Inhibition of Interleukin 1 (IL-1) Binding and Bioactivity In Vitro and Modulation of Acute Inflammation In Vivo by IL-1 Receptor Antagonist and Anti-IL-1 Receptor Monoclonal Antibody

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Summary

Recombinant human interleukin 1 receptor antagonist (IL-1ra) and 35F5, a neutralizing monoclonal antibody (mAb) to the type I mouse IL-1 receptor, were examined for their ability to bind to IL-1 receptors (IL-1Rs) on various types of mouse cells and to block immune and inflammatory responses to IL-1 in vitro and in mice. IL-1ra competed for binding of ^{125}I-IL-1α to type I IL-1R present on EL-4 thymoma cells, 3T3 fibroblasts, hepatocytes, and Chinese hamster ovary cells expressing recombinant mouse type I IL-1R. The IC_{50} values for IL-1ra binding (ranging from 2 to 4 ng/ml) were similar to those of IL-1α. In contrast, IL-1ra bound with very low affinity (IC_{50} values ranging from 10 to 200 μg/ml) to cells expressing type II IL-1R, i.e., 70Z/3 pre-B cell line and polymorphonuclear leukocytes (PMN) derived from bone marrow and acute inflammatory exudates. The mAb 35F5 bound specifically to type I IL-1R; no inhibition of ^{125}I-IL-1α binding to cells having type II IL-1R was observed with very high concentrations of antibody. While neither IL-1ra nor 35F5 had intrinsic activity in bioassays using T helper D10.G4.1 cells and mouse thymocytes, both agents blocked the ability of IL-1 to stimulate proliferation of these cells. The effects of IL-1ra and 35F5 on acute inflammatory responses in mice were also evaluated. IL-1ra and 35F5 blocked the local accumulation of PMN after intraperitoneal injection of rIL-1α. The response to IL-1 was inhibited when IL-1ra or 35F5 was administered simultaneously with or before administration of IL-1. IL-1ra and 35F5 also blocked PMN accumulation after intraperitoneal injection of lipopolysaccharide or proteose peptone, suggesting IL-1 is important in mediating responses to these agents. In addition, IL-1ra and 35F5 significantly blocked the ability of IL-1 to stimulate egress of PMN from bone marrow, to induce a transient neutrophilia, and to elevate serum levels of hepatic acute phase proteins, IL-6, and corticosterone. Thus, IL-1ra and 35F5 competitively inhibit the binding of IL-1 to the IL-1R on certain cell types. These two IL-1 receptor antagonists act to inhibit biological responses induced by IL-1 and other inflammatory agents.

The IL-1 proteins, IL-1α and IL-1β, are implicated in numerous physiological and pathological host responses to trauma, stress, and infection (1). Many of the actions of IL-1 are pro-inflammatory and include the ability of IL-1 to stimulate prostaglandin E_2 (PGE_2)[1] synthesis (2, 3), collagenase release (2), bone resorption (4), and PMN accumulation (5–7), and to induce fever (8) and stimulate the acute phase response (9). Thus, these responses create the potential for significant IL-1-mediated pathology in a number of disease states.

Recently, a human monocyte-derived IL-1 inhibitor, designated IL-1 receptor antagonist (IL-1ra), has been identified. The cDNA encoding IL-1ra has been cloned and rIL-1ra protein has been produced in *Escherichia coli* (10–12). The rIL-1ra binds to the IL-1R on EL-4 thymoma cells (10) and blocks several IL-1-stimulated responses in vitro, including: (*a*) PGE_2 release by fibroblasts (10), synovial cells, and chondrocytes (13); (*b*) thymocyte proliferation (12, 13); (*c*) collagenase

[1] *Abbreviations used in this paper:* APP, acute phase protein; CHO, Chinese hamster ovary; C3, complement component 3; IL-1ra, interleukin 1 receptor antagonist; PEC, peritoneal exudate cell; PGE_2, prostaglandin E_2; SAP, serum amyloid P.

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production by synovial cells (13); and (*d*) leukocyte adherence to endothelial cells (12, 14).

A rat mAb, 35F5, to the IL-1R on mouse EL-4 thymoma cells has recently been described (15). Antibody 35F5 was shown in initial studies to block the binding of ^{125}I-IL-1 to IL-1R on mouse T cells and fibroblasts (15) and to diminish host responses to IL-1 and other inflammatory stimuli in vivo (16–18).

Recent studies have shown that at least two types of IL-1R are differentially expressed on the surface of certain types of murine cells and cell lines (15, 19). The type I (T cell, fibroblast) receptor, but not to the type II (B cell, macrophage) receptor, appears to selectively bind IL-1ra (10). Similarly, 35F5 binds solely to the type I IL-1R but not the type II IL-1R (15). The binding of IL-1ra or 35F5 competitively antagonizes the binding of radiolabeled IL-1 (10, 15). In the studies reported here, we have extended the observations of earlier reports on the in vitro activity of IL-1ra and 35F5. In addition, to further our understanding of the biology and pathophysiology of IL-1, the effect of these IL-1 inhibitors on the induction of various acute inflammatory reactions in mice was examined.

Materials and Methods

Mice. C57BL/6 and C3H/HeJ female mice, 6–8 wk of age, were obtained from The Jackson Laboratory, Bar Harbor, ME. Mice were maintained in sterilized cages with filter tops and autoclaved food, water, and bedding. Mice were routinely acclimated for at least 1 wk before use.

Reagents. Human rIL-1ra was obtained from Drs. R. Thompson and R. Hageman, Synergen, Inc., Boulder, CO. Human rIL-1α and rIL-1β, with specific activities of $\sim 3 \times 10^8$ U/mg, as determined in the D10.G4.1 cell proliferation assay, were provided by Dr. S. Roy, Hoffmann-LaRoche Inc. Diluent vehicle for in vivo studies was sterile Dulbecco's Ca^{2+}- and Mg^{2+}-free PBS (Gibco Laboratories, Grand Island, NY). The IL-1 used in these experiments was shown by Limulus amebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA) to contain <0.2 ng endotoxin/mg IL-1. The vehicle contained <0.2 ng endotoxin per 0.1 ml. Proteose peptone (Difco Laboratories, Detroit, MI) was dissolved (3% [wt/vol]) in endotoxin-screened water (Gibco Laboratories), passed through a 0.45-μM filter, and stored at 4°C until use. LPS (*E. coli*, strain 026:B6) and a purified normal rat IgG preparation were obtained from Sigma Chemical Co., St. Louis, MO. Purified rat 35F5 mAb (15) was obtained from Drs. R. Chizzonite and A. Stern, Hoffmann-LaRoche Inc.

Peritoneal Exudate Cells and Bone Marrow Cells. Peritoneal exudate cells (PEC) were collected after cervical dislocation of mice by injecting 4 ml serum-free RPMI 1640 (Gibco Laboratories) into the peritoneal cavity. After vigorous massage of the abdomen, an aliquot of the medium (≥3 ml) was withdrawn and the cell concentration was determined on a cell counter (Coulter Electronics, Hialeah, FL). Cytocentrifuge (Shandon, Inc., Pittsburgh, PA) smears of PEC were stained with Wright's stain for differential counts. Total of numbers of PMN accumulating in the peritoneal cavity were calculated by multiplying total PEC by the percent PMN (determined from differential counts).

To determine the effects of IL-1 administration on bone marrow PMN numbers, bone marrow was harvested from both femurs by flushing with HBSS. A single cell suspension was prepared by gently passing the bone marrow through 18- and then 21-gauge needles. Total nucleated bone marrow cells were determined on a cell counter (Coulter Electronics). Differential counts of bone marrow cells were performed on Wright's-stained cytocentrifuge smears. The percentage of mature PMN (no bands, metamyelocytes, or myelocytes) was used for calculations since preliminary studies showed that injection of IL-1 (at the doses used) resulted in an increase of only mature PMN in the peripheral blood (data not shown). Bone marrow cells for binding assays were prepared as described below.

Binding Assays. Human rIL-1α was radiolabeled with Na^{125}I using Enzymobeads reagent (Bio-Rad Laboratories, Richmond, CA) as described (20). Murine EL-4 thymoma cells (T19-181; American Type Culture Collection, Rockville, MD) were cultured and membrane fractions prepared as described (20). Binding experiments were conducted with EL-4 membranes as described previously (20). Briefly, the IL-1ra, 35F5, or IL-1 proteins (concentrations ranging from 0.01 ng/ml to 6.7 mg/ml as indicated) were pre-incubated with EL-4 membranes for 30 min at 37°C. Radiolabeled IL-1α (30,000 cpm/ml; 20 pM final concentration) was added and the incubation continued for 1 h at 37°C. The assay was terminated by filtration and bound ^{125}I-IL-1α was determined by use of a gamma counter. Murine 3T3 fibroblast cell line (American Type Culture Collection) was cultured, and binding experiments were performed with monolayer cultures of cells as described (21). Briefly, confluent, adherent 3T3 cells were incubated with the unlabeled competitor and ^{125}I-IL-1α (20,000 cpm/ml, final concentration) for 3 h at 4°C. The cells were washed, detached with SDS-containing buffer, and bound ^{125}I-IL-1α was determined.

Chinese hamster ovary (CHO) DHFR$^-$ cells, transfected with a cDNA (provided by Dr. U. Gubler, Hoffmann-LaRoche Inc.) encoding the mouse type I IL-1R and amplified in the presence of methotrexate, express ~6,000 mouse IL-1R/cell (22). Confluent cultures of transfected CHO cells in 24-well tissue culture plates (Costar, Cambridge, MA) were incubated with competing ligand in the presence of ^{125}I-IL-1α for 3 h at 4°C. Bound ^{125}I-IL-1α was determined essentially as described for the 3T3 cell line above.

Membranes were prepared from livers obtained from normal C57BL/6 mice. Individual livers were excised, minced with scissors, and homogenized in 25 ml buffer A (10 mM Tris-HCl [pH 7.2], 1 mM EDTA, 1 μg/ml PMSF) in a Polytron homogenizer (Brinkman, Westbury, NY). The homogenate was passed through two layers of cheesecloth and twice clarified by centrifugation at 450 *g* for 10 min. Crude membranes were collected from the clarified supernatant by centrifugation at 39,000 *g* for 30 min. The membrane pellets were resuspended in buffer A and homogenized in a glass-glass Dounce homogenizer. Aliquots of membranes were stored at −80°C. Binding assays with liver membranes were performed as described above for EL-4 cell membranes.

The 70Z/3 pre-B lymphocyte cell line (American Type Culture Collection) was maintained as described (15). Elicited peritoneal exudate cells were collected by peritoneal lavage from mice injected with proteose peptone (7). Femoral bone marrow cells were obtained from mice treated 5 d earlier with 150 mg/kg cyclophosphamide followed by four daily subcutaneous injections of IL-1 (10 μg/kg). Such bone marrow cells were >50% late-stage granulocytes (metamyelocytes, bands, mature neutrophils) by morphological criteria (23). Binding assays with 70Z/3, peritoneal exudate cells, and bone marrow cells were performed in 12 × 75-mm polypropylene tubes as described (7, 15). Cells were incubated with IL-1ra, 35F5, or IL-1 proteins (concentrations ranging from 0.01 ng/ml to 6.7 mg/ml) in the presence of ^{125}I-IL-1α for 3 h at 37°C, and bound ^{125}I-IL-1α was determined by centrifugation over oil.

Hematology. Peripheral white blood cells (WBC) were enumer-

ated on a cell counter (Coulter Electronics). Differential WBC counts were performed on Wright's-stained smears. Absolute PMN numbers were calculated by multiplying the total WBC by the percent PMN obtained from differential counts.

Acute Phase Protein Determinations. Concentrations for serum amyloid P and complement component 3 (C3) were determined by the "rocket" immunoelectrophoresis method of Laurell (24). Specific antisera and antigen standards were obtained from Calbiochem-Behring Corp., La Jolla, CA, and Organon Teknika Corp., West Chester, PA.

Serum Corticosterone Assay. Mice were quickly (5 s) anesthetized with CO_2 and bled from the retro-orbital plexus. Blood obtained during the first 15 s was allowed to clot at room temperature and serum was assayed for corticosterone with a commercial RIA kit (ICN Biomedicals, Inc., Costa Mesa, CA).

IL-6 Bioassay. Serum levels of IL-6 were determined using a modification of the B9 hybridoma cell assay described previously (25). Cells were treated with twofold serial dilutions of the test sera in 96-well microtiter plates. After a 3-d incubation at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, the wells were pulsed with 0.5 μCi of [^{3}H]TdR and incubated for an additional 18 h. The cells were then harvested onto glass fiber filters and the level of [^{3}H]TdR incorporation was determined using a liquid scintillation counter. IL-6 units are defined as the inverse of the serum dilution that produces half-maximal [^{3}H]TdR incorporation compared with a reference standard.

D10.G4.1 Assay. Mouse T helper D10.G4.1 cells (American Type Culture Collection) were originally described by Kaye et al. (26). Cultures (2×10^4 D10.G4.1 cells suspended in RPMI 1540 containing 5% FCS, 5×10^{-5} M 2-ME, 8 μg/ml gentamicin, 2 mM L-glutamine, and 2.5 μg/ml Con A) were established in 96-well flat-bottomed tissue culture plates. IL-1ra or 35F5 was added to triplicate cultures 1 h before the addition of IL-1α or IL-1β. The plates were incubated, and incorporation of [^{3}H]TdR was determined as described above for the IL-6 assay.

Thymocyte Proliferation Assay. C3H/HeJ thymocytes (1.5×10^6) in RPMI 1640 containing 5% FCS, 2.5×10^{-5} M 2-ME, 8 μg/ml gentamicin, and 6 μg/ml PHA were added to 96-well microtiter plates. IL-1ra or 35F5 was added to triplicate cultures 1 h before the addition of IL-1α or IL-1β. The plates were incubated, and incorporation of [^{3}H]TdR was determined as described for the IL-6 assay.

Statistical Analysis. Data are presented as the mean ± SD of three to five mice per group. The differences between treatment groups were analyzed by Student's t test (27) and were considered statistically significant at $p < 0.05$.

Results

In Vitro Studies with IL-1ra

Inhibition of Receptor-Ligand Interactions. IL-1ra was examined for its ability to inhibit binding of ^{125}I-IL-1α to a number of cells and cell lines (Table 1). The IC_{50} values for IL-1ra binding (i.e., concentration of competitor inhibiting binding of ^{125}I-IL-1α by 50%) ranged from 2 to 4 ng/ml in experiments performed with mouse EL-4 thymoma, 3T3 fibroblast, and CHO cells expressing recombinant mouse type I IL-1R. These IC_{50} values for IL-1ra were similar to those obtained for IL-1α and IL-1β with these same cell types.

The binding of these three ligands to additional cell types was examined further. Both IL-1α and IL-1β bound with high affinity to IL-1R present on the mouse 70Z/3 cell and to protease peptone-elicited mouse peritoneal neutrophils and to granulocytes and granulocyte progenitors obtained from mouse bone marrow. In contrast, IL-1ra bound with very low affinity to these cell types (IC_{50} values ranging from 10 to 200 μg/ml). Inhibition of binding by the IL-1 proteins and IL-1ra was specific since concentrations of IL-2 up to 1 mg/ml were found to have no effect on the binding of ^{125}I-IL-1α to any of the cell types (data not shown).

Table 1. *Inhibition of Binding of ^{125}I-IL-1α to IL-1R on Cells and Cell Lines by IL-1 Proteins, IL-1ra, and 35F5 antibody*

	Inhibition of binding of ^{125}I-IL-1α			
Cell or cell line (type)	IL-1α	IL-1β	IL-1ra	35F5
	IC_{50}, ng/ml			
EL-4 (thymoma)*	2.0	10	2.0	20
3T3 (fibroblast)‡	1.0	2.0	4.0	ND
CHO cells w/ rIL-1R‡	1.5	10	2.0	20
Hepatocytes*	2.0	ND	2.0	20
PMN from:				
Peritoneal cavity‡	10	ND	1×10^5	$>6.7 \times 10^6$
Bone marrow‡	3.0	ND	2×10^5	$>6.7 \times 10^6$
70Z/3 (pre-B cell)‡	14	ND	1×10^4	$>6.7 \times 10^6$

Competitive inhibition binding experiments were performed with ^{125}I-IL-1α and indicated cells and cell lines as described in Materials and Methods. IC_{50} values were determined from graphed binding data of complete competitive inhibition dose-response curves for each indicated competitor.

* Assays performed with membrane preparations.

‡ Assays performed with intact cells.

The anti-IL-1R mAb 35F5 was examined for its ability to block the binding of ^{125}I-IL-1α to IL-1R (Table 1). Antibody 35F5 inhibited the binding of IL-1 to the type I IL-1R on T cells, fibroblasts, hepatocytes, and CHO cells expressing type I mouse IL-1R with an affinity comparable with that of IL-1ra, taking into consideration the ~10-fold difference in molecular weight of the two inhibitors. In contrast to IL-1ra, however, antibody 35F5 is highly specific for type I IL-1R, as concentrations of 35F5 up to 6.7 mg/ml failed to inhibit the binding of IL-1 to IL-1R on 70Z/3 cells and inflammatory and bone marrow–derived PMN (Table 1). This finding was corroborated by studies in which no binding of radiolabeled ^{125}I-35F5 to IL-1R on these cells was detected (data not shown).

Effect of IL-1ra and 35F5 on IL-1-stimulated T Cell Responses. IL-1ra inhibited, in a dose-dependent manner, the co-mitogenic effect of IL-1 on D10.G4.1 cells and mouse thymocytes (Table 2). A 1,000-fold molar excess of IL-1ra over IL-1 was needed to completely block the response to IL-1α in bioassays. However, the inhibition by IL-1ra was specific since higher concentrations of IL-1 could overcome the in-

Table 2. *Inhibition of IL-1-stimulated D10.G4.1 and Thymocyte Proliferation by IL-1ra and 35F5*

Exp.	Additions to culture		Cell proliferation ([^{3}H]TdR incorporation)	
	IL-1α	IL-1ra	D10.G4.1	Thymocytes
	ng/ml		*cpm × 10^{-3}*	
1	0	0	3	87
	0.12	0	49	220
	0.12	1.2	39	143
	0.12	12	20	100
	0.12	120	2	60
	12	0	52	225
	12	120	51	173
	IL-1α	35F5	D10.G4.1	Thymocytes
	ng/ml		*cpm × 10^{-3}*	
2	0	0	7	19
	0.12	0	60	38
	0.12	10	46	12
	0.12	100	22	7
	0.12	1,000	5	12
	12	0	61	60
	12	1,000	63	24

D10.G4.1 cells or C3H/HeJ thymocytes were preincubated with the indicated concentrations of IL-1ra or 35F5 for 60 min at 37°C before addition of IL-1 as described in Materials and Methods.

hibitory effect of IL-1ra. The inhibitory effect of IL-1ra on IL-1β-induced proliferation was similar to that seen with IL-1α (data not shown).

mAb 35F5 was also tested in these in vitro assays. Antibody 35F5 demonstrated a dose-dependent inhibition of proliferation of both D10.G4.1 cells and thymocytes at low concentrations of IL-1 (Table 2). These effects of 35F5 were reversed at higher concentrations of IL-1.

In Vivo Studies with IL-1ra and 35F5

Inhibition of IL-1-stimulated Induction of IL-6 by IL-1ra. IL-1ra inhibited the induction of IL-6 by IL-1α in a dose-dependent manner when IL-1ra was injected simultaneously with IL-1 (Table 3). The inhibition was maximal when >100-fold excess of IL-1ra over IL-1 was administered. Furthermore, inhibition was observed when 10 mg/kg of IL-1ra was administered simultaneously with or 1–2 h before 10 μg/kg of IL-1; no inhibition was seen when IL-1ra was given 4 h before IL-1 (Fig. 1). Administration of 35F5 to mice also blocked induction of IL-6 by IL-1 (data not shown), confirming previous studies (18).

Inhibition of Acute Phase Protein Synthesis by IL-1ra and 35F5. The effect of IL-1ra on IL-1-stimulated increases in

Table 3. *IL-1ra Inhibits IL-1-induced Elevation of IL-6 Levels in Serum in a Dose-dependent manner*

Treatment		
IL-1α	IL-1ra	Serum IL-6
μg/kg		*U/ml*
0	0	<20
10	0	3,850 ± 1,050
10	1	3,350 ± 1,400
10	10	1,950 ± 850*
10	100	1,200 ± 550*
10	1,000	300 ± 150*

C57BL/6 mice (four/group) were injected subcutaneously with the indicated dose of IL-1ra together with 10 μg/kg IL-1. The mice were bled 3 h later and the levels of serum IL-6 were determined.
* $p < 0.05$ compared with IL-1 alone.

the hepatic acute phase proteins (APP), serum amyloid P (SAP) and complement component 3 (C3), was examined. A single injection of IL-1ra given simultaneously with or 15 min before IL-1 significantly attenuated the increases in SAP and C3 observed 24 h after IL-1 injection (Fig. 2). When IL-1ra was injected 15 min after IL-1, the IL-1-stimulated levels of SAP and C3 were not significantly reduced. Thus, IL-1ra maximally inhibited IL-1-stimulated APP synthesis when given simultaneously with IL-1.

Significant reductions in IL-1-stimulated SAP levels were also observed upon administration of 35F5 antibody to mice. Increases in serum SAP levels were blocked in a dose-dependent manner by 35F5 (Table 4, Exp. 1). The inhibition of the IL-1-stimulated increase in SAP was observed when 35F5 was administered before, but not after, IL-1 (Table 4, Exp. 2). Anti-

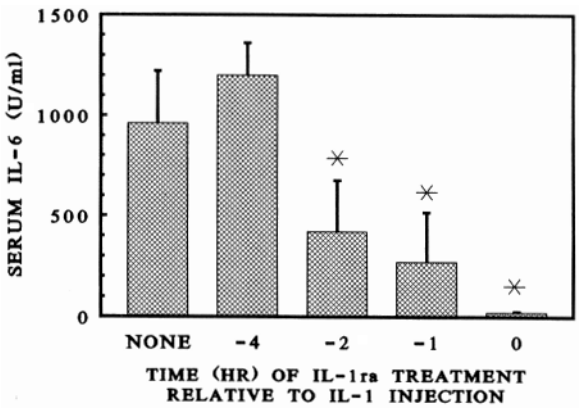

Figure 1. Effect of time of IL-1ra injection on ability to block induction of serum IL-6 by IL-α. C57BL/6 mice (four/group) were injected with IL-1ra (5 mg/kg, s.c.) at various times relative to injection of IL-1α (5 μg/kg, s.c.). Mice were bled 3 h after IL-1 injection and serum IL-6 levels were determined. Control (PBS-injected) mice had no detectable (<20 U/ml) IL-6 (data not shown). * $p < 0.05$ compared with mice receiving no IL-1ra.

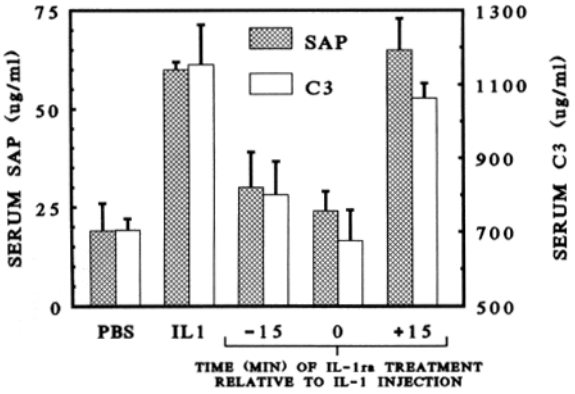

Figure 2. Inhibition of IL-1-stimulated acute phase protein synthesis by IL-1ra. C57BL/6 mice (three/group) were injected with PBS or IL-1 (5 μg/kg, s.c.). Additional groups of mice were injected with IL-1 (5 μg/kg, s.c.) and IL-1ra (10 mg/kg, i.v.) at various times relative to the injection of IL-1. Mice were bled 24 h later and concentrations of serum SAP and C3 were determined by immunoelectrophoresis as described in Materials and Methods. Reductions in SAP and C3 levels in mice receiving IL-1ra at −15 or 0 min were statistically significant ($p < 0.02$) compared with levels in mice receiving IL-1 alone.

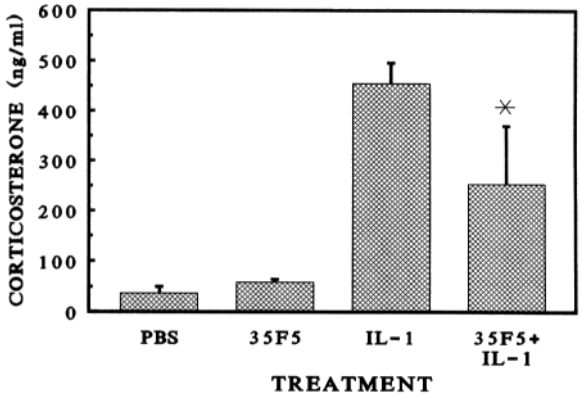

Figure 3. Inhibition of corticosterone release by 35F5. C57BL/6 mice (four/group) were injected intravenously with 35F5 (5 mg/kg) 30 min before intraperitoneal injection of IL-1 (5 μg/kg) or PBS, and serum samples were obtained 2 h later. Serum corticosterone levels were determined by RIA as described in Materials and Methods. * $p < 0.02$ compared with IL-1 alone.

body 35F5 has also been shown to modulate SAP levels in mice during inflammation induced by other agents (17).

Inhibition of IL-1-stimulated Adrenal Glucocorticoid Release by 35F5. Antibody 35F5 was tested for its ability to block increases in serum corticosterone induced by IL-1 (Fig. 3). Compared with PBS-injected control mice, mice injected with 5 μg/kg of IL-1 alone showed an increase in serum corticosterone of ~10-fold. This IL-1-stimulated increase was significantly ($p < 0.02$) blocked in mice treated with 5 mg/kg of 35F5. Injection of 35F5 alone did not alter serum corticosterone. Injection of mice with IL-1ra also blocked IL-1-stimulated increases in serum corticosterone (data not shown), in agreement with an earlier report (12).

Table 4. *Inhibition In Vivo of IL-1-stimulated Acute Phase Protein Synthesis by the anti-IL-1R mAb, 35F5*

Exp.	Treatment	SAP
		μg/ml
1	PBS	12.3 ± 3.5
	IL-1α	75.3 ± 14.2
	35F5 (0.1 mg/kg) + IL-1α	55.8 ± 4.3*
	35F5 (0.5 mg/kg) + IL-1α	49.8 ± 1.5*
	35F5 (2.5 mg/kg) + IL-1α	47.5 ± 6.1*
	35F5 (10.0 mg/kg) + IL-1α	38.3 ± 1.5*
2	PBS	6.5 ± 1.3
	IL-1α	59.3 ± 8.0
	35F5 (−4 h) + IL-1α	32.0 ± 2.7*
	35F5 (−1 h) + IL-1α	41.5 ± 6.9*
	35F5 (+1 h) + IL-1α	58.0 ± 8.8
	35F5 (+4 h) + IL-1α	51.3 ± 5.2

In exp. 1, C57BL/6 mice (four/group) were injected subcutaneously with various doses of 35F5 antibody 4 h before subcutaneous injection of IL-1α (5.0 μg/kg). Mice were bled 24 h after IL-1 injection for SAP determinations. In exp. 2, C57BL/6 mice (four/group) were injected subcutaneously with 35F5 (10 mg/kg) at the indicated times before or after subcutaneous injection of IL-1α (5.0 μg/kg). Mice were bled 24 h after IL-1 injection for SAP determinations.
* $p < 0.05$ compared with IL-1.

Suppression by IL-1ra and 35F5 of IL-1-induced Neutrophilia and Release of PMN from Bone Marrow. IL-1ra and 35F5 were tested for their ability to block IL-1-induced neutrophilia in mice. Treatment of mice with 10 mg/kg of IL-1ra eliminated both the relative and absolute increases in peripheral blood PMN in response to 5 μg/kg of IL-1 (Table 5, Exp. 1). Injection of IL-1ra alone caused no change in peripheral blood leukocyte numbers (data not shown). In addition to stimulating an increase in numbers of PMN in the blood, injection of IL-1 induced a reciprocal decline in numbers of PMN in the bone marrow. This depletion of bone marrow PMN was largely blocked by treatment with IL-1ra. Treatment of mice with 10 mg/kg of 35F5, but not a control preparation of purified rat IgG, blocked both relative and absolute neutrophilia induced by injection of IL-1 (Table 5, Exp. 2).

Inhibition of IL-1-induced PMN Accumulation by IL-1ra. To study the effect of IL-1ra on IL-1-induced PMN accumulation, mice were treated with IL-1ra at various times relative to the injection of 5.0 ng/kg of IL-1α. The data in Fig. 4 demonstrate that IL-1ra must be administered at the time of, or shortly before, IL-1 injection in order to inhibit PMN accumulation in response to IL-1. Thus, accumulation of PMN in response to IL-1 was significantly inhibited in mice treated with IL-1ra at the same time as or 10 min before IL-1 injection. The inhibitory effects of IL-1ra were markedly diminished when IL-1ra was administered 30 min before IL-1. Furthermore, IL-1ra was unable to block the PMN influx when given 10 min after IL-1. When tested over a range of doses (50 pg/kg to 5 mg/kg), injection of IL-1ra alone did not elicit

Table 5. *Inhibition of IL-1-induced Neutrophilia and Release of PMN from Bone Marrow by IL-1ra and 35F5*

Exp.	Treatment	PMN in blood		PMN in bone marrow	
		%	$\times 10^3/\mu l$	%	$\times 10^6$/Femur
1	Control	14.0 ± 3.0	1.1 ± 0.4	12.0 ± 1.7	4.0 ± 0.8
	IL-1α	35.7 ± 1.5*	2.1 ± 0.3*	4.2 ± 1.3*	1.2 ± 0.6*
	IL-1ra + IL-1α	12.3 ± 3.5‡	0.8 ± 0.2‡	8.0 ± 0.9‡	2.6 ± 0.5‡
2	Control	6.2 ± 1.8	0.5 ± 0.1	ND	ND
	IL-1α	27.2 ± 6.4*	1.8 ± 0.4*	ND	ND
	IgG + IL-1α	29.4 ± 4.7*	1.8 ± 0.2*	ND	ND
	35F5 + IL-1α	10.0 ± 3.4‡	0.8 ± 0.2‡	ND	ND

In Exp. 1, mice (three/group) were injected intraperitoneally with 0.1 ml PBS (control), 0.1 ml PBS containing either 0.5 μg/kg of IL-1α or 0.5 μg/kg of IL-1α, plus 5 mg/kg of IL-1ra. In Exp. 2, mice (five/group) were injected subcutaneously with 10 mg/kg of purified rat IgG or 35F5 antibody 18 h before intraperitoneal injection with 5 μg/kg of IL-1α. Mice were bled 4 h after IL-1 injection and absolute numbers and percentages of PMN in peripheral blood and in femoral bone marrow were determined.

* $p < 0.05$ compared with control.

‡ $p < 0.05$ compared with IL-1α alone.

PMN influx (data not shown). Other routes of administration for IL-1ra were also highly effective at blocking localized peritoneal responses to IL-1. Parenteral (subcutaneous or intravenous) administration of IL-1ra just before intraperitoneal injection of IL-1 also blocked accumulation of PMN (data not shown).

Inhibition of LPS- and Proteose Peptone–induced PMN Accumulation by IL-1ra and 35F5. The effects of IL-1ra and 35F5 on acute inflammatory responses to LPS and proteose peptone were studied. Mice were injected with IL-1ra or 35F5 and then injected with LPS (5 μg/kg) or sterile proteose peptone (1.0 ml, 3% solution). As shown in Fig. 5, both IL-1ra and 35F5 significantly inhibited PMN influx induced by both of these agents, suggesting that IL-1 mediates this PMN response to LPS and proteose peptone. In repeated experiments, IL-1ra and 35F5, even at high dose (50 mg/kg), only partially blocked (55–80%) the response to LPS and proteose peptone, whereas the PMN influx in response to IL-1 can often be virtually eliminated with IL-1ra (McIntyre et al., unpublished observations). These findings suggest that factors in addition to IL-1 may be involved in regulating PMN accumulation in vivo in response to LPS and proteose peptone.

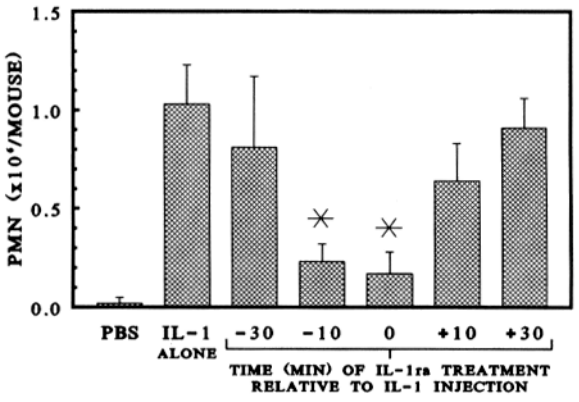

Figure 4. Time of IL-1ra injection affects ability to inhibit PMN influx. Mice (three/group) were injected intraperitoneally with either PBS (control) or IL-1 (5 ng/kg, all other groups). Individual groups were injected intraperitoneally with IL-1ra (5 mg/kg) at various times relative to IL-1 injection as shown. PEC were collected from all mice 4 h after IL-1 injection and total PMN determined. * $p < 0.005$ compared with IL-1 alone.

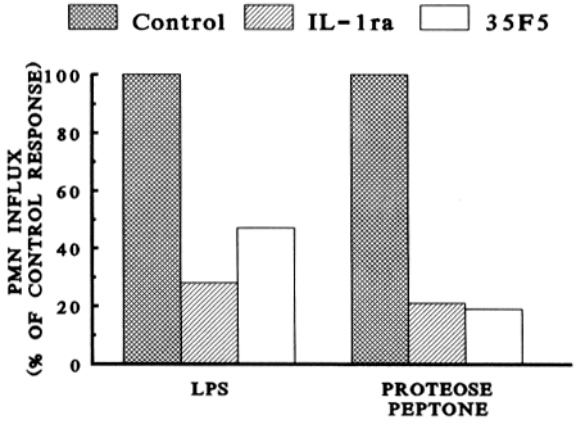

Figure 5. Inhibition by IL-1ra of peritoneal accumulation of PMN stimulated by LPS or proteose peptone. Mice (four/group) were injected with IL-1ra (5 mg/kg, i.v.; and 5 mg/kg, i.p.) or 35F5 (5 mg/kg, i.p.) at 10 min before intraperitoneal injection of LPS (5 μg/kg). Mice were injected with IL-1ra (20 mg/kg, i.p.) or 35F5 (5 mg/kg, i.p.) at 10 min before intraperitoneal injection of 1.0 ml proteose peptone (3% [wt/vol]). Total PMN in the peritoneal cavities were determined at 4 h. The data are expressed as a percent of the PMN response in the absence of inhibitor (LPS alone: 1.52 ± 0.49 × 10^6 PMN/mouse; proteose peptone alone: 1.84 ± 0.53 × 10^6 PMN/mouse). Values for mice treated with either IL-1ra or 35F5 were significantly lower than for mice receiving LPS or proteose peptone alone ($p < 0.05$).

Discussion

A previous study by Hannum et al. (10) showed that IL-1ra binds to IL-1R present on EL-4 thymoma cells with an affinity approximately equal to that for human IL-1β. Our experiments show that IL-1ra also inhibits binding of ^{125}I-IL-1α to membranes prepared from EL-4 cells with an IC_{50} of ~2.0 ng/ml. This value is comparable with those obtained for IL-1α and IL-1β (2.0 and 10.0 ng/ml, respectively). In addition, IL-1ra bound with high affinity to the IL-1R present on the 3T3 fibroblast cell line and to mouse rIL-1R expressed by CHO cells. The IL-1R present on EL-4 and 3T3 cells is identical to that which has been cloned and expressed in CHO cells and has been tentatively designated as the T cell/fibroblast, or type I, IL-1R (15, 19). A second type of IL-1R, designated the B cell/macrophage or type II IL-1R, is present on murine macrophage and B cell lines and on PMN and PMN progenitors (15, 19). Studies by Hannum et al. (10) and Carter et al. (12) suggest that IL-1ra binds to the type I IL-1R but not to the type II IL-1R. In this report, high concentrations of IL-1ra were evaluated in competitive inhibition experiments using 70Z/3 pre-B cells, proteose peptone–elicited peritoneal exudate cells, and bone marrow cells. Our results show that IL-1ra blocks binding of radiolabeled IL-1α to the type II mouse IL-1R at very high concentrations. An IC_{50} of 30 μg/ml was obtained for 70Z/3 cells. A concentration of 200 μg/ml of IL-1ra was found to inhibit binding of IL-1α to proteose peptone–elicited peritoneal exudate cells or bone marrow cells by 50%. Previous work has shown that PMN in the peritoneal exudate cell population (7) and granulocytes and granulocyte progenitors in bone marrow (K.P. Parker, K.W. McIntyre, and P.L. Kilian, unpublished observations) are the cells expressing IL-1R. We conclude from these binding experiments that IL-1ra binds with high affinity to the type I IL-1R and with a greatly reduced affinity to the type II IL-1R.

The receptor binding experiments with 35F5 confirm and extend previous observations (15) that this antibody specifically recognizes the type I IL-1R and shows no detectable inhibitory activity on type II IL-1R even at high concentrations (Table 1). This finding has important implications for the interpretation of the in vivo experiments with IL-1ra reported here. In the responses examined, a large molar excess of IL-1ra over IL-1 (10^2- to 10^5-fold) is often necessary to achieve significant inhibition. This raises the question of whether the IL-1ra at these high doses may bind to type II IL-1R in vivo, thereby inhibiting IL-1-induced responses through interaction with type II IL-1R. However, this possibility is unlikely inasmuch as the type I IL-1R-specific 35F5 antibody reproduces the inhibitory activities of IL-1ra in vivo (and in vitro). These findings confirm the proposition that suppression of these IL-1-stimulated responses is mediated through interactions with the type I IL-1R.

The studies on stimulation of thymocyte and D10.G4.1 cell proliferation by IL-1 suggest that very few IL-1Rs need to be occupied by IL-1 in order to elicit a biological response. Proliferation of D10.G4.1 cells is stimulated by IL-1 concentrations that are substantially lower than the IC_{50} obtained for IL-1 in receptor binding assays. Similar findings were reported for IL-1-stimulated production of IL-2 by EL-4 cells (28) and suggest that a large proportion of IL-1R on cells are present as "spare receptors". Based on these findings, it would seem likely that near saturation of IL-1R with IL-1ra would be required to achieve antagonist activity (13). This, in turn, probably explains why a high molar excess of IL-1ra or 35F5 over IL-1 was needed to block the effects of IL-1 in vivo.

APP synthesis by the liver during inflammation is regulated by the concerted action of multiple soluble mediators. The presence of IL-1, IL-6, and adrenal glucocorticoids is required for maximal stimulation of APP synthesis by hepatocytes in vitro (29). This condition is met in vivo where injection of IL-1 into mice stimulates increased levels of both IL-6 and glucocorticoids. However, IL-1ra (Fig. 1) and 35F5 (18) prevent the induction of IL-6, and both IL-1ra (12) and 35F5 (Fig. 3) block corticosterone release. In addition, these type I IL-1R antagonists block the binding of IL-1 to IL-1R on liver cells (Table 1). Thus, the combined action of the three signals important for the stimulation of hepatic APP synthesis is suppressed by IL-1ra and 35F5, resulting in inhibition of this IL-1-stimulated response (Fig. 2, Table 4).

The appearance of PMN at sites of inflammation is the result of a coordinated series of events. These steps may include release of PMN from bone marrow into the circulation, their adherence to endothelial cells, and then their subsequent migration into adjacent tissues. The release of PMN from bone marrow and the subsequent neutrophilia induced by IL-1 injection are suppressed by IL-1ra (Table 5). However, IL-1ra failed to reduce IL-1-stimulated neutrophilia in other studies (12) using a higher dose of IL-1 than that used here. That failure of IL-1ra to inhibit neutrophilia (possibly due to an insufficient molar excess of IL-1ra) led to the suggestion that the type II IL-1R was mediating this response (12). However, our current data with IL-1ra and 35F5 demonstrate that IL-1-stimulated neutrophilia is indeed blocked by type I IL-1R antagonists.

Adhesion of PMN to endothelial cells is a step crucial in PMN accumulation (30, 31). Adhesion is augmented by IL-1 through increased expression of adhesion glycoproteins on endothelial cells (31), and IL-1ra pretreatment of endothelial cells blocks this response (14). Accumulation of PMN is further stimulated by the localized generation of factors chemotactic for PMN. The PMN chemotactic factor IL-8 (33) is synthesized by fibroblasts (34, 35) and endothelial cells (36) upon exposure to IL-1. Since these cell types express type I IL-1R, IL-1ra may be able to block this response as well. Thus, in mediating its inhibition of PMN accumulation, IL-1ra may act at three steps in the inflammatory pathway by: (*a*) blocking PMN release from bone marrow and the resulting neutrophilia; (*b*) blocking endothelial cell/PMN adhesion; and (*c*) blocking the generation of specific PMN chemotactic factors.

Our results also demonstrate that IL-1ra and 35F5 block PMN accumulation in response to the nonspecific stimuli LPS and proteose peptone. This holds significance for the mechanism of action of various stimuli in mediating acute inflammatory cell infiltration. The ability of IL-1ra and 35F5 to inhibit PMN influx after injection of proteose peptone or LPS strongly suggests that IL-1 is an important participant in these

responses. However, IL-1 does not appear to be the sole responsible mediator since IL-1ra and 35F5 fail to completely block PMN accumulation. Other factors such as complement component fragments and leukotrienes are chemotactic for PMN, and these factors may be generated by IL-1-independent mechanisms after injection of proteose peptone or LPS.

In summary, our experiments show that human rIL-1ra binds with very high affinity to the type I IL-1R and with a greatly reduced affinity to type II IL-1R. In contrast, 35F5 anti-IL-1R mAb is highly specific for the type I IL-1R. IL-1ra and 35F5 antibody inhibit a wide variety of IL-1-stimulated physiological responses in vivo. Both IL-1ra and 35F5 also modulate responses to nonspecific inflammatory stimuli, implying a role for IL-1 in these models. Furthermore, these data demonstrate that the type I IL-1R represents the predominant signaling pathway for many host responses attributed to IL-1. The role of the type II IL-1R in normal or pathological conditions is unclear. Elucidation of its physiological function awaits the identification of antagonists (or agonists) specific for the type II IL-1R.

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