

## PIG INTERLEUKIN 1

### Purification of Two Immunologically Different Leukocyte Proteins that Cause Cartilage Resorption, Lymphocyte Activation, and Fever

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Interleukin 1 (IL-1)<sup>1</sup> is a term that was originally introduced as a synonym for lymphocyte-activating factor (1). It is now becoming clear that proteins characterized as lymphocyte-activating factors may have a number of biological effects. When injected into animals they cause fever (2, 3) and increased circulating levels of acute phase proteins (4). In cell cultures they have been found to stimulate proliferation of dermal fibroblasts (5), and to increase the amounts of prostaglandin and latent collagenase produced by certain types of fibroblast and by chondrocytes (6–8). In organ cultures, they apparently stimulate cartilage (7) and bone resorption (7, 9) and promote proteolysis in voluntary muscle (10). In mature cartilage the chondrocytes respond to IL-1 by breaking down their surrounding proteoglycan matrix (7); in the calvariae of newborn mice there is a complex response to IL-1 that results in bone being resorbed through the recruitment and action of osteoclasts (11). Although much of the evidence that lymphocyte-activating factors can affect nonlymphoid cells has been obtained with impure proteins, experiments carried out with supposedly homogenous preparations of IL-1 have tended to support the idea that a single protein can cause all the various effects (7, 12–14).<sup>2</sup> Such proteins could act as important hormones of inflammation by altering the activity of cells of connective tissues and the immune system, as well as contributing to systemic features such as fever, the acute phase response, and breakdown of muscle protein.

We have previously reported the complete purification of an acidic protein from pig mononuclear leukocytes that was purified on the basis of its ability to stimulate explants of cartilage to break down their proteoglycan matrix (15). This protein was originally called catabolin, and had been initially identified as a product of synovial tissue in organ culture (16). Subsequently, the pure leukocyte protein was shown to activate lymphocytes (7), to be pyrogenic in rabbits, and to increase circulating levels of serum amyloid A protein in mice.<sup>2</sup> Clearly, this protein is a form of pig IL-1.

<sup>1</sup> *Abbreviations used in this paper:* Con A, concanavalin A; DME, Dulbecco's modification of Eagle's medium; FCS, fetal calf serum; HPLC, high performance liquid chromatography; IL-1, interleukin 1; PG, prostaglandin; PHA, phytohemagglutinin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

<sup>2</sup> Saklatvala, J., S. J. Sarsfield, M. J. Staruch, W. I. Cranston, R. F. Hellon, and Y. Townsend. Homogeneous acidic pig interleukin 1 (catabolin) causes fever and production of serum amyloid A as well as breakdown of proteoglycan in cartilage. Submitted for publication.

Besides the acidic IL-1, lectin-stimulated pig mononuclear leukocytes also produce a basic protein that stimulates cartilage resorption. This second protein accounts for about half of the total cartilage-resorbing activity produced by the leukocytes. Here we report the complete purification of both proteins, and we show that the basic protein is also a form of IL-1, since it is pyrogenic and activates lymphocytes. The purification of the acidic IL-1 is an improvement over the one previously described (15) since, by avoiding the use of reverse phase, high performance liquid chromatography (HPLC), it yields a protein of higher specific activity. Antisera raised against each form of IL-1 only react with that form and not the other. This immunological difference between the two forms means that they may be different proteins. We will refer to the acidic protein as IL-1/5 and the basic one as IL-1/8, based on their approximate pI.

## Materials and Methods

### *Materials*

Dulbecco's modification of Eagle's medium (DME), fetal calf serum (FCS), and RPMI 1640 were from Imperial Laboratories (Europe) Ltd., Salisbury, United Kingdom. Concanavalin A (Con A), chondroitin sulphate (whale), and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) were from Sigma Chemical Company Ltd., Poole, UK. Phytohemagglutinin (PHA), grade HA16, was from Wellcome Research Laboratories, Beckenham, UK; dimethyl methylene blue from Serva Feinbiochemica GmbH, Heidelberg, Federal Republic of Germany; nitrocellulose membrane from Bio-Rad Laboratories, Richmond, CA; peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins from DAKO Ltd., High Wycombe, Bucks., UK; rabbit anti-PGE<sub>2</sub>-bovine serum albumin from Steranti Research, St. Albans, UK; and both [5,6,8,11,12,14,15(n)-<sup>3</sup>H]PGE<sub>2</sub> and [methyl-<sup>3</sup>H]thymidine were from Amersham International plc, UK. Ultrogel AcA54 was from LKB Produkter, Bromma, Sweden, and hydroxyapatite from BDH Chemicals Ltd., Poole, UK. Pharmalyte ampholine, Polybuffer exchanger PBE94, Polybuffer 74, Mono S and Mono Q columns, and the fast liquid chromatography system were from Pharmacia (Great Britain) Ltd., Hounslow, Middlesex, UK. Pellicon cassette ultrafiltration system fitted with a 10,000 mol wt cut-off membrane (PTGC 000 05) was from Millipore (UK). Limited and Diaflo ultrafiltration cells and PM10 membranes were from Amicon, C3H/He Ola mice were from Olac (1976) Limited, Bicester, Oxon, UK. Dialysis was carried out in Visking tubing that had been boiled in 0.1 M NaHCO<sub>3</sub>/10 mM EDTA and washed well before use.

### *Assay of Proteoglycan Release from Cartilage*

Discs (2 × 1 mm) of cartilage from the bovine nasal septum of freshly killed animals were precultured for 3–5 d in DME containing 5% heat-inactivated bovine calf serum at 37°C in CO<sub>2</sub>/air 1:19. They were then transferred to 96-well microtiter plates (one disc per well) and each was maintained in 150 μl of the same culture medium in which the sample under test was incorporated. After a further culture period (16–40 h) the medium was removed and its content of chondroitin sulphate was measured by use of the metachromatic dye, dimethyl methylene blue. Each sample was tested on four discs and results were expressed as the mean release of glycosaminoglycan per disc. The procedure has been fully described (15).

To estimate recoveries and purification factors, an arbitrary unit of activity was defined as the amount required to increase threefold the release of chondroitin sulphate from a disc of cartilage during 16 h culture. This unit was used to calculate the data in Table I, all the measurements being made on the same batch of cartilage.

### *Preparation, Concentration, and Gel Filtration of Pig Leukocyte Culture Medium*

Blood was collected from pigs as they were slaughtered at a local abattoir. 10 ml/L of a sterile solution of 50% (wt/vol) trisodium citrate was added as anticoagulant. 332 ml of

a sterile (autoclaved) solution of 3% (wt/vol) gelatin dissolved in phosphate-buffered saline (1 mM Na H<sub>2</sub>PO<sub>4</sub>/18 mM Na<sub>2</sub>HPO<sub>4</sub>/145 mM NaCl, pH 7.4) containing 1 mM EDTA was added to each liter of blood, mixed well, and the erythrocytes allowed to sediment for 30 min at 37°C. The leukocyte-rich plasma was decanted and centrifuged at 400 g for 15 min at room temperature. The supernatant was discarded and the cells were resuspended in 0.83% (wt/vol) NH<sub>4</sub>Cl (~40 ml for cells from 1 L of blood) to hemolyze the remaining erythrocytes. After 10 min the suspension was diluted 10-fold with the phosphate-buffered saline containing 1 mM EDTA and was centrifuged as before. The cells were next washed three times by resuspending and centrifuging them in the same buffer. Finally they were resuspended at  $50 \times 10^6$  cells/ml in DME containing Con A (0.05 mg/ml) and were cultured at 37°C in CO<sub>2</sub>/air (1:19) in rectangular glass dishes (30 × 22 × 3 cm; 350 ml/dish) for 40 h.

After culture, the supernatant was decanted, clarified by centrifugation, and stored at -20°C. 601 batches (from ~200 L of blood) were concentrated to 600 ml by ultrafiltration in a Pellicon cassette system fitted with a membrane of 10,000 mol wt cut-off (PTGC 000 05). The concentrated solution was clarified by centrifugation (10,000 g for 10 min) and solid NaCl was added to a final concentration of 0.5 M. It was chromatographed at room temperature on an Aca54 column (9 × 100 cm) equilibrated with 20 mM sodium phosphate buffer, pH 7.4, containing 0.5 M NaCl (flow rate, 300 ml/h). Fractions (150 ml) were assayed for their ability to stimulate release of proteoglycan from cartilage. Active material eluted as a single peak at a position corresponding to  $M_r$  15,000–25,000 as previously described (15). The active fractions were pooled and concentrated by ultrafiltration over a PM10 membrane (cut-off,  $M_r$  10,000) in a 21 Diaflo cell. The buffer was changed to 25 mM histidine (adjusted to pH 6.4 with HCl) by ultrafiltration.

#### *Purification of IL-1/5 (Fig. 1)*

**Chromatofocusing.** A column (1.6 × 100 cm) of Polybuffer PBE 94 exchanger was equilibrated with 25 mM histidine, pH 6.4, at 4°C. The active material from the Aca54 column was applied to the bed and the column was eluted with the histidine buffer until all the nonadsorbed protein had passed through. This was saved since it contained the basic IL-1 (see below). The column was then eluted with Polybuffer 74, which had been diluted 1:8 and adjusted to pH 4.0 with HCl. 28-ml fractions were collected and assayed for activity on cartilage. Two peaks of active material were found, a smaller one eluting at about pH 5.0 and a larger one at pH 4.6. This separation has been described and illustrated (15).<sup>2</sup>

**Hydroxyapatite chromatography.** The active fractions eluting at pH 4.6 were pooled, adjusted to pH 7.0 with a few drops of 5 M NaOH, and pumped onto a hydroxyapatite column (1.5 × 2 cm) equilibrated with water. The unbound protein was discarded. Active material was eluted with 150 mM sodium phosphate buffer, pH 7.0.

**Cation-exchange chromatography on Mono S (Fig. 2).** A Mono S HR 5/5 column was equilibrated with 50 mM formate (HCOOH/HCOONa) buffer, pH 4.0, containing 0.1 M NaCl. Active protein that eluted from hydroxyapatite was dialyzed against this buffer and applied to the column, which was then eluted at 1 ml/min with a linear, 28 ml gradient of 0.1–0.35 M NaCl dissolved in the formate buffer. Although fractions 14–22 were active on cartilage, most of the activity was in 14 and 15.

**Anion exchange chromatography on Mono Q (Fig. 3).** A Mono Q HR 5/5 column was equilibrated with 20 mM ethanolamine adjusted to pH 9.0 with HCl. Fractions 14 and 15 from Mono S (Fig. 2) were diluted 1:5 in this buffer and their pH adjusted to 9.0; they were applied to the column which was then eluted at 1 ml/min with a linear, 28 ml gradient of 0–0.25 M NaCl dissolved in the formate buffer. The acidic IL-1 eluted as a sharp peak in fraction 22.

#### *Purification of IL-1/8*

**Cation exchange chromatography at pH 4.0 on Mono S (Fig. 4).** A Mono S HR 10/0 column was equilibrated with 50 mM formate buffer, pH 4.0, containing 0.1% Brij 35. Protein that had not bound to the chromatofocusing bed (see above) was dialyzed against

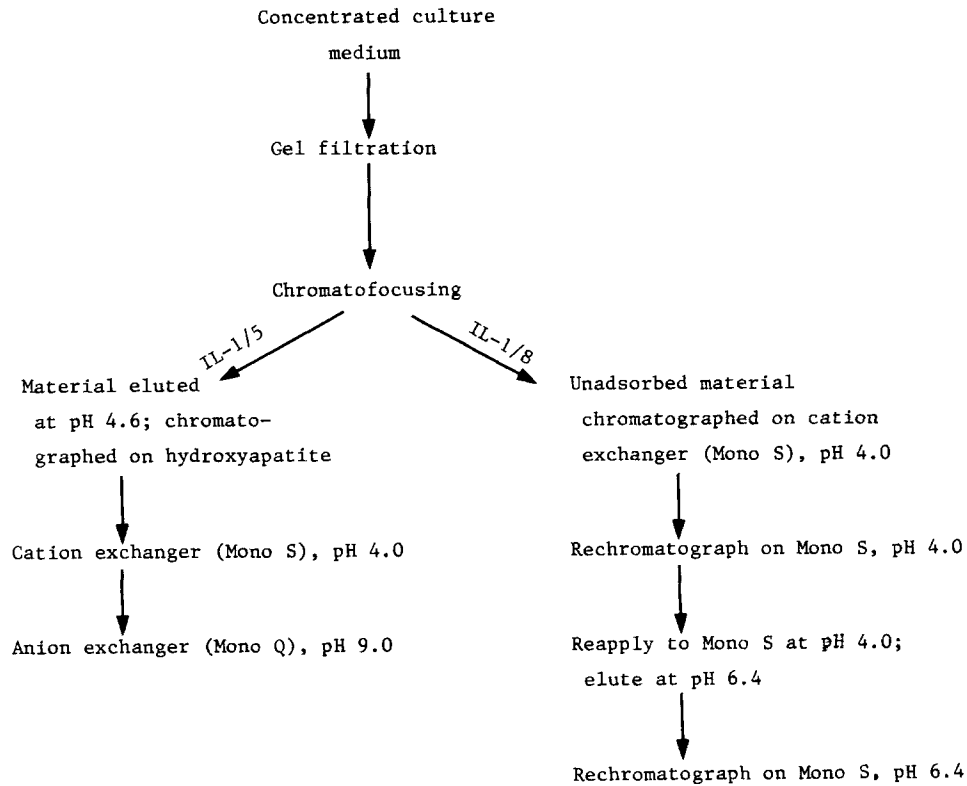


FIGURE 1. Purification scheme for pig IL-1s.

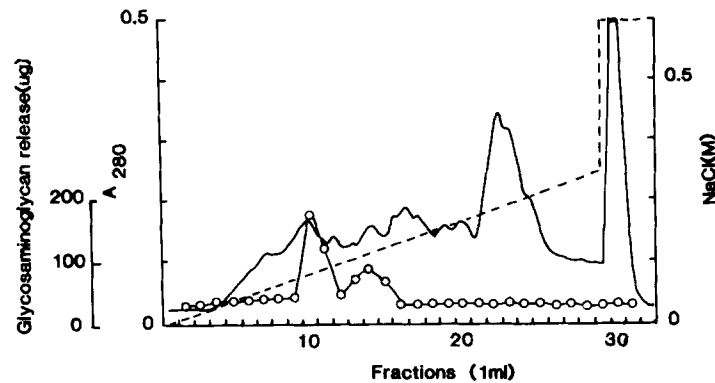


FIGURE 2. Chromatography of IL-1/5 on Mono S. Active material from hydroxyapatite was chromatographed at pH 4.0 as described in Materials and Methods. Fractions were assayed on cartilage at  $0.5 \mu\text{l/ml}$ . Results are shown as micrograms of glycosaminoglycan released per disc during 16 h culture (means of four discs) (O).  $A_{280}$  (—); molarity of  $\text{MNaCl}$  (---).

this buffer and applied to the column. The column was eluted at a flow rate of 3 ml/min with a 230 ml linear gradient of 0–1 M NaCl dissolved in the equilibrating buffer. Fractions (7.5 ml) were collected and assayed on cartilage. A sharp peak of active material was eluted at  $\sim 0.5$  M NaCl (Fig. 4a). These fractions were pooled, diluted 1:2 with formate buffer, and rechromatographed under the same conditions, except that the

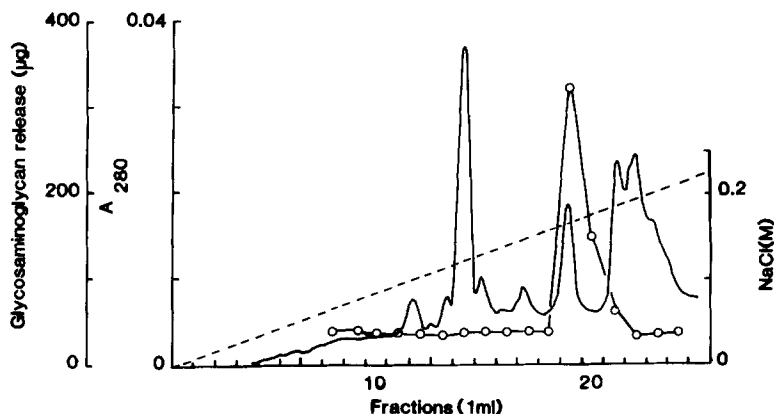


FIGURE 3. Chromatography of IL-1/5 on Mono Q. Fractions 10 and 11 from Mono S (Fig. 2) were chromatographed as described in Materials and Methods. Fractions were assayed at  $0.1 \mu\text{l/ml}$  for 16 h. Symbols are as for Fig. 2.

gradient (460 ml) was run from 0.31 to 0.68 M NaCl. The fractions active against cartilage indicated by the bar were pooled (Fig. 4b).

*Cation exchange chromatography at pH 6.4 on Mono S (Fig. 5).* The pool of active fractions that had been rechromatographed at pH 4.0 were next diluted 1:5 with the 50 mM formate buffer, pH 4.0 (without Brij 35) and applied to a small Mono S column (HR 5/5, 1 ml bed volume). The active protein was adsorbed by the exchanger and was eluted as a sharp peak when the buffer was changed to 50 mM sodium phosphate, pH 6.4 (Fig. 5a). The active material was next dialyzed for 24 h against this phosphate buffer. The column (after washing with 1 M NaCl) was also equilibrated with it. Surprisingly, when the sample was reapplied to the bed, the active component now bound to it and was eluted as a sharp peak coinciding with a protein peak on a gradient (28 ml) of 0–0.25 M NaCl (Fig. 5b).

#### Protein Concentration

Absorbance at 280 nm was measured and protein concentrations were estimated assuming  $E_{1\text{cm}}^{1\%} = 10$ . The values for the pure IL-1s were calculated from the area of their peaks on the final chromatograms. Molarities expressed are based on these measurements and assume  $M_r = 21,000$ .

#### Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis (SDS/PAGE) and Western Blot

SDS-PAGE was carried out in vertical slab gels of 12.5% total acrylamide concentration with a glycine/2-amino-2-methylpropane-1,3-diol (Ammediol) HCl buffer system (17). Samples were boiled for 5 min in the upper gel buffer, containing 1% SDS and 0.5% 2-mercaptoethanol before electrophoresis. Gels were stained for protein with Coomassie Brilliant Blue G250.

For Western blots, proteins were transferred electrophoretically to nitrocellulose paper. This was then soaked for 1 h at  $37^\circ\text{C}$  in blocking buffer (wash buffer [10 mM Tris/HCl, 0.9% NaCl, pH 7.4] plus 1% bovine serum). After washing three times it was incubated overnight with antiserum diluted 1:100 in blocking buffer. After washing again, the blocking step was repeated. The paper was washed once more, incubated for 2 h with the second antibody (swine Ig to rabbit Ig) diluted 1:200 in blocking buffer and washed again. Finally, bound peroxidase was detected with 4-chloro-1-naphthol (0.5 mg/ml in wash buffer plus 0.01%  $\text{H}_2\text{O}_2$ ) (18).

#### Isoelectric Focusing

This was done in 7.5% polyacrylamide gel cylinders ( $0.4 \times 6$  cm) containing 1% Pharmalyte, pH 3.0–10. The anode solution was 0.2%  $\text{H}_3\text{PO}_4$ , the cathode solution, 0.1%

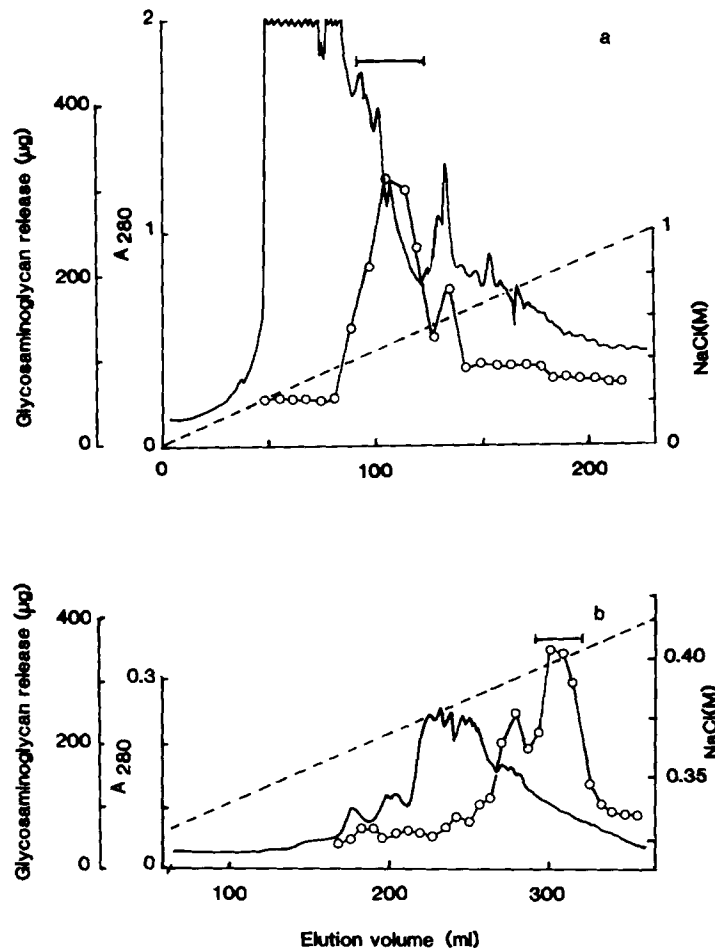


FIGURE 4. Chromatography of IL-1/8 on Mono S at pH 4.0. (a) Active material that had not bound to the chromatofocusing column was chromatographed on the large Mono S column (8 ml) as described in Materials and Methods. Fractions were assayed at  $2 \mu\text{l/ml}$ . Symbols are as for Fig. 2. (b) Rechromatography of fractions indicated by bar in a. Conditions were as in a but NaCl gradient was 0.31–0.68 M. Symbols are as in Fig. 2. See Materials and Methods for details.

NaOH. Focusing was carried out for 1 h to a limit voltage of 300 V. Gels were stained for protein with Coomassie Brilliant Blue G250 (20). One gel was sliced for pH measurement. The slices (3-mm) were each eluted in 1 ml of degassed water for 2.5 h and the pH of the eluates measured. Some gels were eluted to test for activity. Each slice (3-mm) was placed in a small bag of dialysis tubing and crushed. 1 ml DME was added to each bag and the contents were dialyzed for 24 h against a large volume of DME. The eluates retained in the dialysis bags were assayed on cartilage.

#### Thymocyte Assay

Thymocytes from C3H/HeOla mice aged 6–12 wk were cultured in flat-bottomed microtiter plates ( $1.5 \times 10^6$  cells in 0.2 ml per well) in RPMI 1640 containing PHA (1  $\mu\text{g/ml}$ ) and  $10^{-5}$  M 2-mercaptoethanol and the sample being tested. After 48 h, cultures were pulsed for 16 h (0.5  $\mu\text{Ci/well}$ ) with [ $^3\text{H}$ ]thymidine. Subsequently, cells were harvested, washed, and their uptake of  $^3\text{H}$  measured in a scintillation counter.

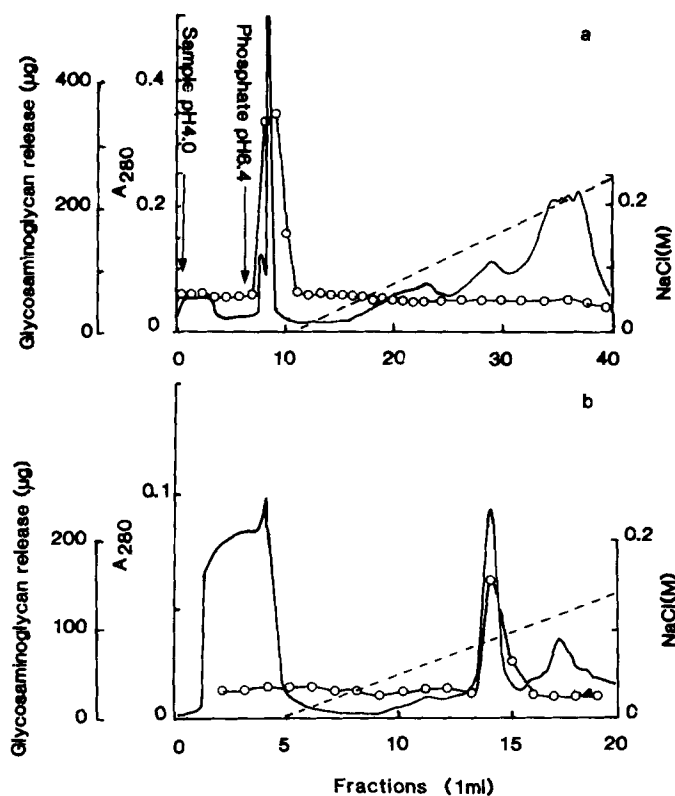


FIGURE 5. Chromatography of IL-1 on Mono S at pH 6.4. (a) Active fractions indicated by the bar in Fig. 4b were chromatographed on the 1-ml Mono S column as described in Materials and Methods. Active material eluted when the buffer was changed to 50 mM phosphate, pH 6.4. Fractions were assayed at 0.2  $\mu$ l/ml. (b) Active peak from a was dialyzed against the 50 mM phosphate buffer, pH 6.4 and rechromatographed on the Mono S column as described in Materials and Methods. Symbols are as for Fig. 2.

#### *Fibroblast Culture*

Pig synovial fibroblasts were obtained by enzymic digestion of synovial tissue from metacarpophalangeal joints as detailed previously (7). Fibroblasts were cultured in DME containing 10% FCS and maintained at 37°C in CO<sub>2</sub>/air (1:19).

#### *Assay of Prostaglandin*

PGE<sub>2</sub> was assayed by specific radioimmunoassay using rabbit anti-PGE<sub>2</sub>-bovine serum albumin exactly as described (7).

#### *Assay of Collagenase*

Collagenase was measured by digestion of [<sup>14</sup>C]acetylated skin collagen fibrils (20). Before assay, samples of serum-containing medium were treated for 10 min with trypsin (100  $\mu$ g/ml) at 37°C, followed by soybean trypsin inhibitor (500  $\mu$ g/ml) for 20 min. This procedure activates latent collagenase and saturates the serum proteinase inhibitors (21).

#### *Pyrogen Assays*

These were carried out on New Zealand White rabbits (2–2.5 kg) as previously described.<sup>2</sup> Intracerebroventricular injections of test solutions were made through fitted headplates. Animals were restrained in stocks in a room maintained at 20  $\pm$  1°C, and

their rectal temperatures were recorded by means of in-dwelling thermocouples. Samples for injection were diluted in sterile pyrogen-free saline as necessary. Endotoxin contamination was checked by the limulus ameobocyte lysate assay.

#### Immunization

5  $\mu$ g of IL-1/5 in complete Freund's adjuvant was injected into each popliteal lymph node of a rabbit. After 10 wk, the animal was injected subcutaneously with a further 10  $\mu$ g of IL-1/5 in incomplete Freund's adjuvant in the popliteal region. The animal was killed 3 wk later and its serum was collected.

10  $\mu$ g of IL-1/8 in complete Freund's adjuvant was injected intradermally into multiple sites along the back of another rabbit. After 5 wk the animal was reimmunized with a further 10  $\mu$ g of protein (in incomplete Freund's adjuvant) and serum was collected 10 d later.

### Results

*Purification of Acidic and Basic ILs.* Fig. 1 illustrates the purification schemes for the two forms of IL-1, the results of which are summarized in Table I. From 31.4 g of leukocyte protein starting material we obtained 15  $\mu$ g of IL-1/5 and 50  $\mu$ g of IL-1/8. The final specific activities of the proteins were very similar and the degree of purification was 110,000–120,000 in relation to the starting material. The amount of protein recovered was ~18% (IL-1/8) and 6% (IL-1/5) of the total activity of the starting material. On the basis of the distribution after chromatofocusing, IL-1/8 actually accounted for about 40–50% of the activity of the starting material, so the real recovery of this protein from the procedure was probably 35–40%. Similarly, the true recovery of IL-1/5 was calculated to be ~10–12%. The lower recovery of the acidic protein may partly be due to its charge heterogeneity, as a result of which only the major form can be purified.

TABLE I  
*Purification of Pig IL-1 Proteins*

Purification step	Total protein <i>mg</i>	Total activity (arbitrary units)	Specific activity	Purification factor	Recovery of activity
Concentrated medium	31,450.0	715,000	23	1	100
Ultrogel AcA 54	4,100.0	660,000	161	7	92
Chromatofocusing (pH 4.6 peak)	252.0	140,000	556	24	20
Hydroxyapatite	10.4	110,000	10,577	466	15
Mono S	0.32	66,000	206,250	9,085	9
Mono Q	0.015	40,000	2,660,000	117,180	6
Chromatofocusing (un- adsorbed)	1,320.0	314,000	238	11	48
Mono S, pH 4.0	8.8	200,000	22,730	1,001	28
Mono S, pH 4.0 (re- chromatography)	1.8	164,000	91,111	4,013	23
Mono S, pH 4.0/pH 6.4	0.36	152,000	422,222	18,600	21
Mono S, pH 6.4	0.05	126,000	2,520,000	111,013	18

Purification was monitored with the cartilage bioassay for release of proteoglycan. See Materials and Methods for details.



Fig. 6 shows the appearance of the two proteins on SDS-PAGE. Both migrated as single bands of identical  $M_r$  21,000. When the proteins were run in the same lane (Fig. 6, lane *d*), they ran together as a single band. It was not possible to elute any active IL-1/8 from the gels, although this had been done successfully for IL-1/5 (16). The detergent apparently destroys the biological activity of the protein. After isoelectric focusing in a polyacrylamide gel, IL-1/8 focused as a doublet at pH  $\sim$ 8.3. Active material was eluted at this position from a parallel gel (Fig. 7).

*Dose Response of Pig IL-1s on Cartilage and Thymocytes.* The proteins were active on cartilage in the range of 10–100 pM during a 15 h incubation (Fig. 7). Longer incubations increased the sensitivity of this assay: at 40 h incubation, the dose response was 1–10 pM (Fig. 7). IL-1/5 was much more active on murine thymocytes (10–100 pM) than IL-1/8 which, even at 30 nM, only augmented the uptake of [ $^3$ H]thymidine 2.5-fold (Fig. 8).

*Stimulation of Production of PGE and Collagenase by IL-1/8.* IL-1/8 was stimulatory to prostaglandin synthesis by pig synovial fibroblasts at a concentration of 50–100 pM (Table II). Concomitant increases in levels of latent collagenase were detected in the culture medium. The protein also stimulated PGE production by human dermal fibroblasts, but higher concentrations of IL-1 were needed

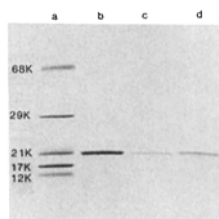


FIGURE 6. SDS-PAGE of pig IL-1s. (a) Molecular weight standard proteins; (b) 500 ng IL-1/8; (c) 200 ng IL-1/5; (d) 150 ng IL-1/8 plus 150 ng IL-1/5.

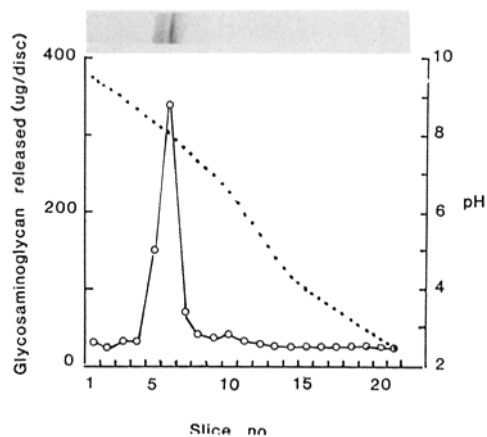


FIGURE 7. Isoelectric focusing of pig IL-1/8. 2.4  $\mu$ g of IL-1/8 was focused as described in Materials and Methods and was stained with Coomassie Brilliant Blue R250. A parallel gel containing 0.2  $\mu$ g of IL-1/8 was sliced and the eluates of the crushed slices were assayed on cartilage after diluting 1:5 in culture medium (O). pH (---).

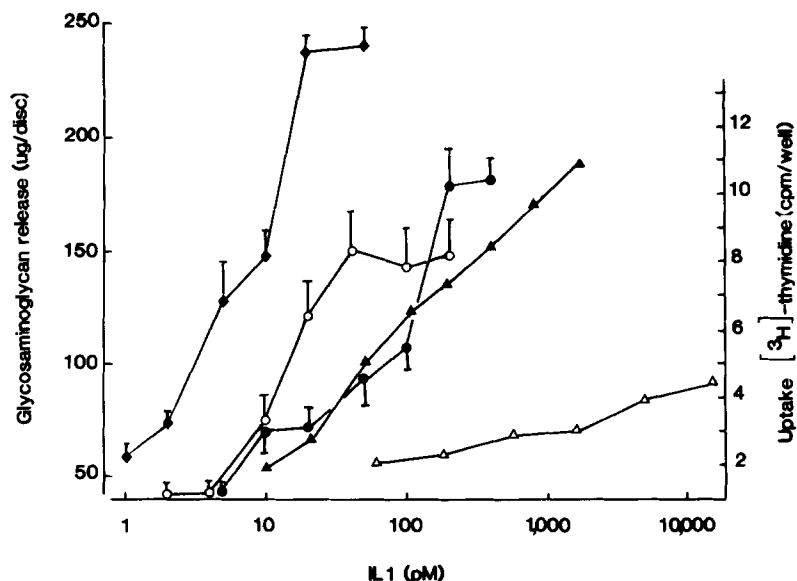


FIGURE 8. Dose response curves of pig IL-1s in bovine cartilage and mouse thymocyte assays. IL-1/5 ( $\blacktriangle$ ) and IL-1/8 ( $\triangle$ ) were assayed for augmentation of mouse thymocyte proliferation and results are shown as uptake of [ $^3\text{H}$ ]thymidine (mean cpm/well). The same batches of protein were also assayed on bovine nasal cartilage for 16 h (IL-1/5,  $\bullet$ ; IL-1/8,  $\circ$ ). IL-1/5 was also assayed for 40 h ( $\blacklozenge$ ). Bars represent SEM.

TABLE II  
Effect of ILs on Production of Prostaglandin- and Trypsin-activatable  
Collagenase by Fibroblasts

Stimulant	nM	Pig synovial fibroblasts		Human fore- skin fibro- blasts
		PGE <sub>2</sub>	Collagenase	PGE <sub>2</sub>
		ng/ml	U/ml	ng/ml
0	—	7.7 $\pm$ 1	1	0.7 $\pm$ 0.1
IL-1/8	0.05	98 $\pm$ 25	3.6 $\pm$ 1	—
	0.25	290 $\pm$ 20	13.5 $\pm$ 1	15.6 $\pm$ 2
	1.0	670 $\pm$ 131	35.4 $\pm$ 2.3	70.5 $\pm$ 5
	8.0	—	—	177 $\pm$ 19
IL-1/5	0.25	—	—	253 $\pm$ 16

Confluent fibroblasts in 30-mm petri dishes were stimulated for 48 h with the indicated dose of IL-1. Results are given as mean  $\pm$  SEM for groups of five dishes. Pig fibroblasts were at passage 5, human at 20.

to obtain a response from these cells than from the pig synovial fibroblasts (Table II).

*Pyrogenicity of Basic IL-1.* Although IL-1/8 was active at low concentration on chondrocytes and fibroblasts, it was only a weak activator of mouse lymphocytes. Since lymphocyte activation is a defining bioassay, we were uncertain of the identification of the protein as a form of IL-1. We therefore tested its ability to

cause fever since this is another effect associated with IL-1 (Table III). Injection of 5 ng into the rabbit cerebral ventricle caused an increase in rectal temperature of 1.47°C after 2 h. Significant fever followed injection of as little as 1 ng. After boiling, 5 ng was only as active as 0.5 ng. Such heat inactivation and the low level of endotoxin contamination (2 pg/5 ng IL-1/8) indicate that the fever was caused by the protein.

*Immunological Difference Between Pig IL-1s.* The IL-1s were electrophoresed in adjacent lanes on SDS-PAGE and electrophoretically transferred to nitrocellulose paper. Each IL-1 was stained by its respective antiserum, but neither antiserum stained both IL-1s. (Fig. 9). Each antiserum inhibited its own IL-1 but not the other (Table IV). The antiserum to IL-1/5 was much more strongly inhibitory than that to IL-1/8, a 1:10,000 dilution of the former being as effective as a 1:50 dilution of the latter.

### Discussion

Pig mononuclear leukocytes stimulated with Con A make at least two proteins that demonstrate the types of biological activity associated with IL-1. These

TABLE III  
*Pyrogenicity of Pig IL-1/8 in Rabbits*

Dose per animal ng	Change in rectal temperature at:		
	0.5 h	1 h	2 h
5	0.28 (0.03)	0.73 (0.16)	1.47 (0.15)
2	0.13 (0.12)	0.56 (0.11)	1.3 (0.10)
1	0.02 (0.02)	0.28 (0.05)	0.87 (0.21)
0.5	-0.07 (0.04)	-0.05 (0.13)	0.45 (0.22)
5 (heated)*	0.04 (0.03)	-0.05 (0.13)	0.45 (0.16)

Results show change in rectal temperature ( $^{\circ}\text{C} \pm \text{SEM}$ ) in rabbits at times indicated after intracerebroventricular injection. The amount of endotoxin contained in the highest dose was  $\sim 2$  pg as measured by limulus amoebocyte lysate assay.

\* Heat inactivation was carried out by boiling the sample for 30 min.

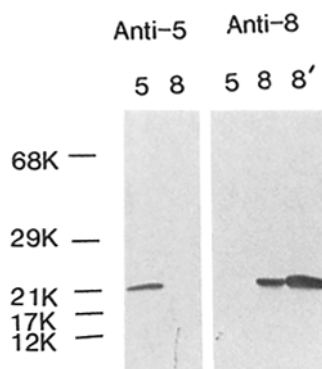


FIGURE 9. Western blot of pig IL-1s. Samples were electrophoresed in SDS-PAGE, transferred to nitrocellulose paper, and stained with antisera as described in Materials and Methods. (Lane 5), 60 ng IL-1/5; (lane 8 and 8') 20 and 60 ng of IL-1/8. Left-hand piece of nitrocellulose was stained with rabbit antiserum to IL-1/5, the right with antiserum to IL-1/8.

TABLE IV  
*Inhibition of IL-1s by Antisera in Cartilage Bioassay*

Antiserum to:	Antiserum dilution	Percent inhibition	
		IL-1/5	IL-1/8
IL-1/5	1:50	98	0
	1:3,300	96	—
	1:10,000	89	—
	1:33,300	29	—
	1:100,000	0	—
IL-1/8	1:50	0	84
	1:100	—	48
	1:200	—	38
	1:500	—	35
	1:1,000	—	0

Antisera were preincubated with either IL-1/5 or IL-1/8 (both at 2 ng/ml) in the cartilage bioassay culture medium for 6 h at the indicated dilutions. The samples were then assayed directly on cartilage discs for 24 h as described in Materials and Methods. Results are shown as a percent inhibition of the IL-1 assay without antiserum. Preimmune serum or normal rabbit serum had no effect on IL-1s in this bioassay.

proteins have identical  $M_r$  as determined by SDS-PAGE but different isoelectric points. Our purification of IL-1/5 was an improvement over the previous one (15), since the avoidance of reverse phase HPLC (which had been carried out at pH 2.0 and exposed the IL-1 to 55% acetonitrile) resulted in a nearly 10-fold improvement in recovery of activity as well as a higher overall purification factor. Even so, only ~15–20  $\mu\text{g}$  of homogeneous IL-1/5 was obtained from 200 L of blood. The complete purification of IL-1/8 was made possible by its unusual behavior on the Mono S column. When applied to the column in 50 mM formate, pH 4.0, it was eluted immediately upon the buffer being switched to 50 mM phosphate, pH 6.4. But when both sample and column were first equilibrated with the phosphate buffer, the IL-1/8 bound to the exchanger and was eluted by a salt gradient. Compared with IL-1/5, significantly greater quantities of IL-1/8 can be isolated: recent batches (200 L of blood) have yielded up to 80  $\mu\text{g}$ .

The final specific activities of the IL-1s against cartilage were similar. In contrast, the IL-1/5 was more active than IL-1/8 on mouse thymocytes and human fibroblasts. The relatively weak effect of the IL-1/8 on mouse lymphocytes called into question its identification as an IL-1, but it was as potent a pyrogen in rabbits as IL-1/5.<sup>2</sup>

The close similarity in their biological effects and the fact that they migrate identically on SDS electrophoresis suggest that the two forms of IL-1 are closely related, if not the same polypeptide. It was therefore surprising that the rabbit antisera made against each IL-1 apparently did not crossreact with the other, as judged by either Western blot or inhibition of biological activity. Besides this immunological difference the two proteins have quite different pIs. If there were a single IL-1 polypeptide chain, the most likely posttranslational modification sufficient to lower the pI by 3.5 U would be glycosylation, but such a degree of glycosylation would likely cause a change in mobility in SDS-PAGE. The appar-

ently identical  $M_r$  in SDS-PAGE and the similarity in biological activity of the IL-1s suggest that there is only one protein, but the immunological difference and the disparity in charge suggest that there are two. Amino acid sequencing will answer this question.

Molecular heterogeneity of IL-1s has been reported in several species. Human IL-1 has been found to be heterogeneous in molecular weight and charge, although all the forms are similar in their biological activity (22). Generally, human IL-1 has been found to be of  $M_r$  15–17,000 and to exist in two main isoelectric forms, one of pI  $\sim$ 7.0 and the other, pI  $\sim$ 5.0 (8, 22, 23). Of the human IL-1s purified to homogeneity, one has  $M_r$  14,500 and pI 7.0 (12), another,  $M_r$  17,000 and heterogeneous pI (four isoelectric forms at pH 5–6.5 [13]), and a third,  $M_r$  17,500 and an unreported pI (14). Rabbit IL-1s demonstrate a similar charge heterogeneity with the main forms at pI  $\sim$ 5 and  $\sim$ 7 (3, 24). There is also evidence that they are immunologically distinct, since an antiserum to the neutral form did not crossreact with the acidic one (25).

In light of this biochemical heterogeneity, it is interesting that the cDNA sequences reported for IL-1s of mouse (26) and human (27) origin show little similarity, except that they code for polypeptides of similar size ( $\sim$ 31,000  $M_r$ ) which are thought to be precursors of IL-1. There may be two types of IL-1, although it would be surprising if they did not show considerable homology.

### Summary

Two forms of interleukin 1 (IL-1) were purified to homogeneity from the culture supernatants of pig buffy coat leukocytes stimulated with concanavalin A. The two proteins had identical  $M_r$  of 21,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, but one, which had previously been purified as a cartilage-resorbing protein, had pI 5 (IL-1/5) and the other, pI 8.3 (IL-1/8). After initial gel filtration and separation by chromatofocusing IL-1/5 was purified by chromatography on hydroxyapatite and the ion exchangers, Mono S and Mono Q; IL-1/8 was purified by chromatography at pH 4.0 and pH 6.4 on Mono S. Purification was monitored by a cartilage proteoglycan release assay and both proteins had a final specific activity  $\sim 10^5$  times that of the leukocyte culture medium. Medium conditioned by cells from 200 L of blood yielded  $\sim 15$   $\mu$ g of IL-1/5 and 50  $\mu$ g IL-1/8. IL-1/8 augmented mouse thymocyte proliferation, stimulated synovial fibroblasts to produce prostaglandin E and latent collagenase, and was pyrogenic upon intracerebroventricular injection into rabbits. IL-1/5 has previously been shown to possess all these activities. An antiserum to each IL-1 was raised in rabbits. Each antiserum inhibited its respective IL-1 in a cartilage bioassay and stained it upon Western blotting. Neither antiserum inhibited or stained the other IL-1. We conclude that pig leukocytes make two immunologically distinct forms of IL-1 that have identical  $M_r$ , demonstrate the same range of biological activity, but differ in isoelectric point.

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