# PURIFICATION TO HOMOGENEITY AND AMINO ACID SEQUENCE ANALYSIS OF TWO ANIONIC SPECIES OF HUMAN INTERLEUKIN 1

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IL-1 is the term used to describe a group of factors, primarily macrophage-derived, that exhibit a multitude of specific in vitro and in vivo effects. These effects include stimulation of murine thymocyte proliferation in vitro, IL-2 production, fever, fibroblast proliferation, enzyme and prostaglandin production by chondrocytes and synoviocytes, acute-phase protein production by hepatocytes, bone resorption, fluctuations in circulating levels of metal ions, and chemotactic and chemokinetic effects on leukocytes (reviewed in reference 1). It has been previously reported that IL-1 activity is primarily associated with several charged proteins having isoelectric points (pI) of 6.8, 5.4, and 5.2 (2–5). Partially purified preparations of the neutral (pI 6.8) and anionic forms (pI 5.4/5.2) of IL-1 have been compared in a number of biological assay systems, with the result that all appeared to exhibit an identical spectrum of activities (6). This led to the conclusion that the various charged species of IL-1 must be closely related, perhaps differently processed, products of the same gene (6).

Recently, several groups have cloned IL-1 cDNAs of murine (7) and human origin (8, 9). However, when the amino acid sequence deduced from the murine IL-1 cDNA (7) was compared with that deduced from the first reported cDNA for human IL-1 (8), only ~30% homology was observed (9, 10). Since murine IL-1 is an anionic molecule (11) and the predominant human form is neutral, it seemed possible that a similar lack of homology might also exist between the anionic and neutral human IL-1 species. The subsequent report of an additional cDNA for human IL-1 (IL-1-$\alpha$, reference 9) supported this hypothesis. The deduced amino acid sequence for the IL-1-$\alpha$ cDNA displayed only 30% homology with the amino acid sequence deduced from the first reported human cDNA (since designated IL-1-$\beta$), but 60% homology (9) with the murine sequence, suggesting that it represented the human equivalent of murine IL-1, presumably an anionic form.

These findings indicate that there are at least two human genes coding for molecules possessing IL-1 activity and that the IL-1-$\alpha$ cDNA is likely to encode one or more of the anionic species of human IL-1. However, no direct evidence relating the IL-1-$\alpha$ cDNA, which was cloned using hybrid selection techniques

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and bioassay, to the native charged species of IL-1 is available. The pI 6.8 molecule has been purified to homogeneity by us and others (10, 12–14), and *N*-terminal and internal amino acid sequence analysis proved that the IL-1-$\beta$ cDNA did in fact encode for the precursor of IL-1/6.8 (10). However, the anionic forms have not previously been purified in any amount suitable for characterization and thus the relationship between these proteins and the cloned cDNAs remains unclear.

We here report the purification to homogeneity of two anionic forms of human IL-1 having pIs of 5.2 (IL-1/5.2) and 5.4 (IL-1/5.4). Both IL-1/5.2 and IL-1/5.4 are indistinguishable from IL-1/6.8 in terms of their molecular weights, as determined by SDS PAGE and specific bioactivities in the mouse thymocyte proliferation assay. Nevertheless, four antisera directed against IL-1/6.8 do not crossreact with either anionic protein. Moreover, reverse-phase HPLC of cyanogen bromide (CNBr)[1] peptides of IL-1/5.4 reveal it to be structurally distinct from IL-1/6.8. Amino acid sequence analyses of intact IL-1/5.2 and CNBr peptides of IL-1/5.4 show complete homology with the amino acid sequence deduced from the IL-1-$\alpha$ cDNA.

## Materials and Methods

*Preparation of IL-1-containing Supernatants.* IL-1-containing supernatants were prepared as previously described (14). Briefly, human PBMC were separated by density gradient centrifugation (LSM, Bionetics Laboratory Products, Charleston, SC). Cells were cultured in serum-free RPMI (Gibco Laboratories, Grand Island, NY) at a density of $3 \times 10^9/150$ cm$^2$ in 400 ml of medium for 4 d in the presence of PHA (5 $\mu$g/ml; Sigma Chemical Co., St. Louis, MO) and LPS (1 $\mu$g/ml; *Escherichia coli* 055:B5, Sigma Chemical Co.). Supernatants were concentrated (300 ×) using a Pellicon cassette ultrafiltration apparatus (mol wt >10,000), as previously described (14), then dialyzed against the running buffer for the first ion exchange column.

*Purification of Two Anionic Species of IL-1.* This purification takes advantage of the fact that the anionic forms remain tightly bound to the HPLC-DEAE column (Bio-Gel TSK-DEAE-5-PW [21.5 × 150 mm]; Bio-Rad Laboratories, Richmond, CA) after the elution of IL-1/6.8, as previously described (10). Briefly, 4 ml of dialyzed, concentrated supernatant was loaded onto the DEAE-HPLC column equilibrated in 20 mM Tris/Acetate (pH 8.3). IL-1/6.8 was then eluted from the column with a descending pH gradient (pH 8.3–6.8) at a flow rate of 4 ml/min at room temperature (10). The column was washed extensively with 20 mM Tris/Acetate (pH 6.8) and the anionic forms were then batch-eluted with the same buffer containing 0.3 M sodium acetate. This resulted in the elution of all the remaining IL-1 activity. The column was then washed with 1 M sodium acetate to remove the remaining proteins.

Five of the 0.3 M acetate elutions were pooled, concentrated in a stirred cell using a YM10 ultrafiltration membrane (Amicon Corp., Danvers, MA), and subsequently dialyzed against 20 mM Tris/Acetate (pH 8.3). This material was then rechromatographed on the HPLC-DEAE column. The conditions employed were the following: (*a*) the sample was applied in 20 mM Tris/Acetate (pH 8.3) to ensure complete binding and washed for 30 min at 4 ml/min with this buffer; (*b*) the column was then washed for an additional 30 min with 20 mM Tris/Acetate (pH 6.8); (*c*) the anionic forms of IL-1 were then eluted with a gradient of 0–300 mM sodium acetate in 20 mM Tris (pH 6.8) buffer at a rate of 3 mM/min. The absorbance of the column effluent was monitored at 280 nm and 4 ml fractions were collected. Both the batch eluted material and the gradient eluted fractions were stored at 4°C in the presence of 10 $\mu$g/ml aprotinin (Sigma Chemical Co.), 1 $\mu$M

[1] *Abbreviations used in this paper:* CNBr, cyanogen bromide; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid.

PMSF (Sigma Chemical Co.), and 0.025 M EDTA. IL-1 activity reproducibly eluted as two major peaks, with minor peaks of activity occasionally observed. The active fractions from each of the two major peaks were pooled and concentrated on YM10 membranes to a volume of 1 ml before fractionation by preparative HPLC size-exclusion chromatography (TSK 2000SW [21.5 mm × 60 cm]; Bio-Rad Laboratories). The column was run in 100 mM sodium phosphate (pH 6.8), 20% ethylene glycol, and 0.02% azide at a flow rate of 2 ml/min. 2 ml fractions were collected and assayed. The active fractions were then chromatographed on a Vydac C4 reverse-phase HPLC column (4.6 mm × 5 cm; Rainin) equilibrated in 0.1% trifluoroacetic acid (TFA) in water. The column was eluted with a gradient (0–40%) of acetonitrile containing 0.1% TFA. The gradient was developed rapidly (4 min) to 36% acetonitrile, followed by a 0.25%/min gradient at a flow rate of 0.5 ml/min. The absorbance of the column effluent was monitored at 210 nm and peaks of interest were manually peak-collected using a Frac-100 fraction collector (Pharmacia Fine Chemicals, Piscataway, NJ). The peaks associated with IL-1 activity were rechromatographed by reverse-phase HPLC, using the same conditions as above. The concentrations of the purified proteins were calculated by integration of the absorbance profile obtained at 210 nm using the Nelson Analytical data acquisition system, Model 4416, run on a Hewlett Packard Co. (Palo Alto, CA) 9816 computer. Known amounts of ribonuclease were used to calibrate the integration function of the data aquisition system.

*Isoelectric Focusing.* Isoelectric focusing of IL-1 was performed as previously described (14). The pH gradients were standardized with proteins of known pI, as previously described (14). Gels were cut into 2 mm slices and eluted into 0.5 ml RPMI/10% FCS overnight at 4°C. Samples were assayed for IL-1 activity in the mouse thymocyte proliferation assay at dilutions of 1:5 and 1:20.

*Analytical SDS-PAGE.* Samples (50–150 ng) were diluted into Laemmli sample buffer containing 1 mM dithiothreitol and boiled for 5 min before SDS-PAGE in vertical slab gels containing 15% acrylamide. Gels were stained with silver, as previously described (15). Standard proteins consisting of phosphorylase b (mol wt 94,000), BSA (mol wt 67,000) OVA (mol wt 43,000), carbonic anhydrase (mol wt 30,000), soybean trypsin inhibitor (mol wt 20,100), and alpha-lactalbumin (mol wt 14,400) were run on the same gel slab and molecular weights of the IL-1 species were calculated by linear regression analysis of the molecular weights of the standards.

*Thymocyte Proliferation (Lymphocyte Activating Factor [LAF]) Assay.* IL-1 activity was monitored using the mouse thymocyte comitogenic proliferation assay performed as previously described (14) using 6–8-wk-old C3H/HeJ male mice. Half-maximal units of bioactivity were calculated as previously described (10).

*Preparation and Assay of Anti-IL-1/6.8 Antisera.* Antisera were raised in rabbits to KLH-conjugated synthetic peptides, as previously described (16). Antiserum to intact IL-1/6.8 was raised in rabbits by immunization with 3 μg of pure IL-1/6.8 (10) in saline injected into the popliteal lymph nodes (day 0), followed by a subcutaneous injection with 3 μg in CFA (day 21). Rabbits were boosted three times by intramuscular injection of 3–10 μgs IL-1/6.8 in IFA on days 43, 57, 82, and 152 and they were bled on day 163. Antibody binding was assayed using ELISA or Western analysis, as previously described (16).

*CNBr Cleavage of IL-1/5.4.* Reverse-phase–purified IL-1/6.8 and IL-1/5.4 (4 μg in 100 μl) were brought to 50% TFA (vol/vol), and 1 mg of CNBr in 10 μl water was added. After incubation for 24 h in the dark at room temperature, the entire sample was diluted 1:5 in water and injected directly onto an Aquapore Bu-300 reverse-phase column (2.1 mm × 3 cm; Brownlee Labs, Santa Clara, CA) equilibrated in 0.1% TFA/90% water/10% acetonitrile. Peptides were eluted from the column with a 2%/min gradient of acetonitrile at a flow rate of 0.2 ml/min. Peaks were collected into polypropylene tubes and stored at 4°C.

*Amino Acid Sequence Analysis.* Intact IL-1/5.2 or IL-1/5.4 peptides were applied by repeated application to polybrene-coated filters and sequenced using an automated gas-phase sequencer employing Edman chemistry (model 470A; Applied Biosystems, Inc., Foster City, CA). The ATZ amino acids were treated in the conversion flask with

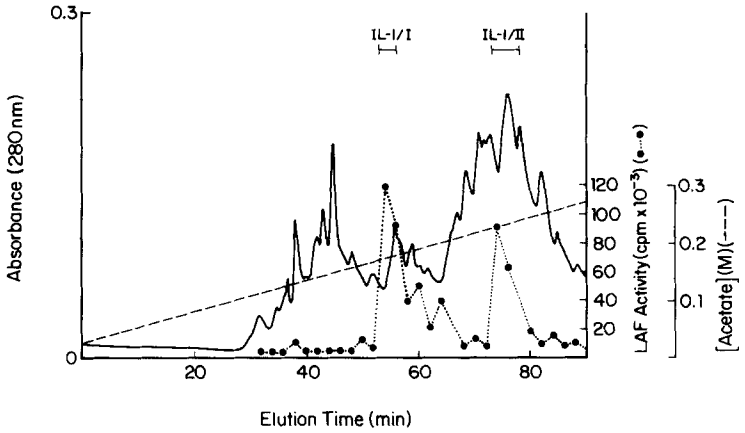


FIGURE 1. DEAE/HPLC chromatography of anionic species of human IL-1. 0.3 M sodium acetate–eluted material from five column runs was reapplied and eluted with a sodium acetate gradient at a rate of 3 mM/min, as described in Materials and Methods. Absorbance was monitored at 280 nm and fractions were assayed at a dilution of 1:8,000 in the thymocyte proliferation (LAF) assay. The fractions under the horizontal bars (IL-1/I and IL-1/II) were pooled for further purification steps.

methanolic-HCl to form the methyl esters of the phenylthiohydantoin (PTH) derivatives of glutamic acid and aspartic acid. The PTH amino acids were identified by isocratic separation on a Zorbax Bioseries PTH/HPLC column (Dupont Co., Wilmington, DE) as per the manufacturer's instructions.

## Results

*Purification of IL-1/5.2 and IL-1/5.4.* As previously reported (10), IL-1/6.8 is purified on a DEAE/HPLC column using a pH gradient elution protocol (pH 8.3–6.8). After this step, a significant amount of IL-1 activity is still firmly bound to the column. In preliminary experiments, all the remaining activity was found to elute in 0.3 M sodium acetate (pH 6.8). The amount of activity recovered at this step was ~20% of the total activity of the starting material. To accumulate enough material for further purification, five batches eluted with 0.3 M sodium acetate were then pooled and concentrated.

Fig. 1 shows the absorbance and activity profiles of this material when reapplied to the DEAE/HPLC column and eluted with a shallow gradient of sodium acetate (see Materials and Methods). The two areas of thymocyte proliferation activity indicated by the horizontal bars (designated IL-1/I and IL-1/II) were separately pooled and concentrated in preparation for size-exclusion chromatography. Both of these anionic species of IL-1 eluted from a preparative TSK2000 column in nearly identical elution volumes (126–130 ml), as measured by thymocyte stimulating activity (data not shown). This elution volume represented a mol mass of 17–18 kD when compared with standards of known mol mass, and was identical to that seen with IL-1/6.8. Fig. 2 (*A* and *C*) shows the absorbance profiles when the two anionic species eluted from the TSK2000 column were separately chromatographed on a Vydac C4 reverse-phase column equilibrated in 0.1% TFA in water. Fig. 2 (*B* and *D*) shows the corresponding activity profiles obtained when the peak-collected fractions were tested for IL-1 activity. Both anionic IL-

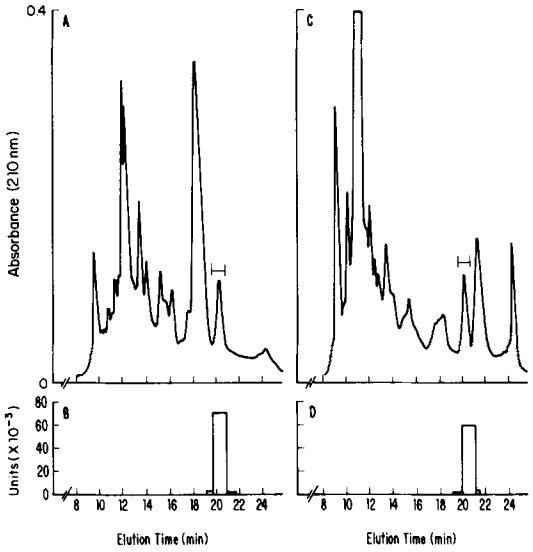


FIGURE 2. Reverse-phase HPLC of anionic species of human IL-1. Active fractions from ion-exchange HPLC (IL-1/I and IL-1/II; see Fig. 1) were each further purified by size-exclusion HPLC (data not shown). The active fractions from these runs were chromatographed on a Vydac C4 reverse-phase column as described in Materials and Methods (*A* and *B*, IL-1/I; *C* and *D*, IL-1/II). Absorbance was monitored at 210 nm (*A* and *C*) and activity (U/fraction) was measured in the murine thymocyte assay (*B* and *D*).

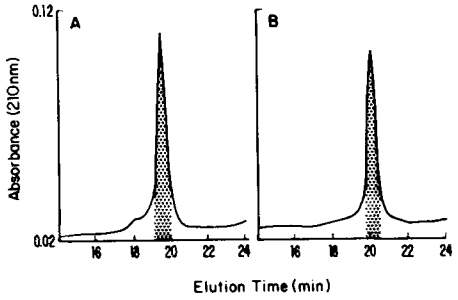


FIGURE 3. Rechromatography of anionic species of human IL-1 on reverse-phase HPLC. The peaks corresponding with the active fractions from the Vydac C4 column (Fig. 2) were rechromatographed using the same protocol as in Fig. 2. (*A*) IL-1/I, (*B*) IL-1/II. The stippled areas represent those portions of the peaks that were collected for subsequent analysis.

1s had similar, but not identical, retention times of ~20 min, which represented an acetonitrile concentration of ~39%. In contrast, IL-1/6.8 elutes in ~33% acetonitrile under identical gradient conditions (data not shown). Injection of a mixture of the pure anionic species yielded a double peak (data not shown), indicating that the slight difference in retention time observed when the species were chromatographed separately was not due to run variation. Rechromatography of the peaks in Fig. 2 corresponding with IL-1 activity is shown in Fig. 3. >95% of the integrated area was found under single, sharp, symmetrical peaks in each case. The stippled areas represent those portions of the peaks that were collected for subsequent analyses. SDS-PAGE of these materials is shown in Fig. 4. Both preparations gave single, silver-staining bands (lanes *3* and *4*) with an

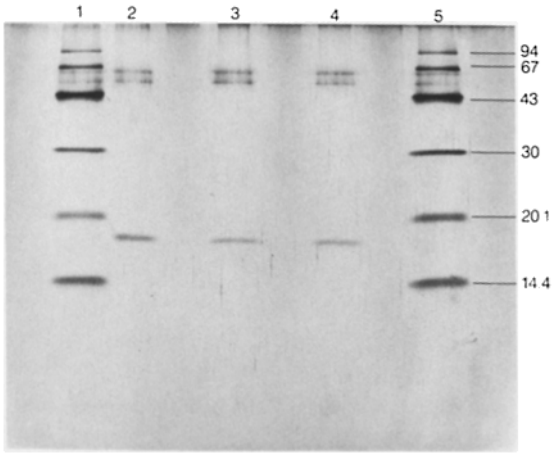


FIGURE 4. Silver-stained SDS-PAGE of anionic and neutral human IL-1 species. Materials represented by the stippled areas in Fig. 3 were subjected to SDS-PAGE along with IL-1/6.8, purified as previously described (10), and standard proteins of known molecular weights (see Materials and Methods). (Lanes *1* and *5*) standard proteins, (lane *2*) IL-1/6.8, (lane *3*) IL-1/I (Fig. 3*A*), (lane *4*) IL-1/II (Fig. 3*B*). Molecular weights shown are $\times 10^{-3}$.

apparent mol wt of 17,500. This mol wt is indistinguishable from that of IL-1/6.8 (lane *2*). Two high mol wt bands are also seen. Their presence in all the loaded lanes, including those containing the mol wt markers, indicates that they represent contaminants in the sample buffer. A doublet of this mobility is often seen on silver-stained gels (17). Finally, the pure proteins were subjected to isoelectric focusing in separate lanes to determine their respective isoelectric points. Peaks of IL-1 activity corresponding with pIs of 5.2 and 5.4 were observed (Fig. 5*B*). These peaks were found to coincide exactly with a double peak of activity observed when batch-eluted material from the first DEAE column was subjected to isoelectric focusing in a third lane (Fig. 5*A*).

The amount of activity represented by the pure IL-1/5.4 and IL-1/5.2 accounts for ~6% of the activity found in the material batch-eluted with 0.3 M sodium acetate. The total amount of IL-1/5.2 and IL-1/5.4 recovered from crude, unconcentrated supernatant was ~0.8 μg/liter.

*Activity of Purified IL-1 Species in the Mouse Thymocyte Assay.* Pure preparations of IL-1/6.8, IL-1/5.4, and IL-1/5.2 were assayed for their ability to stimulate the proliferation of mouse thymocytes in the presence of PHA. Fig. 6 illustrates a representative experiment in which all three IL-1 species were found to stimulate thymocyte proliferation in a dose-responsive manner. Specific activities, as defined by that amount of IL-1 which gives half-maximal stimulation of thymocyte proliferation, were indistinguishable among all three charged species. The concentrations necessary for half-maximal stimulation, calculated from linear regression of the data shown, averaged 430 pg/ml (range, 425–438 pg/ml), or $\sim 2 \times 10^{-11}$ M. This represents a specific activity in the thymocyte proliferation assay (0.2 ml volume) of $1.2 \times 10^{7}$ half-maximal U/mg. Identical results were obtained with three other purified preparations of IL-1/5.2 and IL-1/5.4.

*Antigenic Differences between IL-1/6.8 and the Anionic IL-1 Species.* Antibodies directed against either intact IL-1/6.8 or synthetic peptides of IL-1/6.8 were

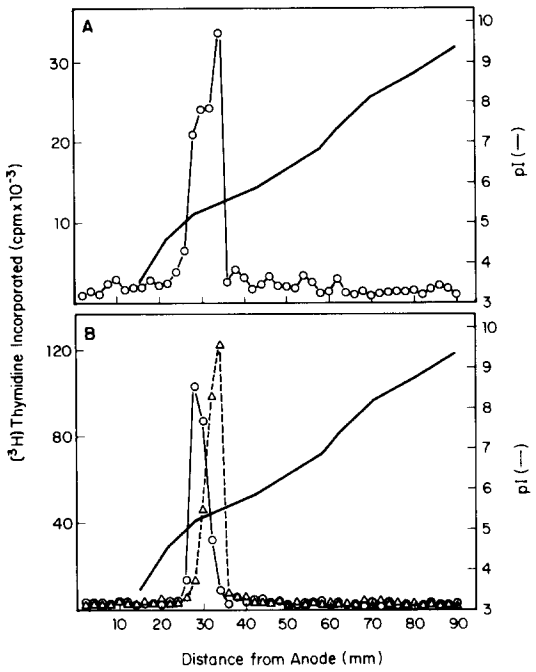


FIGURE 5. Isoelectric focusing of anionic human IL-1 species. (*A*) 15 μl of 0.3 M sodium acetate–eluted material containing both anionic forms of IL-1. (*B*) IL-1/I (○) and IL-1/II (△). 100 ng of the materials shown in Fig. 3, *A* and *B* were subjected to isoelectric focusing in separate lanes. Gels were cut into 2-mm slices and eluted into 0.5 ml RPMI/FCS. Samples were assayed in the mouse thymocyte assay at a 1:5 dilution (*A*) or a 1:20 dilution (*B*). The pH gradient was determined by focusing 11 standard proteins of known pI in an adjacent lane.

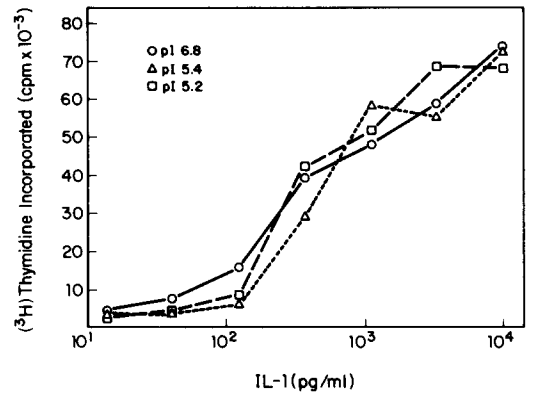


FIGURE 6. Titration of IL-1/6.8, IL-1/5.4, and IL-1/5.2 in the mouse thymocyte proliferation assay. Materials shown in Fig. 3, *A* and *B*, as well as pure IL-1/6.8, were assayed for their ability to stimulate the proliferation of murine thymocytes in the presence of PHA. Protein concentration was determined by measuring absorbance at 210 nm in comparison with known amounts of RNase as described in Materials and Methods.

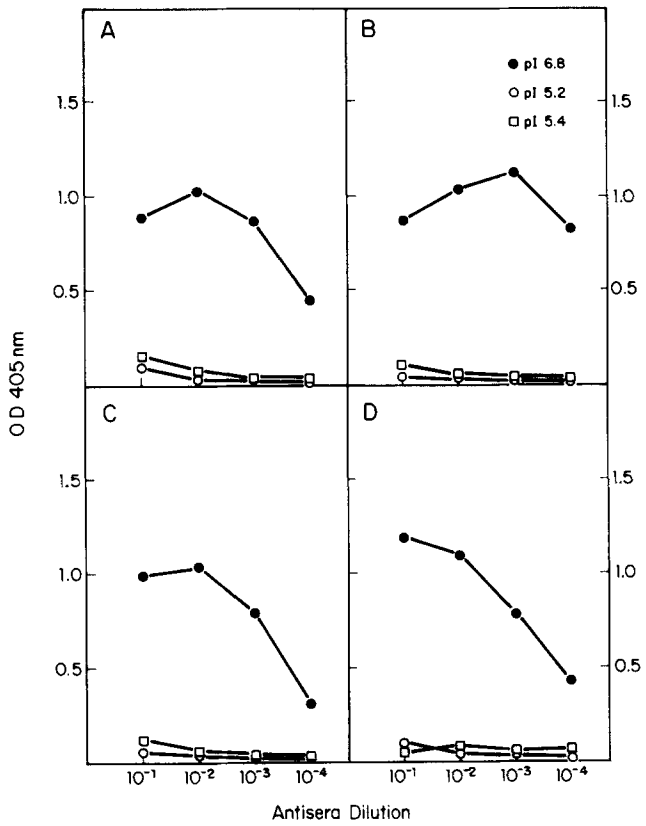


FIGURE 7. ELISA, testing ability of four (*A–D*) anti-IL-1/6.8 antisera to crossreact with IL-1/5.2 and IL-1/5.4. 10 ng of each species were allowed to bind to the wells before addition of the diluted antiserum and other reagents (see Materials and Methods). (*A*) anti-*N*-terminal peptide; (*B*) anti-internal peptide; (*C*) anti-*C*-terminal peptide; (*D*) anti-IL-1/6.8.

tested for their ability to bind to equal amounts of immobilized IL-1/6.8, IL-1/5.4, and IL-1/5.2 in an ELISA. As shown in Fig. 7, no cross-reactivity could be shown with any of the four antisera tested. All four antisera were also strongly reactive with IL-1/6.8 on Western analysis, but were completely nonreactive with equal amounts of IL-5.2 and IL-5.4 (data not shown). These results suggest that little or no antigenic similarity exists between the neutral and anionic species of human IL-1.

*Amino Acid Sequence Analysis of IL-1/5.4 and IL-1/5.2.* Attempts at direct *N*-terminal sequence analysis of IL-1/5.4 were unsuccessful. Therefore, preparation of CNBr peptides of IL-1/5.4 was performed as described in Materials and Methods. As can be seen in Fig. 8*A*, three prominant peptides were separated by reverse-phase HPLC. The ascending baseline is characteristic of the gradient protocol used, and the large peak at 22 min was also present in the control reaction mixture without protein. The reverse-phase chromatograph of the CNBr peptides of IL-1/5.4 (Fig. 8*A*) was markedly different from that of IL-1/6.8 CNBr peptides generated and chromatographed under identical conditions (Fig. 8*B*). The indicated peaks in Fig. 8*A* (I, II, and III) were sequenced and the

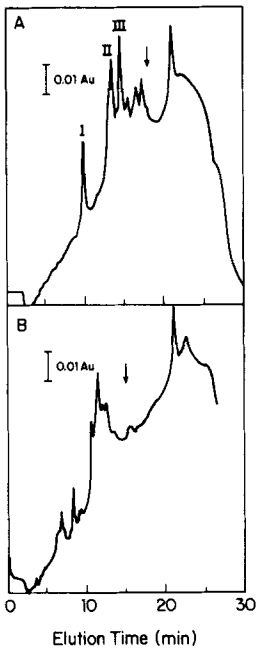


FIGURE 8. Reverse-phase HPLC of CNBr peptides of IL-1/5.4 (*A*) and IL-1/6.8 (*B*). Both IL-1 species were treated with CNBr as described in Materials and Methods. I, II, and III in *A* indicate the peptides that were subsequently sequenced and shown in Fig. 9. In each panel, the arrow indicates that time at which the corresponding intact protein eluted. Data acquisition during the run shown in *B* was stopped at 26 min.

**A** IL-1/5.4

**Peptide I:**

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (Gly) | Ala | Tyr | ? | Ser | Ser | ? | Asp | Asp | Ala | ? | Ile | Thr | Val |
| Ile | Leu | Arg | Ile | ? | | | | | | | | | |

**Peptide II:**

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pro | Glu | Ile | Pro | ? | (Thr) | Ile | ? | (Gly) | ? | Glu | ? | Asn | Leu |
| Leu | Phe | Phe | ? | Glu | ? | | | | | | | | |

**Peptide III:**

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Arg | Ile | Ile | ? | Tyr | Glu | Phe | Ile | Leu | Asn | Asp | Ala | Leu | Asn |
| Gln | (Ser) | Ile | Ile | (Arg) | Ala | Asn | Asp | Gln | Tyr | Leu | (Thr) | Ala | Ala |
| Ala | Leu | ? | Asn | Leu | (Asp) | Glu | Ala | ? | ? | (Phe) | ? | | |

**B** IL-1/5.2

**Amino Terminus:**

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Leu | Ser | Asn | Val | (Lys) | Tyr | Asn | Phe | Met | Arg | Ile | Ile | ? | Tyr |
| Glu | Phe | Ile | Leu | Asn | Asp | Ala | Leu | Asn | Gln | (Ser) | Ile | Ile | Arg |
| (Ala) | Asp | Asp | Gln | Tyr | Leu | ? | Ala | Ala | Ala | Leu | His | ? | |

FIGURE 9. (*A*) Amino acid sequences of CNBr peptides of human IL-1/5.4. Amino acids in parentheses indicate tentative identifications. I, II, and III refer to the peaks in Fig. 8. (*B*) Amino terminal sequence of IL-1/5.2. Complete homology was observed between peptide III of IL-1/5.4 and the amino terminal sequence of IL-1/5.2, except for the underlined residues.

results are shown in Fig. 9*A*. Direct *N*-terminal sequence analysis of IL-1/5.2 was then performed. The results are shown in Fig. 9*B*. The amino acids shown in Fig. 9*A* and *B* correspond exactly with the amino acid sequence deduced from

| | | | | | | | | | 10 | | | | | | | | | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Met | Ala | Lys | Val | Pro | Asp | Met | Phe | Glu | Asp | Leu | Lys | Asn | Cys | Tyr | Ser | Glu | Asn | Glu |
| | | | | | | | | | 30 | | | | | | | | | 40 |
| Asp | Ser | Ser | Ser | Ile | Asp | His | Leu | Ser | Leu | Asn | Gln | Lys | Ser | Phe | Tyr | His | Val | Ser |
| | | | | | | | | | 50 | | | | | | | | | 60 |
| Gly | Pro | Leu | His | Glu | Gly | Cys | Met | Asp | Gln | Ser | Val | Ser | Leu | Ser | Ile | Ser | Glu | Thr |
| | | | | | | | | | 70 | | | | | | | | | 80 |
| Lys | Thr | Ser | Lys | Leu | Thr | Phe | Lys | Glu | Ser | Met | Val | Val | Val | Ala | Thr | Asn | Gly | Lys |
| | | | | | | | | | 90 | | | | | | | | | 100 |
| Leu | Lys | Lys | Arg | Arg | Leu | Ser | Leu | Ser | Gln | Ser | Ile | Thr | Asp | Asp | Asp | Leu | Glu | Ala |
| | | | | | | | | | 110 | | | | | | | | | 120 |
| Ala | Asn | Asp | Ser | Glu | Glu | Glu | Ile | Ile | Lys | Pro | Arg | Ser | Ala | Pro | Phe | Ser | Phe | **Leu** |
| | | | | | | | | | **130** | | | | | | | | | **140** |
| **Asn** | **Val** | **Lys** | **Tyr** | **Asn** | **Phe** | **Met** | **Arg** | **Ile** | **Ile** | Lys | **Tyr** | **Glu** | **Phe** | **Ile** | **Leu** | **Asn** | **Asp** | **Ala** |
| | | | | | | | | | 150 | | | | | | | | | 160 |
| **Asn** | **Gln** | **Ser** | **Ile** | **Ile** | **Arg** | **Ala** | **Asn** | **Asp** | **Gln** | **Tyr** | **Leu** | **Thr** | **Ala** | **Ala** | **Ala** | **Leu** | **His** | **Asn** |
| | | | | | | | | | 170 | | | | | | | | | 180 |
| **Asp** | **Glu** | **Ala** | Val | Lys | **Phe** | Asp | Met | **Gly** | **Ala** | **Tyr** | Lys | **Ser** | **Ser** | Lys | **Asp** | **Asp** | **Ala** | Lys |
| | | | | | | | | | 190 | | | | | | | | | 200 |
| **Thr** | **Val** | **Ile** | **Leu** | **Arg** | **Ile** | Ser | Lys | Thr | Gln | Leu | Tyr | Val | Thr | Ala | Gln | Asp | Glu | Asp |
| | | | | | | | | | 210 | | | | | | | | | 220 |
| Pro | Val | Leu | Leu | Lys | Glu | Met | **Pro** | **Glu** | **Ile** | **Pro** | Lys | **Thr** | **Ile** | Thr | **Gly** | Ser | **Glu** | Thr |
| | | | | | | | | | 230 | | | | | | | | | 240 |
| **Leu** | **Leu** | **Phe** | **Phe** | Trp | **Glu** | Thr | His | Gly | Thr | Lys | Asn | Thr | Phe | Thr | Ser | Val | Ala | His |
| | | | | | | | | | 250 | | | | | | | | | 260 |
| Asn | Leu | Phe | Ile | Ala | Thr | Lys | Gln | Asp | Tyr | Trp | Val | Cys | Leu | Ala | Gly | Gly | Pro | Pro |
| | | | | | | | | | 270 | | | | | | | | | |
| Ile | Thr | Asp | Phe | Gln | Ile | Leu | Glu | Asn | Gln | Ala | | | | | | | | |

FIGURE 10. Comparison of amino acid sequences of IL-1/5.2 and IL-1/5.4 with the amino acid sequence deduced from cDNA IL-1-$\alpha$ (9). Amino acids in bold type indicate those residues confirmed by amino acid sequence analysis of CNBr peptides of native IL-1/5.4 (Fig. 9*A*) and *N*-terminal sequence analysis of IL-1/5.2 (Fig. 9*B*).

the cDNA reported by March et al. (9). Those amino acids of the deduced sequence of IL-1-$\alpha$ cDNA, which have now been confirmed by direct amino acid sequence analysis of the purified protein, are in bold type in Fig. 10.

## Discussion

We have described the purification to homogeneity of two anionic forms of IL-1. These IL-1 species were more difficult to purify than the pI 6.8 species because: (*a*) they exist in the starting supernatant in 5–10-fold lesser amounts (this report and references 3 and 9), and (*b*) many of the contaminating proteins contained in the starting supernatant display behavior similar to that of the anionic IL-1 species during ion-exchange chromatography. Separation of the anionic IL-1 species from each other was accomplished by the use of a shallow gradient of sodium acetate for elution from the DEAE/HPLC column (Fig. 1). The use of chloride as a counter-ion resulted in poor separation of the two anionic species and a much higher level of contaminating proteins. Attempts to more selectively elute the anionic species of IL-1 by means of a descending pH gradient (6.8–4.0), such as the approach used for IL-1/6.8 (10), were also unsuccessful because only a small proportion of the bound activity eluted (unpublished observations). Subsequent purification of the sodium acetate eluted peaks of activity by size exlusion, and reverse-phase chromatography (Figs. 2 and 3) was sufficient to yield material that was homogeneous by SDS-PAGE (Fig. 4). The estimated recovery of the anionic species, though low, is similar to that found for porcine catabolin (18) and murine IL-1 (19), two other anionic IL-1 molecules. Most of the apparent loss of activity occurred during the anion-

exchange HPLC step. This may be due, at least in part, to the loss of other potent thymocyte proliferation factors (e.g., IL-2) or the separation of unidentified cofactors during this step. Loss of bioactivity in partially purified preparations during storage was a difficulty in preliminary studies, but this problem was overcome by the addition of aprotinin, PMSF, and EDTA to fractions obtained from the ion-exchange and size-exclusion HPLC columns. These protease inhibitors were readily resolved from IL-1 on reverse-phase HPLC, the final step in the purification protocol.

The only previously reported purification of anionic human IL-1 (12) yielded a protein (presumably a mixture of IL-1/5.4 and IL-1/5.2) of 14,500 mol wt, which was ~500-fold less active on a mass basis than our pure material. No additional biochemical characterization of this material was presented. Other authors (20, 21) report the purification of human IL-1 without separating the neutral and anionic charged species from each other. Kronheim et al. (22) have purified a 17,500 mol wt protein having IL-1 activity. Analytical IEF of this material yielded four silver-staining bands, suggesting that the various charged species of IL-1 had not been separated from each other. Therefore, the current report is the first in which the two native anionic IL-1 proteins have been separately isolated in pure form.

The pure proteins are characterized as having mol wts identical to IL-1/6.8 (17,500, Fig. 4) and pI of 5.4 and 5.2 (Fig. 5). These characteristics identify these proteins as those low pI IL-1 species previously described in the IL-1 literature (2–6). The specific activities of the purified anionic IL-1s, as determined by half-maximal stimulation in the thymocyte comitogenic assay (Fig. 6), were identical to that of IL-1/6.8 ($\sim 1.2 \times 10^7$ u/mg [10, 13]) and in the same range as that reported for pure murine IL-1 ($\sim 2 \times 10^7$ U/mg [7]) and porcine catabolin ($\sim 5 \times 10^6$ U/mg [23]), two other anionic IL-1 molecules. It was found that IL-1/5.4 and IL-1/5.2 behaved similarly, though not identically, when subjected to reverse-phase chromatography (Fig. 3). In contrast, the behavior of IL-1/6.8 on reverse-phase differed significantly from the anionic species. The striking differences between the peptide maps of IL-1/6.8 and the IL-1/5.4, their substantially different behavior on reverse-phase HPLC, as well as the inability of four different anti-IL-1/6.8 antisera to crossreact with IL-1/5.2 and IL-1/5.4, clearly show the significant structural and antigenic differences between these proteins.

Recently the cloning of two human IL-1 cDNAs ($\alpha$ and $\beta$) has been reported (8, 9). That the $\beta$ cDNA encodes the major species of human IL-1 having a pI of 6.8 has been conclusively shown by direct comparison of the amino acid sequence of the pure native protein with that deduced from the nucleic acid sequence of the cDNA (9, 10, 13). However, the relationship of the $\alpha$ cDNA to the different charged species of IL-1 was not established. We have sequenced the amino terminus of intact IL-1/5.2 and three CNBr peptides of IL-1/5.4 (Fig. 9, *A* and *B*). Comparison of the amino acid sequences as obtained from the pure IL-1 species with that deduced from the IL-1-$\alpha$ cDNA clearly shows that IL-1/5.2 and IL-1/5.4 are encoded by the IL-1-$\alpha$ cDNA. 74 residues were identified. The amino terminal sequence of IL-1/5.2 (Fig. 9*B*) corresponds with residues 119–158, while peptides I, II, and III of the IL-1/5.4 (Fig. 9*A*) correspond with

residues 169–186, 208–226, and 128–166 deduced from the IL-1-$\alpha$ cDNA (Fig. 10). Each of the peptides follows a methionine, indicating that they are located internally within the sequence of the mature IL-1/5.4 molecule. It is also clear from these data that mature human IL-1/5.2 and IL-1/5.4 are derived from the *C*-terminal portion of the 31 kD precursor encoded by the IL-1-$\alpha$ cDNA. Thus, as in the case of IL-1/6.8, most of the processing of the precursor molecule occurs at the amino terminal end.

Our attempts to sequence the amino terminus of the mature IL-1/5.4 were unsuccessful, presumably due to the presence of an as yet unidentified blocking group. Similar difficulties were encountered with murine IL1 (7). However, after CNBr cleavage, the amino terminal peptide of murine IL-1 was susceptible to Edman degradation, and the apparent amino terminus was identified as Ser 115. The corresponding residue in the IL-1-$\alpha$-deduced sequence is Ser 113 (9). We were able to sequence the amino terminus of IL-1/5.2 and found Leu 119 to be the amino terminal residue. Cleavage of the Phe-Leu linkage by a chymotrypsin-like enzyme such as cathepsin G (24) is probably responsible for this terminus. Such cleavage may occur intracellularly, in the culture supernatant, or during the isolation procedure. In any event, the full activity of IL-1/5.2 in the thymocyte assay as compared with IL-1/5.4, IL-1/6.8, and murine IL-1 (7) suggests that the amino acid residues that lie 5′ to Leu 119 are not essential to bioactivity.

The amino acid sequences obtained have clarified the relationship between the two anionic species of human IL-1. As mentioned above, the amino terminus of IL-1/5.4 is apparently blocked while the amino terminus of IL-1/5.2 is susceptible to Edman degradation. Comparison of the amino acid sequences determined for the two species shows that peptide III of IL-1/5.4 is homologous with the amino terminal sequence of IL-1/5.2, beginnning with Arg 128. The homology is complete for all confirmed amino acid residues with one exception; residue 148 sequenced as Asn (underlined in Fig. 9*A*) in the case of peptide III, but as Asp (underlined in Fig. 9*B*) in the amino terminal sequence. This may account for the small difference in net charge between the two acidic species and may be due to either a single base change at the nucleic acid level or, more likely, deamidation, a noncatalytic process that occurs spontaneously during storage of proteins (25).

Although the presence of additional IL-1 genes cannot be eliminated, it is now clear that the majority of the IL-1 activity, which resides in species having pI of 6.8, 5.4, and 5.2, can be accounted for by the products of two genes. The products of these genes, while indistinguishable from each other in the murine thymocyte assay, are structurally and antigenically distinct based on peptide analysis, primary amino acid sequence, and reactivity with antisera specific for IL-1/6.8. Now that these native IL-1 molecules are obtainable in pure form, it will be possible to determine their relative specific activities on other target cells. In addition, it shall also be possible to determine whether these different IL-1 species interact with a single receptor site or different receptor sites that use similar pathways of cellular activation.

## Summary

Two anionic species of human IL-1 have been purified to homogeneity. These molecules were characterized as having pI of 5.4 and 5.2 and molecular weights identical to IL-1/6.8 (17,500). The specific activities of IL-1/5.4 and IL-1/5.2, as measured in the mouse thymocyte comitogenic assay, were identical to that of IL-1/6.8, namely $1.2 \times 10^7$ U/mg, with half-maximal stimulation observed at $2 \times 10^{-11}$ M. IL-1/5.4 and IL-1/5.2 were found to be antigenically distinct from IL-1/6.8 in an ELISA. IL-1/5.4 was structurally distinct from IL-1/6.8 based on reverse-phase HPLC or CNBr peptides. Intact IL-1/5.2 and three intact CNBr peptides of IL-1/5.4 were sequenced, with the identification of 74 amino acid residues. These sequences were found to correspond exactly with the amino acid sequence deduced from the IL-1-$\alpha$ cDNA reported by March et al. (9).

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## References

1. Durum, S. K., J. A. Schmidt, and J. J. Oppenheim. 1985. Interleukin 1: an immunological perspective. *Ann. Rev. Immunol.* 3:263.
2. Lachman, L. B., M. P. Hacker, and R. E. Handschumacher. 1977. Partial purification of human lymphocyte activating factor (LAF) by ultrafiltration and electrophoretic techniques. *J. Immunol.* 119:2019.
3. Schmidt, J. A., S. B. Mizel, D. Cohen, and I. Green. 1982. Interleukin 1, a potential regulator of fibroblast proliferation. *J. Immunol.* 128:2177.
4. Ihrie, E. J., and D. D. Wood. 1985. Biochemical heterogeneity of human interleukin-1. *Lymphokine Res.* 4:169.
5. Krakauer, T. 1984. Structural analysis of the charge heterogeneity of human interleukin 1. *Arch. Biochem. Biophys.* 234:371.
6. Wood, D. D., E. K. Bayne, M. G. Goldring, M. Gowen, D. Hamerman, J. L. Humes, E. J. Ihrie, P. E. Lipsky, and M. J. Staruch. 1985. The four biochemically distinct species of human interleukin 1 all exhibit similar biologic activities. *J. Immunol.* 134:895.
7. Lomedico, P. T., U. Gubler, C. P. Hellmann, M. Dukovich, J. G. Giri, Y.-C. E. Pan, K. Collier, R. Semionow, A. O. Chua, and S. B. Mizel. 1984. Cloning and expression of murine interleukin-1 cDNA in *Escherichia coli*. *Nature (Lond.)*. 312:458.
8. Auron, P. E., A. C. Webb, L. J. Rosenwasser, S. F. Mucci, A. Rich, S. M. Wolff, C. A. Dinarello. 1984. Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. *Proc. Natl. Acad. Sci. USA.* 81:7907.
9. March, C. J., B. Mosley, A. Larsen, D. P. Cerretti, G. Braedt, V. Price, S. Gillis, C. Henney, S. Kronheim, K. Grabstein, P. J. Conlon, T. P. Hopp, and D. Cosman. 1985. Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature (Lond.)*. 315:641.
10. Cameron, P. M., G. Limjuco, J. Rodkey, C. Bennett, and J. A. Schmidt. 1985. Amino acid sequence analysis of human interleukin 1 (IL-1). Evidence for biochemically distinct forms of IL-1. *J. Exp. Med.* 162:790.
11. Mizel, S. B. 1979. Physicochemical characterization of lymphocyte-activating factor (LAF). *J. Immunol.* 122:2167.
12. Krakauer, T. 1984. Purification to homogenicity of biologically active human interleukin 1. *Prep. Biochem.* 14:449.
13. Dewhirst, F. E., P. P. Stashenko, J. E. Mole, and T. Tsurumachi. 1985. Purification

and partial sequence of human osteoclast-activating factor: identity with interleukin 1 beta. *J. Immunol.* 135:2562.

14. Schmidt, J. A. 1984. Purification and partial biochemical characterization of normal human interleukin 1. *J. Exp. Med.* 160:772.
15. Morissey, J. N. 1981. Silver stains for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* 117:307.
16. Limjuco, G., S. Galuska, J. Chin, P. Cameron, J. Boger, and J. A. Schmidt. 1986. Antibodies of predetermined specificity to the major charged species of human interleukin 1. *Proc. Natl. Acad. Sci. USA.* In press.
17. Ochs, D. 1983. Protein contaminants of sodium dodecyl sulfate-polyacrylamide gels. *Anal. Biochem.* 135:470.
18. Saklatvala, J., V. A. Curry, and S. J. Sarsfield. 1983. Purification to homogeneity of pig leukocyte catabolin, a protein that causes cartilage resorption *in vitro. Biochem. J.* 215:385.
19. Mizel, S. B., and D. Mizel. 1981. Purification to apparent homogeneity of murine interleukin 1. *J. Immunol.* 126:834.
20. Kock, A., and T. A. Luger. 1984. Purification of human interleukin 1 by high-performance liquid chromatography. *J. Chromatogr.* 296:293.
21. Matsushima, K., S. K. Durum, E. S. Kimball, and J. J. Oppenheim. 1985. Purification of human interleukin 1 from human monocyte culture supernatants and identity of thymocyte comitogenic factor, fibroblast-proliferation factor, acute-phase protein-inducing factor, and endogenous pyrogen. *Cell. Immunol.* 92:290.
22. Kronheim, S. R., C. J. March, S. K. Erb, P. J. Conlon, D. Y. Mochizuki, and T. P. Hopp. 1985. Human interleukin 1. Purification to homogeneity. *J. Exp. Med.* 161:490.
23. Saklatvala, J., S. J. Sarsfield, and Y. Townsend. 1985. Pig interleukin 1: purification of two immunologically different leukocyte proteins that cause cartilage resorption, lymphocyte activation, and fever. *J. Exp. Med.* 162:1208.
24. Wilcox, P. E. 1970. Chymotrysinogens-Chymotrypsins. *Methods Enzymol.* 19:64.
25. Robinson, A. B., and C. J. Rudd. 1974. Deamidation of glutaminyl and asparaginyl residues in peptides and proteins. *In* Current Topics of Cellular Regulation. Vol. 8. B. J. Horecker and E. R. Stadtman, editors. Academic Press, New York. 247–295.