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Separation by cation-exchange high-performance liquid chromatography of three forms of Chinese hamster ovary cell-derived recombinant human interleukin-2

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SUMMARY

Purified recombinant (r) interleukin 2 (IL-2) produced by a transformed Chinese hamster ovary cell line shows a single peak when analysed by reversed-phase high-performance liquid chromatography, but it can be resolved into three forms by sodium dodecyl sulphate polyacrylamide gel electrophoresis. These three forms were successfully isolated by narrow-bore ion-exchange chromatography through optimization of the elution conditions. The addition of *n*-propanol as an organic modifier to the mobile phase proved to be essential for the recovery of the protein from the column in a yield of 90% or better based on protein quantification and biological activity determination. This chromatographic method was used for the purification of these three rIL-2 forms which represent variable glycosylation of a single polypeptide chain. A comparison of the biological activities using the murine CTLL-2 cell proliferation assay showed that the specific activities of the three forms are similar.

INTRODUCTION

Interleukin-2 (IL-2), an activated T-lymphocyte lymphokine that plays an important role in the immune response[1,2], is a glycoprotein heterogeneous with respect to charge and size; this heterogeneity is caused by variable glycosylation on a single polypeptide chain[3–5]. Recently, the carbohydrate structures of the two major forms of IL-2 produced by peripheral blood lymphocytes have been determined as the tetrasaccharide NeuAc(α2–3)Gal(β1–3)[NeuAc(α2–6)]GalNAc-ol for the form called N2 and as the trisaccharide NeuAc(α2–3)Gal(β1–3)GalNAc-ol for the N1 form (NeuAc = neuraminic acid; Gal = galactose; GalNAc = N-acetylgalactosamine)[5]. Both structures are O-linked to threonine 3 of the polypeptide chain. Stimulated T-lymphocytes also secrete a nonglycosylated IL-2, the M form[6].

Using partially purified material it was shown that variable glycosylation and sialylation accounted for heterogeneity of size and charge but made no difference to the *in vitro* biological activity of the lymphokine[7]. However, no further comparative

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studies were done to extend these observations probably owing to the difficulty in producing and purifying sufficient material of each form from T-lymphocytes.

We have previously described the isolation of a transformed Chinese hamster ovary (CHO) cell line that secretes large amounts of glycosylated recombinant (r) IL-2[8]. As the CHO-derived rIL-2, like natural IL-2, is heterogeneous with a variable sialic acid content, we searched for a simple procedure to separate the different glycovariants. Methods such as isoelectric focusing or sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) can be used to analyse the forms[7,9,10] but are not suitable for purification. Chromatofocusing has been used for IL-2 purification[3,8,11] but the method is time consuming and not well adapted for low-level protein detection because of the interference of the polyampholytes present in the mobile phase. Recently, a reversed-phase high-performance liquid chromatographic (RP-HPLC) method for the separation of the different natural IL-2 forms was described, but it allowed only a partial separation of these forms[5].

We describe here the development of a rapid narrow-bore cation-exchange HPLC (CEX-HPLC) method that separates the different glycosylated rIL-2 forms and the partial biochemical characterization of these forms.

EXPERIMENTAL

Production of glycosylated recombinant interleukin-2

Glycosylated rIL-2 was produced by a transformed CHO cell line and purified to homogeneity as described previously[8].

High-performance liquid chromatography

All chromatographic separations were carried out at room temperature on a Hewlett-Packard HP1090 HPLC system equipped with a diode-array detector. RP-HPLC was performed on a BU 300 column (100 × 2.1 mm I.D. (Brownlee) and developed with a 15-min linear gradient from 40 to 60% acetonitrile–0.1% trifluoroacetic acid (TFA) at a flow-rate of 0.4 ml/min. Protein monitoring was performed at 220 nm.

CEX-HPLC was performed on a CX 300 column (100 × 2.1 mm I.D.) (Pierce) with a sodium acetate eluting buffer. The pH and organic modifier (*n*-propanol) were adjusted as described under Results and Discussion. A 15-min linear gradient from 20 to 100 m*M* sodium acetate and a flow-rate of 0.4 ml/min were used throughout. Elution was monitored at 280 nm and peaks were quantified according to their areas.

SDS-PAGE

SDS-PAGE was carried out as described by Laemmli[12] on 15% polyacrylamide gel; the acrylamide: bisacrylamide ratio was set at 29:1 to improve the separation in the 12 000–17 000-dalton range. Gels were silver stained (Bio-Rad Labs. kit) and scanned with a 2202 Ultroscan laser densitometer (LKB).

Neuraminidase treatment

Samples of rIL-2 were digested overnight at room temperature with *V. cholerae* neuraminidase (Calbiochem) in 50 m*M* sodium acetate buffer (pH 5.5) containing 154 m*M* sodium chloride and 4 m*M* calcium chloride. One unit of enzyme was used for

100–200 μg of rIL-2. TFA was added (1% final concentration) to stop the reaction, then the samples were stored at −20°C until chromatographic analysis.

Sialic acid determination

Sialic acid was liberated from rIL-2 by mild 1-h acid hydrolysis with 50 m*M* sulphuric acid, then quantified by HPLC on a Aminex HPX-87H column (300 × 7.8 mm I.D.) (Bio-Rad Labs.) with 5 m*M* sulphuric acid as the eluent at a flow-rate of 0.5 ml/min as described elsewhere[13]. Monitoring was done at 195 nm. The sialic acid standard was obtained from Calbiochem.

Protein determination

The concentration of the rIL-2 was determined either by amino acid analysis as previously described[8] or with a Bio-Rad Labs. kit using bovine serum albumin as the standard.

Biological assay

The activity of IL-2 was measured by the spectrophotometric assay described by Mosmann[14]. Units are expressed with reference to the standard provided by the National Cancer Institute Biological Response Modifier Program.

RESULTS AND DISCUSSION

Purified rIL-2 produced by a transformed CHO cell line[8] shows a single peak on RP-HPLC using a C_4 column and an acetonitrile gradient at pH 2.1 (Fig. 1). However, it can be resolved into three bands by SDS-PAGE, two major bands of 16 500 and 16 000 dalton and a minor band of 15 000 dalton (Fig. 2, lines a and h). These three bands have been separated by chromatofocusing and have been proved to be derived from post-translational modifications of a single polypeptide chain[8]. The time-consuming chromatofocusing separations resulted in low and variable recoveries and, for further characterization, an additional purification step was often required to eliminate the polyampholytes. Recently an RP-HPLC method based on a hexyl

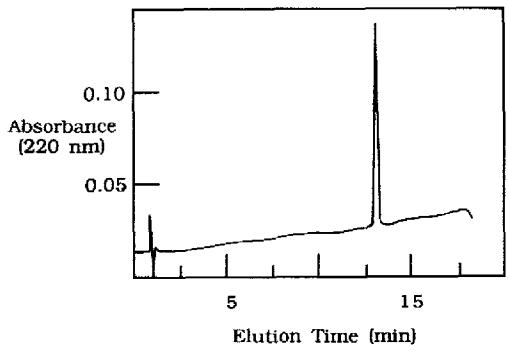

Fig. 1. Reversed-phase HPLC of rIL-2. Approximately 3 μg of purified rIL-2 were injected onto a BU 300 column (100 × 2.1 mm I.D.) and eluted with a linear gradient from 40 to 60% acetonitrile containing 0.1% TFA at a flow-rate of 0.4 ml/min. The single peak eluting at 13 min was collected for SDS-PAGE analysis.

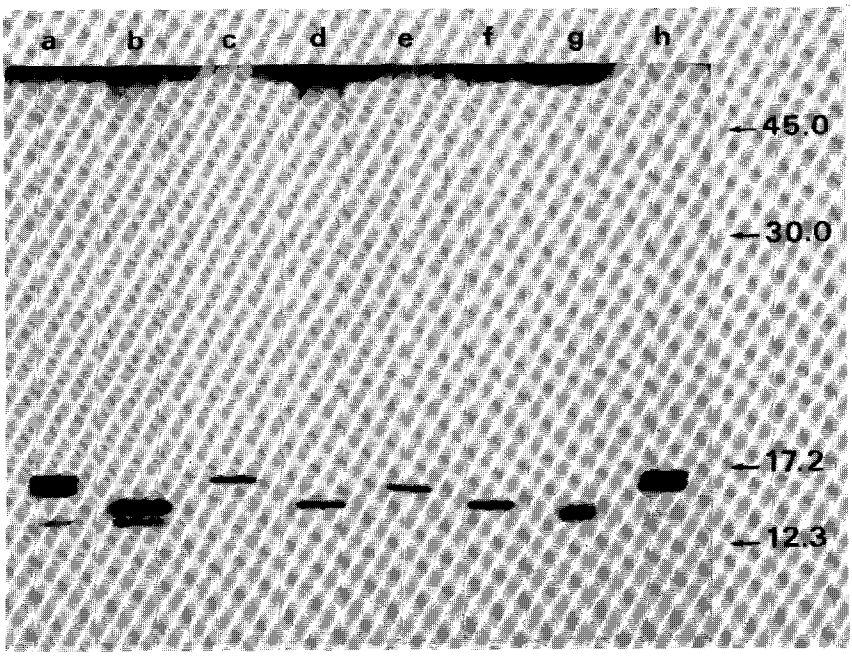

Fig. 2. SDS-PAGE. Lanes: a and h = purified glycosylated rIL-2; b = glycosylated rIL-2 treated with neuraminidase; c and d = rIL-2 eluting in peak I (Fig. 4) before and after neuraminidase treatment; e and f = rIL-2 eluting in peak II (Fig. 4) before and after neuraminidase treatment; g = rIL-2 eluting in peak III (Fig. 4). Values on the right hand side indicate molecular weights in kilodalton.

column using a propanol gradient at pH 4.3 for the separation of the different natural IL-2 forms was described, but it allowed only a partial separation of the different glycosylated forms[5]. Because of these problems and taking into account the rapidity of HPLC, we developed and optimized a CEX-HPLC system for the separation of the different sialylated forms of IL-2.

The first attempts to isolate the IL-2 forms on a silica-based cation-exchange column with an aqueous salt gradient resulted in a very poor recovery; however, better than 90% recovery, based on protein determination and biological activity, was obtained when an organic modifier, *n*-propanol, was used as a mobile phase additive (Table I). Concentrations of *n*-propanol up to 60% were tested, with optimum resolution between 30 and 45% (Fig. 3). Quantitative recovery of the IL-2 was not obtained if *n*-propanol was replaced with acetonitrile, suggesting a strong hydrophobic interaction of this protein with the chromatographic support. This behaviour can be attributed to the hydrophobicity of IL-2, as several other small proteins when chromatographed on the same silica based column were recovered nearly quantitatively without the need for additives (results not shown). The effect of the pH of the mobile phase was also studied; sharper peaks and good resolution were obtained at pH 6.5. At a pH higher than 7.6, the components were barely retained and poorly separated; at a pH lower than 6.5 the resolution was maintained even if the retention times were increased. pH 6.5 was chosen because it proved to be very convenient for

TABLE I

SEPARATION OF rIL-2 FORMS BY CEX-HPLC: TYPICAL RECOVERY IN A PREPARATIVE EXPERIMENT

360 μg of purified rIL-2 (determined by amino acid analysis[7]) were loaded onto the CX 300 column and eluted with a 30-min linear gradient from 20 to 100 m*M* sodium acetate in 40% *n*-propanol. Each indicated fraction was collected and the IL-2 content was determined by amino acid analysis. The values are means of three determinations. Peaks are labelled according to Fig. 4.

Material	*Amount of IL-2 (μg)*	*Recovery (%)*
Starting material	360	–
Eluting material:		
Peak I	155 ± 15	43
Peak II	155 ± 15	43
Peak III	43 ± 5	12
Total	353	98

storing the glycosylated IL-2 forms, as lower pH values favoured a slow desialylation of the molecules[5].

A flow-rate of 0.4 ml/min and a gradient of sodium acetate from 20 to 100 m*M* in 15 min were chosen because they resulted in a good compromise between resolution and separation time.

Neuraminidase treatment of the rIL-2 shifted the electrophoretic mobility of the 16 500- and 16 000-dalton forms to 15 500 dalton, leaving unchanged the 15 000-dalton band (Fig. 2, line b), indicating that sialic acids are covalently linked to the CHO-derived rIL-2 as previously described[8]. In order to identify the eluting material in peaks I, II and III from the CEX-HPLC (Fig. 4A), each peak was collected and analysed by SDS-PAGE; the material in peaks I, II and III gave single bands of 16 500,

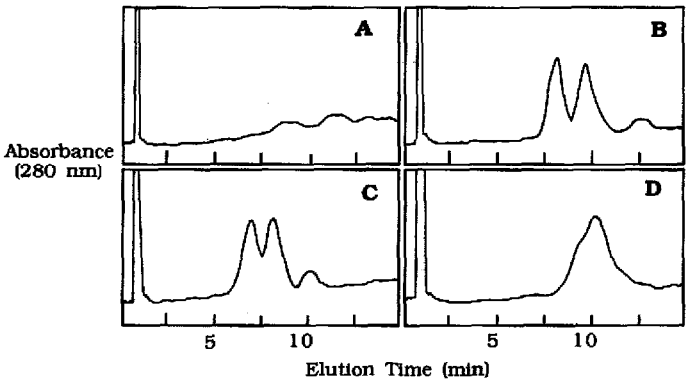

Fig. 3. Effect of *n*-propanol on the CEX-HPLC separation of rIL-2. Approximately 4 μg of purified rIL-2 were injected onto a CX 300 column (100 × 2.1 mm I.D.) and eluted with a 15-min linear gradient from 20 to 100 m*M* sodium acetate buffer (pH 6.5) in the presence of (A) 15%, (B) 30%, (C) 45% and (D) 60% *n*-propanol.

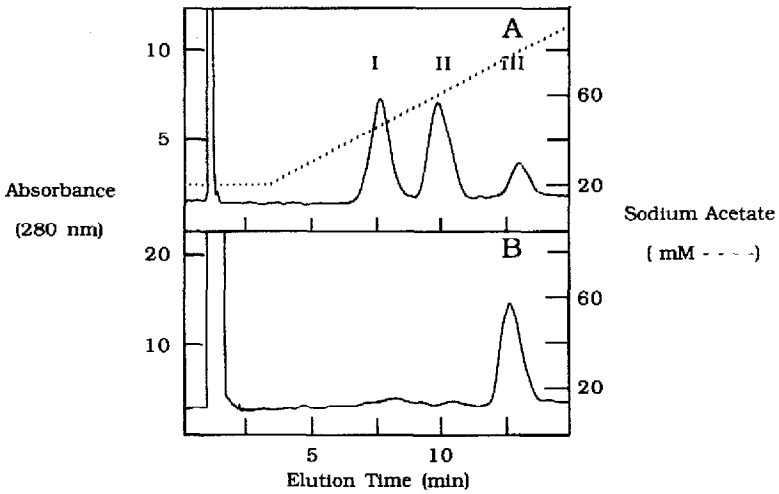

Fig. 4. Neuraminidase treatment of rIL-2. Approximately 4 μg of purified rIL-2 were injected (A) before and (B) after neuraminidase treatment onto a CX 300 column (100 × 2.1 mm I.D.) and eluted with a 15-min linear gradient from 20 to 100 m*M* sodium acetate buffer (pH 6.5) in the presence of 30% *n*-propanol. Individual peaks, labelled I, II, and III, were collected for SDS-PAGE analysis.

16000 and 15000 dalton, respectively (Fig. 2, lines c, e and g). Neuraminidase treatment of each isolated peak confirmed the presence of sialic acid covalently linked to the forms eluting in peaks I and II as the molecular weights, M_r, were shifted, in both cases, to 15500 dalton (Fig. 2, lines d and f) while the M_r of the material in peak III remained unchanged (not shown). The changes in electrophoretic mobility were accompanied by a shift in the eluting positions of peaks I and II into III (Fig. 4B). In addition, when the neuraminidase-treated 15500-dalton molecules of peaks I and II were treated with an O-glycanase specific for the Gal(β1–3)GalNAc conjugates, the M_r values were reduced to 15000 dalton without any changes in the CEX-HPLC behaviour (results not shown). Sequence analysis of the material eluting in peak III showed the expected NH_2-terminal Ala–Pro–Thr–Ser–Ser–Ser–Thr–Lys–Lys; the NH_2-terminal sequences of the material eluting in peaks I and II were identical with that determined for the material eluting in peak III, but no amino acid was identified in the third position, confirming our previous results that this threonine residue is the attachment point of the sugar moiety[8].

These results are compatible with a carbohydrate structure for the rIL-2 eluting in peak I as that of the natural IL-2 form called N2[5] containing the tetrasaccharide NeuAc(α2–3)Gal(β1–3)[NeuAc(α2–6)]GalNAc O-linked to the threonine in position 3 of the polypeptidic chain and for the recombinant material eluting in peak II as that of the natural IL-2 form called N1[5], identical with N2 but missing one sialic acid. The rIL-2 in peak III corresponds to non-glycosylated rIL-2 (M form). Additional information that supports the assignment of these structures was obtained by fast atom bombardment (FAB) mass spectrometric analysis of the purified amino-terminal tryptic peptides of each of the separated forms[15].

We compared CEX-HPLC with SDS-PAGE for IL-2 analysis. Both techniques provide similar resolution and correlate well in the quantification of the different

TABLE II

COMPARISON BETWEEN SILVER-STAINED SDS-PAGE DENSITOMETRY, CEX-HPLC AND SIALIC ACID DETERMINATION FOR THE QUANTIFICATION OF THE DIFFERENT FORMS OF rIL-2

Method	*IL-2*			*Sialic acid content (mol/mol IL-2)*
	N2 (%)	*N1 (%)*	*M (%)*	
SDS-PAGE	44	44	12	1.32[a]
CEX-HPLC	41	44	15	1.26[a]
Sialic acid	–	–	–	1.18[b]

[a] Deduced from the percentage of each form.
[b] Determined after mild acid hydrolysis as described under Experimental.

sialylated and non-sialylated forms with the amount of sialic acid released from the rIL-2 after mild acid hydrolysis (Table II). However, CEX-HPLC is advantageous because it is rapid and offers a simple way to recover the isolated forms. In fact, as the same resolution was observed with loads ranging from 2 to 200 μg (for loads of 200 μg or more, a 30-min gradient was used), we used this chromatographic system to purify each CHO-derived rIL-2 form for further characterization of their specific biological activities.

The biological activities of the purified forms were determined on a murine IL-2-dependent cytotoxic T cell line. In this system, the three forms showed similar specific activities [N2, $(2.1 \pm 0.2) \cdot 10^7$; N1, $(1.9 \pm 0.2) \cdot 10^7$; M, $(1.8 \pm 0.2) \cdot 10^7$ IU/mg (means ± S.D., $n = 3$)], suggesting that these forms interact similarly with the high-affinity IL-2 receptor present on this cell line and responsible for the cell proliferation as previously described[7]. However, as this proliferation assay may overlook small structural differences, more precise IL-2 receptor binding studies are being undertaken with the purified forms.

In conclusion, we have developed a simple chromatographic method for the separation of the different glycosylated and non-glycosylated rIL-2 forms. The availability of these highly purified forms of IL-2 will allow not only a better understanding of the structural function of these post-translational modifications, but also a more complete comparison in different biological systems *in vitro* and *in vivo*. The latter are of particular interest because, as has been shown for many others glycoproteins[16], the sialic acid residues can be critical for the survival of this molecule in the circulation.

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