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A mechanism for the antiinflammatory effects of corticosteroids: The glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial–leukocyte adhesion molecule 1 and intercellular adhesion molecule 1

(neutrophils/endothelium/inflammation)

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ABSTRACT **Corticosteroids are the preeminent antiinflammatory agents although the molecular mechanisms that impart their efficacy have not been defined. The endothelium plays a critical role in inflammation by directing circulating leukocytes into extravascular tissues by expressing adhesive molecules for leukocytes [e.g., endothelial–leukocyte adhesion molecule 1 (ELAM-1) and intercellular adhesion molecule 1 (ICAM-1)]. We therefore determined whether corticosteroids suppress inflammation by inhibiting endothelial expression of adhesion molecules for neutrophils (polymorphonuclear leukocytes). Preincubation of endothelial cells with endotoxin [lipopolysaccharide (LPS), 1 μg/ml] led to a 4-fold increase in subsequent adherence of polymorphonuclear leukocytes ($P < 0.0001$, $n = 10$) to endothelial cells, an increase that was markedly attenuated when endothelial cells were treated with dexamethasone ($IC_{50} < 1$ nM, $P < 0.0001$, $n = 6$ or 7) during preincubation with LPS. Moreover, the steroid receptor agonist cortisol (10 μM), but not its inactive metabolite tetrahydrocortisol (10 μM), diminished LPS-induced endothelial cell adhesiveness. Further evidence that the action of dexamethasone was mediated through ligation of corticosteroid receptors [human glucocorticoid receptors (hGRs)] was provided by experiments utilizing the steroid antagonist RU-486. RU-486 (10 μM), which prevents translocation of ligated hGR to the nucleus by inhibiting dissociation of hGR from heat shock protein 90, completely aborted the effect of dexamethasone on adhesiveness of endothelial cells ($P < 0.0005$, $n = 3$). Treatment of endothelial cells with LPS (1 μg/ml) stimulated transcription of ELAM-1, as shown by Northern blot analysis, and expression of membrane-associated ELAM-1 and ICAM-1, as shown by quantitative immunofluorescence (both $P < 0.001$, $n = 9$). Dexamethasone markedly inhibited LPS-stimulated accumulation of mRNA for ELAM-1 and expression of ELAM-1 and ICAM-1 ($IC_{50} < 10$ nM, both $P < 0.001$, $n = 4–9$); inhibition of expression by dexamethasone was reversed by RU-486 (both $P < 0.005$, $n = 4–6$). As in the adhesion studies, cortisol but not tetrahydrocortisol inhibited expression of ELAM-1 and ICAM-1 (both $P < 0.005$, $n = 3$ or 4). In contrast, sodium salicylate (1 mM) inhibited neither adhesion nor expression of these adhesion molecules. These studies suggest that antagonism by dexamethasone of endotoxin-induced inflammation is a specific instance of the general biological principle that the glucocorticoid receptor is a hormone-dependent regulator of transcription.**

Although glucocorticoids are among the most potent and widely used antiinflammatory agents, the mechanisms by which they reduce inflammation are unknown. Various hypotheses have been proposed; these include "allosteric" effects on enzymes (1), redirection of lymphocyte traffic (2), direct inhibition of various phospholipases (3), induction of such proteins as lipocortin (4), inhibition of the transcription of various cytokines and metalloproteases (5–14), and our own earlier suggestion that glucocorticoids stabilize lysosomal and other cellular membranes (15, 16). However, none of these hypotheses is sufficient to account for the well-known pharmacologic effects of glucocorticoids in humans: leukocytosis (17), inhibition of leukocyte recruitment to inflamed areas (18, 19), retention of lymphocytes in the lymphatic circulation with shrinkage of peripheral lymph nodes, and the promotion of microbial infection (2).

Recent studies have suggested that endothelial cells can direct the traffic of leukocytes into inflamed and infected areas (heterotypic adhesion) via the regulated expression of surface adhesive molecules [e.g., GMP140, endothelial–leukocyte adhesion molecule 1 (ELAM-1), intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule (VCAM) (20–23)]. In a complementary fashion, leukocytes also express proteins [CD11a-c/CD18, L-selectin, or lectin/epidermal growth factor cell adhesion molecule 1 (LECAM-1)] on their surface that mediate their specific localization to sites of inflammation (24, 25). Agents that modulate the interaction of leukocytes with the endothelium may, therefore, possess potent antiinflammatory properties.

We now present data compatible with the hypothesis that glucocorticoids—at nanomolar concentrations—inhibit the expression of adhesive molecules ELAM-1 and ICAM-1 by endotoxin-activated endothelial cells and thereby interfere with the traffic of leukocytes into inflamed areas. Pretreatment of endothelial cells with corticosteroids prevents endothelial cells from becoming more adhesive for neutrophils [polymorphonuclear leukocytes (PMNs)] and diminishes stimulated expression of ICAM-1 and ELAM-1, molecules critical for neutrophil adhesion. Moreover, these data make it likely that corticosteroids regulate ELAM-1 at the transcriptional level.

MATERIALS AND METHODS

Materials. Lipopolysaccharide (LPS, *Salmonella typhimurium*) was obtained from Calbiochem and *N*-formylmethionylleucylphenylalanine was obtained from Vega Biochemi-

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Abbreviations: ELAM-1, endothelial–leukocyte adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; PMN, polymorphonuclear leukocyte; LPS, lipopolysaccharide; HUVEC, human umbilical vein endothelial cell; IL, interleukin; TNF, tumor necrosis factor; hGR, human glucocorticoid receptor; FITC, fluorescein isothiocyanate.

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cal. Collagenase, dexamethasone, hydrocortisone, tetrahydrocortisol, and sodium salicylate were obtained from Sigma. RU-486 was a gift to Herbert Samuels (Roussel-Uclaf). Medium 199, RPMI 1640, and fetal bovine serum were obtained from GIBCO. Ficoll/Hypaque was purchased from Nyegaard (Oslo). All other salts and reagents were of the highest quality that could be obtained.

Monoclonal Antibodies. In these studies the monoclonal antibodies used included antibodies directed against ICAM-1 (84H10, AMAC, Westbrook, ME), ELAM-1 (BMA 4D10, Accurate Chemicals, Westbury, NY, and H18/7, a generous gift of Michael Bevilacqua, San Diego), MOPC21, and UPC10, nonbinding isotype controls. Fluorescein-labeled anti-IgG, goat anti-mouse fluorescein isothiocyanate (GAM-FITC) was obtained from Coulter Immunology. Endothelial cells were also stained with rhodamine-labeled *Ulex europaeus* agglutinin I (Vector Laboratories); all monoclonal antibody studies were performed in ice-cold phosphate-buffered saline (PBS) with 0.02% sodium azide and 0.025% bovine serum albumin.

Culture of Endothelial Cells. Human umbilical vein endothelial cells (HUVECs) were cultured as described (26). All experiments were performed on endothelial cells in their third passage.

Incubation of Endothelial Cells with Pharmacologic Agents. Endothelial cells were stimulated by incubation with LPS (1 μg/ml) in a medium consisting of RPMI 1640/10% fetal bovine serum for 4 hr at 37°C in a 5% CO_2 atmosphere with or without other agents as indicated. The monolayers were then washed three times.

PMN Adherence to Endothelial Monolayers (Heterotypic Adherence). PMNs (150,000 per well), isolated from whole blood as described (27), suspended in RPMI 1640 medium, were added to monolayers of endothelial cells and incubated for 10 min at 37°C in a 5% CO_2 atmosphere and adherence was determined as described (28). Pretreatment of unstimulated HUVECs with each of the agents used did not affect basal PMN adherence (data not shown). The highest concentration of diluent used (ethanol, 0.1%) also had no effect on PMN adherence to LPS-stimulated endothelium.

In some experiments PMNs were labeled with ^{111}In and after incubation of labeled PMNs with endothelium for 10 min at 37°C adherence was determined by a previously described method (29) and expressed as % adherence.

Expression of ELAM-1 and ICAM-1. After incubation for 4 hr with various stimuli and agents the HUVECs were removed from wells by exposure to EDTA (0.01%, wt/vol) in PBS for 10 min at 37°C in a 5% CO_2 atmosphere followed by gentle scraping with a rubber policeman and trituration. Cells were resuspended in ice-cold saline containing sodium azide (0.02%) and saturating concentrations of antibodies, incubated for 30 min at 4°C, washed and counterstained with fluorescein-labeled anti-IgG for 30 min at 4°C, washed, and fixed in formaldehyde (3.7% in PBS). HUVECs were then analyzed with a FACScan (Becton Dickinson) (30). In these experiments the fluorescence of cells stained with an isotype control antibody (MOPC21) was 33 ± 13.

Analysis of Message for ELAM-1. HUVECs were incubated without or with stimuli in the presence and absence of dexamethasone (0.1 μM) for 3 hr at 37°C. Following treatment with collagenase/EDTA [0.1%/0.01% (wt/vol) 10 min at 37°C] cells were suspended and washed, and mRNA was isolated by use of the FastTrack kit following the instructions provided (Invitrogen, San Diego). After electrophoresis through agarose the mRNA was transferred to nitrocellulose and hybridized with full-length ^{32}P-labeled cDNA probes for ELAM-1 (generously supplied by Tucker Collins) and actin (Stratagene) under conditions of high stringency. Labeled cDNA for ELAM-1 hybridized with a single band of 3.65 kilobases (kb) and labeled cDNA for actin hybridized with a single band of 2.0 kb. Autoradiograms were prepared and then analyzed by laser densitometry (31).

Statistical Analysis. The significance of differences among and between experimental treatment groups was determined by means of the appropriate level of analysis of variance and determination of separate post-hoc variances by means of the CSS Software (Statsoft, Tulsa, OK) using an IBM-compatible computer.

RESULTS

Glucocorticoids Prevent Adhesion of PMNs to LPS-Stimulated Endothelial Cells (Heterotypic Adhesion). Activation of HUVECs with LPS or cytokines [interleukin 1 (IL-1) and tumor necrosis factor α (TNF-α)] causes endothelial cells to bind unstimulated PMNs more avidly (20, 21, 30, 32). When we treated HUVECs with LPS (1 μg/ml) we found, as expected, that ≈4-fold more PMNs adhered to treated than untreated HUVECs (50 ± 3 vs. 13 ± 2 PMNs per high-power field, $P < 0.0001$). Dexamethasone inhibited PMN adhesion to the LPS-stimulated HUVECs in a dose-dependent manner ($IC_{50} < 1$ nM, $P < 0.005$, Fig. 1).

Glucocorticoid Receptors Mediate the Effects of Glucocorticoids on Adhesion of PMNs to HUVECs. To determine whether the modulation of endothelial adhesiveness by dexamethasone was receptor-mediated we studied the effects of RU-486, a noncompetitive antagonist of glucocorticoid receptors (33, 34). RU-486 (10 μM) completely reversed the effect of dexamethasone on LPS-stimulated adhesiveness of HUVECs (Fig. 1, $P < 0.0005$). In other experiments 100-fold higher concentrations of the less potent steroid receptor agonist cortisol (IC_{50} = 100 nM, n = 2), but not its inactive metabolite tetrahydrocortisol (0.1 and 10 μM), diminished the LPS-stimulated increment in adhesiveness (data not shown). Further, neither indomethacin (10 μM) nor sodium salicylate (1 mM) affected the LPS-stimulated increment in endothelial adhesiveness.

Glucocorticoids Modulate Expression of ELAM-1 and ICAM-1 on LPS-Stimulated Endothelium. We next determined whether the effect of corticosteroids on endothelial cell adhesiveness resulted from diminished expression of ELAM-1 or ICAM-1. After stimulation by LPS, HUVECs increased expression of ELAM-1 by 321% ± 68% (SEM, $P < 0.0001$, n = 16) and ICAM-1 by 250% ± 38% ($P < 0.0001$, n = 16, Fig. 2).

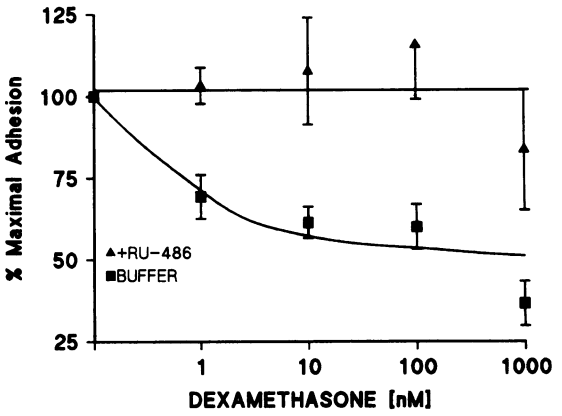

FIG. 1. Inhibition by dexamethasone (0.1–1000 nM) of PMN adhesion to LPS-stimulated HUVECs. HUVECs were stimulated, as above, in the presence or absence of various doses of dexamethasone and RU-486 (10 μM). Adherence of PMNs to unstimulated endothelium was determined and subtracted from the PMN adherence to stimulated endothelium and then expressed as a percentage of net adherence of PMNs to untreated endothelial monolayers. In the absence of dexamethasone RU-486 did not affect adhesion of PMNs to either unstimulated (122% ± 16% of control, n = 4) or LPS-treated endothelium (114% ± 11% of control, n = 4).

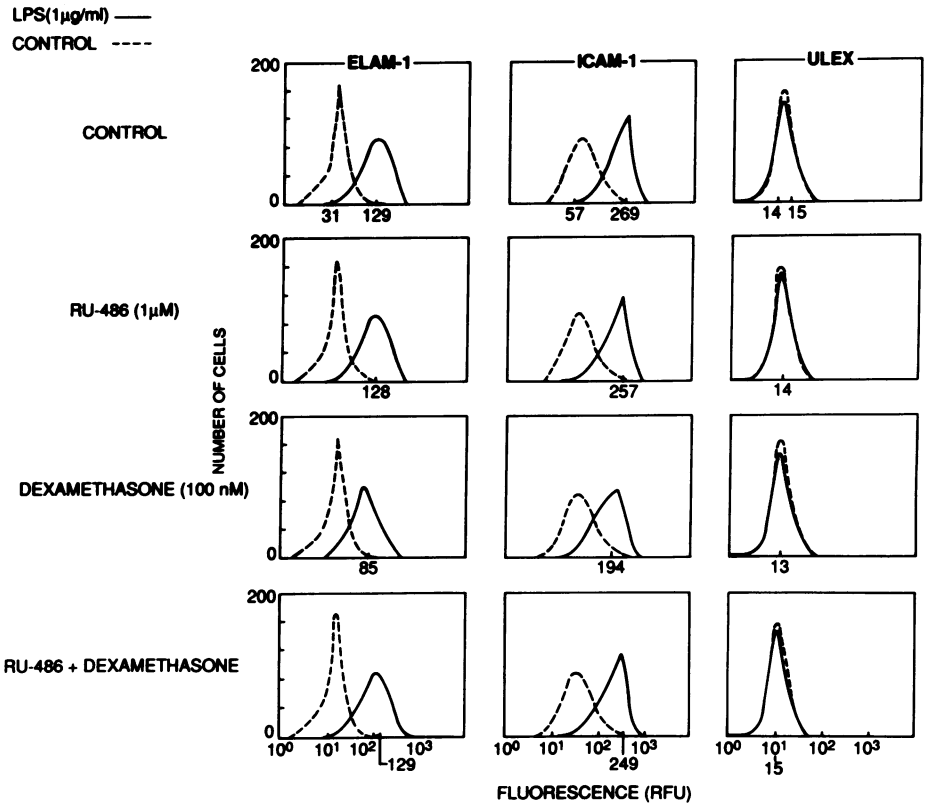

FIG. 2. Inhibition by dexamethasone of the increased surface expression of ICAM-1 and ELAM-1 by LPS-stimulated HUVECs. Fluorescence histograms are as follows: top row, resting and activated (LPS, 1 μg/ml) HUVECs stained with antibodies directed against ICAM-1, ELAM-1, and *Ulex europaeus* agglutinin I; second row, resting and activated HUVECs activated in the presence of RU-486 (10 μM); third row, HUVECs activated in the presence of dexamethasone (0.1 μM); and bottom row, resting and activated HUVECs activated in the presence of dexamethasone (0.1 μM) and RU-486 (10 μM). Mean fluorescence of cells stained with FITC-labeled antibody (MOPC-21) alone was 10 relative fluorescence units (RFU). Shown is a representative experiment of seven, the mean results of which are presented in Figs. 3 and 4.

Dexamethasone (IC_{50} < 1 nM) inhibited the LPS-stimulated expression of ELAM-1 and ICAM-1 ($P < 0.00001$ and $P < 0.00001$, respectively, Fig. 3) without altering basal expression of these molecules (data not shown). Dexamethasone did not alter binding of rhodamine-labeled *Ulex europaeus* agglutinin I to the endothelial cell surface (Fig. 2). Similarly, none of the compounds studied affected the nonspecific binding of antibodies MOPC21, UPC10 (isotype controls), or FITC anti-mouse IgG (data not shown). As expected, RU-486 completely reversed the effect of dexamethasone on the LPS-stimulated expression of ICAM-1 and ELAM-1 ($P < 0.004$, Figs. 2 and 4).

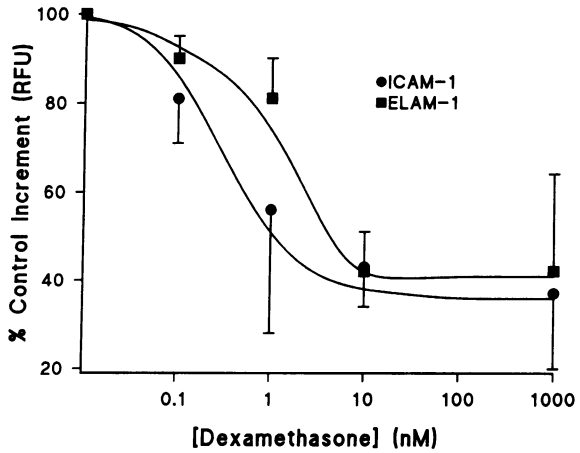

FIG. 3. Inhibition by dexamethasone (0.1 nM–10 μM) of the up-regulation of ICAM-1 and ELAM-1 expression on LPS-stimulated HUVECs. The results shown represent the means ± SEM of 4–10 experiments. RFU, relative fluorescence units.

Although RU-486 appeared to diminish LPS-stimulated up-regulation of ICAM-1 (Fig. 4), the difference observed was not statistically significant. Similarly, cortisol, but neither tetrahydrocortisol nor sodium salicylate, inhibited expression of ICAM-1 and ELAM-1 ($P < 0.003$, Fig. 5). None of the agents studied affected binding of *Ulex europaeus* agglutinin I to HUVECs (Fig. 2).

Glucocorticoids Prevent Accumulation of Message for ELAM-1 in Response to LPS and IL-1α But Not in Response to TNF. To further define the mechanism by which glucocorticoids inhibit up-regulation of adhesive molecules, we studied the effect of dexamethasone (0.1 μM) on the level of mRNA for ELAM-1 in HUVECs. Treatment of HUVECs with LPS, IL-1α (20 units/ml), and TNF (50 units/ml) stimulated a marked increase in detectable message for ELAM-1 in HUVECs (Fig. 5). Dexamethasone (0.1 μM) did not affect basal levels of mRNA for ELAM-1 but markedly inhibited the LPS- and IL-1α-stimulated increase in message. In contrast, dexamethasone did not affect the TNF-stimulated increment in detectable ELAM-1 message, an observation that suggests that glucocorticoids do not directly affect stability of message for ELAM-1. Although these findings need to be fortified by further studies of the effects of glucocorticoids on the stability of ELAM-1 message and the rate of transcription of ELAM-1 by stimulated endothelium, the results suggest that glucocorticoids act at the transcriptional level.

Dexamethasone Does Not Reverse the Effect of TNF-α on Endothelial Adhesiveness for PMNs. Since dexamethasone did not affect the level of message for ELAM-1 in TNF-α-stimulated HUVECs, we sought to determine whether this was reflected in the adhesiveness of TNF-α-treated endothelium for PMNs. As previously reported, TNF-α (50 units/ml)

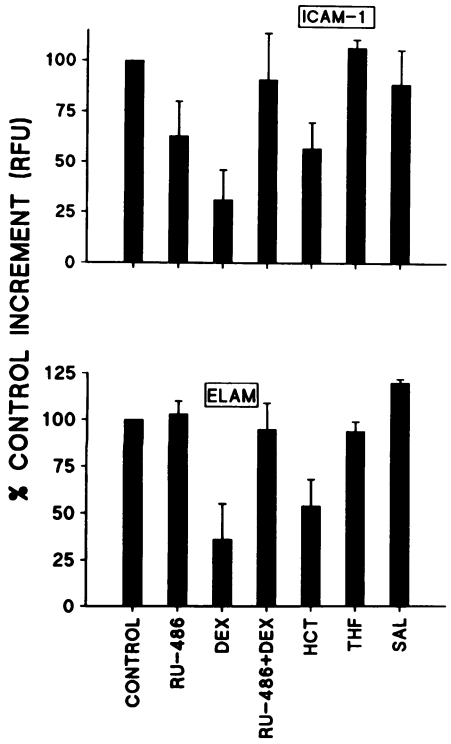

FIG. 4. Corticosteroids inhibit the LPS-stimulated expression of ICAM-1 and ELAM-1. HUVECs were incubated with LPS (1 μg/ml) in the presence of dexamethasone (DEX, 100 nM), RU-486 (10 μM), dexamethasone plus RU-486, cortisol (HCT, 10 μM), tetrahydrocortisol (THF, 10 μM), or sodium salicylate (SAL, 1.25 mM). (*Upper*) Effect of the various compounds tested on the LPS-induced increment in expression of ICAM-1 expressed as a percentage of the increment induced by LPS alone. (*Lower*) Effect of these same compounds on the LPS-stimulated increment in ELAM-1 expression. Shown are the means ± SEM of 3–10 experiments. RFU, relative fluorescence units.

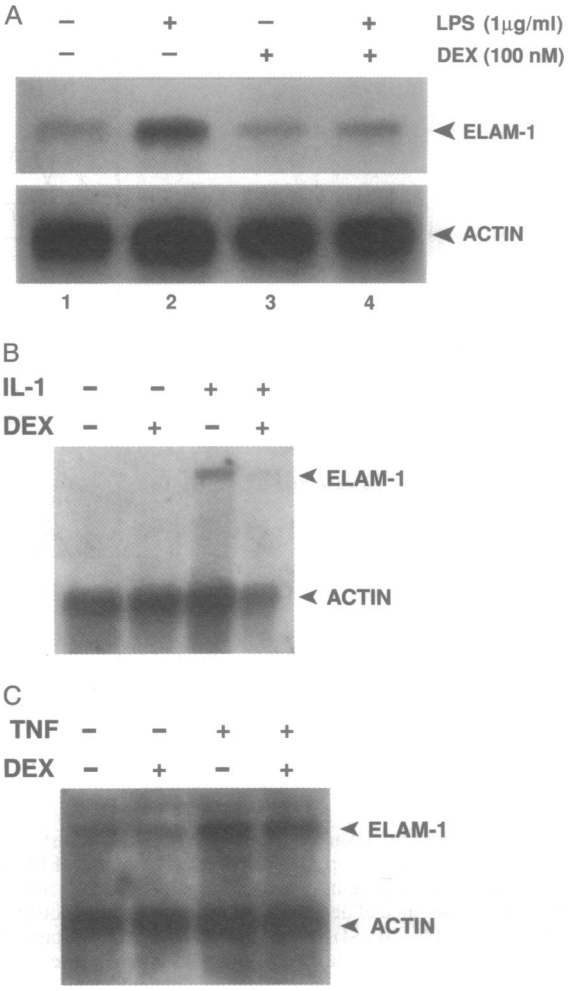

FIG. 5. Dexamethasone inhibits the stimulated increase in mRNA for ELAM. (*A*) HUVECs were incubated without (−) or with (+) LPS (1 μg/ml) in the presence (+) and absence (−) of dexamethasone (DEX, 0.1 μM). When normalized for the content of actin mRNA, LPS stimulated a 234% increase in mRNA for ELAM. Dexamethasone did not affect the basal level of mRNA for ELAM-1 (98% of control) but completely inhibited the LPS-stimulated increase in mRNA for ELAM-1 (90% inhibition). Similar results were found in a second experiment. (*B*) HUVECs were incubated with (+) or without (−) IL-1α (20 units/ml) in the presence (+) or absence (−) of dexamethasone (0.1 μM). When normalized to the content of actin mRNA, dexamethasone inhibited the IL-1α-induced increase in mRNA for ELAM-1 by 95% without affecting basal levels of mRNA for ELAM-1 (104% of control). Similar results were found in a second experiment. (*C*) HUVECs were incubated with (+) or without (−) TNF-α (50 units/ml) in the presence (+) or absence (−) of dexamethasone (0.1 μM). When normalized to the content of actin mRNA, dexamethasone did not affect either basal mRNA for ELAM-1 (97% of control) or the TNF-α-induced increment in mRNA for ELAM-1 (110% of control). Similar results were found in a second experiment.

rendered the endothelium more adhesive to PMNs (23% ± 1% adherence vs. 9% ± 2% adherence, $n = 4$, $P < 0.01$) and dexamethasone did not diminish the increased adhesiveness of TNF-stimulated endothelium for PMNs (21% ± 1% adherence, $n = 4$).

DISCUSSION

We show here that one important mechanism by which glucocorticoids may affect the inflammatory response is modulation of the capacity of the endothelium to respond to an inflammatory stimulus. Glucocorticoids, acting at their cytoplasmic receptors, diminish the LPS-stimulated increase in endothelial adhesiveness for resting PMNs and diminish transcription and expression of pro-inflammatory adhesive molecules on the surface of the endothelium. Since recent studies have increasingly pointed to the central role of the endothelium in directing the traffic of leukocytes into inflamed areas, our observations bear directly on the mechanism for the antiinflammatory effects of glucocorticoids. Moreover, our results help to explain the dramatic leukocytosis observed in patients taking therapeutic doses of corticosteroids [concentrations similar to those studied here (17)].

RU-486 stabilizes the association of steroid receptors with heat shock protein 90 in the presence of ligand, which prevents translocation of glucocorticoid receptors to the nucleus and thereby blocks transcription of genes containing glucocorticoid-responsive elements (33, 35–40). Our demonstration that RU-486 reverses the effects of dexamethasone on the adhesive qualities of HUVECs and the expression of adhesive molecules on their surface is therefore most consistent with the hypothesis that the effects of steroids on the endothelium are mediated through glucocorticoid receptors. Further confirmation is provided by the observation that cortisol, but not its inactive metabolite tetrahydrocortisol, inhibits endothelial cell responses to LPS. The absence of a glucocorticoid-responsive element in the gene for ELAM-1 suggests that glucocorticoids must either interfere directly with a transcriptional regulator of ELAM-1 transcription or induce the synthesis of a second regulatory element. Montgomery and co-workers (41) have demonstrated that NFκB regulatory elements are necessary (but not sufficient) for transcription of ELAM-1. Thus the induction by human glucocorticoid receptor (hGR) of the synthesis of a counterregulatory element such as IκB could

account for the inhibition of ELAM-1 expression by dexamethasone. Steroid–receptor complexes also participate in protein–protein interactions with *jun*, preventing its interaction at AP-1 regulatory sites of the 5′ flanking regions of appropriate genes (36, 37), although the AP-1 site present in the gene for ELAM-1 does not appear to participate in the regulation of ELAM-1 (41).

Our results appear to differ from those of Bochner *et al.* (5), who found that prolonged (24 hr) treatment of microvascular endothelium from foreskin with glucocorticoids did not prevent LPS from modulating PMN adhesion, whereas, as we report here, treatment of HUVECs for 4 hr dramatically diminished adhesiveness. The inefficacy of prolonged treatment with corticosteroids may have been due to "desensitization" of hGR (i.e., complete depletion of hGR in the cytosol) in the presence of high concentrations of agonist (42). Alternatively, occupancy of hGR may only transiently transactivate genes that regulate ELAM-1 transcription or translation, permitting subsequent activation of ELAM-1 transcription by LPS.

We were surprised to observe that dexamethasone did not inhibit accumulation of mRNA for ELAM-1 induced by TNF-α. In parallel studies Ghezzi and Sipe (43) found that dexamethasone inhibited LPS-stimulated, but not TNF- or IL-1-stimulated, serum amyloid protein A secretion in mice. Thus LPS, TNF, and IL-1 may induce the transcription of ELAM-1 by several different mechanisms, only some of which are sensitive to corticosteroids.

These experiments permit us to suggest a mechanism for the antiinflammatory effects of glucocorticoids: acting via their receptor, glucocorticoids prevent the recruitment of leukocytes at inflammatory loci by inhibiting the display of adhesive molecules on the surface of the endothelium. This hypothesis rests on three separate lines of evidence: *functional*, at nanomolar concentrations glucocorticoids inhibit endotoxin-stimulated increases of endothelial adhesiveness for leukocytes; *phenotypic*, at similar concentrations they inhibit the stimulated expression of adhesive molecules on the surface of HUVECs; *genotypic*, glucocorticoids inhibit accumulation of mRNA for ELAM-1 in endotoxin- and IL-1-stimulated cells. In addition to these direct receptor-mediated effects of glucocorticoids on the capacity of HUVECs to localize the inflammatory response, glucocorticoids also inhibit the ligand-induced release of cytokines (IL-1, IL-3, and TNF-α) by endothelial and other inflammatory cells (5–12). These findings suggest the general hypothesis that corticosteroids act as antiinflammatory agents by diminishing, directly and indirectly, the ability of HUVECs to direct leukocyte traffic into inflamed or infected tissue. Our data also provide a reasonable explanation for the opposing effects of endotoxin and glucocorticoids in infection and immunity (15, 16).

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