Production of Interleukin-1 Receptor Antagonist and Interleukin-1β by Peripheral Blood Mononuclear Cells Is Differentially Regulated

By Debra D. Poutsiaka, Burton D. Clark, Edouard Vannier, and Charles A. Dinarello

We studied the relationship between the production of the 23-Kd interleukin-1 receptor antagonist (IL-1ra) and IL-1β in cultures of human peripheral blood mononuclear cells (PBMC) using a specific radioimmunoassay for IL-1ra that had a sensitivity of 166 ± 11 pg/mL. PBMC cultured without human serum made little IL-1ra or IL-1β. In the presence of 1% AB serum, there was no increase in IL-1β (0.25 ± 0.13 ng/mL) but IL-1ra production increased sevenfold to 3.4 ± 0.5 ng/mL. IgG (2.5 to 100 µg/mL IgG) or granulocyte-macrophage colony-stimulating factor (GM-CSF) (1 to 100 ng/mL) had no significant effect on IL-1β production but increased IL-1ra production up to 18-fold (18.2 ± 3.9 ng/mL). Using endotoxin as a stimulant, 82% ± 2% of IL-1ra was secreted in comparison with 52% ± 9% of IL-1β. Culture conditions of PBMC influenced the production of IL-1ra but not IL-1β. Rocking endotoxin-stimulated PBMC produced 75% less IL-1ra but the same amount of IL-1β when compared with PBMC cultured in stationary plastic tubes. Rocking IgG- or GM-CSF-stimulated PBMC also produced 75% to 80% less IL-1ra. GM-CSF or IL-1β at concentrations that elicited submaximal production of IL-1ra potentiated IgG-induced IL-1ra production. The production of IL-1ra and IL-1β are under differential regulation because serum, IgG, and GM-CSF were potent stimuli for the production of IL-1ra but not IL-1β, and the prevention of cell-cell contact of PBMC reduced IL-1ra but not IL-1β production.

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Materials and Methods

Materials. Ficoll type 400 and endotoxin (Escherichia coli, serotype 055:B5) were obtained from the Sigma Chemical Company (St Louis, MO). Hypaque-M, 90%, was obtained from Winthrop Pharmaceuticals (New York, NY). Sterile pyrogen-free water and 0.9% saline were obtained from Abbott Laboratories (Rockford, IL). RPMI 1640 culture medium was obtained from Whittaker M.A. Bioproducts (Walkersville, MD). Ultrafiltration filters were obtained from Fresenius AG (Bad Homburg, Germany). Polypropylene tubes, 12 x 75 mm, were obtained from Becton Dickinson Laboratories (Lincoln Park, IL).13 In the di-iodinated form of the Bolton-Hunter reagent was obtained from Dupont-New England Nuclear (Boston, MA). Econo-Pac 10DG desalting columns and Tween-20 were obtained from Bio-Rad Laboratories (Richmond, CA). MaxiSorp 96 microtiter plates were obtained from Nunc-Immunoplate (Roskilde, Denmark). Polyacrylamide gels (10%) were obtained from Pierce (Rockford, IL). Monoclonal antihuman IL-1p and polyclonal rabbit antihuman IL-1a were the gift of Richard Dondero (Cistron, Pine Brook, NJ). Human recombinant IL-1β was a gift of Tohoku University School of Medicine, Sendai, Japan. Human recombinant GM-CSF was a gift of Dr Steven Clark (Genetics Institute, Cambridge, MA). Human recombinant IL-1ra3 was a gift of Dr Robert Thompson (Synergen, Boulder, CO). Sheep antirabbit IgG was obtained from Ventrex Laboratories (Portland, ME). Monoclonal antihuman IL-1β and polyclonal rabbit antihuman IL-1β were the gift of Richard Dondero (Cistron, Pine Brook, NJ). ImmunoPure alkaline phosphatase-conjugated goat antirabbit IgG and p-nitrophenol phosphate were obtained from Pierce (Rockford, IL).

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Address reprint requests to C.A. Dinarello, MD, New England Medical Center, Division of Geographic Medicine and Infectious Diseases, Department of Medicine, 750 Washington St, Boston, MA 02111.

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INTERLEUKIN-1 (IL-1) exhibits many different effects in vivo and in vitro. Specific inhibitors of the various biologic activities of IL-1 have been described since the early 1980s.2 The “IL-1 inhibitor” was initially described as a 23- to 25-Kd protein purified from the urine of patients with monocytic leukemia3,4 or from human monocytes cultured on immune complex-coated surfaces.5 Natural IL-1 inhibitor blocked the activity of IL-1 in several bioassays.6,7 The IL-1 inhibitor blocked the binding of IL-1 in a competitive fashion to receptors on T cells and fibroblasts.8 This IL-1 inhibitor was purified from adherent monocytes10 and subsequently was cloned.11 As opposed to IL-1β, the cloned protein lacked agonist activity in inducing prostaglandin E2 in human dermal fibroblasts.9 Therefore, it was renamed the interleukin-1 receptor antagonist (IL-1ra). An identical protein was cloned from U937 cells and reported as the IL-1 receptor antagonist protein (IRAP).12

The measurement of IL-1 inhibitors has been based on a reduction in the activity of IL-1 in various bioassays. This approach does not allow for specific and independent measurements of IL-1 and IL-1ra. Without independent assays, the relationship between IL-1 and IL-1ra in health and disease states cannot be fully explored. Recent studies used an enzyme-linked immunosorbant assay (ELISA) for IL-1ra to study the differential gene expression of IL-1ra and IL-1β.13

We describe here a specific and sensitive radioimmunoassay (RIA) for the measurement of IL-1ra. Using this assay and a specific ELISA for IL-1β, we have studied the regulation of the production of IL-1ra and IL-1β proteins from peripheral blood mononuclear cells (PBMC) cultured in the presence of endotoxin, granulocyte-macrophage colony-stimulating factor (GM-CSF), IgG, or IL-1. In addition, we examined the role of cellular contact in the production of IL-1ra and IL-1β.

Materials and Methods

Materials. Ficoll type 400 and endotoxin (Escherichia coli, serotype 055:B5) were obtained from the Sigma Chemical Company (St Louis, MO). Hypaque-M, 90%, was obtained from Winthrop Pharmaceuticals (New York, NY). Sterile pyrogen-free water and 0.9% saline were obtained from Abbott Laboratories (Rockford, IL). RPMI 1640 culture medium was obtained from Whittaker M.A. Bioproducts (Walkersville, MD). Ultrafiltration filters were obtained from Fresenius AG (Bad Homburg, Germany). Polypropylene tubes, 12 x 75 mm, were obtained from Becton Dickinson Laboratories (Lincoln Park, IL).13 In the di-iodinated form of the Bolton-Hunter reagent was obtained from Dupont-New England Nuclear (Boston, MA). Econo-Pac 10DG desalting columns and Tween-20 were obtained from Bio-Rad Laboratories (Richmond, CA). MaxiSorp 96 microtiter plates were obtained from Nunc-Immunoplate (Roskilde, Denmark). Polyacrylamide gels (10%) were obtained from Amersham (Arlington Heights, IL). XAR-5 film was obtained from Kodak (Rochester, NY). An intensifying screen, Cronex Lightening Plus, was obtained from Dupont (Wilmington, DE).

Human IgG, clinical grade suitable for intravenous injection, was a gift of Hyland Laboratories (Duarte, CA), and human recombinant IL-1ra was a gift of Dr Peter Lomedico (Hoffman-LaRoche, Nutley, NJ). Human recombinant IL-1β was a gift of Dr Alan Shaw (Glaxo Institute for Molecular Biology, Geneva, Switzerland). Human recombinant GM-CSF was a gift of Dr Steven Clark (Genetics Institute, Cambridge, MA). Human recombinant IL-1ra was a gift of Dr Robert Thompson (Synergen, Boulder, CO). Sheep antirabbit IgG was obtained from Ventrex Laboratories (Portland, ME). Monoclonal antihuman IL-1β and polyclonal rabbit antihuman IL-1β were the gift of Richard Dondero (Cistron, Pine Brook, NJ). ImmunoPure alkaline phosphatase-conjugated goat antirabbit IgG and p-nitrophenol phosphate were obtained from Pierce (Rockford, IL).

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Study population. The study was approved by the Human Investigation Review Committee of the New England Medical Center Hospitals. Informed consent was obtained from each subject. Healthy male and female volunteers 20 to 50 years of age taking no medications were recruited from laboratory personnel.

Preparation of PBMC. PBMC were isolated from human blood as described previously. Some experiments used human AB serum that had been collected under sterile, pyrogen-free conditions, heated at 56°C for 30 minutes, filtered (0.22-μm pore size), and then frozen at −70°C. PBMC were incubated in polypropylene tubes at 37°C in a humidified atmosphere containing 5% CO₂. In experiments where tubes were rocked, the first hour of incubation occurred in the incubator with gentle agitation every 20 minutes, to equilibrate the concentration of CO₂. Tubes were tightly sealed with paraffin film and cultured at 37°C on a rocking table set at 20 cycles/minute. Control experiments showed that tightly sealing the culture tubes had no adverse effect on IL-1α or IL-1β production in stationary tubes (data not shown). Cells were cultured with the following: human AB serum, endotoxin, human IgG, human recombinant IL-1α, human recombinant IL-1β, and human recombinant GM-CSF. After 20 to 24 hours of incubation, cultures were frozen at −70°C and subjected to three cycles of freeze-thawing before assay for IL-1β or IL-1α. In some experiments, cells were separated from supernatants by centrifugation at 1,500g for 10 minutes at room temperature. The resulting cell pellets were resuspended in 1 mL of fresh culture medium. The cell and supernatant fractions were frozen as described previously.

Radiolabeling of IL-1α. Human recombinant IL-1α was radioiodinated with ¹²⁵I by the method of Bolton and Hunter. Radiolabeled protein was separated from unbound ¹²⁵I by chromatography on a desalting column equilibrated with 0.05 mol/L phosphate buffer, pH 7.5, containing 0.25% gelatin. The specific activity, calculated from the disintegrations per minute (dpm) comprising the protein peak, assuming a 70% recovery, ranged from 15 to 77 μCi/μg.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Approximately 10,000 cpm of ¹²⁵I-IL-1α was solubilized in 15 μL of loading buffer (62 mmol/L Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 1% 2-mercaptoethanol, 0.0005% bromphenol blue) and heated at 100°C for 3 minutes. Prestained protein molecular weight standards (myosin, M, 30,000; phosphorylase b, M, 92,500; bovine serum albumin [BSA], M, 69,000; carbonic anhydrase, M, 30,000; trypsin inhibitor, M, 21,500; and lysozyme, M, 14,300) were used on each gel. The gel was dried with heat in vacuo and autoradiography was performed by exposure of XAR-5 film for 2 days at −70°C using an intensifying screen.

RIA for IL-1α. A polyclonal anti-IL-1α antiseraum was raised in New Zealand white rabbits immunized with IL-1α. Dilutions of anti-IL-1α from 1:100 to 1:6,400 were added to ¹²⁵I-IL-1α (10,000 cpm). After 24 hours at room temperature, immunoprecipitates were formed by the addition of sheep antirabbit IgG and polyethylene glycol, at respective final concentrations of 1% and 3%. After 1 hour at room temperature, immunoprecipitates were recovered by centrifugation at 1,500g for 15 minutes at room temperature. Dilutions of anti-IL-1α precipitating approximately 20% of the added ¹²⁵I-IL-1α were used in the RIA.

Twee-20 and phenylmethylsulfonyl fluoride at final concentrations of 0.1% and 1 μmol/L, respectively, were added to PBMC samples, which were then kept on ice for 1 hour. Undiluted or diluted PBMC samples were added to RIA buffer (0.01 mol/L phosphate-buffered saline [PBS], pH 7.4, 0.25% BSA, and 0.05% sodium azide) containing 0.1% Tween-20. Diluted anti-IL-1α was added to the samples, which were kept at room temperature overnight. On the second day, ¹²⁵I-IL-1α (10,000 cpm) was added to each sample. On the third day, immunoprecipitates were formed, recovered, and counted as described above.

The maximum specific binding was calculated by subtracting nonspecific binding (cpm precipitated in tubes containing ¹²⁵I-IL-1α but no anti-IL-1α or unlabeled IL-1α) from total binding (cpm immunoprecipitated from tubes containing ¹²⁵I-IL-1α and anti-IL-1α but no unlabeled IL-1α). The percent specific binding for each sample was calculated by dividing the specific binding of the sample by the maximal specific binding and multiplying by 100. All samples and standards were run in duplicate. IL-1α concentration in each sample was read on a logit plot of the percent specific binding versus the log concentration of a diluted IL-1α standard from the linear portion of the curve (usually between 35% and 85% specific binding).

Determination of IL-1β. Human IL-1β was quantified using an ELISA. Standard curves and IL-1β concentrations in samples were determined using Microplate Manager software (Bio-Rad Laboratories) on a Macintosh SE computer (Apple Computer, Inc, Cupertino, CA). The sensitivity of the assay (limit of detection at the 95% confidence level) was 40 to 80 pg/mL.

Statistical analysis. Statistical analysis was performed using Stat-View software (Abacus Concepts, Inc, Calabasas, CA) on a Macintosh SE computer. Two-tailed paired t-tests and analysis of variance (ANOVA) using Fisher’s least significant difference were used. Data are expressed as the mean ± standard error of the mean (SEM). Differences were considered significant for P < .05.

RESULTS

SDS-PAGE analysis of IL-1α. Radiolabeled IL-1α was resolved by SDS-PAGE analysis on a 10% polyacrylamide gel to observe its integrity and purity. The autoradiogram in Fig 1 shows a single band of M, 18,000, indicating that

![Fig 1. Autoradiograph of SDS-PAGE analysis of ¹²⁵I-IL-1α. Radiolabeled IL-1α, 10,000 cpm, was resolved by SDS-PAGE on a 10% polyacrylamide gel.](image-url)
IL-1ra had not undergone aggregation or breakdown during radioiodination.

**RIA for IL-1ra.** The sensitivity of the assay was 166 ± 11 pg/mL, determined from five standard curves. The variation in six replicates of a PBMC sample was 5.9%. The assay was specific because the presence of IL-1α or IL-1β (10 ng/mL) did not interfere with the measurement of IL-1ra (Fig 2). IL-1α (10 ng/mL) added to each tube in the RIA for IL-1α or IL-1β had no influence on either assay (data not shown).

**Influence of standard culture conditions on IL-1ra production.** Fresh, uncultured, and lysed PBMC contained no IL-1ra (data not shown). To study the effect of normal human serum on IL-1ra production, PBMC were cultured in the presence or absence of 1% AB serum. Total IL-1ra and IL-1β were measured (Fig 3). In serum-free medium, PBMC produced small amounts of IL-1β or IL-1ra. PBMC cultured in the presence of serum produced sevenfold more IL-1ra compared with PBMC in serum-free medium (P < .05). Maximal production of IL-1ra (13.6 ± 0.8 ng/mL) was observed in the presence of the lowest concentration of AB serum tested (0.25%). There was no change in the production of IL-1β when serum was added. All subsequent experiments were performed in the absence of serum.

**Differential induction of IL-1ra and IL-1β production by IgG and GM-CSF.** PBMC were cultured in the presence of increasing concentrations of human IgG, GM-CSF, or IL-1α. After 24 hours, total IL-1ra and IL-1β were measured. As shown in Fig 4A, human IgG was a potent stimulus for IL-1ra production. As little as 1 μg/mL IgG induced significant IL-1ra production compared with baseline (P < .01). In contrast, the presence of up to 100 μg/mL IgG had little effect on IL-1β production in the same cultures.

GM-CSF was also a potent stimulus of IL-1ra production (Fig 4B). Significant augmentation of IL-1ra production over baseline was observed in cultures containing as little as 1 ng/mL (P < .05). Over the range of concentrations tested, there was no effect of GM-CSF on the production of IL-1β.

The production of IL-1 and other cytokines is influenced by IL-1, interferon, and IL-1ra. The effect of IL-1 on IL-1ra production is unknown. Therefore, PBMC were cultured with IL-1α or IL-1β (0.1 to 100 ng/mL) for 24 hours and total IL-1ra was measured. Although there were increases in IL-1ra production over baseline levels, they did not reach statistical significance (data not shown).

The effect of cell-cell contact on IL-1ra production. Culture conditions of monocytes influence their functional state. Therefore, PBMC in serum-free medium or stimulated with human IgG or GM-CSF were cultured in either stationary or rocking polypropylene tubes. After 24 hours, the cultures were harvested and assayed for total IL-1β or IL-1ra. The data from three experiments are expressed as the mean ± SEM.
hours, total IL-1ra was measured. Both IgG (Fig 5A) and GM-CSF (Fig 5B) were potent stimuli for IL-1ra production in stationary cultures (some of the data are displayed in Fig 4). However, in the presence of IgG, rocking cultures of PBMC produced significantly lower levels of IL-1ra. The same phenomenon was observed in cultures of PBMC in the presence of GM-CSF, although the reduction was less.

We also compared the effect of these culture conditions on the production of IL-1β and IL-1ra in PBMC stimulated by endotoxin. After 24 hours, total IL-1β and IL-1ra were measured. Endotoxin was a strong stimulus for IL-1ra production in stationary cultures (Fig 6A). However, there was a substantial reduction in IL-1ra production in rocking cultures of endotoxin-stimulated PBMC. In contrast, rocking had no significant effect on endotoxin-induced IL-1β production (Fig 6B).

Distribution of IL-1ra and IL-1β between cellular and supernatant compartments. PBMC in stationary polypropylene tubes were cultured with endotoxin (10 ng/mL). After 24 hours, cells were separated from supernatants by centrifugation. Each fraction was assayed for IL-1β and IL-1ra. The results are shown in Fig 7. Similar to previous results,26,27 the distribution of IL-1β between cellular and supernatant fractions was variable. In contrast, the distribution of IL-1ra between cellular and supernatant compartments was consistent. Approximately 80% of the IL-1ra was secreted into the supernatant. The differences in IL-1ra content between the cell-associated and supernatant fractions was statistically significant (P < .001). The difference between the proportions of IL-1ra and IL-1β in the cell-associated fraction was statistically significant as was the...
difference between the proportions in the supernatants (P < .05).

Potentiation of IgG-induced IL-1ra production by GM-CSF or IL-1β. Because low (picogram) levels of cytokines exist in vivo,28-31 we questioned whether potentiation of IgG-induced IL-1ra production by GM-CSF or IL-1 existed in vitro. PBMC were cultured in stationary tubes for 24 hours with IgG (1 μg/mL) in the presence or absence of GM-CSF (0.1 ng/mL). These concentrations stimulated submaximal production of IL-1ra when used separately (Fig 4). In addition, PBMC were cultured with IgG (1 μg/mL) with or without IL-1β (100 ng/mL). The results are shown in Fig 8. Potentiation of IgG-induced IL-1ra production was observed in the presence of GM-CSF (Fig 8A) or IL-1β (Fig 8B).

DISCUSSION

We have investigated the relationship between IL-1ra and IL-1β production by PBMC using an RIA sensitive and specific for IL-1ra. This RIA enabled the independent measurement of IL-1ra in the same samples containing IL-1, a complicated task when using bioassays. The primary finding of these studies was the existence of differential regulation of the production of IL-1ra and IL-1β. This conclusion is based on (1) human AB serum, human IgG, and GM-CSF induced IL-1ra but not IL-1β; (2) the prevention of cell-cell contact substantially decreased IL-1ra production but had no effect on IL-1β production; and (3) approximately 80% of IL-1ra produced by PBMC was secreted into the medium whereas only 50% of IL-1β was secreted. We also observed the potentiation of IgG-induced IL-1ra production by GM-CSF or IL-1β. The existence of differential regulation of IL-1ra and IL-1β production was in agreement with studies on the synthesis of mRNA for each protein.13

This and other studies suggest that IL-1ra production would occur in tissue rather than in the circulation. Such a view is supported by observations that the production of IL-1ra or other IL-1 inhibitors similar or possibly identical to IL-1ra is increased under conditions associated with monocyte differentiation. For example, IL-1ra or IL-1 inhibitor production increased in macrophage-monocyte cultures maintained for several weeks with or without GM-CSF32 or in freshly obtained PBMC stimulated with GM-CSF, as in the present study. In contrast, the prevention of cell-cell contact (as might occur in the circulation) resulted in a marked decrease in IL-1ra production.

The mechanism for the stimulation of IL-1ra production is unclear. Stimulation by IgG presumably occurs via the Fc receptor. Therefore, it is likely that conditions which increase Fc receptor expression and/or function, such as adherence24 or exposure to cytokines such as interferon or GM-CSF,33 augment IgG-induced IL-1ra production. The observed potentiation of IgG-induced IL-1ra production by GM-CSF may be explained by such a phenomenon. It is unlikely that augmentation of Fc receptor function is the mechanism whereby IL-1β potentiated IgG-induced IL-1ra production because IL-1 does not change Fc receptor function or expression on monocytes.33

It is also unclear why the production of IL-1ra by rocking PBMC regardless of the stimulus was less than that by stationary PBMC. However, it was apparently not through an injurious effect of rocking because IL-1β production by the same cells was not affected. It is possible that when cell-cell contact or adherence to culture vessels was prevented, there was reduced synthesis of a necessary factor for IL-1ra production. Because induction of IL-1β by endotoxin was unaffected by rocking, a reduction of endotoxin receptors is an unlikely explanation. Alternatively, the prevention of cell-cell contact or adherence to culture vessels might have stimulated the production of a factor that inhibited IL-1ra production.

There is 26% homology between the sequences of IL-1ra and IL-1β.11 However, there appears to be a signal sequence encoded by the IL-1ra gene which is lacking in the sequence encoded by the IL-1β gene. This implies different mechanisms of secretion for the two proteins.51 Disparity between the amount of IL-1ra secreted and the amount of IL-1β secreted may be explained by this structural difference.

The relevance of the differential regulation of the production of IL-1β and IL-1ra observed in vitro to the pathogenesis of disease is unknown. Certainly, IL-1β is implicated in several disease states.1 These include septic or endotoxin shock,29,34 inflammatory bowel disease,35 and acute myelogenous leukemia.36 It is unknown if conditions thought to
involve increased IL-1 activity have an accompanying deficiency in IL-1ra production. The antagonism of IL-1 by exogenous IL-1ra may afford a therapeutic option in these diseases. In view of this, exogenous IL-1ra (1) blunted septic shock induced by *Escherichia coli* in rabbits and baboons (Dr Lyle Moldawer, personal communication, November 1990); (2) reduced mortality induced by endotoxin in rabbits; (3) blunted fever induced by IL-1 in rabbits; (4) decreased the inflammation seen in immune complex colitis; and (5) reduced the spontaneous production of GM-CSF by and proliferation of acute myelogenous leukemia cells.41

Intravenous Ig is effective in disorders characterized by increased activity of the immune system. These include graft-versus-host disease,15 Kawasaki syndrome,24 and immune thrombocytopenic purpura.46,46 One plausible mechanism of action of Ig is the in vivo induction of IL-1ra production. Given the therapeutic potential of IL-1ra, further in vitro and in vivo work is warranted.

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Production of interleukin-1 receptor antagonist and interleukin-1 beta by peripheral blood mononuclear cells is differentially regulated

DD Poutsiaka, BD Clark, E Vannier and CA Dinarello