Journal of Clinical Laboratory Analysis 23 : 40–44 (2009)

A Rapid Enzyme-Linked Immunosorbent Assay With Two Modes of Detection for Measuring Cytokine Concentration

Keishi Miwa,[1]* Naoko Shibayama,[2] Takeshi Moriguchi,[3] Junko Goto,[3] Masahiko Yanagisawa,[3] Yoshiaki Yamazaki,[1] Giman Jung,[1] and Kenichi Matsuda[3]

[1]Toray Industries, Inc., New Frontiers Research Labs, Kamakura, Kanagawa, Japan
[2]Toray Industries, Inc., Specialty Material Research Labs, Shiga, Japan
[3]Department of Emergency and Critical Care Medicine, University of Yamanashi Hospital, Yamanashi, Japan

Interleukin (IL)-6 and IL-8 were measured in 101 serum samples collected from eight intensive-care unit patients using a polystyrene-based stick enzyme-linked immunosorbent assay (STICKELISA) system. This system consisted of an immobilized-antibody ELISA stick and a noncontact spectrophotometer. Cytokine concentration was detected by two ways: first, rapidly and semi-quantitatively by naked-eye observation of the color change and second, quantitatively using the spectrophotometer for accurate concentration determination. The spectrophotometric assay enabled the quantitation of as little as 100 pg/mL cytokine and took only 45 min to complete. There was a good agreement between the STICKELISA observations and data obtained using a plate ELISA system. The agreement between STICKELISA naked-eye observation and plate ELISA determination was 94 and 85% for IL-6 and IL-8, respectively. The correlation coefficients between the STICKELISA spectrophotometric determination and plate ELISA determination were 0.88 and 0.91 for IL-6 and IL-8, respectively, in a 0.1–5 ng/mL cytokine concentration range. These results demonstrate that the STICKELISA system is a simple, rapid, and quantitative method for bedside cytokine measurement in critical-care settings. J. Clin. Lab. Anal. 23 : 40–44, 2009.

Key words: cytokine; STICKELISA; critical care; rapid detection; colorimetric

INTRODUCTION

High-risk systemic inflammatory response syndrome (SIRS), sepsis, and septic shock pose serious clinical challenges in the critical-care setting (1). Despite the development of intensive-care techniques and an improved understanding of the underlying pathophysiology of these severe inflammatory disorders, mortality rates still remain high in affected patients. Inflammatory cytokines are frequently overproduced in septic patients; in particular, the proinflammatory cytokines interleukin (IL)-6 and IL-8 have been suggested to play a causal role in the pathogenesis of this disease (2–4). Accordingly, a rapid method for measuring these cytokines would enable a quick diagnosis, allowing timely administration of anti-cytokine therapy and improving clinical outcome.

Immunostick systems have previously been used for rapid enzyme-linked immunosorbent assay (ELISA) assays (5); however, the results of these assays were assessed by the naked eye and could not accurately quantify protein concentrations. Rossi et al. (6) reported a quantitative immunostick assay that used soluble substrate, but this system required additional equipment. In this study, we developed a simple and rapid stick-type ELISA (STICKELISA) that easily measured cytokine concentrations by two ways using a single ELISA stick. First, this STICKELISA enables rapid semi-quantitative cytokine detection without additional equipment. Second, the STICKELISA can be used with

*Correspondence to: Keishi Miwa, Senior Researcher, New Frontiers Research Labs, Toray Industries, Inc. 6-10-1, Tebiro, Kamakura, Kanagawa 1248-8555, Japan. E-mail: Keishi_Miwa@nts.toray.co.jp

Received 29 July 2008; Accepted 20 October 2008
DOI 10.1002/jcla.20287
Published online in Wiley InterScience (www.interscience.wiley.com).

a noncontact spectrophotometer to accurately quantitate cytokine levels.

MATERIALS AND METHODS

Patients and Blood Samples

This study was reviewed and approved by the institutional research review boards of both Yamanashi University and Toray Industries, Inc., and was conducted in the Department of Critical Care Medicine of Yamanashi University Hospital. Blood samples were obtained from 30 healthy volunteers in Toray Industries, Inc., as well as from eight patients admitted to the intensive-care unit of Yamanashi University Hospital between October 2006 and September 2007 (Table 1). Written informed consent was obtained from the volunteers and patients before enrolment.

Reagents

Plate ELISA kits for IL-6 and IL-8 were purchased from Kamakura Technoscience Ltd. (Kanagawa, Japan). Anti-IL-6 polyclonal antibodies (goat), HRP-labeled anti-IL-6 monoclonal antibodies, anti-IL-8 polyclonal antibodies (rabbit), and HRP labeled anti-IL-8 monoclonal antibodies were also purchased from Kamakura Technoscience Ltd. These antibodies were identical to the antibodies used in the plate ELISA kits. Standard IL-6 and IL-8 antigens were purchased from BioSource (Invitrogen, Carlsbad, CA).

Stick Preparation

Purified polyclonal anti-IL-6 or anti-IL-8 antibody was diluted in phosphate-buffered saline (PBS, pH 7.2) to a concentration of 2 μg/mL, and a 0.5 mL aliquot was placed in a polypropylene tube. A polystyrene stick was then placed in the tube and incubated for 18 hr at 4°C. The unbound antibody was rinsed off, and the stick was blocked by incubation for 2 hr at 25°C with 1 mL of PBS plus 0.5% bovine serum albumin fraction V (BSA; Serological Proteins Inc., Kankakee, IL). After blocking, the stick was soaked in conservation treatment solution at room temperature for 1 hr. After rinsing with distilled water, the stick with immobilized antibodies was stored at 4°C until use.

STICKELISA Protocol

Serum samples (200 μL) were put in test tubes containing reaction buffer (200 μL). Reaction buffer was PBS (pH 7.2) plus 2 μg/mL horseradish peroxidase-conjugated anti-IL-6 or anti-IL-8 monoclonal antibodies, 0.25% BSA, 0.05% Tween-20, and 10 μg/mL goat IgG. The immobilized-antibody sticks were immersed in this mixture for 30 min at room temperature. The sticks were rinsed with distilled water and then soaked for 15 min at room temperature in substrate solution (1 mL) containing 3,3′,5,5′-tetramethylbenzidine (Promega Corp., Madison, WI). Color development was terminated by washing the stick with distilled water.

Measurements

The concentration of IL-6 or IL-8 was determined by the surface color of the stick using naked eye semi-quantitative (NESQ) observation or by a spectrophotometric quantitative (SCMQ) method using a specially modified computer-controlled noncontact type spectrocolorimeter with a width of 200 mm, depth of 115 mm, and height of 115 mm (JX7-500, Color Techno System Co., Tokyo, Japan). The color readings were compared with standard samples, which were serial dilutions of standard IL-6 or IL-8 in serum from healthy human volunteers. For quantitative measurements, color values were read with the spectrocolorimeter and color differences (ΔE) were calculated using the CIE-L$^*a^*b^*$ uniform color space theory (7), which correlates spectrophotometric values with cytokine concentration. The color difference was calculated as $\Delta E = ((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)^{1/2}$, with ΔL^* representing lightness; a^* representing the color position difference between magenta and green; and b^* representing the color position difference between yellow and blue.

TABLE 1. Clinical Data of Patients Included in this Study

Patient	Gender	Age	Sample number	Background	SIRS/sepsis
1	M	95	5	Cardiopulmonary arrest	Non-SIRS
2	F	80	5	Pneumonia	Non-SIRS
3	M	52	8	Severe acute pancreatitis	SIRS
4	M	57	8	Necrotizing fasciitis	SIRS
5	F	77	16	Necrotizing fasciitis	Septic shock
6	M	64	14	Acute leukemia	SIRS
7	F	58	17	necrotizing fasciitis	Septic shock
8	F	66	28	Severe acute pancreatitis	SIRS

SIRS: systemic inflammatory response syndrome.

RESULTS

Development of the STICKELISA System

We developed the STICKELISA system to rapidly quantify IL-6 and IL-8 concentrations. This system relies on an immobilized-antibody polystyrene stick on which a sandwich ELISA antigen–antibody reaction occurs on the surface. By measuring the surface color development with a spectrophotometer after allowing the stick to react with the cytokine in a serum sample, it was possible to accurately quantify cytokine concentration. Optimal reagent concentrations and incubation times for the STICKELISA were determined using known cytokine standards diluted in heparinized plasma from healthy human volunteers. Four-color parameters were measured: lightness (L^*), the color position difference between magenta and green (a^*), the color position difference between yellow and blue (b^*), and the color difference (ΔE). The relationship between ΔE, L^*, and a^* and the cytokine concentration was consistently linear in the concentration range of 100–5000 pg/mL (Fig. 1), with correlation coefficients of 0.94, 0.93, and 0.93, respectively. A mean absorbance value of +2 standard deviations at a cytokine concentration of 0 pg/mL was obtained from 20 determinations. On the basis of this value, calculated from ΔE as shown in Figure 1A, this assay system was considered reliable for quantitating as little as 54 and 47 pg/mL IL-6 and IL-8, respectively. The coefficient of variation (CV) values were obtained from five determinations using the SCMQ assay with three concentrations of IL-6 and IL-8: 500, 2,500, and 5,000 pg/mL. The CV values for the IL-6 measurements were 16, 8, and 5%, respectively, for the three concentrations; the CV values for IL-8 measurements were 15, 7, and 2%. At concentrations higher than 5,000 pg/mL, color development was saturated and the linearity of the calculation curve disappeared for both cytokines. Cross-reactivity was not observed for IL-1β, IL-4, IL-10, IL-12, TNF-α, and IFN-γ (data not shown).

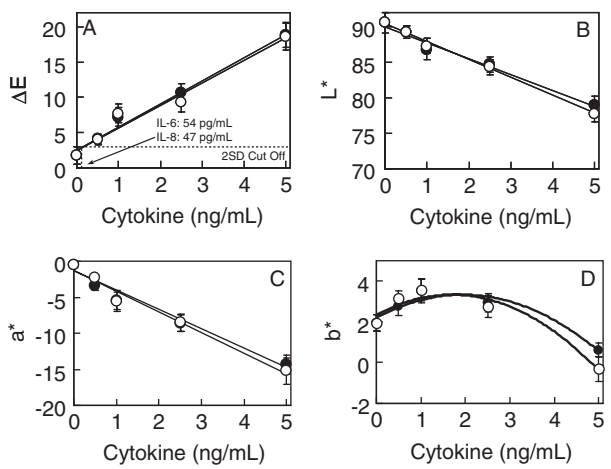

Fig. 1. Standard curves for IL-6 (●) and IL-8 (○) using four parameters. The correlation coefficients between IL-6 concentration and (**A**) the color difference (ΔE), (**B**) lightness (L^*), (**C**) the color position difference between magenta and green (a^*), and (**D**) the color position difference between yellow and blue (b^*) were 0.94, 0.91, 0.92, and 0.79, respectively; the correlation coefficients between IL-8 concentration and ΔE, L^*, a^*, and b^* were 0.94, 0.93, 0.93, and 0.84, respectively. This was within a 100 pg/mL–5 ng/mL cytokine concentration range. Data are expressed as mean ± 2 SD. The lowest concentrations for quantitation as determined from the mean $\Delta E \pm 2$SD were 54 and 47 pg/mL for IL-6 and IL-8, respectively.

Measurement of Cytokine Concentrations in Clinical Samples

With NESQ observation, color development could not be detected for either cytokine when the STICKELISA was performed using plasma from healthy volunteers. Using the SCMQ method to measure 25 samples, we determined that IL-6 and IL-8 concentrations in healthy human plasma were 30 ± 67 and 27 ± 68 pg/mL, i.e., below the quantitation limit.

Plasma IL-6 and IL-8 levels were examined in a total of 101 samples from eight ICU patients (4 males and 4 females). These patients were 52–95 years of age (median age, 66 years) and had primary diagnoses of SIRS ($n = 4$), septic shock ($n = 2$), or non-SIRS ($n = 2$) (Table 1). The NESQ observations classified cytokines in these samples as being in one of three concentration ranges. For IL-6, the concentration ranges were less than 1,000, 1000–5000 pg/mL, and greater than 5,000 pg/mL. For IL-8, the concentration ranges were less than 500, 500–2500 pg/mL, and greater than 2,500 pg/mL. As shown in Figure 2, the overall concordance of serum cytokine concentrations measured by STICKELISA and

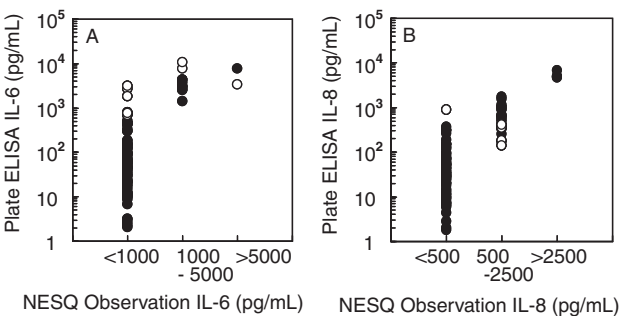

Fig. 2. The correlation between STICKELISA (NESQ; naked-eye observation) measurements and plate ELISA measurements in 101 serum samples collected from 8 patients. The overall concordance of serum cytokine concentrations measured by both methods was 91 and 85% for IL-6 (**A**) and IL-8 (**B**), respectively. The concentration range for the comparison was 1–5 ng/mL for IL-6 and 0.5–2.5 ng/mL for IL-8. A solid circle (●) indicates a sample in which the concentration range matched between STICKELISA and plate ELISA; an open circle (○) indicates samples in which different results were obtained.

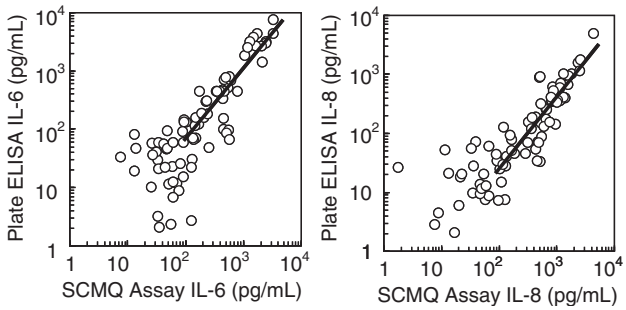

Fig. 3. The correlation between STICKELISA (SCMQ; spectrophotometrical assay) measurements and plate ELISA measurements in 101 serum samples collected from 8 patients. The serum concentration of IL-6 and IL-8 was measured using both STICKELISA and plate ELISA. The slope and correlation coefficients were 1.4 and 0.94, and 0.78 and 0.86, for IL-6 (**A**), and IL-8 (**B**), respectively, using a concentration range of 100–5,000 pg/mL. Each data point is represented as an open circle (○) and the regression curve as a line.

plate ELISA was 91% (92 samples, Fig. 2A ●) and 85% (86 samples, Fig. 2B, ●) for IL-6 and IL-8, respectively.

The same sticks used for NESQ observation were used for spectrophotometric measurements, and cytokine concentrations were calculated from the ΔE values. As shown in Figure 3, the correlation coefficients between the SCMQ and plate ELISA measurements were 0.78 and 0.86 for IL-6 and IL-8, respectively. The slopes of the calibration curves were 1.4 and 0.94, respectively, within the concentration range of 100–5,000 pg/mL.

DISCUSSION

In this study, we report the development of a STICKELISA system to measure cytokine concentration in human serum samples. It is a rapid, simple, and quantitative assay that enables cytokine measurement by two modes of observation and is appropriate for use when either rapid semi-qualitative measurements or accurate measurements of cytokines are needed. This system is based on a stick ELISA with a precipitating substrate; color differences that correlate with cytokine concentration can be assessed by visual inspection or by using a noncontact type spectrocolorimeter. An immunostick assay using a soluble substrate was reported previously. Although that assay is rapid and simple, adsorption cannot be observed with the naked eye and requires a spectrometer.

As shown in Figure 1, the ΔE values calculated from the lightness and color position values correlated with cytokine concentration in a linear manner. Because the ΔE value is calculated from both lightness and color characteristics, and because changes in ΔE reflect the change observed by the naked eye rather than only L^* (lightness) or a^* values (color), we selected ΔE as the key parameter for cytokine quantification. With ΔE, the lower limits of detection of the STICKELISA were 54 and 47 pg/mL for IL-6 and IL-8, respectively, and the linearity of the calibration curve was very good ($R = 0.94$) within the concentration range of 0.1–5 ng/mL. In addition, these immunological reactions were not affected by serum components by several pharmaceuticals administrated in the critical-care setting, or by other cytokines. These findings demonstrate that it is possible to measure cytokine concentration both with the naked eye and with a spectrocolorimeter using a single ELISA stick, and that the STICKELISA is a sensitive and reliable assay for use in a clinical setting.

Clinical sample measurements indicated 94 and 85% agreement between NESQ and plate ELISA for concentrations of IL-6 and IL-8, respectively, and the correlation coefficient between the SCMQ and plate ELISA was 0.88 and 0.91 for concentrations of IL-6 and IL-8, respectively, within a concentration range of 0.1–5 ng/mL. We speculate that the differences in results using the STICKELISA vs. the plate ELISA could be owing to uneven color development on the surface of the sticks; however, the mechanism responsible for uneven color development is not clear.

In conclusion, the STICKELISA system is a simple, rapid, and quantitative ELISA system with a high correlation with conventional plate ELISA and would be a practical tool for use in critical-care settings. Using the STICKELISA system, cytokines or other biochemical factors could be measured quickly in the field or in clinics with minimal equipment, and the stick could be stored until more accurate quantification could be performed.

We are currently developing more convenient and highly sensitive tools using lab-on a-chip ELISA technology. The chip will enable serum separation and 10 pg/mL detection sensitivity.

ACKNOWLEDGMENTS

The authors thank Mr. Masashi Higasa and Dr. Shingo Hiramatsu for the excellent technical support.

REFERENCES

1. Tracy KJ. Physiology and immunology of the cholinergic antiinflammatory pathway. J Clin Invest 2007;117:289–296.
2. Carlet J. Rapid diagnostic method in the detection of sepsis. Infect Dis Clin North Am 1999;13:483–494.
3. Oda S, Hirasawa H, Shiga H, Nakanishi K, Matsuda K, Nakamura M. Sequential measurement of IL-6 blood levels in patients with systemic inflammatory response syndrome (SIRS) sepsis. Cytokine 2005;21:169–175.
4. Kurt AN, Aygun AD, Godekmerdan A, Kurt A, Dogan Y, Yilmaz E. Serum IL-1beta, IL-6, IL-8 and TNF-alpha levels in early diagnosis and management of neonatal sepsis. Mediators Inflamm 2007(ID31397):1–5.

5. Carrera E, García T, Céspedes A, et al. Immunostick colorimetric ELISA assay for the identification of smoked salmon, trout and bream. J Sci Food Agric 1997;74:547–550.
6. Rossi CL, Tsang VC, Pilcher JB. Rapid, low-technology field- and laboratory-applicable enzyme-linked immunosorbent assays for immunodiagnosis of Schistosoma mansoni. J Clin Microbiol 1991;29:1836–1841.
7. CIE Supplement No.2 to Publication (CIE No.15 E-1.3.1). Colorimetry: Uniform Colour Spaces, Colour Difference Equations and Metric Colour Terms. Paris: Central de la CIE, 1978.