Radioimmunoassay of Interleukin-6 in Plasma
Anna-Maija Teppo,¹ Kaj Metsärinne,¹,² and Frej Fyhruast¹,²

We present a double-antibody radioimmunoassay for determining human interleukin-6 (IL-6) in biological fluids. The detection limit of the assay is 20 ng/L (B, 2 SD). Bound radioactivity in the range of 30% to 90% of the B, counts corresponds to IL-6 concentrations of 100 to 14 000 ng/L. Analytical recovery of IL-6 added to EDTA-treated plasma averaged 25% more than that added to serum. The plasma concentration of IL-6 was therefore ~85 ng/L more than the concentration in simultaneously drawn serum. The mean serum concentration of IL-6 in 45 healthy subjects was 83 ng/L (range 20–290 ng/L), in 20 patients with multiple myeloma 303 ng/L, in 20 patients with rheumatoid arthritis 234 ng/L, and in 13 patients with systemic lupus erythematosus 183 ng/L. Markedly increased (>3000 ng/L) concentrations of IL-6 were found in sera of patients with meningococcus meningitis and infectious peritonitis.

Additional Keyphrases: reference range · cytokines · multiple myeloma · rheumatoid arthritis · systemic lupus erythematosus · bacterial meningitis · peritonitis

Interleukin-6 (IL-6) is a cytokine produced by various cells, notably monocytes (I, 2). In cultured human fibroblasts and osteosarcoma cells, the production of IL-6 is stimulated by tumor necrosis factor and interleukin-1 (3).

The biological function of IL-6 is not completely settled. It acts as an endogenous pyrogen (4, 5), induces B-cell differentiation and enhances B-cell immunoglobulin secretion (6, 7), stimulates hepatic synthesis of acute-phase protein by inducing a liver-specific protein that binds to the promoter of the acute-phase gene (8–11), and regulates hematopoiesis (12–14). These findings suggest that IL-6 plays a central role in host-defense mechanism (15, 16).

Various bioassays have been used to measure IL-6 activities in diseases. Increased serum IL-6 activities have been found in autoimmune diseases (17–19) in plasma cell dyscrasias, where the concentration of IL-6 seems to reflect the activity of disease (20); in acute-phase situations (5, 21, 22); in HIV-infected patients (23, 24); and in urine of patients with IgA nephropathy (25). In addition, because of their abnormal expression of IL-6, increased serum IL-6 activities are expected to be found also in patients with Castleman disease, cardiac myxoma, and mesangial proliferative glomerulonephritis (16).

To study the role of IL-6 in acute-phase situations and in various other diseases, we have developed a radioimmunoassay for quantifying the concentrations of IL-6 in human serum and plasma. The method can be applied to study the concentrations of IL-6 in other biological fluids also.

Materials and Methods

Patients. We assayed serum IL-6 in 45 healthy subjects and in 103 patients. The clinical groupings of the patients are shown in Table 1.

Interleukin-6. We used Escherichia coli-derived recombinant human IL-6 from Janssen Biochemica (Beersse, Belgium) with a molecular mass of 26 kDa and a specific activity of 10¹² arb. units/g of protein, as measured by bioassay with B9 hybridoma cells (1). The preparation was diluted in assay buffer and stored at ~70 °C.

Antiserum to IL-6. Rabbit anti-human IL-6 antiserum (no. LP-716; Genzyme Corp., Boston, MA) had a total protein concentration of 1 g/L. Reported, 1 mg of LP-716 neutralizes ~1000 units of natural or recombinant human IL-6 in the B9 cell proliferation assay (1). This antiserum showed no significant cross-reactivity with interleukin-1, interleukin-1β, tumor necrosis factor-α, tumor necrosis factor-β, interferon-α, or interferon-γ. The antiserum was stored at −35 °C.

¹²⁵-I-labeled interleukin-6. We iodinated IL-6 by the method of Bolton and Hunter (26), and purified it by gas–liquid and "high-performance" liquid chromatography (Amersham International, Amersham, U.K.). Specific activity was 1000–1500 Ci/mol. The tracer was diluted with assay buffer and stored at 0 °C.

Reagents. Assay buffer was phosphate-buffered saline, pH 7.3 (per liter: 10 g of bovine albumin, 50 mmol of phophate, and 150 mmol of NaCl). Micro Sepharose-anti-rabbit IgG ("Decanting Suspension 3") was from Pharmacia (Uppsala, Sweden). Quantikine™ human IL-6 enzyme immunoassay kit was from Research and Diagnostics Systems (Minneapolis, MN).

Blood sampling. Venous plasma samples were col-

| Table 1. Characteristics of Healthy Control Subjects and Patients |
|---------------------------------|-----------------|-----------------|
| Sex, Age, years |
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| Healthy controls | 45 | 30/15 | 44 | 21–85 |
| Multiple myeloma | 20 | 13/7 | 69 | 42–85 |
| Meningococcus meningitis | 18 | 11/7 | 1 | 0.6–1.9 |
| Systemic lupus erythematosus | 13 | 0/13 | 45 | 25–69 |
| Rheumatoid arthritis | 20 | 9/11 | 48 | 23–74 |
| Infectious peritonitis | 32 | 15/17 | 48 | 26–87 |

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lected in prechilled polyethylene tubes containing 1 mL of 150 mmol/L Na₂EDTA reagent per 10 mL of blood. Serum samples were collected into ice-cold glass tubes. Plasma and serum samples were separated within 30 min and stored at -20 °C until assayed.

**Radioimmunoassay.** Mix 50 μL of standards or serum, 100 μL of antiserum to IL-6 (diluted 200-fold in assay buffer), and 50 μL of 2500-fold-diluted tracer (about 5000 counts/min for 50 μL); incubate for 16 h at 4 °C. Separate bound and free IL-6 by adding 1.0 mL of Decanting Suspension 3, incubate at room temperature for 30 min, centrifuge (1500 × g, room temperature, 10 min), and decant. Measure the radioactivity of the pellets and use a logit-log linear transformation curve for the calculations. Report IL-6 concentrations as the mean (and SD) of duplicate estimations.

**Statistical analyses.** Results were expressed as mean (and SD). Values <20 ng/L (detection limit of the method) were considered to be 10 ng/L in calculations. For statistical analyses, we used linear-regression analysis and Student’s t-test. The nonparametric Mann–Whitney’s U-test was used to evaluate the differences in the IL-6 concentrations between the groups of patients.

**Other methods.** An enzyme-linked immunosorbent assay (ELISA; Quantikine™) was used to measure the concentration of IL-6 in 35 sera from patients with peritonitis.

**Absorption with antisera.** Two sera with initial concentrations for IL-6 of 15 500 and 4300 ng/L were absorbed with immobilized antibodies to IL-6 as follows: To 500-μL aliquots of sera, we added 10 ng of rabbit anti-human IL-6 antibodies (LP-716) bound to Micro Sepharose-anti-rabbit IgG. After incubation at 4 °C overnight, the mixtures were centrifuged, and the supernates were analyzed for IL-6.

**Results**

**Assay Conditions**

Tracer diluted 2500-fold gave a radioactivity measurement of about 5000 counts/min in 50 μL; we used this dilution throughout the study.

To determine the optimum dilution of the antiserum, we incubated 50-μL aliquots of 2500-fold-diluted ¹²⁵I-labeled IL-6 (~5000 counts/min) with 100 μL of serially diluted antiserum. Bound ¹²⁵I-labeled IL-6 was separated by adding 1.0 mL of Decanting Suspension 3. The 200-fold-diluted antiserum bound >60% of the tracer (Table 2), so we used that dilution in further studies.

**Standard curve and detection limit.** Figure 1 shows a typical standard curve and its logit-log transformation. IL-6 doses of 500, 2000, and 7500 ng/L corresponded to 71%, 50%, and 38% tracer binding relative to the zero dose (100% = B₀), respectively. B₀ values ranged between 53% and 57% of the tracer. Nonspecific binding was <4%. The minimum concentration of IL-6 clearly distinguishable from zero (i.e., B₀ - 2 SD) was 20 ng/L.

**Precision of the assay.** To calculate within-run and between-run precision (CV), we analyzed during four months aliquots of two serum samples (IL-6 at 250 and 900 ng/L) stored frozen (-35 °C) in two parallel determinations, each in 16 separate assays, for the between-run assessment, and 10 parallel determinations for assessing within-run precision. Within- and between assay CVs were respectively 12.3% and 15.5% for the 250 ng/L sample, and 11.8% and 14.6% for 900 ng/L.

**Conditions for blood sampling.** To find the optimum conditions for blood sampling, we analyzed in parallel the concentrations of IL-6 in serum and EDTA-plasma from 13 apparently normal persons and from 32 patients with bacterial peritonitis. In the normal subjects the

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**Table 2. Effect of Antiserum Dilution on Binding of Tracer**

<table>
<thead>
<tr>
<th>Dilution of antiserum, n-fold</th>
<th>B/T, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>86.5</td>
</tr>
<tr>
<td>100</td>
<td>72.0</td>
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<tr>
<td>200</td>
<td>54.9</td>
</tr>
<tr>
<td>400</td>
<td>31.0</td>
</tr>
<tr>
<td>800</td>
<td>18.8</td>
</tr>
<tr>
<td>1600</td>
<td>11.8</td>
</tr>
<tr>
<td>3200</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Tracer was diluted 2500-fold. The bound tracer was separated by adding 1.0 mL of Decanting Suspension 3.

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Fig. 1. Standard curves for RIA of IL-6: (a) B/B₀ ratio corrected for nonspecific binding of tracer; (b) standard curve after log/log transformation.
mean concentration of IL-6 in serum was 28 ng/L (SD 28 ng/L), and in plasma 54 ng/L (SD 31 ng/L). In patients with peritonitis the mean concentration of IL-6 in serum was 294 ng/L (SD 297 ng/L), and in plasma 408 ng/L (SD 330 ng/L). Figure 2 shows the regression line between the concentrations of all sera and plasma. The concentration of IL-6 in EDTA-plasma is ~90 ng/L more than the concentration in simultaneously collected serum samples. Keeping this in mind, either serum or plasma can be used for quantifying IL-6; we prefer the use of EDTA-plasma. However, for this study, we assayed serum samples.

Analytical recovery of IL-6. We added recombinant IL-6, 560 ng/L, to 13 sera (mean initial content of IL-6, 30 ng/L, SD 28 ng/L) and EDTA-plasma (mean initial content 60 ng/L, SD 31 ng/L) from normal persons, and to aliquots (12 each) of an acute-phase serum (initial concentration of IL-6, 170 ng/L) and EDTA-plasma (initial concentration 220 ng/L). All aliquots were analyzed directly and after freezing at -20 °C. Analytical recoveries ranged from 74% to 132% from plasma, from 64% to 126% from normal serum, and from 70% to 115% and from 89% to 153% from acute-phase serum and plasma, respectively (Table 3). Recoveries were higher from plasma (P <0.05) than from serum, but no differences between results for normal and acute-phase samples or between results for fresh and frozen samples were noted.

Absorption experiments. To evaluate the specificity of the assay, we absorbed two sera with 10 ng of immobilized antibodies. The absorption reduced the concentration of IL-6 from 15 500 to 1500 ng/L, and from 4300 ng/L to a value below the detection limit of the assay. A repeat absorption of the first serum reduced its concentration of IL-6 also to a value below the detection limit of the assay. Thus it seems that the assay is measuring only IL-6.

Clinical Results

Concentration of IL-6 in normal sera. The mean serum concentration of IL-6 in apparently healthy subjects was 88 ng/L (SD 71 ng/L). In nine sera (20%) the concentration was <20 ng/L. We noticed no difference in IL-6 concentrations between men and women and no corre-

### Table 3. Analytical Recovery of IL-6 Added to Various Samples

<table>
<thead>
<tr>
<th>Added</th>
<th>Total*</th>
<th>n</th>
<th>Without freezing*</th>
<th></th>
<th>After freezing*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Found, ng/L</td>
<td>Recovery,</td>
<td>Found, ng/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Normal serum</td>
<td>590 (28)</td>
<td>13</td>
<td>590 (170)</td>
<td>99.5 (24.4)</td>
<td></td>
</tr>
<tr>
<td>Normal plasma*</td>
<td>560</td>
<td>13</td>
<td>790 (270)</td>
<td>128.3 (38.6)</td>
<td>670 (110)</td>
</tr>
<tr>
<td>Acute-phase serum</td>
<td>730 (50)</td>
<td>12</td>
<td>690 (90)</td>
<td>94.0 (15.4)</td>
<td>880 (130)</td>
</tr>
<tr>
<td>Acute-phase plasma*</td>
<td>780 (60)</td>
<td>12</td>
<td>900 (120)</td>
<td>116.0 (15.0)</td>
<td></td>
</tr>
</tbody>
</table>

* Mean (and SD).

*EDTA-treated.
lation with age.

**Concentration of IL-6 in patients.** The mean concentration of IL-6 in all groups of patients was significantly higher than in healthy controls. The mean concentration in patients with myeloma was 303 ng/L (P < 0.05), in patients with systemic lupus erythematosus 183 ng/L (P < 0.001), in patients with rheumatoid arthritis 234 ng/L (P < 0.001), in children with meningococcus meningitis 2170 ng/L (P < 0.01), and in patients with peritonitis 670 ng/L (P < 0.01) (Figure 3). Values >225 ng/L (mean ± 2 SD of results for the controls) were found in six patients with multiple myeloma (30%), in two of 13 patients with lupus (14%), in eight of 20 patients with rheumatoid arthritis (40%), in 15 of 42 sera with peritonitis (in 90% of those with acute infection, and in 19% of those in remission), and in five of 18 patients (28%) with meningococcus meningitis.

**Comparison with ELISA.** In 35 sera from patients with peritonitis, the results obtained by our radioimmunoassay (y) and by ELISA (x) correlated somewhat (r = 0.80), the regression equation being y = 0.879x + 793.6 ng/L (Figure 4). Samples with high IL-6 concentrations gave similar results in both assays, whereas samples with lower IL-6 concentrations gave values in the RIA that averaged sevenfold greater than those in the ELISA.

**Discussion**

Thus far, IL-6 has been measured by various bioassays (1, 3, 4, 7, 17, 20-23, 25, 27) and by enzyme immunoassays (24, 28, 29). The bioassays may be affected by synergistically acting cytokines (4), which casts doubt on the specificity of available bioassays for IL-6. Moreover, plasma and other body fluids may contain inhibitors that can interfere with determinations of IL-6 by bioassays, and the bioassays can be affected by drugs taken during disease (15, 18). The bioassays are far too slow—the IL-6 determination usually takes about five days—and impractical for many clinical purposes; furthermore, they are not easily used in normal clinical laboratories. Specific and rapid methods are needed for accurately measuring IL-6.

The sensitivity of the radioimmunoassay we describe (20 ng/L) is good enough for determining IL-6 in normal serum. The assay can measure concentrations as great as 20 000 ng/L in undiluted samples. The assay may also be applied to determine the concentrations of IL-6 in urine and other biological fluids. To study the concentration of IL-6 in normal serum, and in situations where IL-6 is only slightly increased, we incubated samples at 4 °C overnight. However, in urgent situations, e.g., in serial measurements of IL-6 for monitoring renal transplant patients, where the IL-6 concentrations in acute rejection exceed normal by 100-fold (21), much shorter incubations at higher temperatures could be used.

The concentration of IL-6 in EDTA-plasma averaged 90 ng/L greater than in serum. Because the analytical recoveries of IL-6 from plasma were about 25% higher than from sera, the difference in the plasma and serum concentrations may be due to the differences in recoveries.

The specific activity of the recombinant human IL-6 used to standardize our assay was determined with the B9 cell bioassay (1 pg of IL-6 corresponding to ~1 unit of IL-6; see Materials and Methods) (1). This made it possible to express our values as units/mL, and to compare the values we found with those obtained by B9 cell bioassay. In healthy persons the mean concentration of IL-6 measured with our RIA (83 units/mL, n = 45) is about 10-fold greater than that (~10 units/mL) obtained with the B9 cell bioassay (21-23). Also, in patients with rheumatoid arthritis, our values (mean 234 units/mL, n = 20) are more than 10-fold the values (mean 17.8 units/mL, n = 54) found by bioassay (17). However, Waage et al. (27), using bioassay, found in 45% of such patients IL-6 values ranging from 50 to 350 units/mL, values comparable with those by our assay.

In addition to the intact IL-6 (21-25 kDa), another form of IL-6, also biologically active, with a molecular mass of 5-7 kDa, has been found in serum (21). Thus, our assay may measure both forms of IL-6, and perhaps some additional biologically inactive aggregates or split products.

When comparing our results with those obtained by ELISA, our values in healthy persons (mean 83 ng/L) are ~10-fold greater than those (~10 ng/L) reported earlier (24). However, we found a good correlation between the results obtained by the presented radioimmunoassay and those obtained by ELISA. Interestingly, in serum with high concentrations of IL-6, approximately equal results were obtained by both assays, whereas in serum with a slightly increased concentration of IL-6, the value obtained by RIA was about sevenfold greater than that obtained by ELISA.

Some inhibitory property of serum commonly contributes to the risk of false-negative results in bioassay measurements of IL-6 activity in serum (24), and also interferes with the determination of IL-6 by ELISA (28, 29). Matsuda et al. (30) reported that serum IL-6 is bound to α2-macroglobulin. Even though this complexed IL-6 retains its biological activity (30), the binding of
the large α2-macroglobulin to IL-6 could be expected to interfere with the determination of IL-6 by enzyme immunoassays. In fact, we noticed (results not shown) that, as measured by ELISA, results for the IL-6 standards diluted in sera were <50% of those for standards diluted in assay buffer. Furthermore, serum samples diluted fivefold in assay buffer showed 50–80% higher concentrations than sera assayed undiluted. These findings suggest that serum contains an inhibitor that may result in ELISA measurements that are five- to 10-fold too low.

In normal serum, IL-6 is found in the 450- to 550-kDa fraction, whereas in AIDS patients with high concentrations of circulating IL-6, most of the IL-6 molecules are in the free form, with a molecular mass ~25 kDa (24). We thus speculate that, in sera with low concentrations of IL-6, the inhibitor(s) in serum is enough to "block" the IL-6 molecule, whereas in sera with a high concentration of IL-6, most of the IL-6 molecules are unbound and measurable also by ELISA. This theory would explain why the results obtained by our RIA and by ELISA were equal for sera with high concentrations of IL-6.

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References