Hematologic and Immunomodulatory Effects of an Interleukin-1 Receptor Antagonist Coinfusion During Low-Dose Endotoxemia in Healthy Humans


Endotoxin is a component of gram-negative bacteria that causes hematologic and immunologic changes through its induction of cytokines. Interleukin-1 receptor antagonist (IL-1Ra) is a naturally occurring inhibitor of IL-1 that competes with IL-1 for occupancy of cell-surface receptors but possesses no agonist activity. We investigated the ability of human recombinant IL-1Ra to block the effects of low-dose endotoxin. Fourteen healthy male volunteers between 18 and 30 years old were injected intravenously with 3 ng/kg Escherichia coli endotoxin. Concurrent with the injections, nine volunteers received a 3-hour continuous intravenous infusion of IL-1Ra. The other five subjects were given a 3-hour infusion of saline. Volunteers injected with endotoxin experienced a threefold increase in circulating neutrophils over baseline. This neutrophilia was significantly reduced by 48% in subjects administered endotoxin plus IL-1Ra (P = .0253). Ex vivo mitogen-induced peripheral blood mononuclear cell proliferation decreased by greater than 60% at 3 and 6 hours after endotoxin injection (P = .0053). This endotoxin-induced reduction in mitogen response was reversed in subjects coinfected with IL-1Ra (P = .0253). Endotoxin-induced symptoms, fever, and tachycardia were unaffected by IL-1Ra. IL-1 appears to be an important mediator in endotoxemia because some of its hematologic and immunomodulatory effects can be blocked by IL-1Ra.

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Materials and Methods

Volunteer selection. The protocol and consent forms for this study were approved by the Human Investigation Review Committee of New England Medical Center and Tufts University Health Sciences. Fourteen healthy human immunodeficiency virus antibody-negative male volunteers between 18 and 30 years old entered the study after giving informed consent. Subjects were not blinded. Eligibility requirements included the absence of underlying disease on history, physical examination, bloodwork, electrocardiogram, or chest radiograph. Volunteers abstained from taking oral cyclooxygenase inhibitors during the 2 weeks preceding the study.

Study design. After a 10-hour overnight fast, volunteers received 3 ng/kg of US standard reference endotoxin (lot EC-5, Escherichia coli 0113; Bureau of Biologics, Food and Drug Administration, Bethesda, MD) as an IV bolus injection into the antecubital fossa. Simultaneously, a 3-hour IV infusion of human recombinant IL-1Ra or 0.9% sterile sodium chloride (Abbott Laboratories, North Chicago, IL) was started in the other arm. Total doses of IL-1Ra were 1 mg IL-1Ra/kg of body weight (3 subjects), 5 mg/kg (3 subjects), and 10 mg/kg (3 subjects). Five volunteers served as controls and received only saline.

Clinical evaluation. Vital signs (temperature, pulse, blood pressure, and respirations) were monitored every hour, beginning before the endotoxin and continuing for 12 hours. Volunteers completed a symptom check list 6 hours after the endotoxin injection.

Blood samples for complete blood count (ie, hemoglobin, white blood cell count with differential, platelets) and serum cortisol were drawn immediately before the infusion, hourly for the next 6 hours, and again 12 hours after the injection of endotoxin. Absolute neutrophil count (ANC) was defined as the total number of polymorphonuclear leukocytes plus band forms per cubic millimeter. Blood chemistries (serum amyloid A, insulin, growth hormone, epinephrine, fibrinogen, factor VIII, and erythrocyte sedimentation rate) were obtained immediately before and 6 hours after the endotoxin infusion. Twenty-four hours after endotoxin injection, vital signs, symptom check list, complete blood count, serum cortisol, and blood chemistries were repeated.

Pharmacokinetics. Blood was collected in sterile, vacuum blood collection tubes containing EDTA (Becton Dickinson, Rutherford, NJ). The concentrations of IL-1Ra were determined using a specific radioimmunoassay with a lower detection limit of 0.3 ng/mL. Endotoxin concentrations were measured using the Limulus amebocyte lysate assay.

Results

Endotoxin is a constituent of the outer cell membrane of gram-negative bacteria. Patients with gram-negative bacteremia often have demonstrable endotoxemia. When injected in low doses into healthy human volunteers, endotoxin induces fever, leukocytosis, and a decrease in ex vivo T-cell proliferation, but not hypotension. However, these changes are not entirely caused by the direct effects of endotoxin, but rather, result in part from endotoxin-induced cytokines. Humans administered intravenous (IV) interleukin-1α (IL-1α), IL-1β, or tumor necrosis factor (TNFα) also develop fever and leukocytosis.

IL-1 receptor antagonist (IL-1Ra) is a 22-kDa glycoprotein previously called the "IL-1 inhibitor." IL-1Ra has 19% amino acid identity with IL-1α and 26% amino acid identity with IL-1β. The binding of IL-1 to the type I IL-1 receptor on T cells as well as the type II IL-1 receptor on neutrophils and B cells is inhibited by IL-1Ra. IL-1Ra has no agonist activity in vitro.

Recently, we reported that healthy human volunteers experience no adverse effects when infused with sufficient human recombinant IL-1Ra to achieve a plasma level of 29 μg/mL. Based on these results we administered IL-1Ra concurrently with a low dose of endotoxin to healthy male volunteers. In this report, we describe the ability of IL-1Ra to reduce the neutrophilia and reverse the transient inhibition of ex vivo mitogen-induced peripheral blood mononuclear cell proliferation characteristic of endotoxemia.

Materials and Methods

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erford, NJ) before and 0.5, 0.75, 1, 1.5, 2, 3, 3.25, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 10, 11, and 12 hours after the infusion was begun. Platelet-poor plasma was separated from the blood samples and was stored at -70°C. Subsequently, plasma samples were assayed for IL-1Ra using an enzyme-linked immunosorbent assay. The limit of detection of the assay was 1 ng/mL. Volume of distribution at steady state and plasma clearance of IL-1Ra were estimated for each plasma IL-1Ra concentration versus time curve using noncompartmental analysis based on statistical moment theory.22 IL-1Ra plasma half-lives were estimated by curve-fitting (RSTIP; MicroMath Scientific Software, Salt Lake City, UT) the postinfusion IL-1Ra plasma disappearance.

Plasma cytokine assays. Blood was collected in sterile, vacuum blood collection tubes containing EDTA immediately before and 1, 2, 3, 4, 5, 6, 12, and 24 hours after endotoxin injection. Samples were placed on ice, and plasma was separated using the methods of Cannon et al38 and then stored at -70°C. All samples were assayed for IL-1β,23 IL-6,24 IL-8,25 TNFα,26 or granulocyte colony-stimulating factor (G-CSF) (Quantikine Human G-CSF Immunoassay; R & D Systems, Minneapolis, MN). The limit of detection for each radioimmunoassay (RIA) was 40 to 80 pg/mL for IL-1β, 20 to 40 pg/mL for IL-6, 20 to 40 pg/mL for IL-8, and 40 to 80 pg/mL for TNFα. Previous studies have shown no cross-reactivity with various cytokines in these RIA. The limit of detection for the G-CSF enzyme-linked immunosorbent assay was 80 pg/mL.

Isolation of peripheral blood mononuclear cells (PBMC). Immediately before and 3, 6, and 24 hours after the bolus injection, blood was drawn into syringes containing heparin (20 U/mL; final concentration; LymphoMed Inc, Rosemont, IL). PBMC were isolated by centrifugation through Ficoll (Sigma Chemical Co, St Louis, MO) and Hypaque (90%; Winthrop Laboratories, New York, NY). Preparations of Ficoll-Hypaque used sterile, nonpyrogenic water (Abbott). PBMC were washed twice in 0.9% sodium chloride before being resuspended.

Fluorescent-activated cell-sorting analysis (FACS) of PBMC. PBMC were resuspended in FACS buffer consisting of phosphate-buffered saline containing 0.1% bovine serum albumin (Fraction V; Sigma) and 0.1% sodium azide (Sigma). Then 5 x 106 cells were aliquoted into each of four tubes. After centrifugation, supernatants were discarded and the pellets were mixed with 10 μL of anti-CD3, anti-CD4, anti-CD19, or anti-CD56 mouse-antihuman monoclonal antibody (Becton Dickinson). After a 30-minute incubation at 4°C, the cells were washed before being combined with goat-anti-mouse fluorescein isothiocyanate-conjugated antibody (Becton Dickinson) for another 30 minutes. Finally, PBMC were washed, fixed in FACS buffer with 0.1% formalin, and analyzed with a fluorescent-activated cell sorter.

Mitogen-induced PBMC proliferation. PBMC were resuspended in RPMI 1640 (Sigma) containing 10 mmol/L L-glutamine, 24 mmol/L NaHCO3 (Mallinkrodt, Paris, KY), 10 mmol/L HEPES (Sigma), 100 U/mL penicillin, and 100 μg/mL streptomycin (Irvin Scientific, Santa Ana, CA); pH 7.4. RPMI was ultrafiltered using polysulfone hollow fiber filters (F40; Fresenius AG, Bad Homburg, Germany) to remove substances capable of inducing cytokine production.24 The cell suspension was supplemented with 2% vol/vol/glycine-inactivated (56°C, 45 minutes) human AB serum. Phytohemagglutinin (PHA) was purchased from Difco Laboratories (Detroit, MI); Concanavalin A (Con A) was obtained from Sigma. As previously described,19 1.5 x 106 PBMC were aliquoted into the wells of 96-well polystyrene flat-bottom cell culture plates (Costar Corp, Cambridge, MA) and either RPMI, Con A (final concentration 3 μg/mL), or PHA (final concentration 8 μg/mL) was added. After a 24-hour incubation at 37°C in 5% CO2, 100 μCi/mL [3H]thymidine (New England Nuclear, Boston, MA) was added to each well. Eighteen hours later, the plates were frozen at -70°C. All cultures were harvested on the same day and β-radioactivity determined. The standard deviation of counts per minute of triplicate wells was consistently less than 15%.

Statistics. Mean peak plasma IL-1Ra levels, half-lives, and volume of distribution are expressed as the mean ± standard deviation. Other results are shown as the mean ± standard error of the mean. Some data are expressed as the percentage change compared with baseline measurements obtained just before endotoxin injection (time = 0). Data were analyzed using the Kruskal-Wallis test (χ2 approximation).

RESULTS

Clinical evaluation. In volunteers injected with endotoxin only, transient chills and generalized myalgia developed after 1 to 2 hours. Fever peaked 4 to 5 hours after injection (an increase of 1.6 ± 0.1°C). Pulse was maximal at 3 to 4 hours (an increase of 27 ± 5 beats/min); no significant changes in mean arterial pressure were detected. At each time point there were no clinically significant differences in symptoms or vital signs between subjects receiving endotoxin or endotoxin plus IL-1Ra. Hematologic evaluation. Volunteers developed transient neutropenia at 1 hour, followed by neutrophilia, which was maximal (threefold over baseline) between 5 and 6 hours after the endotoxin injection. Between 2 and 6 hours, ANCs were lower in all IL-1Ra dose groups than in volunteers receiving endotoxin alone (Fig 1). As shown in Fig 1C, the ANC at 6 hours was 48% less in the group receiving 10 mg/kg IL-1Ra than in the group receiving endotoxin alone (P = .0253). In the 10 mg/kg IL-1Ra dose group this reduction in endotoxin-induced neutrophilia persisted for 12 hours after the endotoxin injection (P = .0253). Platelet counts decreased in all groups. There were no significant differences in platelet counts when comparing the IL-1Ra dose groups with the group receiving endotoxin alone.

Biochemical evaluation. When compared with baseline, no changes in insulin, growth hormone, fibrinogen, and erythrocyte sedimentation rate were detected in any group. Before the injection of endotoxin, serum amyloid A levels were less than 5 μg/mL. Twenty-four hours after endotoxin injection, serum amyloid A was 154 ± 33 μg/mL in the group receiving endotoxin alone. In the group receiving a coinfusion of 10 mg/kg IL-1Ra, serum amyloid A increased to 117 ± 40 μg/mL (P = .18 when compared with the group injected with endotoxin alone). Although serum cortisol, epinephrine, and factor VIII levels increased after the injection of endotoxin, these changes were not significantly affected by IL-1Ra.

Plasma cytokines. All groups experienced significant increases in plasma IL-1β, IL-6, IL-8, TNFα, and G-CSF after endotoxin injection (Table 1). Maximal plasma cytokine levels were reduced 21% for IL-1β, 25% for IL-6, 23% for IL-8, 36% for TNFα, and 35% for G-CSF in the group receiving 10 mg/kg IL-1Ra compared with the volunteers injected only with endotoxin. However, these differences did not achieve statistical significance.

Mitogen-induced PBMC proliferation. At both 3 and 6 hours after endotoxin injection, Con A-induced PBMC pro-
EFFECTS OF IL-1RA ON EXPERIMENTAL ENDOTOXEMIA

**Table 1. Maximum Plasma Cytokine Levels**

<table>
<thead>
<tr>
<th>Plasma Cytokine</th>
<th>Endotoxin</th>
<th>Endotoxin + 10 mg/kg IL-1Ra</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>98 ± 9</td>
<td>77 ± 1</td>
</tr>
<tr>
<td>IL-6</td>
<td>694 ± 220</td>
<td>520 ± 140</td>
</tr>
<tr>
<td>IL-8</td>
<td>999 ± 352</td>
<td>773 ± 197</td>
</tr>
<tr>
<td>TNFα</td>
<td>517 ± 235</td>
<td>333 ± 162</td>
</tr>
<tr>
<td>G-CSF</td>
<td>654 ± 214</td>
<td>427 ± 134</td>
</tr>
</tbody>
</table>

**Fig 1.** Effect of IL-1Ra on endotoxin-induced neutrophilia. ANC were obtained immediately before and for 24 hours after a 3 ng/kg bolus of endotoxin alone (C) or with a coinfusion of IL-1Ra (E). Data are expressed as percent change in ANCs from time zero. The graphs depict the results of (A) 1 mg/kg, (B) 5 mg/kg, or (C) 10 mg/kg IL-1Ra coinfusion. *Indicates \( P = .0053 \) when comparing the ANC of subjects injected with endotoxin alone (n = 5) with the ANC of subjects who received endotoxin plus a coinfusion of IL-1Ra (n = 3 at each dose).

**Fig 2.** Effect of IL-1Ra on endotoxin-induced reduction in Con A-induced PBMC proliferation. PBMC were isolated from volunteers either immediately before or 3, 6, or 24 hours after a 3 ng/kg bolus injection of endotoxin. Cells were stimulated in vitro with Con A (3 μg/mL). At each time point, data were expressed as percent change in PBMC proliferation from time zero. *Indicates \( P = .0053 \) when comparing cell proliferation at time zero in subjects injected with endotoxin alone with cell proliferation in these same subjects after endotoxin injection (n = 5, [II]). **Indicates \( P = .0253 \) when comparing cell proliferation at time zero in subjects injected with endotoxin alone with cell proliferation in subjects who received a coinfusion of 10 mg/kg IL-1Ra (n = 3, [III]).

Proliferation was reduced by greater than 60% in subjects administered endotoxin alone (\( P = .0053 \)) (Fig 2). In contrast, this reduction in mitogen response was reversed in the group receiving 10 mg/kg IL-1Ra (\( P = .0253 \)). A similar reversal was seen with PHA-induced PBMC proliferation (data not shown). These findings were not a result of quantitative changes in lymphocyte counts or lymphocyte phenotypic subsets (as determined by FACS for CD3, CD19, and CD56) when comparing the IL-1Ra dose groups with the group receiving endotoxin alone (data not shown).

**Pharmacokinetics.** Mean peak plasma IL-1Ra levels were 2.4 ± 0.9 μg/mL for the 1 mg/kg dose group, 15.3 ± 5.2 μg/mL for the 5 mg/kg dose group, and 29.2 ± 7.2 μg/mL for the 10 mg/kg dose group. The dose of IL-1Ra did not
affect the pharmacokinetics. Postinfusion plasma IL-1Ra levels decreased quickly, exhibiting an initial half-life of 19 ± 6 minutes and a terminal half-life of 102 ± 18 minutes. IL-1Ra distributed into a steady-state distribution volume of 12.2 ± 2.7 L. Plasma clearance for IL-1Ra was 169 ± 12 mL/min. Previously described healthy volunteers receiving IL-1Ra alone had a plasma clearance for IL-1Ra of 151 ± 5 mL/min. When compared with the group receiving IL-1Ra alone, the group receiving the coinfusion of IL-1Ra and endotoxin had an increased IL-1Ra clearance of borderline significance (P = .056).

**DISCUSSION**

Granulocytosis is probably the most sensitive indicator of endotoxemia in humans, because it occurs at subpyrogenic doses. Therefore, a priori we considered endotoxin-induced neutrophilia to be the response most likely to be affected by IL-1Ra. Consistent with previous investigations, we detected a transient neutropenia followed by a threefold increase in circulating neutrophils in volunteers administered endotoxin. Endotoxin-induced neutrophilia was significantly reduced in volunteers given a coinfusion of IL-1Ra. As previously reported, an infusion of 10 mg/kg IL-1Ra alone did not affect peripheral blood neutrophil counts. In the present study, IL-1Ra may be preventing IL-1-induced synthesis of molecules such as CSF-2 which mediate the release of granulocytes from the bone marrow. The persistence of some neutrophilia, despite exogenous IL-1Ra, may be caused by insufficient levels of the antagonist as well as by endogenous TNFα or G-CSF, which also cause neutrophilia.

In contrast to its effects on endotoxin-induced neutrophilia, IL-1Ra did not prevent the neutropenia. This finding is not surprising because the neutropenia is unlikely to be caused by IL-1. Neutropenia occurs 1 hour after endotoxin injection and before IL-1 is significantly elevated in the circulation. In humans, an infusion of recombinant human IL-1α or IL-1β does cause a transient decrease in peripheral leukocyte counts within 1 to 2 hours after infusion. However, due to the rapidity of its onset, the neutropenia seen in our study is probably mediated directly by endotoxin.

Lymphocyte proliferative responses to mitogens are reduced after endotoxemia, burns, cardiac surgery, and trauma. In our subjects, low-dose endotoxin was associated with a 60% transient reduction in mitogen-induced ex vivo PBMC proliferation; a coinfusion of IL-1Ra prevented this reduction. However, as previously described, in healthy volunteers administered only IL-1Ra, levels of antagonist as high as 29 μg/mL did not reduce proliferative responses in PBMC stimulated ex vivo with T-cell mitogens. This is consistent with studies showing that the addition of IL-1Ra in vitro does not reduce human T-cell proliferation induced by mitogens, soluble antigens, or allogeneic determinants. Because 10 mg/kg IL-1Ra administered in vivo to our volunteers reversed the reduction in proliferation induced by endotoxin, this effect of endotoxin appears to be mediated by IL-1. The inability of lower doses of IL-1Ra to exert an effect suggests this phenomenon is dose dependent. Reversal of endotoxin-induced changes in PBMC proliferation by IL-1Ra may be caused by inhibition of IL-1-induced prostaglandin synthesis. Indomethacin abrogates the expected decrease in PHA-induced lymphocyte proliferation after endotoxemia or surgery.

Plasma levels of IL-1β, IL-6, IL-8, TNFα, and G-CSF in the group that received endotoxin were elevated as compared with saline-injected controls. However, in subjects receiving endotoxin plus a coinfusion of 10 mg/kg IL-1Ra, decreased cytokine levels were measured; these decreases did not achieve statistical significance. Because the magnitude and kinetics of cytokine production in experimental endotoxemia exhibit considerable inter-individual variability, the lack of significance may be caused by the relatively small sample size.

During endotoxemia in human subjects, a 100-fold molar excess of IL-1Ra over IL-1β is found in the circulation. In this study, administering 10 mg/kg IL-1Ra resulted in a 200,000-fold molar excess of IL-1Ra over IL-1β. An unresolved question is whether the level of endogenous IL-1Ra is sufficient to reduce the severity of disease. In vitro, molar ratios of IL-1Ra to IL-1 from 1 to 5 block 50% of IL-1-induced effects in human cells. In PBMC, 1 μg/mL IL-1Ra inhibits endotoxin-induced cytokine synthesis. However, a 10,000-fold molar excess of IL-1Ra over endogenous IL-1 is required to block shock and death in animals with endotoxemia.

In the present study, plasma levels of IL-1Ra obtained 1 hour after the injection of endotoxin were 26.5 ± 11.8 μg/mL in the group receiving 10 mg/kg IL-1Ra. The lower levels of IL-1Ra found in the circulation during the first half hour (10.6 ± 2.7 μg/mL) after endotoxin were still probably sufficient to block the IL-1β (<100 pg/mL) circulating during this period. Paradoxically, the low dose of endotoxin may not have stimulated sufficient IL-1 to observe the maximal effect of IL-1 receptor blockade. This concept is supported by studies in baboons which show the effects of IL-1Ra on lethal E. coli-induced shock are more prominent than its effects on sublethal endotoxemia. Furthermore, similar results have been reported in rabbits with Staphylococcus epidermidis-induced hypotension. Therefore, the clinical responses to a low dose of endotoxin not blocked by IL-1Ra were likely caused by other cytokines such as TNF and IL-6. Nevertheless, our data show that IL-1 is an important contributor to the neutrophilia and reduction in PBMC mitogen response characteristic of low doses of endotoxin.

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