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The Development of Anti-Interleukin-2 Antibodies in Patients Treated with Recombinant Human Interleukin-2 (IL-2)

MARK ALLEGRETTA,[1] MICHAEL B. ATKINS,[1] ROY A. DEMPSEY,[1] EDWARD C. BRADLEY,[2] MICHAEL W. KONRAD,[2] ANN CHILDS,[2] SIDNEY N. WOLFE,[2] and JAMES W. MIER[1]

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Approximately 65% (11/17) of cancer patients participating in an ongoing Phase I clinical trial with recombinant interleukin-2 developed nonneutralizing serum IgG anti-interleukin-2 antibodies within 1 month of initiating therapy. These antibodies could be detected using any of several standard techniques including immunoblots and enzyme-linked immunosorbent assays. Western blot analysis and retention experiments with protein A-Sepharose indicate that the antibodies are specific for interleukin-2. The interleukin-2 mutein utilized in this clinical trial (des-ala-ser$_{125}$ r-IL-2) differs from the major species of the human T cell-derived lymphokine in that it lacks the N-terminal alanine of the native molecule, is not glycosylated, and possesses a serine–cysteine substitution at position 125. Another recombinant interleukin-2, identical to the mutein except that it retains the cysteine at position 125 (des-ala-cys$_{125}$ r-IL-2), strongly competes with the mutein in competitive enzyme-linked immunosorbent assays, suggesting that the amino acid substitution is not responsible for the recognition of the molecule by serum antibodies. Conversely, nonrecombinant T cell-derived interleukin-2 fails to compete in these assays and is not retained by protein A-Sepharose columns when mixed with high-titer antiserum. These results suggest that the anti-interleukin-2 serum antibodies generated in the course of treatment do not react with the nonrecombinant lymphokine but recognize epitopes peculiar to recombinant forms which are not dependent on the amino acid substitution at position 125. The failure of the antibodies to neutralize the biological activity of recombinant interleukin-2 (IL-2) in lymphocyte proliferation assays and to bind to the native lymphokine suggests that they may not affect IL-2-dependent cellular immune functions *in vivo*.

[1]Division of Hematology–Oncology, Department of Medicine, New England Medical Center, Boston, Massachusetts 02111.
[2]Cetus Corporation, Emeryville, California.

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INTRODUCTION

Interleukin-2 (IL-2) was initially described as a T cell-derived growth factor specific for lectin and antigen-activated T lymphocytes (1, 2). It has since been shown to augment the cytolytic activity of murine and human natural killer (NK) cells *in vitro* (3, 4) and to stimulate the release of soluble mediators from helper T cells (5). Interleukin-2 also induces the proliferation of NK cells (6) and the generation of lymphokine-activated killer (LAK) cells, which lyse a wide range of tumor cells resistant to unstimulated lymphocytes (7–10). The intraperitoneal injection of IL-2 enhances murine cytolytic T-cell and NK-cell activity against lymphoma cell lines (11), and the injection of high doses of recombinant lymphokine has recently been reported to cause regression of metastatic tumors (12).

The cloning of the human IL-2 gene, modified by site-directed mutagenesis to encode a serine rather than cysteine at position 125, and its expression in *Escherichia coli* (13, 14) have enabled the production of large quantities of IL-2 for clinical testing and over 30 trials with this IL-2 mutein are currently underway. The amino acid substitution was chosen to prevent inappropriate intra- and intermolecular disulfide bridge formation, which markedly reduces the stability and biological activity of native sequence IL-2. This structural alteration in the IL-2 molecule, the well-known immunogenicity of other lymphokines such as interferon (15, 16), and other considerations led us to

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suspect that the IL-2 utilized in our Phase I clinical trial might elicit antibodies to IL-2. This investigation was therefore undertaken to determine the frequency, specificity, and clinical significance of anti-IL-2 antibodies in patients with refractory cancer treated with recombinant IL-2.

MATERIALS AND METHODS

Serum Samples

A Phase I clinical trial of recombinant interleukin-2 (IL-2) was initiated at our institution in August 1984. Patients enrolled in the study had malignancies of various histologic types refractory to conventional therapy. Patients were treated weekly with intravenous bolus injections of recombinant IL-2 in escalating doses ranging from 10,000 to 1,000,000 units/M^2. Sequential serum samples were obtained prior to entry into the study and at weekly intervals during the course of treatment. Aliquots of serum were frozen at −20°C until assayed. Seropositive samples repeatedly used in the various assays were sterilized by filtration and preserved with 0.01% thiomerosol. Pooled serum from healthy donors served as a control for the various assays.

Interleukin-2 Preparations

The interleukin-2 administered to the patients participating in the Phase I clinical trial was provided as a lyophilized powder by Cetus Corporation (Emeryville, CA). This recombinant material (des-ala-ser_{125} r-IL-2) differs from the major species of IL-2 produced by lectin-stimulated human lymphocytes at the N terminus, where it lacks an alanine residue, and at position 125, where a serine is substituted for a cysteine residue (14). Furthermore, the recombinant species is not glycosylated. This IL-2 mutein has biological activity which is indistinguishable from that of native-sequence recombinant or nonrecombinant IL-2 in a variety of *in vitro* proliferation and lymphocyte activation assays (17). One unit per millimeter, the concentration that induces 50% maximal [^{3}H]thymidine incorporation in activated T cells, corresponds to a concentration of approximately 0.35 ng/ml. The preparations used in the aforementioned clinical trial were negative for endotoxin (<0.01 ng/mg protein in the *Limulus* assay) and yielded a single 15-kD protein band on $NaDodSO_4$–polyacrylamide gel electrophoresis. Recombinant IL-2 preparations with the normal cysteine at position 125 but lacking the N-terminal alanine (des-ala-cys_{125} r-IL-2) were also provided by Cetus Corporation for various *in vitro* assays. Nonrecombinant IL-2 was purified from the conditioned medium of human peripheral blood mononuclear cells stimulated by the ionophore meserein (LC Services, Woburn, MA) by sequential reverse-phase high-pressure liquid chromatography and gel filtration with Sephadex G-75 (Pharmacia). The resultant lymphokine preparation had a specific activity of over 50,000 U/mg protein.

Enzyme-Linked Immunosorbent Assay (ELISA) for Anti-IL-2 Antibodies

The wells of a 96-well microtiter plate (Nunc Immunoplate) were coated with IL-2 by incubation with a 50 m*M* Na_2CO_3, 50 m*M* $NaHCO_3$ solution, pH 9.5 (coating buffer), containing 2.5 μg/ml des-ala-ser_{125} r-IL-2 (Cetus Corporation, Emeryville, CA) for 18 hr at 4°C. The plates are washed four times with phosphate-buffered saline containing 0.5% Tween-20 and 0.01% thiomerosol (PBS-Tween) and then incubated at room temperature with 100 μl of a 1–10% solution of serum in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum. After 2 hr, the plates were again washed four times with PBS-Tween and then incubated at room temperature with a 1:1000 dilution of affinity-purified goat anti-human IgG-specific Fab'_2 fragments conjugated with alkaline phosphatase (Sigma). After 1 hr, the plates were again washed four times with PBS-Tween, and 100 μl of a 1.0 mg/ml para-nitrophenyl phosphate solution (Sigma) in substrate buffer containing 2 m*M* $MgCl_2$ was added to each well. The absorbance at 410 nm was recorded with a Dynatech ELISA tester.

In some experiments, serial 1:2 dilutions of serum were assayed in the above ELISA. Anti-IL-2 antibody titers were defined as the reciprocal of the minimal serum dilution yielding an ELISA value equal to that obtained with a 1.0% dilution of normal serum. Nine normal donors were used to define the background for these quantitative assays. The ELISA reactivities of the nine normal sera were similar and, in contrast to those of seropositive IL-2 recipients, were relatively constant over a wide range of serum concentrations. Titers over 200 were considered positive.

A modification of the above was employed to determine the cross-reactivity of native and recom-

binant IL-2 preparations in a competitive ELISA. Ninety-six-well flat-bottom polystyrene plates (Dynatech, Immulon 1) were coated with des-ala-ser$_{125}$ r-IL-2 by dissolving the IL-2 (5 μg/ml) in $NaCO_3$ buffer (0.1 *M*, pH 9.6) and adding 100 μl to each well. The plates were incubated at 4°C overnight and then washed four times with PBS containing 0.05% Tween-20. The patients' sera were diluted in PBS containing 0.5% BSA and 0.05% Tween-20 and mixed with a constant volume of a diluted IL-2 preparation. The two recombinant preparations were diluted with 0.1% $NaDodSO_4$ in 50 m*M* Na_2HPO_4, pH 7.0. Twenty microliters of diluted des-ala-cys$_{125}$ r-IL-2 or des-ala-ser$_{125}$ r-IL-2 was mixed with 1 ml of serum diluted 1:4000. Native IL-2 was diluted with PBS and mixed with an equal volume of serum diluted 1:2000. The serum-IL-2 mixtures were incubated for 90 min at room temperature, after which 100 μl of the mixtures was placed in coated ELISA wells. After 45 min, the wells were washed three times with PBS-0.5% Tween, and 100 μl of goat anti-human IgG HRP conjugate (Cappel) diluted 1:1000 in PBS containing 0.5% BSA and 0.05% Tween-20 was added to the wells. After 1 hr, the wells were again washed and then developed by adding first 100 μl of *o*-phenylene diamine solution (1 mg/ml *o*-phenylene diamine in 0.1 *M* Na-citrate, pH 5.0) and then 30 μl of 30% H_2O_2. The development was stopped with 50 μl of 1.0 *M* H_2SO_4 and the plates were read at 492 nm. The absorbance in the ELISA of controls done with hyperimmune and ELISA (−) serum in the absence of added IL-2 were arbitrarily assigned values of 100 and 0%, respectively, and values obtained with the various hyperimmune serum-IL-2 mixtures normalized accordingly.

Immunoblot Assays

Ten microliters of 4 mg/ml IL-2 in 0.5 *M* NaCl/20 m*M* Tris–HCl, pH 7.5 (Tris-buffered saline; TBS), was applied as a small dot onto nitrocellulose paper (BioRad). The paper was then incubated in 3% gelatin while shaking gently with a rotary shaker for 1 hr at room temperature. The nitrocellulose was then transferred to a 10% solution of serum in TBS with 1% gelatin. After 2 hr at room temperature, the paper was rinsed with distilled water and washed three times with TBS containing 0.05% Tween-20 (TBS-Tween). The paper was subsequently incubated with a 1:1000 dilution of rabbit anti-human IgG, IgM, and IgA antibodies conjugated with horseradish peroxidase (Accurate Chemicals, Westbury, NY) in TBS with 1% gelatin. After 1 hr, the paper was rinsed with distilled water and washed three times with TBS-Tween. The substrate for the peroxidase reaction was made by dissolving 1 mg of 3,3′,5,5′-tetramethyl benzidine (Sigma) in 100 μl of DMSO which was then diluted to 10 ml in 0.1 *M* Na-citrate/0.2 *M* Na_2HPO_4, pH 5.0. This reagent and then 30 μl of 30% H_2O_2 were added and the color was allowed to develop. Dark spots were visible at the sites of Ig deposition within 10 min.

Western Blot Analysis of Anti-IL-2 Antibodies

Fifteen micrograms of des-ala-ser$_{125}$ r-IL-2 was incubated with 3 mg of human serum albumin (Armour Pharmaceutical Co.) in 1% $NaDodSO_4$ for 2 hr. The proteins were then separated by $NaDodSO_4$–polyacrylamide gel electrophoresis and transferred to nitrocellulose paper using a Trans-blot cell (BioRad) (18). The paper was cut into longitudinal strips and the transfer of both proteins verified by staining one strip with amido black. The remaining strips were then treated as described above for immunoblot assays.

Detection of Anti-IL-2 Antibodies with Protein A-Sepharose

Des-ala-ser$_{125}$ r-IL-2 was diluted to a concentration of 2000 U/ml in RPMI-1640 medium containing 50 μg/ml human serum albumin (HSA). The IL-2 solution was then mixed 2:1 with pretreatment serum or serum obtained from the same patient after several weeks of IL-2 injections and kept at 4°C for 18 hr. Seventy-five microliters of the IL-2 serum mixtures was loaded onto 0.3-ml columns of protein A-Sepharose 4B (Sigma) previously equilibrated with the above albumin solution. After 1 hr, the columns were washed with approximately 6 column vol of a detergent solution containing 0.5% NP-40, 1.0 *M* LiCl, and 0.15 *M* NaCl in 0.2 *M* Tris–HCl, pH 8.0, and then with RPMI-1640 culture medium containing 50 μg/ml HSA. These washes were combined (pool 1). To verify that all of the nonspecifically bound IL-2 had been eluted, the column was again washed sequentially with the above detergent- and HSA-containing solutions and the two rinses were combined (pool 2). The column was then rinsed twice with 50 m*M* glycine, 0.15 *M* NaCl, 1 m*M* EDTA, pH 2.5 (pools 3 and 4). The various fractions were dialyzed, filter-sterilized,

and assayed for IL-2 activity in a standard [^{3}H]thymidine incorporation assay utilizing PHA-activated, IL-2-dependent human T-lymphocytes as indicator cells (19). The amount of IL-2 present in the various column eluates was determined by multiplying the IL-2 concentration by the pool volume. Similar experiments were performed with T cell-derived IL-2.

Partial Purification of Human Immunoglobulin

Thirty milliliters of serum from a seropositive patient was collected and the immunoglobulin precipitated by adding 45% ammonium sulfate and stirring at 4°C overnight. The suspension was then centrifuged at 30,000*g* for 45 min, and the precipitate was resuspended in 5 ml of PBS and dialyzed five times against a 100-fold volume of PBS. The sample was filter-sterilized and 1.0 ml was applied to a 120-ml Sephacryl 200 column (Pharmacia) equilibrated with PBS. One-milliliter fractions were collected and assayed for anti-IL-2 antibodies with the previously described ELISA and the positive fractions were combined. The immunoglobulin preparation was concentrated fourfold using a PM-10 ultrafiltration membrane (Amicon, Beford, MA) and the high molecular weight retentate assayed for protein concentration using a Bradford protein assay kit (BioRad) with affinity-purified human IgG (Accurate Chemicals) as a standard. The IgG concentration was determined with an ELISA. Briefly, serial 1:5 dilutions of a known concentration of human IgG and the pooled fractions from the Sephacryl column were prepared in coating buffer and 100 μl was added to duplicate wells of a 96-well microtiter plate. The plates were stored at 4°C for 18 hr and washed four times with PBS-Tween as described above for the ELISA for anti-IL-2 antibodies. The plates were then incubated with a 1:1000 dilution of goat anti-human Ig specific antibody conjugated with alkaline phosphatase (Sigma). After 2 hr at room temperature, the plates were washed again with PBS-Tween, the para-nitrophenyl phosphate substrate was added, and the absorbance at 410 nm was read in a spectrophotometer. Immunoglobulin G concentrations were calculated by plotting linear regression lines of the serial dilutions. This assay was able to detect as little as 10 pg of IgG.

Interleukin-2 Neutralization Assay

IL-2 activity was determined by using a [^{3}H]thymidine incorporation assay with phytohemagglutinin-activated, IL-2-dependent, human T lymphoblasts as indicator cells (19). In the neutralization assays, 1.0 U/ml des-ala-ser$_{125}$ r-IL-2 was incubated for 2 hr with various dilutions of unfractionated serum or partially purified human Ig before addition to the indicator cells. Some serum preparations had been previously heated to 56°C for 1 hr to destroy an inhibitor of T-cell proliferation. DMS-1, an IgG$_1$ kappa monoclonal antibody with IL-2 neutralizing activity (Genzyme, Boston, MA) (20), and an affinity-purified rabbit anti-IL-2 heteroantiserum were used as positive controls.

RESULTS

Detection of Anti-IL-2 Antibodies

Serological and clinical data from the Phase I trial are displayed in Table I. Eleven of 17 patients developed serum anti-IL-2 antibodies which were detected with the ELISA. The titer did not correlate with the diagnosis, duration of treatment, or total amount of IL-2 administered. The temporal course of development of IgG antibodies to IL-2 is illustrated in Fig. 1. Of eight patients whose serum was subjected to weekly serologic studies, four became seropositive in the ELISA within 1 month of initiating treatment.

Immunoblots were also used to screen sera for anti-IL-2 antibodies (Fig. 2). In each instance, the intensity of staining in the immunoblot correlated with the reactivity in the ELISA, although the immunoblot was generally more sensitive, as some sera marginally positive in the ELISA were unequivocally positive with the immunoblot.

Western blots were performed to verify that the reactivity in the ELISA and immunoblot assay was directed against the IL-2 molecule and not a nonspecific detergent–protein complex. As shown in Fig. 3, a mixture of des-ala-ser$_{125}$ r-IL-2 and a gross excess of human albumin was incubated in $NaDodSO_4$, fractionated by $NaDodSO_4$-PAGE, and transferred to nitrocellulose paper. Strips of the paper were either stained with amido black, which demonstrated the presence of a 15-kD band (IL-2) and a wide 68-kD band corresponding to albumin, or developed with serum and anti-human Ig as described for immunoblots, in which case only the

Table I. Development of Serum Anti-IL-2 Antibodies in Patients Receiving Recombinant IL-2

Patient	Age	Tumor	Treatment duration (weeks)	Dose range (U/M^2)[a]	IL-2 antibody titer[b]
1	53	Breast	21	10,000–500,000	14,000
2	62	Unknown primary	9	10,000–50,000	800
3	63	Breast	3	10,000	40
4	45	Melanoma	5	10,000–25,000	1,400
5	56	Head and neck	33	25,000–1,000,000	240
6	46	Renal cell	13	25,000–100,000	1,000
7	46	Colon	18	25,000–500,000	640
8	57	Melanoma	4	50,000	200
9	40	Lung (non-small cell)	31	50,000–500,000	1,280
10	58	Lung (small cell)	8	100,000–250,000	40
11	45	Renal cell	16	100,000–500,000	80
12	58	Melanoma	14	500,000–1,000,000	800
13	54	Pancreas	9	500,000–1,000,000	60
14	67	Rectum	9	500,000	380
15	65	Lung (non-small cell)	15	1,000,000	160
16	67	Colon	19	500,000–1,000,000	80
17	51	Pancreas	17	1,000,000	10,000

[a]One unit is approximately 0.35 ng.
[b]The titer is the reciprocal of the minimal serum dilution yielding an ELISA absorbance equal to that of an average of normal sera assayed at a 1% dilution. Titers displayed correspond to the last serum sample obtained from each patient during treatment.

lower molecular weight band was visualized with the horseradish peroxidase reagent, confirming that the antibodies reacted with IL-2.

The development of serum anti-IL-2 antibodies was also demonstrated by incubating IL-2 with serum obtained both prior to and after several weeks of IL-2 treatment and subsequently removing the immune complexes by passing the mixture over protein A-Sepharose. The nonadherent flow-through fractions and the acid eluate were dialyzed against PBS and assayed for IL-2 activity in the microtiter [^{3}H]thymidine incorporation assay. As

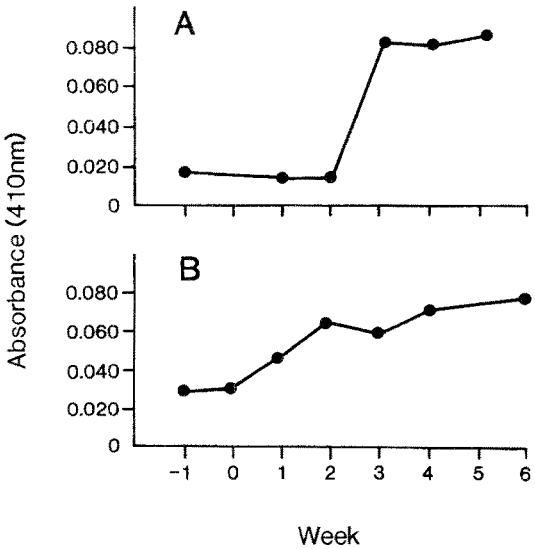

Fig. 1. Enzyme-linked immunosorbent assays (ELISAs) of sequential serum samples from two patients participating in a Phase I clinical trial with recombinant IL-2. IgG antibodies against IL-2 are evident within 3–4 weeks of initiating therapy with weekly intravenous injections of IL-2.

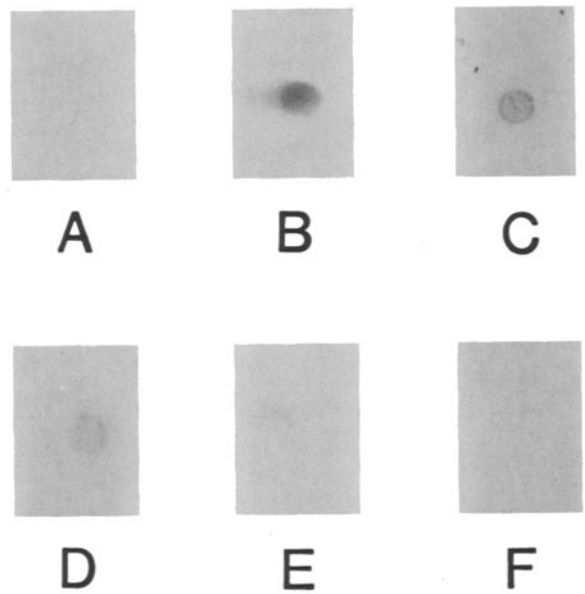

Fig. 2. Antibodies against IL-2 detected by an immunoblot technique with normal serum (A) and serum from patients receiving weekly injections of r-IL-2 (B–F). Stain intensity correlated with antibody titer as determined by ELISA. B and C correspond to the most seropositive patients (Table I), with antibody titers of 14,000 and 10,000, respectively.

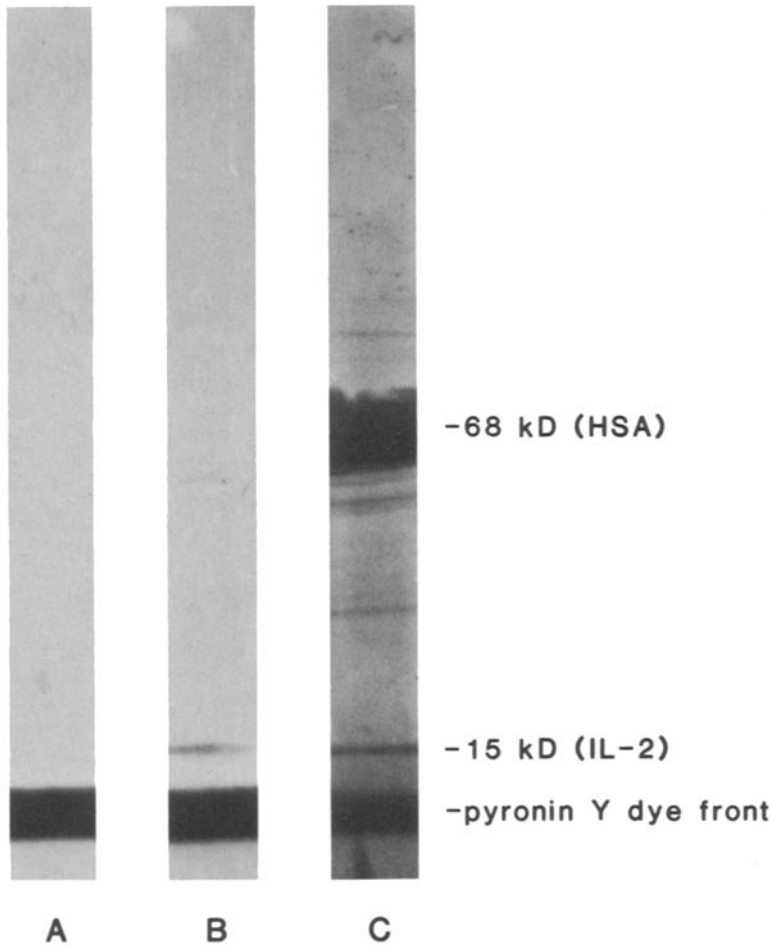

Fig. 3. Detection of anti-IL-2 antibodies with Western blots. A mixture of HSA and r-IL-2 was subjected to $NaDodSO_4$–PAGE and the proteins were transferred to nitrocellulose. The nitrocellulose was cut into longitudinal strips which were developed with pretreatment (A) or hyperimmune (B) serum and a rabbit anti-human Ig antibody–horseradish peroxidase conjugate as the second-step reagent. In lane C, the nitrocellulose strip was stained with amido black to confirm the transfer of proteins from the polyacrylamide gel.

illustrated in Table II, little IL-2 was retained by the protein A-Sepharose column when pretreatment serum was used, whereas nearly half (42%) of the IL-2 was retained when posttreatment serum was employed.

Antibody Specificity

To determine if the serum anti-IL-2 antibodies were directed against an epitope dependent on the serine–cysteine substitution at position 125, competitive ELISAs were performed with des-ala-ser_{125} r-IL-2 immobilized on the Immulon plates and both unsubstituted and altered-sequence r-IL-2 added to the antibody (+) serum. As shown in Fig. 4, des-ala-cys_{125} r-IL-2 is interchangeable with des-ala-ser_{125} r-IL-2 in competitive ELISAs. In similar experiments with highly purified T cell-derived IL-2, no such competition was observed (Table III). These results corroborate those from the protein A-Sepharose experiments which suggested that the anti-IL-2 antibodies are almost entirely directed against epitopes peculiar to recombinant IL-2 preparations.

Neutralization Assays

The effects of human serum on IL-2-induced T-cell proliferation are shown in Fig. 5. IL-2 (1 U/ml) was preincubated with various sera or par-

Table II. Retention of IL-2 Immune Complexes by Protein A-Sepharose[a]

		IL-2	
	Pool	Concentration (U/ml)	% recovered
Expt 1			
Pretreatment serum	1	34	92
Des-ala-ser$_{125}$ r-IL-2	2	<3	
	3	5	8
	4	<3	
Posttreatment serum	1	26	58
Des-ala-ser$_{125}$ r-IL-2	2	<3	
	3	19	42
	4	<3	
Expt 2			
Posttreatment serum	1	27	96
Lymphocyte-derived IL-2	2	0	
	3	1.2	4
	4	0.6	

[a]IL-2 activity was detected in the acid glycine wash (pool 3) when hyperimmune serum was mixed with r-IL-2 prior to applying the IL-2-serum mixture to a protein A-Sepharose column. Minimal IL-2 was detected in these fractions when pretreatment (ELISA negative) serum was employed or when T lymphocyte-derived IL-2 was substituted for the recombinant lymphokine.

Table III. Competitive ELISAs with T Cell-Derived and Recombinant IL-2[a]

	IL-2 (U/ml)	% reduction in absorbance
Expt 1	80,000	−9
	15,000	−2
	*18,000 (r-IL-2)	53
Expt 2	30,000	9
	10,000	9
	3,000	8

[a]As in Fig. 4, data are expressed as the percentage reduction in absorbance in ELISAs with des-ala-ser$_{125}$ r-IL-2 immobilized on the Immulon plates and various concentrations of T cell-derived IL-2 added to the serum. In Experiment 1, *des-ala-ser$_{125}$ r-IL-2 served as a positive control.

tially purified Ig prior to addition to the indicator T cells in [^{3}H]thymidine incorporation assays. As previously reported, human serum contains an inhibitor that can be removed by heating to 56°C for 1 hr (21) or by sequential fractional ammonium sulfate precipitation and gel filtration. The preheated samples and the partially purified Ig preparation had no effect on IL-2-induced isotope incorporation at concentrations as high as 1 mg/ml.

DISCUSSION

Approximately 65% of the patients enrolled in a Phase I clinical trial with recombinant IL-2 developed serum anti-IL-2 antibodies. The first few patients entered in the study received weekly intravenous injections of only 10,000 units/M^2, a much lower dose than that undergoing evaluation in cur-

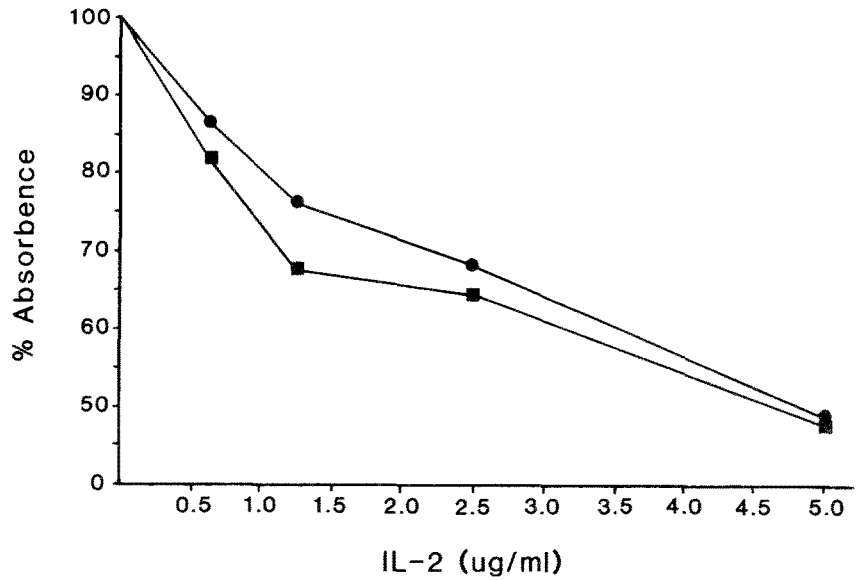

Fig. 4. Competitive ELISAs with des-ala-ser$_{125}$ r-IL-2 immobilized on Immulon plates and either des-ala-ser$_{ser}$ (●——●) or des-ala-cys$_{125}$ (■——■) r-IL-2 added to ELISA (+) serum. The recombinant IL-2 preparations reduce the reactivity of the serum in the ELISA equally, suggesting that serum anti-IL-2 antibodies are not directed against an epitope dependent on the serine–cysteine substitution at position 125.

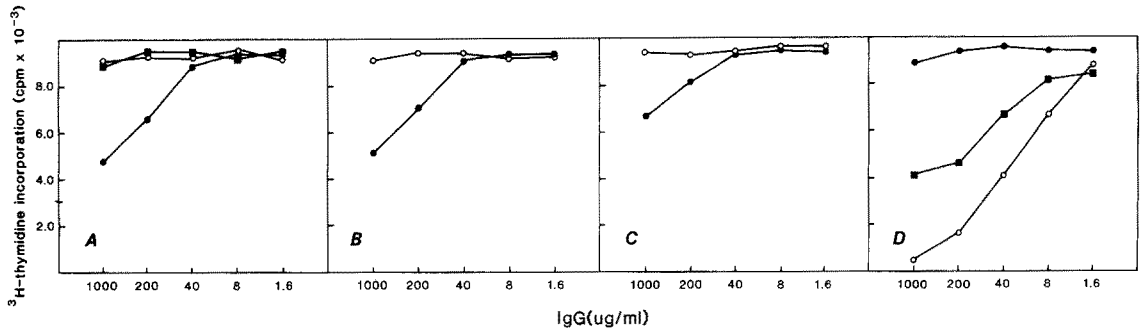

Fig. 5. Neutralization of IL-2 activity. Serial dilutions of unheated serum (●—●), serum heated to 56°C for 1 hr (○——○), and partially purified Ig (■——■) from two ELISA (+) patients (A and B) and a seronegative donor (C) were added to IL-2 (1 U/ml) and the activity of the mixtures was determined in a standard [^{3}H]thymidine incorporation assay. Sera from the two ELISA (+) patients had anti-IL-2 antibody titers of 1600 and 90, respectively, at an IgG concentration of 1000 μg/ml. IL-2 alone at concentrations of 1.0 and 200 U/ml (maximal stimulation) yielded cpm of 9330 and 24,682, respectively. The inhibitory effects of these sera were eliminated by heat treatment and were not detected in partially purified Ig preparations. The effects of affinity-purified Ig from a rabbit prior to (●——●) and after (○——○) immunization with recombinant IL-2 and that of a neutralizing murine monoclonal antibody DMS-1 (■——■) on IL-2-induced isotope incorporation are displayed in D.

rent trials (21, 22). Furthermore, many of these patients were removed from study after only three or four injections because of overt tumor progression. The development of antibodies to IL-2 is most likely dependent on the dose given as well as the duration of therapy, and it is possible that nearly all patients would develop antibodies if they remained on study for longer periods of time. However, other factors, including the immunologic competence of the patient, are clearly important, as some of the persistently seronegative patients were treated for an extended duration.

The development of anticytokine serum antibodies is not without precedent. Cancer patients treated with interferon, for example, routinely develop antiinterferon antibodies (15, 16). Furthermore, in certain circumstances, endogenous lymphokines may be immunogenic, as patients with systemic lupus erythematosis have been reported to develop spontaneously antiinterferon antibodies (23). The recombinant interleukin-2 utilized in the clinical trial lacks the N-terminal alanine of the native molecule and has an amino acid substitution at position 125. Due its hydrophobicity and lack of a carbohydrate component, it has a tendency to aggregate at neutral pH in serum (unpublished observation). All of these structural features would be expected to enhance the immunogenicity of the preparation.

Although the epitopes recognized by the serum antibodies are not known, it is evident from the competitive ELISAs (Fig. 4) that they are not dependent on the serine substitution at position 125. Indeed, the antibodies are equally reactive with recombinant material possessing a cysteine at this site. The inability of native IL-2 to compete in these assays suggests that a large percentage of the antibody molecules does not interact with the native lymphokine but recognizes a determinant peculiar to recombinant preparations. These antibodies may bind to regions of the molecule that are denatured due to the detergents used in purification procedures or regions that are conformationally altered because of aggregation. The antibodies were reactive, however, with the 14-kD protein band in Western blots (Fig. 3), suggesting that aggregation is not crucial, although this monomeric material may have reaggregated when transferred to nitrocellulose. The Western blots also exclude the possibility that the antibodies were directed against a $NaDodSO_4$–protein complex, as the HSA band was not visualized in these experiments. The lack of reactivity of the antibodies against the native IL-2 in the competitive ELISAs was corroborated by the protein A-Sepharose experiments with hyperimmune sera in which a substantial percentage of the recombinant, but not native, IL-2 preparation was retained by the gel.

Serum contains a variety of substances which inhibit lymphocyte proliferation. Some of these inhibitors are complex lipoproteins thought to be products of suppressor T cells (24, 25). The inhibitors in human serum are heat labile and, as shown in Fig. 5, can also be separated from immunoglobulin by sequential ammonium sulfate precipitation and gel filtration. Although serum from seropositive

patients inhibits the biological activity of IL-2, our experiments implicate components other than immunoglobulin in the neutralization, as neither preheated serum nor partially purified Ig fractions had any effect in the IL-2 assays.

The injection of rabbit anti-murine IL-2 antibodies inhibits the *in vivo* induction of cytolytic T lymphocytes in mice repeatedly immunized with allogeneic cells, suggesting that antilymphokine antibodies might be generally immunosuppressive (26). It was therefore crucial to determine the clinical significance of the anti-IL-2 antibodies generated in our patients. The inability of the anti-IL-2 antibodies present in our patients' sera to bind to native IL-2 in the competitive ELISAs or to neutralize the biological activity of IL-2 suggests that they may have little effect on IL-2 generated *in vivo* at a site of inflammation or lymphocyte-tumor cell interaction.

Bolus injections or prolonged infusions of high doses of either lymphocyte-derived or recombinant IL-2 induce fever, acute-phase protein synthesis, and the release of pituitary hormones associated with the response to stress in recipient patients (20, 27). Serum complement levels do not decline with the advent of IL-2-induced fever. Furthermore, these toxic side effects are often observed at the time of the first treatment, prior to the development of anti-IL-2 antibodies. These observations suggest that circulating immune complexes do not mediate any aspect of the acute-phase response associated with IL-2 treatment. These data and the results of the neutralization experiments cited above argue that the development of anti-IL-2 antibodies in cancer patients participating in clinical trials may not inhibit IL-2-dependent cellular immune processes or contribute to the toxicity of the lymphokine.

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