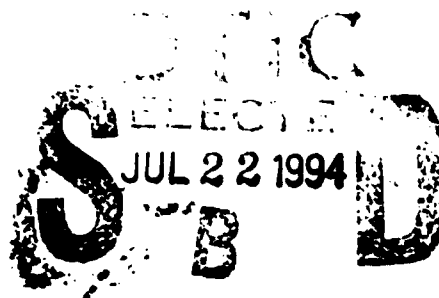


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**A SENSITIVE, SPECIFIC IMMUNOBIOASSAY FOR QUANTITATION
OF HUMAN INTERLEUKIN 6**

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ABSTRACT

Picogram quantities of human interleukin 6 (h-IL-6) were detected by a two-step method. A microtiter plate coated with anti-h IL-6 monoclonal antibody was used to capture the IL-6 present in biological samples. An IL-6- dependent B cell line (7TD1) that proliferates in response to IL-6 was added to the captured IL-6. The lower limit of detection for this immunobioassay with serum or cell culture supernatants was 5 pg/ml. The specificity of the assay was achieved by the antibody used in the first step. The sensitivity was provided by the IL-6-dependent cell line. The method also allows for the removal of inhibitors, metabolites, antagonists or activating agents used to induce IL-6. This immunobioassay has the advantage over other current methods in that it measures immunoactive as well as biologically active IL-6.

(KEY WORDS: immunobioassay, human interleukin 6, IL-6)

INTRODUCTION

Interleukin-6 (IL-6) is a member of a class of pleiotropic cytokines involved in regulating hematopoiesis and immune responses. It can stimulate growth and differentiation of T

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lymphocytes, stimulate the maturation of B lymphocytes and B-cell-derived hybridoma cell lines to produce immunoglobulin, stimulate hematopoietic cells, and induce the synthesis of various acute-phase proteins by hepatocytes (1). Because of these diverse biological activities, IL-6 has been proposed as a mediator in infectious diseases (2, 3, 4) and various inflammatory diseases (5), including autoimmune disorders (6). Because multiple mediators, both stimulatory as well as inhibitory, are often present in biological fluids and culture supernatants, the development of a specific and sensitive method to detect IL-6 may provide insights in the pathogenesis of various diseases.

This report describes a specific and sensitive measure of IL-6 activity (using commonly available reagents) by combining the human IL-6 capture step, as in an ELISA, with the cell proliferation of a IL-6-dependent cell line. We describe the use of this assay with cell culture supernatants and serum samples from patients with Korean hemorrhagic fever (KHF). We compare this method to an ELISA that used monoclonal and polyclonal antibodies to IL-6.

MATERIALS AND METHODS

Materials

Human recombinant IL-6 (sp. act. 1×10^6 U/ μ g), monoclonal antibody to human IL-6 (0.1 mg/ml), and goat anti-human IL-6 (1 mg/ml) were purchased from Collaborative Research Inc. (Bedford, MA). Peroxidase-conjugated, swine anti-goat antibody, and

3,3',5,5'-tetramethylbenzidine (TMB) substrate were purchased from Boehringer Mannheim (Indianapolis, IN) and Kirkegaard and Perry (Gaithersburg, MD), respectively. Unless specified, all other reagents were from Sigma (St. Louis, MO).

Preparation of Samples with IL-6 Activity

Human peripheral blood mononuclear cells (PBMC) were isolated by ficoll-hypaque density gradient centrifugation of heparinized blood from normal donors. PBMC were plated at 1×10^7 cells per well in 24-well plates in RPMI 1640 tissue culture medium containing 10% heat-inactivated fetal bovine serum (FBS) and allowed to adhere for 2 h at 37°C. Non-adherent cells were removed by four vigorous washes of the cultures. Adherent PBMC were stimulated with 2 $\mu\text{g}/\text{ml}$ of lipopolysaccharide (LPS) (Escherichia coli 055:B5, Difco, Detroit, MI) for 24 h.

Frozen, lyophilized serum samples obtained from patients diagnosed with hemorrhagic fever during the Korean conflict were rehydrated with sterile water to their original volume. Samples were tested for IL-6 activity both in the ELISA, bioassay and the immunobioassay.

ELISA for Measuring Levels of Human IL-6

Levels of IL-6 were determined by an ELISA using monoclonal antibody (mAb) to h IL-6 as the capture antibody. Nunc plates 96-well (C-96 Maxisorp) were coated with 0.07 $\mu\text{g}/\text{well}$ of mAb at 4°C for 18 h. Plates were washed three times with wash buffer

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(PBS containing 0.05% Tween 20). Blocking buffer, 100 μ l/well of 1% bovine serum albumin in PBS was added and the plates were incubated for 1 h at 37°C. These plates can be stored semi-dry, after removal of blocking buffer, in sealed plastic bags for up to 6 months.

Recombinant human IL-6 (rh IL-6) was diluted to the indicated concentration in 10% FBS and used as calibration standards. Tested samples or standards (100 μ l) were added to antibody-coated plates in duplicate and incubated for 2 h at 37°C. Plates were washed three times with wash buffer. Polyclonal, goat anti-h IL-6 antibody (100 μ l/well of 4.3 μ g/ml) was added and plates were incubated at 37°C for 105 min. Plates were washed four times with wash buffer. Peroxidase-conjugated anti-goat IgG at 1:2,000 dilution (100 μ l per well) was added and plates were incubated at 37°C for 20 min. Plates were washed four times with wash buffer. After the addition of TMB and hydrogen peroxide as substrates (prepared according to manufacturer's directions), bound enzyme was measured at 450 nm (MR600 Microplate Reader, Dynatech, Alexandria, VA).

Bioassay of Human IL-6

The murine IL-6-dependent hybridoma cell line, 7TD1, was obtained from ATCC (Rockville, MD). The passage and culture conditions of this cell line were as described previously (4). For bioassay, serial twofold dilutions of samples or standards were plated with 1×10^4 cells per well in 96-well Costar plates with RPMI supplemented with 10% FBS, 2mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 50 μ M of 2-

mercaptoethanol (bioassay medium). After 3 days incubation at 37°C in a 5% CO₂ incubator, plates were pulsed with 1 µCi of ³H-thymidine per well (³H-TdR) (specific activity = 2 Ci/mmol, Amersham, Arlington Heights, IL) for the final 5 h of incubation. Incorporated radioactivity was collected on filter paper and counted (LKB scintillation counter). Dose-response curves were analyzed by a method previously described for IL-6 (7). Briefly, the following two equations were used stepwise to generate the half-maximal response.

$$N/L = s \times (M - N) \quad \text{and}$$

$$\ln (T/1-T) = a \times \ln (L) + \ln (k)$$

where N = the observed response in cpm, M = maximal response (cpm) attainable in the assay, L = concentration of added IL-6, s = proportionality constant, T = N/M, a, k = coefficients estimated by regression.

Immunobioassay for Human IL-6

All solutions used were sterilized by filtration and all procedures were carried out under sterile conditions. Costar plates 96-well were coated with monoclonal antibody and blocked as described above for ELISA, except PBS was the wash buffer. Samples or standards (100 µl) were applied to wells in triplicate and incubated at 37°C for 2 h. Plates were washed three times with 10% FBS in RPMI. Washed 7TD1 cells (1 x 10⁴) in bioassay medium were added to each well. After 3 days, cultures were pulsed with 1 µCi of ³H-TdR per well for the final 5 h of incubation. Proliferation data were analyzed in the same manner as for the bioassay described above.

Other cytokines interleukin 1 (IL-1), tumor necrosis factor α (TNF α) and gamma interferon (IFN γ), when tested at 100 ng/ml, did not cross-react in either the ELISA or immunobioassay.

RESULTS AND DISCUSSION

ELISA for Human IL-6

Recombinant hIL-6 standards were prepared to determine the sensitivity of a sandwich ELISA. Figure 1 represents a typical standard curve with a linear range of 20 to 1,000 pg/ml. Adding human serum (tested up to 25%) did not alter the sensitivity of this method. Various cytokines (IL-1 α , IL-1 β , TNF α , IFN γ) at 1,000-fold higher concentrations were negative in this assay, which demonstrated the specificity of this ELISA. The practical lower detection limit of this method was 50 pg/ml of hIL-6.

We used this method to investigate whether IL-6 could be detected in serum samples from patients with KHF. This was done because the clinical features of the disease involve fever, shock, and renal dysfunction; thus, IL-6 may be involved in the pathogenesis of this disease. Thirty-one percent of 98 serum samples from KHF patients had >50 pg/ml of IL-6 by ELISA (8).

Two samples that were positive for IL-6 in the ELISA were examined to determine the correlation between immunoreactive IL-6 and biologically active IL-6. The biological activity of KHF sample 1 correlated well with the immunoreactivity determined by the ELISA (Figure 2); however, KHF sample 2 was negative in the bioassay (Figure 2, middle panel).

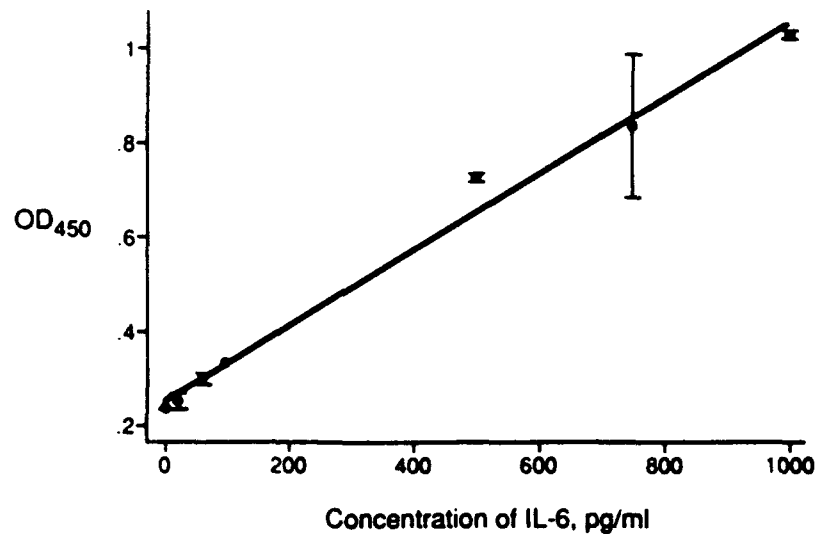


FIGURE 1. Calibration curve of a standard preparation of human IL-6 by ELISA.

Immunobioassay for Human IL-6

We devised a method combining the ELISA and the bioassay to measure the biological activity of IL-6. The result of this method using a standard preparation of rhIL-6 is presented in Figure 3. This method was more sensitive: the lower detection limit was 5 pg/ml. Because of the saturating effect at higher concentrations of IL-6, we used an validated method of determining the maximum of the bioassay for each sample. This method had been applied to quantitate the biological activity of IL-1 and IL-6 (7,9). The dilution yielding half-maximal responses were accurately computed and compared with standard hIL-6. Both KHF sample 1 and sample 2 had similar IL-6 concentrations in the immunobioassay and the ELISA (Figure 2: top and bottom panel).

To illustrate an additional feature of this immunobioassay, we further assayed the IL-6 produced by LPS-stimulated PBMC by

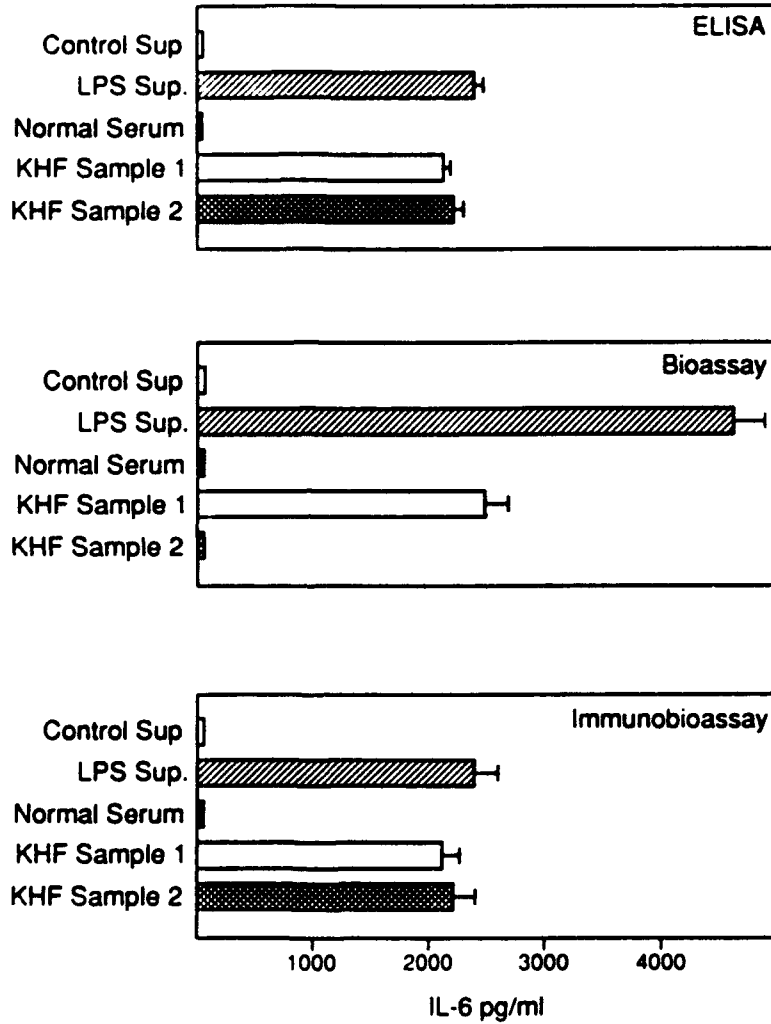


FIGURE 2. Bar graph representing the IL-6 levels determined by the three methods: ELISA, bioassay, and immunobioassay of five illustrative samples: unstimulated PBMC culture supernatant, LPS-stimulated PBMC supernatant, normal serum control, KHF sample 1, and KHF sample 2.

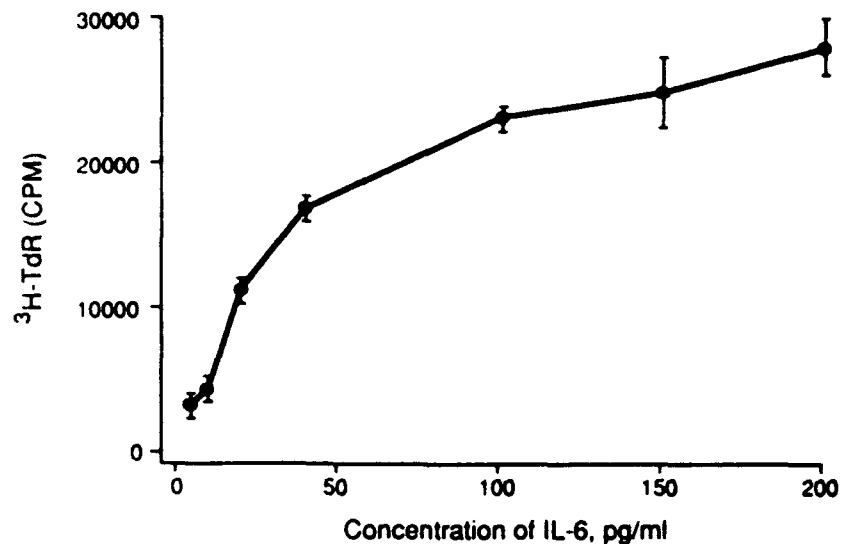


FIGURE 3. Calibration curve of a standard preparation of human IL-6 by immunobioassay.

three different methods: ELISA, bioassay, and immunobioassay. Because LPS by itself stimulates the proliferation of 7TDI cells (unpublished observation), and remains present in the supernatants, the bioassay yielded spuriously high estimates of the concentration of IL-6 in LPS-stimulated PBMC supernatants, 3,940 pg/ml vs 2,070 pg/ml by ELISA. However, the immunobioassay gave a concentration of IL-6 similar to that of ELISA (2,150 pg/ml vs 2,070 pg/ml). The capture of IL-6 in the first step of this method effectively removed the LPS present in the supernatant; consequently the subsequent bioassay only detected the IL-6 present. Because the immunobioassay measurements agreed with the ELISA method, the different IL-6 levels determined by the ELISA and bioassay of KHF sample 2 may have been due to the presence of inhibitory substances present in serum. The levels of IL-6 measured by ELISA and immunobioassay were similar, which indicate the presence of biologically active IL-6 in these

samples. However, because of the high sensitivity of the immunobioassay (5 pg/ml), only a few microliters of serum are needed for this assay.

The two common methods currently used to detect of IL-6 in biological samples are ELISA or bioassays. ELISA plates are commercially available, with a minimum sensitivity of 20 to 50 pg/ml. However, they are prohibitively expensive. Bioassays of the various biological activities of IL-6 are more sensitive; the cell proliferation assay with hybridoma cell lines was the easiest to perform. However, bioassays are subject to interfering substances such as stimulants, metabolites, inhibitors, or other cytokines present in biological samples. In this paper, we combined ELISA with a bioassay to devise a method with high sensitivity and specificity for the detection of biologically active IL-6. Recently, a similar immunobioassay was also devised for measuring human interleukin 2 (10).

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