

Purification and Characterization of Interleukin 1 Receptor Level Antagonist Proteins from THP-1 Cells*

(Received for publication, February 15, 1990)

Michael J. Bienkowski^{‡§}, Thomas E. Eessalu[‡], Ann E. Berger[‡], Scott E. Truesdell[¶], John A. Shelly[¶], Alice L. Laborde[¶], Heidi A. Zurcher-Neely^{||}, Ilene M. Reardon^{||}, Robert L. Heinrikson^{||}, John G. Chosay^{**}, and Daniel E. Tracey^{**}

From the Departments of [‡]Cell Biology, [¶]Chemical and Biological Screening, ^{||}Biopolymer Chemistry, and ^{**}Hypersensitivity Diseases Research, The Upjohn Company, Kalamazoo, Michigan 49007

Culture medium conditioned by phorbol 12-myristate 13-acetate-differentiated THP-1 cells contained interleukin 1 (IL-1) antagonist activity as measured by inhibition of both IL-1 β binding to receptors on YT cells and inhibition of IL-1/phytohemagglutinin-stimulated IL-2 synthesis by LBRM-33-1A5 T cells. Based on their ability to compete for ¹²⁵I-IL-1 β binding to receptors on YT cells, four distinct antagonist proteins were purified from THP-1 cell conditioned medium using a combination of ion-exchange, hydrophobic interaction, and size exclusion chromatographies. The four proteins had different isoelectric points with molecular masses in the range 22–26 kDa and had similar specific activities for inhibition of IL-1 β binding to cell surface receptors (K_i values 0.33–0.64 nM) and for inhibition of IL-1/phytohemagglutinin-stimulated IL-2 synthesis by 1A5 cells (IC_{50} values 25–100 pM). Amino-terminal sequence analysis of the two major forms (25 kDa/pI 5.1 and 22 kDa/pI 5.8) revealed complete identity for the first 27 residues in both forms. Based on the results of peptide mapping, amino acid compositional analysis and immune blotting, all of the forms were deduced to be variants of a common protein. Deglycosylation of the antagonist proteins with *N*-glycanase converted them to a common form (22 kDa/pI 5.8), indicating that the four isoforms represent glycosylation variants of a common protein and that asparagine-linked oligosaccharides are responsible for the observed size and charge heterogeneity.

Interleukin 1 (IL-1)¹ α and β are cytokines with limited sequence homology, but which bind to the same receptor and have common effects on a large number of target cells (reviewed in Refs. 1 and 2). A role for IL-1 inflammation has been well documented (3, 4). IL-1 stimulates the synthesis of

inflammatory eicosanoids in macrophages, fibroblasts, synovial cells, and chondrocytes and the production of collagenase by the latter two cell types (4, 5). It is also found in elevated levels in joint fluid of patients with active arthritis and is believed to contribute to leukocyte activation and tissue destruction (6). Given that IL-1 is a principal mediator of inflammation, interfering with its activity represents an approach for developing a disease modifying therapy for chronic inflammatory diseases. Regulation of IL-1 activity can, in principle, occur at the level of synthesis (transcription and/or translation), processing, secretion, IL-1 receptors, or receptor signal transduction. Feedback regulation of IL-1 synthesis has been shown to involve the brain-pituitary-adrenal axis since IL-1 induction of adrenocorticotropin release results in increased synthesis of glucocorticoid steroids which in turn block IL-1 synthesis (7–9). IL-1 antagonist factors which inhibit various *in vitro* activities of IL-1 have been described from a number of diverse sources (reviewed in Ref. 10), but those synthesized by mononuclear cells or found in febrile urine have been characterized in the most detail (11–15). IL-1 antagonists of 22–25 kDa have been partially purified from medium conditioned by adherent monocytes stimulated with immobilized immune complexes (11) and from GM-CSF stimulated human monocytes (12). Inhibitors having similar physical properties have also been partially purified from febrile urine (13–15). In each case, these partially purified antagonists have been shown to inhibit various *in vitro* activities of IL-1. Inhibition of IL-1 receptor binding has been demonstrated for both the monocyte- (12, 16) and urine-derived antagonists (17). Two reports concerning the purification and characterization of IL-1 antagonist proteins from IgG-stimulated human monocytes (18) and GM-CSF-stimulated U937 cells (19) were recently published. The human monocyte antagonist proteins were resolved into three forms (18–22 kDa) with pI values in the range 4.5–5.3. The results of sequence analysis and deglycosylation experiments verified that the three forms represent glycosylation variants of a common protein (18). A 25 kDa/pI 5.0 IL-1 antagonist glycoprotein has been isolated, sequenced, and cloned from U937 cells (19) and is identical to the protein cloned from human monocytes (20).

We have demonstrated the synthesis and secretion of IL-1 antagonist proteins by PMA-differentiated THP-1 cells. Conditioned medium from these cells yielded four different IL-1 antagonist proteins which were purified based on their ability to inhibit ¹²⁵I-IL-1 β binding to receptors on YT cells. The four forms varied in isoelectric points, but all had similar specific activities for inhibition of IL-1 receptor binding and inhibition of IL-1/phytohemagglutinin-stimulated IL-2 synthesis by murine LBRM-33-1A5 T-cells (1A5). Structural

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§ To whom correspondence should be addressed. Tel.: 616-385-7001.

¹ The abbreviations used are: IL-1, interleukin 1; GM-CSF, granulocyte-macrophage colony-stimulating factor; PMA, phorbol 12-myristate 13-acetate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IEF, isoelectric focusing; LAF, lymphocyte activating factor; FBS, fetal bovine serum; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; PTH, phenylthiohydantoin; GuHCl, guanidine hydrochloride; TPCK, *N*-tosyl-L-phenylalanyl chloromethyl ketone; TFA, trifluoroacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

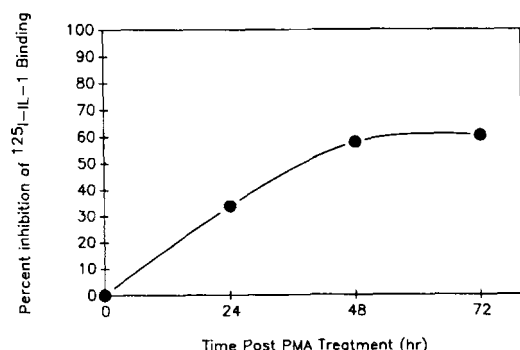


FIG. 1. Kinetics of IL-1 antagonist production by differentiated THP-1 cells. THP-1 cells (5×10^5 /ml) were treated with 100 nM PMA in RPMI 1640/10% fetal bovine serum for 48 h and the adherent (differentiated) cells washed with phosphate-buffered saline. The differentiated cells were then cultured in RPMI 1640/1% fetal bovine serum for various times and IL-1 antagonist activity quantified in the receptor binding assay.

analysis of the two major forms revealed that they had identical amino-terminal sequences. Based on comparisons of peptide maps, amino acid compositions, immunological cross-reactivities, and the results of deglycosylation experiments, all four forms were shown to be glycosylation variants of a common polypeptide. Comparison of the peptide sequences obtained from the THP-1-derived IL-1 antagonist proteins with those reported for the human monocyte antagonist (18) and predicted from cDNAs (19, 20) revealed that they are all similar if not identical proteins.

EXPERIMENTAL PROCEDURES

RESULTS²

Conditioned medium from THP-1 cells does not contain detectable amounts of IL-1 antagonist activity unless the cells are first differentiated with PMA. The conditions for maximal production of IL-1 antagonist activity were determined (PMA concentration and time of PMA treatment) and the kinetics of secretion of IL-1 antagonist activity under these conditions are shown in Fig. 1. Treatment of the cells with 100 nM PMA for 48 h caused the cells to growth arrest, adhere to the culture dish, and begin to constitutively produce IL-1 antagonist activity as measured by inhibition of ¹²⁵I-IL-1 binding to receptors on YT cells (Fig. 1) and in the 1A5/HT-2 assay (data not shown). The activity accumulated in the supernatant and reached a steady state level 48 h post-differentiation. The cells were then mass-cultured under these conditions to provide starting material for purification. The details of the purification scheme are shown in Figs. 2–8 and Table 1 in the Miniprint Section.

The purification protocol that we developed resolved four distinct IL-1 antagonist proteins, referred to as Peaks A, B, C, and D (26 kDa/pI 4.9, 25 kDa/pI 5.1, 25 kDa/pI 5.7, and 22 kDa/pI 5.8, respectively) based on their order of elution from a Mono S column at pH 6.0 (Fig. 6). The activity of each of these forms was profiled by measuring the concentration-dependent inhibition of ¹²⁵I-IL-1 β binding to receptors on YT cells. The results, shown in Fig. 9, demonstrate that all four antagonists had similar potency for IL-1 β displacement with IC_{50} values in the range 0.60 to 1.15 nM (IL-1 β 0.16 nM). Hill

plots of the binding data revealed a slope near unity, indicating noncooperative binding of the antagonist. Given this simple binding model, K_i values were calculated from the IC_{50} values using the Cheng-Prusoff relation (28). The K_i values varied over the range 0.33 to 0.64 nM where the K_i for homologous IL-1 β competition was 0.08 nM under these conditions.

The structural relationships among the four IL-1 antagonist isoforms were determined by a combination of peptide mapping, sequencing, and immune blotting. The two forms recovered in the highest yield (Peak B, 25 kDa/pI 5.1, and Peak D, 22–21 kDa/pI 5.8) were selected for sequence analysis. Direct sequencing of the Peak B material gave a unique sequence for 27 cycles, shown in Table 2. Additional sequencing was performed on tryptic peptides derived from digestion of S-pyridylethylated Peak B protein. The tryptic peptides were resolved by reverse phase HPLC and the sequences of the major peptides are also shown in Table 2. Two of the peptides, KMQAFR and KTFYLR, exactly matched regions of the amino-terminal sequence of the Peak B protein. The Peak D material, prepared by pooling the 19-kDa GF3000 fractions and Mono S Peak D protein, consistently migrated as a closely spaced doublet on SDS-polyacrylamide gel electrophoresis (Fig. 7) and could be resolved into two pI forms (5.8 and 4.8) by isoelectric focusing (Fig. 8). Direct sequencing of the Peak D material showed that it was composed of a 40/60 mixture of intact protein (same sequence as the Peak B protein) and a truncated form that was missing the first six amino acids (des-6 antagonist). Removal of the peptide RPSGRK, presumably by a trypsin-like cleavage, removes 3 basic residues and accounts for the more acidic form noted in isoelectric focusing analysis. The combined sequencing data from the Peak A and Peak D proteins represents a total of 44 residues, which accounts for approximately 30% of the expected protein sequence for these two isoforms. The remaining two antagonist proteins (Peak A and Peak C proteins) were compared to the Peak B and D proteins by Western blotting using a polyclonal antiserum raised against recombinant human IL-1 antagonist protein obtained by *Escherichia coli* expression of a cDNA cloned from U937 cells (19). The results are shown in Fig. 10. The specificity of the antiserum was demonstrated by the lack of cross-reaction with either IL-1 α or IL-1 β (lanes 1, 9, and lanes 2, 10, respectively) and the strong reaction with the recombinant antigen (lanes 3 and 8). Peaks A, B, C, and D proteins (lanes 7–4, respectively) all showed reactions that were comparable to that of a similar loading of the recombinant antigen. Finally, we performed amino acid compositional analysis on all four forms and the compositions were identical within experimental error (data not shown).

The conclusion from the structural and immunochemical studies was that each of the four forms had similar if not identical protein sequences. The source of the size and charge heterogeneity was shown to be due to the asparagine-linked oligosaccharides using N-glycanase treatments. The results of digestion of Peak A–D proteins with N-glycanase are summarized in Table 3. Removal of the asparagine-linked oligosaccharides converted Peak A, B, and C proteins to a common 22-kDa form while the Peak D protein was unaffected by this treatment (no asparagine-linked oligosaccharides). Peak A, B, and C proteins were all converted to the same pI form (5.8) as the Peak D protein, indicating that the asparagine-linked oligosaccharides are the source of both the charge and size heterogeneity.

² Portions of this paper (including "Experimental Procedures," Table 1, and Figs. 2–8) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

FIG. 9. Receptor competition curves for Mono S Peaks A–D. Serial dilutions of IL-1 antagonist proteins Mono S Peaks A–D in the range 0.01–10 nM (based on amino acid composition) were incubated with YT cells in the presence of a constant amount of ^{125} I-IL-1 β (50 pM) as described under “Experimental Procedures” and bound label quantified by γ counting.

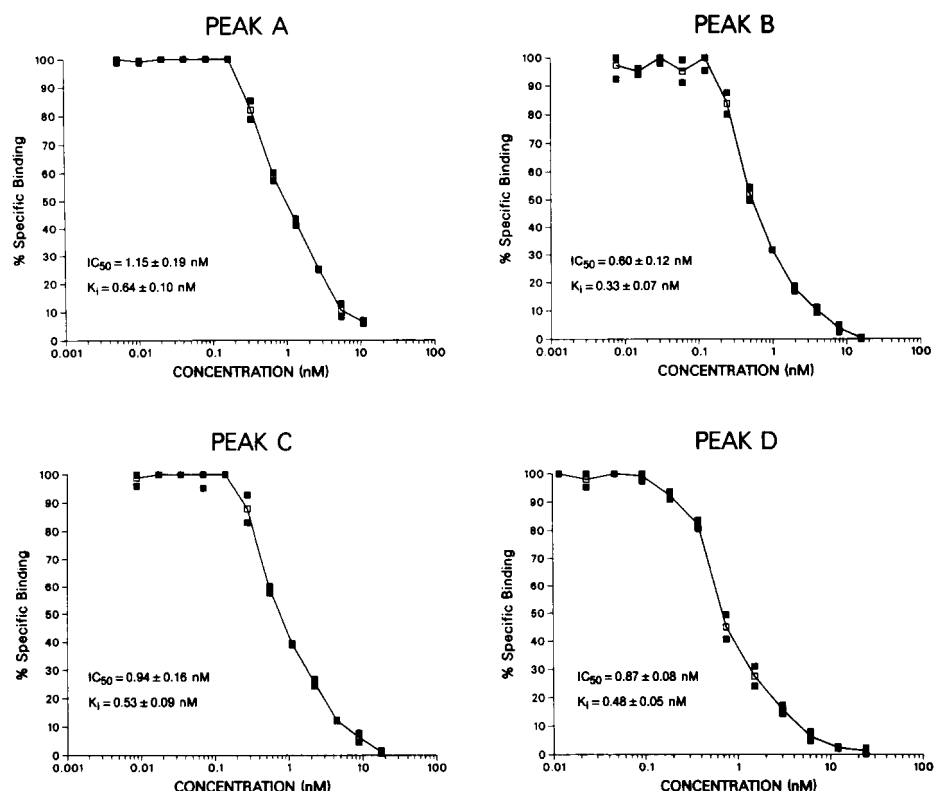


TABLE II
Summary of IL-1 antagonist peptide sequences

Sample	Amino-terminal sequence
Peak B	RPSGRKSSKMQAFRIWDVNQKTFYLRN
Peak D	RPSGRKSSKMQAFRIWDVNQ (40%) SSKMQAFRIWDVNQKTFYLR (60%)
Peak B	Tryptic peptides ^a
	T1 (21.23 min) KMQAFR
	T3 (29.28 min) KTFYLR
	T4 (31.47 min) (K) ^b FYFQE
	T5 (33.42 min) (R)FAFIR
	T8 (43.05 min) (K)IDVVP

^a Denatured and S-pyridothylated Mono S Peak B was digested with *N*-tosyl-L-phenylalanyl chloromethyl ketone-trypsin and mapped by reverse phase HPLC. The major peaks detected at 215 nm were sequenced by automated gas phase Edman degradation with on-line detection of the phenylthiohydantoin derivatives from each cycle. Mono S Peak D (10 μ g, 0.5 nmol) was sequenced directly for 20 cycles.

^b Amino-terminal residue assigned based on the specificity of trypsin and comparison with a protein sequence predicted from cDNA (19).

DISCUSSION

The human monocyte cell line THP-1 constitutively produces IL-1 antagonist proteins only after cell differentiation by treatment with PMA. Differentiation of these cells results in growth arrest, adherence to the culture dish, and the appearance of macrophage phenotypic markers (29). Since the undifferentiated cells produce IL-1 (30) and not IL-1 antagonist, synthesis of the antagonist may be a property associated with macrophage maturation. The production of IL-1 antagonist activity by unstimulated monocyte-like cell lines (murine P388D1 and human M20 cells) has been reported (31, 32). Induction of IL-1 antagonist activity synthesis in peripheral blood mononuclear cells can be accomplished by culturing the cells on adherent immune complexes (11, 16) or by culturing the cells under conditions which facilitate differ-

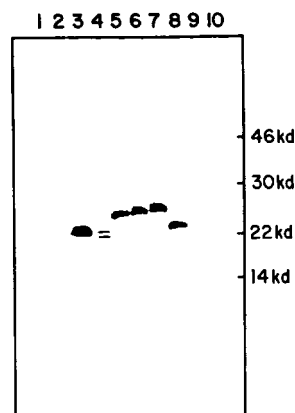


FIG. 10. Western blot analysis of IL-1 antagonist proteins. Samples containing 0.2 or 1.0 μ g of the various proteins were run on a reducing 15% SDS-polyacrylamide gel, electrotransferred to nitrocellulose, and probed with a 1/500 dilution of rabbit anti-human IL-1 antagonist protein. Immune complexes were visualized using peroxidase-conjugated second antibody. Lanes 1 and 10 contain rhIL-1 α (1.0 and 0.1 μ g, respectively); lanes 2 and 9 contain rhIL-1 β (1.0 and 0.1 μ g, respectively); lanes 3 and 8 contain recombinant IL-1 antagonist (1.0 and 0.1 μ g, respectively). Lanes 4, 5, 6, and 7 contain Peak D, Peak C, Peak B, and Peak A proteins (0.1 μ g/lane), respectively.

entiation into macrophages (extended time in culture and stimulation with GM-CSF) (12). The kinetics of IL-1 antagonist production induced by adherent immune complexes or IgG (11, 16) reached a steady state after 24 h, similar to the rate of production by differentiated THP-1 cells. Synthesis of IL-1 antagonist activity by unstimulated cultured mononuclear cells (12) required much longer times (4–6 weeks) and stimulation with GM-CSF increased both the rate and absolute amount of IL-1 antagonist activity produced (12). Addition of recombinant human GM-CSF (50 units/ml) to PMA-differentiated THP-1 cells did not enhance IL-1 antagonist production.³

³ A. E. Berger, unpublished observations.

TABLE 3

Summary of deglycosylation experiments

Samples containing approximately 1 μ g of protein were digested with *N*-glycanase as described under "Experimental Procedures." The reaction products were then analyzed and the protein bands visualized by silver staining.

Protein	Mass ^a		pI ^b	
	-N-Glycanase	+N-Glycanase	-N-Glycanase	+N-Glycanase
	<i>kDa</i>			
Peak A	26	22	4.9	5.8
Peak B	25	22	5.1	5.8
Peak C	25	22	5.7	5.8
Peak D	22	22	5.8	5.8

^a Analyzed by SDS-polyacrylamide gel electrophoresis.

^b Analyzed by isoelectric focusing.

The exclusive use of nondenaturing chromatography steps during the purification resulted in good recoveries of biologically active antagonist proteins. The four pI forms of IL-1 antagonist proteins that we isolated were also detected in similar ratios by preparative isoelectric focusing and bioassay of THP-1 conditioned medium,⁴ so they do not appear to be artifacts of the purification procedure. Whether all four forms are secreted by the cells or whether they are formed by extracellular processing is unknown. The des-6 antagonist protein does appear to be formed by proteolysis in the supernatant since the yield of the des-6 protein increases with time of storage at 4 °C. The physical properties reported here for the various forms of the THP-1 derived IL-1 antagonist proteins are similar to those previously determined for other IL-1 antagonists using partially purified preparations. The monocyte-derived antagonists described above have molecular masses in the range 22–25 kDa (11, 12, 16), while the pI values of these proteins have not been reported. Antagonist proteins from P388D1 cells have been resolved into forms with pI 5.3 and 6.0, and both have similar molecular masses in the range 40–60 kDa (31). The antagonist protein from the M20 cell line was reported to be 52 kDa (32). Based on the detailed comparison of the four THP-1 IL-1 antagonist proteins by physical (M_r , pI), chemical (sequencing and amino acid composition), glycoprotein (*N*-glycanase), immunological (Western blotting), and biological (receptor competition and 1A5 bioassay) analyses, we conclude that all four forms are variants of a single polypeptide. We have also purified the same four IL-1 antagonist proteins from medium conditioned by adherent human peripheral blood monocytes that had been stimulated with either IgG or GM-CSF, so the appearance of multiple antagonist forms is not peculiar to monocytic cell lines⁵ (19). After the completion of this work, a report describing the characterization of three IL-1 antagonist proteins (IL-1ra-x, - α , and - β) from stimulated human monocytes was published (18). These three antagonist proteins have physical properties similar but not identical to those reported here (18, 22, and 22 kDa, pI values in the range 4.5–5.3). Based on the results of partial sequence analysis, deglycosylation experiments, and receptor competition studies, these authors conclude that the three forms represent glycosylation variants of the antagonist protein (18). Comparison of the THP-1-derived IL-1 antagonist amino acid sequences reported here with the composite sequence obtained by combining sequences obtained from IL-1ra-x, IL-1ra- α , and IL-1ra- β (18) reveal complete identity (45/45 residues) between the antagonists isolated from two distinct sources. All of these peptide

sequences are also present in IL-1 antagonist sequences predicted from U937 cell or human monocyte cDNAs (19, 20), so the THP-1-derived antagonist proteins are very closely related if not identical to those isolated and sequenced from IgG-stimulated human monocytes (18) or GM-CSF stimulated U937 cells (19). The detailed characterization of the four pI forms from THP-1 cells, including M_r and pI determination, partial sequence analysis, the preparation of specific antiserum, and the determination of K_i values for receptor competition should facilitate comparison of antagonist proteins derived from other sources.

Each of the four THP-1-derived IL-1 antagonist proteins had similar specific activities for inhibition of IL-1 β receptor binding and the K_i values were 4–8-fold higher than determined for homologous competition. Hill plots of the binding data gave a straight line with a slope near unity, indicating single-site competitive binding with no cooperativity. These data, coupled with the fact that we have not been able to detect a high affinity interaction between IL-1 β and the antagonist protein (data not shown), suggest that the antagonist works by directly competing at the IL-1 binding site. Furthermore, the high specific activity for the Peak D proteins indicates that neither glycosylation or the first 6 amino-terminal residues are required for IL-1 receptor binding activity. Based on the K_i values determined by receptor competition, the expected specific activities for inhibition of IL-1-dependent processes are in the range 2.5–5.0 $\times 10^6$ inhibitory units/mg protein (using the standard lymphocyte-activating factor unit as a reference). The specific activities of the Peak A, B, and C proteins in the 1A5 assay were greater than 2 orders of magnitude lower than the values expected from the receptor competition data. These specific activities are comparable to a similar antagonist protein from U937 cells and to rhIL-1 antagonist prepared by expression in *E. coli* (19). The latter protein has a specific activity of 8.8 $\times 10^5$ inhibitory units/mg protein in a thymocyte proliferation assay (19) so it appears that the specific activities are assay-dependent. The Peak D proteins showed weak agonist activity in the 1A5 assay despite having potent activity in the receptor binding assay. The agonist activity was due to contamination of this pool with IL-1, which has a specific activity approximately 1000 times higher in the 1A5 assay. Absorption of the Peak D sample with a combination of rabbit anti rhIL-1 α and rhIL-1 β antibodies coupled to Sepharose beads prior to bioassay removed the agonist activity. Although several reports concerning the inhibition of IL-1 receptor binding by antagonist proteins are available (12, 16–18, 20), accurate K_i values were not determined.

The stoichiometry of antagonist receptor binding and the inhibition of IL-1 driven cellular responses (18, 19) suggest that these proteins are natural regulators of IL-1 activity. Synthesis of both IL-1 and an IL-1 antagonist by the same cell type (monocytes) may involve feedback regulation, without the necessity for factors derived from accessory cells. Induction of IL-1 antagonist activity by factors that facilitate macrophage maturation (PMA, GM-CSF) (12, 16, 17) suggests that a balance between monocyte IL-1 synthesis and macrophage IL-1 antagonist synthesis may be involved in the regulation of IL-1 activity. In support of a feedback regulatory mechanism, we have demonstrated IL-1-dependent induction of IL-1 antagonist expression in U937 cells.⁶ Further studies on the factors which regulate the balance between IL-1 and IL-1 antagonist activities are in progress.

Acknowledgments—We would like to thank M. R. Deibel for com-

⁴ M. R. Deibel, unpublished observations.

⁵ T. E. Eessalu, unpublished observations.

⁶ M. J. Bienkowski, manuscript in preparation.

municating the results of the preparative isoelectric focusing experiment, A. W. Yem for providing a sample of rhIL-1 antagonist, Janet Lynn for cell culture support, and Jan Peterson for preparation of the manuscript.

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SUPPLEMENTARY MATERIAL

TO
PURIFICATION AND CHARACTERIZATION OF IL-1 RECEPTOR LEVEL ANTAGONIST
PROTEINS FROM THP-1 CELLS

by
Michael J. Bienkowski, Thomas E. Eessalu, Ann E. Berger, Scott E. Truesdell,
John A. Shelly, Alice L. Laborde, Heidi A. Zurcher, J. Ilyse M. Reardon,
Robert L. Heinrichson, John G. Chosay, and Daniel E. Tracey

EXPERIMENTAL PROCEDURES

Materials

Cells and Cell Culture

THP-1 cells were obtained from J. Minowada, Hayashibara Biochemical Laboratories, Okayama, Japan. YT-NCI cells were obtained from K. Matsushima (NCI). LBRM-33-1A5 (1A5) cells, and HT-2 cells were obtained from the American Type Culture Collection (Rockville, MD). THP-1, YT-NCI, and 1A5 cells were maintained in suspension cultures in RPMI medium containing 100 µg/ml streptomycin/100 U/ml penicillin/2 mM glutamine (complete medium) with 10% FBS at 37°C in 5% CO₂. HT-2 cells were maintained in complete medium supplemented with 10% rat cell polyclone. 5% FBS and 47 µM 2-mercaptoethanol.

Reagents for cell culture were obtained from Irvine Scientific. Low endotoxin FBS was from Hyclone. Phytohemagglutinin, benzamide, phorbol-12-myristate, complete Freund's adjuvant, incomplete Freund's adjuvant were from Sigma. 4-Vinylpyridine, 1,10-phenanthroline and 4-chloronaphthol were from Aldrich. Fast-flow chromatography resins (Q-Sepharose, S-Sepharose, and Phenyl-Sepharose), TSK-GF3000 and Mono S columns, and precast IEF gels were from Pharmacia-LKB. Vydac C4 column was from the Nest group. N-glycanase was from Genzyme. Chemicals for SDS-PAGE and protein quantification were from BioRad. ¹²⁵I-Bolton-Hunter reagent was from Amersham. rhIL-1β was produced by expression in *E. coli* as previously described (21). rhIL-1β was iodinated as described by Dower (22). rhIL-1β (8 µg in 40 µl PBS) was added to a tube containing 4 mCi ¹²⁵I-Bolton-Hunter reagent. After 2 hr at 4°C, the reaction was quenched by addition of 1 µl 1 M glycine ethylester and the ¹²⁵I-rhIL-1β purified by chromatography on a Biogel P-6DG column eluted with PBS containing 10% glycerol and 0.25% gelatin. Specific activities were typically 3000 Ci/mmol.

Determination of IL-1 Antagonist Activity

IL-1 antagonist activity was quantified in either a whole-cell receptor competition assay or a coupled cell assay that measures IL-1 dependent IL-2 synthesis. Receptor binding assays were conducted in 96 well plates containing 1.0 × 10⁵ YT-NCI cells, 50 pM ¹²⁵I-IL-1β and various dilutions of the antagonist in a total volume of 0.2 ml. The reactions were incubated for 1 hr at ambient temperature and unbound radioactivity was removed by filtration. IL-1 agonist or antagonist activity was measured as a decrease in bound ligand. Non-specific binding was determined in the presence of a 1000 fold molar excess of unlabeled IL-1β and was typically < 10%. For determination of the IC₅₀ and K_i, concentration dependent inhibition of IL-1β binding was measured and the values were calculated using probit-log transformation to linearize the competition curve data.

The LBRM-33-1A5 IL-1 antagonist assay was modified from Gillis and Mizel (23) as described previously (24). 1A5 cells (1 × 10⁵ cells/well) were costimulated with rhIL-1β (0.01-0.03 Units/ml), 2 µg/ml phytohemagglutinin, and various dilutions of the antagonists in a total volume of 0.2 ml. After 24 hours in culture, IL-2 levels in the cell supernatants were quantified by mitogenesis (3H-thymidine incorporation) in the IL-2 dependent cell line HT-2. IL-1 antagonist activity was measured by a decrease in IL-2 production where 1 inhibitory unit of IL-1 antagonist activity is the amount required to inhibit 1 unit of IL-1β. A unit of IL-1 activity is the standard LAF unit that gives half maximal proliferation in a murine thymocyte assay (25).

Preparation of THP-1 Cell Conditioned Medium

For production of IL-1 antagonist activity, THP-1 cells were differentiated by treatment with PMA. Suspension cultures of cells were harvested by centrifugation (600 × g, 7 min), resuspended to 5 × 10⁵ cells/ml in RPMI 1640/2 mM L-glutamine/100 U/ml penicillin/100 µg/ml streptomycin/20 mM HEPES/100 nM PMA/7% FBS and seeded at 250 ml/650 cm² roller bottles. After 48 hours, the medium was removed, the adherent cells washed with PBS, and 250 ml of fresh medium containing 1% low endotoxin FBS and lacking PMA was added per bottle. After an additional 48 hours of culture, the medium, adjusted to 1 mM benzamide, was harvested by filtration through a 0.45 µm membrane, and either stored at -20°C or processed immediately.

Purification of IL-1 Antagonist Proteins

Conditioned medium was concentrated and then dialyzed by spiral fiber filtration through a YM 10 membrane. The supernatant was typically concentrated by a factor of 5 and then exchanged into 20 mM Tris-HCl (pH 8.0). The dialyzed supernatant was loaded (10 ml/min) onto a 300 ml column of Q-Sepharose Fast Flow that had been equilibrated in 20 mM Tris-HCl (pH 8.0) (3 liters total) followed by stepwise elution with 0.25, 0.50, and 1.0 M NaCl in 20 mM Tris-HCl (pH 8.0) (3 liters total) followed by the elution profile was monitored by absorbance at 280 nm and by assay of the individual fractions in the receptor binding assay. Pooled active fractions from the Q-Sepharose column were concentrated and exchanged into 20 mM MES-Na (pH 6.0) by repeated Amicon pressure filtration using a YM-10 membrane. The dialyzed pool was then loaded onto a 100 ml S-Sepharose Fast Flow column that had been equilibrated in 20 mM MES-Na (pH 6.0). When the 280 nm baseline returned to zero, the column was eluted stepwise with 0.25, 0.50, and 1.0 M NaCl in 20 mM MES-Na (pH 6.0). The elution profile was monitored as described above and the active fractions (0.25 M NaCl wash) pooled. The active pool from the S-Sepharose column was adjusted to 1.0 M (NH₄)₂SO₄ by slow addition of solid ammonium sulfate and loaded onto a 50 ml Phenyl Sepharose column previously equilibrated in 20 mM MES-Na (pH 6.0) containing 1.0 M (NH₄)₂SO₄. The column was then eluted with a linear gradient from 1.0 M to 0 M (NH₄)₂SO₄ in MES-Na (pH 6.0) and the elution profile monitored as described above following dialysis of the individual fractions since the (NH₄)₂SO₄ interferes with the receptor binding assay. Pooled active fractions from the Phenyl Sepharose column were concentrated to 5.0 ml using a stirred cell Amicon with a YM 10 membrane and 2.0 ml aliquots were chromatographed on a 2.5 × 60 cm TSK GF3000 column. The column was eluted with PBS at 3.0 ml/min and the elution profile monitored as described above. Pooled active fractions from the GF3000 column were dialyzed into 20 mM MES-Na (pH 6.0) overnight at 4°C and loaded onto a 0.5 × 5.0 cm Mono S column equilibrated in the same buffer. The column was then eluted at 1.0 ml/min using a shallow gradient of NaCl in 20 mM MES-Na (pH 6.0) over 0 to 0.15 M over 30 min followed by stepwise elution with 0.5 and 1.0 M NaCl. The elution profile was monitored as described above and the pooled active fractions were stored at 4°C.

Analytical Methods

Protein concentrations were estimated using the Bradford method with BSA as the standard or by amino acid composition analysis of the purified proteins (for K_i determinations). SDS-PAGE analysis was performed using 15% reducing gels according to Laemmli (26). Analytical electrofocusing polyacrylamide gels (5% acrylamide, pH range 3.5 to 9.5) were run on Ampholine Pagelates (LKB). In all cases, protein bands were visualized by silver staining (27).

Peptide Mapping and Sequencing

Aliquots of the purified proteins corresponding to approximately 500 pmol were sequenced directly using an Applied Biosystems Model 470B Gas Phase Sequencer with on-line detection of the PTH amino acids. For tryptic peptide mapping, a sample of protein purified on the Mono S column (Peak B; 25 µg, 1 nmol) was first denatured by treatment with 6 M GuHCl/0.25 M Tris-HCl (pH 8.5)/0.1% 2-mercaptoethanol for 30 min at 37°C. 4-Vinylpyridine was added (0.1% vol/vol) for 2 hr and the reaction mixture was desalted by dialysis against 20 mM ammonium bicarbonate (pH 7.8). The dialyzed sample was then digested with TPCK treated trypsin (1% w/wt) for 24 hr at 37°C and mapped by reverse phase HPLC on a 0.4 × 25 cm Vydac C4 column eluted with a linear gradient from 0 to 70% solvent B over 90 min at 1.0 ml/min (solvent A: 0.1% TFA in water; solvent B: 10% TFA/90% water/30% acetonitrile). Peptide peaks were detected by absorbance at 215 nm, collected, and sequenced as described above.

Deglycosylation Studies

The effect of N-glycanase on the various antagonist forms was investigated to determine if the carbohydrate was the source of the observed size and charge heterogeneity. Protein samples (1 µg protein) were heated (3 min) to 100°C in the presence of 0.5% SDS, 0.1 M 2-mercaptoethanol. The samples were then adjusted to 0.2 M NaPO₄ (pH 8.6), 10 mM 1,10-phenanthroline, 125% NP-40/10 U/ml N-glycanase and incubated overnight at 37°C. The reaction products were then analyzed directly on either reducing SDS-PAGE or IPF gels and the bands visualized by silver staining.

Antiserum Preparation and Western Blotting

Polyclonal antiserum was raised by immunization of New Zealand White Rabbits with denatured IL-1 α (0.1% SDS) recombinant IL-1 antagonist protein obtained by expression in *E. coli* (19). Animals were immunized with 150 µg antigen in complete Freund's adjuvant by intradermal injection followed by a boost using 75 µg antigen in incomplete Freund's adjuvant. Reducing SDS-PAGE gels were electroblotted to nitrocellulose (0.2 A, 5 hr) and probed with a 1:500 dilution of IL-1 antagonist antiserum. Immune complexes were visualized using peroxidase conjugated second antibody and 4-chloronaphthol as the substrate.

RESULTS

IL-1 antagonist activity in various fractions generated during the purification was routinely quantified by either a receptor binding assay or a bioassay that measures IL-1 dependent IL-2 synthesis. The receptor binding assay detects competitive binding between [¹²⁵I] IL-1 β and antagonists or agonists to receptors on YT cells as a decrease in bound radiolabeled ligand. The 1A5/HT2 bioassay measures antagonism of IL-1 dependent stimulation of IL-2 synthesis by 1A5 cells as a decrease in IL-2 production when titrated on the IL-2 dependent cell line HT-2. Because the receptor binding assay has a high sample through-put and rapid turnaround time (<24 hr), it was routinely used to monitor purification. The inability of the receptor binding assay to distinguish between antagonist and agonist (i.e., IL-1) required the backup use of the 1A5/HT2 assay on pooled receptor binding assay active fractions.

PURIFICATION

Purification was routinely performed starting with 10 liters of THP-1 cell conditioned medium and the results from a representative run will be described below. The conditioned medium was concentrated by a factor of 5 (maximum concentration that did not result in protein precipitation) and exchanged into 20 mM Tris-HCl (pH 8.0) by spiral fiber filtration. Pilot purification studies performed on the concentrate revealed that the IL-1 antagonist activity was retained by ion exchangers at pH 8.0 and by cation exchangers at pH 6.0, so these steps were used in series. The pH 8.0 concentrate was chromatographed on a Q-Sepharose anion exchange column using gradient elution and the elution profile monitored by both absorbance at 280 nm and by the receptor competition assay of the individual fractions. As shown in Figure 2, numerous peaks of protein were detected at 280 nm but all of the IL-1 antagonist activity was associated with a small peak that eluted at approximately 0.13 M NaCl. The active fractions were pooled, buffer exchanged into 20 mM MES-Na (pH 6.0), and then chromatographed on a S-Sepharose cation exchange column previously equilibrated in the same buffer. The elution profile was monitored as described above and the results are shown in Figure 3. The majority of the protein did not bind to the column under these conditions while all of the IL-1 antagonist activity eluted when the column was washed with 20 mM MES-Na (pH 6.0)/0.25 M NaCl. The active fractions were pooled, adjusted to 1.0 M (NH₄)₂SO₄ and chromatographed on a Phenyl Sepharose hydrophobic interaction column equilibrated in 20 mM MES-Na (pH 6.0)/1.0 M (NH₄)₂SO₄. The elution profile was monitored as described above and the results are shown in Figure 4. Elution of the column with a decreasing gradient of (NH₄)₂SO₄ resolved several peaks of protein with the IL-1 antagonist activity eluting as a broad peak near the end of the gradient, separated from the majority of the protein contaminants. SDS-PAGE analysis of the active fractions from the Phenyl Sepharose chromatography revealed that although fractions 5-9 had activity, fractions 8 and 9 were substantially enriched for a 25 kDa band whose intensity correlated with the antagonist activity. Since the majority of the contaminants in fractions 8 and 9 were larger than 25 kDa (see Figure 7) the pooled material was fractionated with respect to size by chromatography on a preparative TSK GF3000 column. As shown in Figure 5, the IL-1 antagonist activity eluted as a partially resolved doublet and was well separated from the major protein peak. The elution positions corresponded to molecular weights of 25 and 19 kDa. SDS-PAGE analysis of the fractions eluting on the trailing edge of the 19 kDa peak contained a closely spaced doublet that migrated at 21 kDa while the remainder of the active fractions required further purification. Since previous cation exchange runs on S-Sepharose revealed separation of several zones of IL-1 antagonist activity when gradient elution was performed, the pooled 25 kDa active fractions from the size exclusion step were chromatographed on Mono S and the results are shown in Figure 6. Elution using a shallow gradient of NaCl in 25 mM MES-Na (pH 6.0) resolved 4 zones of IL-1 antagonist activity, referred to as Peaks A-D in increasing order of elution. The specific activities of these four proteins were similar as judged by the relative heights of the peaks of absorbance and the bioactivity. The material eluting last from the Mono S column, Peak D, had a composition identical to the 19 kDa pool from the previous step, as these fractions were combined to yield the Peak D material. SDS-PAGE analysis of pooled fractions obtained at various stages of purification is shown in Figure 7. The majority of the contaminants present in the Q-Sepharose and S-Sepharose pools were derived from the 1% fetal bovine serum used to culture the cells. A 25 kDa band was first noted in the Phenyl Sepharose pool while the TSK GF3000 pool showed a broad band centered at 25 kDa. This broad band was due to the presence of overlapping protein bands and this pool was resolved into 4 distinct proteins by chromatography on Mono S. The proteins in Peaks A, B, and C migrated with apparent molecular weights of 28, 25, and 25 kDa, respectively. The Peak D material which was composed of the 19 kDa GF3000 pool and a small amount of Peak D from the Mono S run, ran as a closely spaced doublet corresponding to apparent molecular weights of 21 and 20 kDa. Based on the densitometry of silver stained gel shown in Figure 7, Peak A, B, and C proteins were greater than 98% pure. Analysis of the proteins in Peaks A-D by isoelectric focusing (Figure 8) gave pI's of 4.9 (Peak A material), 5.1 (Peak B material), 5.7 (Peak C material), and 5.8-4.8 (Peak D material).

Based on the 1A5 assay (Table 1), the crude supernatant contained 1,680 units of IL-1 antagonist activity at a specific activity of 0.3 units/mg. The activity could not be detected in the concentrated supernatant but was recovered in a 295% yield in the Q-Sepharose pool. This increase was presumably due to removal of IL-1. Good recoveries of bioactivity (304%) and a 190 fold increase in the specific activity of the S-Sepharose pool was obtained. The apparent drop in recovery at the Phenyl Sepharose step was probably due to incomplete removal of the excess ammonium sulfate (toxic in the bioassay) since the recovery increased after the following chromatographic step. Good recoveries were obtained in the last two chromatographic steps and the final specific activities for Peaks A, B, and C were 6,971, 15,967, and 5,407 units/mg. The Peak D material showed weak agonist activity in the 1A5 assay, and the agonist activity was removed by absorption of the sample with IL-1 specific antisera.

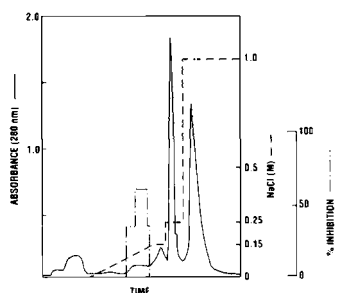


Figure 2. Q-Sepharose Chromatography of THP-1 Conditioned Medium. Concentrated THP-1 cell conditioned medium (5X) that had been dialyzed into 20 mM Tris-HCl (pH 8.0) was chromatographed on a Q-Sepharose column using gradient elution as described in the Experimental Section. The elution profile was monitored by absorbance at 280 nm and by receptor binding assay (50 µl) of the individual fractions.

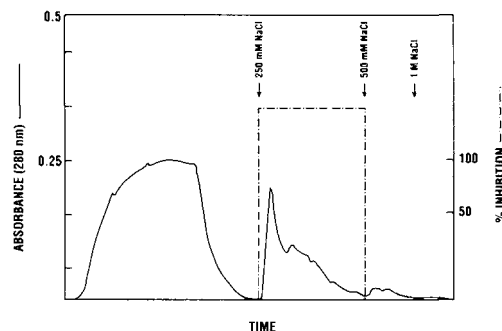


Figure 3. S-Sepharose Chromatography of Pooled Active Fractions from Q-Sepharose. Pooled active fractions from Q-Sepharose chromatography were concentrated, dialyzed into 20 mM MES-Na (pH 6.0) and chromatographed on S-Sepharose using step-wise elution as described in the Experimental Section. The elution profile was monitored by absorbance at 280 nm and by receptor binding assay of the individual fractions (50 µl).

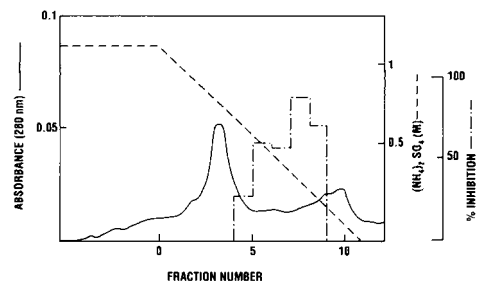


Figure 4. Phenyl Sepharose Chromatography of Pooled Active Fractions from S-Sepharose. Pooled active fractions from S-Sepharose were adjusted to 1.0 M (NH₄)₂SO₄ and chromatographed on Phenyl Sepharose using gradient elution as described in the Experimental Section. The elution profile was monitored by absorbance at 280 nm and by receptor binding assay of aliquots of the individual fractions that had been dialyzed into PBS (to remove the ammonium sulfate which causes non-specific inhibition in the receptor assay).

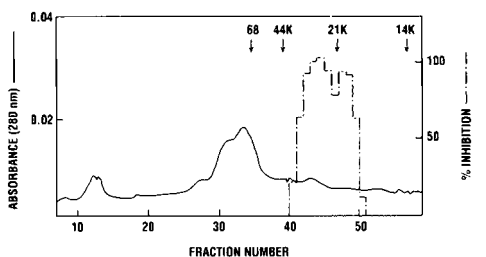


Figure 5. TSK GF3000 Size Exclusion Chromatography of Phenyl Sepharose Active Fractions. Pooled active fractions from Phenyl Sepharose were concentrated and then fractionated by size exclusion HPLC as described in the Experimental Section. The elution profile was monitored by absorbance at 280 nm and by receptor binding assay of the individual fractions (5 µl).

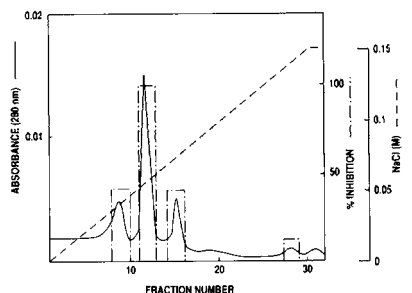


Figure 6. Mono S Chromatography of TSK GF3000 Pool. Pooled active fractions from the TSK GF3000 column corresponding to 25 kDa were dialyzed into 20 mM MES-Na (pH 6.0) and then chromatographed on Mono S using gradient elution as described in the Experimental Section. The elution profile was monitored by absorbance at 280 nm and by receptor binding assay of the individual fractions (5 µl). Peaks A-D correspond to the proteins eluting in peak fractions 9 (Peak A), 12 (Peak B), 15 (Peak C) and 27 (Peak D), respectively.

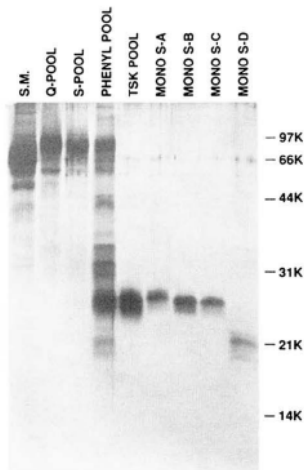


Figure 7. SDS-PAGE Analysis of Fractions During Purification. Aliquots of pooled fractions obtained at various stages of purification (containing either 10 μ g protein (lanes 1-4) or 1 μ g protein (lanes 5-9)) were run on a reducing 15% SDS-PAGE gel and the protein bands visualized by silver staining.

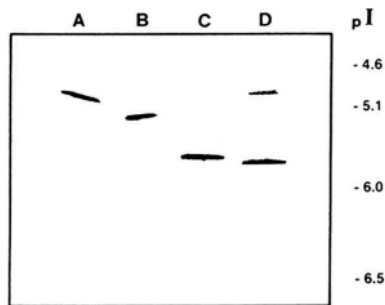


Figure 8. Isoelectric Focusing of Purified IL-1 Antagonist Proteins. Aliquots of the purified fractions (1 μ g) were focused on analytical electrofocusing polyacrylamide gels (pH range 3.5 to 9.5) and the protein bands visualized by silver staining.

Table 1
THP-1 Antagonist Purification Table Based on the 1A5-HT2 Bioassay

Fraction	Total Volume ml	Total Protein mg	Total Activity ^(a)		Specific Activity IU/mg	% Yield	Fold Purification
			IU/ml	Total IU			
Crude Supernatant	10,500	5,700	0.16	1,680	0.3	100	1
Concentrated Supernatant	2,000	5,620	N.D. ^(b)	0	0	0	0
Q-Sepharose Pool	800	384	62	4,960	13	295	43
S-Sepharose Pool	150	90	34	5,100	57	304	190
Phenyl Sepharose Pool	3.0	3.6	69	207	58	12	193
TSK-GF3000 Pool	30	0.583	45	1,350	2,316	80	7,720
Mono S - Peak A	2.0	0.035	122	244	6,971	15 ^(c)	23,236
Mono S - Peak B	2.0	0.060	479	958	15,967	57 ^(c)	53,223
Mono S - Peak C	2.0	0.027	73	146	5,407	9 ^(c)	18,023
Mono S - Peak D	3.3	0.102	N.D. ^(b)	---	---	---	---

^(a)One inhibitory unit of activity (IU) gives 50% inhibition of one thymocyte unit of IL-1 β stimulated IL-2 production by 1A5 cells.

^(b)N.D. = antagonist activity not detected. Mono S Peak D showed weak agonist activity in this assay.

^(c)The sum of Mono S Peaks A - C represents an overall yield of 80% relative to the crude supernatant.