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A new highly sensitive immunoassay for cytokines by dissociation-enhanced lanthanide fluoroimmunoassay (DELFLIA)

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Non-isotopic immunoassays for human tumor necrosis factor α (TNF α) and human interleukin-6 (IL-6) were established by employing the dissociation-enhanced lanthanide fluoroimmunoassay (DELFLIA) system based on the time-resolved fluoroimmunoassay technique with europium-labeled antibody. Compared to enzyme-linked immunosorbent assays and bioassays, the sensitivity and range of measurement were significantly increased by applying the DELFLIA systems to TNF α and IL-6. TNF α was measurable from 100 fg/ml to 10 ng/ml with the TNF α -DELFLIA and IL-6 was measurable from 100 fg/ml to 1 ng/ml with the IL-6-DELFLIA.

Key words: Dissociation-enhanced lanthanide fluoroimmunoassay; Time-resolved fluoroimmunoassay; Tumor necrosis factor α ; Interleukin-6; Cytokine

Introduction

Many cytokines were originally described as antigen-nonspecific immunoregulatory factors secreted by immunocompetent cells, such as macrophages, T cell and B cells (Yoshizaki, 1981;

Kishimoto, 1984, 1989; Beutler, 1989; Matsuzaki, 1990). Cloning of the genes and studies with recombinant cytokines have revealed that cytokines play important roles not only in immune responses but also in inflammatory phenomena (Horii, 1989; Kern, 1989; Nishimoto, 1989, 1990; Wage, 1989; Yoshizaki, 1989; Ohzato, 1992), and, moreover, that they are produced by different kinds of cells and tissues in addition to the immunocompetent cells (Horii, 1988; Beutler, 1989; Yoshizaki, 1990; Kishimoto, 1989). For a complete understanding of the physiological and pathological roles of cytokines it will be important to measure accurately the cytokine levels in biological specimens.

Recently, the dissociation-enhanced lanthanide fluoroimmunoassay (DELFLIA) was developed by Hemmilä et al. (1984). The DELFLIA

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Abbreviations: DELFLIA, dissociation-enhanced lanthanide fluoroimmunoassay; Eu, europium; TNF α , tumor necrosis factor α ; IL-6, interleukin-6; nhTNF α , natural human TNF α ; rIL-6, recombinant IL-6; MH60 cells, MH60BSF2 cells; Cl-4 cells, SKW6Cl-4 cells; PBMNC, peripheral blood mononuclear cells; LPS, lipopolysaccharide; IFN, interferon; ELISA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay.

system was based on the theory of time-resolved fluoroimmunoassay which uses europium (Eu)-labeled antibody (Soini, 1979; Hemmilä, 1984). The emission wavelength of the free chelating Eu is 615 nm and is not affected by either the exciting wave length (340 nm) because of the long Stokes shift (over 250 nm) or by the wavelength of background natural fluorescence (350 nm–600 nm). Additionally, because of the long fluorescence decay time (over 10^4 s longer than the average background fluorescence), measurement of time-resolved fluorescence should detect the specific fluorescence caused by the reduction in background fluorescence (Lövgren, 1985). Furthermore, as the molecular weight of the labeling Eu chelate is very small (MW 151.96) in comparison with alkaline phosphatase or peroxidase (MW 80,000), which are used in the enzyme-linked immunosorbent assay (ELISA), it only slightly influences the antigen-binding activity of the antibody (Lövgren, 1985). In this study, we have established a highly sensitive DELFIA assay system with wide working ranges for tumor necrosis factor α (TNF α) and interleukin 6 (IL-6). To the best of our knowledge this represents the first application of DELFIA to immunoassays for cytokines.

Materials and methods

Natural human TNF α (nhTNF α) and anti-TNF α antibodies

nhTNF α was purified by phenyl-Sepharose column chromatography, chromatofocusing on PBE-94 and affinity chromatography on the monoclonal antibody-conjugated Sepharose column as described previously (Fukuda, 1988). Purity was checked by column chromatography and SDS-PAGE analysis. A Japanese standard reference for TNF α was recently established. One JRU of TNF α activity is equivalent to 500 pg of nhTNF α (Yamazaki, 1986). The mouse monoclonal anti-TNF α antibodies, 3D6(IgM), mAb-1(IgG), mAb-3(IgG), mAb-4(IgG), mAb-5(IgG), mAb-6(IgG) and mAb-7(IgG) were obtained from the hybridomas which were fused with SP2/0 and the spleen cells of nhTNF α -immunized BALB/c mice (Fukuda, 1988).

Recombinant human IL-6 (rIL-6) and anti-IL6 antibodies

The production and purification of rIL-6 and its specific activity (5×10^6 U/mg) were described in a previous paper (Tonouchi, 1988). The details of the polyclonal goat anti-IL-6 antiserum and the monoclonal murine anti-IL-6 antibodies including 2D5 were also described previously (Shimamura, 1991).

Europium labeling of the antibody

Eu-labeling of the antibody was performed according to Mikkala et al. (1989). Briefly, 680 μ g/500 μ l of monoclonal anti-TNF α antibody (3D6), dialyzed against labeling buffer (0.5 M carbonate buffer, pH 9.6) with a DIAFLO PM-10 membrane (Amicon Corp., Panvers, MA), was mixed with 180 μ g of Eu-labeling reagent (N_1 -(*p*-isothiocyanatobenzyl)-diethylenetriamine N_1, N_2, N_3, N_3 -tetraacetic acid chelated with Eu^{3+} ; Pharmacia, Sweden). The mixture was incubated at 22°C with gentle shaking for 24 h. The Eu-conjugated antibody was separated by gel filtration on a tandem-connected G2000SW column (Tosoh, Japan) equilibrated with Tris-HCl buffer (50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN_3 , pH 7.75), and then identified by calculating the molar amount of bound Eu^{3+} in the immunoglobulin. Labeled $^{27}\text{Eu}^{3+}/3\text{D6}$ and $^{14}\text{Eu}^{3+}/2\text{D5}$ antibodies were obtained and were stored at 4°C in Tris-HCl buffer containing 0.1% BSA and 0.05% NaN_3 . The labeled antibodies were stable and could be used for more than half a year after Eu labeling.

DELFIA for TNF α and IL-6

In the DELFIA for TNF α or IL-6, one of the mouse monoclonal antibodies was adsorbed to the walls of 96 well microstrips (Labosystems, Finland) by the addition of 200 μ l/well of antibody solution in coating buffer (0.1 M NaHCO_3 with the pH adjusted to 9.6 with 1 M Na_2CO_3) at 4°C for 24 h. After removing the antibody solution, non-specific binding was blocked with Tris buffer containing 1% BSA, 1 mM MgCl_2 , 0.15 M NaCl, 0.005% Tween 20, and 0.02% NaN_3 (pH 8.1) at 4°C for 24 h. The blocking solution was removed and the microstrips were washed three times with the washing buffer (DELFIA wash

concentrate, Wallac Oy, Turku, Finland). Then 200 μ l of standard nhTNF α , rIL-6 or appropriately diluted samples in assay buffer (0.1 M Tris-HCl with 0.15 M NaCl, 0.05% NaN₃, 20 μ M diethylenetriaminepentaacetic acid, 0.5% BSA, 0.05% bovine γ -globulin and 0.01% Tween 40, pH 7.75; Pharmacia, Sweden) were added to the wells, followed by incubation at 4°C for 24 h. After three washings of the microstrip with washing buffer, Eu-labeled antibody (20 ng/ml) was added to each well (200 μ l/well) and incubated for 4 h at room temperature. The microstrips were then washed six times with washing buffer, and enhancing solution (0.1 M acetone-potassium hydrogen phthalate, pH 3.2, 15 μ M 2-naphthyltrifluoroacetate, 50 μ M tri(*n*-octyl)-phosphine oxide, 0.1% Triton X-100; Pharmacia, Sweden) was added to the microstrips which were then incubated at room temperature for 15 min with gentle shaking. Fluorescence in each well was measured at 615 nm with an 1230 ARCUS fluorometer (LKB Wallac, Finland).

ELISA for TNF α

In the ELISA for TNF α , the same pair of antibodies were used as in the TNF α -DELFA, except that peroxidase-labeled 3D6 was used instead of Eu-labeled 3D6. Colour was developed in the usual with *o*-phenylenediamine and H₂O₂. The absorbance at 490 nm was measured in a microplate reader (ImmunoReader NJ-2000, Nippon InterMed K.K., Tokyo, Japan).

Bioassay for TNF α and IL-6

TNF α levels in samples were assayed by the cytolytic activity of TNF α against L-M cells as described previously (Yamazaki, 1986). Briefly, after L-M cells in serially diluted nhTNF α solution were incubated at 37°C for 48 h, cytotoxicity was measured by the spectrophotometric absorbance of methylene blue, a vital dye.

IL-6 in the sample solution was measured by proliferation of the murine B cell hybridoma cell line, MH60BSF2 (MH60) and by immunoglobulin production of the human B cell line, SKW6Cl-4 (Cl-4). Details of these bioassays have been described in previous papers (Yoshizaki, 1984; Matsuda, 1988).

Culture supernatants of peripheral blood mononuclear cells (PBMNC)

Culture supernatants of peripheral blood mononuclear cells (PBMNC) separated from venepuncture blood were harvested for the detection of TNF α and IL-6. Various quantities of PBMNC were cultured in 2 ml of medium at 37°C for 24 h with or without 50 μ g/ml of lipopolysaccharide (LPS, Difco, Laboratories, Detroit), or 2×10^5 /ml of PBMNC were cultured with LPS for various time periods.

Results

Establishment of TNF α -DELFA

In order to select the most appropriate immobilized anti-TNF α antibody, the polyclonal rabbit antibody and the six mouse monoclonal antibodies were adsorbed to the wells. Specific binding was determined by the addition of Eu-labeled 3D6 (27 Eu³⁺/IgM protein) with or without 200 μ l of 1 ng/ml of nhTNF α . As shown in Table I, two antibodies, mAb-5 and mAb-7, were conspicuously effective as immobilized antibodies. Next, to determine the amounts of the immobilized and the Eu-labeled antibodies required to establish a

TABLE I
SELECTION OF IMMOBILIZED ANTIBODY ^a

Immobilized antibody	Fluorescence (cps)	
	TNF α (+)	TNF α (-)
Rabbit polyclonal Ab	18,136	16,465
Mouse mAb-1	671	647
Mouse mAb-3	530	478
Mouse mAb-4	1,295	587
Mouse mAb-5	70,013	579
Mouse mAb-6	600	536
Mouse mAb-7	74,234	648

^a Microstrips were coated with monoclonal anti-TNF α antibody at a concentration of 1 μ g/ml and blocked in 1% bovine serum albumin. 200 μ l of TNF α (1 ng/ml) were added to the wells. Following the incubation, 20 ng/ml of europium labeled anti-TNF α antibody (3D6) were added to each well. After incubation at room temperature, the microstrips were washed and enhancing solution was added to microstrips. After 15 min incubation at room temperature, fluorescence was measured in each well using the 1230 ARCUS fluorimeter.

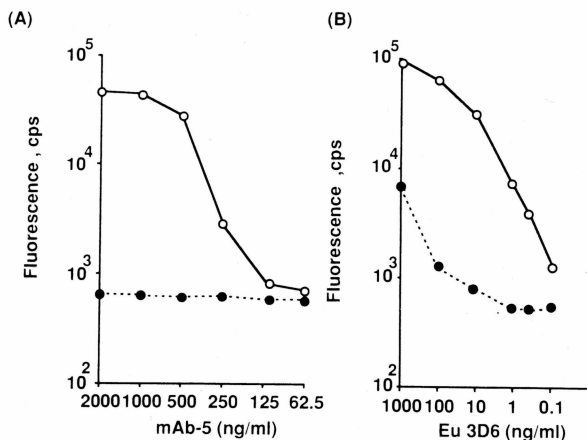


Fig. 1. Determination of the dosage of both immobilized antibody (A) and Eu-labeled antibody (B) for use in the TNF α -DELFI assay. A: serially diluted anti-TNF α antibody, mAb-5, was coated following the addition of 1 ng/ml nhTNF α (○—○) or medium (●—●). The second antibody, Eu-3D6, was used at 50 ng/ml. B: 1 μ g/ml of mAb-5 as the immobilized antibody and 1 ng/ml of nhTNF α (○—○) or medium (●—●) were used. Serially diluted Eu-3D6 was then added to each well. Fluorescence (cps) was measured with a 1230 ARCUS fluorimeter after the addition of enhancing solution.

sensitive TNF α -DELFI assay, two kinds of dose-dependent curves were obtained. Fig. 1A shows the dose-dependent curve for the coating antibody, mAb-5, and Fig. 1B shows that for the Eu-labeled antibody, Eu-3D6. Ultimately, 1 μ g/ml of mAb-5 and 10 ng/ml of Eu-3D6 were identified as optimum concentration. The specificity of this TNF α -DELFI was confirmed by measuring other cytokines, such as IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-8 and IFN- γ (Fig.

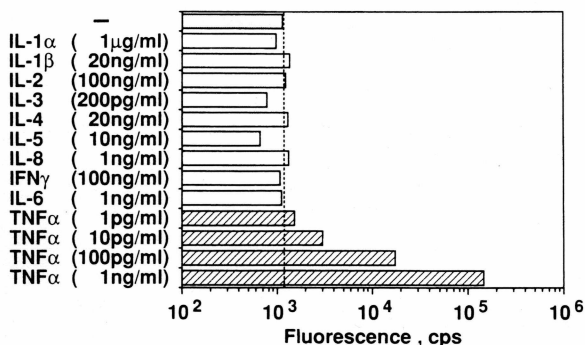


Fig. 2. Specificity of the TNF α -DELFI assay. Nine cytokines as well as nhTNF α were assessed by the TNF α -DELFI assay. None of the cytokines except TNF α , resulted in elevation of fluorescence.

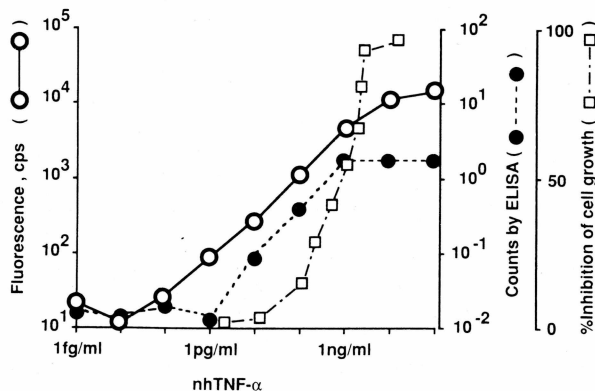


Fig. 3. Comparison of the TNF α -DELFI assay with an ELISA and a bioassay. nhTNF α diluted serially was assessed by the three assay systems in order to produce a standard curve. TNF α -DELFI (○—○) and ELISA (●—●) were used with the same set of antibodies. The bioassay consisted of a cytotoxicity assay utilizing L-M cells (□—□).

2) instead of TNF α . None of these cytokines affected this assay system.

Comparison of TNF α -DELFI with ELISA and bioassay

To compare the sensitivity and range of measurement of the TNF α -DELFI with those of the ELISA and bioassay, nhTNF α was assessed by ELISA utilizing the same pair of antibodies as used for the TNF α -DELFI and the L-M cytotoxicity assay. As shown in Fig. 3, nhTNF α could be detected and measured from 100 fg/ml to 10 ng/ml in one measuring experiment with TNF α -DELFI. In comparison, the measuring range was 10 pg/ml to 1 ng/ml for ELISA and 100 pg/ml to 2 ng/ml for the bioassay. These data suggest that the TNF α -DELFI is a highly sensitive TNF α immunoassay with a wide working range.

Establishment of IL-6-DELFI

The immobilized antibody and Eu-labeled antibody for the establishment of IL-6-DELFI were selected by the same method as that used for TNF α -DELFI. Ultimately, 10⁵-fold diluted goat anti-IL-6 antiserum was chosen as the immobilized antibody, as well as 20 ng/ml of the Eu-labeled mouse anti-IL-6 antibody, Eu-2D5 (14 Eu³⁺/IgG protein). The specificity of the IL-6-DELFI was checked with other cytokines, IL-

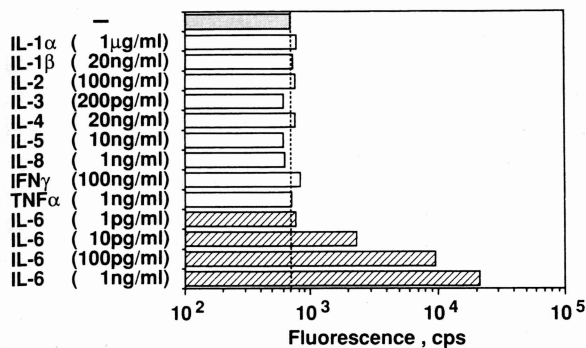


Fig. 4. Specificity of the IL-6-DELFA. Nine other cytokines as well as IL-6 were assessed by the IL-6-DELFA. No significant elevation of fluorescence (cps) was observed with any of the nine cytokines.

1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-8, IFN- γ , and TNF α (Fig. 4). None of these cytokines showed any cross-reactivity in this assay.

Comparison of IL-6-DELFA with bioassays

To compare the sensitivity and the range of detection of the IL-6-DELFA with two kinds of bioassay, rIL-6 was measured by the production of immunoglobulin (IgM) from Cl-4 cells and by the proliferation of MH60 cells. As shown in Fig. 5, the IL-6-DELFA was able to measure rIL-6 from 100 fg/ml to 1 ng/ml. On the other hand, the range of the proliferation assay with MH60 cells was from 100 fg/ml to 10 pg/ml, and that of the production assay with Cl-4 cells was from 10

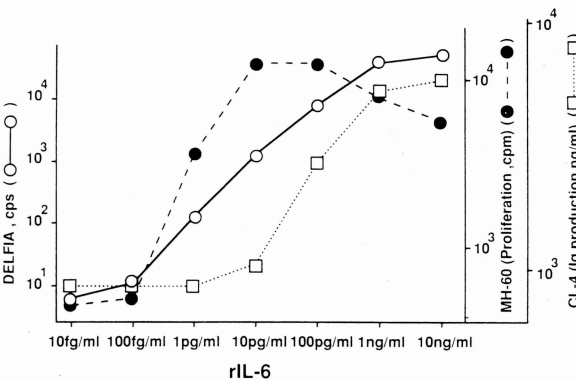


Fig. 5. Comparison of the IL-6-DELFA with two bioassays. Serially diluted rIL-6 was assessed by the IL-6-DELFA (O—O) and compared with the two bioassays, which were based on the proliferation of MH60 cells (●---●) and on the induction of immunoglobulin by Cl-4 cells (□····□).

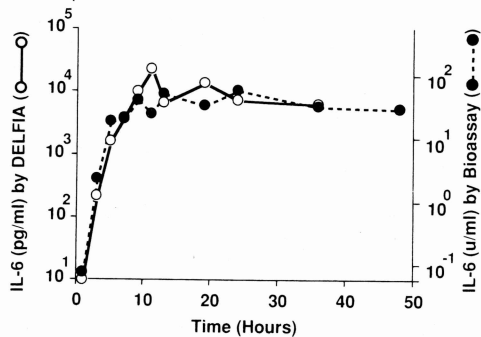


Fig. 6. Kinetics of the amounts of IL-6 in the culture supernatants of LPS-stimulated PBMC. The amounts of IL-6 were detected by both IL-6-DELFA (O—O) and the bioassay using MH60 cells (●---●). The production of the culture supernatants is described in the materials and methods section.

pg/ml to 1 ng/ml of rIL-6. These studies also indicated that the IL-6-DELFA system could measure levels of IL-6 as low as those measured by the proliferation system and over a wider range than those obtained with the bioassays.

Correlations of the amounts of IL-6 detected by IL-6-DELFA with those detected by bioassay

In order to evaluate the reliability and utility of the IL-6-DELFA IL-6 in each culture supernatant of PBMC was measured by both the IL-6-DELFA and proliferation assays. Fig. 6 shows the kinetics of the amounts and the activity of IL-6 in the culture supernatants as assessed by

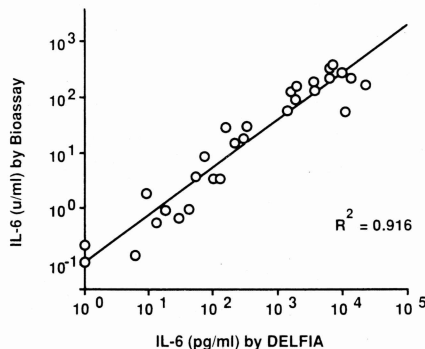


Fig. 7. Correlation of the amounts of IL-6 detected by the IL-6-DELFA with those detected by the bioassay. The amounts of IL-6 in each culture supernatant were measured by both IL-6-DELFA and the bioassay utilizing the proliferation of MH60 cells. One unit of IL-6 activity was calculated as 200 pg of rIL-6.

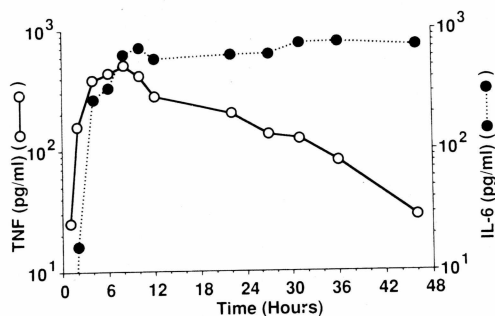


Fig. 8. Kinetics of the amounts of TNF α and IL-6 in the culture supernatants of LPS-stimulated PBMNC. TNF α and IL-6 were assessed by TNF α -DELFI (○—○) and IL-6-DELFI (●—●) in each culture supernatant. The culture supernatants were obtained by the same methods as those in Fig. 7. The kinetic curves obtained were based on single measurements with the DELFI assay.

IL-6-DELFI and the bioassay. The correlation of IL-6 detected by both assay systems is shown in Fig. 7. The R^2 value for the correlation was 0.916 ($P < 0.0001$).

TNF α and IL-6 in culture supernatants

The levels of TNF α and IL-6 in the culture supernatants of PBMNC were assayed by TNF α -DELFI and IL-6-DELFI, respectively. The kinetic curves shown in Fig. 8 suggest that the DELFI system could be used to assay a wide range of cytokines.

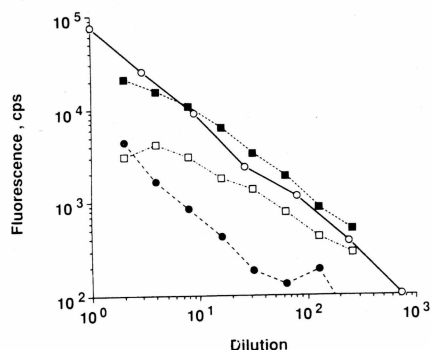


Fig. 9. TNF α in serially diluted serum detected by the TNF α -DELFI. The three serum samples #1(■---■), #2(□---□), and #3(●---●) were diluted from 3^1 - to 3^8 -fold and TNF α in each diluted sample was assessed by the TNF α -DELFI. The standard curve (○—○) was obtained by serially diluting nhTNF α (1 ng/ml).

TNF α in serum

In order to investigate whether DELFI could be used for the measurement of TNF α in serum, three serum samples were serially diluted, and the fluorescence in each diluted sample was detected at 615 nm with an 1230 ARCUS fluorometer. As shown in Fig. 9, the detected fluorescence curves of the sera were parallel with that of a serially diluted preparation of nhTNF α . These findings suggest that the TNF α -DELFI is able to measure TNF α in serum.

Discussion

Although commonly used to measure cytokines, ELISA, RIA and bioassay, all have, certain problems. For example, ELISA is not as sensitive as RIA, while radioisotopes are necessary for the RIA system. Furthermore, the specificity, absolute amounts and reproducibility must always be considered in the case of a bioassay. For this reason, there has been a need for a sensitive, specific and nonisotopic assay system, e.g. enzyme-, chemiluminescence- or fluorescence-based immunoassays. The problem has been overcome by the use of time-resolved fluorescence and labels with a long fluorescence decay time. The DELFI system was developed to provide sensitive detection with time-resolved fluorimetry of europium, one of the lanthanides, dissolved in water (Soini, 1979). Subsequently time-resolved immunofluorometric assays were described for the measurement of two peptide hormones, thyroid stimulating hormone and human chorionic gonadotropin (Lövgren, 1985). The results obtained suggested that very sensitive assays could be established when antibodies were labeled with europium.

In this study we have developed a highly sensitive and wide-range immunoassay for measuring both TNF α and IL-6 with the DELFI system. The sensitivity of these assays was 100 fg/ml and the linear range was more than 10^4 -fold.

We have also established the value of the DELFI system for the detection of the cytokines in serum. However the existence of soluble forms of cytokine receptors, antibodies to cytokines and unknown factors may be a source

of problems with such immunoassay. For example, the IL-6-DELFIA might be affected by unknown serum factors, which might contain the soluble form of the IL-6 receptor and associated gp130 molecules, antibodies to IL-6, α_2 -macroglobulin or other factors. We are currently investigating these possibilities.

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