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Rat interleukin-10: production and characterisation of biologically active protein in a recombinant bacterial expression system.

Abstract

Interleukin-10 is an anti-inflammatory Th1 immunosuppressive cytokine, the active form of which is a non-covalent homodimer, and which exhibits species-specificity both with respect to structure and biological activity. The rat homologue of IL-10 shares 73% identity with human IL-10 at the amino-acid sequence level, and has, in addition to the two disulphide bonds present in

human IL-10, a fifth, unpaired cysteine (cys-149). Preparation of rat IL-10 by bacterial expression followed by solubilisation and refolding in a glutathione redox system, results in a molecule in which cys-149 is almost entirely oxidised, existing either as disulphide dimer or as a mixed disulphide with glutathione, and which has less than 1% of the activity of the native (cys-149-SH) form of the molecule. Site directed mutagenesis of rat IL-10 to replace cys-149 with tyrosine produces a molecule which readily adopts the active conformation upon solubilisation and refolding, and which is recoverable in good yield from bacterial expression systems. Comparison of the biological activities of rat IL-10tyr149 and commercial rat IL-10 preparations confirms that the activity of native-sequence rat IL-10 is either reduced or absent. It is proposed therefore that the biosynthetic analogue rat IL-10tyr149 is a more useful molecule to investigate the biological actions of IL-10 in the rat.

Full text

INTRODUCTION

Interleukin-10 (IL-10) is a Th1-immuno-suppressive cytokine, originally identified in mouse Th2 supernatants as an inhibitor of cytokine synthesis [1], and subsequently cloned from human cDNA [2], and renamed IL-10. IL-10 is now known to play a major role in regulating the synthesis of Th1 cell-derived cytokines such as interferon gamma, IL-2 and tumour necrosis factor-alpha.

The human IL-10 gene encodes a single protein of 178 amino acids, including an 18-residue hydrophobic signal sequence which is cleaved on secretion, leaving a mature protein of 18,647 Da. The mature protein contains 4 cysteine residues, which have been shown to form two disulphide bonds, between Cys-12 and Cys-108, and Cys-62 and Cys-114, respectively [3]. The protein also contains a single, N-linked glycosylation site, which has been shown to be unoccupied [4].

X-ray crystallography studies subsequently revealed that IL-10 exists as a non-covalent homodimer in the form of a V-shaped structure, with each arm of the V consisting of 4 helices of 1 sub-unit inter-chelated with 2 helices of the other [5]. Overall, the molecule bears a close resemblance to the active dimeric form of interferon gamma. The homodimer is now also known to be the biologically active form of IL-10.

The IL-10 homologues have now been identified from a number of other species, in addition to human and mouse, including rat [6], pigs, dogs and sheep. Mouse and human IL-10 show 72% homology at the amino-acid level, whilst rat IL-10 shares 83% identity with mouse IL-10 and 73% identity with human IL-10. One inter-species difference likely to be of significance to the production of active IL-10 by recombinant DNA expression is the existence of an additional cysteine residue at position 149 in both the rat and mouse homologues, which replaces the tyrosine residue present at position 149 in human IL-10.

Human and mouse IL-10 have been prepared by expression in recombinant *E. coli* followed by solubilisation, refolding and purification from inclusion bodies [3, 7]. Although recombinant rat IL-10 is available commercially, there have been no reports describing its preparation or its biological properties. The known species-specificity in both biological and immunological properties of other cytokines suggest that it is desirable, where possible, to use homologous reagents. Since the rat is the species of choice in many investigations into fever, trauma and inflammation, there is a requirement for specific rat reagents, with detailed biological and immunological characterisation.

In this paper we describe bacterial expression, purification and characterisation of rat IL-10, and the identification of conjugates with the unpaired thiol (cysteine 149), formed during re-folding, which compromise both the structure and biological activity of the molecule. We also describe preparation and characterisation of a specifically mutated form, rat IL-10^{tyr149}, where the unpaired cysteine is replaced with the tyrosine present in the human homologue, which can be more readily recovered in stable, active form from the bacterial expression system. We describe comparison of the biological properties of these molecules with human and mouse IL-10, and with commercially available rat IL-10, and the preparation of antisera and a sensitive and specific ELISA for rat IL-10.

MATERIALS AND METHODS

Materials

The lac-inducible, Amp^+ bacterial expression vector PHG253 was a kind gift of Dr R. C. Newton (DuPont-Merck, Wilmington, Delaware, USA). All other reagents were molecular biology or AnalaR grade, unless otherwise stated. Recombinant

rat IL-10 was purchased from R&D systems (Burton Lane, UK) or Peprotech (29 Margravine Rd, London, UK). Human IL-10 was the WHO Reference Reagent (WHO 93/722).

Construction of expression plasmids

Messenger RNA (mRNA) was extracted from endotoxin-activated rat monocytes using the QuickPrep mRNA Purification Kit (Pharmacia Biotech, 23 Grosvenor Road, St Albans, Hertfordshire AL1 3LW, UK). cDNA was transcribed using the First Strand cDNA synthesis kit (Pharmacia Biotech). The coding sequence for the mature rat IL-10 was amplified by PCR (Amplitaq, Perkin-Elmer), using primers designed to engineer the restriction sites Nde-1 and BamH-I at the 5' and 3' ends respectively. The 482 base-pair PCR product was ligated into the vector pHG253 between the Nde-I and BamH-I sites. The pHG-rIL-10 vector was transformed into *Escherichia coli* strain JM109, where the construct was checked by restriction mapping and DNA sequencing by labelled primer extension using T7 DNA polymerase sequencing reagents (Pharmacia Biotech). The sequence of the plasmid from the ribososme binding site to the start codon was 5'-AGGAGATTAAATCATATG-3'.

The expression plasmid coding for the sequence variant rat IL-10^{tyr149} was constructing by site-directed mutagenesis using the Quikchange site directed mutagenesis kit (Stratagene, Hogehilweg, 1101 CB Amsterdam Zuidost, The Netherlands), to change codon 149 from TGC to TAC.

Induction of cultures; isolation, solubilisation and refolding of inclusion bodies

Overnight cultures of recombinant expressing strains, derived from glycerol stocks, were diluted 1:40 into fresh LB-ampicillin medium. Cultures were grown at 37° C with shaking to an E_{600} of 0.6, and induced by the addition of 1 mM isopropyl thiogalactopyranoside (IPTG). After induction and growth for 3 hours, cells were harvested by centrifugation and washed with phosphate buffered saline (PBS). Cell lysates were prepared by successive treatment with lysozyme, deoxycholate and DNase as previously described [8].

Inclusion granules were harvested from the insoluble fraction by differential centrifugation (1,450 x G, 10 min), the granule-containing supernatant being

retained. The pellet was re-homogenised in PBS containing 1mg/ml DOC, and a further 1,450 x G supernatant collected. After three harvests, granules were collected from the combined supernatants by centrifugation (27,000 x G, 20 min). Urea soluble contaminants were removed by re-suspending in three washes of 0.1 M Tris-HCl, pH 8.5 containing 2.5 M urea followed by centrifugation (27,000 x G, 20 min). The final granule preparation was solubilised in 20 volumes of 6 M guanidine hydrochloride in 0.05 M Tris-HCl, pH 8.5, with gentle stirring for 1 hour at room temperature.

The IL-10 was re-folded by diluting to 1.0 M guanidine hydrochloride with 0.05 M Tris-HCl, pH 8.0 containing 2 mM reduced and 0.2 mM oxidised glutathione, 1 mM phenylmethylsulfonylfluoride (PMSF) and incubating at 4° C overnight with gentle stirring.

Ion-exchange extraction of renatured IL-10

Renatured proteins were extracted from the renaturation solution by buffer-exchange into 0.025 M sodium phosphate pH 7.25, either by dilution or dialysis, and binding to SP-trisacryl (Sigma-Aldritch, UK; 1 g resin/10 ml renaturation solution). After washing the resin with 0.025 M sodium phosphate pH 7.25, bound proteins were eluted with 0.025 M sodium phosphate pH 7.25/1.0 M NaCl.

Chromatography

Monomeric and dimeric fractions of IL-10 were prepared by gel filtration on a column (h = 30 cm, Vt = 150 ml) of Superdex 200 (Pharmacia Biotech), equilibrated and eluted in pyrogen-free Dulbecco's PBS (Sigma Alditch). Fractionation was carried out at a constant flow rate of 1.8 ml/min, and the Ve for monomeric and dimeric IL-10 identified by calibration with proteins of known molecular weight. Dimeric and monomeric IL-10 eluted at Ve/Vt = 0.76 and 0.86 respectively. Larger scale fractionations were carried out on a column (450 ml) of Sephadex G-75(fine) (Pharmacia Biotech) using identical, scaled-up conditions.

High-performance (cation) exchange chromatography (HPIEC) was performed on Bio-scale S-10 (Bio-Rad Laboratories, Alfred Nobel Drive, Hercules, Ca, USA). The column was equilibrated in 0.025 M sodium phosphate pH 7.2, and proteins eluted using a linear gradient of 0-1.0 M sodium chloride in the start buffer (Flow

rate 4 ml/min, gradient = 2%/min). Analytical scale cation exchange chromatography was performed using Mono-S HR 5/5 (Pharmacia Biotech.), run under identical conditions using scaled-down flow rates.

Mass spectrometry

Analyses of intact purified IL-10 and tryptic digests of IL-10 were performed on a VG platform II mass spectrometer as described previously [8].

Protein determination, SDS electrophoresis and immunoblotting

The concentration of total protein was calculated from the absorbance measured at 280 nm and the predicted molar extinction coefficient of the recombinant protein, using values of 11,110 for rat IL-10 and 12,330 for rat IL-10^{tyr149}. SDS electrophoresis using 12% polyacrylamide gels was performed according to the method of Laemmli [13]. Proteins were detected by Coomassie staining. Immunoblotting was performed by electro-transfer onto PVDF membranes, and immunodetection using antibodies to murine IL-10 (R&D Systems), and peroxidase-labelled second antibodies.

Bioassay of IL-10

IL-10 was assayed by its ability to inhibit the production of intracellular IL-1beta in endotoxin-stimulated rat mononuclear cells. Heparinised plasma was collected by cardiac puncture from normal male rats, diluted 1:1 with RPMI 1640 medium, and aliquots (35 ml) over-layered onto Ficoll Histopaque (1,083, 15 ml, Sigma-Aldritch UK). After centrifugation (400 x G, 40 min, 20° C), the white cell layer was removed into RPMI 1640 (25 ml) and the cells harvested by centrifugation (400 x G, 10 min). After 2 further washes (400 x G), the cells were re-suspended (RPMI/2% low-endotoxin heat-inactivated foetal calf serum) to 2×10^6 cells/ml and dispensed in 0.5 ml aliquots in 24-well tissue culture plates. Endotoxin (10 IU/100 ul/well) and IL-10 dilutions (1-1,000 ng/0.4 ml) were added separately, and plates incubated overnight (37° C, 5% CO_2). After incubation, supernatants were removed, and the attached cells lysed by dilution with 0.5 ml water, and 1 freeze-thawing cycle. Lysates were assayed for IL-1beta (duplicate determinations of each cell incubation) using the rat-specific ELISA described previously [14].

Antibody preparation

A polyclonal antiserum against rat IL-10^{tyr149} was raised in a sheep as described previously [15]. Antibodies were affinity purified on Affigel-immobilised rat IL-10, and biotinylated by N-hydroxy-succinimide conjugation, as described in Rees *et al.*, 1998 [8].

Enzyme-linked immunos or bent assay (ELISA) of rat IL-10

Rat IL-10 ELISAs were carried out in 96-well microtitre plates (Nunc-Immuno Plate, Maxisorp). Wells were coated with immunoaffinity purified sheep anti-rat IL-10 antibodies (2 mug/ml in PBS, 100 mul) by incubation overnight at 4° C in a humidified container. The wells were washed three times in assay buffer (0.01 M phosphate, 0.5 M NaCl, pH 7.2, 0.1% v/v Tween 20, 200 mul) and blocked with 1% w/v ovalbumin in PBS (100 mul) for 1 hour at room temperature with gentle agitation. Wells were washed again in assay buffer and standard (100 mul) added and incubated overnight at 4° C in a humidified container. The wells were washed again in assay buffer and incubated with biotinylated immunoaffinity purified sheep anti-rat IL-10 antibodies (diluted 1:1,000 in assay buffer plus 1% v/v normal sheep serum, 100 mul) for 1 hour at room temperature with gentle agitation. The wells were washed with assay buffer and incubated with avidin-horseradish peroxidase (1:4,000 Dako Ltd, 100 mul) for 15 min at room temperature with gentle agitation. The wells were washed with assay buffer and incubated with *o*-phenylenediamine (0.4 mg OPD + 0.4 mul 30% H_2O_2/ml in substrate buffer, 34.7 mM citric acid, 66.7 mM Na_2HPO_4 pH 5.0, 100 mul) at room temperature until the colour developed. The reaction was stopped by addition of 1 M sulphuric acid and the optical densities at 490 nm determined.

RESULTS

Cloning and characterisation of rat IL-10 expression vectors

cDNA coding for the mature protein sequence of rat IL-10 was amplified and ligated into plasmid vectors using conventional methodology, described in Materials and Methods. Sequence analysis of the resulting construct confirmed the sequence of rat IL-10 cDNA reported by Goodman *et al.* (1992) [6] with a single C to G base change at position 240 (numbering as in Goodman *et al.*, 1992 [6]). This substitution resulted in an amino acid change of leucine to valine at

position 48 of the mature protein sequence. This change was identified in every clone studied, and therefore presumably represents a genuine difference from the published sequence rather than a PCR artefact.

Two lac-inducible bacterial expression vectors were constructed in these studies; pHGrIL-10, and pHGrIL-10^{tyr149}. Both vectors contained an additional ATG sequence at position - 1 of the mature protein, to initiate bacterial translation, and in addition, the pHGrIL-10^{tyr149} vector incorporated a single base change resulting in a cysteine to tyrosine substitution at position 149 (Materials and Methods). Both vectors showed a strong induction of a band at about 18 kD in the presence of the lactose analogue IPTG (Figure 1, lanes 2/3). The band was putatively identified as rat IL-10 by positive immuno-staining with a commercially available anti-mouse IL-10 antibody (not shown). Pilot scale induction and lysis experiments (not shown) revealed the induced protein to be present entirely in the insoluble fraction, and therefore presumably to be in the form of inclusion granules, commonly observed with bacterial expression vectors.

Purification of rat IL-10 homodimer

The active form of IL-10 is known to be a non-covalent homo-dimer. In these studies, biochemically homogeneous dimer preparations of either native (^{cys149}) or rat IL-10^{tyr149} were recovered from induced cultures by sequential cell lysis, isolation of inclusion granules by differential centrifugation, guanidine solubilisation, re-folding/reoxidation, ion-exchange extraction and elution, and gel-filtration chromatography, as described in Materials and Methods (Figure 1). In the case of the native sequence IL-10 (rat IL-10^{cys149}), preparative gel filtration resolved the ion-exchange purified rat IL-10 into dimer and monomer fractions in the approximate ratio 40:60 (not shown). Comparison of non-reduced and reduced SDS PAGE gels (Figure 1A, lanes 5/6) revealed that whilst being biochemically homogeneous with respect to IL-10 protein, the purified dimer fraction of rat IL-10^{cys149} comprised a mixture of covalent and non-covalent dimers. In contrast, the ion-exchange-purified rat IL-10^{tyr149} protein, whilst being resolved by gel filtration into approximately equal proportions of monomer and dimer (not shown) did not form detectable covalent dimers during the refolding and purification process (Figure 1B, lanes 5/6).

Mass spectrometric analysis of dimeric rat IL-10

Electrospray mass spectrometric (ESMS) analysis of dimeric rat IL-10^{cys149} (Figure 2, panel A) yielded a signal for the main component of 18,941.7 D. This is consistent, within 1 dalton, to the predicted amino-acid sequence, with the following modifications; i) removal of the N-terminal methionine, ii) formation of two intra-chain disulphide bonds, and iii) formation of a mixed disulphide between the remaining cysteine and glutathione. The minor peak of 18,637.0 Da is consistent with two disulphide bonds and an un-oxidised cysteine.

Analysis by ESMS of dimeric rat IL-10^{tyr149} (Figure 2, panel B) produced a signal for the main component of 18,697.9, consistent with the predicted amino-acid sequence, with the following modifications; i) removal of the N-terminal methionine and ii) formation of two intra-chain disulphide bonds. ESMS analysis of tryptic peptides (not shown) revealed that the only disulphide bonds present were between Cys-12 and Cys-108, and Cys-62 and Cys-114, confirming the absence of incorrectly paired disulphide forms of the molecule.

Preparation of rat IL-10$^{cys-149-SH}$

In order to isolate significant quantities of the native rat IL-10 protein with cys^{149} in the free-SH form, ion-exchange-purified rat IL-10^{cys149} was fractionated by gradient high-performance ion-exchange chromatography (HPIEC, Materials and Methods, Figure 3). Rat IL-10 eluted as a broad, heterogeneous peak over a wide range of salt concentrations (Figure 3, panel A). Analysis of the biological (cytokine-synthesis inhibiting) activity of these fractions (Figure 3, panel B) showed that the earliest eluting fractions retained the highest activity, with the later eluting fractions becoming progressively less active, and the latest-eluting fractions being essentially inactive. The purified dimer fraction of rat IL-10^{tyr149} did not exhibit significant heterogeneity on analytical HPIEC (not shown) and produced a single major component corresponding approximately in elution time to fraction A in Figure 3.

Analysis by ESMS of fraction A (Figure 2, panel C) produced a major component corresponding to the predicted structure of rat IL-10^{cys149}, with i) removal of the N-terminal methionine, ii) two disulphide bonds and an un-oxidised cysteine (rat IL-10$^{cys149-SH}$).

Total recoveries of dimeric IL-10 achievable were 0.8 mg/l culture for rat IL-10^{tyr149}, and 0.0375 mg/l for rat IL-10$^{cys149-SH}$.

Biological activities of purified rat IL-10 preparations

Rat IL-10 showed a dose-related ability to completely inhibit IL-1beta synthesis in endotoxin-stimulated rat peripheral blood mononuclear cells (Figure 4).

The dimeric forms of rat IL-$10^{cys149\text{-}SH}$ and rat IL-10^{tyr149} (Figure 4, panel A and B) exhibited approximately equivalent biological activities, with ED_{50}s of approximately 100 pg/ml. In contrast, the dimeric fraction of rat IL-10^{cys149} was some two orders of magnitude less active than either (Figure 4, panel A). Human IL-10 exhibited activity on rat cells in these studies, but with at least an order of magnitude-lower potency (Figure 4, panel A). Interestingly, the monomeric form of rat IL-10^{tyr149} was also active, with approximately one log-lower potency than the dimer (Figure 4, panel B).

The biological activities of two commercial preparations of rat IL-10 are shown in Figure 4 (panel C). Preparation A (R&D Systems) exhibited IL-10 activity, but with an ED_{50} some two logs lower than rat IL-10^{tyr149} dimer. Preparation B (Peprotech) did not exhibit IL-10 activity in this system.

Immunological (ELISA) activities of purified rat IL-10

Utilisation of immuno-affinity purified polyclonal antisera to rat IL-10^{tyr149} dimer, in a two-site ELISA format, produced a sensitive ELISA with a dose-response range of 15-1,000 pg/ml (Figure 5). The two dimeric preparations, rat IL-10^{tyr149} and rat IL-$10^{cys149\text{-}SH}$ exhibited essentially identical immunological activities in this assay. Of the two commercial preparations tested, preparation A (R&D Systems) shows complete reactivity, with immuno-potency on a weight basis being approximately similar to rat IL-10^{tyr149}, whereas preparation B (Peprotech) shows approximately ten-fold lower activity.

DISCUSSION

Rat IL-10 is a relatively easy protein to express in bacteria; indeed, in these studies, expression under the control of a simple lac promoter caused the protein to be induced to over 50% of the total cell protein (Figure 1). The complete sequestration of the protein into insoluble inclusion granules, however, necessitated recovery of the protein by a process of solubilisation in 6.0 M guanidine, renaturation and re-oxidation of the disulphides at an

intermediate concentration of guanidine, and recovery of soluble protein from the renaturation solution by ion-exchange extraction and elution. IL-10 is a disulphide-containing protein, and recovery of such proteins is generally facilitated using a redox system comprising reduced and oxidised glutathione, which catalyses disulphide exchange, allowing the protein to pass through incorrect disulphide intermediates, and eventually to adopt its correct conformation. Crucially, rat IL-10 differs from the human homologue in having, in addition to the two disulphide bonds found in human IL-10, a fifth, unpaired, cysteine. Whilst it is possible to recover such unpaired cysteine residues as free thiols from refolded, reoxidised proteins, as in the case of rat IL-6 [8], these studies suggest that the unpaired cysteine in rat IL-10 is rather reactive. The soluble, dimeric protein recovered from refolding the native sequence IL-10 (rat IL-10^{cys149}) exists in two main forms, a covalent disulphide bonded dimer, revealed by SDS PAGE (Figure 1) or as a mixed disulphide with glutathione, identified by ESMS. Only very low levels of the free thiol form of the molecule were detected using ESMS. The biological activity of this preparation is only around 1% of that of the most active forms of IL-10 isolated in these studies (Figure 4), and we conclude, therefore, that the biological activity of either the covalent dimer or the mixed glutathione-IL-10 disulphide is severely reduced.

Bondoc *et al.* (1997) [7] have described the use of recycling free-flow focusing to separate folding variants of human IL-10, and using a comparable approach in these studies, small quantities of a dimeric fraction enriched in the free thiol form of the molecule could be recovered by preparative ion-exchange HPLC. Whilst the biological activity of this preparation was some two-logs greater than the un-fractionated dimer preparation, ESMS analysis indicated that it was still significantly contaminated, both with the glutathione conjugate, and with other, unassigned contaminants. More significantly, overall recovery of this active fraction (0.0375 mg/l culture) was too low to be useful.

In order to eliminate unwanted reactivity at cysteine 149, and thereby to increase the recovery of active IL-10, it was decided to substitute the cysteine at position 149 with an inert amino-acid. Comparison with the sequence and structure of human IL-10 dimer suggested that both the amino-acids surrounding cys149, and those in the area of the interacting helix in the dimer are conserved [5]. It was reasoned, therefore, that replacement of cysteine 149 with tyrosine, the corresponding amino-acid in human IL-10, was least likely to perturb the active conformation. Rat IL-10^{tyr149} prepared in these studies proved

to be a much more stable and easy molecule to handle. The molecule showed no tendency either to form disulphide dimers, glutathione adducts or mixed disulphide intermediates. Around 50% of the soluble, refolded protein was recovered as the active non-covalent dimer and the yield of protein (0.8 mg/l) was both sufficiently high to be useful, and stable between batches.

From comparison of the biological activities of rat IL-10^{tyr149} and rat IL-$10^{cys149\text{-}SH}$, we conclude that rat IL-10^{tyr149} essentially possesses the full biological activity of the native physiological rat IL-10 homodimer. The behaviour of the two preparations is also essentially equivalent in a specific IL-10 immunoassay. It is proposed therefore that the biosynthetic analogue rat IL-10^{tyr149} is a useful molecule with which to investigate the biological actions of IL-10 in the rat.

Whilst most studies of the biological actions of IL-10 have supported an anti-inflammatory role, there have been some inconsistent or even contradictory reports of activity *in vivo*. Allograft rejection, for instance, has been variously shown to be inhibited [9] or augmented [10] by IL-10. In the brain, whilst IL-10 generally opposes the activity of inflammatory mediators (*e.g.* Bluthe *et al.*, 1999 [11]), at least one report has described an also mediated by effect IL-1 [12].

The findings we report suggest that studies on the actions of IL-10 in the rat have been carried out either with heterologous (human) IL-10, with significantly lower activity in the rat, or with rat IL-10 whose activity can vary from reduced to absent. The biological activities of these preparations is unlikely to be consistent, and comparison or interpretation of the data must inevitably be compromised. We suggest that the biosynthetic analogue of rat IL-10, rat IL-10^{tyr149} may be a useful molecule on which to base investigations both of the activity and the regulation of IL-10 in the rat.

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Figures

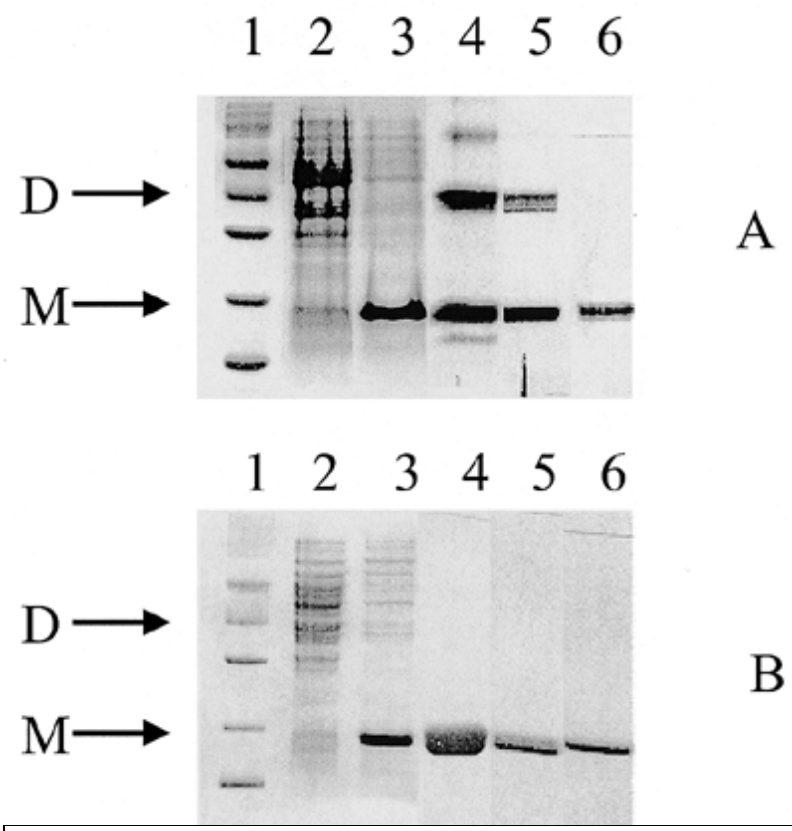


Figure 1

SDS PAGE analysis of rat IL-10 purification.

Rat IL-10 was expressed and purified from plasmids pHGrIL-10 (panel A) and pHG rIL-10^{tyr149} (panel B). Lanes: 1 - molecular weight standards; 2 - un-induced culture, total proteins; 3 - induced culture, total proteins; 4 - ion-exchange (SP-trisacryl) extracted, renatured proteins; 5 - size-exclusion-purified dimer; 6 - size-exclusion-purified dimer. Lanes 1, 2, 3 and 6 were run under reducing conditions (1% beta-mercaptoethanol), lanes 4 and 5 under non-reducing conditions. Migrating positions of dimer (D) and monomer (M) are indicated.

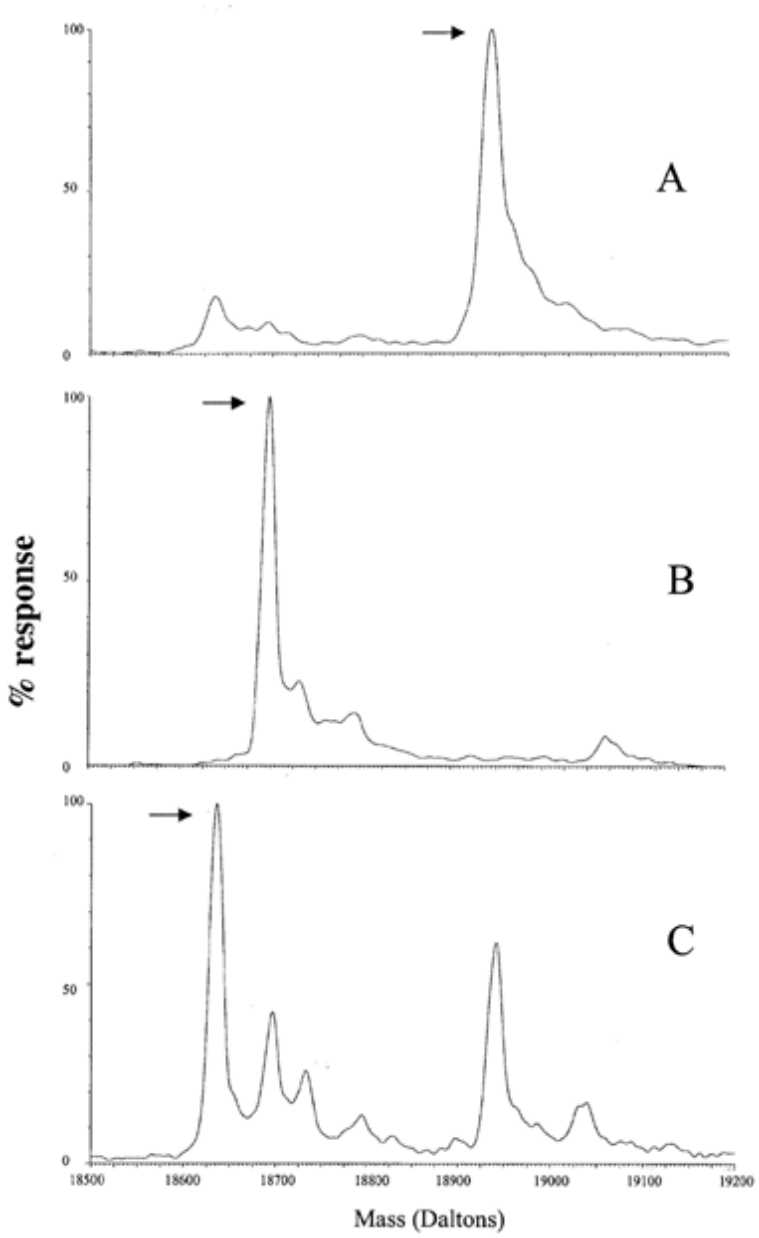


Figure 2

Electrospray mass spectrometric analysis of purified IL-10 dimer preparations.

Deconvoluted mass spectra were obtained as described in Materials and Methods. Panels: A - rat IL-10^{cys149} (dimer purified from plasmid pHGrIL-10); B - rat IL-10^{tyr149} (dimer purified from plasmid pHGrIL-10^{tyr149}); C - rat IL-10$^{cys149\text{-}SH}$ (rat IL-10^{cys149} dimer further purified by high performance ion-exchange chromatography, corresponds to fraction A

from Figure 3). Arrows indicate the major peaks described in the text.

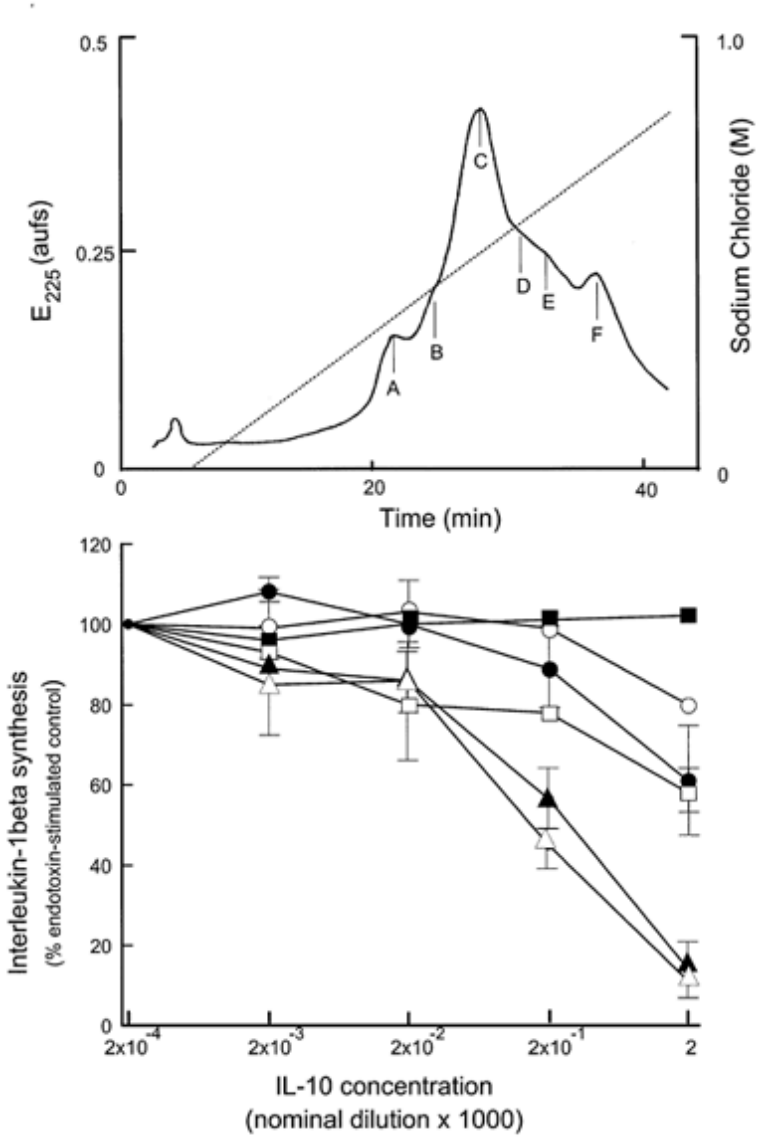


Figure 3

Fractionation by high-performance ion-exchange chromatography of rat IL-10^{cys149} (dimer purified from plasmid pHGrIL-10).

Rat IL-10^{cys149}, prepared by ion-exchange (SP-trisacryl) extraction and elution of solubilised, renatured IL-10 as described in Materials and Methods, was briefly treated

with beta-mercaptoethanol (0.02% v/v, 15 min), and the dimer fraction isolated by Sephadex-G75 gel-filtration (Materials and Methods). The collected dimer fraction (12.5 mg protein) was diluted two-fold with water, and fractionated on Bio-scale-S (Materials and Methods). Elution of proteins was monitored at 225 nm, and fractions A-F collected as indicated (upper panel).

The concentration of each fraction was adjusted to the same nominal value based on E280 measurements, and the biological activity (cytokine-synthesis inhibition, Materials and Methods) of each fraction determined at the dilutions indicated (lower panel). * fraction A, * fraction B, * fraction C, * fraction D, * fraction E, * fraction F.

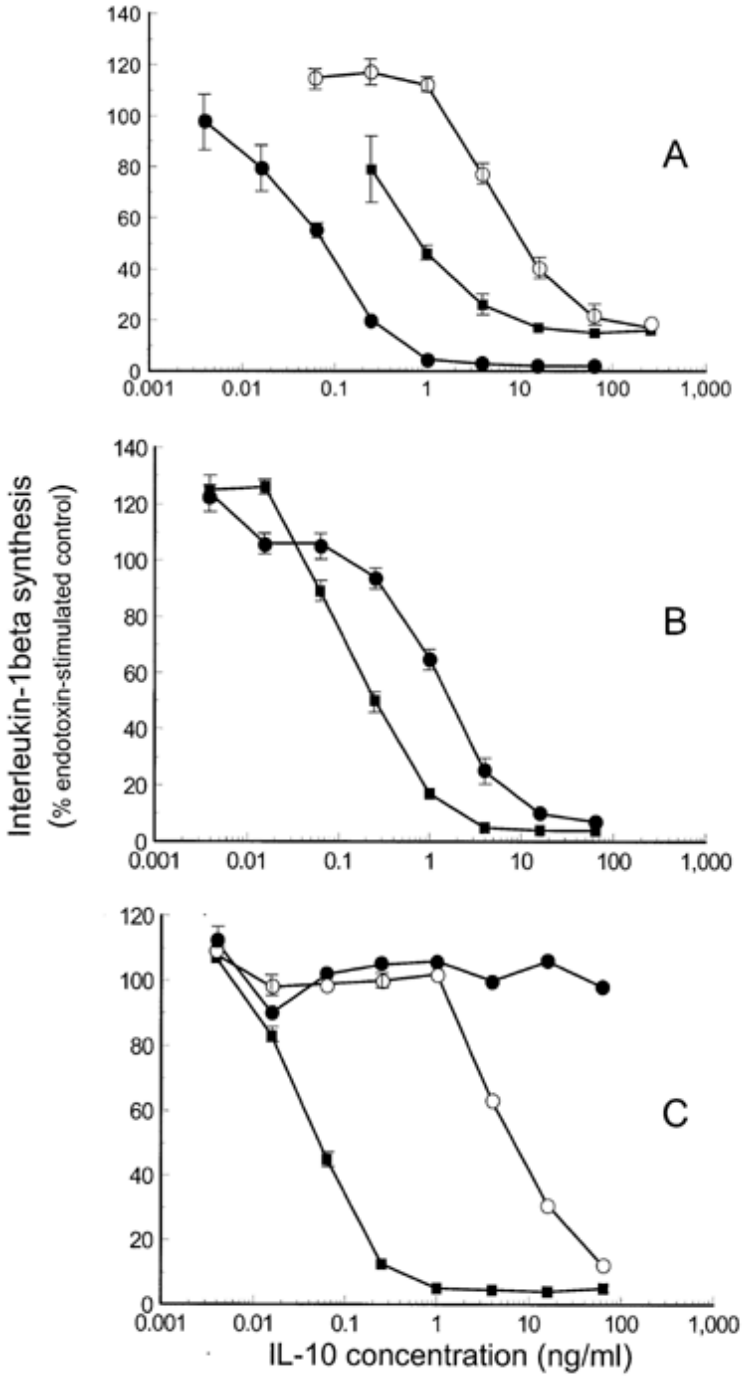


Figure 4

Biological activity of purified IL-10 preparations.

Determined by inhibition of cytokine (IL-1beta) synthesis, as described in Materials and Methods. Data points are the means of triplicate cell incubations ± SEM. Error bars not shown fall within the dimensions of the symbol. Panel A: closed circles, rat IL-10$^{cys149\text{-}SH}$ dimer (see text); closed

squares, human IL-10; open circles, rat IL-10^{cys149} dimer (see text). Panel B: * - rat ; IL-10^{tyr149} dimer; * - rat IL-10^{tyr149} monomer. Panel C: * - rat ; IL-10^{tyr149} dimer, * - commercial rat IL-10 (preparation A); * - commercial rat IL-10 (preparation B).

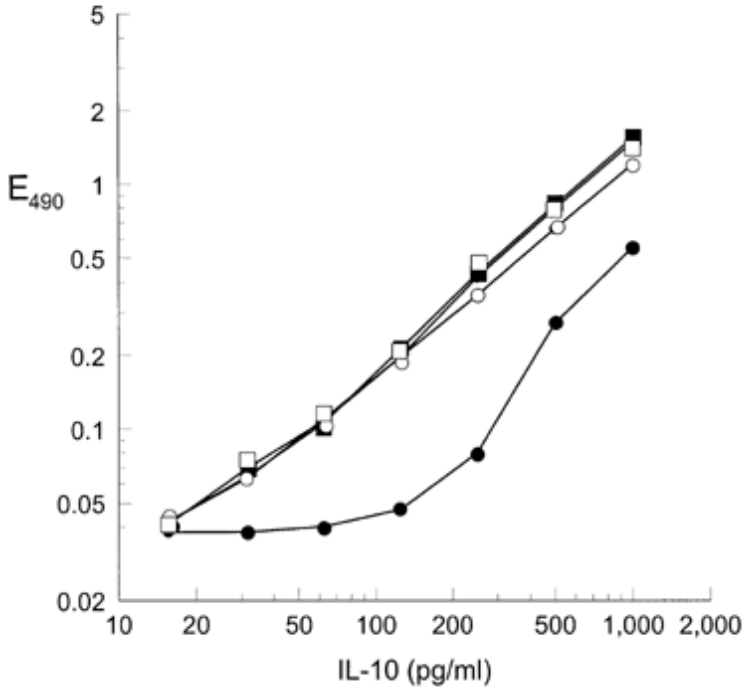


Figure 5

ELISA dose-response curves of purified IL-10 preparations.

Determined as described in Materials and Methods. Data points are the means of triplicate incubations. The mean (within-assay) relative standard deviation (n = 3) in the range 15-1,000 pg/ml was 4.4% (individual error bars are omitted for clarity). Open Squares; rat IL-$10^{cys149\text{-}SH}$; closed squares, rat IL-10^{tyr149}; open circles, commercial rat IL-10, preparation A; closed circles, commercial rat IL-10, preparation B.

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IL-10, recombinant, rat, cytokines.

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