

Soluble Interleukin-2 Receptors Inhibit Interleukin 2-Dependent Proliferation and Cytotoxicity: Explanation for Diminished Natural Killer Cell Activity in Cutaneous T-Cell Lymphomas In Vivo?

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In patients with cutaneous T-cell lymphomas (CTCL), soluble interleukin-2 receptor serum levels (sIL-2R) were determined by ELISA technique, and natural killer cell (NK) activity, by a 4-h chromium-51 release assay. Decrease of NK activity correlated with the augmentation of serum sIL-2R. After a 4-d stimulation with interleukin 2 CTCL patients' peripheral mononuclear cells (PMC) showed an increase of cytotoxic activity similar to that in healthy donors' PMC. Normal donors' PMC demonstrated a diminished IL-2-induced cytotoxic activity in 25% CTCL serum (sIL-2R of 3000, 7330, and 10700 U/ml, respectively) compared to control serum (sIL-2R of 400, 340, and 420 U/ml, respec-

tively). IL-2-dependent proliferation of 2-d phytohemagglutinin (PHA) blasts was lower in CTCL serum than in control serum. sIL-2R was enriched from one CTCL patient's serum by IL-2 affinity chromatography. Transfection of the Tac gene into NIH/3T3 fibroblasts resulted in the production of a recombinant sIL-2R. The presence of enriched native or recombinant sIL-2R inhibited interleukin-2-dependent generation of cytotoxic activity and PHA blast proliferation. We suggest that elevated sIL-2R levels account for diminished NK activity by neutralizing interleukin 2 in CTCL patients. *J Invest Dermatol* 98:50-54, 1992

Receptor shedding is a well-known phenomenon in cellular biology [1-4]. Soluble molecules have been detected for a broad range of surface proteins; they include T-cell antigens such as CD 8 [2], adhesion molecules [3] such as intercellular adhesion molecule-1, and cytokine receptors such as the alpha-chain (p55 kD protein or Tac protein) of the high-affinity interleukin-2 receptor [4]. The physiologic significance of these released surface proteins is still unclear.

Recently, elevated serum levels of soluble interleukin-2 receptors (Tac-protein) in patients with cutaneous T-cell lymphomas (CTCL) were reported [5,6]. These diseases include a heterogeneous group of lymphoproliferative disorders originating in the skin [7]. A stage-related decrease of natural-killer cell (NK) activity in these patients was reported [8,9]. Because interleukin 2 is a potent stimulating factor for NK activity [10], and sIL-2R is able to

bind IL-2 efficiently [11], we analyzed the interactions of NK activity and sIL-2R in vivo in CTCL patients. In order to define the role of the soluble Tac protein in IL-2-dependent proliferation and generation of cytotoxicity, sIL-2R was enriched from the serum of a CTCL patient and a recombinant molecule was produced. Their influence on interleukin 2 in vitro effects was studied.

MATERIALS AND METHODS

Patients Seventeen non-leukemic patients with histologically proved CTCL in different stages were investigated. Patients' characteristics are summarized in Table I. Patients were without systemic treatment or intensive topical treatment for at least 2 weeks before peripheral blood mononuclear cells for Cr-51 release assay and serum for determination of the sIL-2R were taken.

Quantitation of Soluble Interleukin-2 Receptors sIL-2R in the serum or in culture medium were determined by sandwich ELISA (T Cell Sciences Inc., Cambridge, USA) [12]. The test employs two non-competing murine monoclonal antibodies to the alpha-chain of human IL-2R, and was carried out according to the manufacturer's instructions. After the development of the color the ELISA plates were read at 490 nm on a Dynatech MR 700 microplate reader. Units of sIL-2R were calculated from a standard curve constructed on the basis of a supernatant from phytohemagglutinin-stimulated peripheral blood mononuclear cells (T Cell Sciences). Interassay variation was 5%.

Preparation of Peripheral Mononuclear Cells (PMC) Human PMC were obtained from healthy age-matched volunteers and from CTCL patients after informed consent, and separ-

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Abbreviations:

CM: complete medium

CTCL: cutaneous T-cell lymphoma

IL-2: interleukin 2

NK: natural killer cell

PBS: phosphate-buffered saline

PHA: phytohemagglutinin

PMC: peripheral mononuclear cell

sIL-2R: soluble interleukin-2 receptor (Tac-protein)

SL: specific lysis

Table I. Patients' Characteristics and Immunoparameters (sIL-2R, NK activity)^a

Patient	Stage TNM [32]	Histologic Diagnosis	Pretreatment	sIL-2R (U/ml)	Specific Lysis (%)
GE	IIA	Mycosis fungoides	external steroids, electron beam radiation	1,690	5.0
HE	IIB	Mycosis fungoides	external steroids	385	13.0
WA	IA	Mycosis fungoides	external steroids	405	16.5
BA	IIA	Mycosis fungoides	external steroids	330	4.0
HO	IIB	High-grade pleomorphic	MTX, IFN- α , PUVA	538	30.7
ST	IIB	Mycosis fungoides	BCNU topical, external steroids	3,000	6.4
TI	III	Mycosis fungoides	external steroids	1,320	4.0
KA	IIA	Mycosis fungoides	external steroids, IFN- α	3,570	6.0
KAI	IIB	High-grade peripheral	PUVA, external steroids	10,700	6.0
KE	IIA	Mycosis fungoides	external steroids	1,190	21.9
RE	IIA	High-grade peripheral	external steroids	720	15.0
SCH	IA	Mycosis fungoides	external steroids	950	10.5
TR	IA	Mycosis fungoides	PUVA, external steroids	800	42.3
BET	IIA	Mycosis fungoides	none	7,330	2.3
HER	IA	Mycosis fungoides	PUVA, external steroids	1,110	6.9
KÜ	IB	Granulomatous slack skin	external steroids	650	24.7
VA	IIB	Mycosis fungoides	external steroids	300	22.5

^a MTX, methotrexate; BCNU, carmustine, IFN- α , interferon- α 2a.

ated by Ficoll-Hypaque gradient centrifugation. After separation the cells were washed twice and immediately resuspended in complete medium (CM) consisting of RPMI 1640 (Gibco/BRL, Eggenstein, FRG) supplemented with 5% fetal calf serum (Gibco/BRL) or human serum as indicated, 2 mM L-glutamine (Seromed, Berlin, FRG), 10 mM sodium pyruvate (Seromed), 100 U/ml penicillin (Seromed), 100 μ g/ml streptomycin (Seromed), and 50 μ g gentamicin (Seromed). For determination of NK activity, cells were used immediately after preparation in the Cr-51 release assay.

Induction of Cytotoxicity PMC were incubated for 4 d in CM supplemented with different concentrations of recombinant IL-2, kindly provided by Eurocetus, Frankfurt, FRG. The cell concentration was 1×10^6 /ml, conditions were 37°C, 100% humidity, 5% CO₂. Before use the cells were harvested and washed twice with CM. As indicated in the results, in some experiments CM contained 25% allogeneic serum from healthy volunteers or CTCL patients. In the stimulation experiments using recombinant or natural Tac protein PMC were taken from healthy donors and incubated in CM supplemented with 5% autologous serum.

Cr-51 Release Assay Cytotoxic activity was determined in a 4-h Cr-51 release assay as previously described [10], with the "NK-sensitive" erythroblastoma cell line K562 as target cells. At the time of the assay the cell line was cultured in logarithmic proliferation in CM, and without evidence of mycoplasma infection. The effector:target ratio was 40:1. Spontaneous release was assessed by incubating labeled K 562 in medium alone, and total release was evaluated by a solution of SDS. After harvesting the supernatants with a Skatron filter-stick system, radioactivity was measured in a Pharmacia gamma counter. Specific lysis (SL) was calculated as follows:

$$SL(\%) = \frac{\text{Mean experimental release} - \text{mean spontaneous release}}{\text{Mean total release} - \text{mean spontaneous release}} \times 100.$$

Proliferation Assays In order to study the influence of CTCL serum or Tac-protein on IL-2-dependent proliferation, PMC of healthy donors were stimulated with phytohemagglutinin for 2 d. After washing they were incubated with various concentrations of recombinant IL-2 for 3 d. Proliferation was quantified by ³H-thymidine uptake in triplicate as previously described [13] or by a colorimetric assay, which uses hexosaminidase as indicator substrate [14].

Expression System for sIL-2R The gene coding for a soluble Tac species consisting of 223 amino acids and lacking the 28 C-terminal residues (i.e., most of the membrane anchor and the whole intracytoplasmatic domain [15]) was prepared by *B*enI digestion and *B*amHI conversion from a human cDNA clone. It originated from peripheral blood lymphocytes and was modified by inserting the stop-signal bearing oligonucleotide TGACTGCAGTCA into the *N*aeI site within the terminal region of the Tac gene sequence [16]. The isolated modified gene was cloned into the *X*hoI site of the BMGneo-expression vector (kindly provided by F. Melchers, Basel [17]) by filling up with Klenow polymerase and blunt-end ligation (enzymes from Boehringer Mannheim, FRG).

Cultivation of Fibroblast Cells Used for IL-2R Expression NIH/3T3 fibroblasts were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2×10^{-2} M L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Biochrom KG, Berlin, FRG) at 10% CO₂, 37°C, and

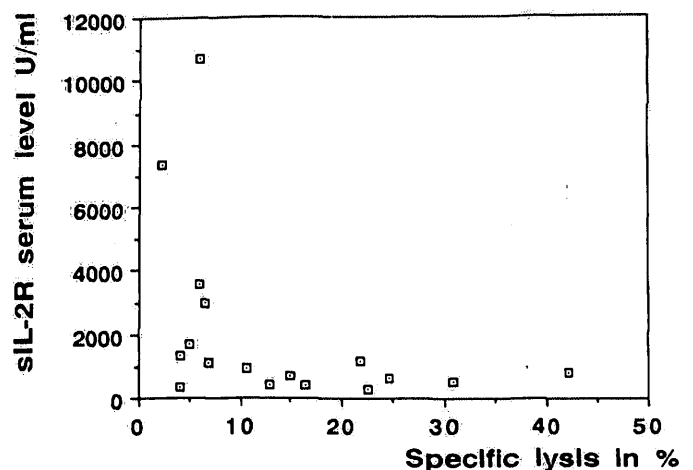


Figure 1. Negative rank correlation ($p < 0.03$) between NK activity (Cr-51 release assay, target cell K 562) and soluble interleukin-2 receptor serum levels in 17 CTCL patients.

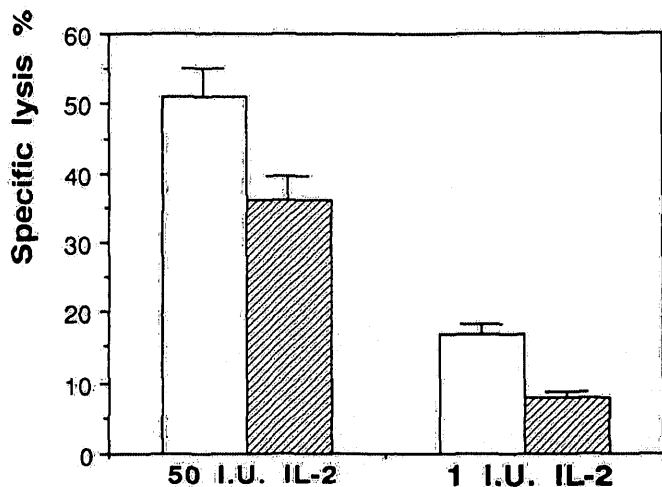


Figure 2. IL-2-induced generation of 4-d natural killer cell activity (specific lysis) is lower in CTCL serum than in healthy donor's serum (Cr-51 release assay, target cell K 562). □, 25% control serum (sIL-2R = 400 U/ml). ▨, 25% CTCL serum (sIL-2R = 3000 U/ml).

100% humidity. For selection, isolation, and maintenance of transfected cells 700 μ g/ml G 418 (Geneticin, Gibco/BRL) were added to the complete medium.

Transfection Stable transfection of NIH/3T3 cells was performed using the calcium-phosphate-precipitation method [18].

Purification of Soluble Tac from Fibroblast Supernatant or CTCL Serum The purification of sIL-2R was performed by IL-2 affinity chromatography. About 80 mg of recombinant IL-2 U 94/T125 [19] were coupled to 4.2 g (dry weight) CNBr-preactivated sepharose 4B (Pharmacia) according to the manufacturer's protocol. A small syringe column was filled with about 1.5 ml swollen affinity matrix and equilibrated with PBS buffer, pH 7.4.

For purification of recombinant sIL-2R about 250 ml of conditioned fibroblast medium were pumped continuously through the column during 14 h at a low flow rate of about 0.5 ml/min. After loading, the column was washed with PBS until the extinction at 280 nm returned to starting levels. Elution was performed with 0.2 M acetic acid/0.2 M NaCl [20,21]. One milliliter fractions of eluate were collected, neutralized with 0.3 vol 1 M Tris/Cl, pH 8, to a final pH of 7 and stored frozen. Tac-free control buffer was treated the same way. The analogous procedure was performed using 20 ml of 1:1 PBS-diluted CTCL serum.

Western Blot Analysis Western blots were performed according to standard procedures [22] under reducing and nonreducing conditions. We used 7G7/B6 (kindly provided by D. L. Nelson, NIH, Bethesda, MD) and R239 (kindly provided by R. J. Robb, Glenoden, PA) as primary antibodies and an alkaline-phosphatase coupled secondary antibody (Sigma, Taufkirchen, FRG).

RESULTS

Statistical Analysis of NK Activity and sIL-2R sIL-2R was found significantly ($p < 0.01$, t test) higher in CTCL patients (mean \pm SD is 2058 \pm 2833 U/ml, $n = 17$) than in age-matched control patients (mean \pm SD is 412 \pm 72 U/ml, $n = 17$). NK activity of fresh PMC was significantly lower in CTCL patients (13.1 \pm 11.5% SL, $n = 17$) compared to healthy controls (28.2 \pm 17.8% SL, $n = 10$) ($p \leq 0.01$, t test).

Statistical analysis of the *in vivo* immunologic findings revealed a negative correlation between NK activity and sIL-2R in CTCL patients (Spearman's rank correlation coefficient is -0.5423 , $p <$

0.03, $n = 17$, Fig 1) as well as in CTCL patients plus 10 control patients, whose sIL-2R and NK activity had been determined simultaneously (Spearman's rank correlation coefficient is -0.4568 , $p < 0.02$, $n = 27$).

Stimulation Experiments Using CTCL and Normal Donors' PMC A 4-d stimulation of PMC (1000 IU IL-2/ml) from CTCL patients resulted in an average increase of cytotoxic activity of 19.2 \pm 16.5% in CTCL patients ($n = 12$) and an increase of 29.9 \pm 12.8% in controls ($n = 10$) (no significant difference).

Stimulation Experiments Using CTCL and Normal Donor's Serum PMC from healthy donors were stimulated in the serum of three CTCL patients (sIL-2R of 3000, 7330, and 10700 U/ml, respectively) and in control serum (sIL-2R of 320, 400, and 420 U/ml, respectively). A 4-d stimulation of a normal donor's PMC with 50 or 5 IU IL-2 in 25% CTCL serum resulted in a reduced induction of cytotoxic activity compared to control serum for an effector:target ratio of 40:1. One typical example is shown in Fig 2. In a similar manner, IL-2-dependent PHA-blast proliferation (hexosaminidase assay [14]) was 60% (50 IU IL-2/ml) or 50% (5 IU IL-2/ml) lower in CTCL serum (sIL-2R = 3000 U/ml) compared to control serum (sIL-2R = 400 U/ml).

Tac Production by Transfected Fibroblasts In order to enhance the basic expression of soluble Tac, 5 μ M CdCl₂ were added to the medium, because the expression of a foreign gene inserted into the BMGneo vector is mediated by a metallothionein promoter [18]. The supernatants of Cd⁺⁺-treated fibroblasts reached soluble Tac concentrations (ELISA) up to 30,000 U/ml during 4 d of incubation, i.e., about 6000 U/24 h/10⁶ cells. 1,000,000 U were determined as about 3 μ g Tac protein.

Purification of sIL-2R With fibroblast supernatants, peak fractions of IL-2 affinity chromatography exhibited soluble Tac concentrations up to about 1,000,000 U/ml. Thus, a single purification step was able to concentrate the protein more than thirtyfold. Tac peak fractions were highly pure, and medium-derived proteins like albumin were removed efficiently (data not shown).

From 20 ml of one CTCL patient's serum (patient ST, sIL-2R = 3000 U/ml), a single purification step resulted in a maximal concentration of 8000 U/ml sIL-2R. The elution profile is shown in Fig 3.

Western Blotting of Purified Recombinant Tac Proteins Western blots were performed using 7G7/B6 (non-reducing conditions) and R239 (reducing and non-reducing conditions) as primary antibodies. The results are summarized in Fig 4. The main

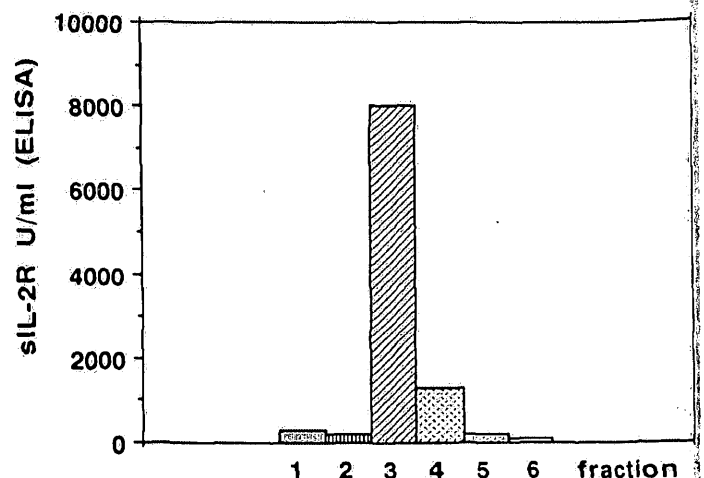


Figure 3. Elution profile of IL-2-affinity chromatography of one CTCL patient's serum determined by ELISA.

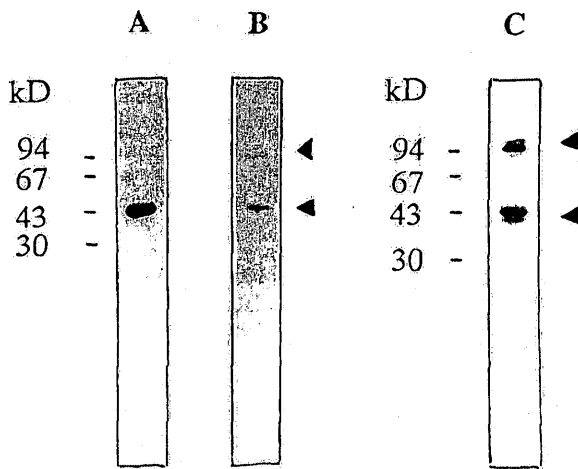


Figure 4. Western blots performed with IL-2-affinity purified sIL-2R. Lanes A, B: R 239 as primary antibody. Lane C: 7G7/B6 as primary antibody. Lane A: reducing conditions. Lanes B, C: Non-reducing conditions.

band at about 45 kD corresponds to the soluble Tac monomer, whereas the band at about 100 kD—present only under non-reducing conditions—obviously is due to dimer formation [21]. Extra bands below and above the main bands probably correspond to glycosylation modifications.

Inhibition of IL-2-Induced Generation of Cytotoxicity and Proliferation by Natural and Recombinant Tac Protein. In the presence of fraction 6 (Fig 3; 25% of total volume), 5 IU IL-2/ml induced a SL of 56.7% in healthy donor's PMC. The addition (25% of total volume) of the peak fraction 3 (Fig 3; sIL-2R = 8000 U/ml) resulted in a SL of 32.3% that presents a 57% inhibition. Proliferation of PHA blasts was reduced by 78.5% in the presence of fraction 3 compared to fraction 6 ($3,429 \pm 478$ cpm versus $15,900 \pm 1,387$ cpm). Healthy donors' PMC developed a reduced enhancement of cytotoxic activity in the presence of recombinant Tac protein in a dose-dependent manner. High amounts of recombinant sIL-2R blocked IL-2 (5 IU/ml)-induced enhancement of cytotoxicity completely. These results are summarized in Fig 5.

IL-2-dependent proliferation of PHA blasts was inhibited in a dose-dependent manner. A 50% inhibition was achieved by a sIL-2R concentration of 18,000 U/ml (50 IU IL-2).

DISCUSSION

Suppressed NK activity in CTCL patients is a well-known phenomenon [8,9]. In this report we provide evidence that sIL-2R may account for the diminished NK activity. Stimulation experiments with IL-2 demonstrated that the response of PMC from CTCL patients to the IL-2 signal resembles that of normal donors' PMC. This supports the notion that serum factors might have an impact on cytotoxic functions in the patients' peripheral blood. Proliferation and cytotoxicity experiments indicated an IL-2-inhibiting activity in the serum of these patients associated with high sIL-2R serum levels. sIL-2R from one CTCL patient's serum was enriched by IL-2 affinity chromatography. This sIL-2R-enriched fraction inhibited IL-2-dependent proliferation more efficiently than the native serum. Therefore, the "IL-2 inhibitor" in CTCL serum seems to be able to bind to IL-2. Because large volumes of CTCL serum were not available, and the presence of other IL-2-binding proteins such as antibodies to IL-2 or shed fragments of the beta-chain of the

high-affinity IL-2 receptor [23] in the purified serum fraction cannot be excluded, transfection experiments were performed to clarify the specific role of the Tac protein on IL-2-dependent effects.

The transfection of a modified Tac gene using a BMGneo expression vector system resulted in the production of a recombinant Tac protein. In order to rule out disturbing factors in the supernatant of the transfected fibroblasts the recombinant protein was purified by an IL-2 affinity column, again. These purified Tac proteins were characterized by Western blotting. As previously described the protein was found in monomeric and dimeric forms [20,21]. The free SH-residue at position Cys 192 could explain the dimer formation of the recombinant soluble Tac species used in our experiments [15,24]. Using this recombinant protein we found a dose-dependent inhibition of IL-2-mediated proliferation and induction of cytotoxicity. These results correspond well to the observation that the released IL-2 receptor is able to bind IL-2 efficiently [11]. However, comparing the neutralizing capacity of recombinant and native sIL-2R, the natural protein seems to be more effective than the recombinant sIL-2R. Possible explanations for this phenomenon include distinct affinities to their ligand due to different degrees of dimer formation or changes in the protein structure by the manner of glycosylation.

As a consequence, sIL-2R seems to be a physiologic inhibitor of IL-2 [25]. Because this cytokine is able to stimulate its production by a positive feed-back mechanism [26], the release of the surface receptor neutralizing the corresponding cytokine might be an elegant method to limit its biologic effects.

Recently, proteins isolated from human urine had been shown to be structurally related to the tumor-necrosis factor-alpha receptor. These proteins were able to bind tumor-necrosis factor-alpha, and provided protection against cytotoxic effects of its ligand in vitro [27]. In addition, the soluble extracellular portion of the interleukin-1 receptor seems to be a regulator of allograft rejection, probably by its biologic efficacy in neutralizing interleukin-1 [28].

So far, soluble receptors also are known to exist for cytokines such as interleukin-1 [28], interleukin-2 [4], interleukin-4 [29], or tumor-necrosis factor-alpha [27] and a broad spectrum of other molecules [2,3]. Therefore, we suggest that receptor shedding might be a simple and widespread principle to regulate biologic functions.

It has been reported that highly elevated serum sIL-2R levels are predictors of an unfavorable prognosis in patients with lymphoproliferative disorders such as lymphoblastic lymphoma [30], Hodgkin

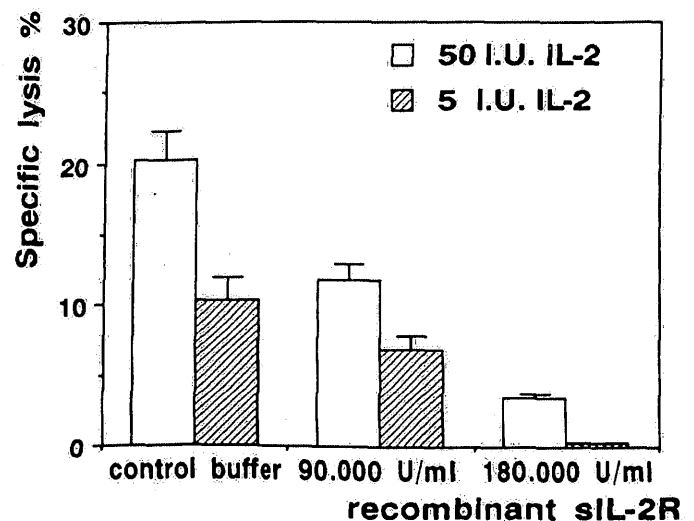


Figure 5. Recombinant Tac protein inhibits IL-2-induced increase of cytotoxic activity in a dose-dependent manner (specific lysis of IL-2-stimulated PMC minus specific lysis of cultured PMC without IL-2 addition, Cr-51 release assay, target cell K 562).

disease [31], or CTCL [5]. We suggest that the elevated levels of this soluble cytokine receptor chain have an impact on IL-2-dependent immunosurveillance.

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