Human Interleukin 1β Is Not Secreted From Hamster Fibroblasts When Expressed Constitutively From a Transfected cDNA

Peter R. Young,* Daria J. Hazuda,* and Philip L. Simon‡

Departments of *Molecular Genetics and ‡Immunology, Smith Kline and French Laboratories, King of Prussia, Pennsylvania 19406-0939

Abstract. To understand the secretion and processing of interleukin-1 (IL-1), a Chinese hamster fibroblast cell line (R1610) was transfected with a human IL-1β cDNA under the control of the SV40 early promoter and linked to the gene for neomycin resistance. After selecting for transfected cells resistant to G418, two clones were found to constitutively express the IL-1β 31-kD precursor which was almost exclusively located in the cytosol. Pulse-chase experiments failed to show any secretion of IL-1 and very little IL-1 activity was detectable in cell supernatants. Furthermore, surface membrane IL-1 activity could not be detected, although low levels of activity could be released upon brief trypsin treatment. Therefore, unlike monocytes, these fibroblast cells lack the mechanism for secreting and processing of IL-1β.

INTERLEUKIN-1 (IL-1)[1] is the collective name given to a group of polypeptide hormones that share a number of properties, including the activation of T cells, stimulation of B cell differentiation, stimulation of acute phase protein synthesis in hepatocytes, stimulation of prostaglandin and protease synthesis in synovial and chondrocyte cells, and augmentation of fibroblast proliferation (10, 38). Furthermore these molecules are endogenous pyrogens. Since the production of IL-1s is stimulated by infection and injury, it is likely that they mediate many of the important responses to insults and inflammation.

Recently, two genes have been isolated from humans which encode the two major isoelectric forms of IL-1 (3, 8, 12, 13, 16, 28, 31). These have been designated α for the pI 5 form and β for the pI 7 form (31). Both proteins are encoded as 31-kD precursors which are subsequently processed and secreted as a mature, active 17-kD carboxyl terminal fragment. However, the full sequence of the 31-kD precursor does not appear to contain the standard hydrophobic signal sequence known to direct the secretion of most exported eukaryotic proteins studied to date (20). It is therefore of interest to understand how this apparently unique class of molecules is secreted from cells.

In many cell types the induction of IL-1, as monitored by the appearance of its activity in the supernatant, requires external stimuli such as PMA or lipopolysaccharide (LPS). This is particularly true of monocytes and monocyte cell lines, which appear to be the predominant source of IL-1 (22, 38). In addition to inducing IL-1 mRNA, these agents cause substantial changes in morphology and reduce the viability of the cells. This has led to speculation that IL-1 may not be actively secreted, but may be passively released by damaged cells (14, 17). One way of addressing this question directly is to ablate the toxic effects of external stimuli by expressing the IL-1 precursor constitutively in a fibroblast cell. Stimulus induced cell damage should be kept to a minimum and we can examine the ability of the IL-1 to be secreted from viable cells.

In this study, we have transfected a vector encoding the full length 31-kD precursor under the transcriptional control of the SV40 promoter into rodent fibroblasts, and have obtained cloned cells which constitutively express high levels of IL-1β precursor. These cells lines have allowed us to assess whether the IL-1 precursor protein encodes the signals necessary for secretion. Furthermore, there have been a few reports suggesting that IL-1 activity can be found associated with the plasma membrane of cells expressing IL-1 (24), which has led to models supporting an independent role for this bound form of IL-1 in T cell activation (25). We have therefore examined these same cell lines for their ability to express membrane IL-1.

Materials and Methods

Plasmid Construction

Cloning of the human Il-1β cDNA and expression of fully active, mature 17-kd IL-1β from it has been described previously (36). The precursor coding region was isolated from the cDNA by cutting first with Acc I, filling in the overhang with Klenow, followed by partial digestion with Nco I. The partially digested 900 bp Nco I/Acc I restriction fragment was then purified by agarose gel electrophoresis, electroelution, and desalting via a prepackaged NACS column (Bethesda Research Laboratories, Bethesda, MD). This fragment was then ligated to the plasmid vector DSP1 (40) which had

1. *Abbreviations used in this paper*: BPV, bovine papilloma virus; galk, galactokinase; IL-1, Interleukin 1; LPS, lipopolysaccharide; tPA, tissue plasminogen activator.

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been cut first with Xba I, filled in with Klenow, then cut with Nco I, followed by phosphatase. The plasmid obtained, DSPIL-1β, placed the IL-1β precursor coding sequence under the control of the SV40 early promoter and RNA start and polyadenylation sites, with the Nco I site religation reconstituting the strong Kozak consensus sequence for initiation of protein translation (21).

This transcription cassette was then transferred into a mammalian cloning vector bovine papilloma virus (BPV)-β globin-neo, which consists of the complete BPV genome, the ampicillin resistance gene of pBR322 for selection in bacteria, and the neomycin resistance gene under the control of the human β-globin promoter and SV40 early polyadenylation regions for selection in mammalian cells via resistance to G418 (Reff, M., personal communication). This was achieved by digesting DSPIL-1β with Sal I, purifying the 1.5-kb fragment containing the IL-1β transcriptional cassette via electrophoresis as above, and ligating to BPV-β-globin-neo which had been previously cut with Sal I and phosphatased. The plasmid obtained was called BPV-IL-1β (see Fig. 1). As controls we used two vectors: BPV galactokinase (BPV-galk) and BPV-tPA which constitutively express the bacterial galk and human tissue plasminogen activator (LPA) genes respectively in BPV constructs similar to BPV-IL-1β (Reff, M., personal communication).

Plasmids used for cloning or transfections were purified via the alkaline lysis method (7), followed by digestion with boiled RNAse A and chromatography through Bio-Gel A-150 (Bio-Rad Laboratories, Richmond, CA) (30) with minor modifications from the published procedures. Restriction enzymes, Calf intestinal phosphatase, DNA polymerase, Klenow, and T4 DNA ligase were obtained from New England Biolabs, (Beverly, MA), Bethesda Research Laboratories or Boehringer-Mannheim (Houston, TX) Deoxyribonuclease I was obtained from Cooper Biomedical (Malvern, PA).

Cell Culture, Transfection, and Cloning

Chinese hamster R1610 cells (galk-, hgprt-) (47) were maintained in DME supplemented with 10% fetal bovine serum, 20 mM L-glutamine, and penicillin/streptomycin (all from Gibco, Grand Island, NY). 10 μg BPVIL-1β DNA was transfected into 1×10^6 cells via the calcium phosphate coprecipitation technique (50). After 16 h, the cells were split 1:3 and grown in the presence of 400 μg/ml of active commercial G418 (Gibco). After several days of selection, the parent cells were completely killed and resistant colonies were large enough to be harvested with cloning cylinders. Two clones, 5.1 and 6.1, which were found to constitutively synthesize IL-1β precursor were henceforward grown under identical conditions to the parent cell line, but were maintained in 400 μg/ml active G418. All cell lines used were found to be mycoplasma free.

Activated human peripheral blood monocytes were prepared from Red Cross buffy coats by centrifugation through Ficoll and Percoll gradients (9), followed by adherence to plastic tissue culture dishes for 1.5 h in RPMI medium (Gibco) containing 1% human AB serum (Irvine Scientific, Santa Ana, CA). All media and reagents used contained less than 0.03 ng endotoxin as determined by Limulus Amoebocyte Lysate (Associates from Cape Cod, MA). The attached monocytes were then activated with 10 ng/ml *Escherichia coli* LPS in 1% human AB serum and RPMI. RNA and protein were prepared from these cells as described below.

Southern Blots

Genomic DNA was prepared from R1610 cells and G418 resistant cell clones by resuspending $\sim 10^7$ cells in 2 ml NTE buffer (0.1 M NaCl, 0.01 M Tris-HCl, pH 8.0, 1 mM EDTA), and then adjusting the suspension to 20 mM EDTA, 0.2% SDS and mixing. After incubation with 100 μg/ml boiled RNAse A (Sigma Chemical Co., St. Louis, MO) at 37°C for 2 h, followed by 100 μg/ml proteinase K for 2 h at 37°C, the solution was gently extracted a few times with phenol/chloroform and dialysed against NTE. Concentration and purity was then assessed by absorbance at 260 and 280 nm.

After restriction endonuclease digestion using the manufacturers recommended conditions, the DNA was fractionated on 0.7 or 1% agarose gels and transferred to 0.45 μm nitrocellulose (Schliecher & Schuell, Keene, NH) according to standard methods (30, 43). Prehybridization and hybridization of the blots were by the method of Wahl et al. (49). Blots were probed with a nick-translated human IL-1β cDNA fragment (36) labeled to $\sim 3 \times 10^8$ cpm/μg with [α^{32}P] dNTPs (>3,000C_i/mmol, Amersham Corp.) (30). For direct comparison of mobility, purified BPVIL-1β plasmid DNA was digested with the same enzyme as the genomic sample to be quantitated, and an appropriate dilution run with digested control R1610 DNA.

Northern Blots

RNA was prepared by the guanidinium thiocyanate-cesium chloride cushion method (30, 35). Poly A+ mRNA from activated monocytes was obtained by oligo-dT chromatography (Collaborative Research) (4). RNA was fractionated on 1.5% formaldehyde-agarose gels (30) and transferred to 0.45 μm nitrocellulose (Schliecher & Schuell) by established methods (48). Prehybridization and hybridization with nick translated probes was performed as outlined above for Southern blots.

Westerns

A rabbit anti-rIL-1β polyclonal antiserum was generated by injecting rabbits with *Escherichia coli* produced 17-kD recombinant IL-1β (19). Protein samples that had been previously boiled in SDS-loading buffer (26) were run on a 15% SDS-polyacrylamide gel (26). For tPA, samples were first boiled in SDS and then denatured in 6 M Urea before electrophoresis on 10% SDS-PAGE in a continuous buffer system to eliminate aggregation of glycosylated protein. Proteins were subsequently transferred to 0.22 μm nitrocellulose (Schliecher & Schuell) via electroblotting (Bio-Rad). The nitrocellulose was then dried, rehydrated in 1 × PBS (0.137 M NaCl, 2.7 mM KCl, 1.3 mM KH_2PO_4, 9.5 mM Na_2HPO_4), and then incubated with a 2% solution of nonfat dried milk (Carnation) in 1 × PBS at 4°C for 1 h. A 1:2,000 dilution of rabbit anti-rIL-1β or rabbit anti-tPA antiserum was then added in 0.5% nonfat milk/1 × PBS and the incubation continued for 16 h at 4°C. The filter was washed 2-3 times with 1 × PBS at room temperature for 20 min each and then incubated with a 1:10,000 dilution of ^{125}I-labeled protein A (0.16 mC_i/ml; ICN) in 0.5% non-fat dried milk and 1 × PBS at room temperature for 1 h followed by four 15-min washes with 1 × PBS at room temperature, before autoradiography.

^{35}S labeling of Precursor and Mature rIL-1β

The genes encoding both the precursor and mature forms of IL-1β were cloned into an inducible *E. coli* expression vector under control of the λ p_L promoter (36, our unpublished studies). Overnight cultures grown at 32°C in modified M56 minimal medium with biotin and ampicillin (50 μg/ml) were diluted 1:10 in fresh medium and grown to an A_{600} of 0.6, at which time the temperature was raised to 42°C by the addition of ½ volume 65°C medium. Cells were incubated for 1 h at 42°C, then centrifuged, and resuspended in an equal volume of unmodified M56 supplemented with 0.2% glucose, 1 μg/ml thiamine, 2 μM amino acids minus cysteine and methionine, biotin, ampicillin, and 20 μCi of Trans^{35}S-label (85% methionine, 15% cysteine, ICN, >1,000 Ci/mmol). The cells were labeled for 20 min at 42°C, centrifuged and the pellets frozen on dry ice.

Before use, the pellets were resuspended in one-fifth volume of lysis buffer (40 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT) plus 2 mM PMSF and sonicated for 30 s using a Branson Sonifier. Sonicates were centrifuged in an Eppendorf microfuge for 30 min. For stability studies, 10 μl of supernatant was then added to dishes containing 1×10^6 6.1 cells and 2.5 ml 1% fetal bovine serum (Gibco) in DME, and incubated for various times before immunoprecipitation as described below.

Immunoprecipitation

Stable R1610 transfected cells containing BPVIL-1β, BPV tPA or BPVgalk were labeled by seeding 1×10^6 cells in a 100 mm dish, and 16 h later washing three times with 1 × PBS before incubating with 5 ml methionine and cysteine free MEM (Gibco) and 1% dialyzed fetal bovine serum (Gibco) for 1 h. After 3 more washes with 1 × PBS, the cells were then labeled for 5 h with 150 μC_i/ml [^{35}S]methionine (>800C_i/mmol, Amersham Corp.) and 165 μC_i/ml [^{35}S]cysteine (>600C_i/mmol, Amersham Corp.) in the above serum and medium. The supernatants were harvested, and the cells washed 3× with 1 × PBS and either lysed immediately in 0.5 ml 1 × RIPA (0.15 M NaCl, 10 mM Tris-HCl, pH 7.5, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM EDTA, 2 mM PMSF) or incubated for a further 20 h with 2 ml fresh MEM (Gibco) containing 1% fetal bovine serum before harvesting as above.

Cell lysates were made 2% milk by adding an equal volume of 4% nonfat dry milk in 1 × RIPA. Protein A-Sepharose beads (50 μl half-packed volume) (Bio-Rad) were added to 0.5 ml of this solution and then incubated at 4°C for 1 h on a rotator. After centrifugation at 10,000 rpm for 10 min in a Beckman model TJ6 tabletop centrifuge, the supernatants were incubated with 1:200 anti-rIL-1β polyclonal antisera (see above) or 1:200 anti-tPA for 1-16 h at 4°C, followed by a further 1 h incubation in the pres-

ence of 50 ml Protein A–Sepharose beads. The beads were centrifuged as described previously, washed twice in 2 ml RIPA buffer, 10 min each, and one time in 1 ml of low salt buffer (20 mM Tris-HCl, pH 7.4, 25 mM NaCl). After the final wash, beads were resuspended in 45 μl of SDS loading buffer and boiled for 5 min. Beads were removed by centrifugation, and one third of supernatant was subjected to electrophoresis on a 15% SDS–polyacrylamide gel (26). Gels were fixed for 1 h in 5% methanol, 10% glacial acetic acid, soaked for 20 min in Amplify (Amersham Corp.) and then vacuum dried before autoradiography.

Media fractions were made 0.5 or 2% milk for tPA or IL-1 detection respectively by the addition of an equal volume of 1 or 4% milk in 1 × RIPA buffer. 100 μl protein A–Sepharose beads were used and the beads were washed twice with 4 ml 1 × RIPA.

Monocytes prepared as described above, were activated with 10 ng/ml LPS for 2 h in methionine-free MEM and 5% fetal bovine serum (Gibco), washed with 1 × PBS, and incubated for a further 1 h in the same medium with 100 μCi/ml [^{35}S]methionine. The cells were then washed twice with 1 × PBS, and either harvested in 1 × RIPA or incubated in 5% fetal bovine serum and RPMI for a further 24 h. The supernatant was removed and diluted with an equal volume of 2× RIPA. Both supernatant and cell lysate were first incubated with 50 μl rabbit preimmune serum for 1 h followed by 50 μl Immuno-Precipitin (formalin-fixed Staph A, BRL) for an additional hour with rotation. The Staph A were then pelleted by centrifugation at 3,000 rpm for 5 min and the supernatant fractions incubated with 1:100 dilution of rabbit anti-IL-1β for 1 h, followed by 50 μl Immuno-precipitin for 1 h with rotation. The samples were then centrifuged at 3,000 rpm for 5 min, and washed four times with 1 × RIPA before resuspension in gel loading buffer and analysis via SDS-PAGE (26).

Immunofluorescence

Cells were seeded at ~5 × 10^5 cells/well in 4-well chamber slides (Falcon). After washing the slides twice with PBS, cells were fixed by incubation in ice cold ethanol for 10 min. Slides were then air dried and the cells blocked by incubation for 1 h at 37°C with nonconjugated goat anti-rabbit IgG (Cappel) diluted 1:250 in 1 × PBS. The cells were washed twice with 1 × PBS and then incubated with polyclonal anti-rIL-1β antisera diluted 1:200 in 1 × PBS. After 1 h at 37°C, slides were washed as before and then incubated with FITC-labeled goat anti-rabbit IgG (Cappel) diluted 1:1,000 for 1 h at 37°C. The cells were washed as above and the slides mounted in glycerol. Slides were examined using phase contrast and fluorescence microscopy (Zeiss Universal).

Fractionation and Harvesting of Cellular Associated IL-1

Approximately 1 × 10^7 cells were fractionated using the method of Matsushima et al. (34) resulting in three fractions: cytosolic, membrane, and particulate, the last representing cellular organelles. The purity of each fraction was assessed via assays for cytosol (lactate dehydrogenase) and membrane (5′ nucleotidase) specific enzymes (5). Lactate dehydrogenase activity was assayed by determining the decrease in A_{340} resulting from the oxidation of NADH (Worthington Catalogue, 1982). 5′-Nucleotidase activity was determined with a Sigma Chemical Co. diagnostic kit (procedure No. 675). Greater than 70% of the total lactate dehydrogenase activity remained associated with the cytosolic fraction. In contrast, less than 20% of the total 5′ nucleotidase activity localized to the cytosol, indicating that the cytosol was only slightly contaminated by membrane components. Quantitation of relative amounts of IL-1β precursor and tPA in each fraction was by densitometric analysis of a Western blot.

To harvest for cell associated IL-1 activity, cells were scraped off tissue culture dishes with a rubber policeman in 1 × PBS, centrifuged at 1,500 rpm in a bench top centrifuge, and washed a second time in 1 × PBS before freezing in dry ice and storage at −70°C. The cells were then thawed in RPMI medium, sonicated, the debris removed by centrifugation, and the supernatants assayed as below. Alternatively, the pelleted cells were resuspended in 0.5 ml 50 mM octaglucopyranoside (octyl β-D-glucoside, Sigma Chemical Co.), and allowed to lyse on ice for 5 min. The lysate was then dialysed 3 times with 1 l RPMI medium (Gibco) at 4°C over a 16 h period. The precipitate that formed was then removed by centrifugation at 10,000 rpm in a Beckman JA13 for 10 min, before assaying for activity.

The protocol for releasing IL-1 activity from 6.1 and R1610 cells was as follows. 60-mm dishes each containing 10^6 cells were washed twice with 1 × PBS, and then 1 ml 0.04% trypsin in 0.5 mM EDTA was added and distributed over all the attached cells. Within 20 s, most of this solution was suctioned off, leaving ~0.1 ml. After incubation with the cells for various time periods, 0.5 ml 0.25% soybean trypsin inhibitor (Sigma Chemical Co.) in RPMI medium was added to the plate, and distributed evenly. The cells were then lifted from the plate via pipetting, and the solution centrifuged at 1,500 rpm in a Beckman TJ-6 centrifuge to remove cells. The supernatant was then assayed for IL-1 activity. The cells were resuspended in 1 × PBS and checked for viability by trypan blue staining. Viability was more than 99% out to 4 min of trypsin digestion.

Assay for IL-1 Activity

IL-1 activity was determined by the EL-4 thymoma assay (42) in which the IL-2 production induced in EL-4 cells costimulated with calcium ionophore A23187 is assayed by measuring [^{3}H]thymidine incorporation in the IL-2 dependent CTL20 cell line. To assay putative surface membrane IL-1 activity, duplicate transfected and control R1610 cells were seeded in two-fold dilutions into a 96-well microtiter dish starting with 1-2 × 10^5 cells. After overnight incubation in 10% fetal bovine serum and DME lacking G418, the supernatants were harvested and tested directly in the EL-4 assay. Meanwhile, the R1610 cells were washed several times with 1 × RPMI before adding 1 × 10^6 EL-4 cells to each well in assay medium, and incubating overnight at 37°C. The supernatants from these coincubations were then incubated with CTL20 cells in the usual manner.

Results

Construction of Vector for IL-1 Expression

To attain high level constitutive expression of the 31-kD IL-1β precursor, we placed the IL-1β cDNA under the control of the SV40 early promoter and polyadenylation sequences, by constructing the plasmid DSPIL-1β (Fig. 1, top). The complete transcription unit was then inserted into

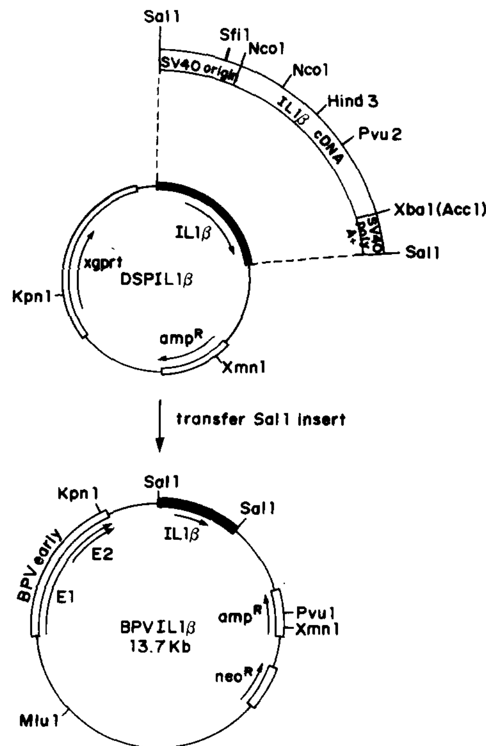

Figure 1. Eukaryotic expression for human IL-1β. (*Top*) DSPIL-1β; (*bottom*) BPVIL-1β. Only key restriction sites are included. See Materials and Methods for detailed description of these plasmids.

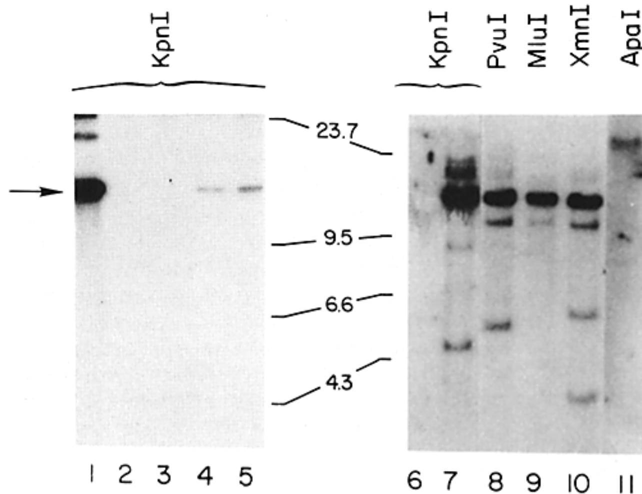

Figure 2. Southern blot of R1610 cell line DNAs. 4 μg of genomic DNA from R1610 cell lines was digested with the indicated restriction enzymes, separated on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to a human IL-1β cDNA probe. (Lane *1*) R1610 cells mixed with 5 ng BPVIL-1β DNA cut with Kpn I; (lanes *2* and *6*) R1610 cells; (lane *3*) R1610 cells transfected with BPVgalk; (lanes *4*, *7–10*, and *11*) 6.1 cells; (lane *5*) 5.1 cells. The arrow marks the migration of the linearized BPVIL-1β vector.

a BPV vector, which contains the complete BPV genome, and the genes for neomycin and ampicillin resistance for selection in mammalian and bacterial cells respectively. This final plasmid was named BPVIL-1β (Fig. 1, bottom).

Transfection and Cloning of Cells Constitutively Expressing IL-1β Precursor

We selected for transfection a Chinese hamster cell line R1610, which had been previously used for its galk-background (47), but was particularly convenient because of its very short cell cycle. Hence, the time required for transfection and cloning in selective media could be kept to a minimum. BPVIL-1β was transfected into R1610 cells via calcium phosphate precipitation (50), and after selection for several days in G418, 20 resistant clones were picked. To further screen amongst these clones for those expressing IL-1β, we initially tested the supernatants from these resistant clones for IL-1 activity using the EL-4 thymoma assay. All 20 clones were negative.

Since it was possible that the cells could synthesize but not secrete the IL-1, we screened cell lysates via Western blot using rabbit polyclonal antisera specific for IL-1β. Two clones, 5.1 and 6.1, were identified which expressed the 31 kD IL-1β precursor (see below), and these were studied further.

Characterization of Transfected DNA

We determined the status of the transfected IL-1 sequences in 5.1, 6.1 and control R1610 genomic DNAs via Southern blot (Fig. 2). Digestion of 5.1 and 6.1 DNA with Kpn I (lanes *4* and *5*), which cuts just once within BPVIL-1β, yielded a 13.7-kb linear IL-1β fragment which comigrated with the linearized vector (lane *1*) and was absent in DNA from untransfected R1610 cells and R1610 cells transfected with a control vector BPVgalk, which expresses the *E. coli* galactokinase gene (lanes *2* and *3*). This indicates that the complete vector sequences have been retained intact in 5.1 and 6.1.

Furthermore, the vector is integrated in multiple tandem repeats, since digestion with enzymes which cut once in BPVIL-1β (Kpn I, Pvu I, Mlu I, and Xmn I) revealed weakly hybridizing junction fragments in addition to the strongly hybridizing linearized vector sequences (lanes *7–10*). Also digestion with Apa I, which does not cut BPVIL-1β, results in a very large DNA fragment (>25 kb). If the vector had been episomal, we would have expected Apa I digestion to yield uncut supercoils, which should run further into the gel.

Characterization of IL-1 mRNA

We checked for synthesis of IL-1β mRNA in 5.1 and 6.1 by Northern blot. As shown in Fig. 3, both cell lines produce IL-1β mRNA with a size of ~1.1 kb, which is absent in untransfected R1610 cells. The size of the mRNA matches that predicted from the vector construct, and is smaller than the endogenous IL-1β mRNA produced in activated human monocytes (Fig. 3, lane *4*) (31). We quantitated the level of IL-1β mRNA by comparison of hybridization intensity in dot blots to a standard IL-1β mRNA synthesized in vitro from an SP6 vector by SP6 polymerase (23). (Smith, M., unpublished data). This indicated that IL-1β constituted ~1% of the total RNA in both transfected cell lines. For comparison, IL-1β represents ~4–9% of the mRNA in activated monocytes (Smith, M., and P. Young, unpublished data).

Synthesis of 31-kD IL-1β Precursor

When cell lysates from 5.1 and 6.1 cells are analyzed by Western blot using rIL-1β specific antiserum (Fig. 4, lanes *3* and *4*), it can be seen that both synthesize high steady state intracellular levels of the predicted 31-kD precursor which is not detected in untransfected R1610 cells (Fig. 4, lane *5*). This protein comigrates with the intracellular product of activated human monocytes (Fig. 4, lane *2*) and precursor IL-1β synthesized in *E. coli* (Fig. 4, lanes *6–9*) (Hazuda, D. J., R. L. Webb, P. L. Simon, and P. R. Young, manuscript submitted for publication) suggesting that neither of the eukaryotic forms is glycosylated (IL-1β contains a potential N-glycosylation site at residue 123 [3, 31]).

As in activated monocytes, there is no evidence for the presence in cell lysates of processed forms of the IL-1β precursor that comigrate with the mature 17-kD form of IL-1β secreted from activated monocytes. Comparison of the intensity of IL-1β precursor in the transfected cell lines and activated monocytes (Fig. 4, lanes *2–4*) to known amounts of purified recombinant IL-1β precursor (Fig. 4, lanes *6–9*)

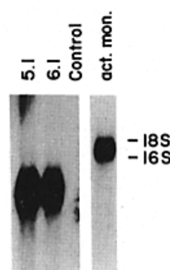

Figure 3. Northern analysis of cell line RNA. 8 μg of total RNA from 5.1, 6.1, and R1610 control cells (*Control*) are compared with 1 μg poly A+ mRNA from LPS activated peripheral blood monocytes (*act. mon.*) The blot was hybridized to a 1.1 kb Pst 1 human IL-1β cDNA insert. Exposure time for the monocyte sample (20 min) was ~20 times less than that for the cell lines (6 h).

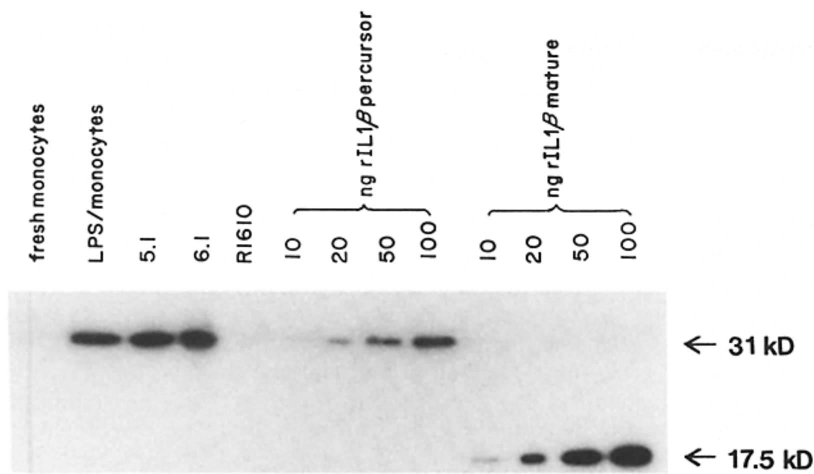

Figure 4. Western analysis of cell line produced IL-1β. After 15% SDS-PAGE, samples were transferred to nitrocellulose and IL-1β detected by incubation with rabbit anti-human 17-kD IL-1β. (Lane *1*) Freshly isolated human purified blood monocytes; (lane *2*) adherent human peripheral blood monocytes activated with 10 ng/ml LPS for 20 h. (Lanes *3–5*) transfected and untransfected R1610 cell lines. (Lanes *6–9*) Different amounts of purified IL-1β precursor as shown; (lanes *10–13*) different amounts of purified mature IL-1β as shown.

allows an approximate quantitation of intracellular IL-1β. Hence, we find that 5.1 and 6.1 make an average ∼4–8 pg IL-1β precursor/cell, compared with the ∼1 pg/cell made by activated monocytes. These amounts are reproducible in different preparations to within a factor of 2, although they do not take account of variations within each cellular population.

To examine whether the IL-1 is secreted from the transfected fibroblasts, 6.1 cells and a control R1610 cell line transfected with BPV-galk were pulse labeled for 5 h with [^{35}S]methionine and [^{35}S]cysteine, and harvested immediately or incubated for a further 19 h in cold medium. IL-1β products were then detected via immunoprecipitation with rabbit polyclonal antisera raised to 17 kD rIL-1β. As can be seen in Fig. 5 *a*, the 31 kD precursor IL-1β can be detected intracellularly at 5 h in BPVIL-1β transfected cells but not in control BPVgalk transfected cells (lanes *1* and *2*). However, neither the precursor or mature form of IL-1β could be detected extracellularly either at 5 h (lane *6*) or upon chasing all the labeled precursor from the cellular fraction (lanes *4* and *8*). A variety of other labeling conditions consistently failed to demonstrate any secretion of IL-1β.

In contrast, when the intracellular IL-1β precursor was labeled to a similar extent in activated human monocytes (Fig. 5 *b*, lane *9*), both precursor, mature and one other processed form of IL-1β were observed in supernatants after a similar chase time (Fig. 5 *b*, lane *10*). The presence of lower molecular weight forms of IL-1β in monocyte cell lysates (Fig. 5 *b*, lane *9*) was not reproducible and appeared to reflect the degree of exposure of the IL-1β precursor to endogenous proteases during lysis. Also, when degradation was observed, as in the present experiment, the lowest molecular weight form of IL-1 consistently migrated faster than mature IL-1β (Fig. 5 *b*, lane *10*), suggesting that the cleavage events occurring in these lysis conditions do not reflect those occurring upon secretion (See reference 19 for more complete details of the kinetics of IL-1 secretion).

Our inability to detect secreted precursor or mature IL-1β in R1610 supernatants was not due to either form being inherently unstable, since incubation of [^{35}S]methionine labeled, *E. coli* synthesized precursor and mature IL-1β in 6.1 supernatants showed only limited degradation of the 31 kD form ($t_{1/2}$ = 14 h) and no degradation of the mature 17 kD form over a 24 h period (Fig. 6). Also, the R1610 cells are not blocked in the ability to secrete proteins since we can detect other labeled proteins in 6.1 supernatants upon pulse labeling cells with [^{35}S]methionine (Fig. 7 *A*). Furthermore, in R1610 cells stably transfected with a BPV vector containing the human tissue plasminogen activator cDNA (BPVtPA), a gene product with a standard amino terminal signal sequence (39), we can readily detect tPA in culture supernatants (Fig. 7 *B*) Hence, R1610 fibroblasts are unlike activated monocytes in that they cannot secrete IL-1β.

Cytoplasmic Localization of IL-1β

Given the failure of the transfected fibroblasts to secrete

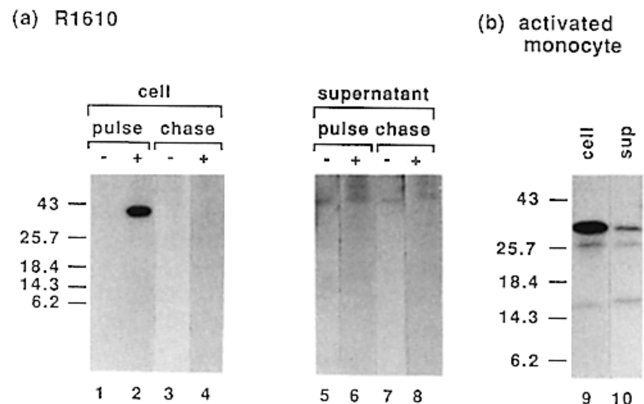

Figure 5. Pulse labeling and immunoprecipitation of R1610 cell lines and activated human monocytes. (*a*) R1610 cell lines were labeled with [^{35}S]methionine and [^{35}S]cysteine for 5 h and harvested (lanes *1*, *2*, *5* and *6*) or chased in nonradioactive medium for a further 19 h (lanes *3*, *4*, *7* and *8*). Lanes *1*, *3*, *5* and *7* (−) Represent control R1610 cells, and lanes *2*, *4*, *6* and *8* (+) represent 6.1 cells. Cell and supernatant fractions are as marked. (*b*) Peripheral blood monocytes activated with 10 ng/ml LPS. Cells were activated for 2 h, labeled with [^{35}S]methionine/cysteine for 1 h (lane *9*), and chased for a further 24 h in nonradioactive medium (lane *10*). The two lanes represent the cell lysate and supernatant fraction as indicated. All samples were immunoprecipitated with a rabbit anti-human IL-1β antiserum before 15% SDS-PAGE and autoradiography. Lanes *5–8* are exposed longer than lanes *1–4* to meet the limits of detection.

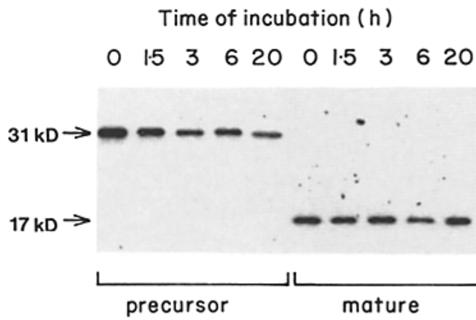

Figure 6. Stability of precursor and mature IL-1β in 6.1 supernatants. *E. coli* expressing precursor and mature human IL-1β were pulse labeled with [^{35}S]methionine, lysed, and centrifuged. The crude soluble fractions were then separately incubated for various times with 6.1 cells in standard culture conditions as shown and the amount of IL-1 remaining determined by immunoprecipitation with IL-1β specific antiserum, SDS-PAGE, and autoradiography.

IL-1β, we examined its intracellular location. This was done in two ways. First, we examined the intracellular distribution of IL-1β in fixed, permeabilized 6.1 cells via indirect immunofluorescence using a rabbit polyclonal antiserum to rIL-1β followed by a fluorescein-conjugated goat anti-rabbit antibody. As shown in Fig. 8, many 6.1 cells stained uniformly throughout the cytoplasm, at higher intensity than control R1610 cells. Preincubation of the rabbit anti-IL-1β antiserum with purified recombinant IL-1β reduced staining of 6.1 cells to background levels, demonstrating that the increased immunofluorescence was specifically due to IL-1β. The lack of staining of all 6.1 cells could result from a lack of clonality after just one round of cloning, but this has not been investigated further.

Secondly, we performed a fractionation of cell lysates into cytosolic, membrane and particulate fractions in the presence of EDTA/EGTA, which should remove all weakly bound membrane-associated proteins (34). Equal amounts of each fraction on a per cell basis were then analyzed by SDS-PAGE followed by immunoblotting with antisera to rIL-1β as above. Fractions were also characterized by enzyme assay (see Materials and Methods). As shown in Fig. 9 *A*, the 31-kD precursor is predominantly in the cytosol.

In contrast, fractionation of BPVtPA transfected R1610 fibroblasts showed that the secreted tPA was found predominantly in the membrane and particulate fractions (Fig. 9 *B*). Thus precursor IL-1β remains in the cytosol and does not transfer into intracellular organelles, in contrast to what has been found for other secreted proteins, but consistent with its lack of a hydrophobic signal sequence.

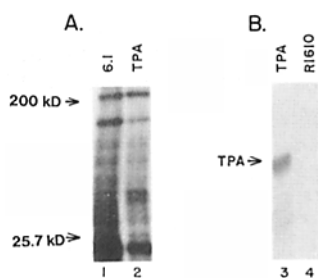

Figure 7. Secretion of other proteins from R1610 cells. Transfected (6.1 and tPA) or untransfected R1610 cells were labeled with [^{35}S]methionine and [^{35}S]cysteine and supernatants harvested as in Fig. 5. (*A*) 10 μl of each supernatant were directly analyzed by 15% SDS-PAGE and autoradiography without immunoprecipitation. (*B*) Supernatants were immunoprecipitated with anti-tPA antiserum before 10% SDS-PAGE and autoradiography. (Lane *3*) BPVtPA-transfected R1610 cell supernatants; (lane *4*) control R1610 cell supernatants.

Activity of Transfected Cells and Cell Supernatants

Although the data indicated that IL-1β was not to be found in significant quantities in either the supernatant or membrane of transfected cells, the methods used above are not as sensitive as assays for IL-1 activity. We therefore tested both cell supernatants and homogenized cell lysates for activity in the EL-4 thymoma assay. We failed to detect any significant activity in either 6.1 or R1610 cell lysates and supernatants, with the exception that sometimes low levels of activity (<2 units/ml) could be found in supernatants of 5.1 or 6.1 freshly plated at high cell concentrations (e.g. 1×10^6 cells/ml). Such activity was never found in control BPVgalk transfected or untransfected R1610 cells plated at similarly high density (data not shown). In contrast, if 5.1 or 6.1 cells were harvested by lysing in 50 mM octaglucopyranoside and then dialyzing out the detergent, we could detect significant levels of activity (Table I), suggesting that the IL-1β being synthesized from the transfected DNA can become active with suitable treatment.

Lack of Surface IL-1 Activity in Transfected Cells

It has been reported that activated monocytes express an active membrane form of IL-1 that can be detected on the surface of fixed cells and membranes, and is therefore believed to exist on the surface of live cells (24). One of the criticisms of using fixed cells is that they might continue to release intracellular material into the supernatant, which could be then mistaken for "membrane" activity. A better test for surface IL-1 activity would be an assay with live cells, in which the potential confusion due to secreted IL-1 has been eliminated, as is the case with the present BPVIL-1β transfected R1610 cell lines.

To test for membrane IL-1 in our transfected cell lines, we looked for IL-2 induction via a direct cell to cell interaction between the transfected fibroblasts and the target EL-4 cells. Hence, we incubated EL-4 thymomas with dilutions of live 5.1, 6.1, BPVgalk-transfected R1610 cells and untransfected R1610 cells. To allow for maximum viability, the transfected and control R1610 fibroblasts were plated one day before the assay, and washed extensively before EL-4 thymomas were added. However, in several attempts we failed to detect activity from any of the cells, whether synthesizing IL-1 precursor or not (data not shown).

Recently, we and others have been able to show that the 31-kD IL-1β precursor has no activity in the EL-4 or other T cell assays (37, Hazuda, D. J., R. L. Webb, P. L. Simon, and P. R. Young, manuscript submitted for publication). It is therefore possible that IL-1β precursor could be expressed on the surface and would go undetected under the present assay conditions. We were also intrigued with the finding that low levels of activity were seen only in the supernatants of transfected cells after splitting cells by treatment with trypsin and replating at high density in fresh medium. This suggested to us that IL-1 activity might be released from the surface of cells by trypsin, perhaps as a result of processing of

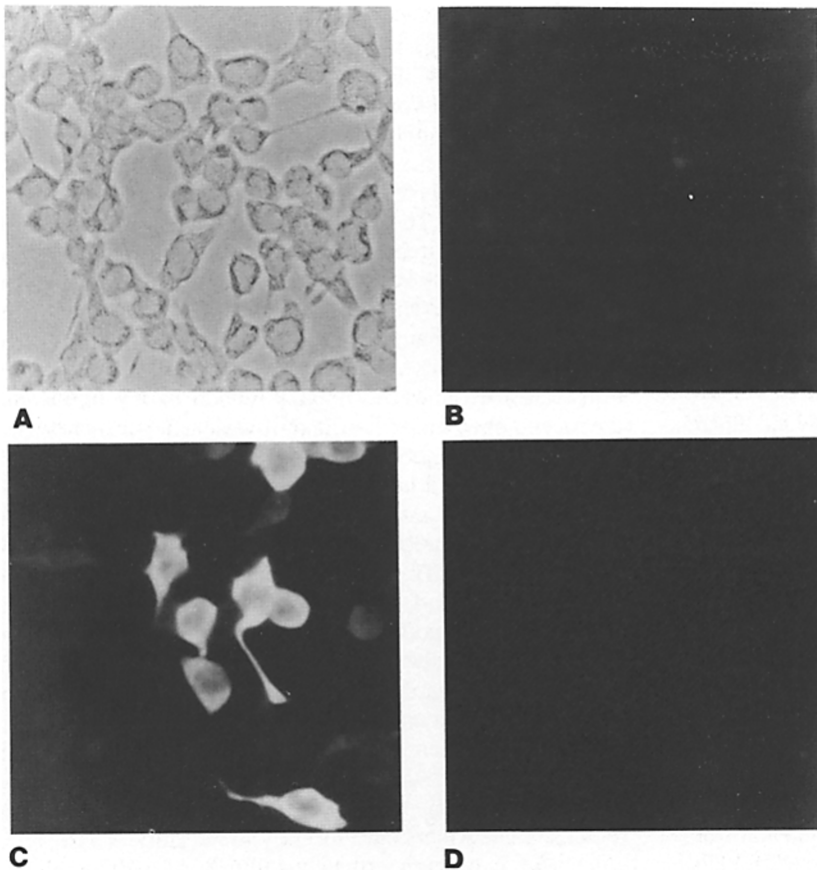

Figure 8. Immunofluorescent localization of fibroblast synthesized IL-1β. Cells fixed with ethanol onto slides were blocked with nonconjugated goat anti-rabbit IgG diluted 1:250 in 1 × PBS before incubation with a polyclonal anti-rIL-1β antiserum (1:200). The cells were then stained with FITC-labeled goat anti-rabbit IgG (1:1,000) and photographed using fluorescent illumination. (*A*) Phase contrast of R1610 cells shown in *B*; indirect immunofluorescence of (*B*) R1610 cells, (*C*) 6.1 cells, (*D*) Same as *C* but using anti-human rIL-1β serum pre-blocked with excess rIL-1β.

the precursor. A similar observation has been reported for a human monocytic cell line (34).

Both transfected and control R1610 fibroblasts were therefore treated with trypsin and proteolysis was halted at various times after addition with soybean trypsin inhibitor. As shown in Fig. 10, activity is released in a time dependent manner. The transitory nature of the IL-1 release is presumably due to the instability of the mature, active form of IL-1 toward further trypsinization (unpublished studies). In contrast, we failed to observe any loss of cell viability via trypan blue staining within the time frame of the experiment.

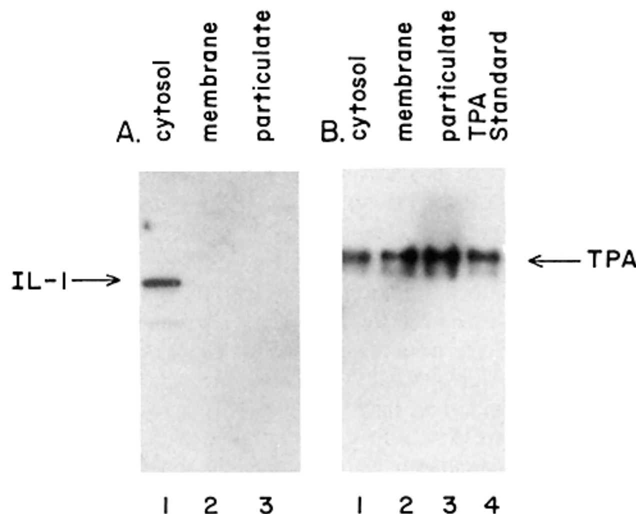

Figure 9. Western blot of fractionated 6.1 and control cells. Cell fractions from 6.1 and BPVtPA-transfected R1610 cells were analyzed via Western blot after SDS-PAGE using rabbit anti-human rIL-1β and rabbit anti-tPA antisera. Cells and fractions are as indicated. (*A*) 6.1 fractions were analyzed on 15% SDS-PAGE; (*B*) tPA fractions (lanes *1-3*) and tPA standard (120 ng, lane *4*) were analyzed on 10% continuous SDS-PAGE after further denaturation in urea. Amounts of each fraction loaded were equivalent on a per cell basis.

Discussion

Most secretory proteins examined to date have been characterized by the presence of a hydrophobic stretch of amino acids known as a signal sequence, which directs the translocation of the protein across the membrane of the endoplasmic reticulum during synthesis. The protein is subsequently ushered out of the cell via exocytosis. Although in the vast majority of secreted proteins the signal sequence is at the amino terminus and is removed upon translocation, there are other proteins such as ovalbumin in which the signal sequence is internal, and is not removed upon translocation (45). Hydropathicity profiles of both IL-1α and IL-1β

Table I. Intracellular IL-1 Activity of R1610 Cell Transfectants

Cell lysate	Activity
	U/10⁶ cells
5.1	24.6
6.1	36.0
R1610	<1.0

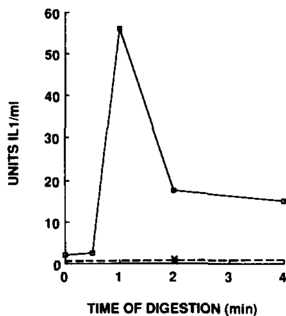

Figure 10. Release of IL-1 activity upon trypsin treatment of transfected R1610 cells. Parallel P60 dishes each containing 10^6 cells were incubated with 0.04% trypsin solution in 0.5 mM EDTA for the times indicated, and the reaction was stopped by adding excess 0.25% soybean trypsin inhibitor. Supernatants were then assayed for IL-1 activity by the EL-4 assay. (*Solid line*) 6.1 cells; (*dashed line*) R1610 cells.

precursors suggest that both proteins apparently lack any region, either amino terminal or internal, which has sufficient hydrophobicity and length to qualify as a signal sequence (3, 16, 28). To study whether IL-1 can be secreted despite these apparent shortcomings, we have examined its expression in transfected Chinese hamster fibroblasts.

Despite high level expression of the IL-1β precursor within the transfected R1610 cells, it is not secreted either as a precursor or the active mature 17-kD form. Instead the IL-1β is present almost exclusively in the cytosol of the transfected cells, and cannot be detected in significant quantities in the membrane or organelle fractions. Furthermore, we know this is a characteristic of IL-1β and not the host R1610 cells, since another secretory protein which does contain an amino terminal signal sequence, human tissue plasminogen activator (tPA), is secreted upon transfection into these cells.

By way of comparison, activated human monocytes also accumulate unprocessed precursor IL-1β intracellularly as judged by Westerns and immunoprecipitates of total cell lysates (see Figs. 4 and 5), and immunofluorescent staining data (6, Hazuda, D. J., and P. R. Young, unpublished data). Unlike the transfected fibroblasts, however, monocytes secrete IL-1β in both the precursor and mature forms (2, 15, 19, Fig. 6). It is therefore likely that hamster fibroblasts lack some cellular factor(s) or state of physiology that allows secretion of release of the intracellular IL-1β.

We believe that the inability to secrete IL-1β may be true for other fibroblast cells, since we have also transfected BALB/*c* 3T3 fibroblasts with BPVIL-1β and obtained cell clones which constitutively synthesize intracellular IL-1β precursor and yet are also unable to secrete IL-1 activity in any significant quantities (unpublished data). This result also seems to pertain to other cell types, including T cells (46), epidermal cells (17, 18, 29, 41), and B cells (32, 33), which have been reported to express endogenous intracellular IL-1 activity and yet fail to secrete it. In particular, it was noted by Hauser et al. (17) that A431 epidermal carcinoma cells only released IL-1 activity into the supernatant as the cells became less viable, suggesting that 'secretion' might really be due to cell 'leakiness'. This finding may also be true for cell types such as glial and glioma cells (11), astrocytes and astrocytomas (11, Lee, J. C., P. L. Simon, and P. R. Young, manuscript submitted for publication), given that the cells are often cultured either for long periods or without serum, conditions which are likely to reduce cell viability.

In all these arguments, it is worth thinking in quantitative terms about the levels of IL-1 produced. In the present case, we calculate that ~4–8 pg of IL-1β precursor is being produced per cell. However, we know that purified recombinant mature IL-1β has a specific activity of $\sim 5 \times 10^8$ units/mg in the EL-4 assay (36), which means that 10^5 transfected fibroblasts should contain 0.5 μg pre IL-1β or $\sim 2.5 \times 10^5$ U of activity. Despite this prediction, we detect no activity in sonicates of these fibroblasts, and only low levels of activity upon cell lysis with the dialysable detergent, octaglucopyranoside. These data are consistent with the finding that the IL-1β precursor is inactive (31, 37). We have confirmed that this is the case by expressing the 31 kd form of IL-1β in *E.coli* (Hazuda, D. J., R. L. Webb, P. L. Simon, and P. R. Young, manuscript submitted for publication). The low levels of activity detected after detergent extraction of the transfected cell lines presumably reflects partial cleavage of the precursor by membrane and/or vesicle derived proteases. Indeed, we have noticed that if activated monocyte cell lysates are harvested in the absence of protease inhibitors, we can detect significant degradation of the precursor, which is otherwise undegraded if such inhibitors are present during the lysis (2, 19). This leads us to believe that the detection of intracellular IL-1 activity may be a function of both the lysis conditions and intracellular protease concentrations as well as the actual levels of IL-1 molecules present. Also important in this regard is the finding that the IL-1α precursor is active (37), in contrast to the observations with IL-1β.

Even though there is no a priori reason to believe that either precursor or mature IL-1 should be membrane bound proteins given their lack of potential hydrophobic spanning regions, a few papers have recently found activity associated either with fixed, nonpermeabilized cells or with membrane fractions (24, 25, 34). We have therefore addressed the question of whether fibroblasts which are constitutively synthesizing IL-1β are able to present it on the outer membrane in an active form. With intact viable transfected fibroblasts, we have been unable to detect cell surface IL-1 activity.

We have also looked for the presence of inactive IL-1β on cell surfaces via immunofluorescence and surface iodination experiments without success (unpublished data). Although this negative result is apparently contradicted by the experiments in which mature, active IL-1 is released from cells upon very brief treatment with trypsin, the latter could be explained in terms of the trypsinization of precursor IL-1β in a few leaky or nonviable cells. Thus even though typically 20–50 U IL-1 are released from 10^6 transfected R1610 cells, this represents only 100 pg mature IL-1β, which is the equivalent of the IL-1 content of 25–50 nonviable cells or $<10^{-2}$% of the population. Since this is beyond even the best viabilities one is likely to achieve, we are unlikely to ever be able to rule out this possibility. Given the comparable levels of IL-1β synthesis in activated human monocytes, we would be concerned that the same caveat would extend to those studies (34). Clearly, this phenomenon is going to be very difficult to answer definitively, given the extraordinarily high activity of the IL-1 molecule. Similar quantitative arguments may also apply to secretion of IL-1 from many of the cell lines that have been studied, where even long incubations often yield very little activity on a per cell basis. This would tend to further support the view that IL-1 is leaked rather than secreted from these cells. Clearly, a more quantitative evaluation similar to that described here may help to resolve this issue. What does appear to be true however, is that monocytes are much more proficient at releasing IL-1

than other cells (19). Whether this is due to unique transport properties or an excessive 'leakiness' awaits further studies of secretion in these cells.

The findings in this paper lend support to a two step model for the secretion of IL-1 from many nonmonocytic cell lines. First, IL-1 is synthesized as a cytosolic protein in response to some external stimulus. Second, IL-1 is released in small quantities from the cell in response to a further stimulation, either by the same or additional agents. This release could be due to some loss in cell viability or transient disruptions in the plasma membrane. Processing then occurs either at this latter step or immediately thereafter via membrane or extracellular proteases.

The key to this model is the very high specific activity of IL-1, which requires the release of very small quantities of mature protein. The model also implies that transcriptional activation by itself is not necessarily enough to allow expression of secreted IL-1. Clearly, the availability of cell lines which constitutively express precursor IL-1 will allow us to test the nature of the steps which are required for release of IL-1. In addition we can begin to ask what role the conserved precursor portion of IL-1 might play in this process, if any, especially since the existence of further secreted proteins which lack conventional 'signal' sequences such as basic fibroblast growth factor (1) suggests that these mechanisms may not be specific to IL-1.

We would like to thank Mitch Reff and John Trill for the gift of tPA-expressing R1610 cell lines, tPA antisera, and purified tPA standard, and Jeff Stadel for helpful comments.

Received for publication 16 February 1988, and in revised form 18 March 1988.

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