

# IDENTIFICATION OF A HIGH-AFFINITY RECEPTOR FOR NATIVE HUMAN INTERLEUKIN 1 $\beta$ AND INTERLEUKIN 1 $\alpha$ ON NORMAL HUMAN LUNG FIBROBLASTS

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While IL-1 was initially described as a lymphocyte-activating factor (1), subsequent studies (2, 3) have shown that IL-1 regulates a variety of nonlymphoid cells involved in the inflammatory response. Prominent among these are connective tissue cells. While early studies demonstrated the ability of IL-1 to stimulate proliferation by human dermal fibroblasts (4–6) and collagenase production by human synovial fibroblasts (7–9), more recent studies have shown matrix resorptive effects on articular cartilage (10) and bone (11–13) in organ culture.

When one fractionates human monocyte culture supernatants by IEF, three major charged species having pI of 6.8, 5.4, and 5.2 are consistently observed whether the fractions are assayed on murine thymocytes or connective tissue cells (4, 5, 14). The pI 6.8 species, which is responsible for ~90% of the biological activity found in human monocyte culture supernatants, has been purified (15, 16), and amino acid sequence analysis (16, 17) shows that it is encoded by an abundant mRNA, the cDNA of which is now referred to as IL-1 $\beta$  (17, 18). The pI 5.2 and pI 5.4 species have recently also been purified (19) and amino acid sequence of these species (19) shows that they are encoded by a tenfold less abundant mRNA, the cDNA of which is now referred to as IL-1 $\alpha$  (17). Even with the insertion of numerous gaps in the amino acid sequences to maximize homology, the two human IL-1 species have only 30% of their amino acid residues in common (17). Nevertheless, comparison of pure pI 6.8 IL-1 and pure pI 5.4/5.2 IL-1 on murine thymocytes shows them to be equally potent with half-maximal stimulation seen at concentrations of 20–34 pM (19). When these same native purified IL-1 species are tested for their ability to stimulate human dermal fibroblast proliferation, they are found to be even more potent, but equally so, with concentrations of only 1–3 pM required for half-maximal stimulation (20).

Recently it has been reported that murine (21) and human (22) lymphoid lines and murine 3T3 cells (23) bear low-affinity receptors for human pI 6.8 IL-1 (IL-1 $\beta$ ) with  $K_d$  of ~200–500 pM (21–23). In particular, the murine T lymphoma line, LBRM-33 1A5, which half-maximally produces IL-2 in response to  $10^{-14}$  M IL-1 $\beta$ , was reported (21, 23) to have 250 sites per cell of similar low affinity. Accordingly, less than a single IL-1 receptor per cell would be occupied when

half-maximal IL-2 production is observed in response to IL-1. This discrepancy calls into question the physiological role of the low-affinity receptors and suggests that considerably higher affinity receptors most likely mediate the biological effects of IL-1 on these target cells.

In the current report, a high-affinity IL-1 receptor is identified on human embryonic lung fibroblasts. As previously noted in the case of human rheumatoid synovial cells (20), these cells produce prostaglandin E<sub>2</sub> at low picomolar concentrations of both IL-1 species, with ~3 pM IL-1 $\beta$  or ~10 pM IL-1 $\alpha$  required for half-maximal stimulation. Equilibrium binding studies with labeled IL-1 $\beta$  give a  $K_d/K_i$  of 10 pM for IL-1 $\beta$ , and IL-1 $\alpha$  can bind to the same site with approximately fivefold lower affinity, that is, with a  $K_i$  of 50 pM. These findings suggest that the biological effects observed at low picomolar concentrations of IL-1 are mediated by receptors with considerably higher affinity than previously reported and are consistent with the existence of a single receptor mediating the biological effects of both IL-1 species.

### Materials and Methods

*Culture of Human Embryonic Lung Fibroblasts (MRC-5).* Human embryonic lung fibroblasts were obtained from the American Type Culture Collection, Rockville, MD, and cultured in 450-cm<sup>2</sup> roller bottles or in 150-cm<sup>2</sup> tissue culture flasks in culture medium consisting of DME containing 10% FCS, 4 mM glutamine, 25 mM Hepes, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37°C in a humidified atmosphere of 7.5% CO<sub>2</sub>/air. The cells were passaged weekly and the medium was changed two additional times weekly. Cells passaged up to four times in our laboratory were used for these studies. In preparation for assays, the cells were removed with 0.5% trypsin/0.2% EDTA, diluted into fresh culture medium, and plated at a density of  $2 \times 10^4$  cells/well in 24-well plates (No. 3524; Costar, Cambridge, MA) or at a density of  $10^5$  cells/well in six-well cluster plates (No. 52795, Nunc, Roskilde, Denmark). Cell number was determined by counting the trypsinized cells in a hemacytometer.

*Measurement of Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in Response to IL-1 Species.* 24 h after MRC-5 cells had been subcultured into 24-well plates, the culture medium was removed from each well by gentle aspiration and was replaced with 0.5 ml of fresh culture medium containing known concentrations of purified IL-1 species (see below). After incubation for 18–20 h, the culture medium was removed, centrifuged at 400 g, and frozen at –70°C until assayed. To measure PGE<sub>2</sub>, 300  $\mu$ l of each sample was extracted three times with 1 ml of ethyl acetate (HPLC grade, Fischer Scientific Co., Pittsburgh, PA). The extracts of each sample were pooled, taken to dryness under a stream of dry nitrogen, and taken up in 50  $\mu$ l of DME/10% FCS. Preliminary experiments with tritiated PGE<sub>2</sub> showed that 85–90% of the labeled material was successfully extracted from culture medium by this procedure. PGE<sub>2</sub> was then assayed by RIA as previously described (24) and the results were expressed as picograms of PGE<sub>2</sub> per culture. Extracts of fresh medium containing <sup>125</sup>I-IL-1 $\beta$  gave no signal in the RIA for PGE<sub>2</sub>, thus permitting assessment of the specific biological activity of the radioligand.

*Purification of Native Human IL-1 $\beta$  and IL-1 $\alpha$ .* Native IL-1 $\beta$  (pI 6.8) and IL-1 $\alpha$  (pI 5.4) were purified from mononuclear cell culture supernatants as previously described (16, 19). The IL-1 species were pure, as determined by SDS-PAGE and silver staining (19). The protein concentration of each of the IL-1 preparations was determined by peak integration at 214 nm as the IL-1 species eluted from a reverse-phase HPLC column, as previously described (19). The integration function of the detection system was calibrated by chromatographing known amounts of pure RNase and constructing a linear calibration plot of integration units versus amount of protein injected. This method has been previously validated by amino acid analysis of purified IL-1 (16).

*Preparation of <sup>125</sup>I-IL-1 $\beta$ .* Pure IL-1 $\beta$  was concentrated to 0.25–0.50 mg/ml by ultrafil-

tration on YM-10 membranes (Amicon Corp., Danvers, MA) and desalted into 20 mM sodium phosphate buffer, pH 7.95, containing 100  $\mu\text{g}/\text{ml}$  of PEG (mol wt = 8,000). 5–10  $\mu\text{l}$  of protein solution was reacted with 2 mCi of monoiodo Bolton-Hunter reagent (New England Nuclear, Boston, MA) for 3 h on ice as previously described (25). These conditions usually resulted in labeling of ~50% of the IL-1 molecules. The radioligand was then extensively reisolated by methods to be published in detail elsewhere.<sup>1</sup> Briefly, the reaction mixture was first chromatographed on an HPLC gel filtration column to remove the unincorporated label. Subsequent HPLC hydrophobic interaction chromatography of the labeled IL-1 $\beta$  preparation resulted in complete resolution of labeled from unlabeled IL-1 $\beta$ . The labeled IL-1 $\beta$  was then collected, pooled, and desalted by HPLC gel filtration chromatography. The amount of protein in the <sup>125</sup>I-IL-1 $\beta$  preparation was determined by integration of the absorbance profile obtained at 214 nm during the final gel filtration chromatography step in comparison with known amounts of RNase, as described above.

*Determination of Radioligand Bindability.* To determine the percentage of radioligand that was bindable, ~10,000 counts of <sup>125</sup>I-IL-1 $\beta$  was added to 0.5 ml of binding buffer (see below) with or without 1 nM unlabeled IL-1 $\beta$ . The solutions were then successively incubated with a series of highly confluent cluster-well cultures of MRC-5 fibroblasts for 1 h each at room temperature with gentle continuous mixing on a platform rocker. The solutions were removed after each adsorption step, counted, and then transferred to fresh cultures of fibroblasts. Counts remaining in solution after each adsorption step were then plotted as a function of passage number.

*Direct and Competitive Binding Experiments.* Before direct binding experiments, the MRC-5 fibroblasts were cultured in six-well cluster plates as described above. After 4–6 d of culture, when the cell density was  $\sim 2 \times 10^6$  cells/well, the medium was removed from each well and replaced with 0.5 ml of binding buffer (DME/5% FCS/0.2% sodium azide) containing measured amounts of <sup>125</sup>I-IL-1 $\beta$  with or without a 50-fold molar excess of unlabeled IL-1 $\beta$ . The plates were then incubated at 20°C for 2 h with gentle continuous mixing. After the incubation period was completed, the supernatants were removed with a Pasteur pipette, counted, and taken as a measure of free counts. In preliminary experiments, the number of free counts was closely approximated by subtracting the number of counts bound (see below) from the total number of counts added ( $\pm 95\%$ ). Therefore, in subsequent experiments, the number of free counts was obtained by using a computer program (see below) that subtracted the number of bound counts from the total number of counts added. To determine the number of counts bound, the plates were washed by dipping them rapidly three times in 3 liters of PBS. Preliminary experiments showed that additional rapid washes did not further reduce the number of counts remaining with the cell monolayer. The bound counts were then solubilized with the addition of 0.3 ml of 2.5 M NaOH per well, transferred to a scintillation vial, and counted. The number of counts bound in the presence of excess unlabeled IL-1 $\beta$  was taken as a measure of nonspecific binding. The number of specific counts bound was calculated by subtracting the number of nonspecific counts bound from the total number of counts bound.

In competitive binding experiments, the cells were overlaid with 2.0 ml of binding buffer containing 4 pM <sup>125</sup>I-IL-1 $\beta$  and known amounts of one of the following unlabeled ligands: IL-1 $\beta$ , IL-1 $\alpha$ , human native tumor necrosis factor  $\alpha$  (reference 26; kindly provided by Dr. Berish Rubin, New York Blood Center, NY), human rIL 2 (Amgen Corp., Thousand Oaks, CA), human rIFN- $\gamma$  (Amgen Corp), or bovine acidic fibroblast growth factor (references 27, 28; kindly provided by Dr. Kenneth Thomas, Merck Research Laboratories, Rahway, NJ). These solutions were then incubated on fibroblasts for 2 h at 20°C and the plates were then washed, solubilized, and counted as detailed above.

*Assessment of Equilibrium.* To determine the time necessary for binding of IL-1 to reach a steady state, 10 pM <sup>125</sup>I-IL-1 $\beta$  in binding buffer was added to replicate wells at time 0. At measured intervals, duplicate wells were washed, solubilized, and counted as

<sup>1</sup> Schmidt, J. A., and J. Chin. Preparation and isolation of monoiodinated human interleukin 1 $\beta$ . Manuscript in preparation.

detailed above. A second series of replicate wells, which were overlaid with 10 pM  $^{125}\text{I}$ -IL-1 $\beta$  and 500 pM unlabeled IL-1 $\beta$  at time 0, were harvested in parallel with the first series of wells to determine the amount of nonspecific binding at each time point. Specific counts bound was then plotted as a function of time.

To assess the proportion of bound counts that were dissociable, MRC-5 cells were incubated with 10 pM  $^{125}\text{I}$ -IL-1 $\beta$  for 2 h. The cells were then washed, solubilized, and counted to determine the number of counts bound. Replicate wells were washed and treated with 200  $\mu\text{l}$  of 2% acetic acid in 0.14 M NaCl (pH 2.5) for 1 min. The supernatant was then removed and counted. The number of counts stripped by acetic acid was taken as a measure of surface-bound radioligand. To ascertain whether the counts removed with acetic acid represented intact radioligand, an aliquot (100  $\mu\text{l}$ ) was precipitated with 10% TCA on ice with 100  $\mu\text{g}$  of BSA added as a carrier. The pellet was washed three times with ice-cold 10% TCA and counted.

**Crosslinking Experiments.** MRC-5 fibroblasts were overlaid with binding buffer containing 100 pM  $^{125}\text{I}$ -IL-1 $\beta$  alone or together with one of the following unlabeled ligands: 1 nM IL-1 $\beta$ , IL-1 $\alpha$ , human rIL 2, human rIFN- $\gamma$ , or human TNF- $\alpha$ . These solutions were then incubated on fibroblasts for 2 h at 20°C as described above. At the conclusion of the incubation period, the plates were washed as described above and then incubated in PBS containing 0.1, 0.2, 0.5, or 1.0 mg/ml of the homobifunctional crosslinker disuccidimidyl suberate (DSS;<sup>2</sup> Pierce Chemical Co., Rockford, IL) for 1 h at 20°C. Subsequently, the plates were washed three times in fresh PBS and the cells were scraped into 50  $\mu\text{l}$  of Laemmli sample buffer containing 15% SDS with or without 10% 2-ME. The samples were boiled for 5 min and electrophoresed on a 7.5% homogeneous gel for 15 h at 5 mA and for 3 h at 20 mA using the discontinuous buffer system of Laemmli. The gels were fixed, stained with Coomassie Blue, dried, and exposed to Kodak XAR-5 film for 7–10 d at -70°C. The gels were calibrated with the following proteins of known molecular mass: myosin, 200 kD;  $\beta$ -galactosidase, 116.2 kD; phosphorylase B, 92.5 kD; BSA, 66.2 kD; OVA, 45 kD; carbonic anhydrase, 31 kD; soybean trypsin inhibitor, 21.5 kD; and lysozyme, 14.4 kD.

**Data Analysis.** Equilibrium binding data were analyzed by the 1986 version of the Ligand (29) family of programs on an appropriately equipped IBM personal computer. Ligand is a nonlinear-curve fitting program that uses the mass-action equation and a weighted least squares statistical algorithm to determine the  $K_d$ , the molar concentration of receptors (R), and nonspecific binding (N). The power of this software as compared with manual or graphical methods has been described in detail elsewhere (29). The salient features of the program with respect to the current study are (a) the ability to compensate for radioligand bindability; (b) the ability to test and compare the goodness of fit obtained with alternative models, e.g., one site versus two site models; and (c) the ability to simultaneously analyze displacement curves obtained for two different unlabeled ligands, e.g., IL-1 $\beta$  and IL-1 $\alpha$ , within an experiment to obtain a more precise and accurate estimate of the parameters shared in common, such as binding capacity and N. Competitive binding data obtained for unlabeled IL-1 $\beta$  and IL-1 $\alpha$  were analyzed using a two ligand/one site model and by equating the nonspecific binding parameters (N1, N2) for both ligands. Graphical output of direct binding data in the Scatchard format was also performed by Ligand.

Plotting and curve fitting of bioassay data and plotting of theoretical binding curves was performed by an IBM PC using Parafit II (Dynacomp, Boston, MA), a nonlinear parameter regression program.

## Results

To determine if human embryonic lung fibroblasts would be suitable target cells for IL-1-R binding studies, it was first determined whether they were biologically responsive to low picomolar concentrations of IL-1 as has been

<sup>2</sup> Abbreviations used in this paper: DSS, disuccidimidyl suberate; N, nonspecific binding; R, molar concentration of receptors.

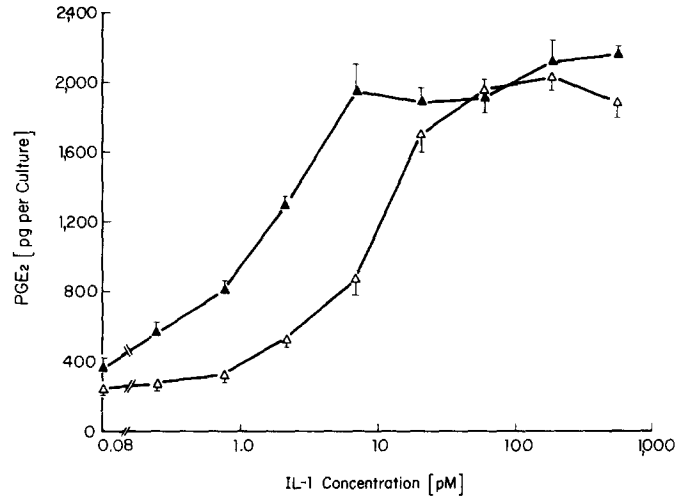


FIGURE 1. Prostaglandin E<sub>2</sub> production by MRC-5 human embryonic lung fibroblasts in response to increasing concentrations of native human IL-1 $\beta$  (filled triangles) or IL-1 $\alpha$  (open triangles). The means ( $\pm$  SEM) of triplicate determinations are plotted.

recently demonstrated (20) for other types of normal human connective tissue cells. As shown in Fig. 1, these cells responded in saturable fashion to low picomolar concentrations of native IL-1 $\beta$  (pI, 6.8) and IL-1 $\alpha$  (pI, 5.4) by secreting five- to eightfold higher amounts of prostaglandin E<sub>2</sub>. IL-1 $\beta$  and IL-1 $\alpha$  stimulated the same maximum level of PGE<sub>2</sub> secretion. In three experiments, half-maximal stimulation of PGE<sub>2</sub> secretion was seen with IL-1 $\beta$  and IL-1 $\alpha$  at concentrations of  $3 \pm 1.2$  pM and  $10 \pm 2.3$  pM ( $\pm$  SEM), respectively. These results suggested that MRC-5 fibroblasts possess functional, high-affinity receptors for both IL-1 $\beta$  and IL-1 $\alpha$ .

To identify the receptor site for IL-1 $\beta$ , native IL-1 $\beta$  was labeled with <sup>125</sup>I-Bolton-Hunter reagent. The Bolton-Hunter technique was chosen because preliminary experiments with other labeling techniques, all of which involved the use of oxidizing agents, showed that even brief exposure of IL-1 to oxidants alone led to its complete loss of bioactivity in the murine thymocyte assay (Limjuco, G., and J. Schmidt, unpublished observations). The conditions of the Bolton-Hunter reaction were chosen so that ~50% of the molecules were labeled. The labeled IL-1 molecules were then completely separated from the unincorporated Bolton-Hunter reagent by HPLC gel filtration chromatography and from the unlabeled IL-1 molecules by HPLC hydrophobic interaction chromatography (see Materials and Methods). As shown in Fig. 2A, HPLC gel filtration chromatography was used as the last step in the isolation procedure to desalt the radioligand. A sharp, symmetrical peak was detected at 214 nm, which corresponded with radioactivity. The specific radioactivity of the labeled material was calculated directly by using the absorbance at 214 nm as a measure of protein concentration (see Materials and Methods). The results of such calculations consistently gave values between 2,000 and 2,400 Ci/mmol, indicating that the labeled material represented monoiodo IL-1 $\beta$ . SDS-PAGE and autoradiography

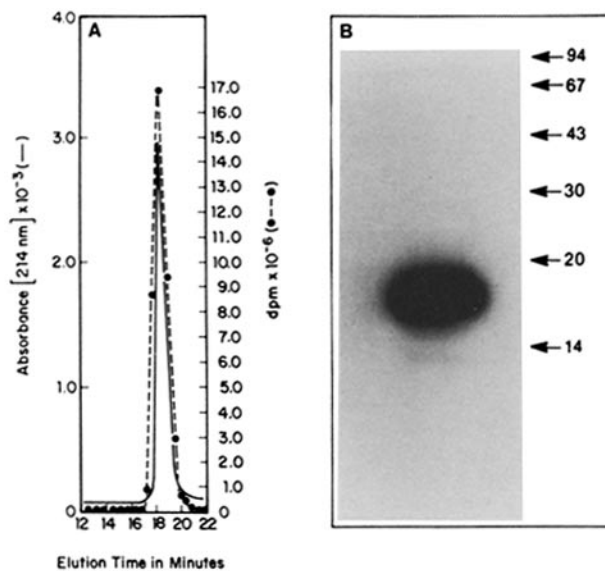


FIGURE 2. Isolation of monoiodo IL-1 $\beta$ . Native human IL-1 $\beta$  was labeled with Bolton-Hunter reagent and the iodinated species were separated from free-label and unlabeled IL-1 $\beta$  by HPLC gel filtration and hydrophobic interaction chromatography (not shown) and then desalted by HPLC gel filtration chromatography (A). The absorbance of the effluent was monitored at 214 nm and the radioactivity (dpm) of 30-s fractions was measured in a gamma counter. SDS-PAGE and autoradiography of the labeled product is shown in B with the positions of molecular mass markers indicated by arrows.

of the purified radioligand (Fig. 2B) showed that all of the counts were associated with a single 18 kD band having the same mobility as human IL-1 $\beta$ .

When various preparations of the purified radioligand were tested for bioactivity on either murine thymocytes or MRC-5 fibroblasts, they were found to retain 40–50% of the specific bioactivity of the unlabeled material (data not shown). More relevant to the calculation of the equilibrium binding constant (30), the radioligand preparations were tested for bindability to MRC-5 fibroblasts by successively adsorbing a limiting amount of radioligand to cultures of MRC-5 fibroblasts in the presence and absence of a 50-fold molar excess of unlabeled IL-1 $\beta$ . The amount of radioligand removed by the cultures minus that amount removed in the presence of excess unlabeled ligand was calculated and taken as a measure of specifically bindable radioligand. As shown in a representative experiment in Fig. 3, 41% of the total initial counts and 55% of the counts remaining after five passages in the presence of excess unlabeled ligand were specifically bound. These percentages represent minimum and maximum estimates of bindability depending on whether damaged radioligand is selectively lost during the transfer of labeled material from well to well. The radioligand that was not adsorbed to cells nevertheless remained TCA precipitable (>95%), thus indicating that proteolysis of radioligand during the course of the experiment was not contributing to decreased bindability. A factor of 0.5 was therefore used to correct the number of free counts in the direct binding experiments (see below).

To assess the time required for binding of  $^{125}\text{I}$ -IL-1 $\beta$  to reach equilibrium,

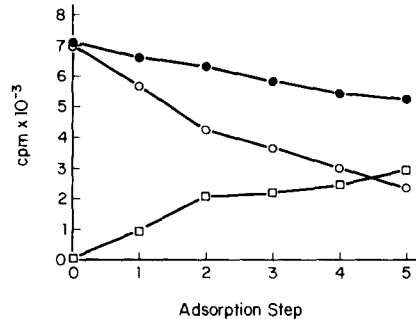


FIGURE 3. Bindability of labeled IL-1 $\beta$ .  $^{125}\text{I}$ -IL-1 $\beta$  (7,000 cpm) was serially incubated for 1 h with cultures of MRC-5 human embryonic lung fibroblasts alone (*open circles*) or in the presence of 1 nM unlabeled IL-1 $\beta$  (*filled circles*). The binding conditions are specified in Materials and Methods. The supernatants were removed after each adsorption step and counted, and the means of duplicate determinations are plotted. The difference between the mean number of free counts obtained with labeled IL-1 $\beta$  alone and the mean number of free counts obtained in the presence of excess unlabeled IL-1 $\beta$  was calculated after each adsorption step (*squares*) and taken as a measure of specifically bindable radioligand. The minimum bindability in this experiment was 41% (2,950 cpm/7,000 cpm) and the maximum bindability was 55% (2,950/5,250 cpm).

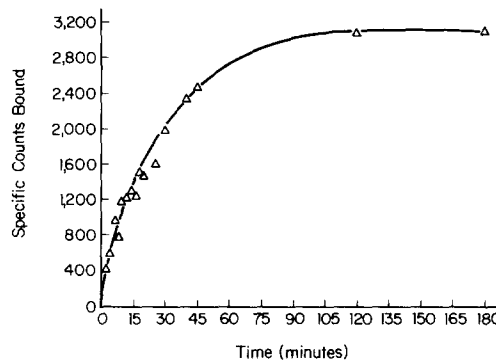


FIGURE 4. Kinetics of binding of  $^{125}\text{I}$ -IL-1 $\beta$  to human embryonic lung fibroblasts. Replicate cultures were incubated with 10 pM  $^{125}\text{I}$ -IL-1 $\beta$  alone or in the presence of excess (500 pM) unlabeled IL-1 $\beta$  as detailed in Materials and Methods. Duplicate cultures from each set were rapidly washed and solubilized at the indicated times. Specific counts bound were calculated by subtracting counts bound in the presence of excess unlabeled IL-1 $\beta$  from counts bound with  $^{125}\text{I}$ -IL-1 $\beta$  alone.

replicate cultures of MRC-5 fibroblasts were incubated with 10 pM  $^{125}\text{I}$ -IL-1 $\beta$  at 20°C in the presence of 0.2% sodium azide. At various times, the cell layers in duplicate cultures were rapidly washed and solubilized to determine the total number of counts bound. The nonspecific-binding component was determined in a parallel series of cultures that were incubated with radioligand and a 50-fold molar excess of unlabeled IL-1 $\beta$  (see Materials and Methods). As shown in Fig. 4, specific binding proceeded moderately rapidly under these conditions and reached a plateau within two hours. By comparison, binding at 4°C proceeded slowly and had not yet reached a steady state by 4 h of incubation (data not shown). To determine if the cell-associated radioligand represented surface-bound or internalized radioligand, stripping experiments were performed. As

TABLE I  
*Recovery of Bound  $^{125}\text{I-IL-1}\beta$  from MRC-5 Fibroblasts*

$^{125}\text{I-IL-1}\beta$	Exp. 1	Exp. 2
Total cpm bound*	2,042	2,278
cpm stripped by acetic acid (%) <sup>‡</sup>	1,965 (96)	1,942 (85)
cpm precipitated by 10% TCA <sup>§</sup>	1,865 (95)	1,876 (97)

Cultures of MRC-5 fibroblasts were incubated with 10 pM  $^{125}\text{I-IL-1}\beta$  for 2 h at 20°C and washed.

\* Total counts bound is the number of counts recovered after the 2-h incubation period when the cell layer was solubilized with 2 M sodium hydroxide. The mean of triplicate determinations is shown.

<sup>‡</sup> Replicate cultures were washed and treated with 2% acetic acid, pH 2.5, for 1 min. The supernatants were removed and counted. The mean of triplicate determinations is shown. The numbers in parentheses indicate the percentage of total cpm bound that were stripped from the cells under these conditions.

<sup>§</sup> The material stripped with acetic acid was supplemented with TCA (10%, final concentration) and BSA (100  $\mu\text{g/ml}$ , final concentration) and allowed to stand on ice for 10 min. The precipitate was washed three times with cold 10% TCA. The mean of triplicate determinations is shown. The numbers in parentheses indicate the percentage of cpm stripped with acetic acid that were TCA precipitable. The radioligand before incubation with cells was 98% TCA precipitable.

shown in Table I in two experiments, 96% and 85% of the counts specifically bound by 2 h at 20°C were removed by a 1-min exposure of the cells to 2% acetic acid, 0.14 M NaCl, pH 2.5, and of those counts removed,  $\geq 95\%$  were TCA precipitable. These results indicate that little or no internalization or degradation of the bound radioligand had occurred under the assay conditions employed.

The results of a representative direct binding experiment are shown in Fig. 5 in which increasing concentrations of  $^{125}\text{I-IL-1}\beta$  were incubated with replicate cultures of MRC-5 fibroblasts for 2 h at 20°C in the presence of sodium azide. The nonspecific-binding component was determined by incubating a parallel series of cultures with radioligand in the presence of a 50-fold molar excess of unlabeled IL-1 $\beta$ . A plot of specific counts bound as a function of radioligand concentration (Fig. 5A) indicates that binding is dose-responsive and saturable. Using a computer-based program using the mass action equation and taking into account the bindability of the radioligand (Ligand, see Materials and Methods), the data in this and two additional direct binding experiments (Table II) were best fit by a single site model with a mean  $K_d$  of  $8.4 \pm 4.1$  pM ( $\pm$  SEM). A two site model with high- and low-affinity binding sites differing in affinity by 2–1,000-fold, fit these data considerably less well (data not shown). A plot of the computational results in the Scatchard format is shown in Fig. 5B. The average number of sites per cell was  $3,100 \pm 1300$  ( $\pm$  SEM). This value was in good agreement with that obtained by dividing the number of molecules of  $^{125}\text{I-IL-1}\beta$  bound at saturation by the number of cells per culture ( $2,100 \pm 600$  sites/cell).

The results of a representative competitive binding experiment are shown in Fig. 6. In these experiments, replicate cultures were incubated with a limiting concentration of  $^{125}\text{I-IL-1}\beta$  (4 pM) and increasing concentrations (0.2–500 pM) of either unlabeled IL-1 $\beta$  or IL-1 $\alpha$  for 2 h at 20°C. Competition by both



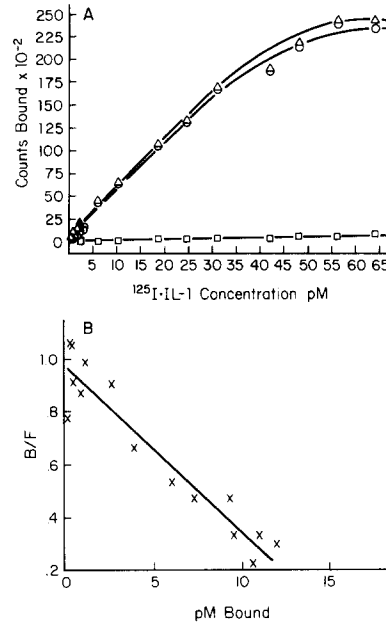


FIGURE 5. Direct binding of  $^{125}\text{I}$ -IL-1 $\beta$  to human embryonic lung fibroblasts. (A) Increasing concentrations of radioligand were incubated with cultures of MRC-5 fibroblasts alone (*triangles*) or in the presence of a 50-fold molar excess of unlabeled IL-1 $\beta$  (*squares*) as detailed in Materials and Methods. Specific counts bound (*circles*) were calculated by subtracting counts bound in the presence of excess unlabeled ligand from counts bound in the presence of radioligand alone. The radioligand concentration (pM) has been corrected for bindability. (B) The data shown in A were analyzed by Ligand and the results were plotted in the Scatchard format. The  $K_d$  obtained in the experiment shown was 15 pM.

TABLE II  
Summary of Results Obtained in Direct and Competitive Binding Experiments

Exp.	Direct binding*		Competitive binding <sup>‡</sup>		
	$K_d$	Receptor concentration (sites/cell)	Competitor		Receptor concentration (sites/cell)
			$K_i$ of IL-1 $\beta$	$K_i$ of IL-1 $\alpha$	
	pM		pM	pM	
1	6.5	1,500	14	23	3,500
2	3.7	2,700	6.8	74	3,200
3	15	5,100	13	53	1,100
Mean $\pm$ SEM	$8.4 \pm 4.1$	$3,100 \pm 1,300$	$11.2 \pm 2.8$	$50 \pm 18$	$2,600 \pm 920$

\* Direct binding experiments were performed by incubating 0.2–100 pM  $^{125}\text{I}$ -IL-1 $\beta$  with MRC-5 fibroblasts as detailed in Materials and Methods. The  $K_d$  and receptor concentration were calculated by Ligand, as described in Materials and Methods.

<sup>‡</sup> Competitive binding experiments were performed by incubating 4 pM  $^{125}\text{I}$ -IL-1 $\beta$  and 0–500 pM unlabeled IL-1 $\beta$  or IL-1 $\alpha$  with MRC-5 fibroblasts, as described in Materials and Methods. The  $K_i$  and receptor concentration were calculated using Ligand and a two ligand/one site model.

unlabeled IL-1 $\beta$  and IL-1 $\alpha$  was observed. Computer-based analysis of the competitive binding data obtained for IL-1 $\beta$  or IL-1 $\alpha$  again showed that each set of data was best fit by a single site model. Using a single site/two ligand model,

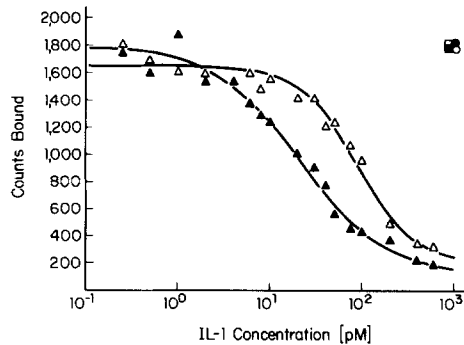


FIGURE 6. Competitive binding of  $^{125}\text{I}$ -IL-1 $\beta$  to human embryonic lung fibroblasts. 4 pM  $^{125}\text{I}$ -IL-1 $\beta$  was incubated with human embryonic lung fibroblasts in the presence of the indicated concentrations of unlabeled IL-1 $\beta$  (filled triangles), IL-1 $\alpha$  (open triangles), human IL-2 (open circle), IFN- $\gamma$  (filled circle), tumor necrosis factor  $\alpha$  (open square), or bovine acidic fibroblast growth factor (filled square) as detailed in Materials and Methods. The competitive binding data obtained for unlabeled IL-1 $\beta$  and IL-1 $\alpha$  were analyzed by Ligand and tabulated in Table II. The  $K_i$  values obtained for IL-1 $\beta$  and IL-1 $\alpha$  in the experiment shown were 13 and 53 pM, respectively.

analysis of three experiments (Table II) gave a mean  $K_i$  of  $11 \pm 2.8$  pM ( $\pm$  SEM) for IL 1 $\beta$ , in good agreement with the  $K_d$  obtained in the direct binding experiments. IL-1 $\alpha$  competed less well than IL-1 $\beta$  with a mean  $K_i$  of  $50 \pm 18$  pM. The same preparations of native IL-1 $\beta$  and IL-1 $\alpha$  used in these experiments were nevertheless found to be equally active on murine thymocytes with half-maximal stimulation observed at 20 pM as previously reported (19). The lower affinity observed for IL-1 $\alpha$  therefore did not appear to be the result of partial inactivation of these preparations as compared with IL-1 $\beta$ . Human IL-2 and human IFN- $\gamma$  gave no inhibition whatsoever at a concentration of 1 nM (Fig. 6). Human tumor necrosis factor  $\alpha$ , which shares some of the same biological properties of IL-1 with respect to connective tissue cells (31), did not compete. Bovine acidic fibroblast growth factor, a molecule which is homologous with human IL-1 $\beta$  at the amino acid level (27, 28), similarly gave no inhibition of binding at a concentration of 1 nM (Fig. 6).

To examine the molecular weight of the IL-1-R molecule and its potential subunit composition, MRC-5 fibroblasts were incubated with 100 pM  $^{125}\text{I}$ -IL-1 $\beta$  for 2 h at 20°C, washed, and incubated with the homobifunctional crosslinker, DSS, for an additional 1 h at room temperature. Afterwards, the cells were washed, solubilized in Laemmli sample buffer with or without reducing agent, and analyzed by SDS-PAGE. As shown in Fig. 7 at a DSS concentration of 0.2 mg/ml,  $^{125}\text{I}$ -IL-1 $\beta$  was associated with a dominant band of 98 kD when electrophoresed under reducing (lane 1) or nonreducing (lane 2) conditions. A fainter band of  $\sim$ 200 kD was also seen in this and one additional experiment. Fainter bands, seen at the top of the running gel, probably represent aggregated material since they constituted a proportionately larger percentage of the labeled material when higher concentrations of crosslinker were used (data not shown). A lower concentration of crosslinker (0.1 mg/ml) gave considerably fainter bands but was otherwise identical to the result obtained at 0.2 mg/ml. Labeling of both the 98 and 200 kD bands was completely blocked by performing the labeling in the

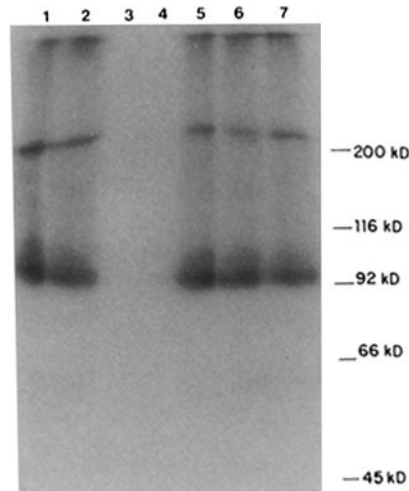


FIGURE 7. Crosslinking of  $^{125}\text{I}$ -IL-1 $\beta$  to human embryonic lung fibroblasts.  $^{125}\text{I}$ -IL-1 $\beta$  (100 pM) was incubated with MRC-5 fibroblasts alone (lanes 1 and 2) or in the presence of 1 nM unlabeled IL-1 $\beta$  (lane 3), IL-1 $\alpha$  (lane 4), human IL-2 (lane 5), IFN- $\gamma$  (lane 6), or TNF- $\alpha$  (lane 7), as detailed in Materials and Methods. The cultures were then treated with DSS and solubilized in sample buffer with (lane 1) or without (lanes 2–7) reducing agent before SDS-PAGE and autoradiography. The positions of molecular mass markers are indicated at right.

presence of 1 nM IL-1 $\beta$  (lane 3) or 1 nM IL-1 $\alpha$  (lane 4). No competition was observed with 1 nM human rIL-2 (lane 5), human rIFN- $\gamma$  (lane 6), or human tumor necrosis factor  $\alpha$  (lane 7).

In Fig. 8, the bioassay results plotted in Fig. 1 have been fitted (see Materials and Methods) and compared with theoretical binding curves generated by the mass-action equation using the mean values for  $K_i$  and R determined in the competitive binding experiments (Table II). This analysis shows that the binding and biological response curves are parallel for both IL-1 species and that ~10–15% of the binding sites must be occupied by IL-1 $\beta$  or IL-1 $\alpha$  for either to induce a half-maximal biological response.

### Discussion

The current work identifies a high-affinity receptor for native human IL-1 $\beta$  and IL-1 $\alpha$  on normal human embryonic lung fibroblasts. While others (21–23) have employed Bolton-Hunter reagent to prepare  $^{125}\text{I}$ -IL-1 $\beta$ , the preparation of radioligand in the current study differs from previous reports in the following important respects. First, advantage was taken of the additional hydrophobicity resulting from the covalent modification of IL-1 $\beta$  by Bolton-Hunter reagent so that labeled IL-1 molecules could be separated from unlabeled molecules by HPLC hydrophobic interaction chromatography. This approach provided material of higher specific radioactivity and permitted unambiguous assessment of the biological activity of the radioligand preparation. The retention of bioactivity obtained with our procedure compares favorably with previous results using the Bolton-Hunter technique and gel filtration chromatography alone where <5% of bioactivity was retained (21). Second, the radioligand was purified in the absence of carrier protein (21, 23) so that the specific radioactivity (Fig. 2) of

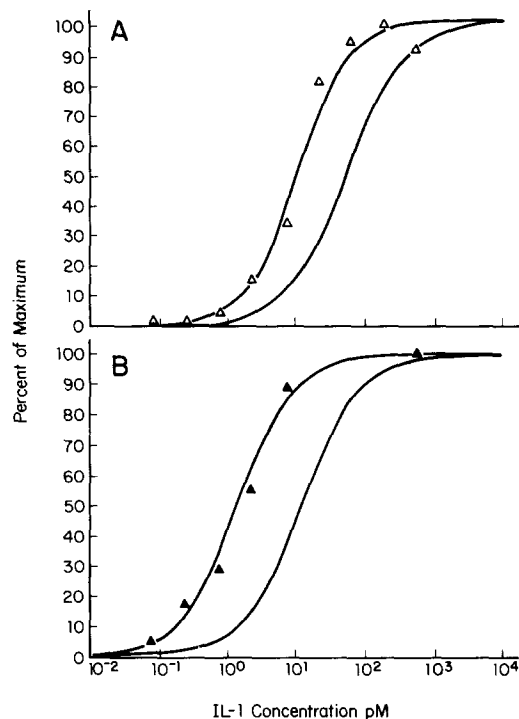


FIGURE 8. Comparison of the biological response and binding curves for native human IL-1 $\alpha$  (A) and IL-1 $\beta$  (B). The bioassay data plotted in Fig. 1 were fitted by Parafit II software as described in Materials and Methods. The positions of the data points used by the curve-fitting routine are indicated by *open* (IL-1 $\alpha$ ) and *filled* (IL-1 $\beta$ ) triangles, respectively. Three of the data points on the plateau of the IL-1 $\beta$  dose-response curve were omitted from the analysis. The *solid curves* to the right in each panel are computer-generated theoretical binding curves for IL-1 $\alpha$  and IL-1 $\beta$  using the mass-action equation and the mean binding parameters ( $K_i$ ,  $R$ ) obtained from the competitive binding experiments tabulated in Table II.

the purified radioligand could be determined directly. Third, the issue of bindability (Fig. 3) was addressed and appropriate corrections in the calculations have been made. Attempts to obtain higher bindability by labeling IL-1 $\beta$  with various phosphokinases, such as has been recently reported for human IFN- $\gamma$  (32), were unsuccessful (Siekierka, J., and J. Schmidt, unpublished observations).

After establishing a set of steady-state conditions (Fig. 4) under which there was little or no evidence of internalization (Table I), the equilibrium binding constant of the receptor was determined in two complementary ways. First, direct binding experiments (Fig. 5, Table II) were performed which, when corrected for bindability, gave a mean  $K_d$  of 8.4 pM for IL-1 $\beta$ . Second, competitive binding experiments (Fig. 6, Table II) were performed which gave a mean  $K_i$  of 11 pM for IL-1 $\beta$  and 50 pM for IL-1 $\alpha$ . Equilibrium binding measurements based on competitive binding experiments are influenced to a much lesser extent by the bindability of the radioligand (30). That the  $K_i$  obtained for IL-1 $\beta$  in the competition experiments is in good agreement with the  $K_d$  obtained in the direct binding experiments therefore provides confirmatory evidence that the estimate of bindability is accurate and that the bindable radioligand interacts with the

receptor in a native or a near-native fashion. The binding curves obtained in both the direct and competitive binding experiments were best fit by models specifying a single family of receptors with the same affinity for IL-1 $\beta$  or IL-1 $\alpha$ . Thus, unlike other receptor systems (e.g., the IL-2-R system; see reference 33), there is no evidence for high- and low-affinity IL-1-R on human lung fibroblasts.

The equilibrium binding measurements are important because they identify a receptor of sufficiently high affinity for native human IL-1 $\beta$  and IL-1 $\alpha$  to mediate the biological effects of these mediators on connective tissue cells. Only 3 pM IL-1 $\beta$  or 10 pM IL-1 $\alpha$  was required to half-maximally activate MRC-5 human embryonic lung fibroblasts, as determined by prostaglandin E<sub>2</sub> secretion (Fig. 1). In comparison, the equilibrium dissociation constants for IL-1 $\beta$  and IL-1 $\alpha$ , as determined in direct and competitive binding experiments, were found to be 8.4–11 and 50 pM, respectively (Figs. 5 and 6; Table II). Two conclusions can be drawn from a comparison of these data (Fig. 8). First, as found in other receptor systems (34), the occupation of a portion of the receptors by either IL-1 $\beta$  or IL-1 $\alpha$  results in a proportionately larger biological response. For example, occupation of 11% of the available sites by IL-1 $\beta$  or 15% of the sites by IL-1 $\alpha$  gave half-maximal increases in prostaglandin E<sub>2</sub> secretion. The three- to five-fold amplification observed on normal human fibroblasts is nevertheless in marked contrast to the extraordinary level of signal amplification (100–100,000-fold; references 21–23, 35) that has been proposed for a variety of lymphoid lines (see below). Second, since the three- to four-fold difference in the specific bioactivities of IL-1 $\beta$  and IL-1 $\alpha$  on MRC-5 fibroblasts (Fig. 1) is similar, within experimental error, to the fivefold difference in the binding affinity for IL-1 $\beta$  and IL-1 $\alpha$ , the data are consistent with a single IL-1-R mediating the biological properties of both IL-1 species. The failure to obtain a better fit of the data with a two-site model would also tend to rule out the existence of a second site with, e.g., higher affinity for IL-1 $\alpha$  and lower affinity for IL-1 $\beta$ . However, crossreactivity by itself is not sufficient evidence to rule out the existence of a second IL-1-R site. For example, insulin-like growth factors I and II, which have similar biological properties and specific bioactivities, have biochemically distinct but crossreactive receptors (36). Quantitative direct and competitive binding studies as well as crosslinking studies with labeled IL-1 $\alpha$  will be required before the possibility of a second binding site can be definitively eliminated.

The crosslinking studies (Fig. 7) suggest that the molecular mass of the IL-1 receptor is ~80 kD, in agreement with the results of a previous study (21) performed on murine cells. As in the competitive binding experiments performed on whole cells, labeling of the 80-kD species was blocked by both IL-1 $\beta$  and IL-1 $\alpha$ , whereas IL-2, IFN- $\gamma$ , and TNF failed to block crosslinkage at a concentration of 1 nM. There was no evidence of subunits bridged by disulfide bonds in that the molecular mass of the receptor was not affected by the addition of reducing agents. A high molecular mass band at ~200 kD was also consistently observed. Like the 80-kD species, this receptor species bound both IL-1 $\beta$  and IL-1 $\alpha$  specifically and was not affected by the addition of reducing agent. This band can represent a second receptor species or, more likely, a receptor dimer formed upon the addition of crosslinking reagent. The formation of dimers in the case of a low-abundance receptor would suggest that the receptor is laterally mobile

in the plain of the membrane and that local aggregation of receptors may occur after ligand binding.

The value obtained in the current study for the equilibrium dissociation constant of IL-1 $\beta$  differs considerably from those reported previously. A number of workers (21–23, 35) working with murine (21, 23, 35) or human (22) lymphoid lines have identified receptors for murine IL-1 (35) and human IL-1 $\alpha/\beta$  with 20–50-fold lower affinity ( $K_d$ ,  $2\text{--}5 \times 10^{-10}$  M) than the receptors found for IL-1 $\beta$  on lung fibroblasts. These target cells, like MRC-5 fibroblasts, are nevertheless reported to require only low picomolar (22, 35) or, in one case (21, 23), subpicomolar ( $10^{-14}$  M) concentrations of IL-1 for half-maximal activation. Accordingly, given the low numbers of receptors identified on these cells (100–250, references 21–23; 1,200, reference 35), few (35) or no (21–23) receptor molecules would be occupied at the low concentrations of IL-1 at which half-maximal biological activation is observed. Similarly, it is difficult to explain how cells bearing receptors of homogeneous affinity respond to IL-1 in dose-responsive fashion when the dynamic range of receptor occupancy between minimum and maximum biological response amounts to the occupation of a few receptors. We therefore propose that the previously reported receptors be considered as having low affinity and suggest that lymphoid cells that respond to low picomolar or subpicomolar concentrations of IL-1 are likely to bear as-yet-unidentified higher affinity receptors, such as those found on fibroblasts, that mediate biological activity.

Recent quantitative molecular studies have strengthened the concept that monocytes/macrophages found in chronic inflammatory lesions might profoundly influence connective tissue metabolism by producing IL-1. Three key facts have emerged from these studies. First, monocytes are highly specialized to make IL-1 $\beta$  in that 1% of the mRNA found in activated cells encodes this molecule (17, 37). Second, only low picomolar concentrations of IL-1 are required to induce eicosanoid production, protease production, and proliferation by connective tissue cells (20). Third, as shown in the current study, connective tissue cells bear high-affinity receptors that enable them to respond to extremely low concentrations of IL-1 in their environment. These data, taken together with numerous biological studies documenting the effects of IL-1 on connective tissues (4–13), suggest that the connective tissue destruction and remodeling that is observed in chronically inflamed tissue may be IL-1 mediated and that blockade of the IL-1-R on connective tissue cells may be useful in the management of chronic inflammatory diseases.

### Summary

Native human IL-1 $\beta$  and IL-1 $\alpha$  stimulated prostaglandin E<sub>2</sub> secretion by human embryonic lung fibroblasts at half-maximal concentrations of  $3 \pm 1.2$  pM ( $\pm$  SEM) and  $10 \pm 2.3$  pM, respectively. In contrast to the 20–50-fold lower affinities previously found for IL-1-R on 3T3 cells as well as murine and human lymphoblastoid lines (ref. 21–23, 35), monoiodo <sup>125</sup>I-IL-1 $\beta$  bound to normal human fibroblasts with a  $K_d$  of  $8.4 \pm 4.1$  pM in direct binding experiments, and with a  $K_i$  of  $11.2 \pm 2.8$  pM in competitive binding experiments. IL-1 $\alpha$  bound to the receptor identified by <sup>125</sup>I-IL-1 $\beta$  with a  $K_i$  of  $50 \pm 18$  pM. The receptor

exhibited homogeneous affinity for IL-1 $\beta$  or IL-1 $\alpha$ . The receptor did not recognize IL-2, IFN- $\gamma$ , tumor necrosis factor  $\alpha$ , a functionally related monokine, or bovine acidic fibroblast growth factor, a structurally related mediator. Comparison of the biological response curves and binding curves obtained for IL-1 $\alpha$  and IL-1 $\beta$  showed that they were parallel and that 10–15% occupancy of the estimated 3,000 sites by either species of IL-1 was sufficient to give half-maximal stimulation of prostaglandin E<sub>2</sub> secretion. Thus, the amount of apparent signal amplification observed on fibroblasts was considerably lower than the 100–100,000 fold amplification previously reported for lymphoid lines. Crosslinking experiments revealed a major band with a corrected molecular mass of ~80 kD and a minor band of ~200 kD. Labeling of these bands was blocked by IL-1 $\beta$  and IL-1 $\alpha$  but not by IL-2, IFN- $\gamma$ , or tumor necrosis factor  $\alpha$ . These results demonstrate that normal human embryonic lung fibroblasts bear IL-1-R of sufficiently high affinity to mediate their biological responsiveness to low picomolar concentrations of IL-1 $\beta$  and IL-1 $\alpha$  and are consistent with the existence of a single receptor mediating the biological properties of both human IL-1 species.

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