A HUMAN URINE-DERIVED INTERLEUKIN 1 INHIBITOR

Homology with Deoxyribonuclease I

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IL-1 is one of the major mediators of immunity and inflammation in mammals. Secreted by mononuclear phagocytes (monocytes, macrophages, and other related cells of this lineage) in response to many inert, immunologic, or microbial agents, IL-1 induces maturation and differentiation in T lymphocytes, induces acute phase serum reactant production by the liver, induces fever via the hypothalamus, increases prostaglandin and collagenase production by cultured synovial cells, and induces fibroblast proliferation and prostaglandin production in vitro (reviewed in reference 1).

The ubiquity and potency of IL-1 in these and other physiologic systems suggest that its effects must be tightly regulated. Accordingly, a number of substances capable of inhibiting the effects of IL-1 have been reported, including molecules that have been derived from human urine (2–5), from normal human white blood cells (6, 7), from human white blood cells in certain disease states (8, 9) or after viral infection (10, 11), from human epidermal cells (12), and from a variety of cell lines (13–16).

We have previously reported that the urine of febrile humans contains large amounts of a 30–40-kD glycoprotein inhibitor of IL-1-induced mouse thymocyte proliferation (3, 17). Based upon differences in size and functional activity, this factor seems to be distinct from two other urine-derived IL-1 inhibitors, uromodulin (18–20), and an inhibitor first derived from the urine of patients with monocytic leukemia (5, 21). However, the febrile inhibitor has not been purified, thereby hindering more exact biological and physiochemical characterization. We have, therefore, undertaken to purify this molecule using methodology that produced reasonable amounts of biologically active material. In this report we describe the successful purification of this factor to homogeneity. It appears to be a glycoprotein of 38 kD in size, with amino acid sequence homology and bioactivity similar to DNase I.

Materials and Methods

Patient Population.

Urine was collected from febrile patients, or patients under treatment or recovering from recent febrile illnesses. The majority were patients with acute or chronic tuberculosis on antimycobacterial therapy. Most were also males because of the relative ease of collection of uncontaminated urine. Patients with documented or suspected HIV infection were excluded, as were all individuals from HIV infection high risk groups (male homosexuals and intravenous drug abusers).

Urine Collection.

All urine voided during a 24-h period was collected into 2-liter plastic containers containing sufficient granular ammonium sulfate, and sodium azide, so that the concentration of saturated ammonium sulfate would be 40%, and sodium azide would be 0.04% when the containers contained 1 liter of fluid. Urines were allowed to stand at room temperature, and were collected daily. Once collected, the urine volumes or amounts of salt were adjusted so that each specimen contained exactly 40% ammonium sulfate, and 0.04% sodium azide. These were pooled and stored at 4°C until processed further. All further procedures were performed at 4°C.

Urine Filtration and Concentration.

Pooled urines were processed in large batches (20–80 liters). Ammonium sulfate–insoluble urine precipitate was first allowed to settle by gravity sedimentation. Supernatant material was filtered through a 1.1-μm Acrodisc cartridge filter (Gelman Scientific, Northbrook, IL). The filtered urine was then concentrated in a Hollow Fiber Concentrator with a 10,000 mol wt cut-off (HP10; Amicon Corp., Danvers, MA). It was concentrated to a final volume of ∼1,000 ml, and then diafiltered against double glass-distilled water until the ammonium sulfate concentration was <1%.

Inhibitor Purification.

All subsequent procedures were performed at 4°C, and all buffers and dialysis fluids contained PEG-1,000 (0.01 g/liter) (Sigma Chemical Co., St. Louis, MO). All buffers and dialysis fluids were made with double glass-distilled water. From the ACA-54 fractionation procedure onward, the preparation was treated at the beginning of each step with 1 mM (PMSF) (Sigma Chemical Co.) to inhibit endogenous protease activity. Preparations were clarified and sterilized by passage through a 0.22-μm filter (Millex-GM; Millipore Continental Water Systems, Bedford, MA) before each column chromatographic step.

Salting Out with Ammonium Sulfate.   The diafiltered urine was first subjected to salting out with 80% saturated ammonium sulfate. After the addition of the appropriate amount of granular ammonium sulfate, the urine was allowed to stand at 4°C overnight with slow mixing. The insoluble material was collected by centrifugation at 10,000 RPM for 30 min in a high-speed centrifuge (RC2B; Sorvall, Newtown, CT). The protein pellet was resuspended in a minimum volume of distilled water (usually 400–800 ml), and dialyzed against 4 liters of distilled water, with four changes over a 48-h period. At the end of this time, the material was clarified by centrifugation (10,000 RPM × 30 min), and the resolubilized proteins in the supernatant were saved.

Ion Exchange Chromatography.   The pooled preparation of urine proteins that were soluble in 40% ammonium sulfate and insoluble at 80% ammonium sulfate were next subjected to fractionation by ion exchange chromatography. Pooled material was buffered with 10 mM sodium phosphate buffer, pH 7.5. This material was applied to a Tris-Acryl DEAE gel (Pharmacia Fine Chemicals, Piscataway, NJ) in a 2.5 × 25 cm column at a flow rate of 40 ml/h. After application of the sample, the column was washed with 10 mM phosphate buffer until the amount of protein eluting from the column, as measured by the absorption at 280 nm (A_{280}), returned to the baseline level. Proteins were eluted from the column using a stepwise gradient of sodium chloride in 10 mM phosphate buffer, pH 7.5 (50 mM NaCl, 250 mM NaCl, and 1 M NaCl). At each step the column was rinsed until the A_{280} returned to baseline. The urine preparation that remained bound after the 50-mM salt wash, but was

eluted by the 250-mM salt wash, was pooled and saved for further purification. This material had previously been found to contain the majority of the urine IL-1 inhibitor activity (17).

Molecular Sieve Chromatography.   The 50–250 mM salt eluate preparation was next subjected to molecular sieve chromatography on an ACA-54 gel (Pharmacia Fine Chemicals). The preparation was first concentrated to a volume of 5–6 ml using a flat membrane filter with a 10,000-mol wt cut-off, in a 50-ml stirred cell (YM-10 membrane, Amicon Corp.). This material was diafiltered with PBS (100 mM NaCl, 50 mM sodium phosphate buffer, pH 7.4). The concentrated material was applied to the top of a 2.5 × 84-cm glass column containing the ACA-54 gel, at a flow rate of 28 ml/h, and the proteins were eluted from the column using PBS. The column was precalibrated using 1 mg/ml protein standards (BSA; mol wt, 67,000; OVA, mol wt, 45,000; chymotrypsinogen; mol wt, 25,000; and cytochrome C; mol wt, 12,500). The proteins that eluted from the column with apparent mol wt between 25,000 and 45,000 were pooled and subjected to further purification.

Hydrophobic Chromatography.   The pooled material from the ACA-54 column was concentrated using a YM-10 filter and brought to a volume of 20–30 ml. This was then diafiltered into 10 mM potassium phosphate buffer, pH 6.4, and made 27% with respect to saturated ammonium sulfate. This sample was applied to a phenyl Sepharose gel (Pharmacia Fine Chemicals) in a 1.7 × 16-cm glass Econo Column (Bio-Rad Laboratories, Richmond, CA) at a flow rate of 12 ml/h. After the sample had been applied, the column was rinsed with 27% ammonium sulfate in phosphate buffer until no more protein eluted from the column. Remaining column proteins were then eluted using a 300-ml linear gradient beginning with 27% ammonium sulfate, in 10 mM phosphate buffer, and ending with 50% ethylene glycol in 4% ammonium sulfate, in phosphate buffer. After the completion of the gradient, the column was stripped with 50% ethylene glycol in 10 mM phosphate buffer. The column fractions containing maximum inhibitor activity were pooled, dialyzed, and concentrated using a YM-10 membrane.

Hydroxyl Apatite Chromatography.   The pooled material from the phenyl Sepharose column was concentrated to a volume of 20–30 ml and diafiltered into 5 mM potassium phosphate buffer, pH 6.8. This material was applied to a hydroxyl apatite gel (Bio-Rad Laboratories) in a 1 × 18-cm glass Econo Column at a flow rate of 12 ml/h. After the sample was applied, the column was washed with starting buffer until no more protein eluted. The remaining bound proteins were then eluted with 0.4 M potassium phosphate buffer, pH 6.8. The proteins that did not stick to the hydroxyl apatite column (5 mM phosphate buffer eluate) were pooled for further processing.

Phenyl Superose Chromatography.   The pooled material from the hydroxyl apatite column containing IL-1 inhibitor was next subjected to hydrophobic affinity chromatography with a phenyl Superose gel using a Fast Protein Liquid Chromatographic (FPLC)[1] apparatus (Pharmacia Fine Chemicals). Pooled material was concentrated to a volume below 10 ml and diafiltered into column running buffer (potassium phosphate buffer, pH 6.4). This was brought to a concentration of 27% saturated ammonium sulfate in phosphate buffer and applied to the phenyl Superose column at a flow rate of 0.5 ml/min. The column was washed with 27% saturated ammonium sulfate, and the retained proteins were then eluted using a 30-ml linear gradient beginning with 27% saturated ammonium sulfate and ending with 10 mM potassium phosphate buffer, pH 6.4. The fractions containing the maximum IL-1 inhibitor activity were pooled and concentrated.

HPLC.   The next step of purification was performed by hydrophobic interaction chromatography (HIC-HPLC) using a high performance liquid chromatographic apparatus, (HP 1090, Hewlett-Packard Co., Palo Alto, CA), equipped with a 4.6 × 200-mm Poly Propyl Aspartamide column (The Nest Group, Southborough, MA). Pooled material from the phenyl Superose Column (20–30 µg) was concentrated on a Centricon 10 (Amicon Corp.) membrane apparatus to a volume of 120 µl or less and diluted to 250 µl with 2 M ammonium sulfate in 100 mM potassium phosphate, pH 6.0, without polyethylene glycol 1,000 (PEG-1,000). The entire sample was injected on the column and eluted at a flow rate of 1.0 ml/min on a 30-min linear gradient from 2.0 M ammonium sulfate in 100 mM potassium phosphate,

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[1] *Abbreviations used in this paper:* FPLC, fast protein liquid chromatography; PEG, polyethylene glycol; HIC, hydrophobic interaction chromatography; PTH, phenylthiohydantoin.

pH 6.0, to 100 mM potassium phosphate, pH 6.0. Fractions of 0.5 ml were collected in tubes containing 0.05 ml of PEG-1,000 (0.01 mg/ml).

Reversed-phase HPLC. HIC-HPLC-purified material was purified to homogeneity for amino acid sequencing by reversed-phase HPLC. The chromatograph (HP 1090) was equipped with a 4.6 mm × 2 cm-column packed with 5 μM, 300 Å Vydac C18 (The Separations Group, Hisperia, CA). Approximately 60 μg of material purified by HIC-HPLC (Fig. 5, peak *IV*) was concentrated to a volume of 500 μl on a Centriprep 10 (Amicon Corp.) membrane apparatus. The sample (250 μl) was injected and eluted at 1.5 ml/min on a linear solvent gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in 75% acetonitrile. Fractions of 0.5 ml each were collected across the peak which eluted at 10.3 min (Fig. 6). Fractions containing a single band with an M_r of 38 kD by SDS-PAGE were subjected to NH$_2$-terminal amino acid sequencing.

Mouse Thymocyte Bioassay.

IL-1 inhibitor activity was measured using a mouse thymocyte bioassay (3). 1-ml aliquots of test samples were prepared for bioassay by dialysis in standard dialysis tubing that had been boiled in double-distilled water with several changes. They were dialyzed at 4°C against ~4 liters of 5 mM Hepes buffer, pH 7.5, with two changes over an 18-h period. Samples were then made isotonic with 10× RPMI, reconstituted with 1% heat-inactivated FCS, and then sterilized by 0.22-μm micropore filtration (Millipore Continental Water Systems). Samples were tested at multiple dilutions depending on their estimated IL-1 inhibitory activity. They were added to a single cell suspension of mouse thymocytes to which PHA (1 μg/ml) (purified PHA; Burroughs Wellcome Co., Research Triangle Park, NY) and human rIL-1α (2 U/ml, Hoffman-La Roche Inc., Nutley, NJ) had been added. Thymocytes were cultured for 72 h, and IL-1 inhibitory activity was measured as previously described (3).

IL-1 inhibitory activity as measured in units/milliliter was calculated as the reciprocal of the last dilution of sample that produced at least 30% inhibition.

SDS-PAGE Analysis and Protein Determinations.

Samples were analyzed for purity using SDS-PAGE analysis, as previously described (17). Aliquots (0.1 ml) of dialyzed samples were lyophilized, reconstituted with sample buffer containing 2-ME, boiled for 5 min, and 25-μl aliquots were subjected to electrophoresis on either 10% SDS-polyacrylamide gels, or 10–20% gradient gels. Gels were stained with silver nitrate. Protein concentrations were measured using a bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL).

Enzymatic Cleavage by Trypsin.

Protein peaks from reversed-phase HPLC were dried in a Speed Vac Concentrator (Savant, Farmingdale, NY). The dried residue was dissolved in 1% ammonium bicarbonate to yield a solution of ~0.5 mg/ml. Trypsin (TPCK treated; Serva Fine Biochemicals Inc., Garden City Park, NY; 1 mg/ml solution in water) was added (2% wt/wt from the protein sample weight) and the mixture was incubated at 37°C. After 6 h a second portion of 2% wt/wt trypsin was added and the incubation was continued overnight. The resulting tryptic peptides were separated by reversed-phase HPLC using a C 18 column (μBondapak; Waters Associates, Milford, MA) (10-μm particle diameter, 3.8 × 300 mm). The mobile phases were 0.1% trifluoroacetic acid in water (buffer A) and in acetonitrile (buffer B). The gradient was run from 0% to 55% B within 55 min. Flow rate was 1 ml/min. Detection was done by UV absorption at 214 and 280 nm, simultaneously. Two of the tryptic peptides were collected manually, dried in a Speed Vac Concentrator, and used for sequencing.

Amino Acid Sequence Analysis.

The dried residue of protein or peptide peaks was dissolved in 75 μl 70% formic acid. This solution was applied in three portions to the cartridge of a gas phase sequenator (model 470 A, Applied Biosystems, Inc., Foster City, CA). Sequencing was done by the program 39APTH, a slightly modified version of the program 02RPTH, which is supplied by the manufacturer. The cleaved phenylthiohydantoin (PTH) amino acids were collected in a frac-

tion collector, dried in a Speed Vac Concentrator, and redissolved in 50 µl of the mobile phase of the HPLC system used for the identification. The HPLC system consisted of a Supersphere RP8 column (Merck & Co., Inc., Rahway, NJ) (4-µm particle diameter, 4.6 × 250 mm) and an isocratic mobile phase of sodium acetate, acetonitrile, and dichloroethane (22). The flow rate was 1 ml/min. Detection was done by UV absorption at 254 nm. Retention times of PTH amino acids in the sequencer samples were compared with amino acid standards (Pierce Chemical Co.).

Assay for Deoxyribonuclease Activity.

The inhibitor (HPLC purified) was compared with bovine DNAase I (D-4513; Sigma Chemical Co.) for the ability to hydrolyze [^{14}C]DNA (CFB. 170; Amersham Corp., Arlington Heights, IL; sp act, 39 µCi/mg). [^{14}C]DNA (0.2 µg) was incubated with 0.03 µg of bovine DNAase I or the inhibitor in a 0.01 M Tris, 4 mM MgCl$_2$ buffer, pH 7.0, in a total volume of 0.1 ml. At 15, 30, 60, and 90 min, the DNA was precipitated at 4°C by 5% TCA, 0.5% Na$_2$H$_2$P$_2$O$_7$. After a 15-min incubation period, the precipitated DNA was harvested onto glass-fiber filter paper using a Skatron cell harvester (Flow Laboratories, Rockville, MD) with a 5% TCA, 0.5% Na$_2$H$_2$P$_2$O$_7$ wash, followed by a 70% ethanol wash. Filters were dried and cpm was determined by β scintillation counting.

Results

Initial Purification.   Purification was begun with a batch of pooled urine collected in 40% saturated ammonium sulfate. This material was filtered, and proteins insoluble in 40% ammonium sulfate were discarded. The solution was then concentrated in a hollow fiber concentrator, diafiltered to remove ammonium sulfate, and brought to a final concentration of ∼1,000 ml. The soluble proteins were then precipitated using 80% saturated ammonium sulfate, and resolubilized in water and dialysed. At this stage, the volume of sample was ∼800 ml. This material was then applied to a large ion exchange column, and the proteins that eluted at sodium chloride concentrations between 51 and 250 mM were collected and pooled. Previous studies and preliminary results indicated that this initial purification method resulted in the highest recovery of inhibitory activity (17).

Chromatographic Purification.   The material from the ion exchange column was next subjected to molecular sieve chromatography on an ACA-54 gel. Proteins eluted from this column with a small void volume peak, followed by a broad, asymmetrical peak ranging in apparent molecular mass from 80 to ∼20 kD (Fig. 1). IL-1 inhibitor activity was found to elute from this column in a symmetrical peak with an
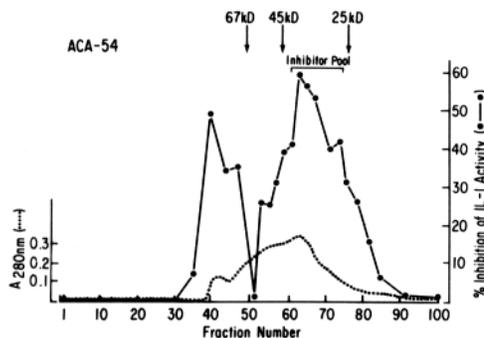
FIGURE 1.   Molecular sieve chromatography. A 6-ml sample of the IL-1 inhibitor–rich pool from the Tris-acryl DEAE step (50–250 mM cut) was applied to the column containing ACA-54 gel and eluted with PBS at a flow rate of 28 ml/h. Fraction size, 3.8 ml.

apparent molecular mass of ~40 kD. A smaller amount of inhibitory activity eluted with the void volume peak. This material was processed separately.

The pooled IL-1 inhibitor containing material from the ACA-54 column was next applied to a phenyl Sepharose column. The majority of the applied proteins adhered to this column and began to elute when ~40% of the gradient had gone through the column (Fig. 2). IL-1 inhibitory activity was found in a symmetrical peak in the latter two thirds of the gradient run. IL-1 inhibitory activity began to elute when the elution buffer contained 15% ammonium sulfate and 25% ethylene glycol and was substantially removed from the column when the elution buffer contained 9% ammonium sulfate and 40% ethylene glycol.

IL-inhibitory material was pooled, concentrated, and subjected to hydroxylapatite chromatography. Preliminary testing with both linear and stepwise gradients indicated that 70–90% of the IL-1 inhibitor activity did not adhere to hydroxylapatite in 5 mM phosphate buffer, and the remainder of the activity eluted in a very broad peak ranging from 10 to 200 mM phosphate buffer. Material was therefore eluted from the column using a two-step gradient. The elution profile consisted of a broad peak of proteins that did not stick to the column in 5 mM phosphate buffer (Fig. 3, fractions *I* and *II*), followed by a very sharp peak as the rest of the proteins were stripped from the column with 0.4 M phosphate buffer (Fig. 3, fraction *IV*). As indicated, ~80% of the IL-1 inhibitory activity did not stick to this column, and the remainder eluted with the high phosphate buffer.

The remainder of the purification was performed on the material that did not stick to hydroxylapatite (Fig. 3, fractions *I* and *II*), since this was found to contain higher specific activity and fewer contaminating proteins. Fractions I and II from the hydroxyl apatite column were pooled, concentrated, and next subjected to hydrophobic affinity chromatography using an FPLC apparatus fitted with a phenyl Superose column. The bulk of the proteins adhered to this column. The elution profile consisted of a broad shallow peak in the early phase of the gradient followed by one to two sharp peaks that eluted close to the end of the gradient (Fig. 4). IL-1 inhibitory activity was found to elute at ammonium sulfate concentrations ranging between 10% and 15%, and corresponded to two small protein peaks that immediately preceded the last peak.
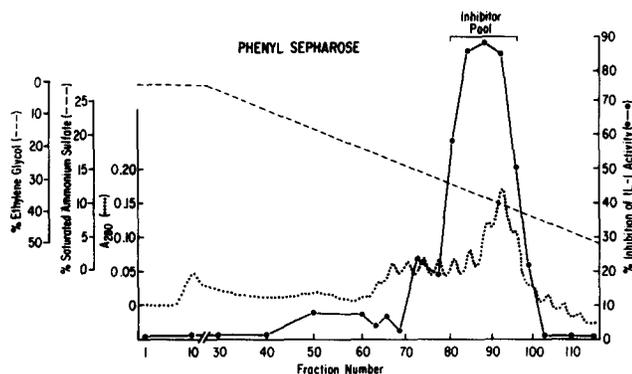
FIGURE 2.   Hydrophobic chromatography. A 7-ml sample of the IL-1 inhibitor pool from the ACA-54 step (see Fig. 1) in 27% ammonium sulfate was applied to a column containing phenyl Sepharose gel and eluted at a flow rate of 12 ml/h as described in Materials and Methods. Fraction size, 3 ml.
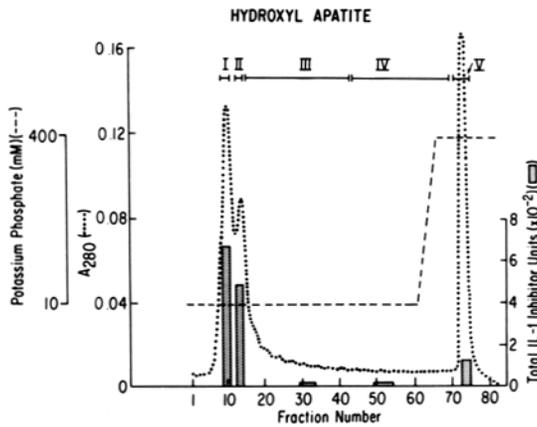
HYDROXYL APATITE

FIGURE 3. Hydroxyl apatite chromatography. A 4.5-ml sample of the IL-1 inhibitor pool from the phenyl Sepharose step (see Fig. 2) in 5 mM potassium phosphate buffer was applied to a column containing hydroxyl apatite gel and eluted at a flow rate of 12 ml/h, as described in Materials and Methods. Fraction size, 3 ml.

The material from the phenyl Superose was pooled, concentrated, and applied to a polypropyl aspartamide column using an HPLC apparatus. This resulted in a UV absorbance (210 nM) elution profile with an early peak, a broader middle region containing three peaks, and a late, sharp peak (Fig. 5, *I*, *III*, and *IV*, respectively) at the end of the gradient. IL-1 inhibitory activity was found to elute exclusively in the last peak.

The inhibitor rich peak from the polypropyl aspartamide column was subjected to a final purification by reversed-phase HPLC. When chromatographed on a C18 column, the inhibitor rich material eluted as a sharp homogeneous peak with a retention time of 10.3 min (Fig. 6). A small, broad peak containing residual contaminating proteins eluted immediately before the inhibitor (9.5 min).

Purification of IL-1 Inhibitor from a Single Batch of Urine. IL-1 inhibitor was next purified from 70 liters of urine using the preceding eight-step sequence.

PHENYL SUPEROSE (FPLC)
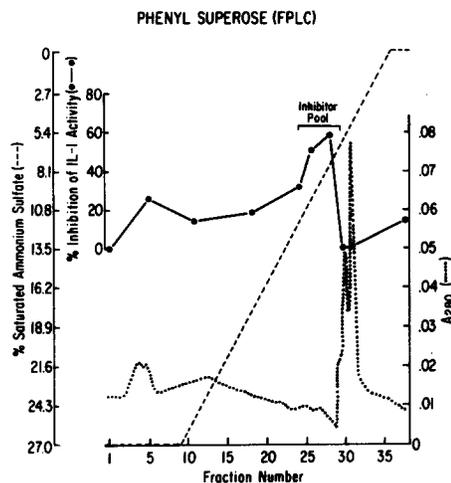
FIGURE 4. Fast protein liquid chromatography. A 1.5-ml sample of the IL-1 inhibitor pool from the hydroxyl apatite column (see Fig. 3) in 27% ammonium sulfate, was applied to a phenyl Superose column using a fast protein liquid chromatographic apparatus and eluted as described in Materials and Methods. Fraction size, 1 ml.

PROPYL ASPARTAMIDE (HPLC)
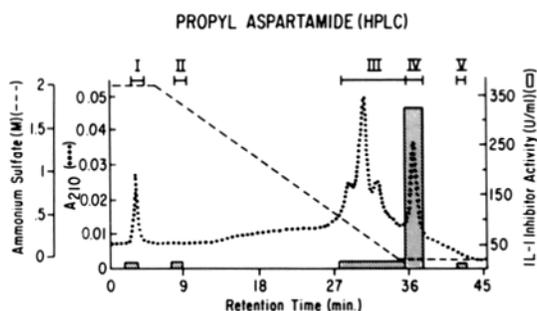
FIGURE 5. Hydrophic interaction chromatography. A 0.5-ml (25 μg) sample of the IL-1 inhibitor pool from the phenyl Superose column (see Fig. 4) in 2 M ammonium sulfate was applied to a polypropyl aspartamide column using an HPLC apparatus, and eluted at a flow rate of 1 ml/min as described in Materials and Methods. Fraction size, 0.5 ml.

IL-1 inhibitor–containing samples from each of the chromatographic steps were subjected to SDS-PAGE, and gels were stained with silver. As expected, early fractions were contaminated with multiple proteins (Fig. 7). However, purification of IL-1 inhibitor activity was associated with increasing concentrations of a 38-kD band. The inhibitor preparation that resulted from the HPLC step (propyl aspartamide) was essentially homogeneous. Bands that appear at 55 and 68 kD (Fig. 7, lanes *3–5*) represent skin keratins that occasionally contaminate SDS-PAGE buffer solutions (23). The preparation resulting from the second HPLC step (reversed phase) also contained only the 38-kD band.

To be certain that the 38-kD protein was the thymocyte inhibitor, material from another purification run (through the propyl aspartamide step) was subjected to SDS-PAGE analysis (Fig. 7, lane *7*). This preparation also contained the 38-kD band, but the higher molecular mass contaminants were not present.

Purification of the inhibitor resulted in a significant increase in specific activity. Pooled material from the ACA-54 column had a sp act of 581 U/mg while the homogeneous propyl aspartamide purified pool had a sp act of 3.4×10^4 U/mg (Fig. 7). The material from the reverse-phase column was not tested for biological activity since the elution conditions destroy inhibitor activity. The propyl aspartamide–purified material from the second run also had a high sp act (9×10^4 U/mg). Overall yield of biologically active inhibitor after the propyl aspartamide step was ~1%.

Amino Acid Sequence. A partial amino acid sequence of the reversed-phase HPLC-purified inhibitor preparation was determined. Information was obtained on the first 23 NH_2-terminal amino acids, as well as on two peptide fragments representing amino acids 48–60 and 61–70 (Fig. 8). Comparison of the unambiguous amino acid
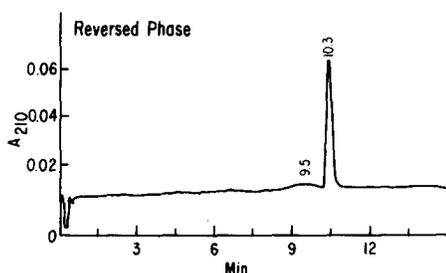
FIGURE 6. Reversed-phase chromatography. A 0.25-ml (30 μg) sample of HIC-HPLC-purified inhibitor (see Fig. 5) was injected on a 4.6 mm × 20 mm Vydac C18 column and eluted at 1.5 ml/min as described in Materials and Methods. Fraction size, 1.5 ml.
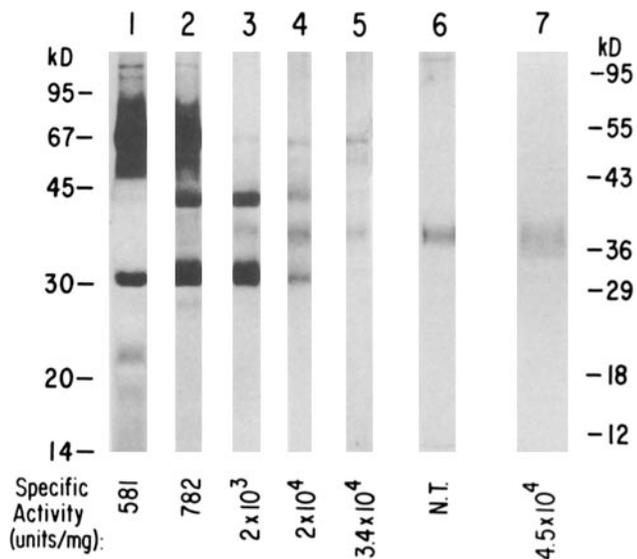
FIGURE 7. SDS-PAGE analysis. Samples of the inhibitor pools from each of the chromatographic steps were diluted 1:10, dialyzed to remove all salts, and lyophilized. Samples were reconstituted with 50 μl of sample buffer containing 2-ME and boiled for 5 min. 25 μl aliquots were then subjected to electrophoresis on a 10% SDS-polyacrylamide gel, fixed, washed, and stained with silver nitrate. Lane *1*, ACA-54; lane *2*, phenyl Sepharose; lane *3*, hydroxyl apatite; lane *4*, phenyl Superose; lane *5*, propyl aspartamide; lane *6*, reversed-phase HPLC; lane *7*, propylaspartamide-purified material from a second purification run (10-20% gradient gel).

sequence with the European Molecular Biology Laboratory (Heidelberg, Federal Republic of Germany) protein data bank revealed that this portion of the inhibitor had at least a 78% homology with bovine DNase I. No human DNase sequence was available for comparison.

DNase Activity of the Inhibitor. Because of the sequence homology with DNase I, the purified inhibitor preparation (propyl aspartamide pool) was analyzed for DNase activity in vitro. Labeled DNA was incubated with equivalent concentrations of either the inhibitor, or a sample of bovine DNase I. After 15, 30, 60, and 90 min, DNA hydrolysis was measured by determining residual TCA-precipitable counts. The inhibitor was found to degrade DNA as actively as the authentic enzyme (Fig. 9). These data, in conjunction with the amino acid sequence findings, strongly suggest that the human urine–derived inhibitor is a type of DNase I.

Discussion

The human urine–derived febrile inhibitor has been purified to apparent homogeneity using a sequence of eight purification steps. Upon SDS-PAGE analysis (reduced) using a sensitive silver stain, this protein appears to be 38 kD in size, and the final material appears to be devoid of any other detectable protein impurities. The purified material is active in vitro in the nanogram range, with a sp act of 34,666 IL-1 inhibitor U/mg. Partial amino acid sequence analysis of the purified inhibitor suggests a close homology with other mammalian (bovine, porcine, and ovine) DNase I enzymes (24). Furthermore, the inhibitor was found to have as potent enzymatic activity as a bovine pancreatic DNase I standard. Finally, preliminary experiments in our laboratory demonstrate that concentrations of bovine pancreatic DNase I preparations exhibit thymocyte inhibitory activity. These findings strongly suggest that the inhibitor of IL-1-induced thymocyte proliferation found in the urine of febrile individuals is a type of DNase I.

1	2	3	4	5	6	7	8	9	10
LEU	LYS	ILE	?	ALA	PHE	ASN	ILE	X	THR
LEU	LYS	ILE	ALA	ALA	PHE	ASN	ILE	ARG	THR

11	12	13	14	15	16	17	18	19	20
PHE	GLY	GLU?	THR	LYS	MET	SER	?	ALA	THR?
PHE	GLY	GLU	THR	LYS	MET	SER	ASN	ALA	THR

21	22	23			48	49	50	51	52
LEU	?	SER?			LEU	LEU	ASP	ASN	LEU
LEU	ALA	SER			LEU	LEU	ASP	TYR	LEU

53	54	55	56	57	58	59	60	61	62
ASN	GLN	ASP	ALA	PRO	ASP	THR	TYR	HIS	TYR
ASN	GLN	ASP	ASP	PRO	ASN	THR	TYR	HIS	TYR

63	64	65	66	67	68	69	70		
VAL	VAL	SER	GLU	PRO	LEU	GLY	ARG		
VAL	VAL	SER	GLU	PRO	LEU	GLY	ARG		

FIGURE 8.   Partial amino acid sequence of the human urine-derived IL-L inhibitor. (?) Could not be identified unambiguously. (X) A cycle where no amino acid could be found.

Available evidence also indicates that the inhibitor is of human origin. Recent experiments in our laboratory revealed that it contains a large amount of N-linked sugars, indicating that it is not derived from bacteria (data not shown). Some viral glycoproteins have been found to inhibit IL-1 (25, 26). However, the close homology between the inhibitor and other mammalian DNase enzymes argues against a viral origin. Other recent experiments in our laboratory indicate that a monospecific rabbit anti-inhibitor antibody does not crossreact with any bovine DNase preparation (data not shown). This fact, in conjunction with the differences between the amino acid sequence of the inhibitor and other mammalian DNase I enzymes already found, make it likely that the inhibitor is a human DNase I.

DNase I-like enzymes have been identified in numerous organs of the rat, including large amounts in lymph node and kidney, and smaller amounts in blood cells (27). Interestingly, most of these tissues contain a 31-kD DNase I, except for kidney, which contains an additional higher molecular mass enzyme of ~36 kD. Although a similar high molecular mass DNase has not yet been reported in humans, it is likely that the human urine–derived IL-1 inhibitor is related to the higher molecular mass form of the rat kidney DNase I.

Several other IL-1 inhibitors have been isolated from human urine. Based on this
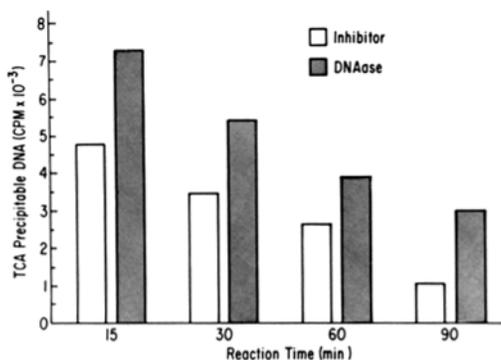
FIGURE 9.   DNAase activity of the inhibitor. Samples of DNAase I and the purified inhibitor were incubated with 0.2 µg of [^{14}C]DNA. DNA hydrolysis was determined by the loss of TCA precipitable counts.

purification as well as previous findings, the febrile inhibitor appears to be distinct from each of these. Our earlier data strongly suggested that it was distinct from uromodulin, the 85-kD molecule that is present in large amounts in the urine of pregnant women (2). The evidence included differences in size and a lack of antigenic crossreactivity (19). We had also noted that the febrile inhibitor exerted its effect late in the activation cycle of thymocytes (19), whereas uromodulin, which appears to act by binding IL-1, only inhibits when added relatively early during thymocyte culture (28). Since there is a close connection between uromodulin and the Tamm-Horsfall glycoprotein (28, 29), the finding in this paper confirming the lower molecular weight of the febrile inhibitor further supports the suggestion that the febrile inhibitor and uromodulin are distinct molecules.

The febrile inhibitor is also distinguishable from the inhibitor first found in the urine of a patient with monocytic leukemia by Dayer and coworkers (5, 21), since the latter molecule is smaller in size (20–25 kD) than the febrile inhibitor. The monocytic inhibitor also appears to block IL-1-induced fibroblast PGE_2 production, while no preparation of febrile inhibitor has ever blocked fibroblast activity (30). Dayer and MacDonald (21) have also reported that this lower molecular weight inhibitor blocks the binding of IL-1 to its receptor while the febrile inhibitor does not interfere with IL-1-R binding when tested either indirectly (19) or directly (D. Rosenstreich, manuscript in preparation).

On the other hand, over the past 10 yr, there have been numerous reports of IL-1 inhibitors derived from various sources that block thymocyte proliferation in vitro (6–16). It is possible that some or all of these may be DNase-like molecules, and the findings presented in this report may help to clarify or reconcile some of these disparate observations. This hypothesis is amenable to experimental verification using established methodology.

There remain a number of interesting and important questions about the IL-1 inhibitor, including its mechanism of action in vitro, its cellular source and tissue of origin, and its physiologic role. Answering many of these questions should be facilitated by its recent purification and identification.

Summary

We have previously reported that the urine of febrile humans contained large quantities of an inhibitor of IL-1-induced murine thymocyte proliferation that was a glycoprotein between 30 and 40 kD in size. In the present study this factor has been purified to homogeneity using a sequence of eight purification steps (ammonium sulfate precipitation, ion exchange chromatography, molecular sieve chromatography, hydrophobic affinity chromatography, hydroxylapatite chromatography, fast protein liquid chromatography, and two HPLC steps). SDS-PAGE analysis indicates that the purified material is a 38-kD molecule. Evidence based on a partial amino acid sequence analysis as well as enzyme studies indicates that this inhibitor is a type of human DNase I.

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