Purification to homogeneity and partial characterization of interleukin 2 from a human T-cell leukemia

(T-cell growth factor/thymocyte stimulatory factor/HPLC/microsequence analysis/peptide mapping)

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ABSTRACT A method utilizing reversed-phase high-performance liquid chromatography has been developed for the purification to homogeneity of interleukin 2 (IL-2) isolated from a human T-cell leukemia. A final purification of 500,000-fold was obtained with a specific activity of pure IL-2 of 10⁹ units/mg. The amino acid analysis of natural IL-2 is strikingly similar to the composition deduced from sequence analysis of a cDNA coding for human IL-2. Protein sequence analysis of CNBr-derived peptides yields data consistent with the sequence proposed from cloned cDNA. The availability of homogeneous IL-2 will allow accurate biological studies of its activity free from the contamination of the numerous lymphokine species that are known to be co-produced with IL-2 during the induction procedure.

Interleukin 2 (IL-2), formerly known as T-cell growth factor, was first described as a lymphokine capable of modulating lymphocyte reactivity and promoting the long-term in vitro culture of antigen-specific effector T lymphocytes (1, 2). IL-2 partially purified from mouse (3, 4), rat (5, 6), and human (7, 8) normal T lymphocytes has been found to retain different types of biological activity, including: (i) marked enhancement of thymocyte mitogenesis (7, 9); (ii) promotion of long-term in vitro proliferation of antigen-specific helper or killer T-cell lines (3, 10); and (iii) induction of cytotoxic T-lymphocyte reactivity and plaque-forming cell responses in cultures of nude mouse spleen cells (7, 9). These activities indicate that IL-2 may be useful in augmenting immune responses and restoring immune-deficient T-cell populations (nude mouse spleen cells) to normal levels of cellular and humoral immunity. Furthermore, these results suggest that IL-2 production and response are important parameters of immunological functions and their measurement may be useful in clinical diagnosis of aberrant immune status.

The sources and techniques that have been used to produce IL-2 have yielded low concentrations of IL-2, with large volumes of conditioned media being required to obtain only small quantities of human IL-2 activity. As a consequence, sufficient quantities of concentrated human IL-2 have not been available for in vivo experiments or for its characterization. Several groups have reported purification procedures for both murine (9, 11) and human (7, 12–14) IL-2, although the purity of these preparations is not clear. The present study reports a procedure for the purification of human IL-2 to homogeneity using several steps of reversed-phase high-performance liquid chromatography (HPLC). Chemical characterization of the purified lymphokine is reported.

**EXPERIMENTAL PROCEDURES**

Source of IL-2. Human leukemia T cells designated Jurkat-H33HJ-JAI (a subclone of the parent Jurkat-FHCRC cell line) (15) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 units of penicillin per ml, 50 μg of gentamycin per ml, and 300 μg of fresh 1-glutamine per ml. The cells were grown in T175-cm² flasks (volume, 150 ml) in a humidified atmosphere of 5% CO₂ in air until saturation (8–10 × 10⁶ cell per ml); they then were harvested, pelleted, and resuspended (in fresh RPMI 1640 medium without serum but with 50 units of penicillin per ml, 50 μg of streptomycin per ml, 50 μg of gentamycin per ml, and 300 μg of fresh 1-glutamine per ml) to a concentration of 2 × 10⁸ cells per ml in fresh T175-cm² flasks. For IL-2 production, the cells were stimulated with the T-cell mitogen phytohemagglutinin M (PHA-M; GIBCO; 1% by volume) and phorbol 12-myristate 13-acetate (PMA; Sigma; at a final concentration of 10 ng/ml) (15). After 24 hr of incubation at 37°C in a humidified atmosphere of 5% CO₂ in air, the suspensions were centrifuged for 15 min at 1000 × g at 4°C, and the resultant supernatant solution was retained as crude conditioned medium. For the purification detailed in Table 1, a total of 50 liters of crude conditioned media was prepared. Despite the fact that such crude supernates were produced serum-free, they contained a relatively large amount of protein (∼1 mg/ml) due to prior growth of the producer cells in serum, the presence of 1% PHA (crude mitogen) in the supernatant solutions, and the liberation of cellular protein into the culture fluid due to death of the Jurkat-H33HJ-JAI cells upon mitogen stimulation (15).

**Ammonium Sulfate Precipitation.** Crude human IL-2 supernatant solutions prepared from several batches of induced H33HJ-JAI cells were pooled and brought to 35% saturation by adding solid ammonium sulfate at 4°C. The solution was kept at 4°C overnight and then was centrifuged at 10,000 × g for 30 min. The precipitate was discarded, and the supernatant solution was brought to 85% saturation by the addition of solid ammonium sulfate at 4°C. The precipitate was isolated by centrifugation at 10,000 × g for 20 min, dissolved in sterile double-distilled water, and dialyzed against 1,000 vol of 0.05 M NaCl/Hepes (25 mM), pH 5.5.

**Ion-Exchange Chromatography on CM Biogel A Column.** Column resin (CM Biogel A, LKB, Bromma, Sweden) was equilibrated in 0.05 M NaCl/Hepes (25 mM), pH 5.5. The column was poured and 1 column vol (100 ml) of 0.05 M NaCl/Hepes (25 mM), pH 5.5, containing 10% fetal calf serum was then applied to the column to block nonspecific binding sites. Serum proteins that bound to the column were...
then eluted off by the addition of 5 column vol of 0.5 M NaCl/Hepes (25 mM), pH 5.5. The column was then reequilibrated with 10 vol of 0.05 M NaCl.

Combining ammonium sulfate-precipitated protein (see above) were applied to a CM column. The column was first washed with 2 vol of 0.05 M NaCl/Hepes (25 mM), pH 5.5, and then eluted with a 500-ml salt gradient from 50 mM to 0.5 M NaCl/Hepes (25 mM), pH 5.5.

**Reversed-Phase HPLC.** The chromatographic system was described previously (16). An automated fluorescence detection system using fluorescamine (Hoffmann-La Roche) monitored the protein in the column effluents (17). Reversed-phase HPLC was carried out using a Magnum 9 Proteol octyl column (9.4 × 250 mm, Whatman), a Vydac C₁₈ column (4.6 × 250 mm, The Sep/a/ra/tions Group, Hesperia, CA), and a Protesil diphenyl column (4.6 × 250 mm). Acetic acid (0.9 M)/pyridine (0.2 M) buffer, pH 4.0, was used with gradients of 1-propanol to elute the proteins (16). Further chromatographic details are given in the figure legends.

**Protein Analysis.** Amino acid analyses were carried out at the picomole level with a fluorescamine amino acid analyzer (18). In preparation for microsequence analysis, the purified protein (18 µg in 500 µl) was reduced and carboxymethylated. This was accomplished by addition of 300 µl of 0.16 M Tris-HCl, pH 8.5/8.5 M guanidine-HCl. The volume was reduced to 400 µl under a stream of nitrogen, and 2 µmol of dithiothreitol was added. The mixture was incubated at 37°C for 4 hr. After reductive cleavage of the disulfide bonds, [¹⁴C]sodium monooiodoacetate (4 µmol) was added and the resulting solution was incubated in the dark at room temperature for 2 hr. The pH was decreased to 4.0 with acetic acid and the mixture was applied to a Supelcosil 308 column (4.6 × 50 mm, Supelco, Bellefonte, PA) that had been equilibrated with 0.9 M acetic acid/0.2 M pyridine, pH 4.0. The column was then washed with 20 ml of the same buffer to remove excess reagents, buffer salt, and denaturing agent. The carboxymethylated protein was removed from the HPLC column by eluting with 40% (vol/vol) 1-propanol/0.9 M acetic acid/0.2 M pyridine, pH 4.0.

CNBr cleavage of the carboxymethylated protein (13 µg) was performed in 70% formic acid (19), and the resultant peptide fragments were isolated by HPLC on a Supelcosil LC-18-DB column (4.6 × 150 mm). Trifluoroacetic acid (0.1%) used with a linear gradient of acetonitrile (80%, vol/vol) was used to elute the peptides.

Microsequence analysis was performed on <100 pmol of the peptide with an Applied Biosystems model 470A protein sequencer similar to that described by Hewick et al. (20). Phenylthiohydantoin amino acids were identified by HPLC using a Beckman/Altex Ultrasphere ODS column and the trifluoroacetic acid/acetonitrile buffer system reported by Hawke et al. (21).

**Assay for Biological Activity.** Material isolated from each of the chromatographic steps was assayed in 100-µl cultures for its ability to stimulate the uptake of [³H]thymidine by IL-2-dependent murine T cells (5, 9). One unit of activity is defined as the amount of IL-2 that induces 50% of maximal thymidine incorporation, as judged against a standard (rat spleen) IL-2 preparation, assayed on every test plate. For example, if a sample induced 50% maximal proliferation at a dilution of 1:20, 1 unit of activity was therefore said to be contained in 1/20th of the 100-µl assay volume or 5 µl. This sample would therefore contain 1,000 ÷ 5 units/ml or 200 units/ml.

**RESULTS**

**Purification of IL-2.** IL-2 was produced by Jurkat-H33HJ-JAI cells, a clone of the parent Jurkat-FHCR cell line, after coulture with PHA and PMA. Fifty liters of supernatant solutions from these cells were collected and the proteins were precipitated by sequential addition of solid ammonium sulfate to a final concentration of 85%. The precipitate was collected by centrifugation and dissolved in 0.05 M NaCl/Hepes (25 mM), pH 5.5.

The dialyzed ammonium sulfate precipitate was placed on a CM Biogel A column, and protein was eluted with a salt gradient (Fig. 1). Peak activity was determined and the active fractions were pooled. This material was pumped onto a semipreparative Proteol octyl column using a trace enrichment technique. The column was then washed with 50 ml of 0.9 M acetic acid/0.2 M pyridine, pH 4.0, buffer. Elution of the proteins was accomplished with a gradient of 0–60% (vol/vol) 1-propanol over 2 hr in the acetate/pyridine buffer, pH 4.0 (data not shown). The typical recovery of biological activity for this purification step was between 60% and 100%.

The fractions containing the major peaks of activity were pooled, diluted 1:1 (vol/vol) with acetate/pyridine buffer, pH 4.0, and pumped onto an analytical Vydac C₁₈ column. Proteins were eluted with a linear 20–40% 1-propanol gradient in acetate/pyridine buffer, pH 4.0, over 8 hr. This procedure yielded a symmetrical peak of fluorescence coinciding with the major peak of IL-2 activity (Fig. 2A). Recovery of biological activity in this step was >90%. A peak of biological activity eluting 15 min earlier in the chromatogram appeared to be a polypeptide of lower specific activity. Upon rechromatography using a different gradient, the biological activity was resolved from the major peak of protein, indicating that the earlier eluting activity may be either an oxidation product of IL-2, differs from it in the degree of glycosylation or primary structure, or may be a completely different protein altogether.

Those fractions (360–366 min) containing the major peaks of activity were once again diluted 1:1 (vol/vol) with the acetate/pyridine buffer and pumped onto a Proteosil diphenyl column. The protein was eluted with a higher concentration of 1-propanol—i.e., a linear gradient of 20–60% (vol/vol) in the acetate/pyridine buffer—because proteins typically bind much tighter to this support than to other reversed-phase supports (16, 22). Therefore, the diphenyl column offers a different type of reversed-phase interaction. A single peak of

![FIG. 1. IL-2 elution pattern from CM Biogel A column. IL-2 was eluted with a salt gradient running from 50 to 500 mM NaCl/Hepes (25 mM), pH 5.5. Samples of each fraction were assessed for their ability to stimulate growth of IL-2-dependent T cells as manifested by [³H]thymidine incorporation. Each cpm incorporated by assay cells in the presence of a 1/3,700 dilution of column fraction. cpm incorporated by assay cells in the presence of a 1/87,000 dilution of column fraction. In the absence of IL-2, assay cells incorporated <200 cpm of [³H]thymidine.](image-url)
fluorescence was again obtained upon this rechromatography, indicating a homogeneous preparation (Fig. 2B). Recovery of activity in this step was >60%. Amino acid analysis was performed on each of the fractions within the peak area to confirm the homogeneity of the polypeptide (see below). Finally, essential homogeneity of this material was confirmed by analytical NaDodSO$_4$/polyacrylamide gel electrophoresis and two-dimensional gel electrophoresis, which yielded a single band ($M$, 14,000) and a single spot, respectively (data not shown).

The overall purification of IL-2 that was achieved is shown in Table 1. The protein content of the Vydac C$_{18}$- and Protei sil diphenyl-purified material was calculated on the basis of the amino acid analysis. A specific activity of $10^9$ units/mg of protein and a final purification of 500,000-fold were obtained. The overall recovery of IL-2 was ~36%.

**Chemical Characterization.** The ability to prepare homogeneous IL-2 allowed for the determination of the amino acid composition and partial sequence analysis of the naturally occurring IL-2 protein. The fractions containing pure IL-2 (4 ml) (Fig. 2B) were diluted with the acetate/pyridine buffer (1:1, vol/vol) and applied to a previously unused diphenyl column. By “pulsing” the protein off the column with 60% 1-propanol in the acetate/pyridine buffer at a slow flow rate, the volume was reduced to 0.5 ml. Chemical characterizations were carried out on this solution of IL-2.

The protein was subjected to carboxymethylation, and its amino acid composition was determined before and after the procedure (Table 2). Simultaneous to the preparation of this report, Taniguchi et al. reported the cloning and sequence analysis of a cDNA coding for human IL-2 (25), from which the primary structure of the mature human IL-2 polypeptide was deduced assuming cleavage of the signal peptide at position 20. The amino acid composition of this structure is shown in Table 2. A comparison of the composition of the naturally occurring protein and the deduced IL-2 shows them to be strikingly similar.

Amino-terminal sequence determination of the IL-2 polypeptide was attempted on 300 pmol by automated Edman degradation. Analysis of the first few cycles yielded no sequence data, suggesting a possible amino-terminal substitution.

To obtain sequence data, carboxymethylated IL-2 was cleaved with CNBr and was subjected to HPLC. The re-

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**Table 1. Purification of IL-2**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein, mg*</th>
<th>Total volume, ml</th>
<th>Total activity, units</th>
<th>Specific activity, units/mg of protein</th>
<th>Purification, fold</th>
<th>Yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Conditioned medium</td>
<td>50,000</td>
<td>50,000</td>
<td>$100 \times 10^8$</td>
<td>$2.00 \times 10^4$</td>
<td>1</td>
<td>100.0</td>
</tr>
<tr>
<td>2 (NH$_4$)$_2$SO$_4$ precipitate</td>
<td>5,000</td>
<td>500</td>
<td>$73.5 \times 10^8$</td>
<td>$1.47 \times 10^4$</td>
<td>7</td>
<td>73.5</td>
</tr>
<tr>
<td>3 CM Biogel A</td>
<td>776</td>
<td>216</td>
<td>$88.9 \times 10^8$</td>
<td>$1.15 \times 10^4$</td>
<td>58</td>
<td>88.9</td>
</tr>
<tr>
<td>4 Proteol octyl$^3$</td>
<td>5</td>
<td>75</td>
<td>$63.7 \times 10^8$</td>
<td>$1.27 \times 10^7$</td>
<td>6,350</td>
<td>63.7</td>
</tr>
<tr>
<td>5 Vydac C$_{18}$</td>
<td>0.056</td>
<td>4</td>
<td>$53.5 \times 10^8$</td>
<td>$9.55 \times 10^6$</td>
<td>477,500</td>
<td>53.5</td>
</tr>
<tr>
<td>6 Proteol diphenyl</td>
<td>0.035</td>
<td>6</td>
<td>$35.8 \times 10^8$</td>
<td>$1.01 \times 10^9$</td>
<td>505,000</td>
<td>35.8</td>
</tr>
</tbody>
</table>

*Protein concentration in individual fractions was assayed by injecting microliter volumes into the postcolumn fluorescamine detection system (23). Bovine serum albumin was used as the calibration standard. The absolute specific activity was determined by amino acid analysis of the homogeneous peak.

$^1$Increase in total units due to elimination of inhibitory factors.

$^3$Step 4 summarizes the results from five chromatographic runs.

$^5$Steps 5 and 6 summarize the results from two chromatographic runs.
Table 2. Amino acid composition of IL-2

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid or asparagine</td>
<td>12.4 (12)</td>
</tr>
<tr>
<td>Threonine</td>
<td>11.6 (13)</td>
</tr>
<tr>
<td>Serine</td>
<td>7.4 (8)</td>
</tr>
<tr>
<td>Glutamic acid or glutamine</td>
<td>16.5 (18)</td>
</tr>
<tr>
<td>Proline</td>
<td>7.4 (5)</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.4 (2)</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.9 (5)</td>
</tr>
<tr>
<td>Carboxymethylcysteine</td>
<td>3.3 (3)</td>
</tr>
<tr>
<td>Valine</td>
<td>4.0 (4)</td>
</tr>
<tr>
<td>Methionine</td>
<td>4.0 (4)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>8.5 (9)</td>
</tr>
<tr>
<td>Leucine</td>
<td>20.8 (22)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.0 (3)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.8 (6)</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.8 (3)</td>
</tr>
<tr>
<td>Lysine</td>
<td>10.6 (11)</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.9 (4)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>ND (1)</td>
</tr>
</tbody>
</table>

Samples (20–30 pmol) were hydrolyzed at 110°C for 22 hr in 100 μl of constant-boiling HCl containing 2.0% thioglycolic acid. Tryptophan was not determined (ND). The results represent means of nine analyses, except for proline and carboxymethylcysteine, which are means of three analyses each. By the method used, the value for proline may be >20% inaccurate (24). Values in parentheses represent the theoretical amino acid composition of IL-2 based upon the primary structure of the polypeptide deduced from sequence analysis of cloned IL-2 cDNA (25).

Solved CNBr peptides are shown in Fig. 3. The amino acid composition of C-1 was shown to be almost identical to that of IL-244,49 based on the polypeptide sequence deduced from IL-2 cDNA (25). Peptide fragment C-2 was tentatively identified as IL-226–40, fragment C-3 as IL-221–43, and fragment C-4 as IL-253–67. The carboxyl-terminal fragment—i.e., IL-2125–153—was not clearly identified on the chromatogram. Due to the hydrophobic nature of this fragment, the possibility exists for the peptide to have bound very tightly to the reversed-phase column. In addition, because this fragment does not contain any lysine residues, it may not react well in the postcolumn fluoroscence detection system.

The amino-terminal sequence of fragment C-1 was determined by automated Edman degradation on 100 pmol of the peptide. Unambiguous results were obtained for the first 14 cycles, with an initial yield of 35%, and the residue assignments were as follows: NH$_2$-Ile-Leu-Asn-Gly-Ille-Asn-Asn-Tyr-Lys-Asn-Pro-Lys-Leu-Thr. This sequence is the same as position 44–57 of the sequence proposed by Taniguchi et al. from cloned human IL-2 cDNA (25).

**DISCUSSION**

IL-2, since its initial use for the growth and maintenance of heterogeneous and cloned T-cell lines in vitro (2, 3, 26), has become both an important research tool and a molecule of keen interest to immunologists and cell biologists. It has also been used to stimulate the growth of many types of antigen-reactive human and murine T-cell lines (10, 27). Since these initial publications, the use of IL-2 as a reagent for in vitro culture of killer, helper, and suppressor T cells has become common.

Biochemically characterized IL-2 has been shown to augment a number of in vitro immune responses, including the generation of antigen-specific cytotoxic T cells (28), the lytic activity of natural killer cells (29), and the development of plaque-forming B cells (9). Likewise, lymphokines or other modifiers that augment T-cell responses have been shown to do so in many instances by facilitating IL-2 production (30–32). Based on the pivotal role that IL-2 has been shown to play regarding T-cell proliferation, this lymphokine may also manipulate in vivo immune responses as a natural mediator of immunity (33, 34).

These data, together with the potential of using IL-2-driven T cells as therapeutic tools, have served to increase scientific interest surrounding IL-2. Attempts to chemically characterize IL-2 progressed rapidly due to the development of advanced separation techniques, such as HPLC, cloned cel-

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**Fig. 3.** CNBr peptides derived from the IL-2 polypeptide. Nine hundred picomoles of carboxymethylated IL-2 was cleaved with CNBr and the resultant peptides were separated by HPLC on a Supelcosil LC-18-DB column (particle size, 3 μm; 4.6 x 150 mm). Elution was carried out using a linear gradient of acetonitrile (---) in 0.1% trifluoroacetic acid at a flow rate of 20 ml/hr. Five percent of the column effluent was diverted to the detection system. CNBr peptides are numbered according to their elution position.
lular sources of the lymphokine (15, 35), and the standardization of an unequivocal bioassay for the detection of IL-2 based on its ability to trigger the proliferation of cloned responsive T cells (5). The availability of the homogeneous IL-2 will now permit biological studies of its activity free from the contamination of impurities and related substances, such as inducing agents and lymphokines, that are known to be co-produced with IL-2 during the induction procedure. There have been earlier reports of highly purified or nearly homogeneous IL-2 preparations based on the appearance of a single band on NaDodSO₄/polyacrylamide gel electrophoresis (6, 8, 14). However, we have found that when material prepared by those procedures is chromatographed by HPLC, it can generally be resolved into several protein peaks, most of which are not associated with the IL-2 activity. As an example, previous reports have referred to a coelution of B-cell growth factor with IL-2 under various chromatographic conditions (36, 37). However, the homogeneous IL-2 contains no detectable B-cell growth factor bioactivity, as measured by its inability to provide a proliferative signal to anti-μ-stimulated human peripheral blood B lymphocytes (unpublished data).

An interesting structural feature of the IL-2 molecule is its high content of leucine residues; >15% of the amino acids comprising IL-2 are leucine residues. In fact, the IL-2 amino acid composition reveals it to be a hydrophobic molecule, which is in accord with its observed elution off reversed-phase supports (Fig. 2). This hydrophobic nature is probably important in the molecule's biological activity in relation to receptor binding and interaction.

Concurrent with the preparation of this report, Taniguchi et al. (25) described a cDNA coding for human IL-2 that had been cloned from a cDNA library prepared from partially purified IL-2 mRNA. The DNA sequence codes for a polypeptide that consists of 153 amino acids including a putative signal sequence. This accomplishment will foster IL-2 research as investigators study the mechanism behind IL-2 gene regulation and expression.

The potential supply of sufficient quantities of pure human IL-2 for use by immunologists and cell biologists who are interested in studying the induction of T-cell proliferation as a model of cell division and differentiation remains a question of chemical purification. This is so whether the source of the material is prepared by bacterial fermentation or by cell culture. The biochemical separation procedures outlined above permit the rapid, efficient purification of human IL-2. These techniques together with appropriate affinity chromatographic procedures may be used to purify both recombinant and naturally produced human IL-2 for biological investigation.

We thank Ms. May Chang for conducting the amino acid analyses, Dr. W. Benjamin for B-cell growth factor bioassays, and Ms. Alice Myers for the preparation of this manuscript.