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Effects of Antirheumatic Drugs on Adhesiveness of Endothelial Cells and Neutrophils

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ABSTRACT. Because disease-modifying antirheumatic drugs might exert part of their effects on adhesion of polymorphonuclear neutrophils (PMN) to endothelial cells, this being the first step for PMN migration to inflammatory lesions, we evaluated such drug effects *in vitro*. Gold sodium thiomalate (GSTM) impaired the ability of interleukin 1β (IL-1β)-stimulated human umbilical vein endothelial cells (HUVEC) to express E-selectin and to bind PMN but had no effect on the expression of intercellular adhesion molecule 1 (ICAM-1) or on hyperadhesivity of *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated PMN. Auranofin (AF) interacted with HUVEC and PMN adhesiveness but in opposite directions: this drug hampered IL-1β-induced HUVEC hyperadhesiveness and expression of E-selectin and intercellular adhesion molecule 1, but augmented PMN adherence and CD18 expression. The net effect of auranofin was a reduction of cytokine-driven adhesiveness and enhancement of formylpeptide-induced adhesion. Salazopyrin did not affect HUVEC or PMN adhesiveness or E-selectin and intercellular adhesion molecule 1 expression. Thus, the gold-containing drugs modulated HUVEC and PMN adhesiveness by different mechanisms but ones involving surface adhesion molecules. BIOCHEM PHARMACOL **56**;12:1661–1669, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. antirheumatic agents; cell adhesion; cell adhesion molecules; neutrophils; vascular endothelium

EC¶ and leukocytes provide adhesion molecules, necessary for migration of leukocytes into the tissues [1–3]. These structures, pivotal for the inflammatory responses in various disorders, e.g. systemic vasculitis and RA, can be activated by means of inflammatory mediators. One such class of stimuli, e.g. histamine, LTB_4 and platelet activating factor, confers a rapidly emerging hyperadhesiveness that is evident within minutes [4–7]. A second class of stimuli, leading to a slowly emerging hyperadhesiveness of EC which becomes evident after some hours, is represented by lipopolysaccharide and cytokines, e.g. IL-1β or TNFα [1–2, 8–11].

Many disease-modifying antirheumatic drugs, used for treatment of RA, have been reported to affect functional responses of EC and PMN. Gold compounds, e.g. AF, interfere with PMN chemotaxis and leukotriene production *in vitro* [12–14], and GSTM has been reported to alter endothelial E-selectin expression and mRNA levels [15–16]. Sulphasalazine, and its metabolites sulphapyridine and 5-aminosalicylic acid, decrease PMN superoxide production, lyzosomal enzyme release and leukocyte adhesion molecule upregulation in response to a variety of agonists [17–19]. Through such effects, antirheumatic drugs may modulate the traffic of leukocytes into inflamed areas and mediate antiinflammatory actions. However, very little has been reported on the adhesive interaction between PMN and HUVEC in relation to these disease-modifying antirheumatic drugs.

The aim of this study was to evaluate the effect of the antirheumatic drugs AF, GSTM and sulphasalazine upon the adhesion between PMN and endothelial cells in vitro. When a significant drug effect was noted in a screening system of PMN and EC together (the combined system), the drug was tested on each cell type alone in order to evaluate if the effect of the drug was primarily on the PMN or on the EC. Moreover, using flow cytometry, such effects were also analyzed with regard to endothelial cell and PMN expression of surface adhesion molecules.

MATERIALS AND METHODS

Chemicals

fMLP, HSA (essential fatty acid-free) and sulphasalazine were obtained from Sigma Chemical Co. IL-1β was from Boeringer Mannheim GmBH. AF was from SmithKline

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¶ *Abbreviations:* AF, auranofin; BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethylester; EC, endothelial cells; FITC, fluorescein isothiocyanate; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GSTM, gold sodium thiomalate; HBSS, Hank's balanced salt solution; HSA, human serum albumin; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon γ; IL-1β, interleukin 1β; LTB_4, leukotriene B_4 (5(S),12(R)-dihydroxy-6,8,10,14-eicoatetraenoic acid); PE, phycoerythrin; PMN, polymorphonuclear neutrophil granulocyte; RA, rheumatoid arthritis; and TNFα, tumor necrosis factor α.

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Beecham and GSTM from Rhone–Poulenc Rorer. Endothelial growth factor was from Collaborative Research Inc. Fetal calf serum (FCS), HBSS and other tissue culture media and chemicals were from GIBCO. BCECF/AM was from Molecular Probes. PBS and formaldehyde were from Apoteksbolaget. Ninety-six-well microtiter plates (Immunolon^{TM}) were from Dynatech Lab. Tissue-culture plasticware were from Nunc and Percoll from Pharmacia Fine Chemicals.

Antibodies

Murine (unlabeled) monoclonal antibodies directed against E-selectin (H18/7), PE-conjugated murine monoclonal antibodies directed against ICAM-1 (LB-2) and murine monoclonal isotype-matched nonbinding control antibodies were from Becton Dickinson Immunocytometry Systems. FITC-conjugated rabbit anti-mouse immunoglobulin antibodies and murine monoclonal FITC-conjugated $F(ab')_2$ fragments directed against CD18 (60.3) were from DAKO A/S.

Culture of HUVEC

EC were obtained from human umbilical veins by treatment with 0.2% collagenase as described [5, 6]. Cells were grown in a culture medium in tissue-culture flasks precoated with gelatin. HUVEC were trypsinized when confluent growth was obtained, resuspended in medium and either seeded into new culture flasks (for a maximum of two passages, once per week) or seeded into 96-well microtiter plates. When new monolayers were established in the culture wells, they were used for adherence assessments. Thus, in this study HUVEC had never been passed more than twice. We have reported that hyperadhesiveness to LTB_4, as well as to platelet activating factor, phorbol myristate acetate and thrombin, declines with an increasing number of passages [5]. Monolayers were identified as homogenous HUVEC by using an indirect immunofluorescence staining for factor VIII-related antigen. These HUVEC did not express CD18 upon immunofluorescence staining with the mAb 60.3 to the common β integrin [5].

Neutrophil Preparations

PMNs were obtained from healthy donors by a one-step discontinuous Percoll gradient centrifugation as described [20]. The purified neutrophils (>95% purity and viability) were resuspended in HBSS with 0.4% of HSA. PMNs, to be stained with the fluorescent probe BCECF/AM, were resuspended in Ca^{2+}- and Mg^{2+}-free HBSS and incubated with BCECF/AM (2 μM) for 20 min at 37°. PMN were washed twice and transferred to regular HBSS (with Ca^{2+} and Mg^{2+}) with 0.4% of HSA (assay buffer) [4, 21].

In Vitro PMN Adherence Assay

Adherence of PMN to HUVEC monolayers or to an albumin-coated plastic surface was assessed as described [4, 21]. Four different basic protocols for adherence were used in this report. In the first two, HUVEC were stimulated with an agonist (IL-1β) that primarily causes hyperadhesiveness in HUVEC, whereas we used an activator of the PMN (fMLP) in the third and fourth protocols. In the first protocol or the combined system, used for screening purposes, HUVEC monolayers were incubated with an antirheumatic drug (or its solvent) for 30 min, and then stimulated with IL-1β for 3 hr. Subsequently, wells were washed twice and the antirheumatic drug (or the solvent) was added again. Finally, 2×10^5 BCECF-stained PMN (in assay buffer) were added to each well and adherence was analyzed after 10 min. The second protocol was a variation on the first, in that the antirheumatic drug was not re-added after washes; consequently, only HUVEC, but not neutrophils, were treated with the drug. In the third protocol, we assessed drug effects when neutrophils alone were stimulated—HUVEC monolayers (in 96-well polystyrene microtiter plates) were incubated with antirheumatic drugs for 30 min, then BCECF-stained PMN were added to each well and allowed to settle for 10 min; subsequently, an agonist for PMN (fMLP) was added (or assay buffer alone for the assessment of spontaneous adherence) and adherence was analyzed after 15 min, which is the optimal time point for detection of PMN hyperadhesiveness to this agonist [5]. Common to all these three protocols, wells were then filled with 37° assay buffer, sealed with tape, inverted and centrifuged at 17 *g* for 5 min to remove nonadherent PMN. The wells were washed gently with assay buffer, filled with 50 μL of lysis buffer (0.1% SDS in H_2O with 50 mM Tris buffer) and fluorescence determined in a microtiter plate fluorometer (Fluoroskan II, Labsystems).

In the fourth protocol, we assessed drug effects on PMN adherence to an albumin-coated plastic surface in polystyrene microtiter plates. BCECF-stained PMN (in assay buffer) were pretreated with antirheumatic drug for 30 min at 37°, subsequently added to each well, and allowed to settle for 10 min at 37°. Then, PMN were stimulated by adding fMLP. Removal of nonadherent PMN and assessment of adherent PMN was performed after 15 min, as stated above.

Test results were expressed as percent increase of adherent cells determined as: [(fluorescence retained by test wells—fluorescence retained by control wells)/fluorescence retained by standards ($=2 \times 10^5$ PMN)] × 100. Tests were run in at least triplicate. The evaluation of the adherence assay with regard to possible interfering factors, e.g. PMN aggregation, has been described previously [5, 21].

Cell Viability

All HUVEC monolayers were inspected prior to and following drug and agonist treatment in order to assess possible injury. None of the drugs tested here or conditions conferred cell damage, measured as release of ^{51}Cr (Bratt J and Palmblad J, unpublished results, and [22]), or lactic dehydrogenase from HUVEC. However, AF caused a dose-

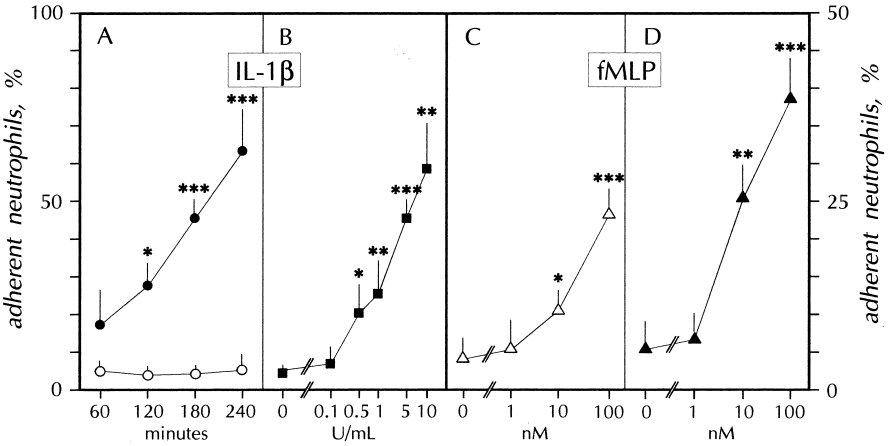

FIG. 1. PMN adhesion to HUVEC or to an albumin-coated plastic surface in response to IL-1β or fMLP. Panel A. Adhesion of PMN to HUVEC monolayers (as given on the left *y*-axis) that had been treated with buffer alone (○) or activated with IL-1β 5 U/mL (●) for the time period indicated on the *x*-axis. PMNs were allowed to adhere to HUVEC for 10 min. Panel B. Dose-response relationships for adherence of PMN to HUVEC monolayers (as given on the left *y*-axis) that had been treated with the indicated IL-1β concentration for 180 min. Panel C. PMN adherence to HUVEC monolayers (as given on the right *y*-axis). In this set of experiments, PMN were allowed to settle on HUVEC for 10 min; subsequently fMLP, at indicated concentrations, were added and adherence was followed for 15 min. This time period has previously been found to be optimal for detection of maximal adherence following fMLP stimulation. Panel D. PMN adhesion to albumin-coated plastic (as given on the right *y*-axis). PMN were incubated with fMLP for 15 min. Data points indicated represent means and SEM for at least five separate experiments performed in triplicate. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared to buffer-treated controls.

dependent contraction of the EC at concentrations of 2 μg/mL or more, similar to what has been noted after additions of various stimuli, e.g. thrombin. Membrane damage, measured as a trypan blue discoloration of >10% of the EC was observed at auranofin concentrations of 10 μg/mL or more.

EC and PMN Expression of Adhesion Molecules

Analysis of HUVEC expression of adhesion molecules was performed as described by Nortamo *et al.* [23], with some modifications. Briefly, HUVEC grown in culture flasks were incubated with GSTM, AF or sulphasalazine in medium containing HBSS with 1% FCS, or with medium alone for 30 min at 37°. Subsequently, HUVEC were incubated with IL-1β or medium for 3 hr at 37°. After trypsinization (in order to prepare single-cell suspensions), washing and resuspension in PBS, HUVEC were incubated with either an anti-E-selectin mAb (H18/7) or a control mAb for 30 min at 4°, and, after subsequent washings, incubated with a secondary FITC-conjugated antibody for 30 min at 4°. Alternatively, HUVEC were incubated with either a PE-conjugated anti-ICAM-1 mAb (LB-2) or a PE-conjugated control mAb for 30 min at 4°. Finally, HUVEC were washed and fixed with 1% formaldehyde. When the surface expression of CD18 on PMN was analyzed, we incubated freshly isolated PMN with AF or medium for 30 min. After washing, PMN were incubated with FITC-conjugated mAb 60.3 for 20 min at 4°. Subsequently PMN were fixed with 1% formaldehyde. Fluorescence was analyzed in a Becton Dickinson FACScan flow cytometer.

Statistical Analysis

Data were analyzed using the Statistica™ software package and Student's two-tailed *t*-test for paired samples. All statistical analyses were based on at least three separate experiments, performed in triplicate, with PMN and HUVEC from different donors.

RESULTS

Spontaneous and Stimulated Adhesion of HUVEC and PMN

The spontaneous adhesion of PMN to HUVEC ranged from 3.4 to 5.4% when followed over a period of 15 to 180 min (Fig. 1A), and PMN adhesion to HSA-coated plastic varied from 5.3 ± 2.1% (mean ± SEM) for 15 min of incubation to 13.0 ± 2.8% at 60 min, when the cells were treated with buffer alone. When HUVEC were stimulated with IL-1β, the adhesiveness for PMN increased in a time- and dose-related fashion, with a 9.7-fold increase when we used 5 U/mL of IL-1β for 3 hr (Fig. 1A and B). Based on the data in Fig. 1A and B, the analyses of drug effects were performed with 5 U/mL of IL-1β and with a stimulation period of 180 min.

When fMLP was added to PMN, either resting on HUVEC or on an albumin-coated plastic surface, the adherence increased in a dose-related manner. fMLP (at 100 nM) resulted in a 5.9-fold increase in PMN adherent to HUVEC and a 3.9-fold increase in PMN adherent to plastic, compared to unstimulated controls (Fig. 1C and D). The peak response for fMLP occurred after 15 min, whereas adherence declined with further incubation periods (data

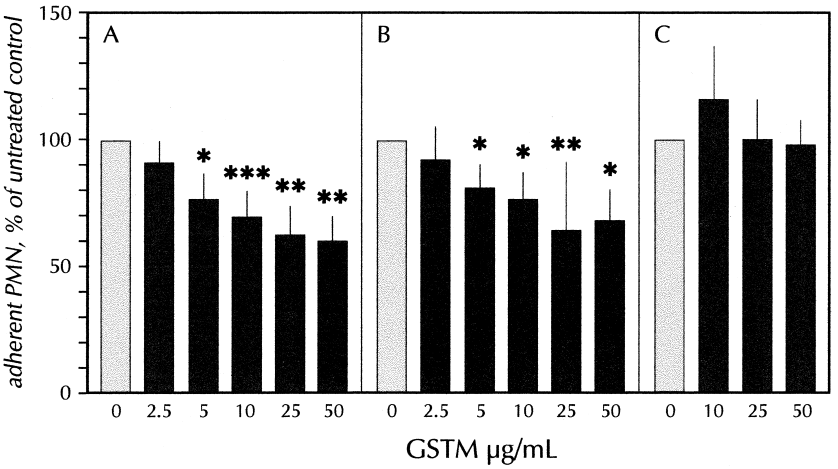

FIG. 2. Effects of GSTM on adhesion of PMN to HUVEC monolayers. Panel A. The combined system (the 1st protocol): HUVEC were treated with GSTM (at indicated concentrations) for 30 min prior to and during subsequent activation with 5 U/mL of IL-1β for 180 min. Then PMN were added (and thus exposed to GSTM during the ensuing adherence) to HUVEC for 10 min. Panel B. HUVEC alone (and not PMN) were treated with GSTM for 30 min prior to and during subsequent activation with 5 U/mL of IL-1β for 180 min. After washing, PMN were added and allowed to adhere to HUVEC for 10 min. This assay is described in the Methods section as the 2nd protocol. Panel C. To HUVEC pretreated with GSTM for 30 min, PMN were added and thus exposed to GSTM for 10 min. Subsequently, PMN were stimulated with 100 nM fMLP for 15 min (according to the 3rd protocol in the Methods section). Data points indicated represent means and SEM for at least three separate experiments performed in triplicate. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to buffer-treated controls.

not shown) [5]. The optimal fMLP concentration for induction of PMN adherence was 100 nM, whereas lower or higher concentrations were associated with lower or only minor further increases of adherence responses ([5] and data not shown). HUVEC did not react with hyperadherence when incubated with fMLP for 5–60 min [21].

Effects of Antirheumatic Drugs on Agonist-induced PMN Adhesion to HUVEC or Albumin-coated Plastic

The effects of antirheumatic drugs on the adhesiveness of HUVEC and PMN were tested in a basic three-step model. First, we tested the effect when both HUVEC and PMN were treated with the antirheumatic drug (the first and third protocols). If any significant effect was noted, the drug was tested on HUVEC or PMN alone (the second and fourth protocols). Modifications of these protocols are given below.

Treatment of the combined HUVEC and PMN system with GSTM reduced the IL-1β-induced hyperadhesivity significantly (Fig. 2A). The effect of GSTM was dose-related with a significant inhibition occurring at 5 µg/mL of GSTM ($P = 0.026$). The maximal inhibitory effect was noted with 50 µg/mL of GSTM, leading to a 40.6% reduction in the number of adhering PMN. Because this adherence protocol did not disclose if GSTM acted on the PMN, HUVEC or both, each cell type was subsequently treated separately with the drug prior to stimulation. The inhibitory effect of GSTM on IL-1β-induced hyperadhesiveness persisted when HUVEC alone (Fig. 2B), but not when PMN alone (data not shown), were treated with GSTM. Treatment of PMN alone with GSTM had no significant effect on fMLP-induced PMN adhesion to either HUVEC (Fig. 2C) or an albumin-coated plastic surface (Fig. 3A). These results pointed to an effect of GSTM on the EC but not on the PMN.

Treatment of the combined system of HUVEC and PMN

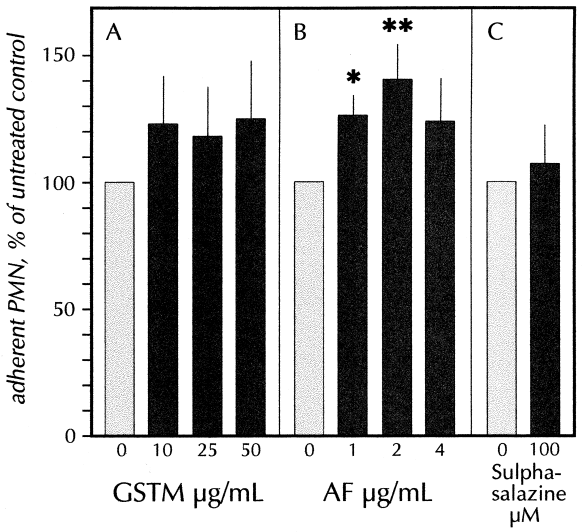

FIG. 3. Effects of GSTM, AF or sulphasalazine on adhesion of PMN to an albumin-coated plastic surface. Panel A: PMN were treated with GSTM (at indicated concentrations) for 30 min, subsequently added to the albumin-coated plastic surface, and stimulated with 100 nM fMLP for 15 min (according to the 4th protocol in the Methods section). Panel B: PMN were treated with AF. All other conditions were identical to panel A. Panel C: PMN were treated with sulphasalazine. All other conditions were identical to panel A. Data points indicated represent means and SEM for at least three separate experiments performed in triplicate. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to buffer-treated controls.

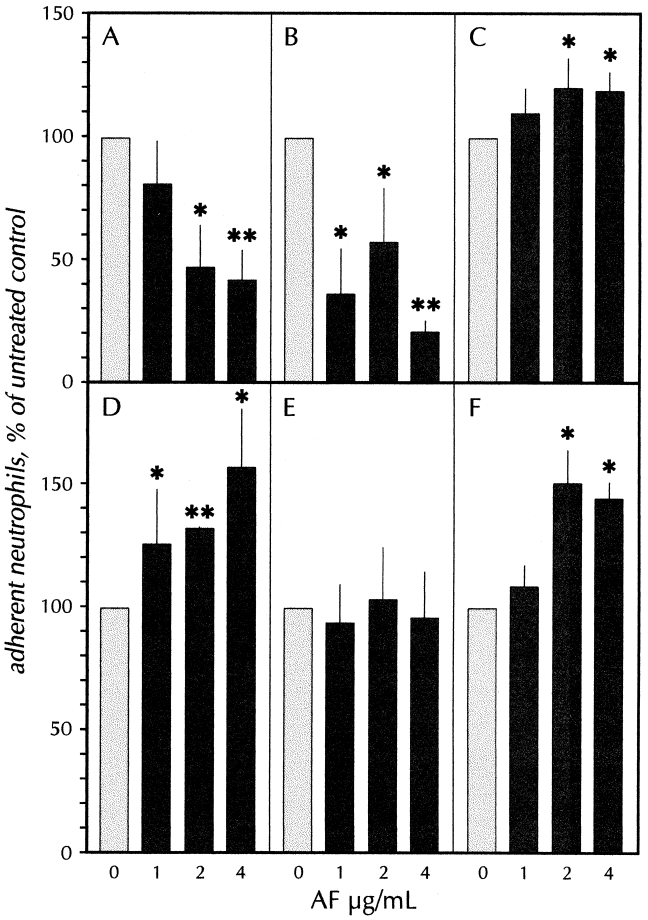

FIG. 4. Effect of auranofin (AF) on adhesion of PMN to HUVEC monolayers. Panel A: The combined system: HUVEC were treated with AF (at indicated concentrations) for 30 min prior to and during subsequent activation with 5 U/mL of IL-1β for 180 min. Then PMN were added (and thus exposed to AF during the ensuing adherence) to HUVEC for 10 min. Panel B: HUVEC alone (and not PMN) were treated with AF for 30 min prior to and during subsequent activation with 5 U/mL of IL-1β for 180 min. After washing, PMN were added and allowed to adhere to HUVEC for 10 min. This assay is described in the Methods section as the 2nd protocol. Panel C: PMN alone (and not HUVEC) were treated with AF for 30 min. Subsequently, PMN were added to HUVEC monolayers that had been stimulated with 5 U/mL of IL-1β for 180 min. Panel D: To HUVEC pretreated with AF for 30 min, PMN were added and thus exposed to AF for 10 min. Subsequently, PMN were stimulated with 100 nM fMLP for 15 min (according to the 3rd protocol in the Methods section). Panel E: HUVEC alone (and not PMN) were treated with AF for 30 min. After washing, PMN were added. After 10 min, PMN were stimulated with 100 nM fMLP for 15 min. Panel F: PMN alone (and not HUVEC) were treated with AF for 30 min. Subsequently, PMN were added to HUVEC monolayers and allowed to settle for 10 min. Finally PMN were treated with 100 nM fMLP for 15 min. Data points indicated represent means and SEM for at least three separate experiments performed in triplicate. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared to buffer-treated controls.

with AF reduced the IL-1β-induced hyperadhesiveness, with a significant inhibition occurring at 2 μg/mL of AF and a maximal 58.6% reduction in the amount of adhering PMN occurring at 4 μg/mL of AF (Fig. 4A). The inhibitory effect of AF not only persisted, but became more pronounced at low drug concentrations, when HUVEC alone were treated with AF (Fig. 4B). In contrast, AF treatment of PMN alone resulted in an enhanced adhesion to IL-1β-activated HUVEC and to albumin-coated plastic when PMN were stimulated with fMLP (Figs. 4C and 3B, respectively). This was associated with a pronounced increase in PMN surface expression of CD18 when PMN were treated with AF at 1 μg/mL (data not shown). Because these results pointed to a more complex effect of AF than observed for GSTM, additional experiments in which the PMN were stimulated with fMLP were carried out. Treatment of the combined system of HUVEC and PMN with AF conferred no inhibitory effect when fMLP was used as agonist; in contrast, an increased adhesiveness was noted (Fig. 4D). Treatment of PMN alone with AF resulted in an enhancement of the fMLP-induced adhesion of PMN to HUVEC (Fig. 4F). When HUVEC alone were treated with AF, no significant effect was seen on the adhesion of PMN to HUVEC induced by fMLP (Fig. 4E).

The adhesion of PMN to HUVEC induced by stimulation of HUVEC with Il-1β was not significantly reduced when the combined system of HUVEC and PMN was incubated with sulphasalazine (at 100 μM, adherent PMN comprised 93.6 ± 13.2% of the cells when compared to controls not treated with drug, i.e. 100%; $P = 0.353$; $N = 7$). fMLP-induced adhesion of PMN either to HUVEC or to an albumin-coated plastic surface was not altered by 100 μM sulphasalazine (108.1 ± 15.5% of controls; $P = 0.391$; $N = 4$).

Effects of Antirheumatic Drugs on Spontaneous PMN Adhesion to HUVEC or Albumin-coated Plastic (Table 1)

GSTM did not affect spontaneous PMN or HUVEC adhesive properties (except for a small enhancing effect in the combined system at the highest tested drug concentration). In contrast, AF treatment of the combined system of PMN and HUVEC resulted in an increased number of PMN adhering to HUVEC. AF treatment of either HUVEC or PMN alone also resulted in an increase of spontaneous adhesion. Furthermore, an increase of spontaneous PMN adhesion to albumin-coated plastic was noted after treatment with AF. Sulphasalazine had no effect either on the combined system of PMN and HUVEC or on PMN adhesion to albumin-coated plastic.

Effects of Antirheumatic Drugs on HUVEC Expression of Adhesion Molecules

EC expression of E-selectin and ICAM-1 was analyzed by flow cytometry. There were no signs of E-selectin surface expression on resting HUVEC (i.e. the fluorescence of anti-E-selectin-antibody labeled unstimulated HUVEC did not differ from HUVEC labeled with the control antibody or nonlabeled HUVEC; Fig. 5A, Table 2). Resting

TABLE 1. Spontaneous adhesion (i.e. without agonist added)

		GSTM			AF			Sulphasalazine
PMN adhesion to	**Drug treatment of**	**10 μg/mL**	**25 μg/mL**	**50 μg/mL**	**1 μg/mL**	**2 μg/mL**	**4 μg/mL**	**100 μM**
HUVEC	HUVEC + PMN	+0.4 ± 0.5 n.s. N = 12	+1.5 ± 1.1 n.s. N = 7	+2.0 ± 1.2 *P* = 0.011 N = 5	+1.8 ± 0.9 n.s. N = 7	+8.4 ± 3.1 *P* = 0.049 N = 4	+18.4 ± 7.5 *P* = 0.011 N = 4	−0.5 ± 0.5 n.s. N = 8
HUVEC	HUVEC	+0.4 ± 1.0 n.s. N = 4	+0.6 ± 1.9 n.s. N = 3	+2.4 ± 1.9 n.s. N = 3	+3.1 ± 1.3 *P* = 0.021 N = 3	+2.1 ± 1.0 *P* = 0.030 N = 3	+2.3 ± 1.3 n.s. N = 3	n.d.
HUVEC	PMN	+1.2 ± 3.0 n.s. N = 3	+0.5 ± 3.2 n.s. N = 3	+0.1 ± 1.0 n.s. N = 3	+2.4 ± 1.9 n.s. N = 3	+4.3 ± 2.1 *P* = 0.039 N = 3	+5.1 ± 2.4 *P* = 0.037 N = 3	n.d.
Albumin-coated plastic	PMN	+3.3 ± 2.6 n.s. N = 4	+1.8 ± 1.7 n.s. N = 3	+1.6 ± 1.2 n.s. N = 3	+12.1 ± 3.9 *P* = 0.004 N = 8	+7.2 ± 5.2 *P* = 0.022 N = 7	+2.5 ± 4.6 n.s. N = 8	+2.9 ± 2.5 n.s. N = 4

Summary of the effects of antirheumatic drugs expressed as % of adhering cells, given as net difference vs buffer treated controls. PMN, polymorphonuclear neutrophils. HUVEC, human umbilical vein endothelial cells. ns, not statistically different vs control. n.d., not determined. N = separate experiments performed in triplicate.

HUVEC, however, exhibited a significant expression of ICAM-1 (Fig. 5E). Incubation of endothelial cells with IL-1β for 3 hr resulted in a dose-related expression of E-selectin and ICAM-1 (Figs. 5A and E). The HUVEC responses were homogeneous in that no subpopulations of less responsive cells were detected.

When HUVEC were treated with GSTM for 30 min prior to and during subsequent incubation with 5U/mL of IL-1β, a significant and dose-dependent reduction in the expression of E-selectin was noted, with a 62% reduction for 25 μg/mL of GSTM (compared with untreated controls, given the value of 100%; Fig. 5B, Table 2). IL-1β-induced expression of ICAM-1 was not affected by GSTM. All cells reacted similarly and there was no evidence of drug-induced HUVEC cytolysis.

Treatment of HUVEC with 1 μg/mL of auranofin resulted in a 48% reduction in the E-selectin expression and a 53% reduction in the ICAM-1 expression induced by IL-1β (Figs. 5C and G, Table 2). AF concentrations greater than 1μg/mL (exceeding therapeutic serum concentrations) were not evaluated because too few cells could be harvested (possibly reflecting the combined injurious effects caused by high AF concentrations, trypsin and multiple washings).

Sulphasalazine had no significant effect on HUVEC expression of E-selectin or ICAM-1 induced by IL-1β (Fig. 5D and H).

DISCUSSION

This study has shown that GSTM and AF modulate adhesive interactions between EC and PMN. GSTM reduced HUVEC (but not PMN) adhesiveness and E-selectin expression. AF also impaired HUVEC adhesiveness and E-selectin expression, but, in contrast to GSTM, it also reduced ICAM-1 expression, and more importantly enhanced PMN adhesivity and CD18 expression, the net effect still being a diminished binding of PMN to cytokine-stimulated HUVEC. Sulphasalazine did not interfere with HUVEC or PMN adhesion, or E-selectin or ICAM-1 expression. The effects of GSTM and AF were noted at concentrations that are obtained in the serum of RA patients during regular treatment.

The method used here, i.e. adhesion of BCECF-labeled PMN to a variety of substrata (quiescent or cytokine-stimulated HUVEC, albumin-coated plastic surface to which resting or fMLP-stimulated PMN adhered), is a sensitive assay for analysis of no-flow adhesive interactions between PMN and HUVEC. The method has been used to demonstrate effects of leukotrienes B_4, C_4 and D_4, lipoxin A_4 as well as thrombin, histamine, bradykinin [4, 21] and IL-1β. The hyperadhesiveness and adhesion molecule expression induced by IL-1β in HUVEC was highly reproducible in our assay system as well as in other investigations [24]. The method also satisfies the need for evaluation of rapid and transient agonist effects, e.g. of LTB_4, which otherwise can be overlooked [4].

The etiology of RA is unclear but T-cells, monocytes/macrophages and cytokines play a key role in the pathogenesis [25–27]. Expression of cytokines such as IL-1 and TNFα in the rheumatoid synovium has been demonstrated [28, 29], with subsequent activation of, for example, adjacent EC. Thus, we used IL-1β as a model for a cytokine agonist in our study. Newman *et al.* [16] noted that TNFα and IL-1β induced similar expression of E-selectin in HUVEC and that the effect of GSTM was identical for the two cytokines. Although IFN-γ increases ICAM-1 expression on human synovial vessel endothelial cells, HUVEC has been shown to be less responsive to IFN-γ in this respect [30]. Treatment of HUVEC with IFN-γ conferred only marginal hyperadhesivity [Heimbürger M, Lärfars G and Bratt J, unpublished results] thus making it less useful in the present context of adhesion interactions.

In our study, GSTM as well as AF impaired endothelial

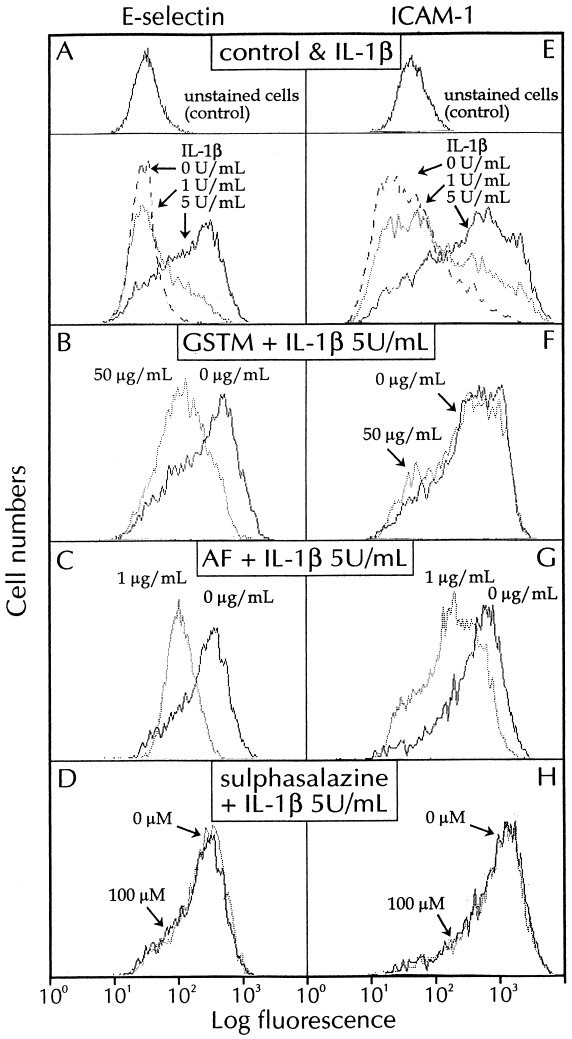

FIG. 5. HUVEC expression of the adhesion molecule E-selectin (left column) or ICAM-1 (right column), assessed by flow cytometry, (see Table 2 for numerical results). Panel A: HUVEC expression of E-selectin before and after stimulation with IL-1β for 180 min. Panel B: HUVEC expression of E-selectin after treatment with GSTM and stimulation with 5 U/mL of IL-1β for 180 min. Panel C: HUVEC expression of E-selectin after treatment with AF and stimulation with 5U/mL of IL-1β for 180 min. Panel D: HUVEC expression of E-selectin after treatment with sulphasalazine and stimulation with 5U/mL of IL-1β for 180 min. Panel E: HUVEC expression of ICAM-1 before and after stimulation with IL-1β for 180 min. Panel F: HUVEC expression of ICAM-1 after treatment with GSTM and stimulation with 5 U/mL of IL-1β for 180 min. Panel G: HUVEC expression of ICAM-1 after treatment with AF and stimulation with 5 U/mL of IL-1β for 180 min. Panel H: HUVEC expression of ICAM-1 after treatment with sulphasalazine and stimulation with 5 U/mL of IL-1β for 180 min. The figure depicts typical experiments that were repeated with similar results being obtained at least three times.

adhesiveness for PMN and endothelial E-selectin expression. GSTM and AF differed in their effects on HUVEC exposure of ICAM-1, where AF was efficient but not GSTM. Thus, the two gold compounds exhibited similarities of action on HUVEC as well as one discrepancy,

TABLE 2. Effects of GSTM, AF and sulphasalazine on HUVEC expression of adhesion molecules induced by IL-1β

	E-selectin		ICAM-1	
GSTM				
50 μg/mL	31.9	N = 4	101.2	N = 3
25 μg/mL	37.7	N = 5	81.5	N = 3
10 μg/mL	48.5	N = 7	88.8	N = 5
5 μg/mL	54.8	N = 4	96.7	N = 3
1 μg/mL	87.3	N = 3	96.7	N = 3
AF				
1.0 μg/mL	57.9	N = 9	49.7	N = 9
0.5 μg/mL	76.2	N = 4	54.4	N = 3
Sulphasalazine				
100 μM	107.6	N = 3	102.2	N = 3
Medium	100.0		100.0	

HUVEC were pretreated with drug or medium alone for 30 min, and IL-1β (5 U/mL) was then added for 3 hr (as described in the Methods section). Effects of drugs on mean fluorescence given as % of medium-treated control. HUVEC, human umbilical vein endothelial cells. N = number of separate tests.

indicating that the drugs might share some target molecules and that AF affects additional molecules. Moreover, AF treatment of PMN increased PMN surface expression of CD18. This effect may explain the observed PMN hyperadhesiveness induced by AF. With respect to GSTM, our results are analogous to those of Corkill and collaborators, who found a reduction in endothelial expression of E-selectin in synovial vessels from patients treated with GSTM for RA [15]. Likewise, Newman et al. described a decrease in E-selectin expression and mRNA levels after treatment of EC *in vitro* [16]; however, the effect on endothelial adhesiveness was not reported.

AF has previously been shown to stimulate a variety of functional responses of PMN at low concentrations (i.e. 1–2 μg/mL) and inhibit responses at higher ones. This has been shown for a whole blood adherence system as well as superoxide and enzyme release, phenomena that have been associated with increased affinity of receptors for fMLP [12, 13]. However, most AF effects relate to agonist-activated PMN functions and not to quiescent cells. As shown here, adhesive effects and CD18 expression were augmented by exposure of resting PMN to AF, suggesting that the drug has intrinsic PMN-activating effects, a notion supported by findings of a rise in cytosolic calcium concentrations in PMN exposed to the drug [31]. The mechanism for this is unknown.

In contrast to the PMN-activating effects of AF, this drug hampered HUVEC adhesive responses and E-selectin and ICAM-1 expression. To the best of our knowledge, this is a novel finding. Previously, AF was shown to impair endothelial-dependent blood vessel relaxation [32], suggesting effects on nitric oxide-associated systems. Because AF inhibits the release of NO from macrophages [33] and reduces PMN-induced cytotoxicity of HUVEC [22], it is tempting to speculate that AF interacts with NO from HUVEC in such a way that adherence is modulated.

Although sulphasalazine inhibits a variety of PMN functional responses to fMLP [17], it is evident that these effects

do not translate to adhesive interactions with HUVEC or a plastic surface within the relatively short treatment period used in our study. It should be noted that Gadangi *et al.* [34] recently reported a reduction in the adhesion between neutrophils stimulated with fMLP and HUVEC, when the latter were pretreated with sulphasalazine. However, in that report HUVEC were pretreated with sulphasalazine for 48 hr compared to 30 min in our corresponding protocol, a difference that might explain the divergent results.

The biochemical and subcellular mechanisms for the drug effects noted here are not known yet. The reduction of mRNA for E-selectin points to an effect that is located proximal to gene activation, but what discrete step(s) of the stimulus-response coupling for IL-β are involved, e.g. the ceramide or the NF-κB cascades, remains to be elucidated.

The results presented herein indicate that interference with endothelial expression of adhesion molecules and the adhesive interaction between neutrophils and endothelial cells are mechanisms that may be responsible for some of the antiarthritic actions conferred by GSTM and AF. In contrast to the gold compounds, sulphasalazine does not exert any significant effect on the adhesion of PMN to EC or on endothelial expression of E-selectin or ICAM-1. Thus, clinical effects of sulphasalazine might better relate to attenuation of other inflammatory systems. The effect of AF is more complex than that of GSTM, augmenting spontaneous adhesion but reducing IL-1β-induced responses. If similar phenomena also occur *in vivo*, they may counteract each other, leading to a lesser effect on inflammatory reactions. The net result of these opposing effects of AF might explain the fact that AF is less efficient than GSTM in the treatment of rheumatoid arthritis [35].

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References

1. Springer TA, Adhesion receptors of the immune system. *Nature* **346:** 425–434, 1990.
2. Carlos TM and Harlan JM, Leukocyte-endothelial adhesion molecules. *Blood* **84:** 2068–2101, 1994.
3. Schleiffenbaum B and Fehr J, Regulation and selectivity of leukocyte emigration. *J Lab Clin Med* **127:** 151–168, 1996.
4. Heimbürger M and Palmblad J, Effects of leukotriene C_4 and D_4, histamine and bradykinin on cytosolic calcium concentrations and adhesiveness of endothelial cells and neutrophils. *Clin Exp Immunol* **103:** 454–460, 1996.
5. Lindström P, Lerner R, Palmblad J and Patarroyo M, Rapid adhesive responses of endothelial cells and of neutrophils induced by leukotriene B4 are mediated by leukocyte adhesion protein CD18. *Scand J Immunol* **31:** 737–744, 1990.
6. Palmblad J, Lerner R and Larsson S, Signal transduction mechanisms for leukotriene B4 induced hyperadhesiveness of endothelial cells for neutrophils. *J Immunol* **152:** 262–269, 1994.
7. Zimmerman GA, McIntyre TM, Mehra M and Prescott SM, Endothelial cell-associated platelet-activating factor: A novel mechanism for signaling intercellular adhesion. *J Cell Biol* **110:** 529–540, 1990.
8. Bevilaqua MP, Pober JS, Wheeler ME, Cotran RS and Gimbrone MA Jr, Interleukin 1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes and related cell lines. *J Clin Invest* **76:** 2003–2011, 1985.
9. Gamble JR, Harlan JM, Klebanoff SJ and Vadas MA, Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor (TNF). *Proc Natl Acad Sci USA* **82:** 8667–8671, 1985.
10. Pober JS, Gimbrone MA Jr., Lapierre LA, Mendrick DL, Fiers W, Rothlein R and Springer TA, Overlapping patterns of activation of human endothelial cells by interleukin 1, TNF and immune interferon. *J Immunol* **137:** 1893–1896, 1986.
11. Bevilaqua MP, Pober JS, Mendrick DL, Cotran RS and Gimbrone MA Jr., Identification of an inducible endothelial-leukocyte adhesion molecule. *Proc Natl Acad Sci USA* **84:** 9238–9242, 1987.
12. Hafström I, Udén A-M and Palmblad J, Modulation of neutrophil functions by auranofin. *Scand J Rheumatol* **12:** 97–105, 1983.
13. Hafström I, Seligmann BE, Friedman MM and Gallin JI, Auranofin affects early events in human polymorphonuclear neutrophil activation by receptor-mediated stimuli. *J Immunol* **132:** 2007–2014, 1984.
14. Parente JE, Wong K, Davis P, Burka JF and Percy JS, Effect of gold compounds on leukotriene B4, leukotriene C4 and prostaglandin E2 production by polymorphonuclear leukocytes. *J Rheumatol* **13:** 47–51, 1986.
15. Corkill MM, Kirkham BW, Haskard DO, Barbatis C, Gibson T and Panayi GS, Gold treatment of rheumatoid arthritis decreases synovial expression of the endothelial leukocyte adhesion receptor ELAM-1. *J Rheumatol* **18:** 1453–1460, 1991.
16. Newman PM, To SST, Robinson BG, Hyland VJ and Schreiber L, Effect of gold sodium thiomalate and its thiomalate component on the *in vitro* expression of endothelial cell adhesion molecules. *J Clin Invest* **94:** 1864–1871, 1994.
17. Kanerud L, Hafström I and Ringertz B, Effect of sulphasalazine and sulphapyridine on neutrophil superoxide production: Role of cytosolic free calcium. *Ann Rheum Dis* **49:** 296–300, 1990.
18. Neal TM, Winterbourn CG and Vissers MCM, Inhibition of neutrophil degranulation and superoxide production by sulfasalazine. *Biochem Pharmacol* **36:** 2765–2768, 1987.
19. Greenfield SM, Hamblin AS, Shakoor ZS, Teare JP, Punchard NA and Thompson RPH, Inhibition of leucocyte adhesion molecule upregulation by TNF α a novel mechanism of action of sulphasalazine. *Gut* **34:** 252–256, 1993.
20. Ringertz B, Palmblad J and Lindgren JÅ, Stimulus specific neutrophil aggregation—evaluation of possible mechanism for the stimulus-response apparatus. *J Lab Clin Med* **106:** 132–140, 1985.
21. Lerner R, Heimbürger M and Palmblad J, Lipoxin A4 induces hyperadhesiveness in human endothelial cells for neutrophils. *Blood* **82:** 948–953, 1993.
22. Bratt J and Palmblad J, Inhibition of neutrophil-dependent cytotoxicity for human endothelial cells by antirheumatic drugs. *J Lab Clin Med* **128:** 552–560, 1996.
23. Nortamo P, Li R, Renkonen R, Timonen T, Prieto J, Patarroyo M and Gahmberg CG, The expression of human intercellular adhesion molecule-2 is refractory to inflammatory cytokines. *Eur J Immunol* **21:** 2629–2632, 1991.
24. Westlin WF and Gimbrone MA, Neutrophil-mediated damage to vascular endothelium—role of cytokine activation. *Am J Pathol* **142:** 117–128, 1993.

25. Klareskog L, Rönnelid J and Holm G, Immunopathogenesis and immunotherapy in rheumatoid arthritis: an area in transition. *J Intern Med* **238:** 191–206, 1995.
26. Arend WP and Dayer J-M, Cytokines and cytokine inhibitors or antagonists in rheumatoid arthritis. *Arthritis Rheum* **33:** 305–315, 1990.
27. Feldmann M, Brennan FM and Maini RN, Role of cytokines in rheumatoid arthritis. *Annu Rev Immunol* **14:** 397–440, 1996.
28. Deleuran BW, Cytokines in rheumatoid arthritis. Localisation in arthritic joint tissue and regulation *in vitro*. *Scand J Rheumatol* **25**(S104)**:** 1–38, 1996.
29. Yanni G, Farahat MNMR, Poston RN and Panayi GS, Intramuscular (i.m.) gold decreases cytokine expression and macrophage numbers in the rheumatoid synovial membrane. *Ann Rheum Dis* **53:** 315–322, 1994.
30. Gerritsen ME, Kelley KA, Ligon G, Perry CA, Shen C-P, Szczepanski A, and Carley WW, Regulation of the expression of intercellular adhesion molecule 1 in cultured human endothelial cells derived from rheumatoid synovium. *Arthritis Rheum* **36:** 593–602, 1993.
31. Wong K, Parente J, Prasad KVS and Ng D, Auranofin modulated cytoplasmic free calcium in neutrophils by mobilizing intracellular calcium and inhibiting protein kinase C. *J Biol Chem* **265:** 21454–21461, 1990.
32. Ohlstein EH and Horohonich S, Selective inhibition of endothelium-dependent relaxation by gold-containing compounds. *Pharmacology* **38:** 93–100, 1989.
33. Gyllenhammar H, Lärfars G, Bratt J and Palmblad J, Antirheumatic gold salts inhibit production of nitric oxide in murine macrophages activated with lipopolysaccharide. *J Invest Med* **44:** 298A, 1996.
34. Gadangi P, Longaker M, Naime D, Levin RI, Phoebe AR, Montesinos MC, Buckley MT, Carlin G and Cronstein B, The anti-inflammatory mechanism of sulphasalazine is related to adenosine release at inflamed sites. *J Immunol* **156:** 1937–1941, 1996.
35. Felson DT, Andersson JJ and Meenan RF, Use of short-term efficacy/toxicity tradeoffs to select second-line drugs in rheumatoid arthritis. A meta-analysis of published clinical trials. *Arthritis Rheum* **35:** 1117–1125, 1992.