ASSAY FOR THE SOLUBLE INTERLEUKIN-2 RECEPTOR
BY SANDWICH ENZYME LINKED IMMUNOSORBENT ASSAY

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SUMMARY: A system for the detection of soluble IL-2R by sandwich enzyme
linked immunosorbent assay (ELISA) was established. This assay system has
good reproducibility, and was found to be specific for soluble IL-2R by
examination of the binding of anti-IgM antibody or anti-IL-2R monoclonal
antibody (mAb) with IgM or soluble IL-2R and inhibition by IL-2 of the binding of
anit-IL-2R mAb to soluble IL-2R. The IL-2R molecules in supernatant of
phytohemagglutinin-stimulated peripheral blood mononuclear cells were of
44,000-61,000 molecular weight as estimated by size of exclusion high
performance liquid chromatography. The results of determinations for soluble
IL-2R with a Eurogenetics kit (sandwich enzyme linked immunosorbent assay)
were significantly correlated with those by the our method.
INTRODUCTION

Interleukin-2 (IL-2) is a hormone-like growth factor, which is synthesized and released by antigen- or mitogen-stimulated T lymphocytes (1). The interaction of IL-2 with high-affinity IL-2R expressed by antigen- or mitogen-activated T cells produces extensive immunopotentiation (2). It was reported that IL-2R is detectable in a soluble form (3). This soluble IL-2R was reported to bind to IL-2 (4) and possibly be involved in the down regulation of IL-2 production. It is detected also in serum, and reported to increase in the serum of patients with lymphoreticular malignancies such as adult T cell leukemia, Hodgkin's disease, chronic lymphocytic leukemia, and hairy cell leukemia (5-8). It has been reported to be found at elevated levels also in the serum of patients with collagen diseases such as rheumatoid arthritis (9). Its level in the serum may possibly reflect in vivo lymphocytic activation and is expected to be useful as an immunological parameter. Therefore, an assay system for soluble IL-2R by sandwich ELISA was established by use of two kinds of monoclonal antibodies (mAb) that recognize epitopes with different IL-2R α-chains. This assay system was examined for the specificity and reproducibility and compared with a commercially available soluble IL-2R assay kit to find whether this system can be used for studying clinical significance of soluble IL-2R.

MATERIALS AND METHODS

Cells: Peripheral blood mononuclear cells (PBMC) were prepared from normal heparinized venous blood by Ficoll-Hypaque density centrifugation as described previously (10). The HTLV-1-positive T cell line, HUT102, was kindly provided by Dr. R. C. Gallo (National Cancer Institute, National Institutes of Health, Bethesda, MD).

Monoclonal antibodies (mAb): Ta60a (β1) mAb and Ta60b (β1) mAb which recognize α-chain of IL-2R (Tac antigen) were prepared as described previously (11). Ta60a recognizes the same epitope as anti-Tac mAb, while Ta60b recognizes a different epitope. These mAbs were purified from hybridoma ascites by gel filtration and diethylaminoethyl (DEAE)-cellulose chromatography.

Culture stimulants: PHA (Wellcome, HA16, Temple Hill, Dartford, England) was used at 4 μg/ml, and pokeweed mitogen (PWM) (Gibco, Grand Island, NY) at a final dilution of 1/100 (v/v).
Cell cultures: PBMC (500,000 cells/ml) were stimulated for 3 days with PHA in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 2 mM of L-glutamine. Cultures stimulated with PWM contained 1 × 10^6 cells in the same medium. Cultures were established in 24-well plates (Nunc, Roskilde, Denmark) by incubation for 7 days at 37°C in a humidified 5% CO2-air atmosphere.

Patients and serum samples: Soluble IL-2Rs were measured in serum samples from 15 patients with systemic lupus erythematosus (SLE), two patients with progressive systemic sclerosis (PSS), two patients with dermatomyositis (DM), and three patients with rheumatoid arthritis (RA). American Rheumatism Association criteria were used for diagnosis of SLE (12) and RA (13), and criteria for PSS and DM were according to Rodnan (14) and Bohan et al. (15), respectively.

Conjugation of horseradish peroxidase (HRP) with Ta60a mAbs: The method reported by Nakane and Kawaoi (16) was modified. Four milligrams of HRP (Boehringer Mannheim, FRG) in 1 ml of 0.3 M sodium carbonate buffer, pH 8.1, was exposed to 0.1 ml of a 1% 1-fluoro-2, 4-dinitrobenzene ethanol solution for 20 min. After dialysis against 0.01 M sodium carbonate buffer, pH 9.5, overnight at 4°C, a 30-min incubation was performed with 1 ml of 0.06 M NaIO4 at room temperature, and 1 ml of 0.16 M ethylene glycol was added. After similar dialysis, 1 ml of 5 mg/ml Ta60a equilibrated with 0.01 M sodium carbonate buffer, pH 9.5, was added. After a 3-hr incubation, the solution was dialyzed against PBS overnight at 4°C, and gel filtered on Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) with PBS as eluant to collect the fractions with antibody activities.

Assay for soluble IL-2R: Each well of a 96-well flat-bottomed Nunc Immunoplate-II was coated overnight with 50 µl of purified Ta60b mAb at 10 µg/ml in 0.05 M carbonate buffer, pH 9.6, or with only carbonate buffer as a background control. After washing, 50 µl of a sample was added to the coated and uncoated wells, incubated for 2 hr at room temperature, washed and then 50 µl of 1/400 dilutions of HRP-conjugated Ta60a mAb was added to all of the wells. After an additional 2 hr incubation, the plates were washed and 100 µl of 2.5 mg/ml o-phenylenediamine in 98 mM Na2HPO4 and 4 mM citric acid were added. After a 30-min incubation, the reaction was stopped with 50 µl of 2 N sulfuric acid, and the absorbance of the wells was determined at 492 nm with an ELISA reader (Sanko Junyaku Co., Ltd., Tokyo). A reference reagent, consisting of the cell-free supernatant of HUT 102 cell lines cultured for 5 days, was used in all of these experiments. The undiluted supernatant was assigned a value of 10,000 U/ml and, as determined by ELISA of serial dilutions of this supernatant, absorbance values were used to generate a reference curve. The absorbance of the test wells was then compared with the standard curve and converted to a numerical value.

Soluble IL-2R determinations with a Eurogenetics kit were made according to the company's manual.
To examine the inhibition of the binding of anti-IL-2R (α-chain) antibody to IL-2R by IL-2, a 96-well microtestplate was coated with 10 μg/ml of Ta60b as described earlier, and then 50 μl of supernatant of cultured HUT 102 cell lines, diluted 4-fold with PBS containing 1% BSA, was added as a source of soluble IL-2R. After a 2-hr incubation at room temperature, the wells were washed, 40 μl of IL-2 (Takeda Pharmaceutical Co., Osaka) diluted to various concentrations with RPMI 1640 medium containing 10% FCS or tumor necrosis factor (TNF: Asahi Kasei Co., Tokyo) as control, was added, and incubated for two hours at room temperature. Then, 10 μl of 1/80 dilutions of HRP-labeled Ta60a mAb was added, and after a 2-hr incubation at room temperature and washing, the substrate was added as described earlier. The percent inhibition by IL-2 of the binding of Ta60a mAb to IL-2R was obtained by the following formula:

\[
\% \text{Inhibition} = \frac{A \text{ in the presence of IL-2 or TNF}}{A \text{ in the absence of IL-2 or TNF}} \times 100
\]

Size-exclusion high performance liquid chromatography (HPLC): HPLC was performed with a Tosoh (CPM system) (Tosoh Co., Tokyo). Ten-fold concentrated culture supernatant of PBMC stimulated with PHA was applied to a size-exclusion column (G 3,000 SW, TSH-Gel, 300 × 7.8 mm, Tosoh Co., Tokyo) and elution performed with 0.05 M phosphate buffer, pH 6.8, at a flow rate of 0.8 ml/min. The column was calibrated with the Pharmacia gel filtration standards (Pharmacia, Uppsala, Sweden).

RESULTS

Repeatability and Reproducibility of Soluble IL-2R Assay

To examine the repeatability (within-assay variation), eight determinations were performed on three serum samples at the same time, and the results were obtained with coefficients of variation (CV) of 7.6%, 5.1% and 7.9% (Table I).

Five repeated measurements using three sera were also made at different times to examine the reproducibility (between-assay variation), and the results were obtained, with CV of 11.5%, 8.6% and 9.4%.
Table I. Repeatability and reproducibility of soluble IL-2 receptor assay

<table>
<thead>
<tr>
<th>Assay</th>
<th>No.</th>
<th>Number of samples</th>
<th>Level (mean ± S.D.)</th>
<th>C. V.(^1) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within assay variation</td>
<td>1</td>
<td>8</td>
<td>328 ± 25</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8</td>
<td>703 ± 36</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8</td>
<td>1103 ± 87</td>
<td>7.9</td>
</tr>
<tr>
<td>Between assay variation</td>
<td>1</td>
<td>5</td>
<td>463 ± 53</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>848 ± 73</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>1487 ± 140</td>
<td>9.4</td>
</tr>
</tbody>
</table>

\(^1\): C. V., coefficient variation.

Fig. 1. Inhibition of binding of anti-IL-2 mAb to soluble IL-2R by IL-2. Culture supernatant of HUT 102 cell lines was added as a source of soluble IL-2R to a Nunc Immunoplate-II coated with Ta60b mAbs (anti-IL-2Rs), to attach soluble IL-2R to the Nunc Immunoplate-II. After washing, IL-2 or TNF was added in various concentrations, incubated and then, HRP-labeled Ta60a mAb was added. The rate of inhibition by IL-2 or TNF of the binding of Ta60a mAb to IL-2R is discussed in the text.
Table II. Specificity of ELISA for soluble IL-2 receptor

<table>
<thead>
<tr>
<th>Coating antibody</th>
<th>Sample</th>
<th>Second antibody</th>
<th>Absorbance at 492 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>PWN sup</td>
<td>HRP anti-IgM</td>
<td>0.010</td>
</tr>
<tr>
<td>None</td>
<td>PWM sup</td>
<td>HRP Ta60a</td>
<td>0.020</td>
</tr>
<tr>
<td>Ta60b</td>
<td>PWM sup</td>
<td>HRP Ta60a</td>
<td>1.632</td>
</tr>
<tr>
<td>Ta60b</td>
<td>PWM sup</td>
<td>HRP anti-IgM</td>
<td>0.020</td>
</tr>
<tr>
<td>Anti-IgM</td>
<td>PWM sup</td>
<td>HRP anti-IgM</td>
<td>1.831</td>
</tr>
<tr>
<td>Anti-IgM</td>
<td>PWM sup</td>
<td>HRP Ta60a</td>
<td>0.042</td>
</tr>
</tbody>
</table>

1: Wells coated with buffer alone, Ta60b (anti-IL-2 receptor antibody) or anti-human IgM antibody received supernatants (sup) of PBMC stimulated with PWM for 7 days, followed by HRP-conjugated Ta60a (anti-IL-2 receptor) or HRP-conjugated anti-human IgM antibody.

Inhibition by IL-2 of Binding of Ta60a mAb to Soluble IL-2R

Culture supernatant of HUT 102 cell lines was added to a Ta60b mAb (anti-IL-2R) coated microplate as a source of soluble IL-2R, followed by the addition of IL-2 or TNF in various concentrations, and then HRP-labeled Ta60a mAb (anti-IL-2R) was added. IL-2 was shown to produce a concentration-dependent inhibition of the binding of Ta60a mAb to soluble IL-2R, but TNF had hardly any effect (Fig. 1).

Specificity of ELISA for Soluble IL-2R

To determine the specificity of soluble IL-2R assays, culture supernatants from PBMCs stimulated for 7 days with PWM were analyzed for IL-2R and IgM for IL-2R and IgM (Table II). Very low absorbance was detected in the uncoated well receiving a sample of either HRP-labeled anti-human IgM or HRP-labeled Ta60a mAb. Wells coated with Ta60b mAb and then receiving samples, followed by HRP-conjugated Ta60a mAb, were strongly positive. Wells coated with anti-IgM and receiving samples and HRP-conjugated anti-IgM were also strongly positive. Those wells that were coated with anti-IgM and receiving samples and
Fig. 2. Estimation of the molecular weight of soluble IL-2R by size-exclusion high performance liquid chromatography. PHA supernatant was eluted from a G 3,000 SE column calibrated with molecular weight standards. Soluble IL-2R in the fractions was measured by ELISA.

HRP Ta60a, or those wells coated with Ta60b mAb and receiving samples and HRP anti-IgM had absorbances comparable with those of uncoated wells. These results show that this assay is specific for the detection of soluble IL-2R.

**Molecular Weight of Soluble IL-2R**

The soluble IL-2R in the culture supernatant of PHA-stimulated PBMC was analyzed by size-exclusion HPLC (Fig. 2). Its molecular weight in the PHA supernatant was 44,000 - 61,000.

**Correlation of Soluble IL-2R Determinations by the Present Assay System and with a Eurogenetics Kit**

Determinations of soluble IL-2R in serum of 22 collagen disease patients by the method we established and with a Eurogenetics kit were examined for correlation (Fig. 3). Although they did not agree exactly, a significant correlation (P<0.001) was noted.
DISCUSSION

The presence of a soluble form of the IL-2R α-chain has been described in serum and also in culture supernatants of activated murine and human T cells (3). We established an assay system for soluble IL-2R using two kinds of mAbs that react with different epitopes of IL-2R α chains. The reproducibility of this assay system was satisfactorily high (Table I). The correlation between determinations of soluble IL-2R by the method we established and with a commercially available kit was examined through measurements of soluble IL-2Rs in serum samples of collagen disease patients by the two methods (Fig. 3). Although the present method did not show predominance over the Eurogenetics kit, a significant correlation was found between them on a level of P<0.001. Therefore, the present assay seems to be as useful for measurement of soluble IL-2Rs as a commercially available kit. Rubin et al. (3) reported that the molecular weight of soluble IL-2R in PHA-stimulated culture supernatant was 45,000 – 50,000 daltons, which virtually agrees with the present result. They reported
also that the molecular weight of IL-2R on the cell surface was somewhat greater than this.

To examine the specificity of the assay system for soluble IL-2R, each well of a microtest plate for ELISA was coated with anti-IL-2R antibody or anti-human IgM antibodies, and into these were added culture supernatant of PWM-stimulated PBMCs. When HRP-labeled anti-IL-2R antibody or anti-human IgM antibody was added, the respective antibody bound specifically with soluble IL-2R or human IgM (Table II).

When culture supernatant of HUT 102 cell line containing soluble IL-2R was added to the wells of an ELISA microplate coated with Ta60b mAb (anti-IL-2R), followed by addition of IL-2 and HRP-labeled Ta60a mAb (anti-IL-2R), the binding of Ta60a mAbs to soluble IL-2Rs was inhibited (Fig. 1). This finding indicates that soluble IL-2R detected by this assay system binds to IL-2, i.e., that this assay system is specific to soluble IL-2R. Rubin et al. (4) reported also that soluble IL-2R can bind to IL-2. There is thus a possibility that soluble IL-2R can down regulate the IL-2R-dependent cellular immune response. Another possibility is that IL-2R is released in soluble form to prevent and regulate excessive IL-2R on the cell membrane, or that the binding of IL-2 to IL-2R extends the half-life of IL-2.

Receptor shedding is seen in cell surface receptors, such as the insulin receptor (18), epidermal growth factor receptor (19) and CD8 (20). If a cell-free receptor retains good affinity, it could mediate the down regulation of the cellular response to the ligand. Elevation of the soluble IL-2R level in serum may be a common phenomenon in a disease induced by an immunological mechanism. Elevation of soluble IL-2R is reported in the serum of, for example, patients with SLE or rheumatoid arthritis (9,21). It has been reported that, in an animal model, the elevation of soluble IL-2R correlates with the autoimmune states (22). It is reported that IL-2 production and reactivity to IL-2 are decreased in MRL-1pr/1pr and NZBXW mice (23), which suggests the involvement of soluble IL-2R, but the physiologic significance of soluble IL-2R remains unknown. Further detailed study of the physiologic significance of soluble IL-2R is necessary. The measurement of soluble IL-2R in serum of patients with immunological disorder may be useful to clarify the clinical meaning of soluble IL-2R.
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REFERENCES


