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Reliability of Soluble IL-2 Receptor Measurements Obtained with Enzyme-Linked Immunosorbent Assay

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1989年から，放射線影響研究所の業績報告書は，従来の日英両文を併記した方式では発行しない．主要な報告書については，今後も日英両文で印刷するが，それぞれ別に発行する．内容が高度に専門的であり，一般の関心が少ないと思われる報告書については英文のみとし，日本文の要約を添付する．

これにより，広島・長崎の原爆電離放射線被曝の人体に及ぼす晩発性生物学的影響に関する最近の知見を今までよりも速やかにお知らせできることと思う．

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財団法人　放射線影響研究所
業　績　報　告　書

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研究課題　2-87

酵素免疫測定法を用いて得られた可溶性インターロイキン2レセプター測定の信頼性§

Reliability of Soluble IL-2 Receptor Measurements Obtained with Enzyme-Linked Immunosorbent Assay

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要　約

酵素免疫測定法（ELISA）を用いて各種自己免疫疾患患者の血清中のヒト可溶性インターロイキン2レセプター（IL-2R）の測定を行った．本法の感度と特性の決定のため，PHA，ツベルクリン精製蛋白誘導体，及びアロ・リンパ球で活性化した末梢血単核球の培養上清と細胞抽出液，並びに各種膠原病患者の血清について可溶性IL-2R測定を行った．その結果，他の研究室からの報告とよく一致する成績を得た．例えば，PHA刺激によって可溶性IL-2Rは最も速やかに多量産生された．また，健常者にも低値ながら可溶性IL-2Rが検出可能であったが，膠原病患者ではその血清値は有意に高かった．

原爆被爆者の血清可溶性IL-2Rについての信頼性の高いELISA測定は，研究計画書RP 2-87「成人健康調査受診者の自己免疫と自己免疫疾患についての研究」において収集されるデータの解釈の際に有用であろう．

§本報告書にはこの要約以外に訳文はない．

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Reliability of Soluble IL-2 Receptor Measurements Obtained with Enzyme-Linked Immunosorbent Assay§

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Summary

Using an enzyme-linked immunosorbent assay (ELISA), human soluble interleukin-2 receptors (IL-2R) were measured in the serum of patients with various autoimmune system diseases. To study the sensitivity and specificity of the assay, soluble IL-2Rs were measured in the culture supernatants and in the cell extracts of peripheral blood mononuclear cells activated with phytohemagglutinin (PHA), purified protein derivative of tuberculin, and allogeneic lymphocytes, as well as in the serum of patients with various collagen diseases. The results correlated well with reports from other laboratories. For example, when stimulated by PHA, the greatest amount of soluble IL-2Rs was produced at the fastest rate. In addition, soluble IL-2R levels in the serum of collagen disease patients were significantly higher than those in healthy persons, who themselves exhibited low levels of detectable soluble IL-2Rs.

It is hoped that reliable ELISA measurements of soluble IL-2Rs in the serum of atomic bomb survivors will assist in the interpretation of data collected during the work described in RP 2-87, a study of autoimmunity and autoimmune diseases in the Adult Health Study.

Introduction

IL-2Rs are expressed on the surface of activated T cells and bind specifically to the cell growth factor, IL-2, which plays an important role in T cell proliferation[1] and in the stimulation of other immunocompetent cells, such as natural killer cell activity,[2] the cytotoxicity of monocytes,[3] and B cell growth.[4]

§**The complete text of this report will not be available in Japanese.**

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Soluble IL-2Rs are released into the culture supernatant of activated T cells[5] and can be detected not only in vitro but also in vivo. It has been reported that elevation of the soluble IL-2R level is observed in the serum of patients with adult T cell leukemia,[6] whose IL-2R (Tac antigen) expression on leukemia cells has increased,[7] and in patients with other lymphoreticular malignancies, such as hairy cell leukemia,[8] Hodgkin's disease,[9] and chronic lymphocytic leukemia.[10] Elevated soluble IL-2R levels have also been reported in sera from patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA).[11] In animal models, elevated soluble IL-2R levels are correlated with the activity of progressive autoimmune disease.[12] Generally, elevated levels of IL-2R in serum might be associated with immune pathogenesis.

Furthermore, it is believed that soluble IL-2Rs in the serum are also released from normal lymphocytes activated in vivo, therefore soluble IL-2R levels in serum can serve as an index of lymphocyte activation. Thus, an attempt was made to establish and confirm a technique for measuring soluble IL-2R in the serum of patients with collagen diseases. The specificity of the measurement system was also studied in preparation for measuring soluble IL-2R in the supernatant of cultured lymphocytes with pokeweed mitogen (PWM). In the future, a study of the measurement will be carried out in order to apply it to sera obtained from the study cohort used by Fujiwara et al.[13]

Materials and methods

Cells. Peripheral blood mononuclear cells (PBMC) were prepared from normal heparinized venous blood by Ficoll-Hypaque density centrifugation as described previously.[14] The HTLV-1 positive T cell line, HUT102, was kindly provided by Dr. Robert C. Gallo, Laboratory of Tumor Cell Biology, NCI.

Monoclonal antibodies (mAb). Ta60a (γ_1) mAb and Ta60b (γ_1) mAb which recognize IL-2R (Tac antigen) were prepared as described previously.[15] Ta60a recognizes the same epitope as anti-Tac mAb, whereas Ta60b recognizes a different epitope. These mAbs were purified from hybridoma ascites by gel filtration and diethylaminoethyl (DEAE) cellulose chromatography.

Culture stimulants. PHA (Wellcome, Temple Hill, Dartford, England) was used at 2 μg/ml, purified protein derivative (PPD) of tuberculin (Connaught Medical Research Laboratories, Toronto, Canada) at 10 μg/ml and pokeweed mitogen (PWM, Gibco, Grand Island, NY) at a final dilution of 1/100 (v/v). PBMCs exposed to 20 Gy of cobalt irradiation with an Isotron-21 (Shimazu Co., Kyoto, Japan) were used as allogeneic stimulator cells.

Cell cultures. Five hundred thousand PBMCs were stimulated with PHA, PPD or 5×10^5 allogeneic lymphocytes in 1 ml of RPMI 1640 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. Triplicate cultures were established in 24-well plates (Nunc, Roskilde, Denmark) by incubation at 37°C in a humidified 5%

CO_2 atmosphere. The cultures were harvested at various time intervals, and the replicates were pooled and centrifuged at 500× g for 10 minutes to separate the cells and the supernatants. After washing, the cells were lysed in phosphate-buffered saline (PBS), pH 7.4, containing 1% Triton X-100 and the proteinase inhibitor, phenylmethyl sulfonyl-fluoride. Supernatants and cell extracts were stored at –20°C until use.

Patients and sera. Soluble IL-2Rs were studied in serum samples from 28 patients (26 females and 2 males, ages 21–57 years, mean ± SD = 36.1 ± 9.4) with SLE, 4 patients with progressive systemic sclerosis (PSS), 1 patient with Sjögren's syndrome, 4 patients with mixed connective tissue disease (MCTD), 1 patient with dermatomyositis (DM), and 7 patients with RA. Patients with collagen diseases other than SLE consisted of 15 females and 2 males, ages 26–67 (mean ± SD = 46.5 ± 10.7). American Rheumatism Association criteria were used for diagnosis of SLE[16] and RA,[17] criteria for PSS, Sjögren's syndrome, MCTD, and DM were according to Rodnan,[18] Fox et al,[19] Sharp,[20] and Bohan et al,[21] respectively.

Serum samples were obtained from 31 healthy persons who had undergone a complete physical examination conducted by the Hiroshima Prefectural Health Association, showing no abnormality in biochemical tests, including those for total protein, GOT, GPT, LDH, γ-GTP, LAP, Alp, ZTT, BUN, creatinin, and uric acid. These healthy persons were age- and sex-matched with patients having collagen disease. The healthy persons numbered 26 females and 5 males, ages 20–53 years (mean ± SD = 39.8 ± 9.6). Serum samples were stored at –20°C until use.

Conjugation of horseradish peroxidase (HRP) with Ta60a mAbs. The method reported by Nakane and Kawaoi[22] was modified. A volume of 0.1 ml of 1% 1-fluoro-2, 4-dinitrobenzene ethanol solution was added to 4 mg of HRP (Boehringer Mannheim, Federal Republic of Germany) in 1 ml of 0.3 M $NaHCO_3$ buffer, pH 8.1, and incubated at room temperature for 20 minutes. Then, the solution was dialyzed overnight at 4°C with 0.01 M $NaHCO_3$ buffer, pH 9.5. After adding 1 ml of 0.06 M $NaIO_4$, the solution was incubated at room temperature for 30 minutes. Then 1 ml of 0.16 M ethylene glycol was added, and dialysis was performed overnight at 4°C using 0.01 M $NaHCO_3$ buffer, pH 9.5. After dialysis, the solution was mixed with 1 ml of 5 mg/ml Ta60a equilibrated with 0.01 M NaHCO3 buffer, pH 9.5, and incubated at room temperature for three hours. Following incubation, the solution was dialyzed overnight at 4°C with PBS, and gel filtration was performed using Sephacryl S-200 equilibrated with PBS to collect fractions with antibody activities.

Assay for soluble IL-2R. Each of a 96-well flat-bottomed Nunc Immunoplate-II was coated overnight at 4°C 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 alone as a background control. Then, PBS containing 1% bovine serum albumin and 0.1% azide was added and incubated for two hours at room temperature. After washing, 50 μl of samples was added to the coated and uncoated wells,

incubated for two hours 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 two-hour incubation, the plates were washed and 100 μl of 2.5 mg/ml 0-phenylendiamine in 98 mM Na_2HPO_4 and 4 mM citric acid was added. After a 30-minute incubation, the reaction was stopped with 50 μl of 2 N H_2SO_4, and the absorbance of the wells was determined at 492 nm by the ELISA reader (Sanko Junyaku Co., Ltd., Tokyo). Five days after 2×10^5/ml of cells was cultured, a reference reagent, consisting of the cell-free supernatant of HUT 102 cell lines was used in all of these experiments. The undiluted supernatant was assigned a value of 10,000 U/ml and, as determined by ELISA performed on serial dilutions of this supernatant, absorbance values were used to generate a reference curve as shown in Figure 1. The absorbance of the test wells was then compared with the standard curve and converted to a numerical value.

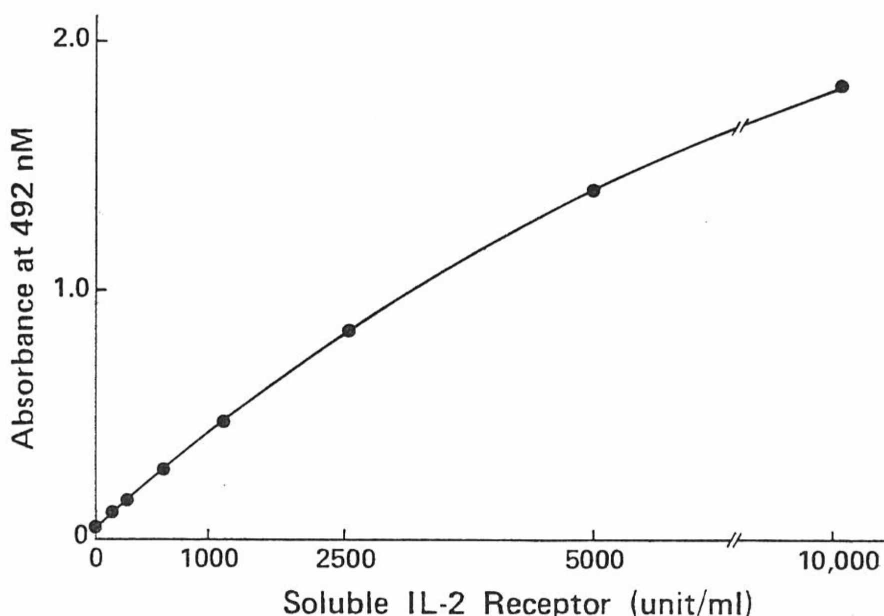

Figure 1. Standard curve for the soluble IL-2R assay. The undiluted supernatant of the HUT 102 cell line (2 × 10⁵/ml) cultured for five days was assigned a value of 10,000 U/ml.

Statistical analysis. Logarithm values were analyzed by Student's t test.

Results

Sensitivity of the ELISA. Various numbers of PBMCs were stimulated with PHA for three days, culture supernatant and cells were collected, and soluble IL-2Rs in the culture supernatant and detergent-extracted cells were measured. The concentration of soluble IL-2Rs in both culture supernatant and cell extract was

dependent on cell number. The minimum cell number for detecting soluble IL-2Rs was found to be 3×10^4 cells/ml and 1.5×10^4 cells/ml in culture supernatant and cell extract, respectively (Figure 2).

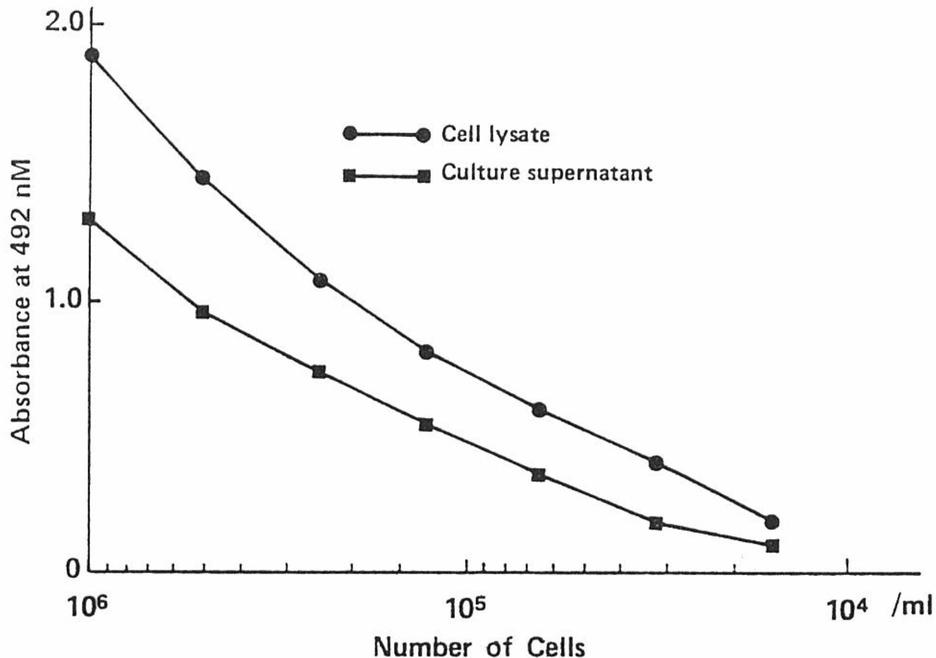

Figure 2. Sensitivity of the ELISA for detecting cellular and supernatant soluble IL-2R. PBMCs were cultured with 2 μg/ml of PHA for three days, and the culture supernatant and the detergent-solubilized cells were analyzed by ELISA.

Specificity of ELISA for soluble IL-2R. To determine the specificity of soluble IL-2R assays, PBMCs were stimulated with PWM for seven days, and IgM and soluble IL-2Rs in the supernatant were analyzed (Table 1). Very low absorbance was detected in the uncoated well receiving a sample of either HRP-labeled antihuman IgM or HRP-labeled Ta60a mAb. The absorbance was high (1.632), when culture supernatant and HRP-conjugated Ta60a mAb were added in the wells coated with Ta60b mAb, whereas it was very low when samples and HRP-conjugated antihuman IgM were added to the wells coated in the same way. The reaction was strongly positive when culture supernatant and HRP-labeled antihuman IgM were added in the wells coated with antihuman IgM antibodies, but there was hardly any reaction when culture supernatant and HRP-conjugated Ta60a mAb were added in the wells coated similarly. These results show that this assay was specific for the detection of soluble IL-2R.

Kinetics of soluble IL-2R in the cell extracts and supernatants. PBMCs at 5×10^5/ml were stimulated with PHA, PPD, or allogeneic stimulator cells, and measurements were made at various time intervals on soluble IL-2Rs in the cell extracts and supernatants. As shown in Figure 3, soluble IL-2R levels in

Table 1. Specificity of ELISA for the soluble IL-2 receptor[a]

Coating antibody	Sample	Second antibody	Absorbance at 492 nM
None	PWM sup	HRP anti-IgM	0.010
None	PWM sup	HRP Ta60a	0.020
Ta60b	PWM sup	HRP Ta60a	1.632
Ta60b	PWM sup	HRP anti-IgM	0.020
Anti-IgM	PWM sup	HRP anti-IgM	1.831
Anti-IgM	PWM sup	HRP Ta60a	0.042

[a]Wells coated with buffer alone, Ta60b (anti-IL-2 receptor antibody) or antihuman IgM antibody-treated supernatants (sup) of PBMC stimulated with PWM for seven days, followed by HRP-conjugated Ta60a (anti-IL-2 receptor) or HRP-conjugated antihuman IgM antibody.

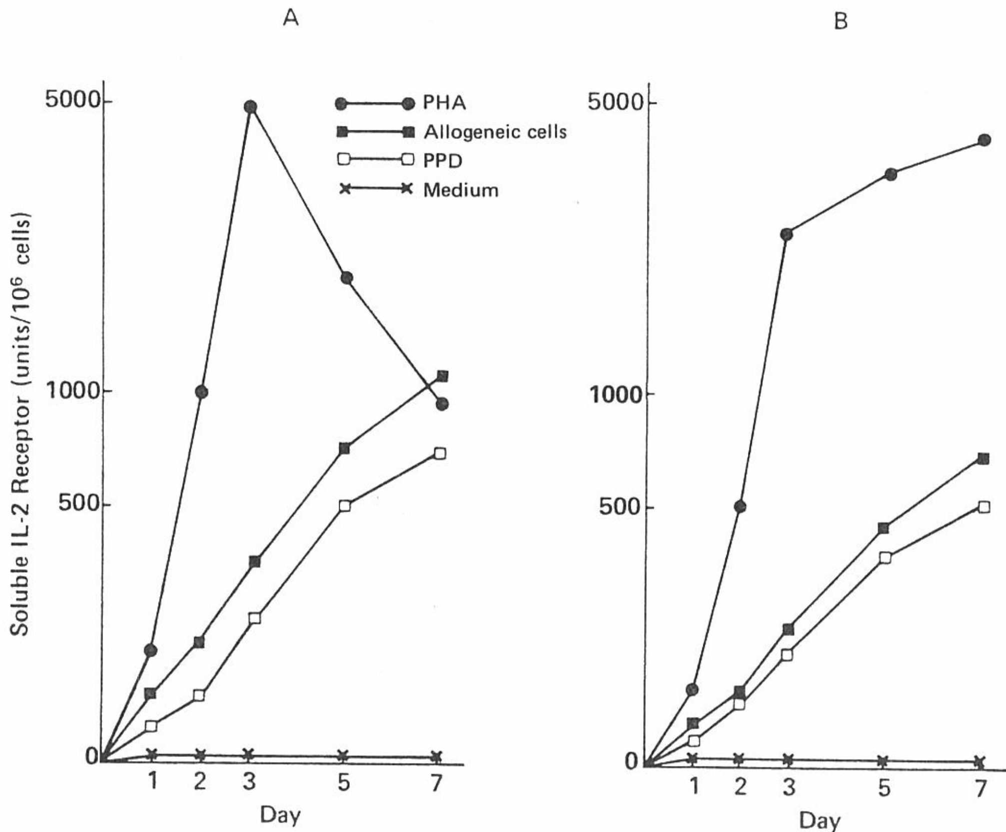

Figure 3. The kinetics of soluble IL-2R in the cell extracts (Panel A) and the supernatants (Panel B). PBMCs were cultured with PHA (•), allogeneic cells (□), PPD (□), or media (×) for various time intervals. Soluble IL-2R in the cell extracts and supernatant were then determined. The results represent the units of IL-2R produced per 5×10^5 cultured PBMCs.

both cell extracts and supernatants rose rapidly to high levels when stimulated with PHA. However, the elevation of the soluble IL-2R levels in cell extracts and supernatants was slow and low when PPD or allogeneic stimulator cells were used as the stimulant. When PBMCs were suspended in the medium as controls, little production of soluble IL-2Rs was observed.

Serum soluble IL-2R levels in patients with various collagen diseases. As shown in Figure 4, soluble IL-2R levels in the serum of patients with various collagen diseases, patients with SLE (484 ± 494 units/ml) or any other collagen diseases (810 ± 702 units/ml) were significantly higher than those of healthy persons (93 ± 78 units/ml, $P<0.001$). In healthy persons, soluble IL-2Rs were detectable, but only at a low level.

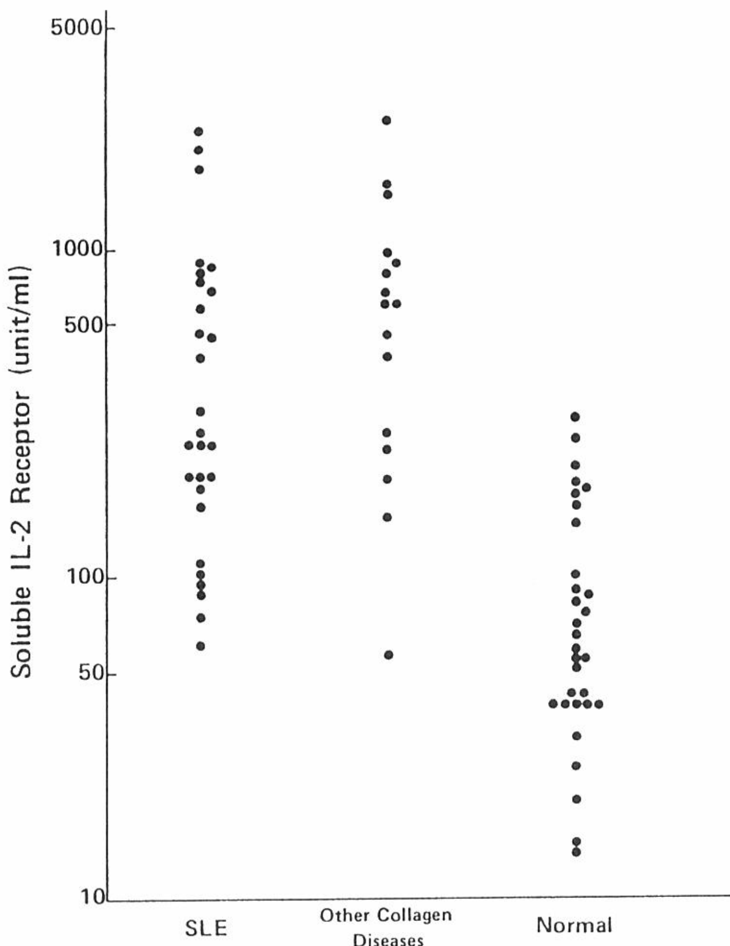

Figure 4. Soluble IL-2R levels in the serum of normal subjects and patients with collagen diseases. SLE: system lupus erythematosus

Discussion

In this study, soluble IL-2Rs were specifically measured in the culture supernatants and in the cell extracts of PBMCs activated with mitogens and allogeneic lymphocytes, as well as in serum, by means of the ELISA technique using two kinds of mAbs that recognize different epitopes of IL-2Rs.

Recently, it was reported that soluble IL-2Rs can bind with IL-2,[23] suggesting that soluble receptors may affect IL-2-dependent immune responses. This may be important in the immunoregulation of diseases, since soluble IL-2R competes with the binding of IL-2 to IL-2Rs on the surface of activated cells. In fact, altered production of soluble IL-2Rs has been reported in patients with immunodeficiency diseases or autoimmune diseases.[11] For example, a high concentration of soluble IL-2Rs has been detected in the serum of those who tested positive for antibodies against the human immunodeficiency virus, and the levels correlated negatively with the ratio of cells with CD4 antigens.[24]

When we applied the methodology described above to the serum of patients with various collagen diseases, the assay, which produced data comparable to those from other laboratories,[11,12] was confirmed, i.e., soluble IL-2R levels in the serum were significantly higher than those of healthy people.

Based on the results of our study, it is felt that ELISA measurements of soluble IL-2Rs in the serum of A-bomb survivors were reliable enough to contribute to the interpretation of data collected during the work described in RP 2-87, a study of autoimmunity and autoimmune diseases in the Adult Health Study.

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