Eur. J. Biochem. *173*, 109–114 (1988)

Expression, renaturation and purification of recombinant human interleukin 4 from *Escherichia coli*

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(Received October 16, 1987/January 7, 1988) – EJB 871157

The lymphokine human interleukin 4 (IL-4) has been expressed from a plasmid in the cytoplasm of *Escherichia coli*. Advantage has been taken of insolubility of the human IL-4 in *E. coli* for rapid purification of this protein in only a few steps. We describe extraction and renaturation procedures which solubilize human IL-4 yielding biologically active protein. The protein was purified to homogeneity by one passage over a gel-filtration column. The refolded human IL-4 was characterized by N-terminal sequence analysis, amino acid analysis and bioassays. The refolded *E. coli*-derived human IL-4 has biological activity on T and B cells and binds to the human IL-4 receptor, comparable to mammalian expressed human IL-4, indicating that the protein is folded correctly.

Interleukin 4 (IL-4), or B-cell-stimulatory factor 1 is a lymphokine initially identified in the supernatant of cells of the mouse thymoma line EL-4 stimulated with phorbol myristate acetate [1]. It is also produced by several T cell lines upon stimulation with concanavalin A or with antigen and antigen-presenting cells [2]. In murine systems, IL-4 has been shown to act on T and B lymphocytes, mast cells and several other hematopoietic lineage cells. It affects the activation, growth, and program of gene expression in these cells [2]. The human homologue of IL-4 has not been found in natural systems but recently has been cloned and characterized based on homology with the mouse cDNA [3]. The human IL-4 stimulates proliferation of anti-IgM-preactivated human tonsillar B cell [3, 4] as well as the proliferation of human T cells and helper T cells clones [3, 5]. It also induces the low-affinity receptor for IgE ($Fc\varepsilon R_L$/CD23) [6]. The human IL-4 consists of 129 amino acids and contains six cysteine residues, with the potential to form three disulfide bonds. The lack of natural human IL-4 requires a recombinant source for further study of its role in the immune system. To obtain a large quantity of this lymphokine, we have cloned and expressed the cDNA in *Escherichia coli*.

Frequently, over-expression of mammalian proteins in bacterial cells leads to the accumulation of aggregated insoluble protein complexes (inclusion bodies) [7–10]. The protein in these complexes is present in scrambled, partially oxidized forms as well as in largely reduced forms [9, 11]. To solubilize and activate these aggregates, the protein often has to be refolded *in vitro*.

Some groups have been successful in refolding recombinant mammalian proteins expressed in *E. coli*; for example, insulin [12], prochymosin [13], urokinase [10] and transforming growth factor α [14]. The success of refolding depends critically on the nature of the expressed polypeptide, the selection of the chaotropic and reducing reagents used to solubilize the aggregates and the conditions under which the solubilized and denatured polypeptide is refolded into the biologically active form [15, 16].

In this paper we describe (a) the expression of human IL-4 in *E. coli*, produced in an aggregated insoluble form; (b) the extraction and refolding of the aggregates; and (c) the purification of the refolded biologically active human IL-4. The purified protein has a specific activity of 5×10^7 U/mg measured by an assay for T cell growth factor (TCGF) and has been characterized by N-terminal sequence analysis, amino acid analysis, and biological activity on T and B cells.

MATERIALS AND METHODS

Recombinant DNA technology

All restriction enzymes, DNA polymerase, kinase and ligase reactions were performed as described by Maniatis et al. [17]. Enzymes were purchased from New England Biolabs and Boehringer Mannheim. Plasmids were isolated according to the alkaline method (small scale [17]) or a modification of this method (large scale [18]).

DNA oligonucleotides were synthesized by phosphoramidite chemistry using an Applied Biosystems 380A synthesizer. DNA sequence analysis was performed using standard dideoxy procedures [19].

Bacterial strain and plasmid construction

E. coli AB1899 *(thr1, leuB6, thi1, argE3, hisG4, proA2, lon1, lacY1, galK2, mtl1, xyl5, ara14, strA31, tsx33, λ-, supE44)* was obtained from Schering Research.

The *Eco*RV – *Bam*HI fragment from the human IL-4 cDNA clone (pcD-hIL-4) [3] was ligated together with the synthetic linkers 5′-d(CGATGCACAAGTGCGAT)-3′ (+ strand) and 5′-d(ATCGCACTTGTGCAT)-3′ (– strand)

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Abbreviations. BCGF, B cell growth factor; IL-4, interleukin 4; PBS, phosphate-buffered saline, 120 mM NaCl, 2.7 mM KCl in 10 mM phosphate buffer pH 7.4; RP-HPLC, reverse-phase high-performance liquid chromatography; TCGF, T cell growth factor.

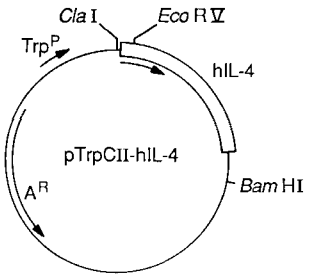

Fig. 1. *Schematic representation of the expression plasmid pTrpC11-hIL4.* Construction of the plasmid is described in Materials and Methods

and *Cla*I/*Bam*HI-digested pTrpC11-mIL2 [18] (Fig. 1). Thus, the mIL-2 sequence present in the TrpC11-mIL2 plasmid was replaced by the human IL-4 sequence. The identity of the final construct (pTrpC11-hIL-4) was confirmed by DNA sequence analysis.

Analytical methods

Guanidine · HCl (sequanal grade) was obtained from Pierce, urea (ultra pure, enzyme grade) from BRL.

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [20]. Proteins were visualized with Coomassie brilliant blue R-250 or by silver stain [21, 22]. Densitometric scanning of stained gels was carried out using an LKB Ultrascan XL laser densitometer.

Protein concentrations were measured by the method of Bradford [23] using bovine serum albumin fraction V (Miles Scientific) as a standard. In a later stage of this study the protein concentration of a sample of pure human IL-4 was determined by amino acid analysis and this sample was subsequently used as a standard for Bradford assay. The use of pure human IL-4 as a standard measured a protein concentration 2.9 times lower than using bovine serum albumin as a standard. The protein concentration necessary for the determination of the specific activity was measured against the human IL-4 standard. Other Bradford assays used bovine serum albumin as a reference.

Purified human IL-4 was analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) on a column (4.6 × 75 mm) of Ultrapore C3 (Beckman) eluted with a gradient of 25–60% acetonitrile in 0.1% trifluoroacetic acid/water.

Amino acid composition was determined by pre-column derivatization with *o*-phthalaldehyde followed by RP-HPLC [24].

Amino-terminal sequence analysis was performed by automated Edman degradation on an Applied Biosystems 470A sequenator [25]. Resultant phenylthiohydantoin derivatives were analyzed by RP-HPLC [26].

Analysis for free thiol groups in the purified protein was done by the method of Ellman [27]. The concentration of human IL-4 in the assay was 0.18 mg/ml.

Immunochemical methods

Antiserum 12-MYO was derived from a rat immunized with a synthetic peptide corresponding to 16 N-terminal amino acids of human IL-4. The monoclonal antibody 11B4

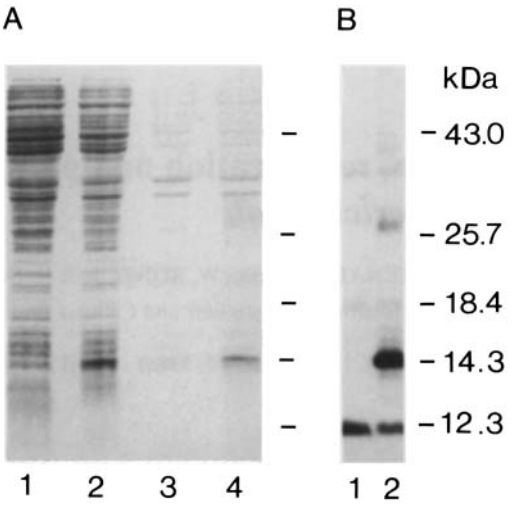

Fig. 2. *Expression of hIL-4.* (A) Cells were prepared as described in Materials and Methods, run on a 15% polyacrylamide gel and stained with Coomassie brilliant blue R-250. Lanes 1 and 2, total cell lysate of cells harboring respectively pTrpC11-hGM (control) and pTrpC11-hIL4; lanes 3 and 4, pellet fraction of sonicated cells carrying the control plasmid (lane 3) or pTrpC11-hIL4 (lane 4). (B) Western blot of samples described in A using the antiserum 12-MYO. Lanes 1 and 2, total cell lysate of cells harboring respectively pTrpC11-hGM (control) and pTrpC11-hIL4. Equivalent amounts of cells were loaded in each lane. Molecular mass markers are indicated

was raised against mammalian-derived recombinant human IL-4 (Chrétien et al., unpublished results).

Western blots were performed as described [18], except that 12-MYO was used at a 100-fold dilution and 11B4, obtained from culture supernatants, was used at a 10-fold dilution.

Human IL-4 obtained by gel filtration chromatography (described below) was assayed by an indirect enzyme-linked immunosorbent assay (ELISA) [29]. Aliquots (1%) of individual column fractions were used to coat the assay plates and 11B4 was used as the primary antibody.

Biological assays

Human IL-4 was tested for T cell growth factor (TCGF) activity on human peripheral blood lymphocytes using a colorimetric assay [3] at a starting dilution of 1:40 of the SDS extracts (described below). The assay medium contained 50 µg/ml gentamicin to prevent growth of *E. coli* cells that survived the extraction procedure. One unit is the amount of human IL-4 that provides a signal 50% of that elicited by saturating amounts of lymphokine.

Extraction of human IL-4 with SDS

E. coli AB1899 cells carrying the TrpC11-hIL4 plasmid were grown in L-broth (1:50 dilution of an overnight culture) to a total absorbance at 560 nm of 2; 1 ml of this culture was centrifuged for 5 min and the cells were resuspended in 500 µl phosphate-buffered saline. Cells were then lysed by sonication (30–50% pulses, 40 W) using a Branson cell disrupter 200. After centrifugation, the pellet was resuspended in 100 µl phosphate-buffered saline containing 1% SDS and incubated for 10 min at 37 °C. The proteins solubilized with 1% SDS were separated from the insoluble fraction by centrifugation

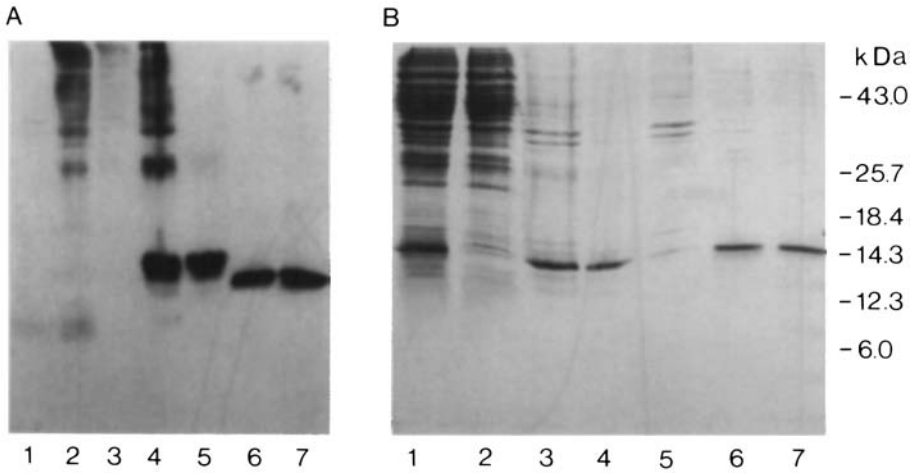

Fig. 3. *Purification of recombinant human IL-4 during the extraction and renaturation procedure.* (A) Western blot under non-reducing conditions; total cell extract (lane 2), supernatant of sonicated cells (lane 3), cell pellet extracted with 5M guanidine (lane 4), 1:9 dilution step (lane 5), dialyzed fraction (lane 6), concentrated fraction (lane 7). Lane 1 shows a negative control. The antibody used was 12-MYO. (B) Coomassie-stained protein gel of the same samples as in A but under reducing conditions; total cell extract (lane 1), supernatant of sonicated cells (lane 2), cell pellet extracted with 5M guanidine (lane 3), 1:9 dilution of 5M guanidine fraction (lane 4), precipitate formed in dilution step (lane 5), dialyzed fraction (lane 6), concentrated fraction (lane 7). Volume loaded in each lane represent equivalent proportions of the total preparation at the particular step; the lanes can therefore be compared directly with regard to human IL-4 yield

for 10 min at 4°C. Centrifugations were performed at 15000 × *g*.

Extraction and renaturation procedure

One liter of *E. coli* AB1899 cells harboring the TrpC11-hIL4 plasmid were grown (1:50 dilution of an overnight culture) in a 2-l conical flask at 37°C in L-broth for 10 h. Cells were harvested by centrifugation at 4500 × *g* for 15 min at 4°C and stored at −20°C overnight.

To lyse the cells, the pellets were resuspended in 30 ml 50 mM Tris/HCl pH 8.0, 50 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride (buffer A), and sonicated (50 50% pulses, 70 W). The cell lysates were centrifuged at 25000 × *g* for 15 min at 4°C. The resulting pellet containing the insoluble human IL-4 protein was resuspended in buffer A supplemented with 5 M guanidine · HCl and 2 mM reduced and 0.2 mM oxidized glutathione. For every 1 g wet pellet, 9 ml buffer was added to a maximum protein concentration of 2.5 mg/ml. After 1 h at room temperature, this solution was added slowly to 9 vol. buffer A (without phenylmethylsulfonyl fluoride) supplemented with 2 mM reduced and 0.2 mM oxidized glutathione, and incubated at room temperature for 1.5–4 h.

The lysate was centrifuged at 2500 × *g* for 15 min at 4°C to remove the precipitate formed during the dilution step. The clear supernatant was dialyzed against phosphate-buffered saline pH 7.4 with three changes of buffer (total dilution 1000-fold) at 4°C to remove the refolding reagents. After removal by centrifugation of the precipitate formed during dialysis, the protein concentration of the dialyzate was increased to 8 mg/ml using an Amicon concentrator with an YM5 filter. Again precipitates formed which were removed by centrifugation.

Gel filtration chromatography

The refolded and concentrated dialyzate was loaded onto a Bio-Gel P-30 (Bio-Rad) column (1.5 × 90 cm) equilibrated with phosphate-buffered saline and eluted at a flow rate of 8 ml/h; the eluate was collected in 2-ml fractions. The column was calibrated with the following molecular mass markers from Pharmacia: ribonuclease (M_r 13700), chymotrypsinogen (M_r 25000), ovalbumin (M_r 43000) and bovine serum albumin (M_r 67000).

RESULTS

Expression and solubility of human IL-4

To confirm the expression of human IL-4, lysates of *E. coli* AB1899 cells transformed with plasmid TrpC11-hIL4 were analyzed by PAGE and Western blotting using 12-MYO (Fig. 2). These lysates (lane 2) contained a band at an M_r of 14500 representing the human IL-4 molecule which was absent in the control (lane 1). The expressed human IL-4 was found in the pellet fraction after sonication and centrifugation and represented the major protein in this fraction (Fig. 2A lane 4). These results indicate that the human IL-4 is expressed as insoluble aggregates.

The aggregates were solubilized with 1% SDS but the specific activity of the extracted human IL-4 as measured by the TCGF assay was low. Partial removal of the SDS by cold precipitation followed by centrifugation a 4°C failed to yield a more active protein (data not shown). These results indicated that such measures were insufficient to renature the molecule fully.

Renaturation and purification of human IL-4

To optimize existing extraction and renaturation procedures [10, 13] for the human IL-4 protein, different denaturants (guanidine and urea) with or without the addition of reduced and oxidized glutathione were tested and the refolded extracts were assayed for TCGF activity. The highest specific activity of human IL-4 was obtained using 5M guanidine as denaturant with the addition of 2 mM reduced and 0.2 mM oxidized glutathione (results not shown). The specific activity

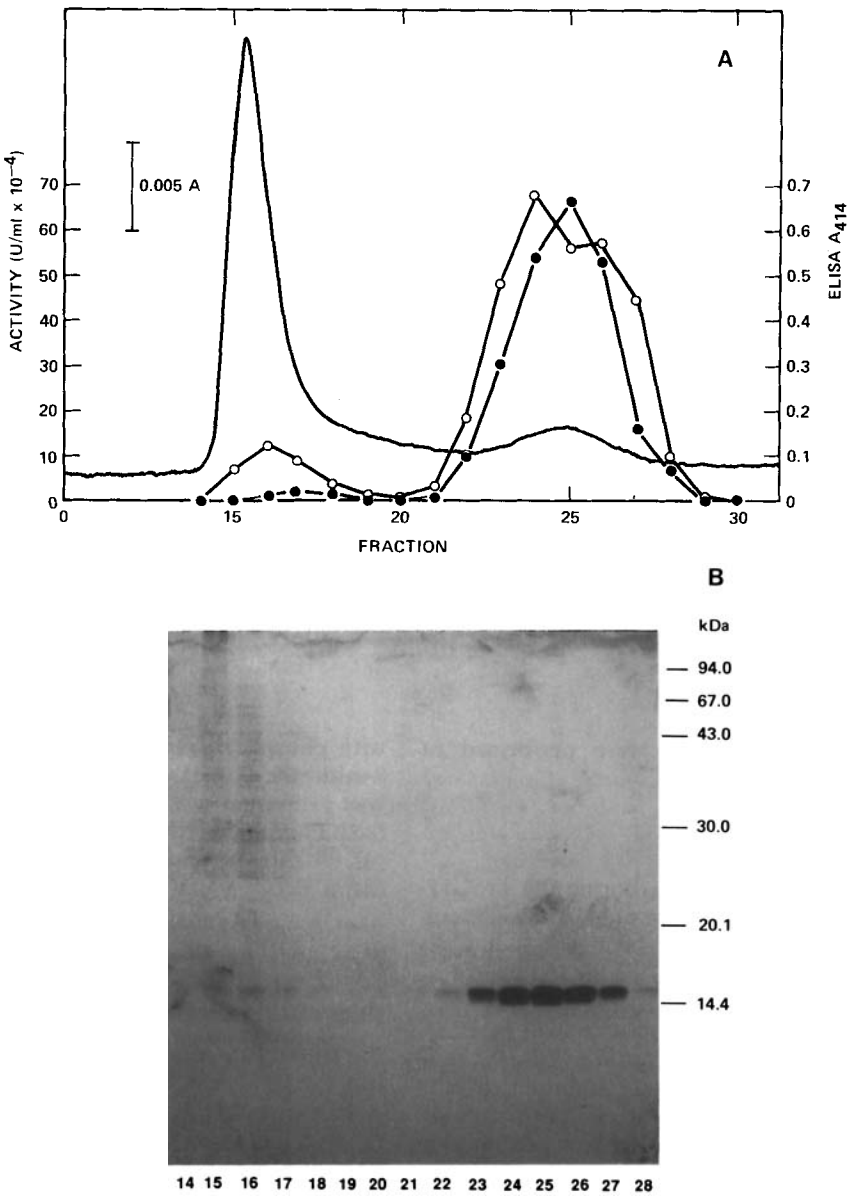

Fig. 4. *Bio-Gel P-30 gel filtration of refolded protein extract.* (A) The column (1.5 × 90 cm) was run at 4 °C in phosphate-buffered saline; 2-ml fractions were collected at a flow rate of 8 ml/h. (——) A_{280}; (○——○) ELISA; (●——●) biological activity. (B) Silver-stained SDS/polyacrylamide gel electrophoresis of column fractions 14–28 in A. Sample loaded into each well contained the equivalent of 10 µl of the corresponding fraction

of human IL-4 was comparable to mammalian expressed human IL-4, indicating that the refolding is complete at this step.

The refolding during the extraction and renaturation procedure was followed. The toxic reagents in the samples during the refolding interfere with the bioassay so that the extent of refolding could not be measured by the specific activity. Therefore another approach was taken to analyze the samples. The degree of reoxidation of human IL-4 was inferred from its migration in SDS-PAGE under non-reducing conditions and compared with the native (oxidized) human IL-4. Formation of intramolecular disulfide bonds, occurring in the oxidized molecule, presumably results in a more compact structure with a faster mobility than the reduced molecule. The samples were analyzed by Western blotting using 12-MYO (Fig. 3A). In the total cell lysate human IL-4 was present as aggregates (lane 2), which were solubilized with guanidine (lanes 4 & 5). This resulted in a molecule with a slower mobility than the native human IL-4 (lanes 6 & 7), indicating that this form is still incompletely oxidized. Only after dialysis was native (oxidized) human IL-4 present. This result was supported by the results of a Western blot of the same samples treated with 11B4. Under non-reducing conditions described above 11B4 recognizes only native human IL-4 (data not shown) and reacted with the human IL-4 only after the dialysis step (data not shown).

During the extraction and renaturation procedure the purity of human IL-4 was increased from 8% to 32% (Table 1 and Fig. 3B). The step during which most of the contaminating proteins were removed was the sonication and centrifugation step separating the soluble proteins from the insoluble human IL-4 (lanes 1 and 2). During the dialysis to remove

Table 1. *Purification of human IL-4 expressed by* E. coli
Protein was determined by the Bradford method. The purity of hIL-4 was determined by densitometric scanning of gel lanes (Materials and Methods) in steps 1 and 2 or by reverse-phase HPLC in the last step

Purification step	Volume	Total protein	Purity hIL-4	hIL-4	Yield
	ml	mg	%	mg	%
1. Total lysate	30	240	8	19.2	100
2. After extraction and refolding (dialyzate)	314	27.5	32	8.8	46
3. P-30 eluate pooled peak fractions	4	5.8	95	5.5	29

the refolding reagents, precipitation of some contaminating proteins occurred (lane 5).

For further purification, the refolded protein was concentrated and passed over a Bio-Gel P-30 gel filtration column (separation range of 2500 – 40000 Da). The absorbance at 280 nm (A_{280}), ELISA and bioactivity profiles of the human IL-4 from this column are shown in Fig. 4A. There were two major peaks in the A_{280} profile, the first of which was the void volume of the column and the second contained proteins eluting with the M_r of human IL-4 (14500). Further characterization of the protein peaks with an ELISA using 11B4 showed the antibody reacting mainly with the proteins eluting in the second peak and to some extent with column fractions from the first protein peak. Bioassays localized activity mainly to the second peak.

Protein in the column fractions was analyzed by silver-stained PAGE (Fig. 4B). Fractions 22 – 28 (peak two) contained only human IL-4 and no contaminating proteins. The void volume peak (lanes 14 – 19) contained mainly high-molecular-mass proteins with some human IL-4 accounting for the ELISA reactions described.

Further characterization of the isolated protein was pursued on pooled column fractions 23 – 27.

Characterization of the human IL-4

To estimate the purity of human IL-4, an aliquot from the gel filtration step was analyzed on a RP-HPLC column. The A_{280} profile gave a major symmetrical peak with some minor contaminants (data not shown). Based on peak integration, the human IL-4 purity was 95%.

The amino acid composition of the purified protein was determined to be the same as human IL-4 [3] with the addition of one mole methionine/mole protein (Table 2). This methionine was added to the mature sequence for the initiation of translation.

The identification of the N-terminal amino acid as methionine indicates that the amino acid specified by the initiation codon is not removed. The results of a further 19 sequenator cycles were consistent with the known N-terminal sequence of human IL-4 [3]. The yield of the polypeptide at the first step was identical to the amount determined by amino acid analysis on a parallel sample.

Ellman's reagent did not react with the purified human IL-4 indicating that all the cysteine residues (six) are disulfide-linked. The sensitivity of the assay for thiol groups was 1 nmol. The amount of human IL-4 present was 10 nmol.

Table 2. *Amino acid composition of recombinant human IL-4*
The expected number of residues is based on the sequence described [3], including the initiator Met (see text). The residues in the recombinant human IL-4 were determined by the method of Lydon et al. [24]

Amino acid	Expected residues	Recombinant human IL-4
Asx	10	8.83
Glx	17	16.30
Ser	8	7.08
His	5	4.83
Gly	3	3.94
Thr	15	12.93
Arg	9	8.90
Ala	8	8.41
Tyr	2	1.72
Met	2	1.99
Val	3	3.48
Phe	6	7.23
Ile	6	6.17
Leu	16	16.98
Lys	12	13.12

The refolded human IL-4 was tested for bioactivity in two different assays. The assay for B cell growth factor (BCGF) measures the proliferative stimulus of human IL-4 on activated B lymphocytes, whereas the TCGF assay measures the proliferation of peripheral blood T lymphocytes. The *E. coli*-derived human IL-4 is able to induce the proliferation of both lymphocytes. The specific activity of the human IL-4 purified from *E. coli* is 5×10^7 U/mg, based on the bioactivity value from the TCGF assay, comparable with the mammalian-derived human IL-4 (2.6×10^7 U/mg, Le et al., unpublished results).

In addition to its bioactivity in the TCGF assay, *E. coli*-derived human IL-4 was assayed for its ability to inhibit the binding of ^{125}I-labeled human IL-4 derived from a mammalian expression system [3]. The *E. coli*-derived human IL-4 competed with the radiolabeled species for the IL-4 receptor on Jijoye cells on an equimolar basis indicating that the two proteins are comparable (Dr J. Banchereau, personal communication).

The purified protein is very stable, even in low concentrations (20 μg/ml) in phosphate-buffered saline pH 7.4. After 4 weeks at room temperature the bioactivity of human IL-4 measured by the TCGF assay had not changed compared to the original determination.

DISCUSSION

In this report we have demonstrated the expression of human IL-4 in the cytoplasm of *E. coli*. Human IL-4 was expressed at high levels and was found as aggregates in the low-speed pellet fraction after cell lysis. Denaturation and refolding of these aggregates was necessary to activate the expressed protein. The human IL-4 can be renatured by extraction and refolding procedures [10, 13] using guanidine and a mixture of reduced and oxidized glutathione. The use of urea in the extraction procedure did not yield active human IL-4 (result not shown), indicating that the choice of denaturant is important.

The protein concentration during the refolding procedure is critical. Too low a protein concentration can result in insta-

bility of the protein [13] and too high a concentration can lead to precipitation of the protein [30, 31]. For human IL-4, the protein concentration during the refolding step should not exceed 0.25 mg/ml. After dialysis the concentration could be increased to at least 8 mg/ml without formation of human IL-4 aggregates.

The human IL-4 protein has six cysteine residues with the potential to form three intramolecular disulfide bonds. It can be concluded from the results described here that the fully oxidized form represents the active molecule. Dialysis against a buffer containing 2 mM reduced glutathione caused a substantial decrease of activity (result not shown), reflecting the importance of the disulfide bonds for activity.

During separation of refolded monomeric human IL-4 from contaminants, an important observation was made regarding the choice of buffer in the recovery of monomeric human IL-4. Equilibration and elution with Tris buffer (50 mM Tris pH 8.0, 50–150 mM NaCl, 1 mM EDTA) yielded mostly aggregated human IL-4 with only a negligible amount of monomeric human IL-4 (results not shown). In contrast, use of phosphate-buffered saline (120 mM NaCl, 2.7 mM KCl in 10 mM phosphate buffer pH 7.4) yielded mostly oxidized and monomeric human IL-4. The use of the latter buffer was adopted and no further studies have been done to examine the effect of different buffers on protein aggregation.

After denaturation and refolding *in vitro*, the human IL-4 has biological activity on B and T lymphocytes. The specific activity obtained from the TCGF assay, and the binding to the human IL-4 receptor are comparable for the *E. coli* and mammalian-derived recombinant human IL-4. This indicates that (a) the human IL-4 derived from *E. coli* is folded correctly and (b) the carbohydrates attached to mammalian-expressed human IL-4 are not necessary for the biological activity of the protein in the assays examined.

In conclusion, we have described here the expression of human IL-4 in *E. coli*, the activation of the aggregated human IL-4 by denaturation and refolding of the protein, and the purification of biologically active human IL-4 by gel filtration. The expression of human IL-4 in *E. coli* means that large quantities of this lymphokine can be produced. Our ability to refold this molecule correctly and recover its biological activities will facilitate further studies of its role in the immune system.

The authors would like to thank Dr Takashi Yokota for providing the human IL-4 cDNA clone, Isabelle Chrétien for providing 11B4 hybridoma supernatants, Karl Pope for providing synthetic DNAs, Dr Paul Trotta of Schering Research for the amino acid analysis data, Dr Jacques Banchereau of UNICET Research Laboratories for the B cell assays, Drs Jolanda Schreurs, John Conaway and John S. Abrams for valuable suggestions during this work, and Drs Gerard Zurawski, Philip W. Ledger and Robert S. Haxo for critically reading the manuscript.

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