

Distinct Clinical and Laboratory Activity of Two Recombinant Interleukin-2 Preparations¹

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ABSTRACT

Interleukin-2 (IL-2) is a potent lymphokine that activates natural killer cells, T cells, and other cells of the immune system. Several distinct recombinant human IL-2 preparations have shown antitumor activity, particularly for renal cell cancer and melanoma. Somewhat distinct immune and clinical effects have been noted when different IL-2 preparations have been tested clinically; however, the regimens and doses used were not identical. To compare these more directly, we have evaluated two clinical recombinant IL-2 preparations *in vitro* and *in vivo* using similar regimens and similar IUs of IL-2. We used the Food and Drug Administration-approved, commercially available Chiron IL-2 and the Hoffmann LaRoche (HLR) IL-2 supplied by the National Cancer Institute. Using equivalent IUs of IL-2, we noted quantitative differences *in vitro* and *in vivo* in the IL-2 activity of these two preparations. In patients receiving comparable IUs of the two preparations, HLR IL-2 induced the release of more soluble IL-2 receptor α into the serum than Chiron IL-2. In addition, more toxicities were noted in patients receiving 1.5×10^6 IU of HLR IL-2 than were seen in patients treated with 1.5×10^6 or even 4.5×10^6 IU of Chiron IL-2. These toxicities included fever, nausea and vomiting, and hepatic toxicity. *In vitro* proliferative assays using IL-2-dependent human and murine cell lines indicated that the IU of HLR IL-2 was more effective than Chiron IL-2 at inducing tritiated thymidine incorporation. Using flow cytometry, we also found quantitative

differences in the ability of these two preparations to bind to IL-2 receptors. These findings indicate that ~ 3 – 6 IU of Chiron IL-2 are required to induce the same biological effect as 1 IU of HLR IL-2.

INTRODUCTION

IL-2⁴ is a 133 amino acid protein that is used clinically for cancer immunotherapy. The initial clinical studies of IL-2 evaluated natural and recombinant preparations (1–3). When these studies were initiated, there was not a uniform standard for calibrating the various IL-2 preparations. Each preparation was calibrated against an “in house” standard, and individual companies defined their own units of IL-2. The International Standard for IL-2 was established in 1988 (4). This standard is available in lyophilized form, 100 IU/vial, to calibrate and standardize other various IL-2 preparations. The IU is defined as the amount of IL-2 that induces 50% of maximal proliferation of an established IL-2-dependent cell line. Quantification of IL-2 content in other preparations is achieved by comparing dose-response curves for the standard and unknown sample using a parallel line analysis, or by computerized software such as the ALLFIT program (5).

Clinical trials of IL-2 have used different IL-2 preparations, each individually calibrated to the International Standard. In addition, these different IL-2 preparations have been given using different schedule and dosing regimens. Unfortunately, there are no published data directly comparing the clinical effects of the different human recombinant IL-2 preparations. Recently, Lentsch *et al.* (6) noted significant differences when they compared systemic toxicities seen in mice given the same number of IUs of either the natural sequence IL-2 (nIL-2; HLR) or IL-2 with the serine amino acid substitution (ser-IL-2; Chiron). We have direct experience with two sequential clinical studies of recombinant IL-2 in which the same constant infusion IL-2 regimen was used in a similar patient population, and where reagent availability required changing from one recombinant product to another at the beginning of the second study. In the first study, using IL-2 manufactured by HLR and supplied by the Biological Resource Branch of the NCI, we determined that 1.5×10^6 IU/m²/day for 4 days/week for 3 weeks was a well tolerated, yet satisfactory outpatient dosing regimen (7). When

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⁴ The abbreviations used are: IL-2, interleukin-2; NK, natural killer; FDA, Food and Drug Administration; HLR, Hoffmann LaRoche; NCI, National Cancer Institute; BRMP, Biological Response Modifiers Program; GM-CSF, granulocyte-macrophage colony-stimulating factor; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; AST, aspartate aminotransferase; LAK, lymphokine-activated killer; IL-2R, IL-2 receptor; sIL-2R α , soluble IL-2R α ; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MFI, mean fluorescence intensity; dThd, thymidine; MTD, maximum tolerated dose; IU, international unit.

Table 1 Toxicities seen with recombinant IL-2 plus GM-CSF

The percentage of patients experiencing the indicated toxicity during either the first week of therapy, or during the 3-week course of therapy for the Phase I trial using HLR IL-2 at 1.5×10^6 IU/m²/day, or the Phase II trial using Chiron IL-2 at 4.5×10^6 IU/m²/day.

Toxicity	Percentage of patients experiencing toxicity			
	Phase I HLR 1.5×10^6 IU/m ² /day 3-week course (n = 6)	Phase I HLR 1.5×10^6 IU/m ² /day Week 1 (n = 11)	Phase II Chiron 4.5×10^6 IU/m ² /day 3-week course (n = 15)	Phase II Chiron 4.5×10^6 IU/m ² /day Week 1 (n = 17)
Fever >38	100	82	80	35 ^a
Hypotension drop >20 mm Hg	67	55	80	29
Performance status drop ≥ 2	17	18	40	12
Weight gain $\geq 5\%$ body mass	50	9	7	0
Nausea/vomiting (any grade)	83	73	80	47
Chills (any grade)	83	82	80	59
AST increase (any grade)	50	45	0 ^a	6 ^a

^a Comparison of patients on the Phase I study versus the Phase II study at either the 1-week time point or after a 3-week course of therapy was statistically significant ($P < 0.05$).

this same 1.5×10^6 IU/m²/day dose of the commercially available Chiron IL-2 preparation was used in the initial three patients receiving Chiron IL-2 in this study, the clinical features associated with the biological effects of IL-2 were dramatically reduced. These clinical features included minimal induction of fever, weight gain, decrease in blood pressure, decreased performance status, and lymphocytosis (data not shown). Thus, subsequent patients received Chiron IL-2 using three times the initial IU dose, or 4.5×10^6 IU/m²/day. The selection of the 3-fold increase in dose was based on communication with other clinical immunotherapy investigators with previous experience with the Chiron preparation, at the NCI and elsewhere, indicating that 3 IU of Chiron IL-2 would correspond to 1 IU of HLR IL-2 (8). Clinical assessments in the two sequential studies reported here suggested that 4.5×10^6 IU/m²/day of Chiron IL-2 induced the anticipated changes seen with 1.5×10^6 IU/m²/day of HLR IL-2. However, even with this dose adjustment, some of these parameters demonstrated that the magnitude of the IL-2 induced change with the Chiron IL-2 was not quite as large as that seen with the HLR IL-2. This suggested that a 3:1 ratio (expressed in IUs) of Chiron:HLR IL-2 may not reflect equipotency.

This study evaluates these *in vivo* clinical data in addition to *in vitro* comparisons of these two recombinant IL-2 preparations to provide quantitative dosing comparisons of these reagents for future *in vitro* and *in vivo* studies. These present studies indicate that 3–6-fold more IU of Chiron IL-2 than HLR IL-2 are needed to induce quantitatively similar effects.

MATERIALS AND METHODS

Clinical Studies. The data on toxicities and immunological effects noted in patients receiving IL-2 were collected in two sequential studies using the same treatment schema, with a change in the source of IL-2. Patients received 4 days of continuous infusion IL-2 for 3 weeks (days 1–4, 8–11, and 15–18), with 12 days of GM-CSF starting on day 8 (7). In addition, for the purposes of comparing IL-2-associated toxicities, we used data obtained from a third study on a large cohort of patients who received an identical 1st week of treatment consisting only of IL-2, with the addition of antibody therapy

during the 2nd and 3rd weeks of treatment (9). Results of some of these trials have been published (7, 9). For the purpose of this comparative analysis, only data for patients during their 1st week of treatment on these separate studies will be evaluated because the only treatment given during the 1st week of these studies was the 96-h constant infusion of IL-2 (hours 0–96).

Sources of IL-2 and Their Assigned Unitage and Specific Activity. Proleukin (Chiron IL-2) is a protein with a molecular weight of 15,300 produced by recombinant DNA technology in *Escherichia coli*. Genetic engineering techniques were used to modify the human IL-2 gene with the recombinant IL-2 differing from the natural product in that it is not glycosylated, the molecule has no N-terminal alanine, and the molecule has serine substituted for cysteine at amino acid position 125. This may affect the aggregation state due to changes in the disulfide bonds. The specific activity of this product is 18 million IU/1.1 mg of protein, as indicated on the package insert (or 16.3 million IU/mg).

The HLR IL-2 used the natural native human gene for IL-2 that was cloned into *E. coli*. The specific activity of the HLR product, as indicated on the product information sheet supplied to the NCI, is 15 million units/1 mg of protein. These Roche units were equivalent to the BRMP interim reference units (10), which were determined to coincide with the IU (4). HLR IL-2 was provided through the Cancer Treatment and Evaluation Program of the NCI.

In Vitro Proliferative Assays. IL-2-responsive cells included the Tf-1 myeloid leukemia cell line transfected with the gene for the IL-2R β chain. This transfected line, designated Tf-1 β , responds to IL-2 using intermediate affinity $\beta\gamma_c$ receptor complexes (11, 12) and, thus, is analogous to the majority of NK cells in IL-2-treated patients, which also use intermediate affinity IL-2Rs (13). PBMCs obtained from patients after completion of a 96-h continuous infusion of IL-2 were also used as responding cells. PBMCs from control donors were cultured for 3 days in 1% PHA to activate high affinity IL-2Rs. These cells were then used as responding cells in the proliferative assays. Tf1- β cells (1×10^4 /well), patient PBMCs, and PHA blasts (1×10^5 /well) were cultured with various dilutions of Chiron or HLR

Table 2 Comparison of IL-2-induced toxicities

The toxicities noted in a large number of patients treated with an identical 1-week course of IL-2 with 1.5×10^6 IU/m²/day for 4 days of HRL IL-2 were greater than the toxicities noted with 4.5×10^6 IU/m²/day for 4 days of Chiron IL-2. These patients all received additional treatment with either GM-CSF or a monoclonal antibody; however, this second agent was not given during the first week of IL-2. Three patients received 1.5×10^6 IU/m²/day of Chiron IL-2, and none of these showed any of the seven toxicities shown here.

Side Effect	HRL 1.5×10^6 Units/m ² /day <i>n</i> = 72 (%)	Chiron 4.5×10^6 Units/m ² /day <i>n</i> = 19 (%)	<i>P</i>
Fever >38	60 (83)	7 (37)	0.0001
Hypotension drop >20 mm Hg	36 (50)	8 (42)	0.5600
Performance status drop ≥ 2	22 (31)	2 (11)	0.1224
Weight gain $\geq 5\%$ body mass	7 (10)	0 (0)	0.4460
Nausea/vomiting, (any grade)	55 (76)	9 (47)	0.0240
Chills (any grade)	56 (78)	12 (63)	0.2550
AST increase (any grade)	39 (54)	3 (16)	0.0027

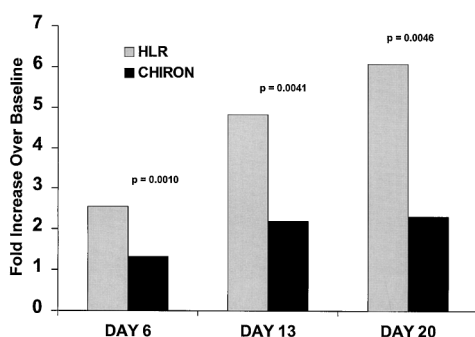


Fig. 1 The rebound lymphocytosis occurring 24 h after completion of a 96-h continuous infusion of IL-2. The mean lymphocytosis seen in 5 patients from the Phase I study using HLR IL-2 at 1.5×10^6 IU/m²/day is compared with the mean of 16 patients on the Phase II study using Chiron IL-2 at 4.5×10^6 IU/m²/day at days 6, 13, and 20. All patients received IL-2 by constant infusion on days 1–4, 8–11, and 15–18, along with GM-CSF on days 8–19.

IL-2 for 72 h, which included an 18-h pulse with 1 uCi of tritiated thymidine.

CTLL-2, a murine IL-2-dependent cell line, was obtained from the American Type Culture Collection (Manassas, VA). These responding cells were used in proliferative assays comparing the activity of the HLR IL-2 and the Chiron IL-2 to the WHO International Standard. This standard is the WHO 1st International Standard for IL-2 (human) 86/504, obtained from the BRMP of the NCI (4). It consists of 100 IU of IL-2 in lyophilized form. The CTLL-2 cells were cultured at 8×10^3 /well with dilutions of IL-2 or IL-2 standard for 20 h, plus a 4 h pulse with [³H]thymidine. All proliferative assays were cultured at 37°C with 5% CO₂. The Packard Filtermate 196 was used to harvest the cultures, and [³H]thymidine incorporation was quantitated with a Matrix 9600 direct β counter using a 5-min counting time. The EC₅₀, the effective concentration necessary to induce 50% of maximal proliferation, was calculated using the ALLFIT program obtained from Jeffrey Rossio (NCI, Frederick, MD; Ref. 5).

Blocking Assay Flow Cytometry. An antibody-IL-2 fusion protein was used to assess the ability of the two recombinant IL-2 preparations to block binding of IL-2 to IL-2Rs (14).

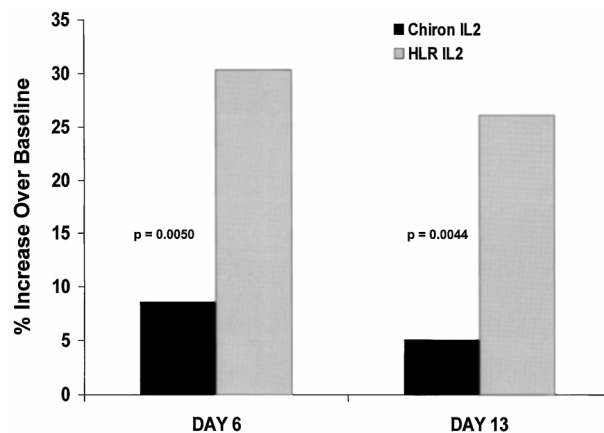


Fig. 2 Increase in CD56⁺ expression with continuous infusion IL-2. The mean increase in the percentage of CD56⁺ cells from baseline was compared at days 6 and 13 for patients in the Phase I study, receiving HLR IL-2 at 1.5×10^6 IU/m²/day (*n* = 6), with the increase noted for patients in the Phase II study, receiving Chiron IL-2 at 4.5×10^6 IU/m²/day (*n* = 12).

Cell bound fusion protein (ch14.18-IL-2; a gift from Drs. Ralph Reisfeld and Steve Gillies) binding via the IL-2R was detected by standard indirect immunofluorescence methods (Becton Dickinson, San Jose, CA). Goat antihuman IgG conjugated to fluorescein (Caltag, San Francisco, CA) was used as the secondary antibody. Excess recombinant IL-2, either Chiron or HLR, was added as a competitive inhibitor.

Statistical Methods. Exact binomial tests were used to compare the percentage of patients experiencing toxicities. The nonparametric Kruskal-Wallis and Wilcoxon tests were used for all other comparisons.

RESULTS

Equivalent IUs of the Two Recombinant IL-2 Preparations Do Not Cause the Same Clinical Toxicities. After completion of a Phase I study of combined GM-CSF and IL-2 (7), a Phase II study was initiated at a well tolerated, biologically active IL-2 dose (1.5×10^6 IU/m²/day) determined in the

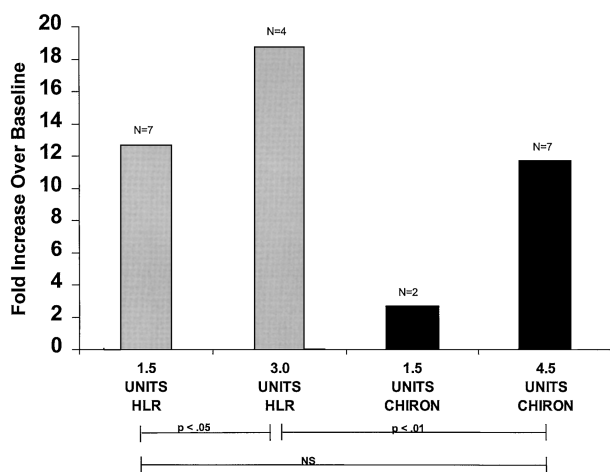


Fig. 3 Increase in the sIL-2R α . The fold increase in the serum CD25 level over the baseline value was determined for patients receiving similar IL-2 constant infusions. There was a significantly greater increase seen with 3.0×10^6 IU/m 2 /day of HLR IL-2 compared with either 1.5×10^6 IU/m 2 /day of HLR IL-2 ($P < 0.05$), or 4.5×10^6 IU/m 2 /day of Chiron IL-2 ($P < 0.01$).

Phase I study.⁵ At the time of the initiation of the Phase II study, the HLR IL-2, which was used in the initial study, was no longer available through the NCI. The FDA-approved and commercially available Chiron “Proleukin” IL-2 was used in the second study. Because this preparation of IL-2 is not identical to the native human IL-2, the differences may effect the level of toxicity induced and the biological changes induced *in vivo* in patients receiving IL-2 as therapy for cancer. That is, equivalent IUs of the Chiron IL-2, as measured in the *in vitro* proliferative assay quantitating IUs, may not necessarily induce equivalent toxicities or immune activation to that induced by an equivalent number of IUs of HLR IL-2. The initial three patients receiving the Chiron IL-2 received the same dose, in IUs, as the dose identified for future analysis from the Phase I study (1.5×10^6 IU/m 2 /day). Unlike patients in the Phase I study, who showed fever and other IL-2-associated toxicities during their treatment with IL-2 (HLR), these three patients experienced virtually no IL-2-related toxicities with the same dose of Chiron IL-2.⁵ Because no toxicities were noted at 1.5×10^6 IU/m 2 /day, the third patient received a second course at 4.5×10^6 IU/m 2 /day, and all subsequent patients received Chiron IL-2 at 4.5×10^6 IU/m 2 /day.

Even with this 3-fold adjustment for “biological” differences, we noted that patients receiving 4.5×10^6 IU/m 2 Chiron IL-2/day were not experiencing the expected constitutional symptoms throughout the course of this treatment compared with patients receiving 1.5×10^6 IU HLR IL-2/m 2 /day in our previous trial.⁵ The patients receiving 4.5×10^6 IU/m 2 /day of Chiron IL-2 seemed to be better tolerating this IL-2 treatment than did patients in previous trials receiving 1.5×10^6 IU/m 2 /day of HLR IL-2. We compared all grade one and greater toxicities seen in patients on the

previous Phase I study receiving 1.5×10^6 IU/m 2 /day of HLR IL-2 with the toxicities noted in the patients on the Phase II study receiving 4.5×10^6 IU/m 2 /day of Chiron IL-2. In the Phase I study, 11 patients received the 1st week of 1.5×10^6 IU HLR IL-2/m 2 /day, and 6 of these patients finished the 3 weeks of treatment without a change in their scheduled GM-CSF (days 8–19). In the Phase II study, 17 patients received the 1st week of 4.5×10^6 IU/m 2 /day of Chiron IL-2, and 15 patients completed the weeks without requiring any change in their GM-CSF. Values for fever, hypotension, nausea, chills, and increased AST were compared for patients in the two studies. The number of patients is small comparing the 3-week course, due to the GM-CSF variable. Only patients receiving identical treatment regimens, aside from the difference in IL-2 source and dose, were compared in Table 1. There was a smaller percentage of patients having an increase in their AST level with Chiron IL-2 when comparing both the 1st week of treatment with IL-2 alone, or the complete 3-week course of treatment with equivalent levels of GM-CSF per comparison groups. In addition, the percentage of patients experiencing fevers was less in those receiving Chiron IL-2 when compared for the 1st week of therapy (Table 1).

In an effort to increase the power of these comparisons, we did a separate analysis that included all patients on these and other Phase I studies receiving 1 week of HLR IL-2 at 1.5×10^6 IU/m 2 /day ($n = 72$) and all patients receiving 1 week of Chiron IL-2 at 4.5×10^6 IU/m 2 /day ($n = 19$) at the University of Wisconsin Comprehensive Cancer Center, during the same time period that the Phase I and Phase II IL-2 plus GM-CSF studies (compared in Table 1) were open. Although these were separate clinical studies, the eligibility criteria for all these studies involved similar parameters for clinical and laboratory assessments. Therefore, the patients in these studies were of similar status, and all received the same treatment during week 1 (1.5×10^6 IU/m 2 /day HLR-IL-2 or 4.5×10^6 IU/m 2 /day Chiron-IL-2 for 4 days) with no other therapy. On the basis of this larger analysis, there were significant differences noted in fever, nausea and vomiting, and increase in AST (Table 2).

Rebound Lymphocytosis. Previous studies using IL-2 have demonstrated that there is an IL-2 dose-dependent increase in the lymphocyte count after the completion of a 96-h continuous infusion of IL-2 (14). This lymphocytosis is associated with activation of NK and LAK cytotoxicity (15, 16). The fold increase from baseline in lymphocyte count was compared for the 6 patients from the Phase I study to the 15 patients from the Phase II study who had the same identical GM dose, with the only difference being the source and dose of IL-2. HLR IL-2 (1.5×10^6 IU/m 2 /day) induced a greater increase in lymphocytosis than 4.5×10^6 IU/m 2 /day of Chiron IL-2. This significant difference is shown in Fig. 1 and was noted over the 3-week course of treatment on day 6 ($P = 0.001$), day 13 ($P = 0.004$), and day 20 ($P = 0.004$).

Increase in CD56-positive NK Cells. IL-2 given systemically induces an increase in circulating NK cells *in vivo* (11, 15, 17). These NK cells can be identified by the CD56 antigen. For both the Phase I and Phase II studies, we measured the increase from baseline in the percentage of CD56+ NK cells. The HLR IL-2 dose of 1.5×10^6 IU/m 2 /day induced a greater increase in CD56+ cells than did the dose of 4.5×10^6 IU/m 2 /day of Chiron IL-2 (Fig. 2). This difference was noted at

⁵ K. M. Hotton, submitted for publication.

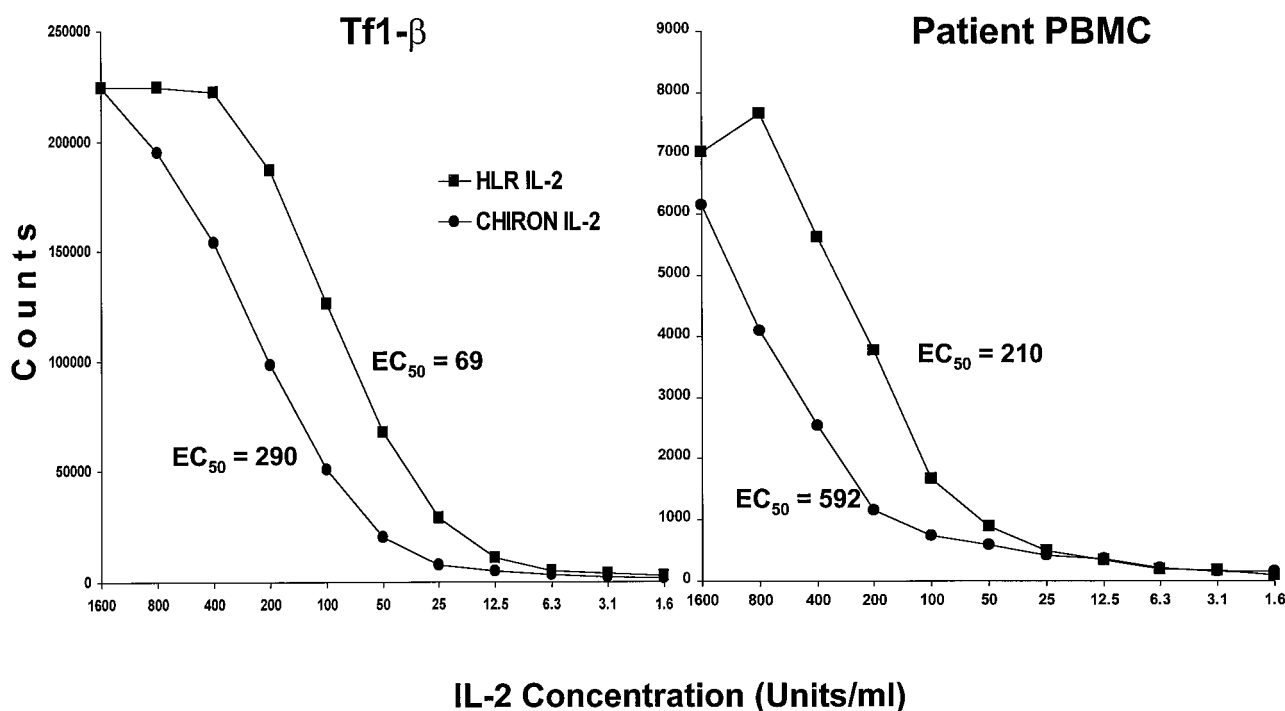


Fig. 4 IL-2-induced proliferative response measured in a 72-h tritiated thymidine incorporation assay. The responding cells are the IL-2-dependent Tf1- β cell line and PBMC obtained from a cancer patient 24 h after completion of a 96-h constant infusion of IL-2. Both cell types respond predominantly through the intermediate affinity $\beta\gamma$ IL-2R. EC_{50} concentrations were determined by the ALLFIT program (5).

6 ($P = 0.005$) and 13 ($P = 0.004$) days in the same group of patients examined in Fig. 1 for the rebound lymphocytosis.

Increase in sIL-2R α . We previously noted that the increase in serum sIL-2R α , associated with continuous infusion IL-2, was an indication of overall stimulation of immune activation (18). We examined the sIL-2R α found in the serum of patients receiving 1.5 and 3×10^6 IU/m²/day of HLR IL-2 and patients receiving 1.5 and 4.5×10^6 IU/m²/day of Chiron IL-2. (Fig. 3) These results reproduced the previously noted dose-dependent increase in sIL-2R α seen with the HLR IL-2 (19). That is, significantly more sIL-2R α was seen in patients receiving 3×10^6 IU/m²/day, compared with those receiving 1.5×10^6 IU/m²/day of HLR IL-2 ($P < 0.05$). In addition, the amount of sIL-2R α induced by 3×10^6 IU/m²/day of HLR IL-2 was greater than that induced by 4.5×10^6 IU/m²/day of Chiron IL-2 ($P < 0.01$). The sIL-2R α level seen with 1.5×10^6 IU/m²/day of HLR IL-2 was 12.8 and was 11.8 for 4.5×10^6 IU/m²/day of Chiron IL-2. These last values are not significantly different.

In Vitro Proliferative Response Induced by the Two Recombinant IL-2 Preparations. With the clinically noted differences in toxicities, degree of lymphocytosis, increase in sIL-2R α , and percentage of CD56-positive cells, we wanted to compare these two IL-2 preparations in *in vitro* proliferative assays in the laboratory. Fig. 4 presents results from an IL-2-induced proliferative assay. The experiment used the TF-1 β cell line, which constitutively expresses the $\beta\gamma$ intermediate affinity IL-2 receptor (11, 12), and PBMC obtained from a patient after a 4-day continuous infusion of IL-2. The majority of cells responding to IL-2 in PBMC populations like this also respond

through the intermediate affinity receptor (13). The effective concentration necessary to induce 50% of maximum proliferation (EC_{50}) is indicated for both IL-2 preparations (5). The EC_{50} for the Chiron IL-2 is ~ 3 – 4 -fold larger than the EC_{50} for HLR IL-2. This indicates that it takes 3–4 times as many IUs of Chiron IL-2 to induce 50% of the maximum proliferative response induced by HLR IL-2.

Does the Absence of Albumin in the Chiron Product Lead to Loss of Activity? Published studies had indicated that the absence of albumin in the Chiron product may have affected the clinical findings that we noted (20–22). The first consideration was that the Chiron IL-2 comes lyophilized, without any additional protein source. The HLR product comes lyophilized, but the lyophilized formulation includes 25 mg of human serum albumin. In the experiment presented in Fig. 5 (*top*), Chiron IL-2 was reconstituted in the University of Wisconsin hospital pharmacy, according to the package insert, with saline for injection. In addition, a separate vial of Chiron IL-2 was also reconstituted with saline supplemented with albumin to achieve the same level of albumin as in the HLR IL-2 formulation. As shown (Fig. 5, *top*), the level of albumin did not substantially influence the IL-2-induced proliferative response for the Chiron IL-2. The HLR IL-2 again induced proliferative responses at lower concentrations. Therefore, the higher EC_{50} for Chiron IL-2 was not solely due to the lack of albumin.

Did the Continuous Infusion Pump System Used Clinically Have a Greater Effect on the Chiron Product than the HLR IL-2? The patients treated on both the Phase I and Phase II protocols received constant infusion IL-2 through a portable

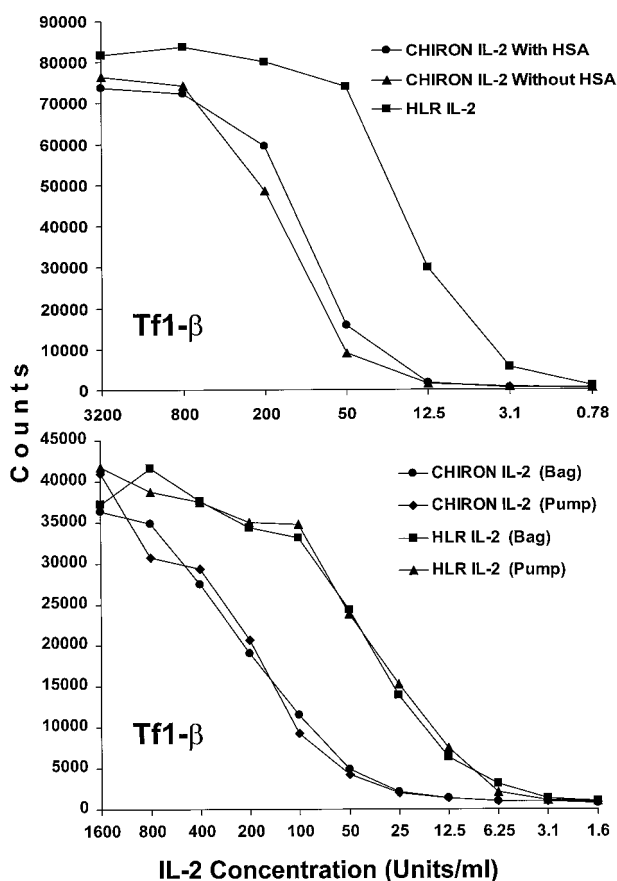


Fig. 5 IL-2-induced proliferative response measured in 72-h tritiated thymidine incorporation assays. *Top*, effect of addition of human serum albumin to the IL-2 suspension. HLR IL-2 formulation includes human albumin such that when reconstituted, according to the manufacturer's specifications, the 1 ml of reconstituted preparation contains 25 mg/ml albumin. Chiron IL-2 was reconstituted according to the package insert (no albumin), and a second vial was reconstituted to contain albumin at 25 mg/ml. *Bottom*, effect of the minipump delivery system. Chiron and HLR IL-2 were prepared in the pharmacy, according to the manufacturer's specifications. The IL-2 was placed in a minipump delivery system set to deliver a standard 24-h dose into a centrifuge tube. The IL-2 remaining in the bag and the IL-2 captured in the tube were diluted accordingly and assessed for stimulation of an IL-2 induced proliferative response.

mini infusion pump system. Previous studies (22, 23) indicated that IL-2 lost biological activity in a pump delivery system. To determine whether the pump system had different effects on the two IL-2 preparations, we used the same pump system to provide specimens for analysis *in vitro*. IL-2 was placed in the infusion pump storage bag, and the system was programmed to deliver a 24-h dose through the small bore plastic tubing, exactly as used clinically for our patients. This 24-h dose was collected over 24 h into a tissue culture tube. After the 24-h delivery, the IL-2 remaining in the storage bag of the pump was compared with IL-2 that had been pumped through the system and collected in the tissue culture tube. Fig. 5 (*bottom*) demonstrates that the pump system did not have any influence on the IL-2 activity for either of the IL-2 preparations. In addition, the HLR

IL-2, independent of whether it had been pumped through the mini pump system, had a 2- and 5-fold lower EC_{50} than the Chiron IL-2 on TF-1 β (Fig. 5, *bottom*) and PHA blasts (data not shown).

Are the Two IL-2 Preparations Binding Equally Well to the IL-2R?: Examination of the Ability of Chiron and HLR IL-2 Preparations to Competitively Block Binding of IL-2 to the CTLL-2 Cell Line. In an effort to determine whether HLR and Chiron IL-2 bind equally well to IL-2Rs, we developed a flow cytometry assay using both IL-2 preparations as competitive inhibitors at equivalent IU concentrations. In this flow assay, we can detect IL-2 binding to IL-2Rs using an IL-2-immunoglobulin fusion protein. The fusion protein binds to the IL-2R on the CTLL-2 cell line via the IL-2 component of the immunoglobulin-cytokine fusion protein. The bound fusion protein can then be detected using a fluorescein-tagged goat antibody against human immunoglobulin (14). We competitively blocked the ability of the IL-2 of the fusion protein to bind to the IL-2R by adding excess soluble recombinant IL-2. We compared equivalent IUs of Chiron and HLR IL-2 for their ability to block fusion protein binding. In experiment 1, presented in Table 3, the fusion protein binding to CTLL-2 cells and developed with goat antihuman-immunoglobulin tagged with FITC resulted in an MFI of 45. When the HLR IL-2 was added to block the binding of the fusion protein, the MFI dropped to 11.0. The same concentration of Chiron IL-2 (IU/ml) only decreased the MFI to 34.9. As a control to ensure that there was not an unidentified factor or protein within the Chiron IL-2 preparation that interfered with the ability of the Chiron IL-2 to block fusion protein binding, Chiron IL-2 was mixed with HLR IL-2 for a blocking assay. The addition of the Chiron IL-2 did not diminish the ability of the HLR IL-2 to block fusion protein binding (bottom line in Table 3). Simultaneous addition of both blocking agents achieved the same level of blocking seen with HLR IL-2 alone as the blocking agent. The first two experiments in Table 3 show similar levels of blocking; the third experiment has a similar pattern, although the initial binding level of the fusion protein to CTLL-2 was lower.

Standardization of the Proliferative Assay with the CTLL-2 Line and the International Standard for IL-2. The proliferative experiments shown above in this study used two cell preparations as IL-2-responding cells. The Tf-1 β cell line and PBMC obtained from patients after a 96-h continuous infusion of IL-2 respond predominantly through an intermediate affinity $\beta\gamma$ IL-2R. In contrast, the initial studies calibrating the first International Standard for IL-2 used the murine IL-2-dependent cell line CTLL-2 (4), which expresses a high affinity $\alpha\beta\gamma$ IL-2R. We, therefore, performed proliferative assays testing the response of the CTLL-2 cell line to HLR IL-2, Chiron IL-2, and the WHO standard for IL-2. Each assay tested a range of IL-2 concentrations to determine the EC_{50} (5). This same experiment was repeated a total of six times (Table 4), and the EC_{50} values for the three preparations and the mean values are shown. Although there is substantial variation between experiments, each assay shows that the EC_{50} for Chiron IL-2 is substantially greater than the EC_{50} for the other two preparations. The mean EC_{50} for the International Standard was 0.86, fairly close to the expected value of 1.0 (the EC_{50} of this assay was used historically to define 1 unit of IL-2). The HLR IL-2

Table 3 IL-2 blocking of ch14.18-IL2 fusion protein

The MFI from flow cytometry experiments compares the ability of HLR IL-2 or Chiron IL-2 to competitively block binding of the ch14.18-IL2 fusion protein to CTLL-2 cells. The results show a decrease in MFI when HLR IL-2 is used to block binding of the fusion protein from 45.3 to 11.0 in experiment 1. In contrast, Chiron IL-2 only blocked binding of the fusion protein from 45.3 to 34.9.

Secondary antibody	Treatment	Exp. ^a 1 (MFI)	Exp. 2 (MFI)	Exp. 3 (MFI)
Goat antihuman-FITC	Control	4.5	7.2	4.5
"	HLR IL-2	4.2	7.0	4.7
"	Chiron IL-2	5.2	7.8	5.1
"	HLR IL-2 + Chiron IL-2	5.4	7.8	5.3
"	ch14.18-IL-2	45.3	41.8	11.6
"	ch14.18-IL-2 + HLR IL-2	11.0	11.7	6.3
"	ch14.18-IL-2 + Chiron IL-2	34.9	30.0	11.0
"	ch14.18-IL-2 + HLR IL-2 + Chiron IL-2	12.2	11.6	6.4

^a Exp., experiment.

Table 4 Quantitative comparison of HLR and Chiron IL-2 against the WHO International Standard

The results of six separate tritiated thymidine incorporation proliferative experiments using CTLL-2 as responding cells are presented. Dilutions of HLR, Chiron, and WHO International Standard IL-2 were made, and the mean EC₅₀ concentration was determined using the ALLFIT program. HLR IL-2 expressed in IUs was not significantly different from the WHO standard ($P = 0.5212$). Chiron IL-2, expressed in IUs, did differ significantly from the WHO standard ($P = 0.0152$). In addition, there was a significant difference between the HLR EC₅₀ and the Chiron EC₅₀ ($P = 0.0022$).

Experiment number	HLR IL-2	Chiron IL-2	WHO International Standard
1	0.9	12.1	2.6
2	0.7	4.0	1.3
3	0.33	1.55	0.22
4	0.39	5.67	0.49
5	0.28	2.19	0.39
6	0.15	2.39	0.31
Mean EC ₅₀	0.73	6.6	0.86

had a mean EC₅₀ of 0.73, and the Chiron IL-2 had a mean EC₅₀ of 6.6. These data indicate that the HLR IL-2 unitage is 84% ($0.73/0.86 = 84\%$) of the value for the WHO standard ($P = 0.52$), which is close to the expected value of 100%. In contrast, the Chiron IL-2 unitage is 7.7-fold greater ($6.6/0.86$) than the International Standard in these assays ($P = 0.015$). This indicates that it takes 7.7 times more IUs of Chiron IL-2 (in IUs as defined by the specific activity on the package insert) to induce 50% of the maximal proliferation of the CTLL-2 cell line, in these six experiments. Fig. 6 shows graphically one of these six experiments where the EC₅₀ of HLR IL-2 = 0.9, the EC₅₀ of the WHO Standard = 2.6, and the EC₅₀ of the Chiron IL-2 = 12.1.

DISCUSSION

Many early clinical trials evaluating IL-2 used preparations from various companies, each having their own product and formulation. These companies included HLR and Cetus (presently Chiron). The dosage of IL-2 for the present Chiron product was originally expressed in Cetus units, and the HLR product was expressed in Roche units. The Roche units were standardized to an interim reference reagent by the BRMP of the NCI (10). The general consensus of the scientific community in the

late 1980s was that 1 Cetus unit was equivalent to ~2.3 Roche units. In 1988, Gearing and Thorpe (4) reported "The International Standard for Human Interleukin-2". At present, we have not been able to identify a published study that directly compares the quantitative clinical effects of the two clinically used reagents, or a study that directly compares both of these two IL-2 preparations to the International Standard, *in vitro*.

According to the package insert for the FDA-approved Chiron clinical reagent, the biological activity determined in a lymphocyte proliferation assay and expressed in IUs, as established by the WHO (1), is 18 million IU/1.1 mg of protein. The recommended dosage and administration sections indicates that proleukin should be given by a 15-min i.v. infusion every 8 h. The insert recommends 600,000 IU/kg (0.037 mg/kg) every 8 h for a total of 14 doses. This is equivalent to 1.8×10^6 IU/kg/day ($\sim 48 \times 10^6$ IU/m²/day) for 4.66 days. Our earlier clinical studies using HLR IL-2 had determined that the MTD as a 4-day continuous infusion given in the hospital was 3×10^6 units/m²/day (24). Studies combining IL-2 with other cytokines or antibodies and administering the IL-2 as an outpatient 4-day continuous infusion established that this MTD was 1.5×10^6 units/m²/day (7, 25).

In an earlier published study, we had indicated that the BRMP unit was equivalent to ~3 IUs (7). This information was obtained from a document distributed by Chiron to clinicians using commercially available Proleukin IL-2 (Chiron IL-2) that stated "... The following is a summary of our current understanding of the conversion of interleukin-2 units: 1 Cetus Unit = 6 International Units, 1 Roche Unit = 3 International Units."

In a personal communication, a representative from Chiron (J. Weaver) indicated that this value, however, was based on clinical perspectives and not the biological assay used to standardized IL-2 units. In the biological assay using the IL-2-dependent cell line CTLL-2 and tritiated-thymidine incorporation, the interim reference standard developed by the BRMP and the WHO IU and the HLR "Roche" units are all equivalent (4).⁶ In initial testing, it was established that the original Cetus unit was equivalent to 3 IUs of IL-2.⁷ To quantitate IL-2 activity, Chiron has further optimized a separate colorimetric assay using

⁶ R. Thorpe and J. Weaver, personal communication.

⁷ R. Thorpe, personal communication.

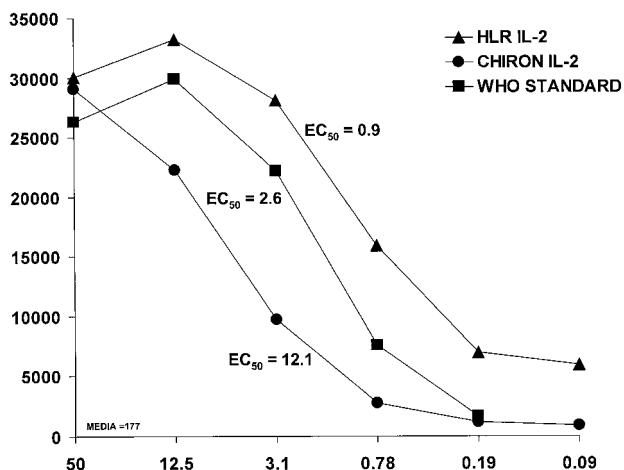


Fig. 6 Comparison of HLR IL-2 and Chiron IL-2 to the WHO International Standard. The murine IL-2-dependent CTLL-2 cell line was used as a responding cell in a 24-h proliferative assay. This experiment, representative of six experiments, shows the WHO Standard to have an EC_{50} of 2.6. The EC_{50} for the HLR IL-2 was 0.9 compared with the EC_{50} for the Chiron IL-2 of 12.1.

the tetrazolium salt MTT to quantitate cell proliferation, and this assay is used to standardize their IL-2 to the IU. The variables optimized in the assay include an initial resuspension of IL-2 in 0.1% SDS before dilution to maintain IL-2 in a nonaggregated form, optimization of kinetics and cell number. As such, the same amount of proleukin formerly corresponding to 1 Cetus Unit of IL-2 is now labeled as equivalent to 6 IUs.⁸

The FDA-approved Chiron IL-2 is a potent immunomodulating agent, used widely for the treatment of renal cell cancer and melanoma. This preparation is not identical to natural human IL-2 due to the serine substitution at amino acid position 125 and the deletion of the N-terminal alanine. These changes may effect the disulfide bonds and aggregation state as well as solubility of the IL-2 molecule, and there may be biological differences in the activities of these two drugs. In addition, the manner in which these two reagents are standardized against the International Standard may effect the specific activity in IU/mg of protein, as noted in the package insert information.

Our data, in addition to the data of Lentsch *et al.* (6), which indicated less toxicity in a murine model for the Chiron IL-2 compared with HLR IL-2 on a unit for unit basis, caution investigators and physicians that the use of an IU, based on an *in vitro* biological assay, may not accurately reflect the dose required for the desired effect. Although both Chiron and HLR IL-2 preparations were labeled with IUs, this study shows that equivalent biological effects are not obtained when using the same number of IUs of these two preparations. At present, the only FDA-approved IL-2 preparation is Chiron IL-2. We caution that if a clinician or laboratory researcher is using Chiron

IL-2 and attempting to compare a dose from a previous study using a separate IL-2 preparation, that researcher should not assume that the same number of IU of one preparation will give the same quantitative biological effects as the other. Our present study indicates that it takes 3–6 times as many IUs of Chiron IL-2 to achieve the same biological effects of a similar number of IUs of HLR IL-2.

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REFERENCES

- Lotze, M. T., Matory, Y. L., Rayner, A. A., Ettinghausen, S. E., Vetto, J. T., Seipp, C. A., and Rosenberg, S. A. Clinical effects and toxicity of interleukin-2 in patients with cancer. *Cancer (Phila.)*, 58: 2764–2772, 1986.
- Rosenberg, S. A., Lotze, M. T., and Mule, J. J. NIH conference. New approaches to the immunotherapy of cancer using interleukin-2. *Ann. Intern. Med.*, 108: 853–864, 1988.
- Kohler, P. C., Hank, J. A., Moore, K. H., Storer, B., Bechhofer, R., and Sondel, P. M. Phase I clinical evaluation of recombinant interleukin-2. *In: R. L. Truitt, R. P. Gale, and M. M. Bortin (eds.), Cellular Immunotherapy of Cancer*, pp. 161–172. New York: Alan R. Liss, Inc., 1987.
- Gearing, A. J. H., and Thorpe, R. The international standard for human interleukin-2 calibration by international collaborative study. *J. Immunol. Methods*, 114: 3–9, 1988.
- Rossio, J. Cytokines and immune cell products. *In: N. Rose, E. deMacario, J. Folds, H. Lane, R. Nakamura, (eds.), Manual of Clinical and Laboratory Immunology*, Ed. 5, pp. 348–356. Washington, DC: ASM Press, 1997.
- Lentsch, A. B., Nakagawa, K., Yoshidome, H., Gerassimides, A., Miller, F. N., and Edwards, M. J. Distinct biological activities of recombinant forms of human interleukin-2 *in vivo*. *Cancer Immunol. Immunother.*, 43: 331–336, 1997.
- Schiller, J., Hank, J., Khorsand, M., Storer, B., Borchert, A., Huseby-Moore, K., Burns, D., Wesly, O., Albertini, M., Wilding, G., and Sondel, P. Clinical and immunological effects of granulocyte-macrophage colony stimulating factor co-administered with interleukin-2: a Phase IB study. *Clin. Cancer Res.*, 2: 319–330, 1996.
- Gause, B. L., Sznol, M., Kopp, W. C., Janik, J. E., Smith, J. W., II, Steis, R. G., Urba, W. J., Sharfman, W., Fenton, R. G., Creekmore, S. P., Holmlund, J., Conlon, K. C., Vandermolen, L. A., and Longo, D. L. Phase I study of subcutaneously administered interleukin-2 in combination with interferon α -2a in patients with advanced cancer. *J. Clin. Oncol.*, 14: 2234–2241, 1996.
- Albertini, M. R., Hank, J. A., Schiller, J. H., Khorsand, M., Borchert, A. A., Gan, J., Bechhofer, R., Storer, B., Reissfeld, R. A., and Sondel, P. M. Phase IB trial of chimeric anti-GD2 antibody plus interleukin-2 for melanoma patients. *Clin. Cancer Res.*, 3: 1277–1288, 1997.
- Rossio, J. L., Thurman, G. B., Long, C., Vargosko, A., and Pinsky, C. The BRMP IL-2 reference reagent. *Lymphokine Res.*, 5, Suppl. 1: S13–8, 1986.
- Sondel, P. M., Kohler, P. C., Hank, J. A., Moore, K. H., Rosenthal, N., Sosman, J., Bechhofer, R., and Storer, B. Clinical and immunological effects of recombinant Interleukin-2 given by repetitive weekly cycles to patients with cancer. *Cancer Res.*, 48: 2561–2567, 1988.
- Farner, N. L., Voss, S. D., Leary, T. P., Gan, J., Hakimi, J., Evans, G., Ju, G., and Sondel, P. M. Distinction between gamma c detection and function in YT lymphoid cells and in the granulocyte-macrophage colony-stimulating factor-responsive human myeloid cell line, Tf-1. *Blood*, 86: 4568–4578, 1995.

⁸ J. Weaver, personal communication.

13. Weil-Hillman, G., Voss, S. D., Fisch, P., Schell, K., Hank, J. A., Sosman, J. A., Sugamura, K., and Sondel, P. M. Natural killer cells activated by interleukin-2 treatment *in vivo* respond to interleukin-2 primarily through the p75 receptor and maintain the p55 (TAC) negative phenotype. *Cancer Res.*, 50: 2683–2691, 1990.
14. Hank, J. A., Surfus, J. E., Gan, J., Jaeger, P., Gillies, S., Reisfeld, R. A., and Sondel, P. M. Activation of human effector cells by a tumor reactive recombinant anti-ganglioside-GD2/interleukin-2 fusion protein (ch14.18-IL2). *Clin. Cancer Res.*, 2: 1951–1959, 1996.
15. Kohler, P. C., Hank, J. A., Moore, K. H., Storer, B., Bechhofer, R., Hong, R., and Sondel, P. M. Phase I clinical trial of recombinant interleukin-2: a comparison of bolus and continuous intravenous infusion. *Cancer Invest.*, 7: 213–223, 1989.
16. Hank, J. A., Kohler, P. C., Weil-Hillman, G., Rosenthal, N., Moore, K. H., Storer, B., Minkoff, D., Bradshaw, J., Bechhofer, R., and Sondel, P. M. *In vivo* induction of the lymphokine-activated killer phenomenon: interleukin 2-dependent human non-major histocompatibility complex-restricted cytotoxicity generated *in vivo* during administration of human recombinant interleukin 2. *Cancer Res.*, 48: 1965–1971, 1988.
17. Meropol, N. J., Porter, M., Blumenson, L. E., Lindemann, M. J., Perez, R. P., Vaickus, L., Loewen, G. M., Creaven, P. J., Wilkes, K. A., Giedlin, M. A., and Caligiuri, M. A. Daily subcutaneous injection of low-dose interleukin 2 expands natural killer cells *in vivo* without significant toxicity. *Clin. Cancer Res.*, 2: 669–677, 1996.
18. Voss, S. D., Hank, J. A., Nobis, C., Fisch, P., Sosman, J. A., and Sondel, P. M. Serum levels of the low-affinity interleukin-2 receptor molecule (TAC) during IL-2 therapy reflect systemic lymphoid mass activation. *Cancer Immunol. Immunother.*, 29: 261–269, 1989.
19. Bogner, M. P., Voss, S. D., Bechhofer, R., Hank, J. A., Roper, M., Poplack, D., Hammond, D., and Sondel, P. M. Serum CD25 levels during interleukin-2 therapy: dose dependence and correlations with clinical toxicity and lymphocyte surface sCD25 expression. *J. Immunother.*, 11: 111–118, 1992.
20. Miles, D. W., Bird, C. R., Wadhwa, M., Summerhayes, M., Balkwill, F. R., Thorpe, R., and Rubens, R. D. Reconstitution of interleukin 2 with albumin for infusion. *Lancet*, 335 (8705): 1602–1603, 1990.
21. Cassidy, J., Poole, C., Sharkie, E., Steward, W. P., and Kaye, S. B. The importance of added albumin during continuous intravenous infusion of interleukin-2 with α -interferon. *Eur. J. Cancer*, 27: 1633–1634, 1991.
22. Vlasveld, L. T., Beijnen, J. H., Sein, J. J., Rankin, E. M., Melief, C. C. J., and Hekman, A. Reconstitution of recombinant interleukin-2 (rIL-2): a comparative study of various rIL-2 muteins. *Eur. J. Cancer*, 29A: 1977–1979, 1993.
23. Tzamos, S. T., Hrushesky, W. J., Wood, P. A., and Przybycien, T. M. Irreversible inactivation of interleukin 2 in a pump-based delivery environment. *Proc. Natl. Acad. Sci. USA*, 93: 5460–5465, 1996.
24. Sosman, J. A., Kohler, P. C., Hank, J. A., Moore, K. H., Bechhofer, R., Storer, B., and Sondel, P. M. Repetitive weekly cycles of recombinant human interleukin-2: responses of renal carcinoma with acceptable toxicity. *J. Natl. Cancer Inst.*, 80: 60–63, 1988.
25. Schiller, J. H., Hank, J., Storer, B., Borchert, A. A., Huseby-Moore, K., Albertini, M., Bechhofer, R., Wesley, O., Brown, R. R., Mrowca Bastin, A., and Sondel, P. M. A direct comparison of immunological and clinical effects of interleukin 2 with and without interferon- α in humans. *Cancer Res.*, 53: 1286–1292, 1993.