in this study is potentially clinically applicable and may provide a new tool with which to assess in vivo immune function.

REFERENCES

1. Denecker NE, Kullberg BJ, Drenth JP, Raemaekers JM, Van der Meer JW. Regulation of the production of pro-inflammatory cytokines and antagonists during chemotherapy-induced neutropenia in patients with haematological malignancies. *Cytokine* 1997;9:702–710.
2. Takamatsu H, Akashi K, Harada M, et al. Cytokine production by peripheral blood monocytes and T cells during haemopoietic recovery after intensive chemotherapy. *Br J Haematol* 1993;83:21–27.
3. Tamura H, Tanaka S, Obayashi T, Yoshida M, Hayashi M, Kawai T. A new sensitive method for determining endotoxin in whole blood. *Clin Chem Acta* 1991;200:35–42.
4. Ishiko O, Hirai K, Nishimura S, et al. Elimination of anemia-including substance by cyclic plasma perfusion of tumor-bearing rabbits. *Clin Cancer Res* 1999;5:2660–2665.
5. Schins RP, Hartingsveldt B, Borm PJ. Ex vivo cytokine release from whole blood. *Exp Toxic Pathol* 1996;48:494–496.
6. Kirikae T, Schade FU, Kirikae F, Rietschel ET, Morrison DC. Isolation of a macrophage-like cell line defective in binding of lipopolysaccharide. *J Immunol* 1993;151:2742–2752.
7. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 1990;249:1431–1433.
8. Corrales I, Weersink AJ, Verhoef J, van Kassel KP. Serum-independent binding of lipopolysaccharide to human monocytes is trypsin sensitive and does not involve CD14. *Immunology* 1993;80:84–89.
9. Wilson BMG, Severn A, Rapson NT, Chana J, Hopkins P. A convenient human whole blood culture system for studying the regulation of tumour necrosis factor release by bacterial lipopolysaccharide. *J Immunol Methods* 1991;139:233–240.
10. De Groote D, Zangerle PF, Gavaert Y, et al. Direct stimulation of cytokines (IL-1 β , TNF- α , IL-6, IL-2, IFN- γ and GM-CSF) in whole blood. I. Comparison with isolated PBMC stimulation. *Cytokine* 1992;4:239–248.
11. De Groote D, Gevaert Y, Lopez M, et al. Novel method for the measurement of cytokine production by a one-stage procedure. *J Immunol Methods* 1993;163:259–267.
12. Yaqoob P, Newsholme EA, Calder PC. Comparison of cytokine production in culture of whole human blood and purified mononuclear cells. *Cytokine* 1999;8:600–605.