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Multiple charge isomers of human recombinant interleukin-1β

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Human interleukin-1β (rhuIL-1β), obtained by DNA recombinant technology, was radiolabelled. Its isoelectric properties were determined by various analytical techniques such as high-voltage ultrathin layer isoelectric focusing (IEF) and chromatofocusing. The rhuIL-1β molecule had a molecular mass of 18 kDa, as determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. When examined by IEF on a polyacrylamide gel of 1 mm thickness in the pH range of 3.5–9.5, it was resolved into two broad bands appearing in the pH range of 6.2–5.8 and 5.5–5.2. Each of the two bands was further resolved into multiple bands when electrofocused on (i) a thinner gel of 0.5 mm thickness and (ii) a narrower pH range of 5–8. Upon chromatofocusing in a liquid column, it was possible to isolate various charged components of rhuIL-1β. However, all these components reacted to the antiserum to rhuIL-1β and displayed a molecular mass of 18 kDa suggesting the charge heterogeneity of rhuIL-1β.

Interleukin-1β, human recombinant; Isoelectric focusing; Chromatofocusing

1. INTRODUCTION

Interleukin-1 (IL-1) is an immunomodulatory protein secreted by activated macrophages and is considered to be one of the key mediators of the body's response to a number of infectious, antigenic and immunologic stimuli [1]. Two different forms of IL-1 have been isolated: IL-1α and IL-1β [2,3]. These two IL-1s share only small stretches of similar amino acids (26% in the case of human IL-1) and are not homologous proteins [4,5]. Both forms of IL-1 have been reported to be synthesized as large precursors with molecular masses of about 30 kDa and are then proteolytically cleaved to smaller mature forms having molecular masses of 17.5 kDa [6–8]. IL-1β is the predominant form of IL-1 as culture supernates and various human body fluids contain more IL-1β than the IL-1α form [9].

Since IL-1 performs a wide variety of inflammation-related activities, several laboratories are actively engaged in finding out ways to block the production and/or activity of IL-1. Recently, substantial amounts of information on the nature of the IL-1 receptor on a variety of cells have become available [10]. In most of these studies related to the characterization of the IL-1 receptor, radiolabelled IL-1s (both natural and recombinant) had been used [11–15]. The two forms of IL-1 (α and β) are generally distinguished by their isoelectric points (pI): the pI of IL-1α is 5.0 whereas the pI of IL-1β is 7.0 [16]. However, controversies have also been raised over whether these two forms should be solely distinguished by their pI values. Thus, Kronheim et al. [17] have reported pI values of 5.6 and 5.5 for human IL-1α and -β forms respectively. Human recombinant IL-1s (both α and β) have been expressed in *Escherichia coli* by a number of investigators [2,17–20]. However, detailed analysis of isoelectric properties of these products have been lacking in the literature. Since IL-1β is the predominant form of IL-1, the present investigation was initiated to investigate properties of recombinant human IL-1 (rhuIL-1β) by applying analytical and preparative techniques such as high voltage isoelectric focusing (IEF) and chromatofocusing. Results presented here indicate that rhuIL-1β exists as multiple charge isomers.

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2. MATERIALS AND METHODS

2.1. *Interleukin-1*

Human recombinant ^{125}I-Interleukin 1β (NEX 232, Lot No. DX 21090) was supplied by DuPont-New England Nuclear (Boston, MA). The rhuIL-1β molecule was genetically constructed and expressed in *Escherichia coli* [21]. The rhuIL-1β molecule was radioiodinated using the Bolton-Hunter reagent [22] to a specific activity of 120–250 μCi/μg (4.44–9.25 MBq).

2.2. *Isoelectric focusing (IEF)*

High performance analytical isoelectric focusing was performed in 1.0 and 0.5 mm thin-layer polyacrylamide gels. Two different pH gradients (pH 3.5–9.5 and pH 5.0–8.0) were used. One millimeter thick gels having a pH gradient of 3.5–9.5 were obtained from LKB (Bromma, Sweden). Ultrathin gels were cast between two glass plates separated by a rubber gasket (0.5 mm thickness). The solutions for gel polymerization were prepared as follows: (a) acrylamide 29.1 g made up to 100 ml, (b) bisacrylamide 0.9 g made up to 100 ml in water, (c) ammonium persulphate 100 mg dissolved in 1 ml of water. Acrylamide solution (3.5 ml) was mixed with bisacrylamide (3.5 ml), ampholine (1.5 ml), ammonium persulphate (0.15 ml, 10% w/v),

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water (12.35 ml) and TEMED (20 μl). Polymerization was complete in 2 h. IEF was performed on a flat bed (LKB, Sweden) cooled to 5°C. When the ampholyte of pH range 3.5–9.5 was used, the anode and cathode wicks were saturated with H_3PO_4 (1 M) and NaOH (1 M), respectively. rhuIL-1β was focused at a constant power of 25 W for 90 min when using the ampholine of pH 3.5–9.5. The time for electrofocusing was increased to 120 min when using the ampholine of pH range 5.0–8.0. Marker proteins for IEF were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

2.3. *Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)*

Aliquots of rhu^{125}I-IL-1β were analyzed by performing SDS-PAGE on a polyacrylamide gel (15%, w/v; 1.5 mm thickness) using the discontinuous buffer system of Laemmli [23].

2.4. *Two dimensional gel electrophoresis (IEF followed by SDS-PAGE)*

After IEF, the gel strip containing rhuIL-1β was cut off and equilibrated for 1 h at room temperature with a solution containing 3.75 ml glycerol, 18.75 ml of Tris-HCl (0.125 M, pH 6.8), 15 ml distilled wwater, 0.75 g SDS, 1.9 ml of 2-mercaptoethanol and a trace of bromphenol blue. Later the gel strip was placed above a stacking gel (4.5%, w/v). It was sealed over the stacking gel with 1% agarose. SDS-PAGE was performed according to the procedure of Laemmli [23] using a resolving polyacrylamide gel of 15% (w/v).

2.5. *Autoradiography*

After IEF gels were fixed in an aqueous solution of trichloroacetic acid (10%, w/v) for 1 h. Subsequently gels were soaked for 1 h in a solution containing ethanol/acetic acid/glycerol/water (25:8:10:57, v/v) and dried in a gel dryer (BioRad, Model 543). Autoradiography of the dried gels was performed at −70°C in a cassette containing Hyperfilm-MP (Amersham).

2.6. *Peptide mapping by limited proteolytic digestion in gels*

rhuIL-1β was focused in the first dimension in a 0.5 mm polyacrylamide gel, the pH gradient being 3.5–9.5. The gel strip after electrofocusing was subjected to peptide mapping by partial proteolysis according to the procedure of Cleveland [24]. The gel strip containing rhuIL-1β proteins after electrofocusing was equilibrated with a solution containing 3.75 ml glycerol, 18.75 ml of Tris-HCl (0.125 M, pH 6.8), 15 ml distilled water, 0.75 g SDS, 1.9 ml 2-mercaptoethanol and a trace of bromphenol blue. It was placed on top of a 4.5% stacking gel and overlayered with a solution containing *Staphylococcus aureus* V8 protease (50 μg/ml in 0.125 M Tris, pH 6.8). Proteolysis was carried out in the stacking gel. Electrophoresis was carried out at 60 V until the tracking dye reached the end of the stacking gel. At that point the current was turned off for 30 min. Subsequently the voltage was increased to 150 V and electrophoresis was carried out until the tracking dye reached the end of the resolving gel. Later the gel was dried and subjected to autoradiography.

2.7. *Chromatofocusing*

rhu^{125}I-IL-1β was chromatographed on a column containing the polybuffer exchanger PBE 94 (10 × 1.5 cm, Pharmacia Fine Chemicals, Uppsala, Sweden). The exchanger was equilibrated by passing 20 bed vols of 0.025 M imidazole-HCl (pH 7.4). An aliquot of rhu^{125}I-IL-1β was mixed with the starting buffer (0.5 ml) and was applied onto the top of the column. Eluted materials were collected in 1 ml fractions in an LKB fraction collector (Multirac 2111) and pH of every fraction was recorded by a pH meter.

2.8. *Affinity chromatography on an anti-human IL-1β-Sepharose 4B column*

Rabbit anti-human IL-1 antibody was generated against recombinant IL-1β and was obtained commercially (Genzyme, Boston). The antibody (1 mg in 1 ml of PBS) was coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the manufacturer's instruction.

The anti-rhuIL-1β Sepharose 4B column was washed four times alternately with high pH (0.1 M $NaHCO_3$, pH 8.3) and low pH (0.1 M acetate buffer, pH 4.0) buffers to remove any excess ligand. An aliquot of rhu^{125}I-IL-1β was applied to the affinity matrix. Unbound materials were washed with PBS. The bound material was desorbed from the column by applying 0.1 M glycine-HCl (pH 2.8). The eluted materials in 1 ml fractions were immediately neutralized by collecting over solid Tris (0.5 g/fraction).

3. RESULTS

The recombinant human IL-1β (rhuIL-1β) was genetically engineered using recombinant DNA techniques and expressed in *E. coli* [21]. The product was detected in the soluble fraction of sonicated *E. coli* and further purified to homogeneity by anion exchange chromatography. Upon examination by SDS-PAGE, the product was found to produce a single band having a molecular mass of 18 kDa (fig.1).

To analyse isoelectric properties, the rhu^{125}I-IL-1β was examined by isoelectric focusing on thin layer polyacrylamide gels. When a gel of 1 mm thickness was used, the molecule was focused into two pH regions: one spot appearing in the pH range of 6.2–5.8 and the other in the pH range of 5.5–5.2 (fig.2a). When the thickness of the gel was reduced to 0.5 mm, each spot was further resolved into multiple bands. Thus, the spot in the pH range of 5.5–5.2 was split into two major and a few minor bands (fig.2b). The spot in the pI range of 6.2–5.8 was split into two sharp bands. When rhu^{125}I-IL-1β was electrofocused using an ampholyte of narrower pH range (5–8), the resolution of these bands was further increased (fig.2c). Two bands were detected in the pH range of 6.2–5.8. Similarly, two distinct bands were observed in the pH range of 5.5–5.2. A few more minor bands became now visible.

The rhu^{125}I-IL-1β, separated by isoelectric focusing in the first dimension, was subjected to SDS-PAGE in the second dimension (fig.3). Two bands, both in the molecular mass region of 18 kDa, were detected. These two bands on the SDS-PAGE gel in the second dimension correspond to the protein bands appearing in the pH regions 6.2–5.8 and 5.5–5.2.

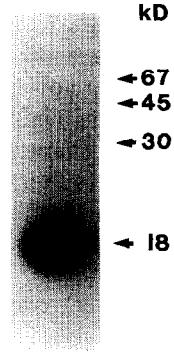

Fig.1. Autoradiograph of ^{125}I-rhuIL-1β after separation by SDS-PAGE on a 15% polyacrylamide gel. SDS-PAGE was performed under reducing conditions according to the procedure of Laemmli [23].

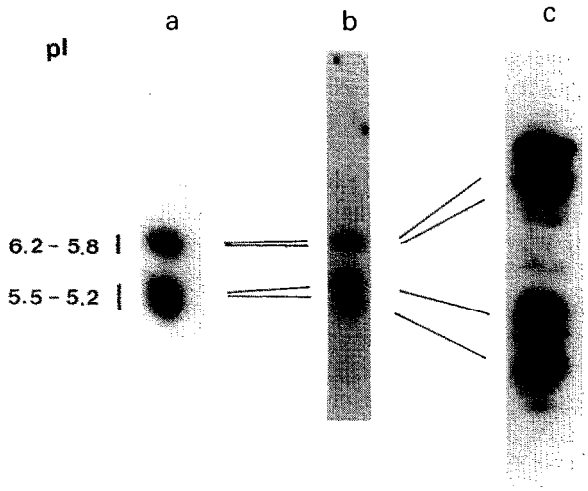

Fig.2. Separation of ^{125}I-rhuIL-1β by high voltage isoelectric focusing (a) in 1 mm thick polyacrylamide gel (ampholyte pH 3.5–9.5), (b) in 0.5 mm thick polyacrylamide gel (ampholyte pH 3.5–9.5) and (c) in 0.5 mm thick polyacrylamide gel (ampholyte pH 5–8). Bands were visualised by autoradiography.

To determine the relationship among protein bands obtained by isoelectric focusing, peptide mapping studies were performed. Proteins separated by IEF in the first dimension were subjected to peptide mapping in gels by digestion with V8 protease from *Staphylococcus aureus*. The resulting products were separated in the second dimension by SDS-PAGE. The enzyme degraded those proteins appearing in the pH regions 6.2–5.8 and 5.5–5.2 (fig.4). There was similarity among these peptide maps. In the second dimension the protein band appearing at 18 kDa had virtually disappeared. Instead protein spots at molecular mass less than 10 kDa were observed.

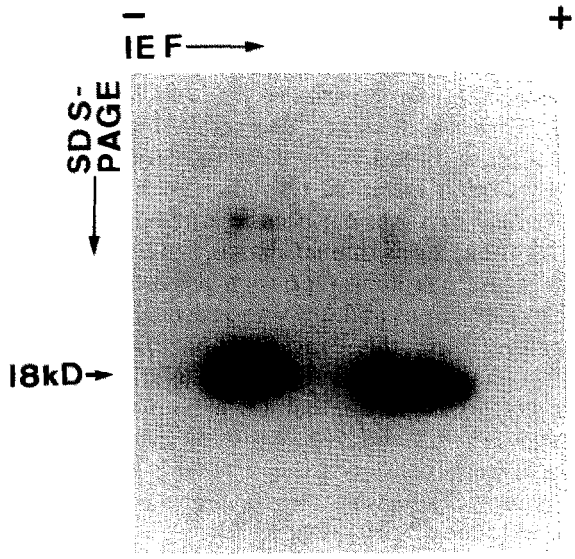

Fig.3. Separation of ^{125}I-rhuIL-1β by 2-dimensional gel electrophoresis. ^{125}I-rhuIL-1β, separated by isoelectric focusing in the first dimension as shown in fig.2c, was subjected to SDS-PAGE in the second dimension in 15% gel under reducing conditions. Bands were visualised by autoradiography.

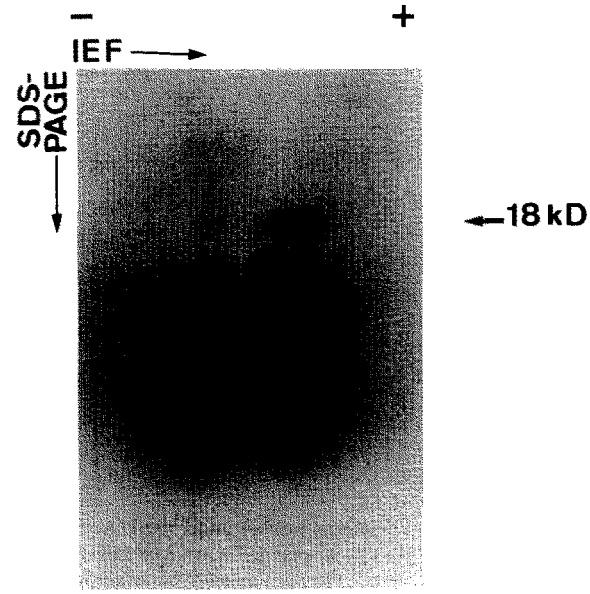

Fig.4. Peptide mapping of ^{125}I-rhuIL-1β in polyacrylamide gels. ^{125}I-rhuIL-1β was separated in the first dimension by isoelectric focusing (ampholyte pH 3.5–9.5) as shown in fig.2b. The gel was subjected to limited proteolysis by *Staphylococcus aureus* V8 protease and separated in the second dimension by SDS-PAGE under reducing conditions on a 15% gel. Bands were visualised by autoradiography.

To isolate various charge isomers, rhu^{125}I-IL-1β was subjected to chromatofocusing using a pH gradient 7–4. The material was separated into two peaks (fig.5a). The peak A appeared to be a sharp one, the peak B was rather broad and trailing was observed in this peak. Materials eluted under these peaks were pooled and were subjected to IEF. It was possible to separate proteins of different charges by chromatofocusing. Thus peak A contained mostly two proteins appearing in the pH range of 6.2–5.8 as determined by IEF on polyacrylamide gels (fig.6a). Traces of the pro-

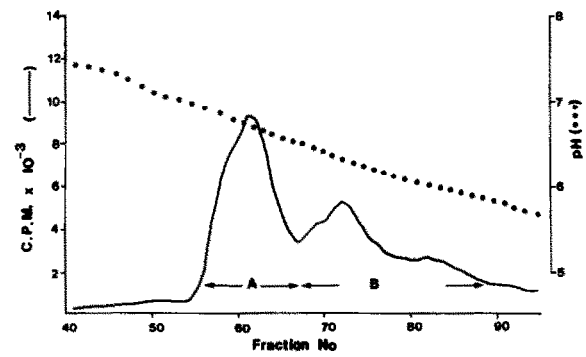

Fig.5. Chromatofocusing of ^{125}I-rhuIL-1β on a column of Polybuffer Exchanger 94 (10 × 1.5 cm) in the range pH 7–4. Elution conditions: start buffer 0.025 M imidazole-HCl, pH 7.4; elution buffer Polybuffer 74 (Pharmacia, Uppsala, Sweden; diluted 1:8) pH 4.0. One ml fractions were collected.

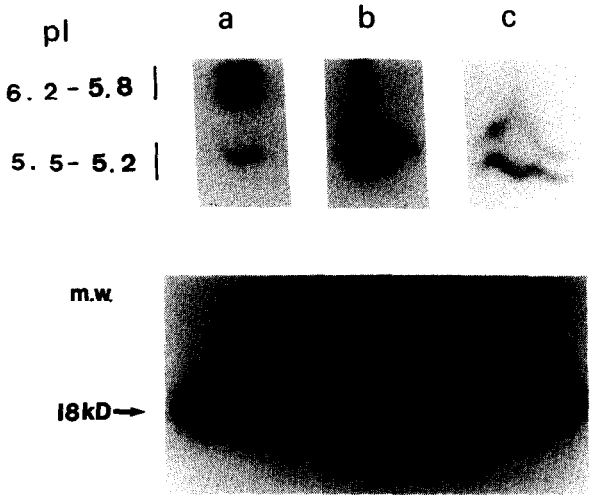

Fig.6. Upper part: (a) fractions pooled under peak A (fig.5) were concentrated and analysed by isoelectric focusing (pH range 3.5–9.5, gel thickness 0.5 mm). Bands were visualised by autoradiography. (b) Early part of the peak B (fig.5; containing fractions 69–75) was pooled, concentrated and analysed by isoelectric focusing (pH range 3.5–9.5, gel thickness 0.5 mm). (c) Later part of the peak B (fig.5; containing fractions 76–90) was pooled, concentrated and analysed by isoelectric focusing (pH range 3.5–9.5, gel thickness 0.5 mm). Lower part: fractions analysed by isoelectric focusing as described in the upper part were also analysed by SDS-PAGE under reducing conditions on a 15% gel. Bands were visualised by autoradiography.

tein of pI 5.5 also appeared in this peak. The early part of the peak B was pooled and analysed by IEF (fig.6b). It contained mostly the protein of pI 5.5 while the trailing limb of this peak consisted mainly of the protein band having pI 5.2 (fig.6c). When pH of each of the fraction obtained by chromatofocusing was monitored, it was observed that the peak A was eluted in the pH range of 7.0–6.5. The peak B had appeared in the pH range of 6.5–5.8. Values of pI obtained by chromatofocusing were higher than those estimated by IEF on

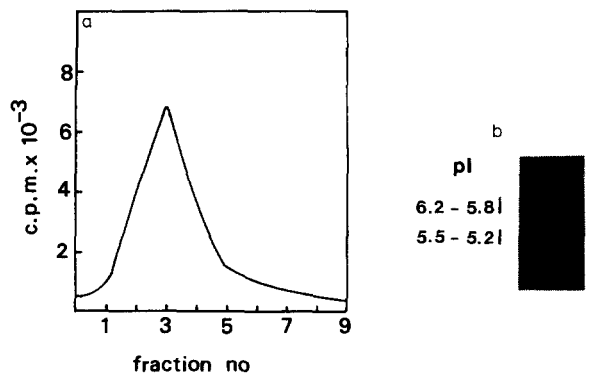

Fig.7. (a) Elution of ^{125}I-rhuIL-1β from an affinity column containing antibodies to rhuIL-1β coupled to Sepharose 4B. Bound materials were eluted by desorption with 0.1 M glycine-HCl (pH 2.8). One ml fractions were collected over solid Tris (0.5 g/fraction). (b) Analysis of the desorbed material from the affinity column containing antibodies to rhuIL-1β by isoelectric focusing (ampholyte pH 3.5–9.5, gel thickness 0.5 mm).

polyacrylamide gels. Contents of the peak A as well as of both the early and the later part of peak B were subjected to SDS-PAGE. All these peaks appeared to contain the protein band appearing at 18 kDa (fig.6, lower part).

To provide evidence that these proteins were indeed IL-1β, ^{125}I-rhuIL-1 was subjected to affinity chromatography over Sepharose 4B-anti-recombinant human IL-1β. The bound material was desorbed by applying 0.1 M glycine-HCl (fig.7a). The desorbed material was examined by IEF using a pH gradient of 3.5–9.5 and visualised by autoradiography. As shown in fig.7b, the material which was eluted from the anti-recombinant human IL-1β-Sepharose 4B column contained proteins focused in the pI range of 6.2–5.8 and 5.5–5.2.

4. DISCUSSION

IL-1β used in this investigation was obtained by using recombinant DNA techniques and expressed in *E. coli* [21]. It had been radioiodinated with the Bolton Hunter reagent [22] and was shown to be immunoreactive against specific IL-1β antisera.

Upon analysis by SDS-PAGE, rhu^{125}I-IL-1β displayed a single band at 18 kDa. When examined by various techniques such as high voltage IEF on thinlayer polyacrylamide gels and chromatofocusing, rhu^{125}I-IL-1β was found to consist of multiple charge isomers. The resolution of rhu^{125}I-IL-1β into different charge isomers depended on the thickness of the separating gel. When examined on a gel of 1 mm thickness, it was resolved into two spots having pI values 6.2–5.8 and 5.5–5.2. When it was tested on an ultrathin polyacrylamide gel having thickness of 0.5 mm, each of the two spots was further resolved into multiple bands. By using an ultrathin polyacrylamide gel of 0.5 mm thickness, it was possible to apply higher field strength as compared to that which could be applied to a 1 mm thick gel. This resulted in an increased resolution as broad spots were resolved into distinct bands. Similar increased resolution of bands applying higher field strengths has been reported in the literature [25]. Although gels thinner than 0.5 mm have been used to separate proteins [26], such gels are more susceptible to distortions and not very practical for handling. Therefore, in this investigation 0.5 mm thick polyacrylamide gels were mostly used. Experiments were performed in ampholines of various pH ranges such as 3.5–9.5 and 5–8. Most of the rhu^{125}I-IL-1β proteins were focussed in the pI range 6.2–5.2. Separation among charged species was improved by the use of an ampholine of pH range 5–8.

These charged species were in fact charged isomers of rhuIL-1β of molecular mass 18 kDa. Thus, when rhuIL-1β was separated by IEF in the first dimension and by SDS-PAGE in the second dimension, only materials producing a sharp band at molecular mass

18 kDa were observed. These charged species were focused into two main pH ranges (pI 6.2–5.8; 5.5–5.2). When analysed by SDS-PAGE in the second dimension two distinct bands at molecular weights 18 kDa were observed. The charge heterogeneity of rhuIL-1β was confirmed by chromatofocusing. By this technique it was possible to separate rhuIL-1β into two major fractions, each containing multiple charge isomers. When subjected to SDS-PAGE, all these components produced identical bands at molecular weight 18 kDa.

Wingfield et al. [18] have prepared recombinant human IL-1β. These authors have also indicated the presence of two charged fractions, separable by chromatofocusing. However, they did not present any data in their report on these charged isomers.

To determine the relation between these charged isomers, peptide mapping studies were performed by endopeptidase isolated from *Staphylococcus aureus* V8. Under the given condition for hydrolysis, the enzyme could cleave on the C-terminal side of glutamyl and aspartyl residues. Identical peptide maps were obtained from these charged species when the enzyme-treated material was separated on the polyacrylamide gel by SDS-PAGE.

That these charged species of rhuIL-1β were indeed isomers was confirmed by isolating these isomers by affinity chromatography on a column of anti-rhuIL-1β. The antiserum, supplied by the manufacturer, had been reported not to be cross reacting with various cytokines such as IL-1α, tumour necrosis factor (α, β) and interferon (α, γ).

Values of pI of rhuIL-1β obtained by high resolution IEF appeared to be slightly more acidic than those obtained from chromatofocusing. Similar anomalies in pI values of IL-1 obtained by IEF and chromatofocusing have been made by Auron et al. [27]. The slightly lower pI values obtained by IEF might be due to the pH gradient shift caused by atmospheric CO_2 during electrophoresis, resulting in a lowering of pH in the gradient [26]. Such problems associated with the gradient drift due to atmospheric CO_2 have been reported by other investigators [28]. Isoelectric points obtained by chromatofocusing may probably reflect more the natural pI values of rhuIL-1β. Thus, rhuIL-1β appears to have two pI maxima: one at pI 6.75 and the other at pI 6.35.

Although pI values obtained by chromatofocusing may represent the more natural state of the compound, the resolving power obtained by high voltage ultrathin IEF is significantly higher. Thus, the peak obtained by chromatofocusing at pI 6.75 was resolved into two distinct charged bands by ultrathin IEF. Similarly the broad peak between pI 6.5–5.8 was resolved into multiple charged bands by IEF. Considering all these factors, it may be advisable to use both chromatofocusing and high resolution IEF to determine pI values as well as the number of charged species present in a product such as rhuIL-1β. Recently several studies have been carried out with radiolabelled rhuIL-1β to identify and characterize the IL-1 receptors on various cell types [10–15]. Some of these studies have utilized rhuIL-1β containing a mixture of charged species such as the one reported in this investigation. Applying chromatofocusing it has been possible to separate the mixture of charged species of rhuIL-1 into more homogeneous fractions. Therefore, it may be advisable to perform future receptor binding studies with more charge homogeneous rhuIL-1β obtained by techniques such as chromatofocusing.

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