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PURIFICATION AND PARTIAL SEQUENCE ANALYSIS OF HUMAN INTERLEUKIN-2 DERIVED FROM PERIPHERAL BLOOD LEUKOCYTES

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SUMMARY: Human peripheral blood leukocyte-derived interleukin-2 (IL-2) was resolved by DEAE-cellulose column chromatography into three peaks of activity, IL-2A, B, and C, with isoelectric points of 7.2, 6.6, and 7.9, respectively. IL-2 A, B, and C were further purified by reverse phase high performance liquid chromatography and resolved into two apparently homogeneous peaks each with identical molecular weight: A-1 and A-2 (Mr17000); B-1 and B-2 (Mr17500); and C-1 and C-2 (Mr14400). The amino acid compositions and partial NH_2-terminal amino acid sequences of these molecular species were consistent with those predicted from IL-2 cDNA sequences derived from Jurkat and peripheral blood leukocytes.

Interleukin-2 (IL-2) is a lymphokine produced by T-cells when they are activated with either lectins or antigens (1,2). It regulates the immune responses and T-cell growth and differentiation (3). Human IL-2 derived from Jurkat, a leukemic T-cell line, has been recently purified to homogeneity by affinity chromatography using monoclonal antibody and high performance liquid chromatography (4-6). Jurkat IL-2 was reported to be a protein with a molecular weight of about 15000. The partial amino acid sequence and amino acid composition of purified Jurkat IL-2 were in good agreement with those predicted from Jurkat-derived cDNA cloned and sequenced by Taniguchi et al. (7).

In contrast, only a little is known about the molecular characteristics of human IL-2 produced by peripheral blood leukocytes (PBL) or tonsillar lymphocytes. Robb and Smith (8) first mentioned the charge and molecular weight

Abbreviations: IL-2, interleukin-2; PBL, peripheral blood leukocytes; Con-A, concanavalin-A; TPA, 12-O-tetradecanoylphorbol-13-acetate; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate.

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heterogeneity of IL-2 derived from tonsillar lymphocytes and suggested that IL-2 was a variably glycosylated protein. The extremely low productivity of IL-2 derived from normal lymphocytes has made it difficult to obtain sufficient amounts of highly purified IL-2 to investigate its physicochemical properties. In this paper, we report the purification to apparent homogeneity of six species of IL-2 derived from PBL and their characteristics including partial NH_2-terminal amino acid sequences.

MATERIALS AND METHODS

Production of IL-2 by human PBL: PBL were separated from the buffy coat of healthy donors (50-100) using dextran as described previously (9). PBL thus obtained were resuspended in a serum-free RPMI-1640 medium at 5 x 10^6 cells/ml, and cultured in a cell factory(A/S Nunc, Roskilde, Denmark) at 37°C for 72hr in a humidified atmosphere of 5% CO_2/air in the presence of concanavalin-A (Con-A) (20 μg/ml; P-L Biochemicals, Inc., Milwaukee, U.S.A.), 12-O-tetradecanoyl-phorbol-13-acetate (TPA, 15ng/ml; P-L Biochemicals, Inc.), and mitomycin-C treated Namalwa cells (5 x 10^5 cells/ml). After incubation the cells were removed by centrifugation and the serum-free supernatant fluid was collected for purification of IL-2.

Determination of IL-2: IL-2 was determined by its ability to maintain an IL-2-dependent murine NK cell line (NKC3) as described previously (9). One unit of IL-2 was defined as the amount of activity present in one ml of our laboratory standard (conditioned medium) which was obtained by incubating PBL (5 x 10^6 cells/ml) at 37°C for 72 hr in an RPMI-1640 medium supplemented with 10% fetal calf serum (M. A. Bioproducts, Maryland, U.S.A.), TPA (15ng/ml) and Con-A (40 μg/ml). The dilution rate that gives a half-maximal incorporation of [^{3}H]-thymidine is about 50 for this standard.

Purification of IL-2: (1) SP-Sephadex column chromatography Culture supernatant (103 l) was acidified to pH 3.5 with HCl and applied to an SP-Sephadex C-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (bed volume of 800 ml) previously equilibrated with 0.02 M sodium citrate buffer (pH 3.5). The column was washed with the equilibration buffer, and proteins were eluted with the same buffer containing 2 M NaCl. Active fractions (468 ml) were pooled and dialyzed against two changes of 30 l of 0.01 M Tris-HCl (pH 8.0). (2)DEAE--Sephacel column chromatography The dialyzed fraction was applied to a DEAE-Sephacel (Pharmacia Fine Chemicals) column (bed volume of 100 ml) previously equilibrated with 0.01 M Tris-HCl (pH 8.0). The column was washed with the same buffer. Proteins were eluted by a linear gradient of NaCl. The gradient was produced by adding 1 l of 0.01 M Tris-HCl (pH 8.0) containing 0.2 M NaCl into 1 l of 0.01 M Tris-HCl (pH 8.0). Active fractions (677 ml) were pooled and concentrated to about 6 ml on a Diaflo YM-5 membrane (Amicon Corp., Massachusetts, U.S.A.). (3) Gel filtration The concentrated solution was chromatographed on an Ultrogel AcA54 (LKB-Produkter AB, Bromma, Sweden) column (2.5 x 96 cm) equilibrated with 0.1 M Tris-HCl (pH 8.0) containing 1 M NaCl. Fractions containing IL-2 activity (36.1 ml) were pooled and dialyzed against 2 l of 0.01 M Tris-HCl (pH 8.0). (4) DE-52 column chromatography The dialyzed fraction was applied on a DE-52 (Whatman Chemical Separation, Ltd., Kent, England) column (bed volume of 20 ml) equilibrated with 0.01 M Tris-HCl (pH 8.0). Proteins were eluted by a linear gradient of NaCl. The gradient was produced by adding 200 ml of 0.01 M Tris-HCl (pH 8.0) containing 0.15 M NaCl into 200 ml of 0.01M Tris-HCl (pH 8.0). By this step IL-2 was resolved into three peaks of activity IL-2A, B, and C. Each fraction was pooled (IL-2A, 24.6 ml; IL-2B, 34.8 ml; IL-2C, 48.0 ml) and concentrated on a Diaflo YM-5 membrane. (5) Reverse phase high performance liquid chromatography (HPLC) The

fractions obtained by the previous step were subjected to a reverse phase HPLC (Varian model 5040 system; Varian Associates, Inc., California, U.S.A.) equipped with an Ultrapore RPSC column (0.46 x 7.5 cm, Beckman Instruments, Inc., California, U.S.A.). Proteins were eluted at a flow rate of 0.8 ml/min with a linear gradient of acetonitrile. Solvents used were A, 0.1% trifluoroacetic acid(TFA)-99.9% water and B, 0.1% TFA-99.9% acetonitrile. The elution program was as follows: time 0 (68%A + 32%B)-25 min (55%A + 45%B)-35 min (45%A + 55%B)-45 min (30%A + 70%B)-48 min (100%B).

SDS-polyacrylamide gel electrophoresis: SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (10) with a 15% polyacrylamide gel containing 0.1% SDS under reducing conditions.

Protein assay: The concentration of proteins was estimated by A_{280} based on the calibration that a protein solution of 1.0 mg/ml gave an absorbance of 1.0 at 280 nm. The protein content in the final preparation was determined by the integration value for a given peak on HPLC obtained by the peak integrator.

Isoelectric focusing: Isoelectric focusing was performed in a dextran gel support using a flat bed apparatus FBE 3000 (Pharmacia Fine Chemicals). The sample was placed on a Sephadex IEF (Pharmacia Fine Chemicals) gel pre-swollen with distilled water containing 5% Pharmalyte (pH 3-10) (Pharmacia Fine Chemicals), 2% dialyzed fetal calf serum and 0.1% polyethyleneglycol 6000. Electrophoresis was carried out at 4 °C for 6 hr at a constant power of 40 W.

Amino acid composition and NH_2-terminal sequence: The amino acid composition of purified IL-2 was determined on 24, 48, and 72 hr hydrolysates with 6 N HCl at 110°C in the presence of 4% thioglycolic acid. Amino acid analysis using o-phthaldialdehyde/2-mercaptoethanol was performed on a Hitachi model 835 amino acid analyzer. The NH_2-terminal amino acid sequence was determined using a gas-phase protein sequencer (model 470A; Applied Biosystems, Inc., California, U.S.A.). The amount of protein used was 0.5-1.1 nmoles for each determination. Phenylthiohydantoin-amino acid derivatives were analyzed on a Varian model 5500 chromatograph equipped with a Micropak-SP column (0.46 x 15 cm) (Varian Associates).

RESULTS

Purification of IL-2 from culture supernatant of human PBL

The serum-free culture supernatant was subjected to SP-Sephadex C-25 and DEAE-Sephacel column chromatography, and gel filtration on an Ultrogel AcA-54 column. The partially purified IL-2 preparation thus obtained was applied on a DEAE-cellulose (DE-52) column and proteins were eluted by a linear NaCl gradient. IL-2 was resolved into three peaks of activity (Fig. 1(A)). The two major fractions eluted at NaCl concentrations of about 0.05 M and about 0.07 M were pooled separately and designated as IL-2A and IL-2B, respectively. The small activity peak recovered before IL-2A was pooled and designated as IL-2C. On isoelectric focusing (Fig. 1(B)), IL-2C was found to be the most basic and migrated as a single activity peak with pI 7.9. The pI values for IL-2A and IL-2B were 7.2 and 6.6, respectively. Almost equal amounts of IL-2A and IL-2B were always obtained by this step in several purification runs. However, in most cases the amount of IL-2C was smaller than the other two and

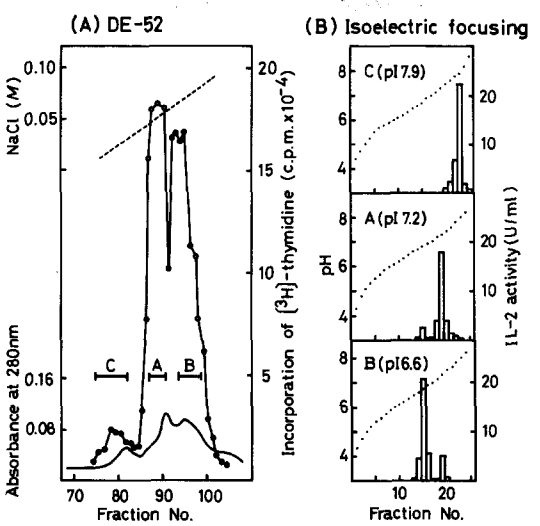

Figure 1. Elution pattern of IL-2 A, B, and C on a DE-52 column (A) and determination of the isoelectric points (B). (A) The eluate on an Ultrogel AcA 54 column was applied on a DE-52 column equilibrated with 0.01 M Tris-HCl (pH 8.0). Proteins were eluted with a linear gradient of NaCl (0-0.15 M). Each 5-ml fraction was collected. ——, A_{280}; ----, molar concentration of NaCl; O—O, IL-2 activity determined by the incorporation of [^{3}H]-thymidine. Fractions C, A, and B denoted by the bars (⊢—⊣) were pooled. (B) Aliquots were subjected to isoelectric focusing on an agarose gel. ·····, pH; ▭, IL-2 activity (U/ml).

varied depending on the batches. IL-2A, B, and C were further purified by reverse phase HPLC with an Ultrapore RPSC column (Fig. 2). The fraction assay for IL-2 activity revealed that IL-2A, B, and C were each resolved into two peaks of activity with exactly the same retention times of 38 and 39 min. The earlier peaks were pooled and designated as A-1, B-1, and C-1; the later peaks were designated as A-2, B-2, and C-2.

Table I shows the summary of purification. The total amounts of the final preparations were about 40 μg starting from 103 l of culture fluid. Purification was at the magnitude of 10^5-fold with a total recovery of about 10%. The specific activities of the final preparations were similar and ranged from 2.3 - 4.6 x 10^4 U/mg. The final IL-2 preparations were subjected to SDS-polyacylamide gel electrophoresis under reducing conditions to give a single band each when stained with silver stain (Fig. 3). The molecular weights were estimated to be 14400 for C-1 and C-2, 17000 for A-1 and A-2, and 17500 for B-1 and B-2.

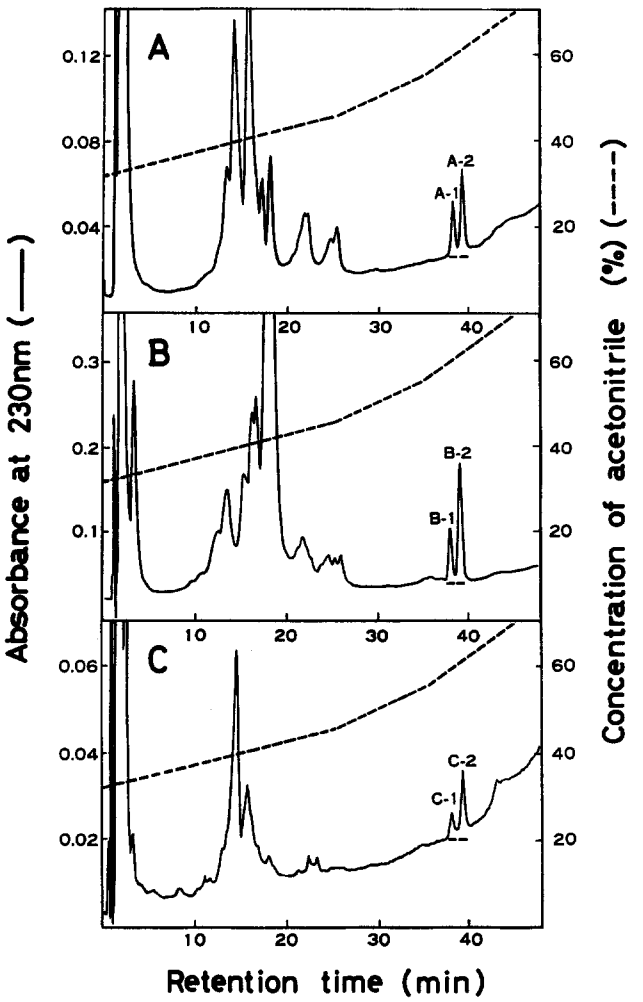

Figure 2. Elution pattern of IL-2 on reverse phase HPLC. An aliquot of the eluate on a DE-52 column (fractions A, B and C) was subjected to HPLC with an Ultrapore RPSC column (0.46 x 7.5 cm). Elution was performed with a gradient of acetonitrile from 32% through 70% in the presence of 0.1% TFA. Flow rate was 0.8 ml/min. ——, A_{230}; ----, concentration of acetonitrile (%). Fractions denoted by the bars corresponding to A-1 and A-2 (A), B-1 and B-2 (B) and C-1 and C-2 (C) were pooled.

Amino acid composition and partial NH_2-terminal amino acid sequence

The amino acid composition was determined by the o-phthaldialdehyde method on an acid hydrolysate (Table II). Although Pro and Trp were not analyzed because of the limitations of the methods applied and values for Gly were not shown because determination of Gly was found not to be reliable, the amino acid compositions were strikingly similar and in good agreement with the values predicted from the cDNA sequence (6,7).

TABLE I. SUMMARY OF PURIFICATION OF IL-2

Purification step		Volume	Total activity	Total protein	Specific activity	Recovery
		ml	U	mg	U/mg	%
Culture supernatant		103000	14523	130810	0.11	100
SP-Sephadex C-25		468	9170	1431	6.4	63
DEAE-Sephacel		677	6908	176	39	48
Ultrogel AcA-54		36.1	6411	17.9	358	44
DE-52	A	24.6	1968	2.20	895	14
	B	34.8	2185	3.50	624	15
	C	48.0	221	0.23	961	1.5
HPLC	A-1	2.0	155	6.3μg	24600	1.1
	A-2	2.0	213	9.1μg	23400	1.5
	B-1	2.1	328	7.1μg	46200	2.3
	B-2	2.1	519	12.2μg	42500	3.6
	C-1	2.0	29	1.0μg	29000	0.2
	C-2	2.0	81	2.2μg	36800	0.6

NH_2-terminal amino acid sequences determined for IL-2 A-1, A-2, B-1 and B-2 were as follows: A-1, Ala-Pro-X-Ser-Ser-Ser-X-X-X-Gln-Leu-; A-2, Ala-Pro-X-Ser-Ser-Ser-X-X-Lys-X-Gln-Leu-Gln-; B-1, Ala-Pro-X-Ser-Ser-Ser-X-Lys-Lys-X-Gln-Leu-Gln-Leu-; B-2, Ala-Pro-X-Ser-Ser-Ser-X-Lys-Lys-X-Gln-Leu-Gln-

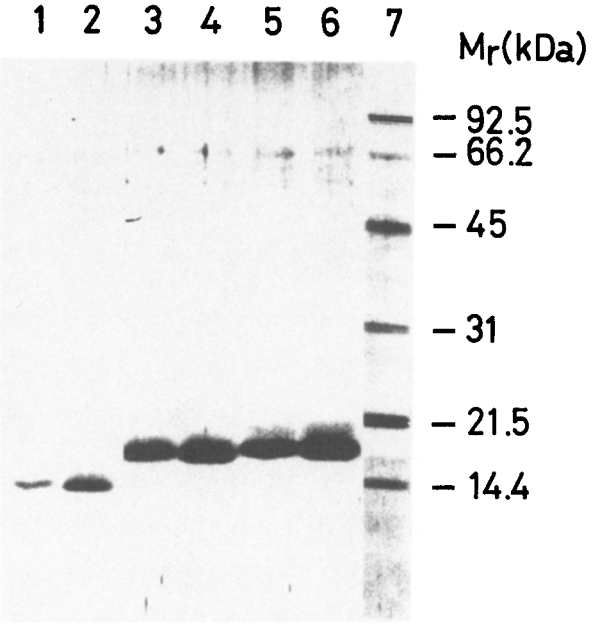

Figure 3. SDS-polyacrylamide gel electrophoresis of IL-2. Electrophoresis was carried out with a 15% polyacrylamide gel containing 0.1% SDS under reducing conditions. Proteins were stained with silver stain. Lanes 1, C-1 (0.03 μg); 2, C-2 (0.10 μg); 3, A-1 (0.25 μg); 4, A-2 (0.25 μg); 5, B-1 (0.25μg); 6, B-2 (0.25 μg); and 7, marker proteins (0.06 μg each).

TABLE II. AMINO ACID COMPOSITION OF IL-2

Amino acid	A-1	A-2	B-1	B-2	C-1	C-2	Values predicted from cDNA sequence
			residues/molecule				
Asp & Asn	12.0	12.0	12.0	12.0	12.0	12.0	12
Thr	11.0	11.7	11.5	12.0	9.0	10.1	13
Ser	9.5	9.6	8.0	8.0	10.8	8.1	8
Glu & Gln	19.0	18.8	18.3	18.2	17.9	18.3	18
Pro	-a)	-	-	-	-	-	5
Gly	-	-	-	-	-	-	2
Ala	6.3	6.0	6.3	5.6	5.9	5.5	5
H-Cysb)	-	-	-	2.6	-	-	3
Val	4.5	4.7	5.7	4.4	4.4	4.3	4
Met	4.5	4.0	3.6	4.0	3.8	4.4	4
Ile	7.9	8.1	7.6	7.9	6.4	7.3	9
Leu	19.5	19.6	18.4	19.6	16.5	19.3	22
Tyr	3.1	3.1	3.2	3.1	3.3	3.0	3
Phe	4.2	5.3	5.7	5.5	5.1	4.7	6
Lys	10.1	10.6	10.9	10.8	9.2	10.2	11
His	2.8	2.8	2.3	2.5	3.0	3.0	3
Arg	4.7	4.2	4.0	4.0	4.3	4.4	4
Trp	-	-	-	-	-	-	1

Hydrolysis was carried out in vacuo at 110°C in 6 N HCl containing 4% thioglycolic acid. Amino acid analysis was performed on a Hitachi model 835 amino acid analyzer using o-phthaldialdehyde/2-mercaptoethanol.
a) Not determined.
b) Determined as cysteic acid after oxidation with performic acid.

15 20
Leu-Glu-X-Leu-Leu-Leu-Asp-(X=unidentified). The sequence predicted from cDNA
1 5 10 15
is Ala-Pro-Thr-Ser-Ser-Ser-Thr-Lys-Lys-Thr-Gln-Leu-Gln-Leu-Glu-His-Leu-Leu-
20
Leu-Asp-. All four species had Ala-Pro at the NH_2-terminus. Thus, as far as examined, the amino acid sequences were consistent with that deduced from the cDNA sequence.

DISCUSSION

Although cloning and sequence analyses of the IL-2 chromosomal gene of Jurkat and normal lymphocytes revealed that there is only a single copy of the human IL-2 gene (11-13), we have purified six molecular species of IL-2 to apparent homogeneity from a serum-free culture fluid of stimulated human PBL. The amino acid compositions for the IL-2 species presented here showed striking similarities and were consistent with that deduced from the cDNA sequence. The NH_2-terminal amino acid sequences for A-1, A-2, B-1, and B-2 all began with Ala-Pro; this indicates directly that the mature IL-2 molecules lack the NH_2-terminal signal peptide of 20 amino acids as recently reported for Jurkat

IL-2 (4). The amino acid composition and the sequence data suggest that the IL-2 molecules (A-1, A-2, B-1 and B-2) with a molecular weight larger than Jurkat IL-2 by 2.0 - 2.5 kDa have polypeptide chains common to that predicted from the cDNA and that the heterogeneity is due to post-translational modification(s). IL-2 C-1 and C-2 are considered to be molecular species that have been modified slightly, if any, judging from their molecular weight.

Similar charge and molecular weight heterogeneity was described for IL-2 derived from tonsillar lymphocytes (8) and human PBL (14,15); however, the molecular weights and pI values are slightly but significantly different from those for our species. The differences may be due to differences of the source of lymphocytes and of the culture conditions employed.

It is worth noting that A-1, B-1, and C-1 were eluted at exactly the same retention time on reverse phase HPLC in spite of the charge and molecular weight heterogeneity among them. This was also the case for A-2, B-2, and C-2. The slight difference in hydrophobicity between peaks 1 and 2 as realized by reverse phase HPLC may have been produced by a modification of the IL-2 molecule such as the oxidation of Met residues. Oxidized forms of parathyroid hormone having methionine sulfoxide residues are known to be eluted earlier than the non-oxidized form on reverse phase HPLC (16). The nature of the difference between peaks 1 and 2 is now under study.

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