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# Soluble Interleukin-6 Receptor Is Released from Receptor-bearing Cell Lines *in vitro*

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**Soluble interleukin-6 receptor (sIL-6R) was found to be spontaneously released from human myeloma cell line U266 cells into culture supernatant, and was quantitatively measured with a fluorescence sandwich enzyme-linked immunosorbent assay employing antibodies specific to IL-6R. The supernatant IL-6R was generated only from IL-6R-positive cell lines; myeloma cell lines RPMI8226 and RPMI1788, and myelomonocytic cell lines U937, THP-1, and HL-60. In contrast, it was not released from the IL-6R-negative cells; T cell line Molt-4 and Burkitt lymphoma cell line Raji. SDS-PAGE analysis of the soluble IL-6R from U266 cells suggested a molecular weight of approximately 50–55 kDa, 25–30 kDa smaller than the mature cell surface receptor. These results suggest that the generation of soluble IL-6R may be a maker of myeloma cells and myelomonocytic cells.**

Key words: Human IL-6 receptor — Soluble interleukin receptor — Recombinant soluble IL-6 receptor — Interleukin-6

Interleukin-6 (IL-6)[7] was initially reported as B cell stimulatory factor-2 derived from T cells, and exhibited the biological activity of induction of immunoglobulin secretion in activated B cells.[1] The cDNA of the cytokine was cloned and IL-6 was shown to be identical with interferon $\beta_2$[2,3] or hybridoma-plasmacytoma growth factor.[4] Further, IL-6 was reported to have multifunctional properties including induction of growth and differentiation of various cells such as T cells,[5–7] hepatocytes and nerve cells[8–10]; induction of multi-CSF activity on hematopoietic progenitor cells[11] and regulation of acute phase responses.[8,9] All IL-6-responsive cell lines have been shown to express IL-6R, and it has been reported that high-affinity IL-6R is present on Epstein-Barr virus-transformed B lymphoblastoid cell lines, but not Burkitt's cell lines.[12,13] Normal resting B cells did not express detectable IL-6R, whereas B cells activated with *Staphylococcus aureus* Cowan I presented several hundred receptors per cell. Normal resting T cells expressed the receptor on the cell surface as detected by using antibodies specific to IL-6R which were prepared by using either soluble human IL-6R released from IL-6R gene-transfected cells or synthetic peptides.[14] These antibodies recognized cell surface IL-6R with the molecular weight of 80 kDa. Moreover, a non-ligand-binding component of IL-6R, gp130 was reported to be associated with IL-6R in the presence of IL-6.[15] This molecule is probably related to the ligand signal transduction of IL-6. It has been reported that soluble IL-6R (sIL-6R) could be detected only in normal human urine and its molecular weight was 50–55 kDa.[16,17] In this study, we identified the soluble form of IL-6R in culture supernatant of IL-6R-bearing cells such as myeloid cell line U266 cells by means of a fluorescence sandwich enzyme-linked immunosorbent assay (FS-ELISA) specific to sIL-6R. Immunoprecipitation analysis followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed the apparent molecular weight of sIL-6R to be 50–55 kDa.

## MATERIALS AND METHODS

**Lymphokine and antibody** Human recombinant IL-6R (rIL-6R) and monoclonal antibody specific to human IL-6R (MT18) were prepared as described previously.[14,18]

**Cell lines** RPMI 8226, RPMI 1788, THP-1, Molt-4, U937 and HL-60 were obtained from the Japanese Cancer Research Resources Bank (Tokyo). The CHO-CN cell line is a CHO cell line transfected with IL-6R gene lacking both the transmembrane and intracytoplasmic segments of human IL-6R.[19] Unless otherwise stated, these cell lines were cultured in RPMI 1640 medium

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[7] Abbreviations: IL-6, interleukin-6; IL-6R, IL-6 receptor; s, soluble; b, biotinylated; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

supplemented with 10% fetal calf serum, 50 $\mu$g/ml streptomycin, 50 U/ml penicillin and 2 m$M$ L-glutamine.

**FS-ELISA for detecting sIL-6R** The FS-ELISA method[20, 21] was used for measurement of sIL-6R. A 96-well microtiter plate (Maxisorp F96, Nunc, Roskilde, Denmark) was coated with 100 $\mu$l per well of purified anti-IL-6R (MT18) at 10 $\mu$g/ml in carbonate buffer, pH 9.6, overnight. Non-specific binding was blocked with 1% bovine serum albumin (BSA, fraction V, Sigma Chemical Co., St. Louis, MO) in Dulbecco's phosphate-buffered saline (PBS) for 3 h at room temperature. The plate was washed with PBS containing 0.1% Tween 20, and 100 $\mu$l of sample was applied to each well. The plate was incubated at 4°C overnight, washed and incubated with biotinylated polyclonal anti-IL-6R guinea pig antibody (b-$\alpha$GV6R) at 2 $\mu$g/ml for 4 h at room temperature. Then it was washed and incubated with 100 $\mu$l per well of adequately diluted streptavidin-$\beta$-galactosidase (Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, MD) for 1 h at room temperature. After washing again, 100 $\mu$l of 4-methylumbelliferyl-$\beta$-galactoside (Sigma) at 0.1 mg/ml in 0.01 $M$ phosphate buffer, pH 7.0, 1 m$M$ $MgCl_2$ was added to each well as a substrate, and the fluorescence intensity was measured with excitation at 360 nm and emission at 450 nm by using a FS-ELISA reader (Flow Laboratories, Rockville, MD). Purified sIL-6R from the supernatant of CHO-CN cells was used as a reference reagent for the IL-6R FS-ELISA.

**Molecular weight estimation** Iodination and immunoprecipitation of sIL-6R were conducted as previously described.[20] SDS-PAGE analysis was performed on 8% gels (1.5 mm thick) under reducing conditions according to Laemmli.[22]

**Flow cytometric analysis for IL-6R** Cultured cells were washed and $1\times10^6$ cells were pre-incubated with heat-inactivated (56°C, 30 min) normal rabbit serum for 30 min, then incubated with either monoclonal anti-IL-6R (MT18) or normal mouse immunoglobulin at 2 $\mu$g/100 $\mu$l for 30 min on ice. The cells were washed with PBS containing 0.5% BSA and 0.1% sodium azide and incubated with fluorescence-conjugated $(Fab')_2$ fragment of goat anti-mouse IgG (H and L chains, Cappel, Malvern, PA) at 2 $\mu$g/100 $\mu$l for 30 min on ice. After washing, the stained cells were analyzed by flow cytometry using a Cyto-Ace cytometer (Japan Spectroscopic Co., Tokyo).

**ELISA for studying the binding ability of shIL-6R to IL-6** Microwell plates were coated with 100 $\mu$l per well of human rIL-6 (10 $\mu$g/ml in 0.1 $M$ sodium carbonate buffer, pH 9.0) and blocked with 1% BSA-PBS. After washing, 100 $\mu$l of test sample was added to each well and the plate was incubated at 4°C overnight. The plate was washed and 100 $\mu$l per well of $\alpha$GV6R (1:1000 diluted anti-sera) was added. The plate was incubated at room temperature for 2 h. The bound antibodies were detected by sequential incubation with alkaline phosphatase-conjugated goat anti-guinea pig IgG and the enzyme substrate solution.[23]

## RESULTS

**FS-ELISA for sIL-6R** We first developed the quantitative assay system for detecting sIL-6R by using the culture supernatant of human sIL-6R transfectant CHO-CN cells. The highly purified sIL-6R from the supernatant of IL-6R-transfected cells (CHO-CN) was employed as a standard in all of these studies. As shown in Fig. 1, sIL-6R content in culture supernatant of both the IL-6R-transfected CHO-CN cells and the non-

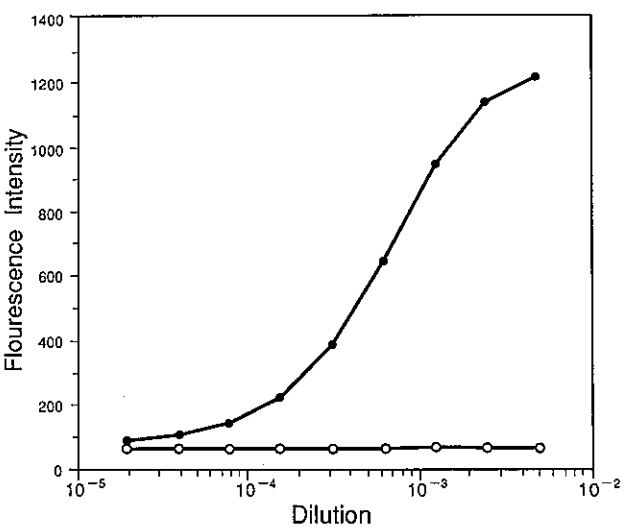


Fig. 1. Dose-response curves of recombinant sIL-6R by using FS-ELISA for sIL-6R. Serial two-fold dilutions of culture supernatants of either CHO-CN cells (●) or CHO cells (○).

Table I. No Effect of Exogenous IL-6 on the Activity of IL-6R FS-ELISA[a,b]

| IL-6 (ng/ml) | sIL-6R (ng/ml) | % of recovered activity |
|---|---|---|
| 0 | 1.10 | 100.0 |
| 10 | 1.22 | 110.9 |
| 100 | 1.18 | 107.3 |

*a*) Various amounts of human recombinant IL-6 were added to 1.1 ng of recombinant IL-6R preparation, and IL-6R activity was assayed by the ELISA.

*b*) Results are expressed as the mean of five independent experiments.

Table II. Study of sIL-6R Generation and IL-6R Expression on Various Cell Lines[a,b]

| Cell line | Cell type | sIL-6R (ng/pl) | | IL-6R |
|---|---|---|---|---|
| | | 24 h | 48 h | % positive cells |
| U266 | Myeloma | 4.04 | 8.52 | 97.1 |
| RPMI8226 | Myeloma | 1.57 | 3.56 | 55.21 |
| RPMI1788 | Myeloma | 0.84 | 2.27 | 30.40 |
| U937 | Myelomonocytic | 2.34 | 6.78 | 96.32 |
| THP-1 | Myelomonocytic | 1.97 | 4.08 | 48.91 |
| HL-60 | Myelomonocytic | 2.07 | 4.17 | 46.90 |
| Molt-4 | T | <0.1 | <0.1 | NS[c] |
| Raji | Burkitt lymphoma | <0.1 | <0.1 | 2.0 |

*a*) Cells ($5\times10^5$ cells/ml) were cultured for 48 h and supernatant sIL-6R generation was measured by the IL-6R FS-ELISA. IL-6R expression was analyzed by using a flow cytometer after staining with MT18 mAb and FITC-anti mouse IgG as described in "Materials and Methods."
*b*) Results are expressed as the mean of independent experiments.
*c*) Not significant (<0.5).

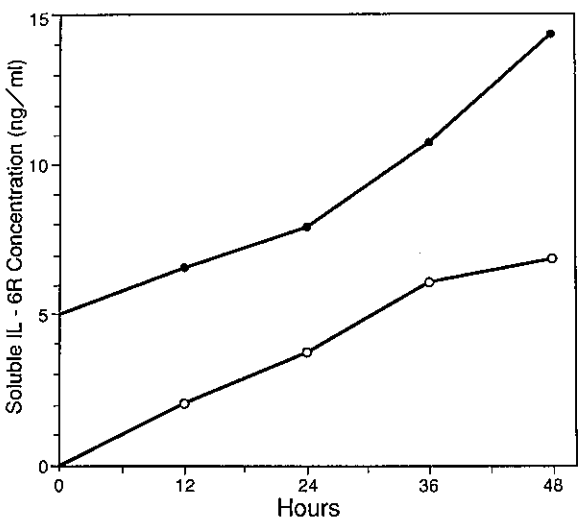


Fig. 2. Kinetic study of the generation of supernatant IL-6R in a culture of U266 cells. U266 cells were cultured at $5\times10^5$ cells/ml in RPMI 1640 medium containing 10% FCS, and analyzed for the levels of supernatant IL-6R by ELISA (○) and cell number (●).

transfected CHO cells was measured by IL-6R FS-ELISA. The fluorescence intensity of the supernatant of CHO-CN cells displayed a sigmoid increase, with increasing amounts of human sIL-6R in the supernatants. The detection limit of this assay was approximately 0.1 ng/ml. Furthermore, a 100-fold excess of IL-6 did not

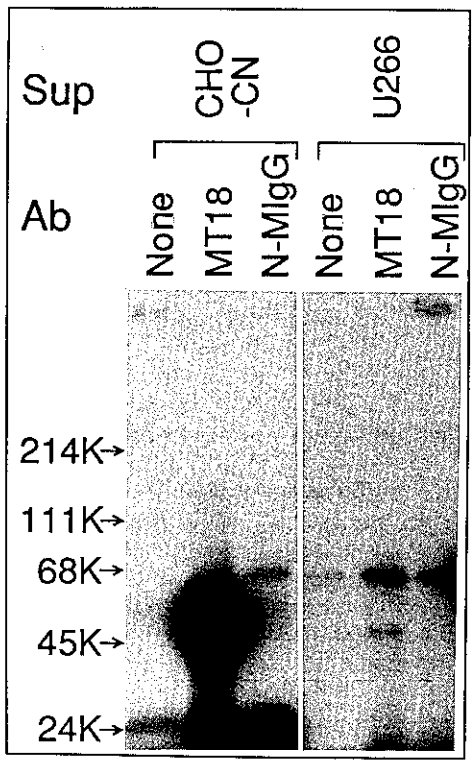


Fig. 3. SDS-PAGE analysis of radiolabeled and immunoprecipitated supernatant sIL-6R by using MT18 antibody. Culture supernatants of either CHO-CN cells or U266 cells were concentrated and radiolabeled with $Na^{125}I$ (None), followed by immunoprecipitation with the IL-6R-specific monoclonal antibody MT18, or with normal mouse IgG (N-MIgG).

affect the measurement of sIL-6R by the assay system (Table I). This assay is specific for the human IL-6R molecule because mouse sIL-6R,[23] human rIL-2, rIL-1$\beta$, rINF, rTNF-$\alpha$, IL-3, IL-4 and human IL-2R showed no reactivity in the ELISA at up to 1 $\mu$g of each soluble mediator per ml (data not shown).

**Spontaneous release of sIL-6R from IL-6R-bearing cell lines** Various cell lines were studied to analyze the relationship between the release of sIL-6R in the culture supernatant of cells, and the expression of IL-6R on the cell surface. As shown in Table II, all IL-6R-bearing cell lines (U266, RPMI 8226, RPMI1788, HL-60, THP-1, U937) spontaneously released nanogram levels of sIL-6R into the supernatant. In contrast, no supernatant IL-6R was detected in the cultures of IL-6R-negative cell lines (Molt-4 and Raji cells). Supernatant IL-6R generation was correlated with the cell surface level of IL-6R expression. Since a large amount of sIL-6R was produced from U266 cells, the kinetics of sIL-6R generation from U266 cells was examined at 12, 24, 36 and 48 h. The sIL-6R

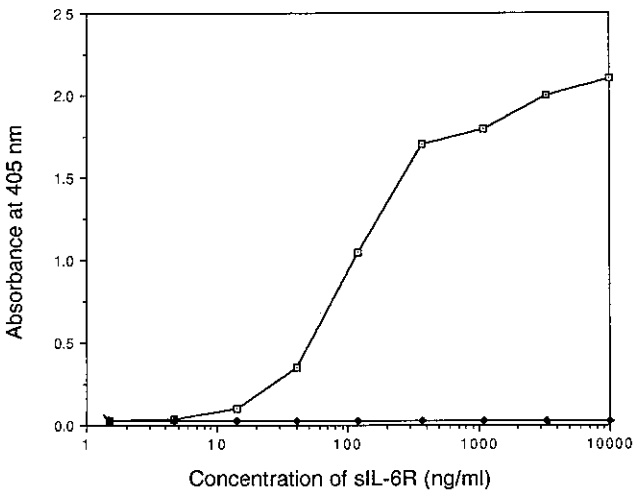


Fig. 4. ELISA for detecting the binding ability of sIL-6R to its ligand. Ten $\mu$g/ml of human recombinant IL-6 (⊡) or BSA (◆) was coated on microwells and the binding ability of sIL-6R was assayed by using recombinant sIL-6R at a concentration from 0.1 to 10000 ng/ml.

was detected in supernatant of 12-h-cultured cells and the level reached approximately 7.0 ng/ml of IL-6R at 36 h of culture (Fig. 2). The cellular viability was more than 98% by trypan blue dye exclusion test. The generation of IL-6R in the supernatant seemed to become larger when the number of U266 cells was bigger (data not shown).

**Molecular weight estimation of sIL-6R** Preliminary studies showed that the sIL-6R was released from U266 cells into the culture medium without fetal calf serum (FCS), and the cellular viability was more than 96% after 36 h incubation. Therefore, U266 cells were cultured at $1\times10^6$ cells/ml in FCS-free medium for 36 h, and the culture supernatant was concentrated approximately 200-fold by using Ultrafree (cut-off point 10,000, Millipore, Bedford, MA). The concentrated samples were radiolabeled, immunoprecipitated with MT18 and subjected to SDS-PAGE. As shown in Fig. 3, the MT18 antibody specifically precipitated a band of approximately 50–55 kDa in the culture supernatant of U266 cells (Fig. 3 lane 5). The result is consistent with the result of immunoprecipitation with MT18 of radiolabeled sIL-6R in CHO-CN cells (Fig. 3 lane 2).

**sIL-6R binds to IL-6** Recombinant sIL-6R was examined by ELISA for the ability to bind to IL-6 on the solid phase. As shown in Fig. 4, sIL-6R bound IL-6 efficiently in a dose-dependent manner. In contrast, no detectable amount of sIL-6R was bound to the solid phase on which BSA was coated instead of IL-6. These results demonstrate that purified recombinant sIL-6R binds IL-6 selectively and quantitatively.

## DISCUSSION

In this study, we showed by developing and using a quantitative hIL-6R ELISA system that soluble form of human IL-6R was released from myeloma cell lines U266, RPMI8226 and RPMI1788, and myelomonocytic cell lines U937, THP-1, and HL-60 into the culture supernatant. The assay system seemed specific for human IL-6R because it did not detect various other cytokines and soluble receptors. Moreover, it was not interfered with by an excess amount of IL-6. It has been reported that the monoclonal antibody MT18 used as the coating antibody in the assay was not inhibited by IL-6 as regards its recognition of IL-6R.[14] Taga *et al.* attempted to characterize human IL-6R by using both radiolabeled IL-6R and two mouse monoclonal antibodies specific to IL-6R, MT18 and PM1,[12] and they demonstrated that IL-6Rs were found on EBV-transformed B cell lines, myeloid cell lines and myeloma cell lines.[12, 14] In this study, the sIL-6Rs were only generated in culture supernatants of constitutively IL-6R-bearing cells. Moreover, the generation of sIL-6R detected by IL-6R ELISA was correlated with the level of receptor expression on the cell surface by flow cytometric analysis. The amount of IL-6R on the cell surface, estimated by Scatchard analysis, has been reported to correlate with the level of IL-6R mRNA.[12, 13] The gene encoding the human cellular IL-6R has been cloned, though no deleted cDNA clone was isolated which lacks the predicted transmembrane segment and intracytoplasmic regions of the molecule.[13] However, both cellular and soluble forms of IL-6R could be the product of the same gene via differential mRNA splicing. Another possible explanation is that sIL-6R is produced by shedding from cell surface receptor via protease digestion at the cell surface. Thus, the mechanism of sIL-6R generation is obscure at present. Additional studies will be required.

Molecular mass analysis of sIL-6R by immunoprecipitation gave a value of 50–55 kDa. Moreover, IL-6R released from a plasma cell line U266 and from a transformed CHO cell line which constitutively expressed sIL-6R[19] showed identical mobility, indicating that the recombinant sIL-6R is electrophoretically indistinguishable from the physiological soluble form of IL-6R. Recently, Novick *et al.* isolated soluble IL-6 receptor with a molecular weight of $50\pm10$ kDa from normal human urine, and showed that its $NH_2$-terminal 30-amino-acid sequence was identical with the $NH_2$-terminal sequence of IL-6R.[16] Thus, the molecular weight of sIL-6R in cell culture supernatants seems to be quite similar to that of sIL-6R in urine, indicating that the sIL-6R might be produced in normal immune responses *in vivo*.

Soluble forms of receptor have been found for several cell surface receptors including insulin,[24] epidermal

growth factor,[25] IL-2R,[26] TNF,[27-29] INF-$\gamma$,[16] Fc$\varepsilon$R II,[30,31] MHC[32-34] and CD8[35] in cell culture supernatants, sera and synovial fluids. The levels of soluble receptors were related to the disease activity, such as soluble IL-2R for rheumatoid arthritis and AIDS, and soluble Fc$\varepsilon$R II for allergy and B cell chronic lymphocytic leukemia. Most of the soluble forms of receptors have also been reported to retain the ligand affinity and could inhibit the cellular responses to the ligand. We have demonstrated by ELISA that recombinant sIL-6R is capable of binding efficiently to purified IL-6. Since this soluble IL-6R molecule can bind to its ligand, the sIL-6R could potentially affect IL-6-dependent biological responses. The release of sIL-6R by various IL-6R-bearing cells might serve for potentiating IL-6 functions[1] by complex formation with IL-6 and binding with cellular gp130 of IL-6R signal peptide.[15] Altered states of immune responsiveness, such as immune deficiency diseases including AIDS and ATL, autoimmune diseases and some lymphoid malignancies are suggested to be states involving unregulated IL-6 responses. Such pathologic states might be associated with increased production of soluble IL-6R.

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