Interleukin-1 Induces Interleukin-8 Secretion From Endothelial Cells by a Juxtacrine Mechanism

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Inflammation is characterized by migration of neutrophils through the endothelium, and the chemokine interleukin-8 (IL-8) appears to be involved. We asked whether adherence of cells bearing a membrane-form of interleukin 1 (IL-1) induces IL-8 secretion from human umbilical vein endothelial cells (HUVEC) and fibroblasts. Human peripheral blood mononuclear cells (PBMC) were stimulated with endotoxin for 12 hours and then fixed for 4 hours with paraformaldehyde. When these cells were added to HUVEC or fibroblasts, IL-8 production was induced. This stimulation by fixed PBMC was attributed to IL-1, because pretreatment of HUVEC or fibroblasts with IL-1 receptor antagonist (IL-1Ra) reduced the induction by 95% and 80%, respectively, \( P < .005 \). Using anti-IL-1α monoclonal antibodies, reduction was complete, whereas anti-IL-1β had no effect. IL-1α was shown on the surface of monocytes by fluorescence-activated cell sorter (FACS) analysis. Blockade of IL-1 receptors on PBMC did not affect the activity of membrane-associated IL-1α, indicating that IL-1 is not anchored to the membrane through its receptors. However, PBMC treated with D-mannose before fixation resulted in a loss of activity; this loss of activity was associated with release of IL-1α, not IL-1β, into the supernatant. Thus, anchoring of IL-1α to the membrane may be via a lectin or mannose receptor-like interaction. Blockade of membrane IL-1α required a 30-fold and fivefold excess of IL-1Ra compared with the amount required to block soluble IL-1β and IL-1α, respectively. We conclude that the fixed PBMC IL-8 inducing activity is almost entirely caused by IL-1, that this represents IL-1α bound to a surface lectin or mannose receptor on the monocyte, and that it functions in inflammation via juxtacrine interactions.

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**MATERIALS AND METHODS**

**Materials.** The following materials were purchased: M199 culture medium, penicillin G-streptomycin solution, L-glutamine, trypsin-EJDTA (GIBCO Laboratories, Grand Island, NY), endothelial cell growth factor (Collaborative Biomedical Products Inc, Bedford, MA), RPMI 1640 culture medium (Whittaker M.A. Bioproducts, Walkersville, MD), fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT), serum plus (Hazleton, Lenexa, KS), sterile 0.9% saline (Abbott Laboratories, Rockford, IL), Hypaque-M 90% (Winthrop Pharmaceuticals, New York, NY), gelatin, PFA, D-mannose, Ficol type 400, LPS from *Escherichia coli* serotype 055:BS (Sigma Chemical Co, St Louis, MO), anti-IL-1α (IGG1, clone alpha 29), anti-IL-1β (IGG1, clone beta 36) monoclonal antibodies (MoAb), biotinylated goat antimouse IgG (Fab), and phycoerythrin-streptavidin complex (Immunotech, Marseille, France), 24-well flat bottom plates and polypropylene tubes (Becton Dickinson Co, Lincoln Park, NJ).

Human IL-8 was a gift from Dr M. Baggiolini, Kocher Institute (Bern, Switzerland). Human recombinant IL-1α (molecular weight 17,000) was a gift from Dr P. Lomedico, Hoffman La Roche (Nutley, New Jersey).
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NJ). Human recombinant IL-1β (mature form) was a gift of Dr A. Tagliabue, Sclavo Research Institute (Siena, Italy). IL-1 receptor antagonist (IL-1Ra) was a gift of Dr. D.E. Tracey, Upjohn Co. (Kalamazoo, MI).

Cell cultures. Human umbilical vein endothelial cells (HUVEC) were prepared as previously described, and used between passages 2 and 4. Cells were grown until confluent in 24-well plates coated with 1% gelatin. Before the experiments, cells were incubated in complete culture medium, consisting of ultrafiltrated RPMI24 100 U/mL penicillin G, 100 µg/mL streptomycin, 1% L-glutamine, and 10% heat-inactivated FCS. Neonatal human dermal fibroblasts were grown in RPMI 1640, supplemented with 10% Fetal Serum, 1% penicillin/1% streptomycin, 100 U/mL penicillin C, and 100 µg/mL streptomycin. The fibroblasts were split into 24-well plates, and grown to confluence in the former medium at 1 ml volumes.

Stimulation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMC) were prepared from freshly drawn heparinized blood by centrifugation on a Ficoll-Hypaque gradient. Using a hemocytometer, cells were adjusted to 10⁶ cells/mL in complete culture medium. PBMC were stimulated with 10 ng/mL of LPS for 1 hour, at 37°C, 5% CO₂, in polypropylene tubes. This was followed by a 14-hour rotation (2.5 rpm/min) at 37°C as previously described. As a control, PBMC were incubated at the same conditions, but without LPS stimulation. After the incubation, the cells were washed three times in RPMI.

Fixation of PBMC. After washing, the PBMC pellets were resuspended in freshly prepared 1% PFA in phosphate-buffered saline, pH 7.4, at room temperature for 4 hours, and then washed three times in RPMI. The fixed PBMC were resuspended in complete culture medium, and 10⁶ cells were added to each well of HUVEC or 2 X 10⁵ cells to each well of fibroblasts. After 20 hours at 37°C, the supernatants were collected, centrifuged for 10 minutes in a microfuge at 4°C, and stored at -75°C for IL-8 assay.

Treatment with D-mannose. PBMC were stimulated with LPS as described above, and incubated with 0.5 mol/L D-mannose or RPMI for 1 hour at 4°C. Supernatants were separated from cell pellets and stored at -75°C before assaying for IL-1α and IL-1β. The pellets were washed three times in RPMI, assessed for viability with trypan blue, and readdressed to 10⁶ cells/mL. The cells were fixed with PFA as described above, and 10⁶ cells/mL were added to the HUVEC for 26 hours. Supernatants were then removed and IL-8 was measured.

Fluorescence-activated cell sorter (FACS) analysis. Staining was performed as described previously, using the following sequence: (1) unconjugated anti-IL-1α and anti-IL-1β MoAb, or an irrelevant IgG1 (20 µg/mL), (2) biotinylated goat anti-IgM (1/50), (3) normal mouse IgG (20 µg/mL) to block residual binding sites for mouse Ig, (4) phycoerythrin-streptavidin complex (1/100). Fluorescence was measured on a FACS analyzer (Epics Profile; Coulter, Hialeah, FL).

Radioimmunoassay for IL-1α, IL-1β and IL-8. IL-8, IL-1α and IL-1β were measured by specific radioimmunoassay (RIA), as previously described.

Statistical analysis. The data were analyzed for statistical significance by Student’s t test. Results are expressed as the mean ± standard error of the mean of three to six experiments unless otherwise specified.

RESULTS

Paraformaldehyde-fixed PBMC induce IL-8 secretion by HUVEC. Stimulation of HUVEC with soluble IL-1β resulted in IL-8 secretion in a dose-dependent fashion (Fig 1, inset). A similar dose-response curve was observed using IL-1α as a stimulant. At 100 pg/mL, IL-1β- (or IL-1α-) induced IL-8 was significant (P < 0.01). Fibroblasts secreted higher amounts of IL-8 when incubated with either IL-1α or β, using 50 pg/mL IL-1β. 90 to 110 ng/mL of IL-8 were secreted into the supernatant compared with control medium.

LPS-stimulated PBMC, after fixation in PFA for 4 hours, induced IL-8 from HUVEC compared with fixed, but unstimulated PBMC (P = 0.0001, Fig 1). The amount of IL-8 produced following stimulation with 10⁶ fixed PBMC was comparable in magnitude to that induced by 100 pg/mL of either soluble IL-1β or IL-1α. The addition of polymyxin B (5 µg/mL) to the cultures did not reduce the effect (three experiments, data not shown), demonstrating that carry-over of LPS into the HUVEC cultures was not the source of stimulation.

IL-1Ra and anti-IL-1α MoAb inhibits the stimulatory effect of fixed PBMC. Both HUVEC and fibroblasts secreted IL-8 in response to LPS-stimulated and fixed PBMC. This could be due to IL-1, TNF, or other LPS-stimulated cytokines. However, blockade of IL-1 receptors on HUVEC or fibroblasts using 10 µg/mL of IL-1Ra before the addition of fixed PBMC inhibited IL-8 secretion by 95% and 80% (P < 0.001 and P < 0.005, respectively). As shown in Fig 2, the presence of anti-IL-1α Ab completely inhibited the induction of IL-8 by LPS-stimulated fixed PBMC (P < 0.002), whereas anti-IL-1β had no effect. The degree of reduction by anti-IL-1α was comparable to that of IL-1Ra. Thus, the effect of LPS-stimulated and fixed PBMC was mainly due to IL-1 and specifically to a surface form of IL-1α.

Stimulated PBMC IL-1α activity is not due to passive IL-1 leakase. LPS-stimulated PBMC were fixed in PFA for 4 hours, incubated in RPMI for 20 hours at 37°C, and separated from the supernatant by centrifugation. As shown in Fig 3, these cells induced large amounts of IL-8 by HUVEC (P < 0.02), whereas the corresponding 20 hour supernatant induced only small amounts of IL-8 (8 ng) compared with control.

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Fig 1. LPS-stimulated and fixed PBMC induce IL-8 secretion by HUVEC. Control represents IL-8 secretion by HUVEC cultured with RPMI alone; ***P = 0.0001, when 10⁶ stimulated PBMC (St. PBMC) were compared with unstimulated PBMC (Unst. PBMC) (mean of six experiments). Inset represents IL-8 secretion by HUVEC cultured with increasing concentrations of soluble IL-1β (mean ± SEM of four experiments). Significant amounts of IL-8 were obtained with concentrations of 100 pg/mL of IL-1. *P < .05, **P < .01, compared with no IL-1β.
medium (6.5 ng, not significant). Thus, it is highly unlikely that passive leakage from stimulated and fixed PBMC during the 20 hours of incubation following fixation accounts for the activity of these cells. When IL-1α was measured in the 20-hour supernatant, it was below the detection limit of 15 pg/mL. However, as depicted in Fig 2, the 20-hour supernatant was below the detection limit of 15 ng, not significant. Thus, it is highly unlikely that passive leakage from stimulated and fixed PBMC was nontsignificantly reduced by pretreatment of HWEC with anti-IL-1α MoAb shown.

Demonstration of IL-1α on the membrane of LPS-stimulated PBMC. Anti-IL-1α MoAb but not anti-IL-1β MoAb inhibits HUVEC IL-8 secretion induced by fixed, LPS-stimulated PBMC. Pretreatment of HUVEC with anti-IL-1α MoAb (+ anti-IL-1 alpha, P < .002) inhibits HUVEC IL-8 secretion induced by stimulated PBMC (St. PBMC) in a similar fashion to pretreatment of HUVEC with IL-1Ra (+ IL-1Ra, P < .002). Pretreatment of HUVEC with anti-IL-1β had no inhibitory effect on HUVEC IL-8 secretion induced by fixed, stimulated PBMC (+ anti-IL-1 beta, P = .3). Mean ± SEM of three experiments are shown.

Effect of D-mannose treatment of PBMC. Stimulated PBMC were treated with D-mannose prior to fixation (St. PBMC + Mann) and in RPMI instead of D-mannose, as a control. After fixation and addition to HUVEC, no decrease in IL-8 secretion was found (data not shown), confirming the specific effect of D-mannose. IL-8 secretion was nearly completely blocked by either D-mannose treatment of PBMC or mannose treated PBMC by FACS analysis. Expression of IL-1α on the membrane of LPS-stimulated PBMC was studied by FACS analysis using anti-IL-1α, anti-IL-1β, or an irrelevant IgG of the same isotype (IgG1). The percentage of labeled monocytes was consistently higher using anti-IL-1α MoAb than anti-IL-1β MoAb or the irrelevant IgG (Table 1). Gating on lymphocytes consistently showed no significant membrane staining using either anti-IL-1α or anti-IL-1β MoAb (data not shown).

Table 1. Direct Demonstration of IL-1α on the Membrane of LPS-Stimulated PBMC

<table>
<thead>
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<th>Experiment no.</th>
<th>Control IgG</th>
<th>Anti-IL-1β</th>
<th>Anti-IL-1α</th>
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<th>Mann+IL-1Ra</th>
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<tr>
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<td>Experiment no. 3</td>
<td>17.5</td>
<td>17.5</td>
<td>20.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
*Percentage of fluorescent cells staining with either anti-IL-1β MoAb, anti-IL-1α MoAb, or an irrelevant IgG1. Results of three different experiments, representative of five experiments, are shown.

Effect of D-mannose treatment of PBMC. Stimulated PBMC were treated with D-mannose prior to fixation (St. PBMC + Mann). IL-8 secretion by HUVEC in these cells was reduced by 90% (P < .05). D-mannose treatment of PBMC induced secretion of over 2,000 pg/mL of IL-8 from HUVEC. A significant decrease (90%, P < .005, Fig 4) in IL-8 synthesis was observed when these cells were treated with D-mannose before fixation. The reduction in IL-8 secretion was not due to D-mannose cell toxicity, because trypan blue exclusion failed to detect nonviable PBMC after a 1-hour incubation with D-mannose. Furthermore, before addition to HUVEC, these PBMC were washed six times to remove any residual D-mannose. In some experiments, LPS-stimulated PBMC were incubated at 4°C for 1 hour in RPMI instead of D-mannose, as a control. After fixation and addition to HUVEC, no decrease in IL-8 secretion was found (data not shown), confirming the specific effect of D-mannose. IL-8 secretion was nearly completely blocked by either D-mannose treatment of PBMC or mannose treated PBMC.
plus pretreatment of HUVEC with saturating concentrations of IL-1Ra (Fig 4).

**IL-1α and IL-1β levels in the supernatants of the D-mannose treated PBMC.** Incubation of PBMC with D-mannose displaced the membrane-associated IL-1 from these experiments were performed with soluble IL-1α (two experiments), 50% inhibition of the IL-8 secretion was observed with IL-1Ra at 6,000 pg/mL for the soluble form and at 30,000 pg/mL for the membrane-bound IL-1, indicating a 50-fold difference. When comparing these experiments with soluble IL-1α (two experiments), 50% inhibition of IL-8 secretion was achieved with IL-1Ra at approximately 1,000 pg/mL for soluble IL-1β, and 30,000 pg/mL for membrane-bound IL-1, indicating a 50-fold difference (Fig 6B).

HUVEC were stimulated for 20 hours with increasing concentrations of either soluble, mature forms of IL-1β, or IL-1α. IL-8 secretion by unstimulated HUVEC was set at 100%. For IL-1β at 25, 50, 100, and 500 pg/mL, there was a 175%, 235%, 255%, and 250% increase whereas for IL-1α stimulation, 127%, 155%, 175%, and 257% increases were measured. These data are means of three wells per IL-1 concentration in one of three representative experiments. Compared with the data shown in Fig 6, the difference between soluble and membrane IL-1 activity on HUVEC cannot be accounted for as differences in soluble IL-1 isoforms.

**Discussion**

In this study, we found that PBMC fixed after LPS-stimulation induced IL-8 secretion by both HUVEC and fibroblasts. This effect was not due to carry-over of LPS from the PBMC cultures because polymyxin B did not reduce IL-8 production. The induction of IL-8 by fixed PBMC on HUVEC or fibroblasts was blocked by the presence of IL-1Ra demonstrating that the activity is due to IL-1. Furthermore, using anti-IL-1α, we observed a complete reduction of this activity, whereas anti-IL-1β MoAb had no inhibitory effect. These results implicate biologically active IL-1α as a form of IL-1 being fixed to the surface of the LPS-stimulated monocyte.

The IL-1 activity of fixed PBMC is due to a membrane-associated form of IL-1α and not to passive leakage of the cytokine. This conclusion is supported by several observations. First, we used a long fixation procedure in PFA, in which we demonstrate no release of biologic activity from these cells even after 20 hours of culture, despite the fact that these cells contain 10 to 15 ng/mL of cytosolic IL-1α. Second, the supernatant from the 20 hour incubation did not contain detectable IL-1α, nor was there a significant reduction using IL-1Ra or anti-IL-1α. Third, in the experiments using mannose to prevent lectin binding, there was no IL-8-inducing activity in the fixed cells despite exposure to millimolar concentrations of D-mannose. Therefore, the reports of membrane leakage after short fixation of PBMC in
PFA is ruled out in these experiments. Immunolocalization studies have shown that IL-1β is exclusively a cytosolic molecule and that IL-1α is at least in part associated with the plasma cell membrane. In agreement with these reports, we observed IL-1α on the surface of stimulated PBMC, using a highly sensitive staining method. Because IL-1 lacks hydrophobic sequences, the mechanism of its membrane-anchoring is unclear. The reader should be mindful that there is no control for the effect of the fixative on the structure of pro-IL-1α. Nevertheless, one possible mechanism could be fixation of IL-1 to its own cell surface receptors, particularly to the type II receptor on monocytes. In such a case, it is difficult to explain how receptor-bound IL-1 would be oriented so that its active site is still available to interact with receptor on the target cells. To rule out this possibility, we blocked IL-1 receptors on PBMC with IL-1Ra and did not observe a decrease in the IL-8-inducing activity, suggesting that membrane-associated IL-1α is not bound to its own receptor.

Posttranslational modifications of the precursor of IL-1α, such as acyl palmytylation, partial mannosylation, or serine phosphorylation, are likely to be involved in the mechanism of association of IL-1α to the membrane. In this study, we found that incubation of the LPS-stimulated PBMC with D-mannose before fixation resulted in a loss of IL-8 induction ability. In D-mannose-treated cultures, IL-1α, but not IL-1β, was found in the supernatants. Although in the present work we did not attempt to study the molecular weight of membrane associated IL-1, these results are similar to several reports showing that membrane-associated IL-1 is the biologically active, 31 kD precursor form of IL-1β. In the present experiments, the amounts of IL-1α displaced by D-mannose were in the range of 50 to 80 pg/10⁶ cells. This is in accordance with previous reports estimating membrane-associated IL-1 to represent approximately 1% to 2% of total cell-associated IL-1α. This relative low amount of membrane associated IL-1α also explains the small but consistent increase in surface staining of IL-1α in stimulated PBMC, whereas there was no increase in IL-1β in the same cells.

These findings also demonstrate a specific effect of the sugar and suggest that a lectin-like mechanism is involved in anchoring IL-1α on the membrane of human monocytes, as previously described for murine macrophages. Another possibility could be the involvement of a D-mannose receptor on the surface of monocytes. Moreover, expression of these receptors is a characteristic of mature macrophages, and membrane-associated IL-1α might also be a
characteristic of rather mature cells of the monocyte lineage because it is mainly expressed on adherent macrophages, or long-term LPS-stimulated macrophages.45,46

Biologically active membrane-associated forms of other important soluble cytokines or growth factors such as TNF,7,11 transforming growth factor-α (TGF-α),40 or platelet activating factor (PAF)46 have been described and appear to have a role in cell to cell interactions in vivo.9,10,11,46 This type of activity is now termed juxtacrine,10 for example, membrane-associated TNF may exert a cytotoxic activity on a target cell,13 or induce polycyclic activation of B lymphocytes during human immunodeficiency virus (HIV) infection.48 In inflammation, PAF, which is coexpressed with P selectin on the surface of endothelial cells, activates PMN and favors their adhesion to the vascular wall through a juxtacrine mechanism.47,49

Functionally, membrane-associated IL-1α may be an important cofactor in antigen presentation to lymphocytes.50 In addition to soluble IL-1β, membrane-associated IL-1α on cells adhering to the endothelium may facilitate PMN extravasation via endothelial IL-8 secretion. In animal models of infection or inflammation, considerably larger amounts of IL-1Ra are required to reduce neutrophil tissue infiltration, compared with the amounts of IL-1Ra required to block exogenously injected IL-1.51 This suggests the existence of a hidden compartment of IL-1, which could be due to membrane-associated IL-1α. In the present studies, a 30-fold excess of IL-1Ra was required to block 50% of the IL-8 secretion induced by membrane-associated IL-1α, as compared with soluble IL-1β. Therefore, based on the amount of IL-1α displaced by D-mannose treatment, stimulation of HUVEC with membrane-associated IL-1α is more potent than soluble IL-1β. These findings may be attributed to differences in the affinity patterns of IL-1α and IL-1β to the endothelium type IL-1 receptor.1 Using soluble IL-1α, however, a fivefold excess of IL-1Ra was still necessary to block 50% of the effect of membrane-associated IL-1, suggesting that membrane-associated IL-1 may be more potent than soluble IL-1.

Unlike IL-1β, IL-1α is usually undetectable in the circulation of patients with endotoxemia or septic shock.21,23 However, our findings as well as previous reports50,54,55 indicate that IL-1α has a highly active role in its membrane-associated form, intracellular. We suggest that membrane-associated IL-1α is important at the tissue level, and could be involved in immunity and inflammation, as well as in cell assembly, differentiation, and growth through juxtacrine interactions.

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