Multiple Biological Activities of Human Recombinant Interleukin 1

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Abstract

Complementary DNA coding for human monocyte interleukin 1 (IL-1), pI 7 form, was expressed in *Escherichia coli*. During purification, IL-1 activity on murine T cells was associated with the recombinant protein. Homogeneous human recombinant IL-1 (hrIL-1) was tested in several assays to demonstrate the immunological and inflammatory properties attributed to this molecule. hrIL-1 induced proliferative responses in a cloned murine T cell in the presence of suboptimal concentrations of mitogen, whereas no effect was observed with hrIL-1 alone. At concentrations of 0.05 ng/ml, hrIL-1 doubled the response to mitogen (5×10^6 half maximal units/mg). Human peripheral blood T cells depleted of adherent cells underwent a blastogenic response and released interleukin 2 in the presence of hrIL-1 and mitogen. hrIL-1 was a potent inflammatory agent by its ability to induce human dermal fibroblast prostaglandin E_2 production in vitro and to produce monophasic (endogenous pyrogen) fever when injected into rabbits or endotoxin-resistant mice. These studies establish that the dominant pI 7 form of recombinant human IL-1 possesses immunological and inflammatory properties and acts on the central nervous system to produce fever.

Introduction

Although the activated monocyte/macrophage produces a great number and variety of biologically active molecules (1), considerable attention has focused on interleukin 1 (IL-1)[1] as an important mediator of host responses to microbial and inflammatory diseases. There presently is increasing evidence that IL-1 is the primary inducer of many components of systemic "acute-phase" responses and is also responsible for local inflammatory changes in various joint diseases (2–4). There are many biological activities attributed to IL-1 including its effects on the endocrinologic, nervous, and immunological systems. In addition, in mesenchymal tissue such as cartilage, bone, muscle, and fibrous tissue, IL-1 induces changes associated with both destructive as well as reparative functions. How a single molecule can affect these seemingly opposite activities in a wide variety of cells remains a challenging biological question.

Initial experiments suggesting a broad role for IL-1 in mediating host responses to infection and injury demonstrated that highly purified monocyte-derived endogenous pyrogens were able to augment T cell proliferation to mitogens and antigens, a property known as lymphocyte activating factor (LAF) (5, 6); in addition to causing fever, IL-1 induced several components of the acute phase response in experimental animals (7). Recent studies from several laboratories using IL-1 purified from mononuclear phagocytes to apparent homogeneity as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) have confirmed the initial observations on the multiple biological activities of IL-1 (8–11). Multiple biological activities of IL-1 are also associated with molecules eluting from gel-filtration chromatography at 30,000–38,000 mol wt (12–14) and with molecules focusing at distinct isoelectric points (~7, 6, and 5) (6, 10, 12, 13); antibodies developed to individual charged forms do not cross react (15). In addition, small fragments of 4,000 and 2,000 mol wt IL-1 derived from human sources have been described and raise the possibility that the active site for some biological activities is segregated in small peptides (16–18).

The sequence of a cDNA coding for human monocyte IL-1 has been recently described (19); the derived polypeptide sequence is a precursor form of 31,000 mol wt and a pI near 5, whereas the mature cleavage peptide has a molecular weight of 17,500 and a pI near 7. Although another human monocyte IL-1 cDNA has been reported (20), it shares nearly 80% homology with the amino acid sequence reported for the murine IL-1 from the P388D cell line (21), which has a pI of 5. It is clear from both protein and messenger RNA (mRNA) measurements that the pI 7 form of human IL-1 is the predominant form. As much as 5% of the total poly A mRNA extracted from human monocytes stimulated with endotoxin codes for this form of IL-1 (22) and this form is also the predominant IL-1 found in human pathological fluids. In this report we describe multiple biological activities of purified human recombinant IL-1 (hrIL-1), which was expressed in *E. coli*, and contained amino acids 71–269 (24,500 mol wt) or 112–269 (17,500 mol wt) of the precursor polypeptide (31,000 mol wt). The recombinant proteins exhibit several biological properties associated with the immunological and inflammatory properties of the molecule.

Methods

Materials. Phytohemagglutinin (PHA) was purchased from Burroughs-Wellcome & Co. (Research Triangle, NC). All endotoxin determinations

1. *Abbreviations used in this paper:* CTLL, cytotoxic T lymphocyte line; FCS, fetal calf serum; hrIL-1, human recombinant interleukin 1; IL-1, interleukin 1; LAF, lymphocyte activating factor; NA, nonadherent; PGE_2, prostaglandin E_2; PHA, phytohemagglutinin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

were made using the Limulus test using lysate with a sensitivity of 20 pg/ml obtained from Associates of Cape Cod (Woods Hole, MA). Polymyxin B was purchased from Pfizer Inc., New York, NY and dissolved in pyrogen-free water.

Purification of human monocyte-derived IL-1. Human platelet-pheresis byproducts were used as a source of mononuclear cells; the mononuclear cells were adjusted to a concentration of 1×10^7/ml in RPMI-1640 (Microbiological Associates, Inc., Bethesda, MD) without methionine containing 1% dialyzed human AB serum. Cells were incubated at 37°C for 1.5 h and the nonadherent cells were removed by vigorous shaking. The adherent cell population was stimulated with opsonized heat-killed *Staphylococcus albus* in the presence of methionine-free medium containing 50 μCi/ml of [^{35}S]methionine and incubated for 36 h. The supernate was removed, centrifuged at 3,000 g, and filtered. The supernate was purified by sequential immunoabsorption, gel-filtration, and chromatofocusing. Details of the purification procedures and the antibody used to make the immunoabsorbent have been published previously (10, 23). IL-1 with pI 6.8–7.2 eluted from the chromatofocusing step as homogeneous 17,500 mol wt determined by SDS-PAGE fluorography (23); fractions were pooled and used as the IL-1 monocyte standard.

hrIL-1. The IL-1 cDNA expression vector was constructed by isolating the 1112 bp *NcoI-XmnI* fragment (bp 295–1407) from the IL-1 cDNA plasmid pcD 12-1-8 (19) and inserting it into an *E. coli* expression plasmid. The hrIL-1 is 223 amino acids long and contains 199 of the 269 amino acid long IL-1 precursor. The amino terminal 24 amino acids are contributed by the expression vector. The hrIL-1 included 46 amino acids of the IL-1 precursor peptide, which are present before the alanine at position 117. This alanine is the N-terminus of the processed IL-1 found in the supernates of stimulated human blood monocytes (24). The predicted molecular weight of the hrIL-1 is 24.5. hrIL-1-producing bacteria were grown at 37°C, lysed, and the hrIL-1 was extracted from the insoluble cellular fraction with 8 M urea. hrIL-1 was purified by sequential ion-exchange and either gel-filtration chromatography or high performance liquid chromatography. The hrIL-1 was stored at −70°C in a 0.15 M phosphate-buffered saline, pH 6.8. The identity of the purified hrIL-1 was confirmed by amino acid composition and the sequence of the amino terminal heptapeptide. Protein concentration was determined by the Bradford method using a bovine serum albumin (BSA) standard. Purity was assessed by SDS-PAGE with Coomassie Blue or silver staining (Fig. 1). The hrIL-1 is estimated to be >99% pure. The endotoxin concentration of the hrIL-1 was ~20 pg/μg of IL-1 protein.

In subsequent experiments for pyrogen assays in rabbits, 41 amino acids of the precursor peptide were removed by enzymatic digestion, which yielded hrIL-1 with an N-terminus at the alanine position 112 and a molecular weight of 17,500. This form of IL-1 is similar to the mature peptide purified from monocytes with the N-terminus at alanine position 117 (24). This recombinant form of IL-1 from amino acid 112–269 was homogeneous on SDS-PAGE and contained 60 pg of endotoxin/mg of IL-1 protein.

LAF assay. For the costimulator assay, the cloned murine helper T cell line, D.10.4G.1 was used (25). Previous studies revealed that this T cell line is sensitive to human monocyte IL-1 preparations (26). Dilutions of IL-1 were made in RPMI containing 5% heat-inactivated fetal calf serum (FCS) and 25 μg/ml of polymyxin B. For the assay, 100 μl of each dilution was added to flat-bottom microtiter wells in triplicate. The T cells were used 10–14 d after the last feeding, washed and suspended in 5% FCS/RPMI at 2×10^5 cells/ml. All wells received 100 μl of the T cell suspension containing 1 μg PHA/ml. The proliferative responses of these cells were measured by uptake of tritiated thymidine after 72 h.

Human T cell responses. Human peripheral blood T cells were purified by a combination of adherence to plastic and nylon wool. T cells (5 $\times 10^6$/ml) were incubated for 3 d in flat-bottom wells in the presence of various concentrations of hrIL-1 and/or PHA (0.5 μg/ml). During the last 12 h, cells were pulsed with [^{3}H]thymidine and then harvested. For IL-2 production, cells were incubated in round-bottom culture tubes for 3 d in 10 mM hydroxyurea and the presence or absence of hrIL-1 and 0.5 μg/ml PHA. The supernatants were dialyzed and assayed for IL-2
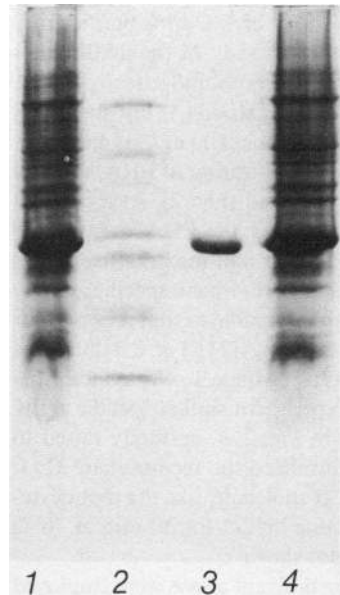

Figure 1. SDS-PAGE of hrIL-1 used in these studies. The gel was loaded with the crude urea extract from the recombinant IL-1 strain (lanes *1* and *4*). Purified hrIL-1 (50 μg) is shown in lane *3*. Lane *2* contains marker proteins.

using an assay based upon incorporation of [^{3}H]thymidine in IL-2-dependent PHA-activated cultured human T lymphocytes (27). Control cultures were stimulated with known concentrations of recombinant IL-2 (Cetus Corp., Berkeley, CA). 2-d supernatants were also assayed for IL-2 using an IL-2-dependent cytotoxic T lymphocyte line (CTLL). All error bars shown in figures represent mean cpm±SD.

Pyrogen assays. A modification of the mouse pyrogen assay (28) was used. C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME) were implanted subcutaneously with radiotransmitting thermosensors (Minimitter, Sun River, OR). Each thermosensor was calibrated prior to implantation. Temperatures were recorded every 10 min using a digital analogue of frequency intervals received from an AM radio (29). Mice were placed in a 34°C infant isolette for 1.5 h and baseline temperature was calculated during 60 min before intraperitoneal injection. Rabbits (New Zealand-derived albino females) weighing 2.5 kg were used for pyrogen testing as previously described (23). Temperatures were recorded every minute using a Kaye Instruments Digistrip II recorder (Bedford, MA). The data represent the first day responses of trained rabbits to hrIL-1. Three rabbits given 1 μg/kg were used in the group that received 10 μg/kg. Rabbits were given daily injections of hrIL-1 in order to induce pyrogenic tolerance; the day 2–5 responses of these rabbits were not used to calculate the mean fevers.

Dermal fibroblast PGE$_2$ production. Newborn human foreskin fibroblast cultures were seeded onto 2-ml flat-bottom wells (Costar, Cambridge, MA) and grown to confluence in RPMI containing 5% FCS as previously described (30). 24 h before the addition of IL-1, cells were washed and incubated overnight in RPMI without serum. IL-1 dilutions were made in serum-free RPMI and were used to replace the overnight medium. After 24 h, fibroblast supernates were removed and frozen at −70°C. Prostaglandin E$_2$ (PGE$_2$) concentration was determined by radioimmunoassay (Seragen Inc., Boston, MA).

Results

Activity of hrIL-1 in the murine LAF assay. The LAF activity of the urea extracts from the recombinant *E. coli* containing the IL-1 insert was compared to urea extracts from recombinant *E. coli* containing the expression plasmid but without the IL-1 sequence. The protein content of the two extracts was adjusted to the same concentration. When diluted, the extracts from the recombinant IL-1-containing strain had considerably more activity than those from the control strain (stimulation indices of 30.6, 15.3, and 3.8 for dilutions of 1:1,000, 1:10,000, and

1:100,000 vs. 8.3, 1.7, and 1.2, respectively). During purification, LAF activity corresponded to the intensity of the 24,000-mol wt protein staining in SDS-PAGE. The homogeneous hrIL-1 was tested on the murine T cell line, D10.G4.1, but there was no proliferative responses to hrIL-1 alone (100 ng/ml); however, in the presence of a suboptimal concentration of PHA, marked increases in thymidine uptake occurred (Fig. 2). At a concentration of 0.05 ng/ml, hrIL-1 doubled the PHA response ($P < 0.05$, Student's paired t test). Using half-maximal responses as the definition of an IL-1 unit, the hrIL-1 has a specific activity of 5×10^6 U/mg protein and is comparable to that reported for recombinant murine IL-1 (6×10^6 U/mg) (21). Fig. 2 *A* illustrates 1 of more than 20 separate assays using these T cells as indicators of hrIL-1 activity and in each experiment similar specific activities were observed. As shown in Fig. 2 *B*, antibody raised to human monocyte IL-1 (23) neutralized the recombinant IL-1. In addition, the recombinant IL-1 molecule, like the monocyte-derived IL-1, is heat labile; heating hrIL-1 for 30 min at 70°C reduced activity by 75% (data not shown).

Human T cell responses. Two different assays were employed using human T cells stimulated in the presence of hrIL-1 and mitogen. A human T cell blastogenesis experiment is shown in Fig. 3 *A* that illustrates that hrIL-1 in the absence of PHA has no effect on T cell proliferation. In the presence of PHA, however, hrIL-1 at concentrations of 100 and 10 ng/ml induced human T cell proliferative responses. In another assay, human peripheral blood T cell IL-2 production was measured during a 3-d incubation. During the incubation period, hydroxyurea was added to reduce IL-2 binding to the T cells. Supernatant and control media were dialyzed and assayed on IL-2-sensitive T cells. As
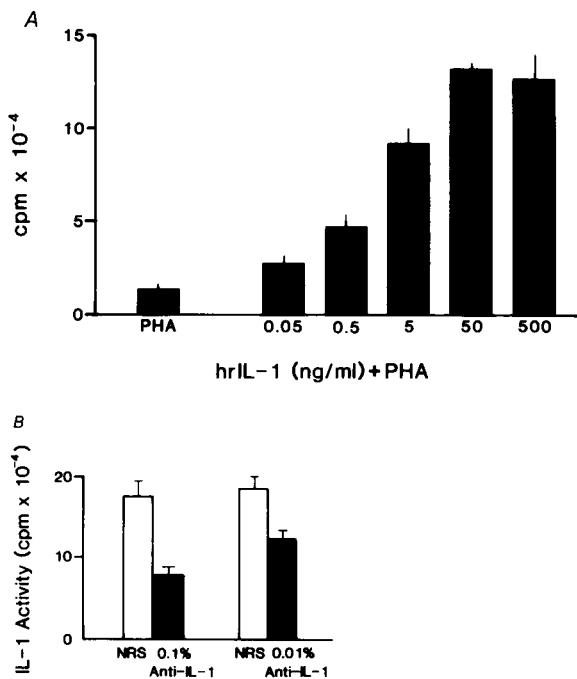
shown in Fig. 3 *B*, hrIL-1 did not induce IL-2 unless PHA was present; significant IL-2 production occurred at IL-1 concentrations of 5 ng/ml.

Additional experiments were carried out in which human T cells depleted of monocytes by adherence followed by two passages over nylon wool. These nonadherent cells (T cells) were then incubated with PHA in the presence of various concentrations of hrIL-1 (as above) for 2 d and the supernatant medium dialyzed before assay. These supernatants were assayed on CTLL cells for the presence of IL-2. In Fig. 4, we show that under these conditions, hrIL-1 in the presence of PHA induced IL-2 from nonadherent (NA) cells at levels comparable to those from unfractionated mononuclear cells incubated with the mitogen. NA cells stimulated with mitogen in the absence of IL-1 produced significantly less IL-2 but this level was severalfold greater than CTLL responses to unconditioned medium (cpm = 346±52).

Fibroblast PGE₂ production. hrIL-1 is a potent inducer of PGE_2 synthesis in human foreskin fibroblasts. As shown in Fig. 5, hrIL-1 stimulated dermal fibroblast PGE_2 production at concentrations of 5–500 ng/ml. Concentrations of 0.5 ng/ml induced small but significant levels ($P < 0.05$). The fibroblasts do not produce PGE_2 under the same conditions when incubated 24 h in the presence of as much as 1 μg/ml of endotoxin (30).

Figure 2. (*A*) Proliferative responses of D10.G4.1 cells to hrIL-1 in the presence of PHA. Concentration of hrIL-1 is indicated under the horizontal axis. (*B*) Effect of antimonocyte IL-1 on hrIL-1. hrIL-1 (5 ng/ml) was incubated with either normal rabbit serum (NRS) or anti-IL-1 at the concentrations (vol/vol) indicated for 2 h at 37°C before being added to the D10.G4.1 cells. PHA = 2,533±322 cpm; the response to 5 ng/ml of hrIL-1 with or without the NRS was within 10%.
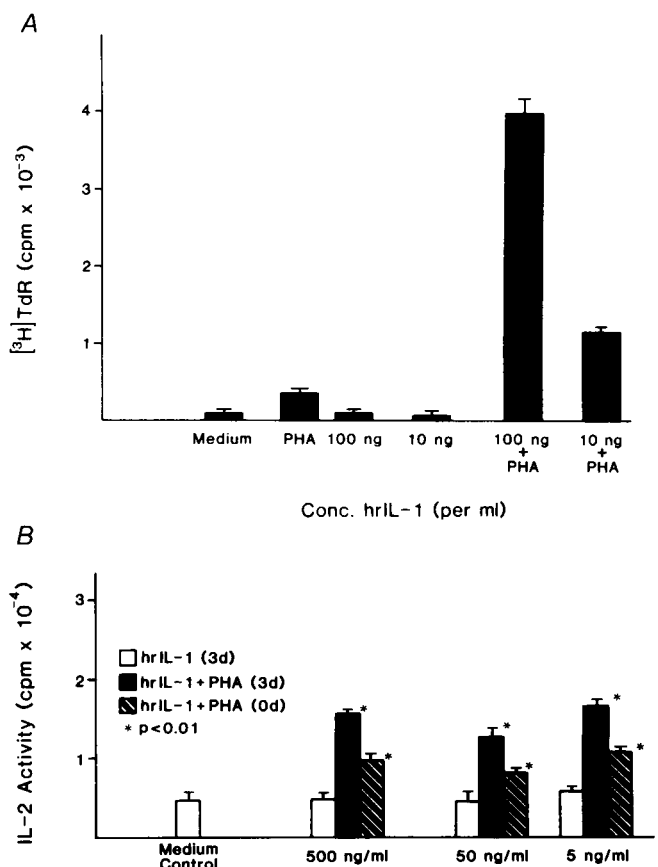
Figure 3. (*A*) Human T cell blastogenic response to hrIL-1 and PHA. Responses of unfractionated cells to 0.5 μg/ml PHA were 9,533±76. Concentration of hrIL-1 is indicated under the horizontal axis. (*B*) Production of IL-2 from human T-cells. Supernates were assayed on T lymphoblasts (27) in quadruplicate. The mixture of PHA plus hrIL-1 was directly assayed without prior incubation with T cells (indicated as 0 d) and the amount of in situ IL-2 generation is reflected by the difference in 3 d and 0 d. The asterisks indicate *, level of significance between the values at 3 d and 0 d using a paired t test.
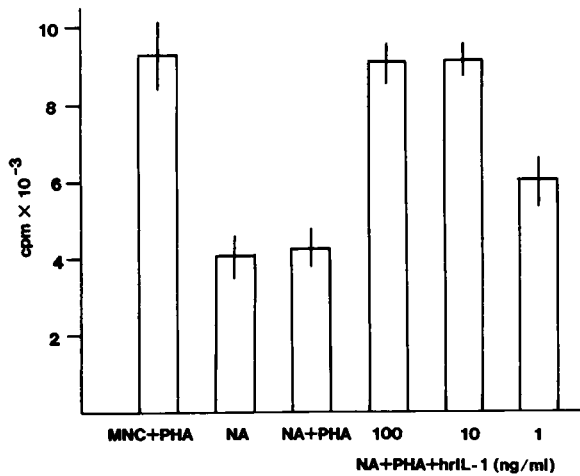
Figure 4. Generation of IL-2 from NA cells stimulated with hrIL-1 and PHA. NA were prepared by adherence and two passages over nylon wool and incubated with or without PHA (see Methods). After 48 h the supernatants were assayed for IL-2 using CTLL cells in quadruplicate. The data show a dilution of supernatants of 1:16.

Fever in mice. Endotoxin-resistant mice (C3H/HeJ) were used in these studies. To rule out endotoxin contamination, the concentration in the purified hrIL-1 was determined by a limulus amebocyte lysate assay that had a sensitivity of 20 pg/ml. Endpoint determinations using the clot-forming method revealed that hrIL-1 contained 20 pg/μg of IL-1 protein. C3H/HeJ mice used under the conditions of this study develop fever (maximum temperature, $T_{max} = 0.4°C$) after the injection of 500 ng per 25-g mouse of *E. coli* endotoxin. As shown in Fig. 6 *A,* the hrIL-1 induces a brisk, monophasic fever in prewarmed mice. Heating the sample resulted in loss of pyrogenicity. The decrease in body temperature that occurred after the injection of heat-inactivated hrIL-1 is frequently observed in studies using prewarmed mice or rats following the injection of nonpyrogenic agents. This may be, in part, due to removal of the animals from their thermoneutral environment during the injection procedure. The dose response for the pyrogenic response is illustrated in Fig. 6 *B.* Similar to previous studies on human monocyte IL-1 in the murine fever model, increasing the dose of pyrogen often produces less fever (28).
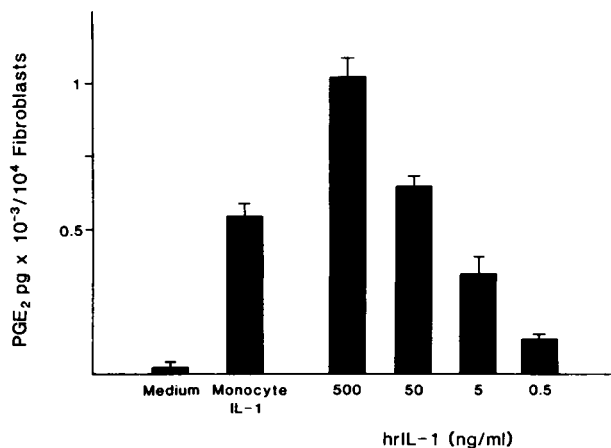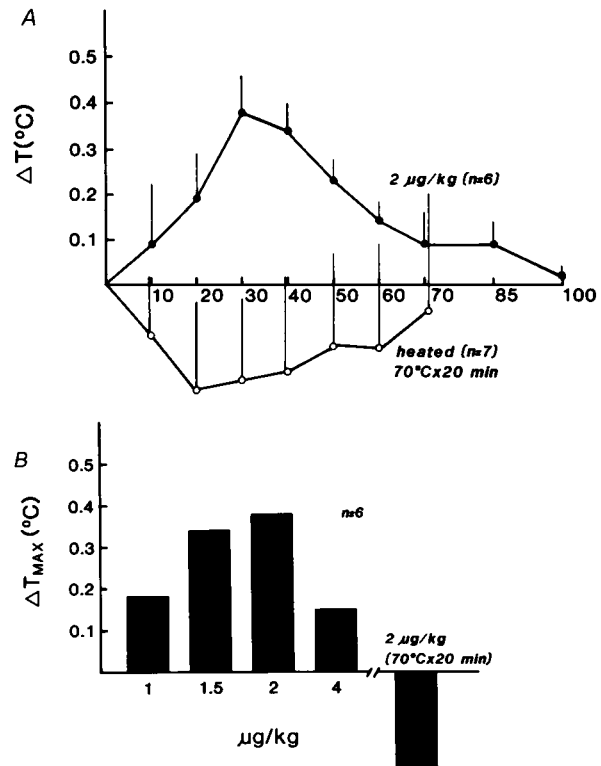

Figure 6. hrIL-1 fever in C3H/HeJ mice. (*A*) Mean fever curve in mice injected with hrIL-1. (*B*) Dose-response of fever in mice given hrIL-1.

Fever in rabbits. Rabbits injected intravenously with hrIL-1 (amino acids 71–269) developed typical monophasic fevers that reached peak temperature elevations ~54 min after intravenous injection (Fig. 7). Preincubation with polymyxin B (250 μg/ml) inhibits the pyrogenic property of endotoxins but did not reduce the fever produced by hrIL-1. Heating for 30 min at 70°C, a procedure that does not affect the biological properties of bacterial endotoxins, results in loss of pyrogenicity of IL-1 derived from mammalian cells (5, 9, 12) and abolished the pyrogenicity of hrIL-1. In other experiments, 5 daily injections of hrIL-1 in rabbits did not induce pyrogenic tolerance (data not shown).
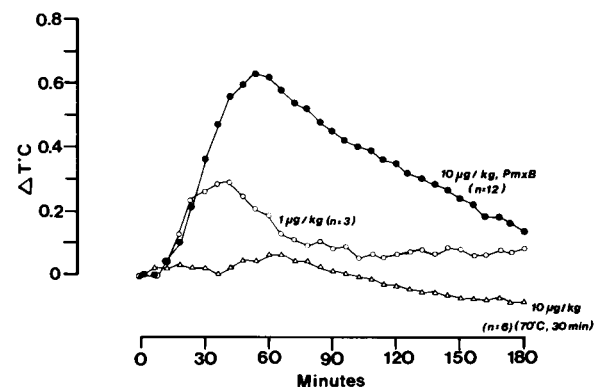

Figure 5. Human dermal fibroblast PGE$_2$ production. hrIL-1 was incubated for 24 h in duplicate wells and PGE$_2$ levels determined as described (30).

Figure 7. hrIL-1 fever in rabbits. Mean fevers following intravenous injections of hrIL-1 (24,500 mol wt) into rabbits. The numbers of rabbits in each group are indicated in parentheses. Heated hrIL-1 was not pretreated with polymyxin B.

In additional fever experiments, the hrIL-1 injected was the shorter form (amino acids 112–269, 17,500 mol wt), which contained ~60 pg of endotoxin/mg of IL-1 protein. This hrIL-1 was 50-fold more pyrogenic in rabbits than the larger hrIL-1 (mol wt 24,500) containing the 46 precursor amino acids (compare Fig. 7 with Fig. 8). The murine T cell responses were similarly increased 50-fold.

Discussion

The object of these studies was to establish that homogeneous preparations of human IL-1 purified from a recombinant strain of *E. coli* are biologically active in assays of both immunological function and inflammatory responses. Although the hrIL-1 used in these studies will need to be further evaluated for its ability to induce physiologic and metabolic changes associated with acute-phase responses, the data presented in this report support the concept that recombinant human IL-1 functions as a factor that promotes mitogen-driven T-cell blastogenesis and IL-2 production as well as induces fever and acts as a mediator of inflammation. Furthermore, these properties are destroyed by mild heating, which is characteristic of monocyte-derived IL-1.

The human IL-1 encoded by the precursor cDNA is unique in that it lacks a clear signal peptide that would indicate a likely processing site for the N-terminus. A murine IL-1 cDNA is similar in that it encodes for a similar size precursor and that it also lacks a signal peptide. Recent studies of the N-terminus amino acid sequence of human monocyte-derived IL-1 with a pI of 7 have indicated that the cleavage of the precursor at the alanine (amino acid 117) is the likely N-terminus of the IL-1 found in supernates of stimulated monocytes (24). The IL-1 expressed in the recombinant *E. coli* and tested in the present study contains 46 of the 116 amino acids that are part of the precursor peptide. Several investigators have isolated peptides with molecular weights between 38,000 and 30,000 employing gel-filtration chromatography, and which exhibit many of the same IL-1 bio-
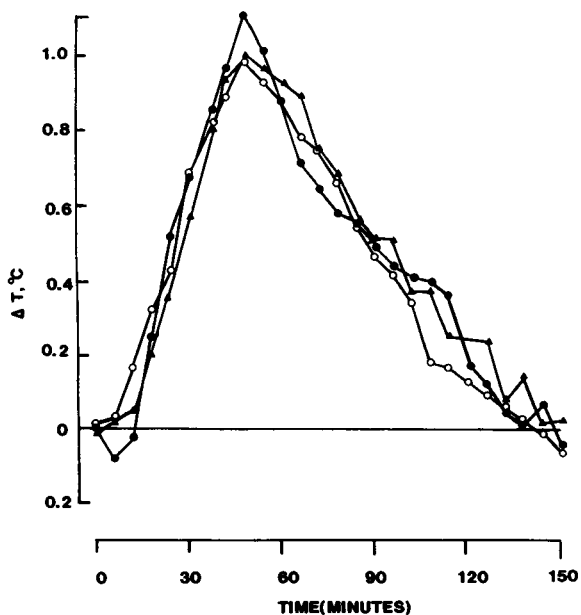
logical activities reported for the 15,000–17,000-mol wt size (12–14, 31, 32). Although some reports indicate that the human IL-1 pI 7 form without cleavage of the precursor peptide has little biological activity (20), the present study establishes that the proximal 46 amino acids of the precursor peptide do not prevent biological activity in the assays used in these studies. This observation supports those of other investigators who have reported multiple biological activities of large molecular weight IL-1s. Since hrIL-1 functions indistinguishably from that of monocyte IL-1, we conclude that the extra amino acids derived from the precursor sequence do not prevent biological activity.

These additional amino acids may affect biological potency of the material, however. Nevertheless, the specific activity of the hrIL-1 from *E. coli* (5×10^6 half-maximal units/mg) is nearly the same as that for recombinant murine IL-1 (21). The murine recombinant IL-1 expressed in that study did not contain any precursor sequences. A recent report on the specific activity of purified human monocyte IL-1 with a pI of 7 indicates a specific activity 4.3×10^7 U/mg protein (8). By removing all but five of the precursor amino acids (N-terminus at alanine position 112 compared with the natural N-terminus at alanine position 117), we observed a 50-fold increase in the specific activity of the hrIL-1 for both murine T cell stimulation as well as in pyrogenicity in rabbits. Cleaving the precursor peptides and producing hrIL-1 with a N-terminus closer to that of the natural IL-1 clearly increased the activities in these models but the shorter hrIL-1 did not have a significantly increased specific activity on dermal fibroblasts (data not shown). This may be due to the fact that some cell culture conditions provide sufficient proteases to cleave the precursor amino acids; alternatively, the precursor polypeptides may affect the potency of IL-1 for certain cells.

The specific activity of hrIL-1 may be affected by several conditions including the denaturization during extraction and purification, the additional amino terminal amino acids, and the lack of glycosylation. To isolate the IL-1 from the recombinant *E. coli*, denaturing conditions were employed. Refolding of the molecule may not be complete or isolation procedures may partially inactivate sites critical for biological activity. The lack of glycosylation by *E. coli* may influence the specific activity or solubility of hrIL-1. The peptide sequence of the IL-1 indicates a likely glycosylation site but the nonglycosylated form used in these studies establishes that the immunologic and inflammatory properties of IL-1 are maintained. Murphy was unable to show that the charge heterogeneity of rabbit IL-1s was due to glycosylated variants (33). Studies presently underway using mammalian cell-derived recombinant IL-1 will be able to clarify this issue further.

Many biological properties of IL-1 are shared with bacterial endotoxins. It is thus important to exclude endotoxin from any IL-1 preparation, particularly recombinant IL-1 produced in *E. coli*. Although the preparations used in the present study had low endotoxin content (20 pg/μg) as determined by the Limulus amebocyte lysate assay, we used several methods to demonstrate that the biological effects of hrIL-1 were not due to any undetected endotoxin. The fibroblast PGE_2 stimulation assay (30) and C3H/HeJ mice are highly insensitive to endotoxins. Heating hrIL-1 to 70°C destroyed its pyrogenicity in mice and rabbits and polymyxin B had no effect on hrIL-1. Nevertheless, endotoxins are potent pyrogens and produce small fevers (minimum pyrogen dose) in rabbits at 3 ng/kg. The shorter hrIL-1 (mol wt 17,500) used in the studies contained ~60 pg of endotoxin/mg of IL-1. At the doses of this IL-1 given to rabbits (500 ng/kg),

Figure 8. hrIL-1 fever in rabbits. Individual rabbit responses to hrIL-1 (mol wt 17,500) following an intravenous injection of 500 ng/kg are shown. Polymyxin B or protein stabilizers were not included.

that would be equivalent to 30 fg endotoxin/kg. Thus, there can no longer be any question that IL-1 is an endogenous pyrogen and that its pyrogenic properties are not due to contaminating endotoxins. These studies clearly demonstrate that hrIL-1 possesses multiple biologic activities attributed to purified material obtained from blood monocytes and that the properties of T cell activation and induction of inflammatory responses reside in a single polypeptide.

Note added in proof. IN additional fever experiments, we injected the 17,500-mol wt hr IL-1 into 6 rabbits and observed a mean fever peak of 0.68±0.07°C following a dose of 100 ng/kg.

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