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Demonstration of Interleukin 1 Activity in Apparently Homogeneous Specimens of the pI 5 Form of Rabbit Endogenous Pyrogen

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Rabbit mononuclear cells from oil-induced peritoneal exudates were purified by centrifugation on Percoll gradients, suspended in tissue culture medium, and stimulated with opsonized *Staphylococcus epidermidis*. The supernatants from these macrophages caused fever when injected intravenously into rabbits (endogenous pyrogen [EP] activity). The EP activity was contained in two protein fractions, with pIs of 7.3 and ca. 5.0. The same fractions caused mouse thymocytes to incorporate tritiated thymidine when incubated in vitro with small quantities of phytohemagglutinin (interleukin 1 [IL-1] activity). The pI 5.0 form of EP was purified to apparent homogeneity by sequential use of ammonium sulfate precipitation, gel filtration, ion-exchange chromatography, hydrophobic chromatography, and high-resolution isoelectric focusing. EP and IL-1 activities were not separable by any of these procedures. Active fractions from isoelectric focusing were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Only one band was visible as judged by a silver staining method, and IL-1 activity could be recovered by renaturing eluates from the same region of sodium dodecyl sulfate gels run in parallel. An estimate of specific activity was made by comparing the intensity of stained bands of EP with the intensity of bands containing known quantities of lysozyme or RNase. By this criterion, the specific activity of purified pI 5 EP was between 17,000 and 58,000°C U/mg of protein, and the specific activity in terms of IL-1 was between 59 million and 360 million U per mg of protein. These observations suggest that both EP and IL-1 activities can be expressed by a single molecular species. The implications of this coincidence are discussed. It was also shown that highly purified pI 5 EP obtained from macrophages stimulated in the presence of ^{14}C-labeled amino acids contained significant ^{14}C radioactivity. This suggests that the pI 5.0 EP, like the pI 7.3 form, was synthesized de novo from amino acid precursors.

Endogenous pyrogens (EPs) were originally described by Beeson (2) and were thought to be secreted by neutrophils. Recent evidence suggests that EPs are in fact secreted only by monocytes and macrophages (13). It has been shown that EPs exist in at least two forms which appear to be chemically and immunologically distinct (23). Considerable progress has been made in purifying rabbit EPs; one species, with a pI of 7.3 and an apparent molecular weight of 15,000, has been purified to apparent homogeneity (24). More recently, the other species has been shown to be a family of proteins with the same apparent molecular weight but several discrete pIs between 4.5 and 5.0 (4).

During the course of purifying these EPs, we became aware of similarities between them and the macrophage factor known as interleukin 1 (IL-1). IL-1 was originally described by Gery et al., who showed that it allowed murine thymocytes to proliferate in the presence of submitogenic quantities of lectins (9). Subsequently, it has been shown that IL-1 is secreted by macrophages stimulated with several different purified bacterial components (26) and that it participates in a wide variety of immunological reactions (1), including those culminating in humoral (15, 18) as well as cell-mediated immunity (8, 19). IL-1 is believed to be an early-acting mediator in immune responses, leading to the secondary generation of other lymphokines such as interleukin-2 (IL-2) (7, 31). Rabbit IL-1 has been shown to exist in two forms, with molecular weights and pI values very similar to those of rabbit EPs (30). Recently, we showed that partially purified rabbit EPs contained IL-1 activity and vice versa; that antibody to the pI 7.3 species of rabbit EP neutralized the corresponding IL-1; and that these same species of IL-1 and EP bound to thiol Sepharose and coeluted with mercaptoethanol (25).

For pI 4.5 to 5.0 EPs and IL-1s, no antibody was available, and there was no suitable affinity column. Evidence that the IL-1s and EPs were identical was restricted to similarities in molecular weight and an identical pattern of heterogeneity of pI. We have, therefore, purified the EP activities in this region to apparent homogeneity and examined the active fractions for IL-1 activity. The results are described in this paper.

MATERIALS AND METHODS

Peritoneal exudate macrophages were obtained from rabbits injected with 50 ml of sterile mineral oil 3 days previously (4). The macrophages were purified by centrifugation on Percoll gradients (13). They were washed twice in minimal essential medium with Hanks salts (GIBCO Laboratories, Grand Island, N.Y.). If they were not to be endogenously radiolabeled, they were put up in RPMI 1640 medium (GIBCO) and stimulated with opsonized washed *Staphylococcus epidermidis* at a ratio of 50 bacteria per cell. If they were to be radiolabeled, the medium used was minimal essential medium with Eagles salts (GIBCO), supplemented with vitamins but without amino acids. A 1-μCi portion of a mixture of 15 ^{14}C-labeled amino acids (ICN Pharmaceuticals Inc., Irvine, Calif.) per ml was added, and the macrophages were stimulated with *S. epidermidis* as described above. No serum was used in either medium, except for those serum components absorbed to the staphylococci. The mixtures of macrophages and bacteria were incubated overnight at 37°C, and the supernatant contained crude pyrogen.

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The methods for pyrogen assay (24), for the pyrogen unit (24), and for the exclusion of endotoxin contamination during experiments were described previously (21, 24).

A 12.7-liter portion of crude ^{14}C-radiolabeled pyrogen was concentrated 10-fold by ultrafiltration on an Amicon UM 05 membrane, and 243 g of ammonium sulfate (Schwartz/Mann, Orangeburg, N.Y.) per liter was added. The mixture was stirred at 4°C overnight and centrifuged at 40,000 × *g* for 30 min. To the supernatant was added 312 g of ammonium sulfate per liter, and the mixture was again stirred overnight at 4°C. The precipitate was collected by centrifugation as described above, redissolved in a minimum quantity of sterile distilled water, and clarified by another centrifugation. The concentrated pyrogen was further purified by gel filtration on Sephacryl S-200, with endotoxin-free acetate buffer (pH 5.0) as described previously (25).

The active fractions from S-200 chromatography were pooled and dialvzed against 50 mM Tris-hydrochloride buffer (pH 8.6) with 1 mM mercaptoethanol. It was then applied to a column of DEAE-A25 Sephadex (Pharmacia Fine Chemicals, Piscataway, N.J.) previously equilibrated with the same buffer. A linear gradient from 50 mM Tris to 50 mM Tris plus 0.2 M NaCl was applied, and fractions were collected. EP activity eluted as several peaks between 0.1 and 0.2 M NaCl; these were combined and made 1.5 M in ammonium sulfate (Schwartz/Mann). The EP was applied to a phenyl Sepharose column (Pharmacia), and after absorption and a short 1.5 M ammonium sulfate wash, a linear gradient from 1.5 M ammonium sulfate and no ethylene glycol to no ammonium sulfate and 60% ethylene glycol in 50 mM phosphate buffer (pH 7.4) was applied.

The active fractions from phenyl Sepharose chromatography were combined and dialyzed against 0.01 M sodium chloride with 1 mM mercaptoethanol. They were then subjected to two isoelectric focusings as described previously (4). A final voltage of 2,000 was maintained for 24 h. pH measurements were done at 0°C with a Beckman pH meter.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed by the method of Laemmli (17), as modified by Jones (14). The gels were 16 cm in length by 0.8 mm thick, and the resolving gel contained 20% acrylamide. Samples of EP from isoelectric focusing columns were not suitable for SDS-PAGE because of the presence of ampholytes. They were, therefore, dialyzed in Spectrapor 4 membranes (Spectrum Medical Industries, New York, N.Y.) against 0.1 M ammonium bicarbonate with 0.01% SDS for 2 days. The contents of each bag was placed in a microfuge tube (Beckman Instruments, Inc., Fullerton, Calif.) and evaporated to dryness with a centifugal evaporator (Savant Instruments, Inc., Hicksville, N.Y.). The pellets were taken up into SDS-PAGE sample buffer (14). Standard protein mixtures for molecular weight calibration (Bio-Rad Laboratories, Richmond, Calif.) were run in parellel with the test samples.

Gels were stained by the silver method of Morressey (22) which, in our hands, would reliably detect 20 to 40 ng of protein. Molecular weight standards were used at 100 ng per protein band.

IL-1 was assayed as described previously (15), except that we used thymocytes from CBA mice at a concentration of 5 × 10^6 cells per ml. In general, IL-1 was quantitatively assayed with reference to a standard preparation of rabbit IL-1 stored in liquid nitrogen. A unit of IL-1 activity was defined as the amount of this standard which caused one-third maximal proliferation in the IL-1 assay. Over 8 months and 11 formal titration assays, the IL-1 activity of the standard preparation was 355 ± 74 U/ml (95% confidence limits). In experiments in which we wished only to demonstrate activity, samples were assayed directly and were expressed in terms of mean tritiated thymidine uptake per well. Both the standard IL-1 preparations and the purified IL-1s reported herein were devoid of IL-2 activity with the CTL 15H cell line and following the method of Gillis et al. (10) (data not shown).

Protein concentrations for all stages of purification except the isoelectric focusing step were performed by using the Bradford Coomassie blue binding assay (3). However, this was not sufficiently sensitive to measure the protein concentration of purified pyrogens in the effluent of isoelectric focusing columns. Therefore, we loaded known amounts of lysozyme or ribonuclease (Worthington Diagnostics, Freehold, N.J.) onto SDS-PAGE gels, and electrophoresed them along with samples of purified EP. The gels were silver stained, and the intensity of the EP bands was compared visually with the density of the lysozyme or RNase bands. This enabled us to estimate protein contents of the EP bands with a maximum probable error of twofold in either direction. Protein contents of the isoelectric focusing column fractions were calculated with the known volumes of EP loaded onto the gels.

IL-1 activity was demonstrated in renatured SDS-PAGE gel eluates by the method of Hager and Burgess (11). Preliminary experiments showed that the recovery of known amounts of IL-1 activity after denaturation and renaturation was over 50%. Before assay, renatured samples of IL-1 were transfered into tissue culture medium plus 5% fetal calf

TABLE 1. Purification of pI 4.8 EP

Purification stage	Protein (mg)	Endogenous radioactivity (cpm)	Pyrogen (U)	IL-1 (U)
First				
Crude	698	3.5×10^8	7,500	1.4×10^7
Sephacryl S-200	14.2	1.4×10^7	3,100	1.1×10^7
DEAE-Sephadex	0.54	8.6×10^5	1,500	8.2×10^6
Phenyl Sepharose	0.11	1.8×10^5	680	3.6×10^6
IEF[a] peak A, pI 4.80	0.0016	3.2×10^4	110	5.8×10^5
IEF peak B, pI 4.95	0.0038	1.1×10^4	79	4.3×10^5
Second				
IEF peak A, pI 4.63	0.0014	3.5×10^3	27	1.2×10^5
IEF peak B, pI 4.70	0.0015	2.1×10^3	25	8.7×10^4

[a] IEF, Isoelectric focusing.

serum by gel filtration on small Sephadex G25 columns. ^{14}C radioactivity was extracted from sliced SDS-PAGE gels in NCS tissue solubilizer (Amersham Corp., Arlington Heights, Ill.) and counted in a Beckman LS-8000 liquid scintillation spectrometer. The results were expressed as counts per minute without corrections to disintegrations per minute.

RESULTS

The purification scheme for the pI 5 form of rabbit EP is shown in Table 1. A second purification, with an identical protocol, was also performed. Again, two principal purified EP peaks were seen in the effluent of the second isoelectric focusing column. Data for these two peaks of the second purification are added at the bottom of Tables 1, 2, and 3.

Crude pyrogen contained some pyrogenic activity due to endotoxin contamination; this was resolved away from the EP activity by gel filtration on Sephacryl S-200. Endotoxin appeared as a peak of pyrogenic activity, without IL-1 activity, in the high-molecular-weight region. There was also a peak of EP activity plus IL-1 activity with an apparent molecular weight of 22,000. At this stage of purification, there was no obvious peak of ^{14}C radioactivity coinciding with the EP and IL-1 peak (Fig. 1).

Crude EP obtained as described was about 85% in the pI 5 form; the pI 7.3 EP was resolved away from the pI 5 EP by chromatography on DEAE-Sephadex. The pI 7.3 form failed to bind to the column and appeared in the void volume. Most of the EP activity did bind to the column and eluted as a broad peak of EP and IL-1 activity, but without an obvious peak of ^{14}C radioactivity.

Chromatography of the pI 5 EP from DEAE on phenyl Sepharose gave a single peak of EP and IL-1 activity which also coincided with a well-marked peak of ^{14}C radioactivity (Fig. 2). Unlabeled contaminating proteins were resolved away from this peak by isoelectric focusing; the first focusing showed a single peak of EP, IL-1, and ^{14}C radioactivity which was resolved into two principal peaks by a second focusing at 2,000 V (Fig. 3). The second batch of EP was purified by an identical protocol (Fig. 4). The final electrofocusing step also showed two principal peaks of EP and IL-1 activities. In the first purified batch, the pIs of these peaks were 4.80 and 4.95; in the second batch, they were 4.63 and 4.70. For each purification, the peak with the lower pI was called peak A, and that with the higher pI was called peak B.

The specific activities of EP and IL-1 at various stages of purification are shown in Table 2. If ^{14}C radioactivity and IL-1 activity are properties of the EP molecule, there should be a reasonably constant ratio between measurements of EP, IL-1, and ^{14}C radioactivity after the initial stages of purification (Table 3).

Samples from the EP peaks in isoelectric focusing columns were run on SDS-PAGE gels and stained with the silver stain. Single bands were seen in both peaks of both purifications (Fig. 5). In both cases, it was possible to elute gel slices, renature the contained proteins, and show that IL-1 activity coincided with the stained visible band (Fig. 6). The apparent molecular weights of the pI 5.0 EPs were 17,200 (peak A) and 17,300 (peak B) as judged by SDS-PAGE.

Stained bands of EP visible on SDS-PAGE gels were cut out, digested with NCS tissue solubilizer, and counted for ^{14}C radioactivity. Each visible band was associated with a single peak of ^{14}C activity (Table 4).

DISCUSSION

The overall EP yield from this purification schedule was 2.5%. However, this value is an underestimate, because some of the pyrogenic activity in crude material was due to endotoxin contamination. The overall yield in terms of IL-1 activity was 7%. Even this is a slight underestimate, because ca. 15% of the EP and IL-1 activities in crude pyrogen was

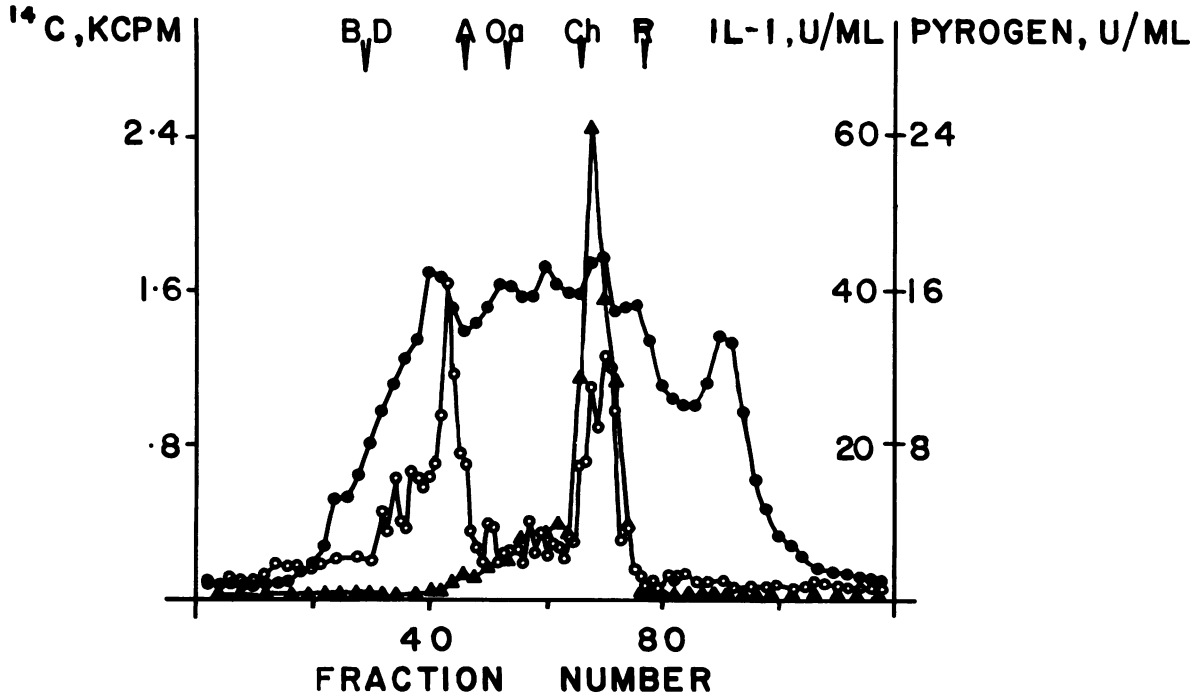

FIG. 1. Gel filtration of crude EP on Sephacryl S-200. Symbols: ○, EP; ▲, IL-1; ●, ^{14}C radioactivity. Abbreviations: BD, blue dextran; A, bovine albumin; Oa, ovalbumin; Ch, chymotrypsin; R, ribonuclease.

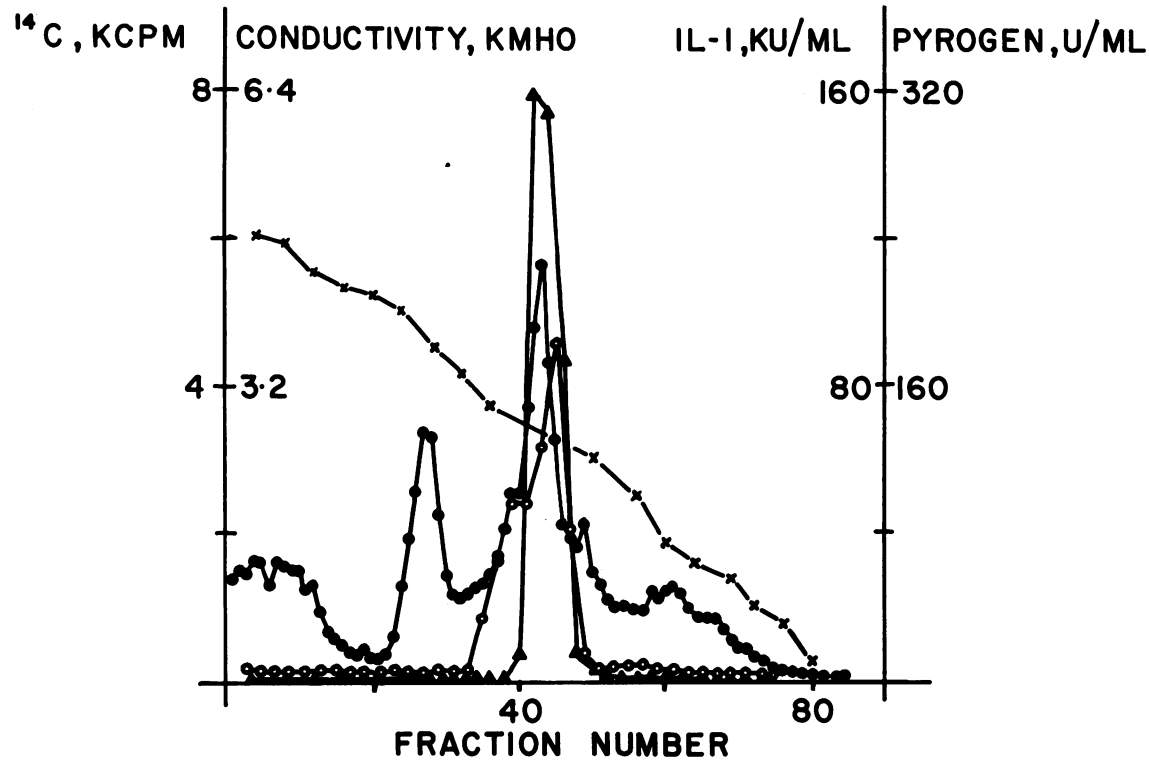

FIG. 2. Chromatography of pI 5.0 pyrogen on phenyl Sepharose. Symbols: ○, EP; ▲, IL-1; ●, ^{14}C radioactivity; ×, conductivity.

due to the pI 7.3 form of EP, which was resolved away from the pI 5.0 form during processing.

In these and previous experiments (4), we have consistently found multiple peaks of EP and IL-1 activities in the pI 5 region of isoelectric focusing columns. The measured pI values of the principal peaks have shown slight variations. We think that these probably represent experimental inaccuracies and that the main peaks are identical from one purification run to another. That was proved to be the case on the two occasions it was tested (4). The cause of the

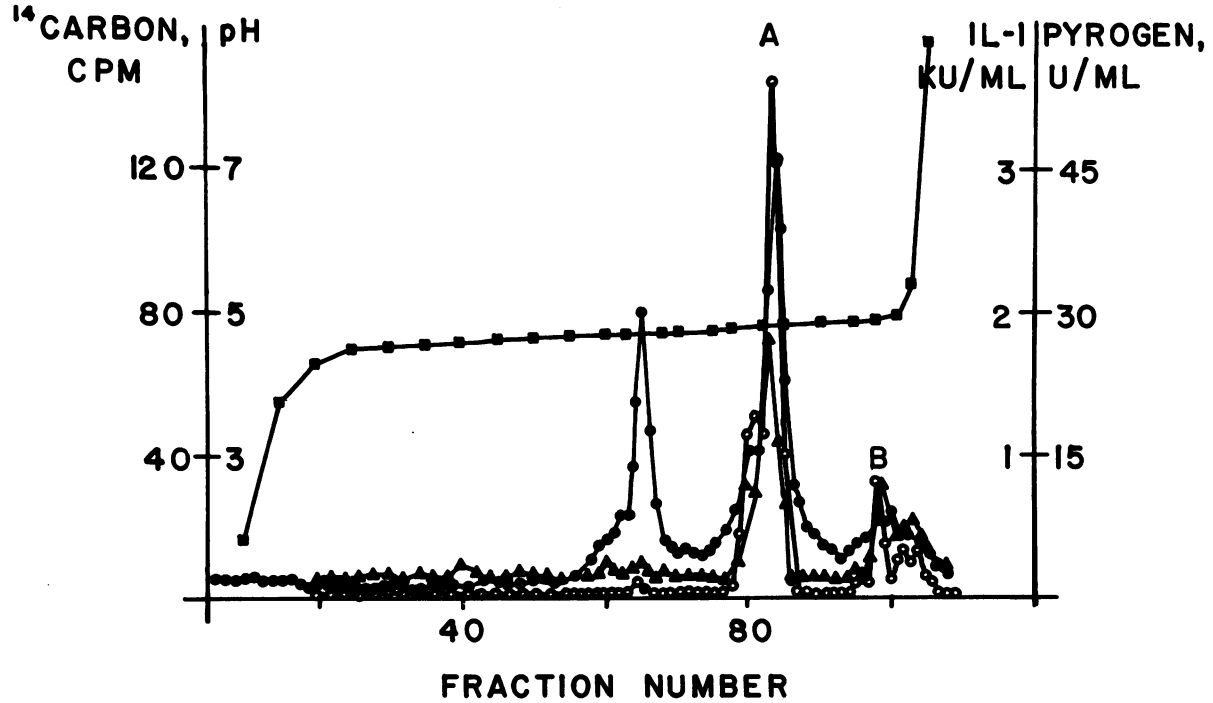

FIG. 3. High-resolution isoelectric focusing of pI 5.0 pyrogen. Symbols: ○, EP; ▲, IL-1; ●, ^{14}C radioactivity; ■, pH. Peaks A and B are marked.

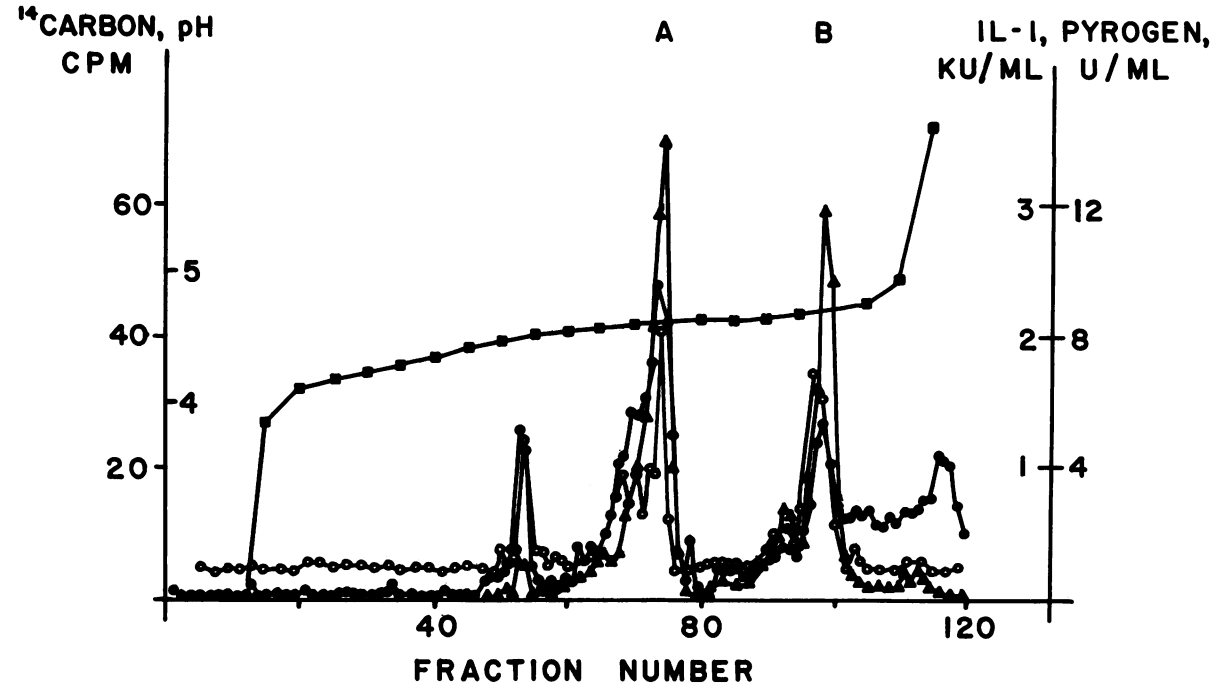

FIG. 4. High-resolution isoelectric focusing of a second batch of pI 5.0 EP. Symbols: ○, EP; ▲, IL-1; ●, ^{14}C radioactivity; ■, pH. Peaks A and B are marked.

microheterogeneity is unknown; there may be a single parent molecule which undergoes post-translational changes such as glycosylation, carbamylation, or limited proteolysis.

The specific activities of purified pI 5.0 EP ranged from 17,000 to 58,000 °C U/mg of protein. These are comparable with the specific activities reported from our laboratory for the pI 7.3 form of EP, which ranged from 20,000 to 33,000 U/mg of protein (24). The IL-1 specific activities ranged from 5.9×10^7 to 3.6×10^8 U/mg of protein. These may be compared with the value of 1×10^6 U/mg of protein obtained for apparently homogeneous specimens of murine IL-1 (20). Our samples of IL-1 were not necessarily any more homogeneous than those of Mizel and Mizel (20); we were assaying rabbit IL-1 on mouse cells, our unit (33% of maximal activity) was about half as large as theirs (50% of maximal activity), we used CBA rather than HeJ cells, the assays were done in different laboratories with different standard preparations, and the methods used to measure protein content were very different. However, it does seem that our IL-1 preparations were at least as active as theirs.

Pacak and Siegert (27) also purified a rabbit EP to apparent homogeneity. The reported pI was 4.2, with a molecular weight of 13,000 to 15,000. Both these values differ appreciably from ours; furthermore, the reported specific activity was 50 ng of protein/kg of rabbit for a fever of 1°C. This value is about 6 times larger than the value we obtained. The purified pyrogen was not tested for IL-1 activity, and whether or not it was identical to one of the EP peaks we isolated is problematic.

Silver staining does not give a constant color yield for all proteins, although we are not aware of any proteins which have been found to be highly resistant to silver staining. Lysozyme and RNase were empirically chosen as standards in the present study and were found to give easily graded

TABLE 2. Specific activities of EP and IL-1 at various stages of purification

Purification stage	Sp act (U/mg of protein)	
	EP	IL-1
First		
Crude	11	2×10^4
Sephacryl S-200	220	8×10^5
DEAE-Sephadex	2,900	1.5×10^7
Phenyl Sepharose	6,000	3.2×10^7
IEF[a] peak A, pI 4.80	58,000	3.6×10^8
IEF peak B, pI 4.95	26,000	1.2×10^8
Second		
IEF peak A, pI 4.63	19,000	8.6×10^7
IEF peak B, pI 4.70	17,000	5.9×10^7

[a] IEF, Isoelectric focusing.

TABLE 3. Ratios of IL-1 activity and ^{14}C radioactivity to EP activity at various stages of purification

Purification stage	Ratio of:	
	IL-1 activity (10^3)/EP activity	^{14}C radioactivity (10^3)/EP activity
First		
Crude	1.9	47
Sephacryl S-200	3.5	4.5
DEAE-Sephadex	5.5	0.57
Phenyl Sepharose	5.3	0.26
IEF[a] peak A, pI 4.80	5.3	0.29
IEF peak B, pI 4.95	5.4	0.14
Second		
IEF peak A, pI 4.63	4.4	0.13
IEF peak B, pI 4.70	3.5	0.09

[a] IEF, Isoelectric focusing.

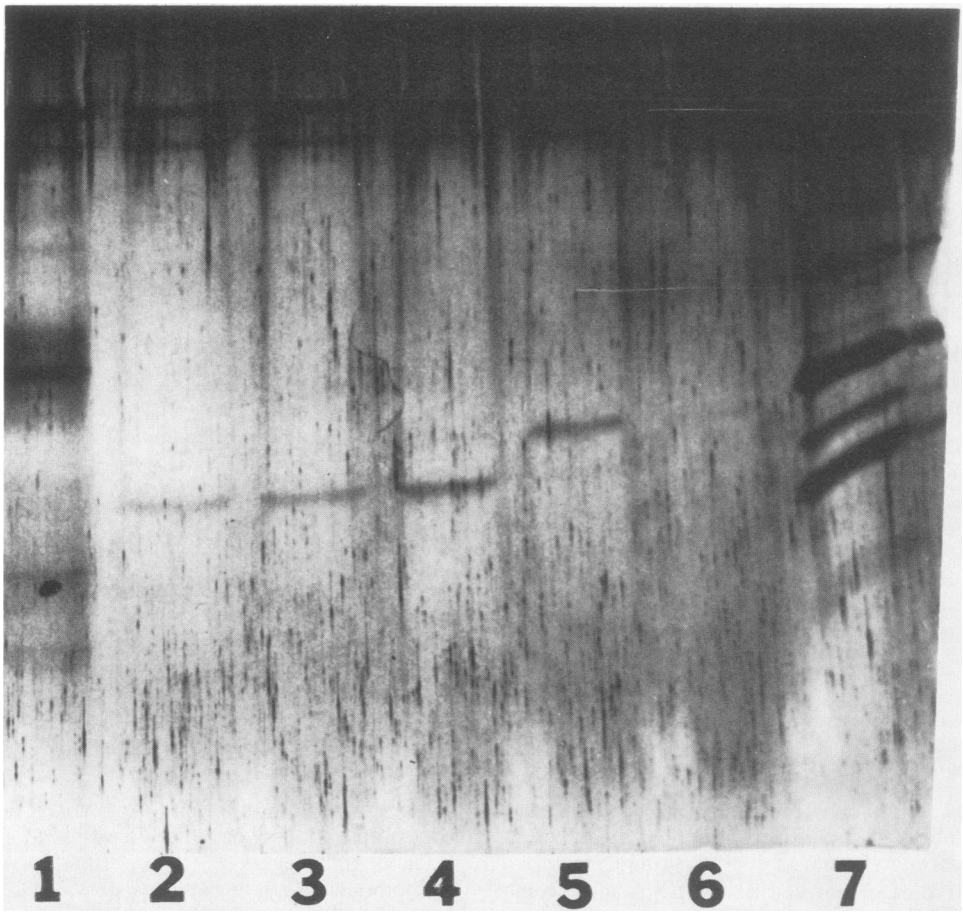

FIG. 5. Silver-stained SDS-PAGE gel of EP peaks shown in Fig. 3. Lane 1, marker proteins, 200 ng per band; 100 μl of fractions 81, 85, 83, and 98 are shown in lanes 2, 3, 4, and 5, respectively. Lane 6, blank; lane 7, marker proteins.

staining responses, especially in the range of 20 to 200 ng per band. This range was used to estimate the protein content of the EP and IL-1 samples. However, the relative color yields of lysozyme, RNase, and EP and IL-1 were unknown, and therefore the figures given for specific activity were only approximations.

Silver staining of gels generated large numbers of artifacts, such as punctillate staining and apparent high-molecular-weight doublets in the 50,000- to 60,000-molecular-weight area. These appearances were seen in lanes loaded only with sample buffer, and they were therefore not attributable to proteins in the samples of purified EP and IL-1.

EP and IL-1 activities were associated throughout the purification procedure. Furthermore, the ratio of IL-1 activity to EP activity was essentially constant at all stages of purification after Sephacryl S-200 chromatography (Table 3). Similar values for this ratio were obtained in a second, completely independent purification. The change in the ratio of IL-1 to EP activity observed at early stages of purification was easily explained; endotoxin in crude material was pyrogenic but had no IL-1 activity, thus lowering the ratio. All other ratios were between 3.5 and 5.5, which seems an acceptable range of variation for the ratio of two biological assays. Inspection of Fig. 4 and 5 also showed that EP and IL-1 activities showed an identical microheterogeneity by high-resolution isoelectric focusing.

SDS-PAGE gels of the active fractions from isoelectric focusing columns reproducibly showed a single band with an apparent-molecular-weight of about 17,000. Renaturation experiments showed that this band contained IL-1 activity. However, the EP assay is 5,000 times less sensitive than the IL-1 assay, and we did not have enough material to demonstrate that the IL-1 band also contained EP activity.

Rosenwasser et al. have used partially purified human EP to successfully replace the requirement for murine IL-1 in a system of antigen-dependent T-cell proliferation (29), and lectin-dependent thymocyte proliferation (28). These authors noted the simlarities between evocative stimulus, cell source, and molecular size and charge for human and murine EP and IL-1. Recently, Lipsky et al. (18) have demonstrated that highly purified human EP is directly stimulatory to B-cells, a property previously ascribed to murine IL-1 (33), and that antisera against human EP inhibit this stimulation. Murphy et al. (25) presented a strong presumptive case for the identity of rabbit EP and IL-1 by using partially purified factors independently prepared for EP or IL-1 activity by different workers. Cell source and molecular size were identical. In addition, both factors occurred in two isoelectric forms, pI 7.3 and pI 5.0. The pI 7.3 form of both EP and IL-1 could be bound and eluted from thiol Sepharose, unlike the pI 5.0 form. The biological activity of both the pI 7.3 forms could be neutralized with antisera raised in goats against the pI 7.3 form of EP. Finally, the pI 5.0 forms of partially purified rabbit EP or IL-1 displayed identical microheterogeneity of pI.

This study represents the first demonstration of the pres-

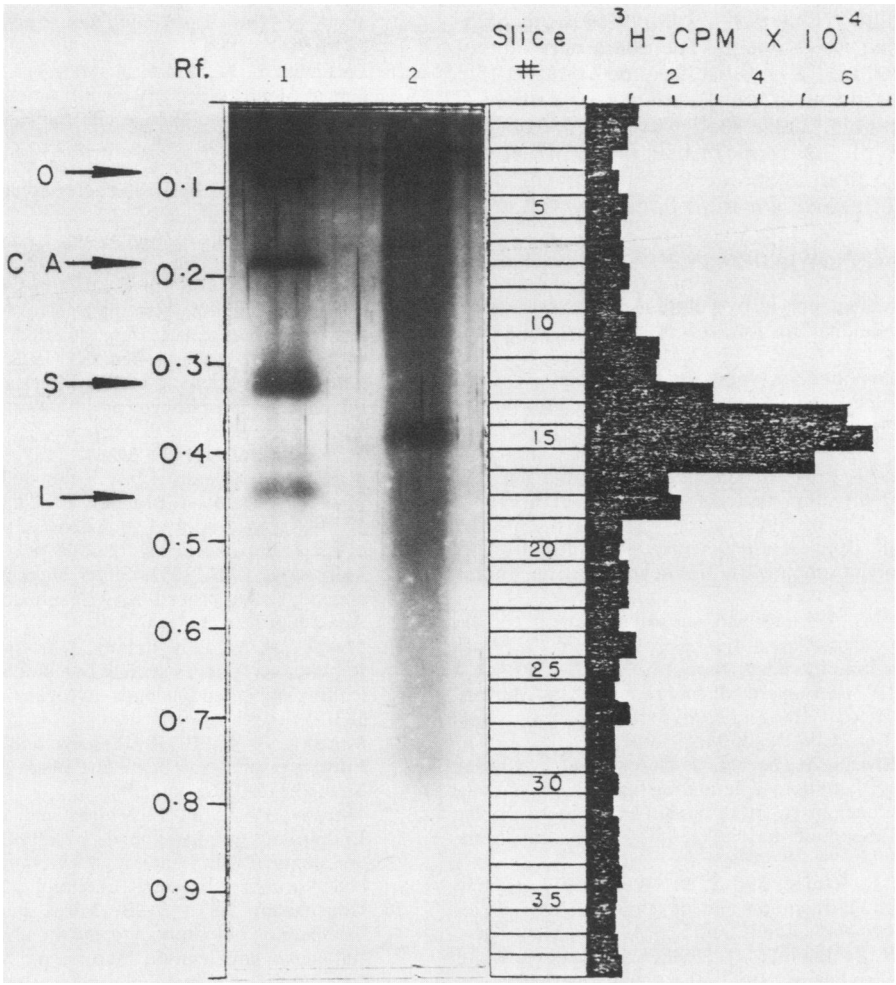

FIG. 6. Correlation of silver-stained band on SDS-PAGE gel with eluted and renatured IL-1 activity. Lane 1, marker proteins. Abbreviations: O, ovalbumin; CA, carbonic anhydrase; S, soybean trypsin inhibitor; L, lysozyme. Lane 2, 100 μl of fraction 99, Fig. 4.

ence of EP and IL-1 activities in an apparently homogeneous preparation. The evidence for this identity can be summarized as follows: the specific activities of EP and IL-1 were comparable with those reported for other homogeneous EPs and IL-1s; the ratio of IL-1 to EP activity did not change during purification; isoelectric focusing column effluents showed an identical microheterogeneity for EP and IL-1 activity; the most active fractions from isoelectric focusing columns showed only one band on SDS-PAGE; and IL-1 activity was demonstrated in that band. All of this supports the view that EP and IL-1 activities are properties of the same molecule.

TABLE 4. ^{14}C radioactivity extracted from 3-mm slices of silver-stained SDS-PAGE gels

Sample	^{14}C (cpm) in fraction no.:[a]		
	81	83	98
Gel slice			
+ 4	108	117	122
+ 3	113	116	120
+ 2	116	119	118
+ 1	116	138	121
Band	179	143	129
− 1	115	117	119
− 2	113	121	123
− 3	115	116	122
− 4	112	117	119

[a] Fractions are as shown in Fig. 3. All vials were counted to a standard error of ±1.5%.

It should be said that there probably are molecules with IL-1 activity which are not pyrogenic, and perhaps vice versa. EPs are known to originate from the cleavage of pyrogenically inactive intracellular precursors which are of higher molecular weight (12). Many of these intracellular precursors do have IL-1 activity (16). Whether this is because thymocytes can cleave the precursors to an active form, or whether the leisurely 3-day IL-1 assay simply allows more time for dissociation of active forms from carrier proteins is unknown. There is also evidence that both EP (6) and IL-1 molecules (32) readily form complexes both with themselves and with serum proteins. It also seems clear that certain substituted muramyl dipeptide derivatives cause macrophages to secrete molecules which have IL-1 activity but are not pyrogenic (5). The size and charge of these proteins have not been investigated.

In our experiments, the ratios of ^{14}C radioactivity to EP activity reached a minimum at the phenyl Sepharose stage and did not change substantially after isoelectric focusing. Peaks of ^{14}C radioactivity aligned with peaks of EP and IL-1

activities. And finally, ^{14}C activity was eluted from SDS-PAGE gels of purified EP (Table 4). The quaternary ammonium solubilizer produced a high background counting rate of between 100 and 120 cpm (Table 4), but in each case a ^{14}C peak significantly above background was eluted from the stained band. The pI 7.3 form of EP is known to be synthesized de novo from amino acid precursors (21), and this now seems to be proved for the 5.0 form as well.

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