AMINO ACID SEQUENCE ANALYSIS OF HUMAN INTERLEUKIN 1 (IL-1)

Evidence for Biochemically Distinct Forms of IL-1

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The term interleukin 1 (IL-1) encompasses macrophage-derived polypeptide factors in man, mouse, and other animals, which stimulate murine thymocyte proliferation (1–3), and in addition activate various target cells involved in chronic inflammation (4–10). The IL-1 produced by the murine macrophage line, P388D1, has a low isoelectric point (pI) and exhibits micro-charge heterogeneity in the range of 4.9–5.2 (11). P388D1-derived IL-1 has been purified (11), and a complementary DNA (cDNA)[1] coding for the precursor form of this molecule has been recently cloned (12). The nucleic acid sequence of this cDNA was validated by sequencing peptides of P388D1-derived IL-1 protein (12).

The study of human IL-1 has lagged behind that of murine IL-1 largely because of the unavailability of a tumor line capable of making large amounts of IL-1. In addition, human IL-1 appears more complex than murine IL-1, in that there are several major charged species (4, 5, 13). Like murine IL-1, two of these species have low pI, 5.1 and 5.3. Porcine IL-1, also known as catabolin, also has a pI in this range (14). In contrast to the mouse and pig, there is, in man, a third type, with a pI of 6.0, and a fourth, dominant species, with a pI of 6.8 (4, 5, 13). The pI 6.8 species has been purified (15), while several of the low-pI human types have been purified by others (16). The biochemical relationships among these various charged species is not known, though their similar bioactivities on fibroblasts (4, 5) and other target cells (17) have suggested to some that they might share similar core structures.

A cDNA putatively coding for a human IL-1 molecule has recently been reported (18). The translation product derived from this cDNA was (*a*) active on D10.G4.1 T cells, a cell line responsive to partially purified preparations of murine IL-1 (19), and (*b*) precipitated by a polyspecific antiserum containing antibodies against several charged species of human IL-1 (20), and several other macrophage-derived products (18). However, the translation product was not shown to be active in the murine thymocyte proliferation assay, the standard assay for all IL-1 molecules. Furthermore, no amino acid sequence analysis of

[1] *Abbreviations used in this paper:* cDNA, complementary DNA; HPLC, high-performance liquid chromatography; IL-1, interleukin 1; PITC, phenyl isothiocyanate; PTH, phenylthiohydantoin; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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purified IL-1 was presented to confirm that the cDNA coded for one or several of the human IL-1 species described previously. Interestingly, comparison of the deduced amino acid sequences of the murine and human cDNA reveals little homology. This observation raises the possibility, despite the similar spectrum of bioactivities exhibited by murine and human IL-1, that there are several biochemically distinct types of IL-1 or, alternatively, that the human cDNA clone represents a novel class of mediator, discovered by molecular genetic techniques, which is largely distinct from the class of mediators known as IL-1.

In this report, the primary amino acid sequences of the amino terminus and three CNBr cleavage fragments of the pI 6.8 species of normal human IL-1 are presented. The data demonstrate conclusively that the recently reported human cDNA codes for the pI 6.8 species of human IL-1. In addition, the identification of the amino terminus of human IL-1 reveals that processing of the IL-1 precursor protein occurs at the amino terminal portion of the molecule, not at the carboxyl terminus, as was originally suspected (18). Most importantly, the data show that, despite the strong functional similarities of all IL-1 molecules, there are biochemically distinct forms of IL-1.

Materials and Methods

Culture Supernatant Preparation. Normal human mononuclear cells were cultured under serum-free conditions, as previously described (15), with the exception that the cells were stimulated with both phytohemagglutinin (5 μg/ml) and lipopolysaccharide (1 μg/ml) for 96 h. The crude culture supernatants were concentrated by ultrafiltration as previously described (15).

Purification of IL-1. The pI 6.8 species of normal human IL-1 was purified from crude concentrated culture supernatants using a modification of a technique previously described (15). The crude concentrated supernatant was dialyzed against buffer A (20 mM Tris acetate, pH 8.3), and loaded on a high-performance liquid chromatography (HPLC) DEAE column (Bio-Gel TSK-DEAE-5-PW [21.5 × 150 mm]; Bio-Rad, Richmond, CA) that was equilibrated in the same buffer. The flow rate was 4 ml/min. After 40 min, a linear descending pH gradient of from 0 to 50% buffer B (20 mM Tris acetate, pH 6.8) was initiated at a rate of 0.5% per minute. The IL-1 bioactivity typically eluted as a sharp peak between 138 and 142 min from the time of injection. The active fractions were pooled, concentrated by ultrafiltration using a YM 10 membrane (Amicon, Lexington, MA) to a volume of 1 ml, and loaded on a Vydac C4 reverse-phase HPLC column (0.4 × 5 cm; Separations Group, Hesperia, CA) equilibrated in 0.1% trifluoroacetic acid (TFA). The flow rate was 1 ml/min. A linear gradient of 0.1% TFA in acetonitrile (0–50%) was used to elute the column. The absorbance of the column effluent was monitored at 210 nm. Fractions were collected by a Pharmacia Frac-100 fraction collector equipped with a programmable peak-collection device.

CNBr Cleavage of IL-1. IL-1 (0.2 nmol), purified by reverse-phase HPLC, was treated with a 3,000-fold molar excess of CNBr for 72 h at room temperature. Preliminary experiments showed that efficient cleavage of test proteins could be obtained in the presence of 0.1% TFA and 40% acetonitrile. Nevertheless, the amount of CNBr required for complete cleavage of IL-1 was 100–1000-fold in excess of published protocols (21). Further experiments with test proteins suggested that the concentration of CNBr required was indirectly proportional to the concentration of the protein reactant, and that conventional protocols, which typically use milligram amounts of protein, significantly underestimate the concentration of CNBr necessary to cleave low (microgram) amounts of protein. Peptides of IL-1 were purified by passage of the CNBr reaction mixture over a Vydac C4 reverse-phase column using the buffer and gradient conditions mentioned above. Peptides were collected as individual peaks using the peak collection feature of the Frac-100

fraction collector. The absorbance of the effluent was monitored at 210 nm and 280 nm simultaneously by a Hewlett-Packard 1040A scanning spectrophotometric detector.

Amino Acid Analysis. Amino acid analysis was used to determine the concentration of purified IL-1 protein. Triplicate samples (25–50 pmoles) were taken to dryness in borosilicate tubes in a Pico-tag work station (Waters Associates, Bedford, MA). The samples were redried twice with 50-μl aliquots of water, and then hydrolyzed under nitrogen with gaseous HCl for 20 h at 105°C. The hydrolysate was derivatized with phenylisothiocyanate (PITC) as previously described (22), to form the phenylthiocarbamyl–amino acid derivatives. These were separated by reverse-phase HPLC as previously described (22), using a C18 microbondapak (Waters Associates) reverse-phase column. The peaks were identified and quantitated by chromatography and integration of standard amino acid mixtures (Pierce, Rockford, IL), which were derivatized with PITC in identical fashion. SEM for each of the 17 assayable amino acids was always <10% of the mean, and usually <5% of the mean. Values obtained for tyrosine, isoleucine, and leucine were particularly reproducible, and were used for mass determinations using values of 4, 5, and 15 moles, respectively, per mole IL-1 (18,000 mol wt).

Sequence Analysis. IL-1 and IL-1 peptides were applied in several applications to polybrene-coated filters and sequenced using an automated gas-phase sequencer employing Edman chemistry (model 470A; Applied Biosystems, Foster City, CA). The ATZ amino acids were treated in the conversion flask with HCl and methanol to form the methyl esters of the phenylthiohydantoin (PTH) derivatives of glutamic acid and aspartic acid. The PTH amino acids were identified by reverse-phase HPLC, and quantitated by integration as previously described (23).

Homology Analysis. The search for homologous regions between the murine and putative human IL-1 cDNA and their respective deduced amino acid sequences was aided by computer analysis on a Digital VAX-11/780 computer, using the Needleman-Wunsch homology search algorithm (24) (Intelligenetics, Palo Alto, CA).

Biological Assay of IL-1. IL-1 was assayed in the murine thymocyte proliferation assay as previously described (15). Samples were assayed in parallel with aliquots of a partially purified preparations of IL-1, which consistently gave 550 half-maximal U/ml. Comparison of the sample and standard preparations at a level of 50% of maximal stimulation was performed as previously described (15).

Results

The human IL-1 used for sequence analysis was purified by a modification of a protocol described previously (15). The modified protocol also used anion-exchange HPLC, but employed a resin-based, pH-stable column (see Materials and Methods), which made it possible to load at higher pH (8.3) and achieve tight binding of the pI 6.8 species of human IL-1. The column was then eluted with a descending pH gradient (pH 8.3–6.8), which provided excellent resolution of IL-1 from nearby contaminants (data not shown). The active fractions obtained from the ion-exchange HPLC column were further purified by reverse-phase HPLC. A sharp, dominant peak corresponding with IL-1 activity was observed (Fig. 1). This material was similar to that isolated by the previous protocol (15), in that it gave a single band having a pI of 6.8 by analytical isoelectrofocusing (data not shown). Furthermore, when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in 15% homogeneous gels under reducing conditions, a single silver-staining band of mol wt 18,000 was observed (data not shown). The material that was previously isolated had a molecular weight of 15,000 using 10–30% gradient SDS-PAGE. IL-1 purified by the previous protocol was found to have a specific bioactivity of 6×10^6 half-maximal U/mg in the murine thymocyte proliferation assay, using absorbance at 210 nm

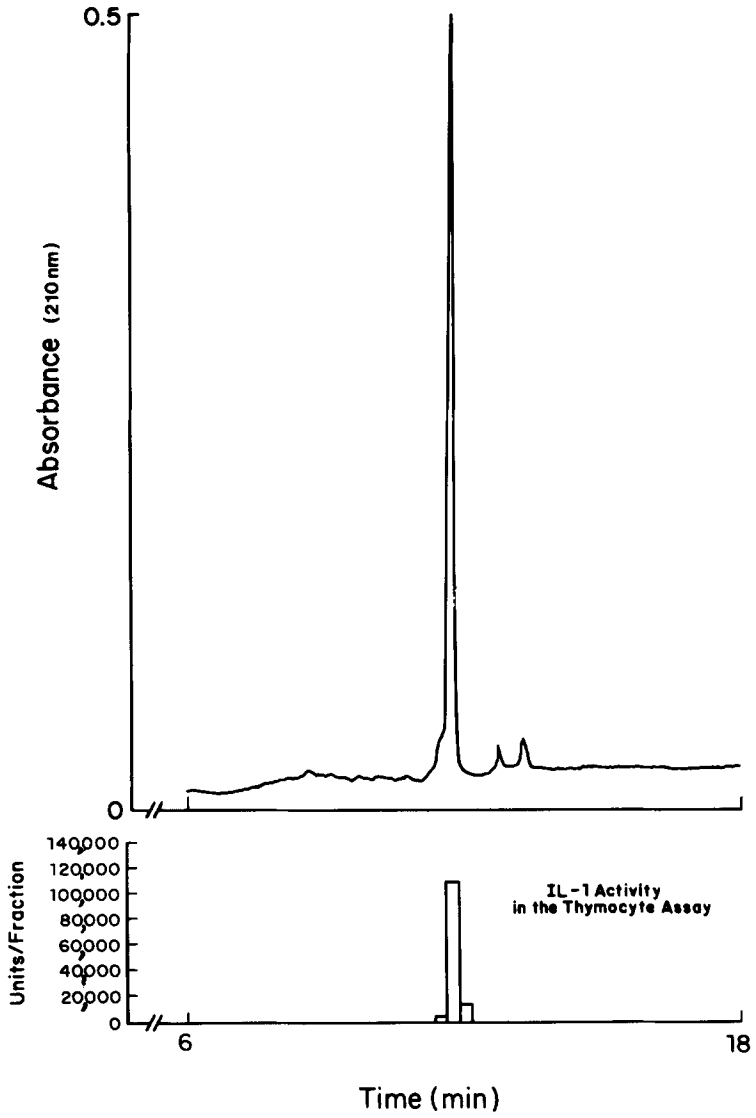

FIGURE 1. Reverse-phase HPLC of human IL-1. Serum-free mononuclear cell supernatants were concentrated and partially purified by anion-exchange HPLC, as described in Materials and Methods. The active fractions were pooled, concentrated, and loaded onto a Vydac C4 reverse-phase HPLC column equilibrated in 0.1% TFA in water. The column was eluted with a linear gradient of 0.1% TFA in acetonitrile (0–50%) at a rate of 2% per minute. The column effluent was monitored at 210 nm (*top*). Fractions were collected using the peak collection feature of a programmable fraction collector, and assayed for IL-1 activity in the murine thymocyte proliferation assay (*bottom*).

as a measure of protein concentration. Material purified by the current protocol gave 1.7×10^7 U/mg, using amino acid analysis as a more accurate measure of protein concentration.

Amino-terminal sequence analysis was performed on 150 pmoles of purified IL-1, as described in Materials and Methods. A single amino terminus was

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                5                  10
ALA-PRO-VAL-ARG-SER-LEU-ASN- X -THR-LEU-

               15                  20
ARG-ASP-SER-GLN-GLN-LYS-SER-LEU-VAL-MET-

               25                  30
SER-GLY-PRO-TYR-GLU-LEU-LYS-ALA-LEU- X -

               35
X -GLN- X - X -ASP
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FIGURE 2. Amino-terminal sequence analysis of human IL-1. 150 pmoles of reverse-phase–purified IL-1 were sequenced by an automated gas-phase sequencer using Edman chemistry. The PTH-derivatives of each residue were identified by reverse-phase HPLC as detailed in Materials and Methods. X designates residues that could not be clearly identified.

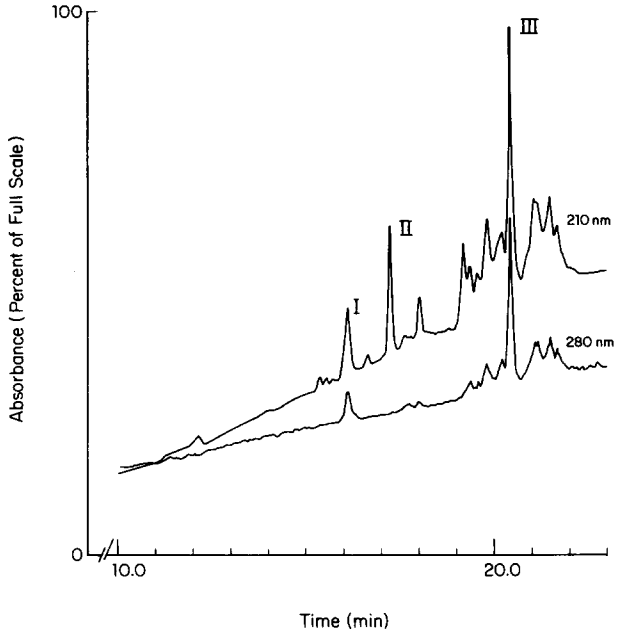

FIGURE 3. Reverse-phase HPLC of CNBr cleavage fragments of human IL-1. Human IL-1 was treated with an excess of CNBr (see Materials and Methods), and the resulting peptides were separated by reverse-phase HPLC and collected as described in the legend to Fig. 1. The column effluent was monitored at 210 nm (0.05 absorbance units, full scale) and 280 nm (0.005 absorbance units, full scale) simultaneously. The peaks identified as I, II, and III were subsequently sequenced.

observed (Fig. 2). Analysis of another preparation of purified material revealed the same, single amino terminus. The yield, as determined by quantitation of the second PTH–amino acid derivative, was 65%. This value was similar to that obtained (25) when highly purified sperm whale myoglobin was similarly analyzed, and suggests that our preparation was not contaminated by proteins having blocked amino termini. 30 of the first 35 *N*-terminal amino acids were unambiguously identified in the two analyses. The seventh residue in the sequence, asparagine, was a potential site for *N*-glycosylation (18), but chromatographed as the free PTH derivative during reverse-phase HPLC.

To obtain internal amino acid sequence, 200 pmoles of purified IL-1 were cleaved with CNBr, as described in Materials and Methods. The resulting peptides were purified and isolated by reverse-phase HPLC (Fig. 3). As expected,

CNBr Peptide I

SER-GLY-PRO-TYR-GLU-LEU-LYS-ALA-LEU- X -
LEU-GLN-GLY- X -ASP- X

CNBr Peptide II

PRO-VAL-PHE-LEU-GLY-GLY-THR-LYS-GLY-GLY-
GLN-ASP-ILE-THR-ASP-PHE-THR- X

CNBr Peptide III

GLU-LYS-ARG-PHE-VAL-PHE-ASN-LYS-ILE-GLU-ILE-
ASN-ASN-LYS-LEU-GLU-PHE-GLU- X -ALA- X

FIGURE 4. Amino acid sequences of three CNBr peptides of human IL-1. X designates residues that could not be clearly identified.

the aromatic content of the peptides was variable, as indicated by their differential absorbance at 280 nm. The indicated peaks (I, II, and III in Fig. 3) were peak-collected and sequenced, as above. The results are shown in Fig. 4. 13 of 16, 17 of 18, and 19 of 21 amino acid residues were identified in peptides I, II, and III, respectively.

The amino acid sequences presented in Figs. 2 and 4 correspond exactly with the amino acid sequence deduced from a cloned cDNA by Auron, et al. (18). Those portions of the deduced sequence that have been confirmed by protein sequence data are capitalized in Fig. 5. Also shown is the alignment of the human translation product with the translation product deduced from the cDNA for murine IL-1 (12). The alignment includes gaps suggested by computer analysis using the Needleman-Wunsch homology algorithm (see Materials and Methods). Only 30% (80 of 270) homology is observed overall. Comparison of the secreted portions of the translation products shows only 29% (45 of 155) homology. In contrast, comparison of the *N*-terminal sequence of human IL-1 with the recently reported *N*-terminal sequence of interferon-inducing factor (26) reveals 63% (24 of 38) homology. Comparison of the nucleotide sequences of the murine and human IL-1 cDNA using the same homology algorithm revealed 43% homology (data not shown).

Discussion

We have isolated the major charged species of normal human IL-1, using an improved version of a previously described (15) purification protocol, made possible by the recent introduction of resin-based, pH-stable, anion-exchange HPLC columns. The ability to load at a pH (8.3) substantially above the pI of IL-1 (6.8) made it possible to achieve tight binding and thus eliminated the need to perform repetitive isocratic runs (15). The revised protocol also succeeded in using reverse-phase HPLC for the final step of the purification. Preparation of samples for sequence analysis by reverse-phase HPLC has been advocated (27) because of the high resolution provided by this technique and the purity and volatility of reverse-phase buffers. Our ability to use this technique for the final isolation of fully active IL-1 is mainly attributed to the introduction of columns specifically designed for labile protein separations (28). In addition, some of the difficulty originally encountered in using these columns as an initial purification step for IL-1 may have been due to the activation of acid hydrolases when crude

supernatants were exposed to low-pH, reverse-phase buffers (our unpublished observations).

Progress has recently been made in defining the nucleic acid sequences of messenger RNA coding for IL-1. Lomedico, et al. (12) have published a cloned cDNA coding for P388D1-derived murine IL-1. This nucleotide sequence was validated by the sequencing of two peptides derived by CNBr cleavage of purified P388D1 IL-1. Nevertheless, the protein encoded by this cDNA had very little homology with the amino acid sequence deduced from a putative human IL-1 cDNA recently reported by Auron, et al. (18). This latter cDNA encoded a protein which, like IL-1, is active on the D10.G4.1 T cell line (19). However, this material was not shown to be active in the murine thymocyte assay, the standard assay for IL-1, nor was the antiserum used to immunoprecipitate the translation products of this cDNA monospecific for human IL-1. It was therefore not possible to interpret the significance of the differences beween the murine and human cDNA because the relationship of the human cDNA to human IL-1 protein was not established.

The amino acid sequence data reported here demonstrates conclusively that the cDNA reported by Auron, et al. (18) does indeed code for human IL-1, and more specifically, for that species of human IL-1 having a pI of 6.8. The amino-terminal sequence analysis shows that the *N*-terminus of the secreted form of IL-1 begins at the residue Ala (117) encoded by the open reading frame. The calculated molecular weight of the remaining polypeptide chain from Ala (117) to the end of the open reading frame (Ser [269]) is 17,380, in good agreement with the molecular weight obtained for our purified material on homogeneous SDS-PAGE. This good correlation between calculated and observed molecular weight is also consistent with the observation that no evidence for *N*-glycosylation was found at Asn (123) during the amino-terminal sequence analysis. Previous studies (16, 29) of murine and human IL-1 have suggested that IL-1 is not a glycoprotein. The peptides I, II, and III correspond exactly with residues 137–151, 247–263, and 212–231 in the deduced sequence. One of these (peptide I) overlaps the *N*-terminal sequence, and corroborates that analysis. Furthermore, all of the peptide sequences follow methionines, as expected, since CNBr cleaves at methionine residues (21). The sequence of one of the peptides, II, is particularly important because it lies within five amino acids of the termination of the open reading frame. This peptide shows that little or no processing of the human IL-1 translation product occurs at the *C*-terminal region of the molecule. This finding, in conjunction with the identification of the *N*-terminus as Ala (117) demonstrates that IL-1 is processed mainly or, perhaps, exclusively, at the amino-terminal end and not at the carboxy-terminal end, as was initially suspected (18). Interestingly, hydrophathicity plots (30) of the portion of the IL-1 molecule that lies 5′ of the secreted *N*-terminus show that IL-1 does not have a hydrophobic domain characteristic of a secretory leader or membrane anchor sequence. This species of IL-1 therefore is not likely to be responsible for the membrane-bound IL-1 activity that has been recently described (31).

These findings now make it possible to compare the translation products of the cDNA reported for human and murine IL-1, using the alignment provided by the amino-terminal analysis reported here. The specific bioactivities of the

secreted forms of both human and murine IL-1 have been independently reported by ourselves (15) and Lomedico et al. (12) to be $1-2 \times 10^7$ half-maximal U/mg in the murine thymocyte proliferation assay. Nevertheless, there is only 29% homology at the amino acid level between these secreted molecules, and only 30% between the complete translation products. This striking disparity between functional and structural relationships raised the possibility that the secondary structures of these two proteins may bring similar or identical amino acids into a similar conformation on the surface of the molecule. Preliminary analysis of this type, however, using the Chou-Fasman algorithm (32) suggests that this is not the case (J. Boger and J. Schmidt, unpublished observations). At the nucleic acid level, 43% homology was observed, suggesting that these two genes diverged from a single parental gene and do not represent two genes of completely different origin converging independently on a single biological function. Such, for example, appears to be the case for egg white lysozyme and T4 lysozyme (32).

Recent findings (26) suggest that the pI 6.8 species of human IL-1 is a member of a closely related gene family. Van Damme et al. have reported the purification and amino-terminal sequence analysis of a human interferon-inducing factor. This material has the same *N*-terminal amino acid as human IL-1 (pI 6.8), and comparison of the first 22 amino acids revealed 63% homology (Fig. 5). This material is active in the murine thymocyte proliferation assay, but appears to have a specific bioactivity that is ~100-fold less than human or murine IL-1 (26).

The differences observed between the pI 6.8 species of human IL-1 and murine IL-1, which has a pI of 4.9–5.2, raise questions concerning the biochemical composition of the low-pI species of human IL-1. The similar spectrum of biological activities shared by all of the charged species of human IL-1 has suggested that they might share similar core structures. However, the striking structural differences between the mouse and human IL-1, despite similar bioactivities, demonstrate that the different human IL-1 species need not have closely related structures. One possibility, suggested by the similar isoelectric points of mouse, pig, and certain of the human IL-1 species is that all of these will constitute a family of related genes, largely distinct at the amino acid level from the gene family represented by the pI-6.8 species of human IL-1, and interferon-inducing factor. Sequence analysis of these other human IL-1 species and cloning of their respective cDNA will clarify these issues.

Summary

The pI-6.8 species of normal human interleukin 1 (IL-1) has been isolated by ion-exchange and reverse-phase high-performance liquid chromatography. The isolated material had a molecular weight of 18,000, and had a specific bioactivity of 1.7×10^7 half-maximal U/mg in the murine thymocyte proliferation assay, values similar to those obtained for murine P388D1-derived IL-1 (12), and human IL-1 isolated by a previously published purification protocol (15). Amino-terminal sequence analysis revealed a single *N*-terminal, and resulted in the identification of 30 of the first 35 amino acid residues. Sequence of three CNBr cleavage fragments of purified IL-1 resulted in the identification of an additional

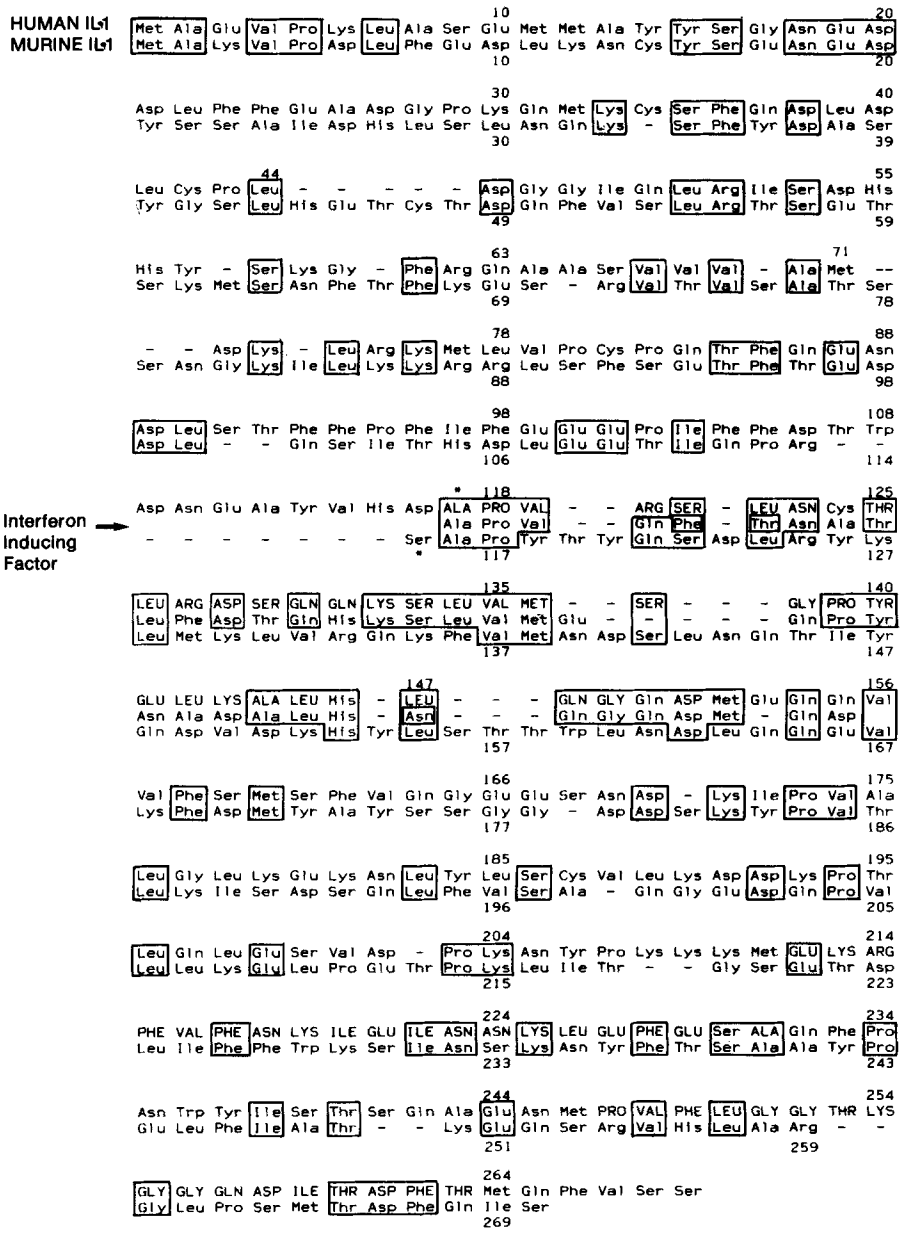

FIGURE 5. Comparison of amino acid sequences deduced from cloned cDNA for human (18) and murine (12) IL-1. The numbers above and below the amino acid sequences are the residue numbers for the complete human and murine translation products, respectively. The *N*-termini (*) of murine and human IL-1, as determined by sequence analysis of the purified, secreted proteins, are indicated. Those amino acids in human IL-1 that were confirmed in this report by sequence analysis of purified protein or peptide are capitalized. Also included is the *N*-terminal sequence of 22,000 mol wt interferon-inducing factor, as described in Ref. 26. Alignments were obtained using the Needleman-Wunsch homology algorithm (see Materials and Methods). Homologous amino acids are enclosed in boxes.

38 residues. All of the sequences agree exactly with those deduced from complementary DNA (cDNA) by Auron, et al. (18), demonstrating that this cloned cDNA, though considerably different from the cDNA reported for murine IL-1 (12), nevertheless codes for the pI-6.8 species of human IL-1. The evidence also shows that the precursor protein for human IL-1 is largely processed at the *N*-terminal end. Little or no processing occurs at the carboxy-terminal end. Sequence homology with interferon-inducing factor (26) suggests that the pI-6.8 species of human IL-1 is a member of a gene family. Although equally potent in the murine thymocyte proliferation assay, murine IL-1 and the pI-6.8 species of human IL-1 are structurally distinct. Further study will answer the interesting question as to the relationship of the other charged species of human IL-1 to these distinct IL-1 classes.

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Note added in proof: March et al. (*Nature* [*Lond.*]. 315:641) have reported the sequence of a cDNA coding for a second human IL-1, which is 62% identical to murine IL-1 at the amino acid level.

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