A Highly Sensitive Enzyme-amplified Lanthanide Luminescence Immunoassay for Interleukin 6, *Lambros M. Bathrellos, Evriklia S. Lianidou,* and Penelope C. Ioannou* (Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, 15771 Athens, Greece; * author for correspondence: fax 301 7231 608, e-mail lianidou@nestor.dc.uoa.gr)

Interleukin 6 (IL-6) is a cytokine that is produced by a wide variety of cells and has pleiotropic biological functions, such as the induction of acute phase proteins in hepatocytes, the terminal differentiation and growth promotion of B cells, the differentiation and activation of T cells and macrophages, and the regulation of expression of other cytokines *(1)*. IL-6 has been implicated in the pathology of several diseases, and its role in inflammation, viral infection, autoimmunity, and cancer has been reviewed recently *(1, 2)*.

Serum IL-6 increases markedly in many pathological conditions, and its accurate and precise determination in biological fluids is of great importance for the early diagnosis of many diseases *(3–7)*. Measurement of IL-6 in biological fluids is mainly based on bioassays and immunoassays *(8–13)*. The presence of natural compounds known to bind IL-6 in biological fluids *(14–18)* and the effect of preanalytical factors *(19)*, as well as the use of an internationally accepted IL-6 standard preparation *(20–21)*, is of great importance for the performance of IL-6 immunoassays.

Here we describe a highly sensitive enzyme immunoassay for the determination of IL-6 in serum and plasma, based on the enzyme-amplified lanthanide luminescence (EALL) detection approach *(22–25)*. An ultra-low detection limit is obtained by the powerful detection system that is based on the combination of enzymatic amplification introduced by the enzyme alkaline phosphatase (ALP) and the formation of a highly fluorescent terbium complex *(26, 27)*, which is monitored by time-resolved or conventional fluorometry.

A monoclonal antibody (anti-IL-6, CLB IL-6/16) and a biotinylated goat polyclonal antibody against human IL-6 were kindly donated by Prof. L. Aarden (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). The IL-6 stock solution (100 μg/L) was prepared by dissolving the lyophilized human recombinant IL-6 international standard, 88/514 (National Institute of Biological Standards and Controls), in 10 g/L bovine serum albumin, in a 50 mmol/L Tris buffer, pH 7.40, containing 0.5 g/L NaN_3 and 9 g/L NaCl, and was kept in aliquots at −20 °C. IL-6 calibrators at a concentration range of 1–2000 ng/L were prepared by appropriate dilutions of the IL-6 stock solution in assay buffer and in IL-6-free plasma. Coating, wash, and blocking solutions and the substrate buffer of ALP were prepared as described previously *(25)*. Assay buffer was 60 g/L bovine serum albumin in 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 50 mL of goat serum per liter. Developing solution was 0.100 mol/L 3-[cyclohexylamino]-1-propanesulfonic acid, pH 12.60, containing 3 mmol/L Tb^{3+} and 3 mmol/L EDTA. The phosphate ester of diflunisal (DIFP) stock solution (10 mmol/L) was prepared in 0.1 mol/L NaOH and kept at 4 °C, whereas the DIFP working solution (500 μmol/L) was prepared just before use by a 20-fold dilution of the stock solution in ALP substrate buffer *(23)*.

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 (Becton Dickinson) and centrifuged at 4 °C. Serum and plasma samples were stored at −20 °C until assayed. All samples were kindly provided by Dr. E. P. Diamandis (Department of Laboratory Medicine and Pathobiology, University of Toronto, Ontario, Canada).

A typical "sandwich type" immunoassay was used. Assay conditions involving all steps, such as antibody concentrations and incubation times, were optimized in respect to the best signal-to-noise ratios, sensitivity, and rapidity. The final optimized assay protocol was the following: (*a*) immobilization of the anti-IL-6 monoclonal (stock solution, 1 g/L), diluted 400-fold (100 μL/well) in coating solution, overnight, wash; (*b*) blocking with blocking solution (200 μL/well) for 2 h, wash; (*c*) addition of the analyte (100 μL/well) diluted with assay buffer (50 μL/well), wash twice (incubation time of 2 h was found to be optimum when time-resolved fluorescence was used as a detection technique, whereas 3.5 h were required to achieve the same sensitivity with conventional fluorescence); (*d*) addition of the biotinylated anti-IL-6 goat polyclonal antiserum (stock solution, 1 g/L) diluted 3000-fold in assay buffer (100 μL/well), incubation for 30 min, wash four times; (*e*) addition of SA-ALP conjugate (stock solution, 1 g/L), diluted 20 000-fold in assay buffer (100 μL/well), incubation for 15 min, wash four times; and (*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.

Time-resolved fluorescence measurements were performed in the Cyber-Fluor 615 Time Resolved Fluorometer/Immunoanalyzer (λexc = 337.1 nm, λem = 615 nm). Conventional fluorescence measurements were performed in the Perkin-Elmer M 512-A fluorescence spectrophotometer (λexc = 284 nm, λem = 546 nm). In this case, 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 before measurement.

The calibration curve for IL-6 has a reportable assay range up to 2000 ng/L. However, when IL-6 calibrators were diluted in IL-6-free plasma, the calibration curve gave a 30% lower slope than that obtained by dilutions in assay buffer. This could be explained by the presence of various IL-6 binding proteins (sIL-6R, α2-macroglobulin, C-reactive protein) in serum and plasma *(14–18)*, which can compete with the assay antibodies for binding to IL-6.

The accuracy of the assay was tested by performing

analytical recovery and dilution experiments. To assess analytical recovery, three plasma and three serum samples supplemented with recombinant human IL-6 (rhIL-6) at three different concentrations were assayed before and after the addition of rhIL-6. When IL-6 calibrators were diluted in a pool of IL-6-free plasma, satisfactory analytical recoveries were obtained (Table 1). Serum dilution experiments showed that both recombinant and native IL-6 are equally recognized by the assay. When we serially diluted three pathological serum samples with high concentrations of IL-6 and one serum sample with rhIL-6, added to a final concentration of 1000 ng/L with a pool of negative control plasmas, the dilution curves were parallel in all cases.

To determine within-run precision of the assay, three serum samples were assayed in six parallel determinations. To determine between-run precision of the assay, aliquots of three serum samples were stored frozen (−20 °C) and analyzed over a period of 2 weeks in three parallel determinations, each in six separate assays. Within-run CVs were 3% to 9% (n = 6) at 10–100 ng/L, and between-run CVs were 5% to 12% (n = 6 over 2 weeks) at 6–85 ng/L. The precision profile of the method in assay buffer and serum, as measured by time resolved fluorescence, is shown in Fig. 1.

The detection limit, defined as the minimal concentration of IL-6 that produces a fluorescent signal equal to the nonspecific background plus 2 SD, was as low as 0.15 ng/L when rhIL-6 calibrators were diluted in assay buffer (n = 12) and 0.5 ng/L when diluted in human plasma (n = 12).

The specificity of the assay is based on the use of well-characterized and selected antibodies *(10)*. However, when 22 apparently positive sera (IL-6, ~20–10 000 ng/L) were assayed after the addition of mouse and goat serum (5%) to the assay buffer, 12 of them were found to contain markedly lower amounts of IL-6 (1–3 orders of magnitude). Therefore, the addition of mouse and goat serum in the assay buffer was shown to abolish false-positive interferences by rheumatoid factors and heterophilic antibodies, whereas the IL-6 calibration curve or the IL-6-positive sera were not affected. These results are in agreement with those reported previously *(10)*.

Table 1. Analytical recovery of IL-6 added to serum and plasma samples.

	IL-6, ng/L			
Sample	Initially present	Added	Found[a]	Recovery, %
Plasma #1	1.9	10	12.6	107
		50	53.6	103
		100	92.8	90.9
Plasma #2	1.5	10	13.8	123
		50	55.8	109
		100	105	103
Plasma #3	2.4	10	12.8	104
		50	55.0	105
		100	91.7	89
Serum #1	2.0	10	12.7	107
		50	60.4	117
		100	104	102
Serum #2	2.6	10	14	114
		50	57.4	110
		100	94.4	91.8
Serum #3	1.8	10	11.9	101
		50	55.7	108
		100	96.6	94.8
Mean recovery				104 ± 9

[a] Mean value of three replicates per specimen.

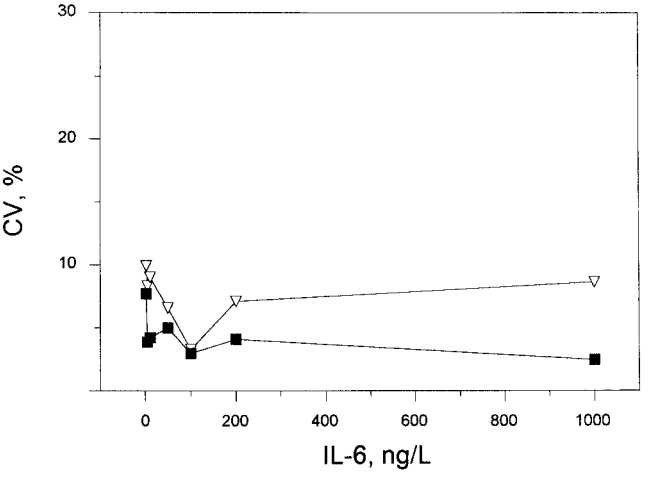

Fig. 1. Precision profile of the IL-6 EALL method (n = 6) in: (■), assay buffer and (▽), serum.

The mean serum concentration of IL-6 in 32 apparently healthy individuals was 3.0 ng/L (SD, 2.7 ng/L), whereas the mean serum concentration of IL-6 in 27 patients with various inflammatory diseases was 72 ng/L (SD, 43 ng/L). We compared the results of our assay with those obtained with a commercially available immunoassay for IL-6, Medgenix Diagnostics ELISA. For 21 serum samples from patients with inflammatory diseases and healthy blood donors analyzed by our EALL immunoassay (x) and by Medgenix Diagnostics ELISA (y), the regression equation was: $y = 1.48x + 3.4$ ng/L, $r = 0.948$, $S_{y|x} = 23.1$. These results indicate that EALL gives lower values for IL-6 than this particular ELISA kit. There is no simple explanation for this, because it has been clearly stated in a recent study *(20)* that even when the same international standards provided by the National Institute of Biological Standards and Controls were used, a wide variation in IL-6 values was obtained for the same samples when analyzed by several commercial ELISA kits.

In conclusion, the EALL immunoassay for IL-6 in serum demonstrates exceptional sensitivity and a much lower detection limit (~0.5 ng/L in serum) in a very short incubation time (~3 h) in comparison with other immunoassays for IL-6 *(8–13)*.

We thank L. Aarden for the generous supply of the IL-6 antibodies and E. P. Diamandis for the use of the Cyber-

Fluor 615 Time Resolved Fluorometer/Immunoanalyzer and for providing the serum and plasma samples used in this study. This work was supported by a research grant from the Secretariat of the Research Committee of the University of Athens.

References

1. Akira S, Taga T, Kishimoto T. Interleukin-6 in biology and medicine. Adv Immunol 1990;54:1–78.
2. Kishimoto T, Akira S, Taga T. Interleukin 6, its receptor. A paradigm for cytokines. Science 1992;258:593–7.
3. Messe J, Eyer D, Donato L, Gallati H, Matis J, Simeoni U. Evaluation of interleukin-6 and soluble receptors of tumor necrosis factor for early diagnosis of neonatal infection. J Pediatr 1996;129:574–80.
4. Dundley DJ, Hunter C, Mitchell MD, Varner MW. Clinical value of amniotic fluid interleukin-6 determinations in the management of preterm labour. Br J Obstet Gynaecol 1994;101:592–7.
5. Stuart RA, Littlewood AJ, Maddison PJ, Hall ND. Elevated serum interleukin-6 levels associated with active disease in systemic connective tissue disorders. Clin Exp Rheumatol 1995;13:17–22.
6. Umeshita K, Monden M, Tono T, Hasuike V, Kanai T, Gotoh M, et al. Determination of the presence of interleukin 6 in bile after orthotopic liver transplantation. Its role in the diagnosis of acute rejection. Ann Surg 1996;223:204–11.
7. Steinmetz HT, Herbertz A, Bertram M, Diehl V. Increase of interleukin 6 serum level preceding fever in granulocytopenia and correlation with death from sepsis. J Infect Dis 1995;171:225–8.
8. Ida N, Sakurai S, Hosaka T, Hosoi K, Kunitomo T, Matsuura Y, Kohase M. An enzyme linked immunosorbent assay for the measurement of human interleukin 6. J Immunol Methods 1990;133:279–84.
9. Teppo AM, Metsarinne K, Fyhrquist F. Radioimmunoassay of interleukin-6 in plasma. Clin Chem 1991;37:1691–5.
10. Helle M, Boeije L, de Groot E, de Vos A, Aarden L. Sensitive ELISA for interleukin-6. Detection of IL-6 in biological fluids: synovial fluids and sera. J Immunol Methods 1991;138:47–56.
11. Peppard JV, Rediske JJ, Koehler JA, Koehne CF. A simple and rapid immunoassay for human interleukin-6. J Immunol Methods 1992;148: 23–8.
12. Ogata A, Tagoh H, Lee T, Kuritani T, Takahara Y, Shimamura T, et al. A new highly sensitive immunoassay for cytokines by dissociation enhanced lanthanide fluoroimmunoassay (DELFIA). J Immunol Methods 1992;148: 15–22.
13. Brailly H, Montero-Julian F, Zuber C, Flavetta S, Grassi J, Houssiau F, van Snick J. Total interleukin-6 in plasma measured by immunoassay. Clin Chem 1994;40:116–23.
14. Yasukawa K, Saito T, Fukunaga T, Sekimori Y, Koishimara Y, Fukui H, et al. Purification and characterization of soluble human IL-6 receptor expressed in CHO cells. J Biochem 1990;108:673–6.
15. Gaillard JP, Bataille R, Brailly H, Zuber C, Yasukawa K, Attal M, et al. Increased and highly stable levels of functional soluble interleukin 6 in sera of patients with monoclonal gammopathy. Eur J Immunol 1993;23:820–4.
16. Montero-Julian FA, Liautard J, Flavetta S, Romagne F, Gaillard JP, Brochier J, et al. Immunoassay for functional soluble interleukin-6 receptor in plasma based on ligand/receptor interactions. J Immunol Methods 1994;169:111–21.
17. Matsuda T, Hirano T, Nagasawa S, Kishimoto T. Identification of α2 macroglobulin as a carrier protein for interleukin 6. J Immunol 1989;142: 148–52.
18. May LT, Vignet H, Kenney JS, Ida N, Allison AS, Sehgal PB. High levels of "complexed" interleukin 6 in human blood. J Biol Chem, 1992;267:19698–704.
19. Thavasu PW, Longhurst S, Joel SP, Slevin ML, Balkwill FR. Measuring cytokine levels in blood. Importance of anticoagulants, processing and storage conditions. J Immunol Methods 1992;153:115–24.
20. Ledur A, Fitting C, David B, Hamberger C, Cavaillon JM. Variable estimates of cytokine levels produced by commercial ELISA kits: results using international cytokine standards. J Immunol Methods 1995;186:171–9.
21. Gaines Das RE, Poole S. The international standard for interleukin-6. Evaluation in an international collaborative study. J Immunol Methods 1993;160:147–53.
22. Evangelista RA, Pollak A, Gudkin Templeton EF. Enzyme amplified lanthanide luminescence for enzyme detection in bioanalytical assays. Anal Biochem 1991;197:213–24.
23. Evangelista RA, Wong HE, Gudkin Templeton EF, Granger T, Allore B, Pollak A. Alkyl and aryl-substituted salicyl phosphates as detection reagents in enzyme amplified fluorescence DNA hybridization assays on solid support. Anal Biochem 1992;203:218–26.
24. Christopoulos TK, Diamandis EP. Enzymatically amplified time resolved fluorescence immunoassay with terbium chelates. Anal Chem 1992;64: 342–6.
25. Lianidou ES, Ioannou PC, Sacharidou E. Second derivative synchronous scanning fluorescence spectrometry as a sensitive detection technique in immunoassays. Application to the determination of a-fetoprotein. Anal Chim Acta 1994;290:159–65.
26. Diamandis EP. Europium and terbium chelators as candidate substrates for enzyme labelled time resolved fluorimetric immunoassays. Analyst 1992; 117:1879–84.
27. Veiopoulou CJ, Lianidou ES, Ioannou PC, Efstathiou CE. Comparative study of fluorescent terbium complexes. Application in enzyme amplified fluorimetric immunoassay for α-fetoprotein. Anal Chim Acta 1996;335:177–84.

Determination of Angiotensin-Converting Enzyme Gene Polymorphisms: Stepdown PCR Increases Detection of Heterozygotes, *Fu-Tien Chiang,[1,2*] Kwan-Lih Hsu,[1] Wei-Ming Chen,[3] Chuen-Den Tseng,[1] Yung-Zu Tseng[1]* ([1] Departments of Internal Medicine and [2] Laboratory Medicine and [3] Graduate Institute of Medical Technology, College of Medicine, National Taiwan University, Taiwan, Republic of China; * address for correspondence: Department of Laboratory Medicine, College of Medicine, National Taiwan University, 7, Chun-Sun South Rd., Taipei 10016, Taiwan, Republic of China; Fax 886-2-23411876)

The angiotensin-converting enzyme (ACE) gene product plays an important role in cardiovascular homeostasis. An insertion/deletion (*I/D*) polymorphism in intron 16 of the *ACE* gene, with insertion polymorphism containing three more Alu-repeat sequences, was reported to be a determining factor of the plasma ACE concentration, and the *D* polymorphism has been found to be associated with certain cardiovascular diseases *(1–5)*. Controversy exists, however, regarding the strength of the association. The diversity of conclusions has been attributed to methodological and technical variations in detection of the polymorphisms *(6, 7)*. The preferential amplification of the *D* allele of the *ACE* gene by the PCR reported by Rigat et al. *(8)* was thought to be one cause. This PCR method occasionally mistyped *ID* heterozygotes as *DD* homozygotes *(9)*. The probability of this mistyping has been estimated to be ~5–10% *(6, 7)*. A confirmatory PCR method, which requires an additional third PCR primer inside the Alu sequence of the *I* allele, was proposed to minimize the mistyping of the *I* allele as a *D* allele *(9)*. Although this PCR technique was reported to be 100% in the typing of *ACE* gene polymorphisms, problems with the preferential amplification of multiplexed PCR are not entirely excluded with this method *(10, 11)*.

A stepdown PCR method, modified from touchdown PCR, has recently been used in several molecular studies *(12)*. This method involves initial PCR annealing temperatures higher than the melting point of the primers, followed by annealing temperatures reduced stepwise to the melting point. This method should result in higher amplification specificity and greater yield. We compared the stepdown PCR with the conventional method from