Total Interleukin-6 in Plasma Measured by Immunoassay

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Determinations of total cytokine concentration in biological fluids by immunoassays face two major problems: the biochemical heterogeneity of the analyte and the interference of cytokine-binding proteins. We developed an ultrasensitive enzyme immunoassay for interleukin-6 (IL-6), using monoclonal antibodies and acetylcholinesterase as the tracer enzyme. The antibodies recognized recombinant and glycosylated forms of IL-6 equally. The antibodies measured dimeric recombinant IL-6, yet we could not detect IL-6 oligomers in plasma samples. We investigated the potential interference of soluble IL-6 receptor (siL-6R), which is present at high concentrations in plasma samples (1 to 2 nmoL/L). Heat treatment of the sample obviated the siL-6R interference. Using calibrators in a plasma matrix, we demonstrated by fractionation, dilution, and recovery experiments that the immunoassay accurately measured total IL-6 in both normal and pathological serum and plasma samples.

Indexing Terms: enzyme immunoassay/acetylcholinesterase/receptors/monoclonal antibodies/cytokines

Circulating interleukin-6 (IL-6) can be detected in plasma samples in various pathological situations (reviewed in 1). High concentrations of IL-6 are detected early in the course of the acute-phase response, before the induction of acute-phase proteins such as C-reactive protein (2). The IL-6 concentration is associated with a subject’s susceptibility to endotoxin (3), indicating the clinical relevance of this analyte for monitoring acute inflammation. IL-6 is also the major paracrine growth factor of multiple myeloma in vivo, as evidenced by the efficacy of therapy with anti-IL-6 antibodies (4). Circulating IL-6 bioactivity has been detected during the course of multiple myeloma, and increased IL-6 is associated with fulminant disease (5). The implication of IL-6 as a growth factor in other oncologic disorders (6), as well as in autoimmune and rheumatoid diseases, is currently being investigated (7).

Measurement of plasma IL-6 is currently based on two methodologies: biosays and immunoassay-type assays. The discovery of IL-6-dependent cell lines, such as the murine plasmacytoma B9, has made possible very sensitive measurement of IL-6 in biological samples (8). The specificity of the bioassay can be tested by means of blocking with anti-IL-6 monoclonal antibodies. However, the extent of the proliferative response depends on the presence of various factors in the biological sample that may potentiate and (or) inhibit the IL-6 response (7), and different IL-6 isoforms may differ quantitatively in their ability to generate an IL-6 response (9, 10). Numerous immunoassays for IL-6 have been designed to circumvent this major drawback and to measure IL-6 molarity in biological samples (11–16); however, lack of sensitivity is a limitation of immunoassays. Another problem is that the monoclonal antibodies used in the assay might differ in their recognition of the different IL-6 isoforms. Immunoassays are sensitive also to interference by plasma components. In the case of IL-6, the soluble form of the p80 component of the IL-6 receptor (siL-6R) binds to IL-6 in solution and is present at a high concentration in serum (17–21). Therefore, sIL-6R might be a major source of interference in immunoassays of IL-6.

We have designed an ultrasensitive enzyme immunoassay (EIA) of IL-6 based on the use of acetylcholinesterase (AchE; EC 3.1.1.7), taking advantage of the high turnover of this enzyme. The antibodies used in this assay were analyzed exhaustively with regard to recognition of circulating IL-6 isoforms and to interference by siL-6R. We conclude that total IL-6 can be measured accurately in plasma samples. Conversely, this assay, with its well-defined monoclonal antibodies, provides a tool to investigate the biochemical heterogeneity of IL-6 in biological fluids.

Materials and Methods
Apparatus

We performed the solid-phase EIA with standard microtitration equipment, including an automated plate washer and a microplate spectrophotometer (both from SLT, Salzburg, Austria). Gel filtration chromatography was performed either with an integrated low-pressure system or by FPLC (both from Pharmacia, Uppsala, Sweden). Cells were transfected by electroporation, with use of a GeneZapper from IBI (Madison, WI). In the bioassay, the cells were harvested with a cell harvester (Skatron, Lier, Norway) and their radioactivity was determined with a Betamatic beta counter (Kontron, Zürich, Switzerland).
Reagents

_Escherichia coli_ derived recombinant human IL-6 (rhIL-6) was obtained from two commercial sources, Prepotech (Rocky Hill, NJ) and CLB (Amsterdam, The Netherlands). Recombinant glycosylated IL-6 produced in transfected murine thymoma BW51.47 was also used (see below). International Standard 88/514 was obtained from the National Institute of Biological Standards and Controls (NIBSC; Potters Bar, UK).

Monoclonal antibodies against human IL-6 were raised in DBA/2 mice immunized with purified rhIL-6 (vanSnick, unpublished data). From the panel of antibodies obtained, we selected for further studies the two most potent in blocking IL-6-dependent cell growth, designated AH64 and AH65. Both antibodies are of the IgG1 subclass. The antibody dissociation constants, determined by Scatchard analysis by one of us (F.A.M.-J.), were 3.7 and 4.8 pmol/L at 4°C for AH64 and AH65, respectively.

AchE was purified by Pradelles and colleagues (CEA, Gif/Yvette, France) from the electric organ of the electric eel _Electrophorus electricus_ by affinity chromatography (22). AchE was used as the globular form, G4, which consists of four catalytic subunits. The AchE activity was measured by the method of Ellman et al. (23). Ellman's reagent consisted of 0.75 mmol of acetylthiocholine, 0.5 mmol of 5,5'-dithio(bis-2-nitrobenzoic acid), and 30 mmol of NaCl per liter of 10 mmol/L potassium phosphate buffer, pH 7.4. In lyophilized form, the reagent was stable for 1 year when stored at 2–8°C. One Ellman unit (1 EU) is defined as the amount of enzyme producing an absorbance increase of 1 A in 1 min at 412 nm in 1 mL of substrate and an optical pathlength of 1 cm; 1 EU corresponds to ~8 ng of AchE.

Unless otherwise stated, the chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany); the cell culture reagents were from Flow (Irvine, Scotland). The buffer used in the immunoassay (EIA buffer) is phosphate-buffered saline (PBS), pH 7.4, containing 20 mmol of phosphate, 0.15 mol of NaCl, 20 g of bovine serum albumin (BSA; Boehringer Mannheim, Mannheim, Germany), and 5 mmol of NaN₃ per liter. Recombinant sIL-6R produced in CHO cells was kindly provided by K. Yasukawa (Tosoh Corp., Tokyo, Japan) (18). Recombinant cytokines used for testing antibody specificity were from Prepotech. Ready-to-use avidin-coated microtiter plates were 96-well microtiter plates (Nunc, Roskilde, Denmark) coated with avidin by a proprietary technology (Immunotech, Marseille, France).

Procedures

**Preparation of anti-IL-6–AchE conjugates.** The anti-IL-6 monoclonal antibody AH64 was covalently coupled to AchE by reaction with the heterobifunctional agent N-succinimidyl-4-((N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC; Pierce, Oud-Beijerland, The Netherlands), by the procedure previously described (24). This method involved the reaction of a thiol group, introduced into the antibody by means of the heterobifunctional agent N-succinimidyl-S-acetylthioacetate (Calbiochem, San Diego, CA), with a maleimido group incorporated into the enzyme after reaction with SMCC. After coupling, the reaction mixture was fractionated by FPLC gel filtration chromatography with a Superdex 200 HiLoad column (Pharmacia). The fractions were tested for their enzymatic activity and their immunoreactivity towards rhIL-6. The immunoreactive fractions were pooled, diluted in EIA buffer, aliquoted, and stored at ~20°C until used.

**Immuoassay procedure.** First, we coated the wells of the ready-to-use avidin-coated microtiter plates (Immunotech) with biotinylated anti-IL-6 antibody, AH65. For this purpose, we biotinylated the antibody with biotin-N-hydroxysuccinimide ester (Boehringer Mannheim) according to the manufacturer's instructions, and incubated a solution of biotinylated antibody at 1 mg/L in EIA buffer for 24 h at 4°C, 200 μL/well. Anti-IL-6-coated plates were stored in the buffer at 4°C, and the wells were rinsed twice before use. The standard immunoassay procedure was as follows: In the coated wells, incubate 100 μL of standard or sample with 100 μL of a 200 EU/L AH64–AchE solution diluted in EIA buffer. Allow the immunological reaction to proceed for 8–12 h at 4°C with agitation on an orbital shaker, then rinse the wells three times with 300 μL of wash solution (0.15 mol of NaCl and 0.5 g of Tween 80 per liter) and add 200 μL of substrate solution per well. After the color reaction has proceeded in the dark for 15 min under standard conditions or for up to 2 h (long development), measure the absorbance at 405 nm with a microplate reader (see Fig. 1, standard curve). Subtract the absorbance of the substrate from all values read. Perform determinations in duplicate. To quantify IL-6 in plasma or serum samples, add polyclonal mouse immunoglobulins (Scantibodies, Santee, CA) to the AH64–AchE solution (final concentration 50 mg/L).

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![Fig. 1. Enzyme immunoassay standard curve.](image-url)

*E. coli* derived IL-6 calibrated with the standard NIBSC 88/514 was diluted in EIA buffer. Substrate incubation time was 15 min. See text for details.
Transfected cell lines producing hIL-6. Murine thymoma cells BW 51.47 (kindly provided by B. Malissen, Marseille) were cotransfected by electroporation with a linearized pHMG-IL-6 vector (25) (kindly provided by J. Content, Institut Pasteur du Brabant, Bruxelles, Belgium) and a plasmid bearing resistance to neomycin. Transfected cells were seeded and grown in the presence of 1.5 g/L neomycin (Gibco, Paisley, Scotland). Neomycin-resistant clones were screened for IL-6 production by using the above immunoassay. Positive colonies were cloned and one of them, BW.C12, was selected for further studies. These cells produced hIL-6, 60 µg/L, under standard culture conditions.

Immunoprecipitation of metabolically labeled IL-6. Transfected and parental cell lines (10⁹ cells/L) were metabolically labeled by 4-h culture in methionine-free RPMI 1640 medium (Vietech, Lyon, France) supplemented with dialyzed fetal calf serum (50 mL/L) and incubated with 0.25 mCi of [³⁵S]methionine (Amer- sham, Bucks, UK). Methionine-labeled supernates were cleared by overnight incubation at 4°C with Affigel 10 (Bio-Rad, Richmond, CA) coated with polyclonal goat antibodies to mouse immunoglobulin (Immunotech). Eluates were then immunoprecipitated with anti-IL-6 or treated with irrelevant control antibody of the same subclass in anti-mouse antibody-coated gel, as described (26). Bound proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

Gel filtration chromatography. Gel filtration chromatography was performed with a 1-m (200-mL) column of Sephacryl S200 HR (Pharmacia). The column was equilibrated with PBS at 5 cm/h, saturated with PBS containing 10 g/L BSA, and calibrated with the following molecular mass standards: murine IgG (150 kDa), BSA (67 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa), and cytochrome c (12.5 kDa). Samples (1 mL) were applied and eluted with PBS at 5 cm/h, and 1-mL fractions were collected and analyzed for IL-6 content by either the immunoassay or the B9 bioassay (see below). BSA was added to each fraction (final concentration 1.5 g/L) to prevent nonspecific binding of IL-6 to the plastic. The gel was recycled by washing with 0.5 mol/L NaOH and a large volume of PBS.

IL-6 bioassay. Biological activity of human IL-6 was evaluated by using the murine IL-6-dependent plasmacytoma cell line B9 (8). B9 cells were cultured for 12 h in the absence of IL-6 and further cultured with IL-6-containing samples for 48 h in microtiter plates in RPMI 1640 medium supplemented with FCS (50 mL/L). The cultures were then incubated with 0.25 µCi of [³H]thymidine (Amer- sham) per well for 8 h. The cells were then harvested, and the radioactivity incorporated in the cell pellets was determined with the beta counter. A standard range of serial dilutions of rhIL-6 was included in every experiment. Specific activity, defined as the IL-6 dose that gave one-half of the maximal B9 proliferation, was ~3–5 ng/mL.

Assay of plasma and serum samples. For serum assays, we collected 2.5 mL of blood in a clean glass tube and let this stand at room temperature for 1 h. We then centrifuged the samples (1700g, 30 min, 4°C), collected the upper two-thirds of the supernates (~1 mL), and stored these at −20°C until assay. Plasma samples were collected into EDTA-containing Vacutainer Tubes (Beckton Dickinson), centrifuged at 4°C without delay, and stored at −20°C until assay.

Before the bioassay, the plasma and serum samples were diluted in culture medium and decomplemented by heating for 20 min at 56°C. Normal samples were obtained from healthy volunteers. Pathological serum samples from sepsis patients were kindly provided by Eric Plaisant (Hopital Ambroise Paré, Marseille).

Results
Specificity of the Assay

The following cytokines were tested for their possible interference in the immunoassay: IL1-α, IL1-β, IL-2, tumor necrosis factor-α, and granulocyte–macrophage colony stimulating factor. The minimal detectable concentrations of these potentially cross-reactive analytes were >1000 µg/L for all cytokines tested. Furthermore, adding these cytokines at 100 µg/L to rhIL-6 solutions did not affect the signal.

Recognition of glycosylated IL-6. Experiments were designed to analyze the recognition of natural glycosylated isoforms of IL-6 by the immunoassay antibodies. First, we immunoprecipitated supernates of the transfected cell lines that produced IL-6 metabolically labeled with [³⁵S]methionine. Both antibodies AH64 and AH65 immunoprecipitated three bands, 21–31 kDa, corresponding to the major glycosylation isoforms described in the literature. Second, we examined the ability of the antibodies to block IL-6 bioactivity in IL-6 from different sources. Both antibodies at 10 µg/mL completely inhibited proliferative activity of (a) E. coli-derived IL-6, (b) supernates of the IL-6-transfected cell line, and (c) fractionated acute-phase serum. Finally, we tested quantitatively the recognition of unglycosylated and glycosylated IL-6 in the immunoassay. For this purpose, we serially diluted in EIA buffer samples of unglycosylated, E. coli-derived rhIL-6 and samples of glycosylated IL-6 from the transfected cell line BW.C12 and analyzed these by the immunoassay. The dilution curves were parallel for all forms tested, indicating that the assay recognized equally well the glycosylated and unglycosylated forms (Fig. 2A).

Recognition of the dimeric form of E. coli-derived rhIL-6. Recombinant E. coli-derived rhIL-6 was used as a model to analyze recognition of multimeric forms of IL-6 by the assay antibodies. The E. coli-derived rhIL-6 (Peprotech) was fractionated by gel filtration, and the immunoreactivity was measured in every fraction. Two major immunoreactive peaks were detected, corresponding to 25- and 48-kDa fractions. Both peaks were stable when refractionated under the same conditions. To further confirm the molecular nature of the two peaks, we fractionated ¹²⁵I-labeled IL-6 and analyzed the 25- and 48-kDa fractions by SDS-PAGE. The autoradiography showed that both peaks migrated as a single 22.5-kDa
band under reducing conditions (data not shown). Eventually, when rhIL-6 samples were heated for 20 min at 56°C and subsequently fractionated, we obtained one single immunoreactive peak at 20 kDa (Fig. 3A). These results demonstrated that the two major immunoreactive peaks consisted of monomeric and dimeric forms of rhIL-6, respectively. The conversion of the purified dimeric form into monomer by heat treatment doubled the immunoreactivity of the sample. This indicates that the EIA actually measures the molarity of the different forms of rhIL-6 rather than the mass concentration. We examined the recognition of the two forms quantitatively in the EIA. The purified dimeric rhIL-6 could be detected in an homologous sandwich assay that used the same antibody for capture and detection of the analyte, whereas the monomeric form was not detected in this system. Dilution curves were parallel for the monomeric and the dimeric forms, in either the homologous or in the standard heterologous EIA, suggesting that both forms were recognized with similar affinity.

**Multimeric forms of natural IL-6.** The recognition of oligomeric forms of IL-6 was analyzed by fractionation of IL-6-containing samples and by means of the homologous sandwich assay system described above. Glycosylated IL-6 produced by BW.C12 transfected cells eluted as a single peak corresponding to a monomeric form (Fig. 3B); using the homologous EIA, we failed to detect oligomeric forms in this preparation. When acute-phase plasma was fractionated under the same conditions, most immunoreactivity eluted as monomeric IL-6, but we detected an additional minor high-Mₘ component (Fig. 3C). Each fraction was assayed for its biological activity in the IL-6 bioassay with cell line B9. The material corresponding to both peaks was found to contain bioactivity. The bioactivity in all fractions was inhibited completely by addition of antibody AH65 at 1 mg/L. Furthermore, the only bioactive fractions were the ones where we detected IL-6 by the heterologous EIA. However, we failed to detect directly the high-Mₘ component by the homologous EIA with AH65 or AH64. In a last attempt to detect multimeric forms of circulating IL-6, we tested sepsis sera samples with the homologous EIA. We did not get any signal in this system.

**Interference by sIL-6R in the assay.** Because the soluble receptor for IL-6 (sIL-6R) was reported to bind IL-6 in solution, we tested sIL-6R for interference with IL-6 determination in the immunoassay. For this purpose, to a 1 μg/L (40 pmol/L) solution of purified monomeric rhIL-6 diluted in EIA buffer we added serial dilutions of purified CHO-derived sIL-6R (in the same buffer), incubated the samples for 12 h at 4°C, and analyzed them by the EIA. The final concentration of sIL-6R after addition to the samples ranged from 0 to 8 nmol/L, which corresponded to an sIL-6R/IL-6 molar ratio of 0 to 200. For the average normal plasma concentration of sIL-6R (reported to ~2 nmol/L; 19–21, 27), we observed a 30% decrease of the signal for IL-6.

In a preliminary experiment, we determined that sIL-6R was a thermosensitive protein and was completely destroyed when heated for 20 min at 56°C, whereas monomeric rhIL-6 was not affected by this procedure. We took advantage of this differential thermosensitivity of IL-6 and sIL-6R in an attempt to dissociate IL-6/sIL-6R complexes and to measure total sample IL-6. When different amounts of sIL-6R were preincubated with rhIL-6 (monomer), as described above, heat treatment of the preformed complexes led to almost complete recovery of the signal in the immunoassay (Fig. 4).

**Measurement of IL-6 in Plasma**

We investigated the accuracy of the immunoassay for the measurement of IL-6 in sera or plasma. In these
Fig. 3. Fractionation of IL-6 from various sources: (A) E. coli-derived rhIL-6; (B) IL-6 derived from BW.C12 transfectants; (C) IL-6-positive serum sample.

(A) The rhIL-6 was fractionated on sephacryl S200 HR chromatography, and the fractions were assayed by the immunoassay. Untreated IL-6 was eluted as a mixture of monomer and dimer (---). After 30 min at 56°C, the same preparation was eluted as a pure monomer (---) (B and C). The samples were fractionated by S200 chromatography, and each fraction was tested either for immunoreactivity with the IL-6 EIA (---) or for bioactivity in the B9 assay (---). For rhIL-6 one B9 unit is equivalent to 3 ng/L.

experiments a standard curve was obtained by diluting E. coli-derived rhIL-6 (Peprotech) with a pool of normal sera that did not contain detectable immunoreactive IL-6. The recombinant molecule used as a standard was calibrated in the immunoassay with NIBSC 88/514 standard. Polyclonal mouse immunoglobulins were added to the anti-IL-6-AchE conjugate solution (final concentration 50 mg/L) so as to eliminate false-positive reactions due to the presence of heterotypic antibodies in human sera. The standard curve in plasma remained linear, but the signal was 35% lower than in EIA buffer. We then investigated two groups of sera and plasma samples: samples from normal healthy volunteers (serum and EDTA-treated plasma) and IL-6-positive serum samples from sepsis patients. The average IL-6 content of normal samples was 7 ng/L. In sepsis samples, we measured concentrations ranging from 200 ng/L to 250 μg/L (average, 3 μg/L). When a saturating amount of IL-6 was present, the sample was diluted with normal serum before immunoassay. The accuracy of the assay was tested in analytical recovery and dilution experiments. In serum and EDTA-plasma from healthy volunteers, the recovery of added E. coli-derived rhIL-6 was satisfying (mean 96.3%, SD 7.0%; 16 samples). Dilution analysis was then performed with samples from sepsis patients. In these experiments, the circulating IL-6 was recognized as well as standard rhIL-6 (Fig. 2B). Finally, the assay linearity was determined by mixing IL-6-containing sera (up to 200 ng/L) with IL-6-negative samples. The immunoassay was found to be linear in this range.

Sensitivity of the Immunoassay

A preparation of E. coli-derived rhIL-6 calibrated with NIBSC 88/514 was serially diluted in either a pool of IL-6-negative normal sera or EIA buffer. Then we analyzed every rhIL-6 solution in 12 simultaneous determinations with the immunoassay. The detection limit, defined as the minimal concentration of IL-6 that produces a signal equal to the nonspecific background plus 2 SD, was as low as 0.3 ng/L when rhIL-6 was diluted in buffer, 3 ng/L when diluted in human plasma. Longer reaction periods led to a decreased SD in the

Fig. 4. Interference by sIL-6R in IL-6 immunoassay.
rhIL-6 (1 μg/L) was incubated in EIA buffer with different concentrations of purified sIL-6R for 12 h at 4°C. Then, samples were either incubated for 20 min at 56°C or not incubated and tested with the immunoassay. Results are expressed as the ratio of IL-6 immunoreactivity in the presence of sIL-6R vs immunoreactivity in the absence of sIL-6R.
signal for the lowest analyte concentrations. This was clearly demonstrated by plotting precision profiles for different reading times (Fig. 5A). Longer incubation times led to a limit precision profile, which may be considered as the intrinsic profile of the immunoassay. Note that the signal remained linear for the lowest concentrations of IL-6, thus allowing quantitative determinations around the detection limit, as outlined in Fig. 5B. In view of the optimal catalytic properties of AChE, which provides a continuous signal for many hours, the working range of the assay may be adapted to the actual concentration of IL-6 in the samples.

Discussion

Cytokines have a very short half-life in vivo (28, 29). Except in acute clinical situations, when the production of cytokine increases dramatically, the concentration of circulating IL-6 is very low, ~5–10 ng/L. Ultrasensitivity (detection limit <1 ng/L) is required for acceptable precision in this range. The ability to measure precisely the minute amounts of circulating IL-6 may open new fields for clinical investigation of IL-6-dependent diseases.

Microheterogeneity of IL-6 has been studied exhaustively by several authors. Different N- and O-glycosylated isofoms have been reported and biochemically characterized (30–33), as well as phosphorylated forms (34). Our experiments do not distinguish between minor isofoms of equal molecular mass. However, as evidenced by immunoprecipitation experiments, the three major isofoms described in the literature are recognized by both of the assay antibodies we used. Moreover, both AH64 and AH65 blocked the biological activity of glycosylated IL-6. We assume, therefore, that the glycosylation sites are outside the antibody epitopes. Macromolecular heterogeneity was also reported: Dimeric IL-6 was found in recombinant preparations (35), and large amounts of high-\(M_r\) immunoreactive and bioactive IL-6 oligomers were reported in plasma samples (9, 10). We chose to study oligomer recognition in the model system of recombinant E. coli-derived IL-6, though this system is not of physiological relevance. Our experiments showed that both assay antibodies recognized dimeric IL-6 as well as monomeric IL-6. From these results we assume that the immunoassay detects naturally occurring IL-6 oligomers. In fractionation experiments with acute-phase plasmas, we detected only a minor high-\(M_r\) immunoreactive component. The elution of B9 bioactivity correlated with the elution of immunoreactivity, indicating that the major active forms were actually found in the main 20- to 30-kDa immunoreactive peak. Taken together, these results prompt us to conclude that the immunoassay detects all IL-6 isofoms and that the predominant circulating form active on the B cell lineage is monomeric. According to May et al. (10), IL-6 is present in plasma at high concentration in an oligomeric form, associated with other plasma proteins. Our results do not rule out this possibility, because these authors also observed that the higher-\(M_r\) forms were not recognized by most anti-IL-6 antibodies and did not activate B9 cells. However, the AH64/AH65 assay does not measure these high-order complexes.

Several molecules were shown to interact with IL-6 and might compete with the assay antibodies for binding to IL-6 and affect the accuracy of analyte quantitation: Soluble IL-6 receptor can complex IL-6 in solution (17–20); more recently, a soluble form of the receptor second-chain gp130 was also demonstrated to bind to IL-6/IL-6R complexes (36, 37); \(\alpha_2\)-macroglobulin can interact with IL-6 (38) as well as with other cytokines (39); other inflammatory proteins such as C-reactive protein and complement components were found associated with IL-6 in multimolecular complexes (10); and finally, anti-cytokine natural autoantibodies were re-
ported (40). We focused on the IL-6/sIL-6R interaction. The IL-6/sIL-6R complexes can interact with membrane-transducing component. In fact, serum sIL-6R can promote the proliferation of IL-6-dependent myeloma cells in the presence of suboptimal concentrations of IL-6 (21), and recombinant sIL-6R can restore an IL-6 response in hepatocytes deprived of membrane IL-6R. Thus, the fraction of IL-6 that is bound to sIL-6R is functionally relevant and must be measured in an IL-6 assay. The average normal concentration of sIL-6R in plasma is 1–2 nmol/L (21, 27), and the dissociation constant of IL-6/sIL-6R interaction is close to 1.5 nmol/L (20, and our own unpublished data). Hence in normal plasma, sIL-6R is in great molar excess to IL-6 (sIL-6R/IL-6 ratio = 2 × 10^4), and −50% of circulating bioavailable IL-6 is then bound to sIL-6R. We observed a 30% decrease of the signal when 2 nmol/L sIL-6R was added to a 10 pmol/L solution of IL-6 in buffer. The standards being diluted in a pool of plasma containing 1.5 nmol/L sIL-6R, the interference due to sIL-6R would be expected to occur only in some samples. The extent of variations in the sIL-6R content remains to be determined. Our results indicate that sIL-6R concentrations in the range of 1–8 nmol/L produce only slight variations of the assay results. Hence, in most cases, the immunoassay measures total IL-6 content. Finally, we have shown that sIL-6R is a thermosensitive protein. This provides a means to cope with sIL-6R interference and to measure total IL-6 directly.

The assay dose/response curve obtained when IL-6 was diluted in plasma was close to the dose/response curve in EIA buffer containing 1.5 nmol/L sIL-6R. Thus, the presence of sIL-6R may account for most of the plasma effect. The accuracy of IL-6 determinations in plasma was further demonstrated by recovery and dilution experiments, with excellent results. Fractionation of plasma samples also indicated that there were no major cross-reactive antigens. Most plasma samples from normal healthy donors contained only minute amounts of immunoreactive IL-6. These results indicate the absence of major plasma interference and (or) cross-reactivity, although such a situation might occur in some pathological samples. A high concentration of polyclonal mouse antibodies was added in the AchE conjugate solution to eliminate false-positive reactions, as described elsewhere (41). The documentation of sample-specific effects, such as the presence of IL-6 autoantibodies, would require further studies with a large number of normal and pathological samples.

Using an AchE-based immunoassay, we could accurately measure concentrations of IL-6 as low as 0.3 ng/L (1.3 × 10^{-14} mol/L) in buffer and 3 ng/L in plasma. The signal-to-noise ratio of the detection system is the critical factor. Ultrasensitivity may be obtained by maximizing specific activity of the tracer antibody and minimizing background noise. Several technical solutions were proposed for ultrasensitive quantitation of IL-6, such as luminescence-based detection systems (11) and amplification steps after conjugate binding (13). The use of AchE conjugate is an alternative to these methods.

The optimal catalytic properties of AchE and especially the high substrate turnover (4.4 × 10^7 mol/h) permit detection in the attomole (10^{-18} moles) range with colorimetric detection (41). The conjugation technique and the coating we used made possible the production of enzyme–antibody conjugates with virtually no nonspecific binding to the antibody-coated solid phase. AchE-based immunoassays of the immunometric type have been previously described for IL-1 (24, 41), as well as competition assays involving a hapten-conjugated enzyme (42, 43).

The biochemistry of the IL-6 system will certainly reveal further complexity. The ultrasensitive EIA we describe measures total IL-6 concentration in plasma samples. This will provide clinical research with a powerful and versatile tool to investigate the IL-6 system in various pathological situations.

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