Antiflammin-2 Prevents HL-60 Adhesion to Endothelial Cells and Prostanoid Production Induced by Lipopolysaccharides

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ABSTRACT

We studied the effect of antiflammin-2 (AF-2) on adhesion molecule expression by HL-60 cells and endothelial (ECV304) cells stimulated by lipopolysaccharides (LPSs), and on leukocyte-endothelial cell interaction in an in vitro coculture system. The action of AF-2 on prostanoid production in these experimental conditions was also tested. LPS increased the adhesion molecule expression, such as lymphocyte function-associated antigen-1 and membrane attack complex-1 on HL-60 cells and E-selectin and intercellular adhesion molecule-1 on ECV304 cells. The LPS-stimulated adhesion molecule expression on HL-60/ECV304 coculture system was higher than on HL-60 or ECV304 cultures. LPS also induced HL-60 adhesion to ECV304 monolayer and thromboxane B_2 and prostaglandin E_2 (PGE_2) production in HL-60 culture and PGE_2 in ECV304 culture. Prostanoid production by HL-60/ECV304 cocultures was higher than by simple cultures. AF-2 inhibited the enhancement of adhesion molecule expression induced by LPSs, especially E-selectin. Thus, AF-2 significantly reduced the HL-60 adhesion to endothelial cells stimulated by LPSs. AF-2 also inhibited prostanoid synthesis by ECV304 cells or HL-60/ECV304 coculture challenged by LPSs. In conclusion, AF-2 reduced HL-60 adhesion to endothelial cells, suggesting that it reduces inflammation by blocking leukocyte trafficking and the subsequent eicosanoid production.

The inflammatory response is characterized by changes in vascular permeability and vasodilatation, resulting in edema and infiltration of leukocytes into the tissues involved. Initiation and perpetuation depend mainly on the specific conditions of cellular contact and the transient or repeated synthesis of soluble mediators (Beekhuizen and Van Furth, 1993; Serhan et al., 1996).

Complex interactions of myelomonocytic cells with endothelial cells are key regulators of leukocyte trafficking during both the early and the later phases of inflammation (Luscin-skas and Lawler, 1994). This is controlled by the expression of surface adhesion molecules, among which E-selectins play a key role in the initial attachment of circulating leukocytes to endothelial cell ligands (Picker et al., 1991). CD18 integrins, membrane attack complex (CD11b/CD18, MAC-1), and lymphocyte function-associated antigen (CD11a/CD18, LFA-1) are believed to be largely responsible for the subsequent tightening of adhesion and transendothelial migration of leukocytes through interactions with their endothelial counter-receptor intercellular adhesion molecules (ICAMs) (Butcher, 1991; Springer, 1994). This response ensures appropriate leukocyte-leukocyte and leukocyte-endothelial interactions and is an index of neutrophil activation both in vitro and in vivo (Shalit et al., 1988; Witthaut et al., 1994).

Prostaglandins and thromboxanes are potent and ubiquitous lipid mediators of microcirculatory alterations and inflammatory reactions. These eicosanoids are synthesized from nonesterified arachidonic acid (AA) by cyclooxygenases (COXs). Both isoforms of COX, COX-1 and COX-2, are expressed by endothelial and leukocytic cells and are regulated by stimuli involved in the pathogenesis of inflammation (Goppelt-Struebe, 1995).

On the basis of computer analysis, Miele et al. (1988) designed several synthetic peptides corresponding to the region of highest similarity between urotoglobin and lipocortin-1. Several reviews on lipocortin-1, urotoglobin, and uroglobin- or lipocortin-derived peptides have attempted to assess the role of these peptides in mediating glucocorticoid-induced effects on inflammation (Flower and Rothwell, 1994; Miele et al., 1994; Perretti, 1994; Moreno, 1997). Thus, these nonapeptides were named antiflammins (AFs); AF-1 for urotoglobin-derived peptide (residues 39–47, MQMKKVLDS) and AF-2 for lipocortin-1-derived peptide (residues 246–254, HDMNKVLDL). These peptides inhibited phospholipase A_2 (PLA_2) in vitro and are effective in classic models of inflammation (Miele et al., 1988; Cabrè et al., 1992; Lloret and Moreno, 1992). We have recently suggested that AFs

ABBREVIATIONS: MAC-1, membrane attack complex-1; LFA-1, lymphocyte function-associated antigen-1; ICAM-1, intercellular adhesion molecule-1; AA, arachidonic acid; COX, cyclooxygenase; AF-2, antiflammin-2; PLA_2, phospholipase A_2; FCS, fetal calf serum; LPS, lipopolysaccharide; PGE_2, prostaglandin E_2; TXB_2, thromboxane B_2.
reduced leukocyte migration partly by blocking leukocyte binding to endothelial cells in vivo (Moreno, 1996). However, the mechanism of this action has not been clarified.

Here we report the effect of AF-2 on adhesion molecule expression by leukocyte and endothelial cells, and on leukocytc-endothelial cell interaction in an in vitro coculture system using human umbilical endothelial cells (ECV304) and human myeloblastic HL-60 cells.

Materials and Methods

Reagents. RPMI 1640, medium 199, fetal calf serum (FCS), penicillin G, streptomycin, and trypsin/EDTA were from Life Technologies (Gaithersburg, MD) AF-2 (mol. wt. 1084) was supplied by Bachem Feinchemikalien (Bubendorf, Switzerland). Purify was >95% as determined by high pressure liquid chromatography; amino acid composition and molecular mass were confirmed by mass spectrometry (data from the manufacturer). AF-2 was stored as lyophilized powder under argon at −20°C. Peptide was never stored in solution. Before opening the flask, it was warmed at room temperature to avoid water condensation and AF-2 was dissolved in either phosphate buffer or cell culture medium before use. Lipopolysaccharides (LPSs) from *Escherichia coli* and sialidase (*Vibrio cholerae*, type III) were supplied by Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

Cells and Cell Culture. Human umbilical vein endothelial cells (ECV304) were obtained from the European Collection of Cell Cultures (Salisbury, UK) and were grown in medium 199 with Earle’s salts, 25 mM HEPES supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. Cells were split at confluence 1 to 3 or at higher passages 1 to 2 every 5 to 7 days and plated on gelatin-coated flasks up to passage 20. Human promyelocytic leukemia cells, HL-60 cells, were from American Type Culture Collection (Rockville, MD) (passage 21) and cultivated in RPMI 1640 with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. These cells were maintained at 2 × 10⁶ to 1 × 10⁶ cells/ml in RPMI 1640 supplemented with 1-glutamine, antibiotics, and 10% FCS.

Measurement of Prostanoid Production. An aliquot of culture medium (0.25 ml) was acidified with 1 ml of 1% formic acid. Prostanoids were extracted in ethyl acetate (5 ml) and, after the aqueous phase was discarded, the organic phase was evaporated in a stream of nitrogen. Prostaglandin E₂ (PGE₂) and thromboxane B₂ (TxB₂) levels in the medium were determined by enzyme immunoassay (Cayman Chemical Co., Ann Arbor, MI), following the manufacturer’s protocols.

Analysis of Adhesion Molecules. HL-60 and ECV304 cells previously scraped off were pelleted and resuspended with diluted specific primary antibody solution. HL-60 cells were incubated with antibodies for LFA-1 or MAC-1 (Boehringer Ingelheim, Ingelheim, Germany; dilution 1:50). ECV304 cells were examined for ICAM-1 (1:10) or E-selectin (1:25) (R&D Systems, Minneapolis, MN). After 30 to 45 min at 4°C, cells were washed twice with 1% bovine serum albumin in phosphate-buffered saline, and fluorescein-labeled goat anti-mouse antibody (Boehringer Ingelheim) was added for 30 min at 4°C. The cells were then washed with PBS, fixed in 1% paraformaldehyde/PBS, and analyzed with a fluorescein-activated cell sorter analyzer (FACScan; Becton Dickinson, Mountain View, CA) as described in Hauser et al. (1993). Antibody binding was determined as mean fluorescence intensity after gating for cells by their characteristic forward and side scatter properties. The results are in relative fluorescence units (RFU). RFU = FUexperimental − FUisotype × 100/ FUcontrol − FUisotype, where FUexperimental and FUcontrol are the fluorescence intensities of stimulated and control cells, respectively, and FUisotype is the fluorescence intensity of class-matched irrelevant antibody.

Assay for HL-60 Adhesion to Endothelial Cells. ECV304 cells were plated and grown to confluence. Therewith, endothelial cells were washed and incubated with HL-60 (2 × 10⁶ cells/well) in the presence of LPS and allowed to attach at 37°C. Nonadherent cells were then removed and the cells were washed three times with cell culture. Adherent cells were fixed in 3% phosphate-buffered formalin. The HL-60 cells adhered to ECV304 cells were counted under a phase-contrast microscope (Nikon, Melville, NY) using a digital photograph system (Sony, Tokyo, Japan), which measured an area of 0.16 mm²/field.

Statistics and Data Analysis. Results are expressed as means ± S.E.M. Differences between control and treated cultures were assessed by analysis of variance test, Student’s *t* test, or one-way analysis of variance followed by the least significant difference test when appropriate.

Results

LPS Stimulates TxB₂ and PGE₂ Production. Determination of prostanoid synthesis in the supernatant of LPS-stimulated cells revealed high levels of TxB₂ and PGE₂ in HL-60 cultures, whereas only PGE₂ levels were enhanced in ECV304 cultures (Figs. 1 and 2).

When HL-60 and ECV304 cells were stimulated in a coculture system, TxB₂ production increased significantly. This increase was proportional to the time of coincubation as shown in Fig. 1. Thus, TxB₂ levels in coculture were extremely high after 4 h. PGE₂ levels in coculture supernatant were higher than PGE₂ produced by ECV304 or HL-60 in simple cultures (Fig. 2).

LPS Induces Adhesion Molecule Expression on HL-60 and ECV304 Cells. Besides these changes in the AA cascade, the expression of adhesion molecules increased. Thus, constitutive LFA-1 and MAC-1 in HL-60 and ICAM-1 and E-selectin in ECV304 cells markedly increased during LPS stimulation. MAC-1 and E-selectin expression were

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**Fig. 1.** TxB₂ synthesis in ECV304 culture (□), HL-60 culture (■), or coculture of endothelial cells (ECV304) with HL-60 cells (●) stimulated by LPS (1 μg/ml). Values are mean ± S.E.M. of the three experiments performed in triplicate. *P < 0.05 versus nonstimulated cells.
maximal 1 h after LPS stimulation, whereas ICAM-1 expression was maximal 8 h after LPS incubation (Table 1). Moreover, the expression of these adhesion molecules significantly increased in LPS-stimulated HL-60/ECV304 coculture. Thus, LFA-1 and ICAM-1 increased by 131 and 145%, 8 h after LPS challenge, whereas MAC-1 and E-selectin increased to 217 and 243%, respectively, 4 h after LPS stimulation.

**LPS Induces HL-60 Adhesion to ECV304 Monolayer.** The enhancement of adhesion molecule expression induced by LPS was associated with an increase in HL-60 adhesion to ECV304 cells, which reached a plateau at 4 h. Figure 3 shows the time course of HL-60 adhesion to ECV304 cells.

**AF-2 Inhibits the Enhancement of Adhesion Molecule Expression, Cell Adhesion, and Eicosanoid Production Stimulated by LPS.** AF-2 inhibited the LPS-stimulated increase of adhesion molecule expression. Thus, LFA-1 and MAC-1 were reduced by 25 and 17%, respectively, in HL-60 cells, whereas ICAM-1 and E-selectin were reduced by 15 and 50%, respectively, in ECV304 cultures (Table 2). The LPS-induced expression of these adhesion molecules in coculture was even more inhibited, especially that of ICAM-1 (43%) and E-selectin (56%) (Table 2). Moreover, Fig. 4 shows that AF-2 induced a dose-dependent inhibition of MAC-1 and E-selectin expression induced by LPS in HL-60/ECV304 cocultures.

These effects of AF-2 in adhesion molecule expression can be correlated with the effect of the nonapeptide on HL-60 adherence to the endothelial monolayer. Thus, AF-2 (10–100 μM) significantly ameliorated the rate of HL-60 adherence to the ECV304 monolayer in a dose-dependent manner (Fig. 3). AF-2 concentrations that markedly reduced adhesion molecule expression and HL-60 adherence to ECV304 induced a significant impairment of LPS-stimulated TxB2 and PGE2 synthesis (Table 3), especially in HL-60/ECV304 cocultures.

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**FIG. 2.** PGE2 synthesis in ECV304 culture (□), HL-60 culture (●), or in coculture of ECV304 and HL-60 cells (▲) stimulated by LPS (1 μg/ml). Values are mean ± S.E.M. of three experiments performed in triplicate. *P < 0.05 versus nonstimulated cells.

**Fig. 3.** Time course of HL-60 adhesion to endothelial (ECV304) monolayer stimulated by LPS (1 μg/ml). HL-60/ECV304 cocultures were pretreated with AF-2 (1 μM, ■; 10 μM, □; 100 μM, ▼) 30 min before LPS stimulation. Effect of AF-2 was compared with cocultures nonstimulated (●) and stimulated by LPS in absence of AF-2 (▴). Values are mean ± S.E.M. of three determinations performed in triplicate.

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**TABLE 1.**

<table>
<thead>
<tr>
<th></th>
<th>Relative Fluorescence Units</th>
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<tr>
<td></td>
<td>LFA-1</td>
</tr>
<tr>
<td><strong>HL-60</strong></td>
<td></td>
</tr>
<tr>
<td>LPS (1 h)</td>
<td>123 ± 9*</td>
</tr>
<tr>
<td>LPS (4 h)</td>
<td>120 ± 11*</td>
</tr>
<tr>
<td>LPS (8 h)</td>
<td>131 ± 12*</td>
</tr>
<tr>
<td>ECV304</td>
<td></td>
</tr>
<tr>
<td>LPS (1 h)</td>
<td>N.D.</td>
</tr>
<tr>
<td>LPS (4 h)</td>
<td>N.D.</td>
</tr>
<tr>
<td>LPS (8 h)</td>
<td>N.D.</td>
</tr>
<tr>
<td><strong>HL-60/ECV304</strong></td>
<td></td>
</tr>
<tr>
<td>LPS (1 h)</td>
<td>156 ± 11*</td>
</tr>
<tr>
<td>LPS (4 h)</td>
<td>176 ± 12*</td>
</tr>
<tr>
<td>LPS (8 h)</td>
<td>185 ± 11***</td>
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N.D., not determined.

*P < 0.05 versus nonstimulated cultures.

**P < 0.05 versus adhesion molecule expression by HL-60 or ECV304 cultures.**
TABLE 2
Effect of AF-2 on adhesion molecule expression on HL-60 and ECV304 cells challenged with LPS
HL-60 (1 x 10^6 cells/ml), ECV304 (1 x 10^6 cells/25 cm^2 culture dish), or HL-60/ECV304 coculture in the above-described conditions were pretreated with AF-2 (100 μM) 30 min before LPS (1 μg/ml) stimulation for 4 h. Adhesion molecule expression was measured as described under Materials and Methods. Values are mean ± S.E.M. of six determinations from two experiments.

<table>
<thead>
<tr>
<th>Relative Fluorescence Units</th>
<th>HL-60</th>
<th>ECV304</th>
<th>HL-60/ECV304</th>
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</thead>
<tbody>
<tr>
<td>LFA-1</td>
<td>121 ± 12</td>
<td>N.D.</td>
<td>173 ± 13</td>
</tr>
<tr>
<td>MAC-1</td>
<td>215 ± 9</td>
<td>N.D.</td>
<td>272 ± 11</td>
</tr>
<tr>
<td>E-selectin</td>
<td>N.D.</td>
<td>120 ± 9</td>
<td>196 ± 12</td>
</tr>
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</table>

In presence of AF-2
| LFA-1                       | 91 ± 5    | N.D. | 122 ± 7*   |
| MAC-1                       | 179 ± 9   | N.D. | 205 ± 9*   |
| E-selectin                  | N.D. | 102 ± 3 | 111 ± 6*  |

N.D., not determined.

TABLE 3
Effect of AF-2 on TxB2 and PGE2 production by HL-60, ECV304, and HL-60/ECV304 cocultures stimulated by LPS
HL-60 (1 x 10^6 cells/ml), ECV304 (1 x 10^6 cells/25 cm^2 culture dish), or HL-60/ECV304 coculture in the above-described conditions were pretreated with AF-2 (10–100 μM) 30 min before LPS (1 μg/ml) stimulation for 8 h. TxB2 and PGE2 were measured as described under Materials and Methods. Values are mean ± S.E.M. of six determinations from two experiments.

<table>
<thead>
<tr>
<th></th>
<th>TxB2 (pg/ml)</th>
<th>PGE2 (pg/ml)</th>
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<tbody>
<tr>
<td>HL-60</td>
<td>1453 ± 121</td>
<td>2153 ± 164</td>
</tr>
<tr>
<td>HL-60 + AF-2 (10 μM)</td>
<td>1376 ± 103</td>
<td>1906 ± 112</td>
</tr>
<tr>
<td>HL-60 + AF-2 (100 μM)</td>
<td>1263 ± 136</td>
<td>1783 ± 152</td>
</tr>
<tr>
<td>ECV304</td>
<td>N.D.</td>
<td>1976 ± 135</td>
</tr>
<tr>
<td>ECV304 + AF-2 (10 μM)</td>
<td>N.D.</td>
<td>1653 ± 86</td>
</tr>
<tr>
<td>ECV304 + AF-2 (100 μM)</td>
<td>N.D.</td>
<td>1123 ± 85*</td>
</tr>
<tr>
<td>HL-60/ECV304</td>
<td>4121 ± 396</td>
<td>8565 ± 372</td>
</tr>
<tr>
<td>HL-60/ECV304 + AF-2 (10 μM)</td>
<td>3754 ± 198</td>
<td>6756 ± 176*</td>
</tr>
<tr>
<td>HL-60/ECV304 + AF-2 (100 μM)</td>
<td>2633 ± 214*</td>
<td>4361 ± 273*</td>
</tr>
</tbody>
</table>

N.D., not determined.

Discussion
The anti-inflammatory activity of AFs has been assessed using several experimental models of inflammation, such as carrageenan rat paw edema (Miele et al., 1988; Ialenti et al., 1990; Lloret and Moreno, 1992), phorbol ester-induced ear edema (Lloret and Moreno, 1995), and oxazolone-induced contact hypersensitivity (Cabré et al., 1992). However, the ability of AFs to inhibit PLA2 or other elements involved in contact hypersensitivity (Cabré et al., 1992). However, the ability of AFs to inhibit PLA2 or other elements involved in
inflammation was questioned. Thus, AFs were not found to significantly inhibit Naja naja naja PLA₂, porcine pancreatic PLA₂, or human synovial fluid PLA₂ (Cabrè et al., 1992). Moreover, AFs did not reduce AA release stimulated by several agonists or modify the cyclooxygenase pathway (Moreno, 1996). However, previous results suggested that the anti-edematous effect and the inhibition of cell influx and eicosanoid production by AFs could be caused by the action of nonapeptides on leukocyte traffic (Lloret and Moreno, 1995).

In this way, N-formyl-Met-Leu-Phe caused profound neutropenia. However, pretreatment of mice with sialidase, an enzyme used to remove carbohydrate moieties, which are counter-receptors for selectins, or DF-2 inhibited the neutropenia caused by the intravenous injection of the formyl peptide (Moreno, 1996).

Endothelial activation primarily attracts leukocytes to the inflammatory site. Qualitative and quantitative functional alteration in the β2-integrins (CD11/CD18) on the leukocyte surface are also induced after cell stimulation (Zimmerman et al., 1992). We determined the effect of AF-2 on the expression of adhesion molecules involved in leukocyte rolling, adhesion, and migration, and as a result, leukocyte influx. LFA-1 and MAC-1 expression quantitatively increased in HL-60 and the ICAM-1 and selectin were enhanced in ECV304 cells after LPS stimulation. The expression of these adhesion molecules was markedly increased in HL-60/ECV304 cocultures. In agreement with previous results reported by Koll et al. (1997), who demonstrated that constitutively present ICAM, vascular cell adhesion molecule-1, and platelet endothelial adhesion molecule-1 were increased during the coculture of HL-60 and epithelial endothelial cells stimulated by phorbol ester. All these biological events were affected by AF-2 in our experimental conditions, and E-selectin expression even more altered. Thus, AF-2 has inhibitory effects on activation-induced changes in adhesion molecule expression as was also reported for glucocorticoids (Cronstein et al., 1992; Filep et al., 1997). This could account for the impairment of HL-60 adherence to ECV304 monolayers stimulated by LPS in the presence of AF-2. Our data are in agreement with recent results reported by Zhou et al. (2000) who observed that AF-1 and AF-2 attenuated changes in L-selectin and CD11/CD18 expression evoked by platelet-activating factor or interleukin-8. Moreover, these authors showed that AFs markedly decreased the polymorphonuclear adhesion to LPS-stimulated coronary artery endothelial cells. Although the effect of AF-2 on HL-60 adhesion to endothelial cells can be associated with the effect on molecule adhesion expression, the possibility that the nonapeptides may cause functional changes in adhesion molecules cannot be excluded.

On the other hand, we showed that AF-2 reduced the PGE₂ synthesis induced by LPS in ECV304 cultures, but especially in HL-60/ECV304 cocultures. In vivo, cellular interactions lead to major changes of the resulting local prostanoied spectrum synthesized in a given tissue compared with individually cultured cell types (Marcus, 1988). Crossover exchange of soluble mediators and probably direct cell contact determine the conditions for specific activation and expression of biosynthetic enzymes (Clark and Brugge, 1995; Dolecki et al., 1995). Moreover, transcellular prostanooid synthesis may substantially modify the specific biosynthetic capacity of cells (Marcus, 1988). Thus, in our experimental conditions, AF-2 significantly inhibited TxB₂ and PGE₂ production in HL-60/ECV304 cocultures stimulated by LPS. Interestingly, AF-2 also inhibited HL-60 adherence to ECV304 cells. Taken together that sialidase treatment reduced HL-60 adherence to ECV304 cells, that it inhibited significantly prostanooid production, and that AA release and the subsequent prostanooid production were triggered by cell adhesion (Lloret and Moreno, 1996), we can consider the hypothesis that prostanooid production induced by LPS in coculture could be the result of LPS stimulation and cell-cell interaction. Thus, the effect of AF-2 on TxB₂ and PGE₂ synthesis by cocultures may be a consequence of the disturbance of HL-60 adhesion to ECV304 cells.

These data could explain the effects of AFs on cell influx and eicosanoid production observed in in vivo experimental models of acute inflammation (Lloret and Moreno, 1995). Recently, we proposed that leukocyte infiltration is involved in eicosanoid synthesis during the inflammatory process (Sánchez and Moreno, 1999). These results correlated the effect of AF-2 on adhesion molecule expression, HL-60 adherence to ECV304 monolayers, and TxB₂ and PGE₂ synthesis in these experimental conditions. In conclusion, antiflammins may be a novel therapeutic approach for host defense and inflammation by blocking leukocyte trafficking and the subsequent eicosanoid production.

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