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Lipopolysaccharide-Induced Leukocyte Rolling and Adhesion in the Rat Mesenteric Microcirculation: Regulation by Glucocorticoids and Role of Cytokines

Kelly L. Davenpeck,* John Zagorski,† Robert P. Schleimer,* and Bruce S. Bochner2*

A common side effect of high dose glucocorticoid therapy is increased susceptibility to bacterial infection, an effect that is in part mediated through inhibition of leukocyte recruitment to infected areas. However, the sites at which glucocorticoids act to prevent the multistep process of leukocyte recruitment have not been fully established. In this study, the effects of the glucocorticoid dexamethasone (DEX) on leukocyte-endothelial interactions, in response to bacterial LPS, were examined utilizing a model of rat mesenteric intravital microscopy. Pretreatment of rats with DEX (0.5 mg/kg) for 18 h or 30 min before stimulation with LPS significantly inhibited LPS-induced leukocyte rolling and adhesion in mesenteric postcapillary venules. Pretreatment with DEX also inhibited LPS-induced changes in expression of L-selectin and a shared epitope of CD11b/c on circulating neutrophils. These effects of DEX may be due to DEX inhibition of IL-1, TNF, and cytokine-induced neutrophil chemoattractant-1 (CINC-1) generation, since antagonists to these mediators were able to mimic DEX effects on leukocyte-endothelial interactions and circulating leukocyte phenotype. These data indicate that inhibition of cytokine- and chemokine-induced leukocyte-endothelial interactions may be a primary mechanism by which glucocorticoids inhibit leukocyte recruitment to bacterial agents and thus increase susceptibility to infection. The Journal of Immunology, 1998, 161: 6861–6870.

Glucocorticoids have potent immunosuppressive effects and are widely used in the management of chronic inflammatory diseases. Despite their therapeutic benefits, glucocorticoid excess results in a variety of side effects, including enhanced susceptibility to bacterial infection. One of the primary mechanisms by which glucocorticoids are thought to suppress the body’s response to bacterial infection is through inhibition of leukocyte recruitment to infected areas (1). Leukocytes play a crucial role in the destruction of opportunistic and pathogenic organisms, and movement of leukocytes out of the circulation into infected LPS-tissues is essential for bacterial killing to occur. Bacterial LPS, a component of the outer wall of most Gram-negative bacteria, is a potent inflammatory agent and plays a primary role in bacterial-induced leukocyte recruitment (2–4). Although glucocorticoids have been demonstrated to inhibit LPS-induced leukocyte recruitment (1, 5, 6), the sites at which glucocorticoids act to prevent the multistep cascade of leukocyte recruitment have not been fully defined.

Recent data concerning the important role of leukocyte and endothelial adhesion molecules in leukocyte recruitment have led to the speculation that glucocorticoid-mediated inhibition of the inflammatory response and, in particular, leukocyte recruitment may be the result of alterations in the expression and/or function of the leukocyte and endothelial adhesion molecules that mediate leukocyte extravasation. In vitro studies examining the direct effect of glucocorticoids on adhesion molecule expression have not yielded definitive data. For instance, Kaiser et al. found that the glucocorticoid budesonide did not inhibit IL-1- or TNF-α-induced expression of E-selectin, ICAM-1, or VCAM-1 on HUVEC (7), while Cronstein et al. found dexamethasone to be effective in inhibiting both LPS- and IL-1-induced synthesis and expression of E-selectin and ICAM-1 (8). Evidence concerning the effects of glucocorticoids on leukocyte adhesion molecule expression are equally inconclusive. Schleimer et al. (9) reported no effect of glucocorticoids on human neutrophil adhesion responses, while in vivo studies have described changes in expression of leukocyte adhesion molecules, particularly β2 integrins and L-selectin, following administration of glucocorticoids (5, 10, 11). Thus, whether glucocorticoids inhibit leukocyte recruitment to sites of inflammation by directly altering adhesion molecule expression remains unresolved.

An alternate and perhaps more likely mechanism by which glucocorticoids may inhibit leukocyte recruitment in response to LPS is through inhibition of inflammatory mediator production and/or release, an effect that could indirectly alter adhesion molecule expression (12). LPS is a potent stimulus for cytokine and chemokine release from several cell types, including monocytes, macrophages, and endothelial cells. In vivo, cytokines such as IL-1 and TNF-α are rapidly released in response to LPS (13–15), and both of these cytokines induce endothelial adhesion molecule expression (7). Glucocorticoids inhibit production of these cytokines (12, 16, 17), as well as chemokines of the C-X-C family such as cytokine-induced neutrophil chemoattractant-1 (CINC-1), which is involved in mediating leukocyte recruitment in response to LPS.

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3 Abbreviations used in this paper: CINC-1, cytokine-induced neutrophil chemoattractant-1; DEX, dexamethasone-21-phosphate; IL-1R, soluble murine IL-1 receptor; TNFR:Fc, soluble human TNF receptor linked to the Fc region of human IgG1; TESTOST, testosterone.
DEX inhibits LPS-induced leukocyte rolling and adhesion

(18–20). Thus, glucocorticoids may inhibit leukocyte recruitment by inhibiting the mediators that induce adhesion molecule-mediated leukocyte endothelial interactions and leukocyte migration.

We have previously demonstrated that superfusion of a single loop of rat mesentry with LPS results in dose- and time-dependent increases in leukocyte rolling and adherence in mesenteric postcapillary venules and that LPS-induced changes in leukocyte rolling and adhesion are largely mediated by both P- and L-selectin (21). The major aims of the experiments described herein were 1) to determine whether glucocorticoids block leukocyte recruitment in response to LPS by inhibiting adhesion molecule-mediated leukocyte-endothelial interactions and 2) to determine whether glucocorticoids affect leukocyte-endothelial interactions through inhibition of the release or actions of cytokines and/or chemokines. To achieve the first aim we utilized an established in vivo model of rat intravitral microscopy to directly examine the effects of the glucocorticoid dexamethasone (DEX) on LPS-induced leukocyte rolling along, and adhesion to, the vascular endothelium. To address the second aim we examined whether antagonists to the cytokines IL-1 and TNF, or to the chemokine CINC-1, given alone or in combination, could mimic the effects of glucocorticoids on LPS-induced leukocyte-endothelial interactions. We demonstrate that either prolonged (i.e., 18 h) or short-term (i.e., 30 min) pretreatment of rats with DEX significantly inhibited the selectin-mediated leukocyte rolling and adhesion induced by superfusion of the mesentery with LPS and prevented LPS-induced alterations in circulating leukocyte adhesion molecule expression. Antagonism of IL-1, TNF, and CINC-1 inhibited LPS-induced leukocyte rolling and adhesion in a manner similar to DEX treatment, thus supporting the hypothesis that glucocorticoid suppression of leukocyte recruitment to LPS is mediated through effects on cytokine generation and/or release.

Materials and Methods

Rat mesenteric intravital microscopy

In accordance with an animal research protocol approved by the Johns Hopkins University Animal Care and Use Committee, male Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) underwent anesthesia and surgical manipulation with exteriorization of ileal mesentery to facilitate intravitral microscopy, as previously described (21). The ileum and mesentery were superfused throughout the experiment with a modified Krebs-Henseleit buffer (KH 2 PO 4 , 1.19 MgSO 4 , 12.5 NaHCO 3 ) (Sigma, St. Louis, MO) heated to 37°C and bubbled with 95% N 2 and 5% CO 2 . A Zeiss Axioskop fixed stage microscope was employed for observation of the mesentery perfused with dexamethasone (i.e., 18 h or 30 min), and their mesentery was superfused with Krebs-Henseleit buffer alone for the entire 2-h period. No significant differences in unstimulated leukocyte rolling or adhesion were seen with either 18 h (n = 4) or 30 min (n = 2) DEX pretreatment when compared with rats not treated with DEX (21). The data presented here refer to groups (i.e., 18 h or 30 min DEX pretreatment) combined (n = 6) and together referred to as “buffer control.” Soluble murine IL-1 receptor (IL-1R, 100 µg/kg) (24) and/or soluble human TNF receptor linked to the Fc region of human IgG1 (TNFR:Fc, 100 µg/kg) (25), generously provided by Drs. Anthony Truitt and Michael Widmer (Inmunex Corporation, Seattle, WA), were given, alone or simultaneously, ten min before LPS superfusion and again following 60 min of superfusion. In other experiments, a neutralizing goat anti-rat CINC-1 polyclonal Ab (19) or control preimmune goat serum was given i.v. (2 mg/rat) ten min before LPS superfusion.

Experimental protocol

Following exteriorization and placement of a loop of ileal mesentery in the superfusion chamber, a 23–42 µm diameter postcapillary venule was chosen for observation. A baseline control recording of 2 min duration was made, and the tissue was then allowed to stabilize for 30 min. If leukocyte rolling or adhesion was observed to increase during this period, the experiment was terminated. Following the 30-min stabilization period, a second video recording (time 0) was made to establish basal values for leukocyte rolling and adherence and for leukocyte rolling velocities. To minimize the influence of preactivation of the tissue, only vessels in which leukocyte rolling was ≤30 cells/min and adherence ≤3 cells/100 µm of venular endothelium were utilized for study. Following stabilization of the tissue, the mesentery was superfused with 1 µg/mL of LPS (from Escherichia coli serotype 0127:B8; Sigma; lot 63H4010) in modified Krebs-Henseleit solution for 120 min as previously described (21). This concentration was shown to be optimal in our previous concentration-response studies and induced similar effects to those seen with LPS from other bacterial serotypes (21). LPS superfusion was initiated immediately following the 0-min video recording, and then subsequent video recordings were made at 30, 60, 90, and 120 min after the initiation of superfusion for determination of leukocyte rolling and adherence and of leukocyte rolling velocity. Arterial blood samples (<100 µl) were obtained at each of the above time points, and circulating total white blood cell (WBC) numbers were determined by light microscopic counting (Uno-pette, Test 5856; Becton Dickinson, Rutherford, NJ), as described (21). Whole blood smears for determination of leukocyte differentials were also made at baseline, 0, and 120 min. Cell differentials were determined by Diff-Quik staining (Shandon, Pittsburgh, PA). In some animals, arterial blood samples (1 ml) were taken before the initial video recording and again after 2 h, and leukocytes were isolated for flow cytometric analysis of leukocyte adhesion molecule expression (See methods below).

For some experiments, rats were pretreated either 18 h or 30 min before LPS superfusion with dexamethasone-21-phosphate (DEX) (Sigma) at a dose of 0.5 mg/kg given as a s.c. injection in 300 µl of sterile PBS. Control rats were pretreated 18 h or 30 min before LPS superfusion with s.c. injections of either 300 µl of PBS alone or with testosterone given at a dose of 0.5 mg/kg (n = 4 for 18 h pretreatment and n = 2 for 30 min pretreatment; data are combined for n = 6 because a similar lack of effect was seen; see Results). To examine the effects of DEX pretreatment on basal or unstimulated leukocyte rolling and adhesion, a group of rats was pretreated with dexamethasone (i.e., 18 h or 30 min), and their mesentery was superfused with Krebs-Henseleit buffer alone for the entire 2-h period. No significant differences in unstimulated leukocyte rolling or adhesion were seen with either 18 h (n = 4) or 30 min (n = 2) DEX pretreatment when compared with rats not treated with DEX (21). The data presented here refer to groups (i.e., 18 h or 30 min DEX pretreatment) combined (n = 6) and together referred to as “buffer control.” Soluble murine IL-1 receptor (IL-1R, 100 µg/kg) (24) and/or soluble human TNF receptor linked to the Fc region of human IgG1 (TNFR:Fc, 100 µg/kg) (25), generously provided by Drs. Anthony Truitt and Michael Widmer (Inmunex Corporation, Seattle, WA), were given, alone or simultaneously, ten min before LPS superfusion and again following 60 min of superfusion. In other experiments, a neutralizing goat anti-rat CINC-1 polyclonal Ab (19) or control preimmune goat serum was given i.v. (2 mg/rat) ten min before LPS superfusion.

Rat leukocyte isolation and flow cytometric analysis of leukocyte adhesion molecule expression

Murine anti-rat CD11a (WT.1, IgG2a, 5 µg/ml), CD11b/c (OX-42, IgG2a, 1 µg/ml), and CD18 (WT.3, IgG1, 5 µg/ml) (PharMingen, San Diego, CA) and the α6 integrin mAb TA-2 (IgG1, 1 µg/ml) (Seikagaku America, Rockville, MD) were purchased and used at the indicated saturating concentrations. A murine anti-rat L-selectin mAb (LAM1-116, IgG1, 3 µg/ml) was generously provided by Drs. Thomas Tedder and Douglas Steeber (Duke University Medical Center, Durham NC). Control nonbinding mouse IgG1 (10 µg/ml) and IgG2a (10 µg/ml) were obtained from Coulter Corporation (Hialeah, FL). Labeling of cells for indirect immunofluorescence was performed essentially as described (26, 27) using saturating concentrations of FITC-conjugated goat anti-mouse secondary Ab (Caltag Laboratories, South San Francisco, CA). Cells were immediately analyzed without fixation using an EPICS Profile II Flow cytometer (Coulter Corporation). Isotype control staining typically yielded values for mean fluorescence of 1–3.

To determine the effects of local LPS superfusion on circulating leukocytes, leukocyte adhesion molecule expression was examined on isolated leukocytes from animals undergoing 2 h superfusion of a single loop of mesentery with LPS (i.e., application of LPS for intravitral microscopy) and compared with adhesion molecule expression on leukocytes from buffer control animals. All animals underwent the same surgical procedures as described for intravitral microscopy, and the mesentery was secured for intravitral microscopic observation as described above. An arterial blood sample (1 ml) was taken, placed in EDTA, and stored at 4°C before the initial video recording and the blood volume replaced with normal saline. Following superfusion of the mesentery with LPS or normal Krebs for 2 h, a second blood sample was obtained. Mixed populations of whole blood...
leukocytes were isolated from EDTA-anticoagulated arterial blood samples. A leukocyte-rich buffy coat was obtained by centrifugation at 400g for 20 min at 22°C, and contaminating red blood cells were removed via hypotonic lysis performed at 4°C. Cell differentials were determined by Diff-Quik staining (Shandon), and viability was confirmed by erythrosin B dye exclusion. Leukocyte adhesion molecule expression was examined for all groups of rats including LPS, DEX-pretreated (18 h and 30 min), DEX-treated buffer controls, IL-1R/TNFR:Fc-treated animals, and anti-CINC-1 Ab treatments, we also examined leukocyte differentials at baseline and at 60 min after LPS superfusion. As reported previously (21), circulating leukocyte numbers increased in both LPS and DEX-treated animals (Table I). In rats pretreated with DEX for 18 h (i.e., buffer control and DEX-treated animals) and red cell velocity in mesenteric postcapillary venules cannot be excluded. However, in an attempt to insure that hemodynamic parameters did not contribute to potential changes in leukocyte rolling and adhesion, vessels within the same diameter range (25 to 40 μm) and with similar red cell velocities to those utilized in non-DEX-treated animals were selectively chosen for observation. Thus, there was no significant difference in venular wall shear rates among the various treatment groups (Table I), and, under these conditions, DEX effects cannot be attributed to shear-related effects.

To determine whether glucocorticoids could inhibit LPS-induced leukocyte recruitment by altering leukocyte-endothelial interactions, rats were pretreated with DEX for either 18 h or 30 min before initiation of LPS superfusion, and effects on leukocyte rolling and adhesion were quantified. DEX pretreatment of rats for 18 h completely inhibited LPS-induced changes in leukocyte rolling and adhesion (Fig. 1, A and B). Values for leukocyte rolling and adhesion in 18-h DEX-pretreated animals in which the mesentery was superfused with LPS were not significantly different from values for buffer control animals. Although initially intended as a control condition, pretreatment of rats with DEX for only 30 min before initiation of LPS superfusion also significantly attenuated leukocyte rolling and completely inhibited leukocyte adhesion (Fig. 1, A and B). Inhibition of leukocyte rolling was not complete, as was seen with 18-h pretreatment, but there was >70% inhibition of leukocyte rolling at all time points. Inhibition of LPS-induced leukocyte rolling and adhesion appear to be specific to glucocorticoids, since pretreatment of rats with the sex steroid testosterone (0.5 mg/kg), either 18 h or 30 min before LPS superfusion, had no effect on LPS-induced leukocyte rolling and adhesion (Fig. 1, A and B).

**DEX effects are not mediated by changes in hemodynamic parameters or circulating leukocyte populations**

We have previously shown that the LPS-induced changes in leukocyte rolling and adhesion observed in this model system occurred in the absence of significant changes in venular wall shear rate (21). Since pretreatment of rats with DEX occurred before set-up and selection of mesenteric venules for observation by intravital microscopy, potential effects of DEX on venular diameter and red cell velocity in mesenteric postcapillary venules cannot be excluded. However, in an attempt to insure that hemodynamic parameters did not contribute to potential changes in leukocyte rolling and adhesion, vessels within the same diameter range (25 to 40 μm) and with similar red cell velocities to those utilized in non-DEX-treated animals were selectively chosen for observation. Thus, there was no significant difference in venular wall shear rates among the various treatment groups (Table I), and, under these conditions, DEX effects cannot be attributed to shear-related effects.

To determine whether changes in the number or differential of circulating leukocytes was responsible for the DEX-mediated decreases in LPS-induced leukocyte rolling and adhesion, we monitored these parameters. As reported previously (21), circulating leukocyte numbers increased in both buffer control and LPS-treated animals (Table I). In rats pretreated with DEX for 18 h (i.e., buffer control and DEX + LPS), circulating leukocyte numbers at baseline were decreased compared with non-DEX-treated rats, although these values did not reach statistical significance when examined utilizing ANOVA. Despite the decreased number of circulating leukocytes at baseline, circulating leukocyte counts increased in these animals in a manner similar to that observed in non-DEX-treated rats. Administration of DEX 30 min before LPS superfusion had no effect on circulating leukocyte numbers at baseline or at any of the later time points. Similarly, administration of testosterone had no effects on circulating leukocyte numbers at any of the time points examined (Table I).

Because glucocorticoids can alter circulating leukocyte populations, we also examined leukocyte differentials at baseline and at

**FIGURE 1.** DEX inhibited LPS-induced leukocyte rolling (A) and adhesion (B). Superfusion of the rat mesentery with LPS (1 μg/ml) resulted in rapid increases in leukocyte rolling and adhesion. Pretreatment of rats with DEX (0.5 mg/kg) 18 h before initiation of LPS superfusion completely inhibited LPS-induced leukocyte rolling and adhesion (n = 6). Pretreatment with DEX for only 30 min also significantly decreased leukocyte rolling and adhesion (n = 6). The nonglucocorticosteroid hormone testosterone (TESTOST, 0.5 mg/kg) given as a control did not affect LPS-induced leukocyte-endothelial interactions when given either 18 h (n = 4) or 30 min (n = 2) before LPS superfusion (data is combined in figure for n = 6 since results were similar). * Values for 18-h and 30-min DEX-treated rats, which are significantly (p < 0.05) different from the LPS + PBS condition.
the termination of the intravital microscopy protocol. Under baseline control conditions, the majority (~80%) of circulating leukocytes in the rat are lymphocytes, with neutrophils making up approximately 10–20% and the remainder monocytes and eosinophils (Fig. 2). Following surgical manipulation and intravital microscopy, the leukocyte differential is changed substantially, with neutrophils making up the majority of circulating cells (~60%; Fig. 2). Similar to changes in circulating leukocyte numbers, a change in leukocyte differential occurs both in the presence and absence of LPS (i.e., buffer control) (21), implying that it is not a direct effect of LPS superfusion of the mesentery. Pretreatment of rats with DEX for 18 h resulted in changes in circulating leukocyte differentials as compared with non-DEX-treated rats (Fig. 2). When rats were pretreated with DEX for 18 h, there was a significant decrease in the percentage of circulating lymphocytes, with a concomitant increase in the percentage of neutrophils. Following surgical manipulation and 2 h LPS superfusion, rats pretreated with DEX for 18 h continued to have a significantly increased percentage of circulating neutrophils and a decreased percentage of lymphocytes, when compared with non-DEX-treated rats. This was not seen in rats pretreated with DEX for only 30 min. Despite the significant increase in the percentage of circulating neutrophils, rats treated with DEX for 18 h had the lowest number of rolling and adherent cells.

**DEX inhibits LPS-induced changes in L-selectin and β2 integrin expression on circulating neutrophils**

Since DEX was demonstrated to significantly decrease leukocyte rolling and adhesion, a series of experiments was performed to determine whether DEX was inhibiting leukocyte-endothelial interactions by altering the expression of L-selectin and/or the β2 integrins on circulating neutrophils. To examine this, mixed leukocyte populations were obtained from whole blood samples taken at baseline (after surgical manipulation) and after 2 h of LPS superfusion; expression of various adhesion molecules was examined by indirect immunofluorescence and flow cytometry. In the first series of experiments, the effect of LPS superfusion on circulating leukocyte phenotype was examined with or without DEX pretreatment. Superfusion with LPS for 2 h significantly altered the phenotype of circulating leukocytes. In particular, LPS superfusion resulted in a significant decrease in the percentage of neutrophils expressing L-selectin (Fig. 3A), although lymphocyte L-selectin expression was not altered (data not shown). The decrease in neutrophil L-selectin expression was accompanied by an up-regulation of the expression of a shared CD11b/CD11c epitope (Fig. 3B). However, expression of the β2 integrin subunit CD18, as well as CD11a and α4 integrin, were not altered by LPS superfusion (data not shown). The effects of DEX on this response are also shown in Figure 3. Both 18 h and 30 min pretreatment with DEX completely inhibited the changes in leukocyte adhesion molecule expression brought about by LPS superfusion of the mesentery (Fig. 3, A and B). In contrast, no significant changes in leukocyte L-selectin or β2 integrin expression were observed in control buffer-superfused animals (Fig. 3, A and B). These data indicate that, unlike changes in circulating leukocyte numbers and differentials, changes in circulating leukocyte phenotype are a direct result of LPS superfusion of the mesentery (i.e., not due to anesthesia or surgical manipulation) and are completely inhabitable by DEX.

To determine whether DEX prevented LPS-induced L-selectin shedding and up-regulation of the CD11b/c integrins in vivo by altering the ability of leukocytes to respond to LPS, we next performed a series of experiments in which whole blood was obtained from control and DEX-treated rats and stimulated ex vivo with LPS. Flow cytometric analysis of changes in leukocyte phenotype

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**Table I. Comparison of venular wall shear rates and circulating leukocyte counts among the treatment groups**

<table>
<thead>
<tr>
<th>Venular wall shear rate (sec⁻¹)</th>
<th>Time (min)</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
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<tbody>
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<td>Buffer control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS + PBS</td>
<td></td>
<td>561.6 ± 59.8</td>
<td>554.9 ± 62.4</td>
<td>544.3 ± 56.8</td>
<td>570.5 ± 69.8</td>
<td>540.9 ± 72.8</td>
</tr>
<tr>
<td>LPS + 18-h DEX pretreatment</td>
<td></td>
<td>541.7 ± 44.3</td>
<td>503.0 ± 44.4</td>
<td>495.6 ± 52.1</td>
<td>516.2 ± 52.1</td>
<td>509.4 ± 58.9</td>
</tr>
<tr>
<td>LPS + 30-min DEX pretreatment</td>
<td></td>
<td>528.2 ± 47.9</td>
<td>517.9 ± 54.4</td>
<td>526.1 ± 57.6</td>
<td>527.2 ± 50.6</td>
<td>532.7 ± 61.9</td>
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<tr>
<td>LPS + testosterone</td>
<td></td>
<td>600.6 ± 47.1</td>
<td>544.0 ± 75.0</td>
<td>593.0 ± 55.3</td>
<td>561.0 ± 54.3</td>
<td>522.0 ± 59.7</td>
</tr>
<tr>
<td>LPS + TESTOST</td>
<td></td>
<td>536.9 ± 33.7</td>
<td>542.0 ± 36.1</td>
<td>522.9 ± 29.1</td>
<td>490.9 ± 29.1</td>
<td>481.1 ± 18.7</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; n = 6 for all groups. Data for testosterone is a combination of 30-min and 18-h pretreatment groups. At a given time point, none of the values were significantly different from buffer control.
was then performed. Stimulation of whole blood obtained from control and DEX (18 h and 30 min)-treated animals with LPS (1 to 1000 ng/ml) for 30 min at 37°C resulted in concentration-dependent shedding of L-selectin from rat neutrophils (Fig. 3C). Pretreatment with DEX for 18 h significantly enhanced ex vivo LPS-induced L-selectin shedding, resulting in a log fold reduction in the concentration of LPS required for this effect. In contrast, ex vivo stimulation of rat neutrophils with LPS did not result in any significant change in $\beta_2$ integrin expression when neutrophils were obtained from either control or DEX-treated rats (data not shown). These data clearly indicate that DEX does not inhibit neutrophil responsiveness to LPS but also indicate that the changes in adhesion molecule expression in vivo may not be a direct result of neutrophil stimulation with LPS.

FIGURE 3. DEX effects on LPS-induced changes in neutrophil expression of L-selectin (A) and a shared CD11b/c epitope (B). Superfusion of a single loop of rat mesentery with LPS for 2 h resulted in a significant decrease in the percentage of neutrophils expressing L-selectin and a significant increase in CD11b/c expression as compared with baseline values. Pretreatment of rats with DEX for 18 h or 30 min completely inhibited in vivo LPS-induced changes in leukocyte L-selectin and CD11b/c integrin expression. *, Values after LPS, which were significantly ($p < 0.05$) different from pre-LPS (0 min) values ($n = 4–5$). C, DEX does not inhibit ex vivo LPS-induced L-selectin shedding. Stimulation of rat whole blood with LPS (30 min, 37°C) resulted in concentration-dependent decreases in L-selectin expression on neutrophils from control and DEX (18 h and 30 min)-treated rats. Neutrophils from rats pretreated with DEX for 18 h displayed significantly enhanced L-selectin shedding in response to LPS in vitro ($n = 5$). *, Values significantly ($p < 0.05$) different from control animals.

Role for IL-1 and TNF in LPS-induced leukocyte rolling and adhesion

Having determined that the ability of DEX to inhibit LPS-induced leukocyte-endothelial interactions was not the result of direct effects of DEX on LPS-induced changes in leukocyte adhesion molecule expression, we performed a series of experiments aimed at examining whether DEX effects on LPS-induced leukocyte rolling and adhesion were mediated indirectly, by inhibition of inflammatory mediators. Because glucocorticoids are potent inhibitors of cytokine production, and LPS induces production of many cytokines including IL-1 and TNF, we performed experiments utilizing soluble IL-1 (IL-1R) and TNF (TNFR:Fc) receptors to determine whether these cytokines were involved in mediating LPS-induced leukocyte rolling and adhesion. Administration of either IL-1R or TNFR:Fc resulted in partial inhibition of leukocyte rolling, although there was a different time course of inhibition for each soluble receptor (Fig. 4A). Soluble IL-1R alone did not inhibit early leukocyte rolling (30 and 60 min) but significantly inhibited leukocyte rolling at later time points (90 and 120 min). Conversely, TNFR:Fc inhibited leukocyte rolling at all time points, although inhibition was most pronounced at the earliest time point (30 min). Both antagonists had significant effects on leukocyte rolling when given alone, but the greatest inhibition of leukocyte rolling was observed when IL-1R and TNFR:Fc were given together (Fig. 4A). IL-1R and TNFR:Fc together significantly decreased leukocyte rolling at all time points, and values for rolling were not significantly different from values in DEX-treated rats.

FIGURE 4. Role for IL-1 and TNF in LPS-induced leukocyte rolling (A) and adhesion (B). Intravenous infusion of TNFR:Fc (100 μg/kg, 10 min prior and 60 min after starting LPS superfusion, $n = 5$) significantly inhibited leukocyte rolling at all time points, while administration of IL-1R (100 μg/kg, 10 min prior and 60 min after starting LPS superfusion, $n = 5$) significantly inhibited leukocyte rolling only at later (90 and 120 min) time points. TNFR:Fc significantly inhibited leukocyte adhesion at 30 and 120 min, while IL-1R inhibited adhesion only at the latest time point. Simultaneous infusion of IL-1R and TNFR:Fc ($n = 5$) significantly inhibited LPS-induced increases in leukocyte rolling and adhesion at all time points. *, Values significantly ($p < 0.05$) different from the LPS + PBS condition.
similar results were found for leukocyte adhesion (Fig. 4B). Administration of either soluble receptor alone resulted in partial inhibition of leukocyte adhesion, while IL-1R and TNFR:Fc given together completely inhibited adhesion at all time points (Fig. 4B). Together, these data demonstrate that both IL-1 and TNF play an integral role in LPS-induced leukocyte-endothelial interactions and are consistent with the hypothesis that DEX inhibits LPS-induced rolling and adhesion by inhibiting production of these cytokines.

Role for cytokine-induced neutrophil chemoattractant-1 (CINC-1) in LPS-induced leukocyte rolling and adhesion

IL-1, TNF, and LPS have all been demonstrated to result in production and release of the neutrophil active chemokine CINC-1 in the rat, and CINC-1 has been demonstrated to play a significant role in LPS-induced leukocyte recruitment (18–20). To determine whether CINC-1, induced by LPS or the cytokines IL-1 or TNF, was playing a role in leukocyte-endothelial interaction in our model, a polyclonal Ab directed to CINC-1 was administered (2 mg/rat) ten minutes before superfusion of the mesentery with LPS. Pretreatment of rats with preimmune goat serum did not significantly alter LPS-induced leukocyte rolling or adhesion (n = 3, data not shown). Since IL-1R and TNFR:Fc given together completely blocked leukocyte rolling and adhesion at the earliest time point (30 min), and anti-CINC antibody had no effect at this time point, it is possible that production of CINC-1 is downstream of cytokine production and thus may be induced by IL-1 and/or TNF. This is further supported by the finding that values for leukocyte rolling and adhesion obtained from rats pretreated with all three antagonists (i.e., IL-1R, TNFR:Fc, and anti-CINC-1 Ab) simultaneously were not significantly different from values obtained when only the cytokine antagonists (IL-1R and TNFR:Fc) were given (data not shown).

Changes in LPS-induced leukocyte rolling and adhesion observed with administration of IL-1R or TNFR:Fc, either alone or in combination, or with administration of anti-CINC-1 Ab were not the result of hemodynamic changes. Neither IL-1R, TNFR:Fc, nor anti-CINC-1 Ab had any effect on venular diameter, RBC velocity, or venular wall shear rates (data not shown).

Role for cytokines and chemokines in systemic changes in circulating leukocyte numbers and phenotype

Since IL-1, TNF, and CINC-1 were all demonstrated to play a role in LPS-induced changes in leukocyte rolling and adhesion, experiments were performed to determine whether these inflammatory mediators were playing a role in in vivo changes in circulating leukocyte phenotype observed with LPS superfusion of the mesentery. Similar to experiments described earlier, mixed leukocyte populations were isolated at baseline and, after LPS superfusion from animals, given either the IL-1R and TNFR:Fc combination or the CINC-1 polyclonal Ab. Expression of L-selectin and the $\beta_2$ integrins was analyzed by flow cytometry. Similar to effects of DEX, administration of both IL-1R and TNFR:Fc in combination, or the anti-CINC-1 Ab, significantly attenuated the LPS-induced decreases in neutrophil L-selectin expression (Fig. 6A). In contrast, IL-1R and TNFR:Fc given in combination, but not anti-CINC-1 Ab, blocked the LPS-induced up-regulation CD11b/c (Fig. 6B). None of the antagonists affected circulating cell numbers or differentials (data not shown).
Discussion

Glucocorticoid suppression of the immune response to infection was first documented over sixty years ago. In a paper that appeared in 1932, Dr. Harvey Cushing described the syndrome, which would later bear his name, of hypercortisolism, and in this manuscript reported an increased susceptibility to infection in association with this syndrome (28). The first report of clinical use of glucocorticoids by Hench et al. (29) for suppression of aberrant immune responses in diseases such as rheumatoid arthritis appeared in the literature in 1950 and was soon followed by case reports and animal studies that showed that administration of exogenous glucocorticoid for the treatment of disease was associated with a wide variety of side effects, including enhanced susceptibility to infection (30, 31). Despite this well-established effect of glucocorticoids, the mechanisms by which glucocorticoids suppress the immune response to infection have not been fully elucidated.

In the present study, we have examined the effects of glucocorticoids on the earliest stages of leukocyte recruitment (i.e., rolling and adhesion) in response to bacterial LPS and have also examined the role of cytokines in LPS-induced leukocyte recruitment. Pre-treatment of rats with DEX (18 h or 30 min) dramatically reduced LPS-induced leukocyte rolling and adhesion in mesenteric post-capillary venules and also inhibited LPS-induced changes in circulating leukocyte phenotype (i.e., L-selectin shedding, CD11b/c integrin up-regulation). The data presented herein concerning the ability of DEX to inhibit LPS-induced changes in leukocyte rolling and adhesion, and in leukocyte phenotype, give new insight into the means by which glucocorticoids block LPS-induced leukocyte recruitment and may also provide new insight into the protective role of glucocorticoids demonstrated in some animal models of sepsis. Although glucocorticoid administration in the absence of antibiotic treatment will enhance bacterial infection, some studies have demonstrated a decrease in mortality due to bacterial sepsis with glucocorticoid treatment (1). These effects are believed to be due to the ability of glucocorticoids to decrease expression of inflammatory mediators, such as TNF, IL-1, and IL-8, that contribute to the hemodynamic instability and organ failure associated with sepsis. Our data imply that some of the protective effects of glucocorticoids in septic shock may also be mediated by inhibition of leukocyte recruitment responses and changes in circulating leukocyte phenotype that accompany the release of these cytokines. However, in blocking leukocyte recruitment responses, one also decreases the ability of leukocytes to kill bacteria. This may play a role in the lack of efficacy in human studies of high dose glucocorticoids for treatment of sepsis, since one of the complications of glucocorticoid therapy is secondary infection (32–34).

We hypothesized that the mechanism by which DEX inhibits leukocyte rolling and adhesion, as well as changes in leukocyte phenotype in our model, is via inhibition of cytokine and chemokine production and/or release. This hypothesis is supported by our findings that antagonists (i.e., soluble receptor or Abs) to specific cytokines (IL-1 and TNF) and chemokines (CINC-1) effectively mimicked DEX effects on LPS-induced leukocyte rolling and adhesion and on changes in circulating neutrophil phenotype. Administration of IL-1R and TNFR:Fc together, and in combination with the anti-CINC Ab, resulted in values for leukocyte rolling and adhesion that were not significantly different from values seen in DEX-treated rats. Further support for this hypothesis is provided by data from previous studies that have found DEX to be ineffective in decreasing leukocyte rolling and adhesion in response to direct tissue stimulation with exogenously applied mediators (35–37). For instance, three different studies have reported that DEX does not decrease leukocyte rolling or adhesion in response to tissue stimulation with chemotactic agents such as leukotriene B4, FMLP, or platelet-activating factor, although DEX does decrease transmigration of adherent leukocytes (35–37). The exception to this is a recent report by Tailor et al. (37) in which DEX partially inhibited leukocyte adhesion in response to IL-1β.

The major difference between our study and these previous studies is the type of stimulus utilized. In each of these studies (35–37), the microcirculatory tissue was directly stimulated with inflammatory mediators, while we stimulated with LPS, a substance known to induce inflammatory mediator synthesis. As such, our model may be more indicative of the normal tissue response to pathogens, where endogenous mediators are produced. This type of model allows for the study of glucocorticoid effects on the production of inflammatory mediators, not just their effects on responses to these mediators. Interestingly, this is supported by data from very early intravital microscopy studies examining these same glucocorticoid effects (38–40). In the 1950s several groups noted that administration of glucocorticoids decreased leukocyte adhesion to the vascular endothelium in various models of inflammation, including thermal injury, tuberculosis infection, and “serum sickness” (38–40). Though not known at the time, inflammation in these models relied on production of endogenous inflammatory mediators, and, similar to our findings, glucocorticoids were very effective in inhibiting leukocyte-endothelial interactions under these conditions.

The most direct means to test whether glucocorticoids exert their effects on leukocyte recruitment by inhibiting cytokine and/or chemokine production would be to measure levels of these mediators in the mesenteric tissues. Unfortunately, the mesentery superfusion model makes this very difficult, since the superfusion buffer dilutes released cytokine by several hundred-fold. Additionally, experiments in which the effects of DEX on endogenously administered cytokines, such as IL-1 and TNF, are examined may give some insight into the mechanism of these DEX effects, but these experiments are complicated by the ability of cytokines, particularly IL-1 and TNF, to induce release of other inflammatory mediators. For instance, in the case of the inhibitory effects of DEX on IL-1β-induced leukocyte adhesion observed by Tailor et al. (37), it is possible that DEX inhibited leukocyte adhesion not by directly altering IL-1 effects, but by inhibiting IL-1-induced production of CINC-1, since IL-1 is the most potent stimulus for production of this chemokine. Similarly, LPS-induced TNF is believed to play a role in LPS-induced IL-1 production, which is itself DEX inhibitable.

One surprising outcome of the present studies, which also warrants further study, is the rapidity with which DEX affected the immune response to LPS. The primary means by which glucocorticoids mediate their actions is through regulation of gene expression (41–43), and numerous genes involved in metabolism, immunological responses, and inflammation, including the genes for IL-1, TNF and CINC-1, are known to be glucocorticoid sensitive. Since gene transcription is the primary mechanism of action for glucocorticoids, the time course for glucocorticoid effects has been felt to be over the course of several hours. Thus, the majority of in vivo and in vitro experiments examining glucocorticoid effects have looked at glucocorticoid actions after prolonged (≥4 h) treatment. In the present study, we demonstrate that a single s.c. injection of DEX 30 min before LPS challenge was extremely effective in inhibiting LPS-induced leukocyte rolling and adhesion and also L-selectin shedding and CD11b/c integrin up-regulation when examined 1–2.5 h later. Since the mechanism for these LPS effects appears to involve production of IL-1, TNF, and CINC-1, these data imply that DEX may alter production and/or release of these inflammatory mediators more rapidly than previously believed. Further studies more closely examining the time course of
glucocorticoid effects on gene regulation and inflammatory mediator production are necessary to determine whether the mechanisms of glucocorticoid action are the same during prolonged vs acute treatment.

The data from our studies utilizing cytokine and chemokine antagonists, beyond their relevance to antiinflammatory mechanisms of glucocorticoids, also provide substantial new insight into the more basic mechanisms of LPS-induced leukocyte recruitment. Although LPS-induced cytokine generation and the role of these cytokines in the development of shock associated with bacterial sepsis in animals and man is well established (13–15), our data extend these findings by demonstrating the microvascular and systemic effects of these cytokines on leukocyte recruitment responses and leukocyte adhesion molecule expression. Superfusion of a single loop of mesentery with LPS resulted in sufficient cytokine production, either systemically or in the local mesenteric environment, to facilitate significant increases in leukocyte-endothelial interactions within 30 min of exposure to LPS. The rapidity with which these cytokines affected changes in leukocyte-endothelial interactions in vivo is in sharp contrast to the majority of in vitro studies in which several hours of endothelial cell stimulation with these cytokines is necessary to induce alterations in leukocyte adhesion responses (44).

Since we have previously demonstrated that the changes in rolling and adhesion in this model system are mediated in large part by P- and L-selectin (21), these data imply that there is a rapid change in the expression and/or function of these molecules in response to LPS-induced cytokine production. The fact that endothelial cells can be rapidly induced to express P-selectin on their surface is not surprising, since P-selectin is stored in the endothelial cell and is rapidly translocated to the endothelial surface in response to various stimuli, including histamine, leukotriene C4, and thrombin (45). What is not clear, however, is whether LPS or cytokines can directly regulate P-selectin expression, particularly in vivo. The ability of LPS to directly induce rapid P-selectin expression remains poorly defined (46, 47), and TNF has not been demonstrated to rapidly (30–60 min) up-regulate P-selectin expression (48). Additionally, the contribution of cytokines in the maintenance of surface P-selectin expression following acute translocation has not been examined. In the case of L-selectin, leukocyte rolling mediated by L-selectin requires induction of the L-selectin ligand on endothelial cells. While both LPS and cytokines have been demonstrated to up-regulate an as yet unidentified endothelial ligand for L-selectin (49), the time course of this action has not been examined. Our data imply that, unlike E-selectin, the ligand for L-selectin may be up-regulated within minutes after exposure to LPS-induced cytokines.

LPS-induced cytokines were also found to modulate adhesion molecule expression on circulating leukocytes in this system. Here we show that superfusion of a single loop of bowel with LPS resulted in significant alterations in L-selectin and CD11b/c integrin expression on circulating neutrophils. Following 2 h of LPS superfusion, ~50% of circulating neutrophils no longer expressed detectable levels of L-selectin, while the circulating neutrophil population as a whole had increased CD11b/c integrin expression. Administration of IL-1R and TNFR:Fc completely inhibited these changes, while anti-CINC polyclonal Ab inhibited LPS-induced L-selectin shedding. Our findings that antagonism of these cytokines can block changes in neutrophil phenotype are consistent with data from previous studies demonstrating the ability of cytokines, such as TNF, and the chemokine CINC-1 to alter L-selectin and/or $\beta_2$ integrin expression on neutrophils (50–52). Additionally, the fact that direct stimulation of rat whole blood neutrophils with LPS in vivo did not result in up-regulation of the shared epitope of CD11b/c detected by the mAb OX-42 may be further indication that effects on neutrophil phenotype seen in vivo were not the result of direct stimulation of circulating leukocytes by LPS. Interestingly, our finding that direct stimulation of rat neutrophils with LPS did not up-regulate expression of CD11b/c is in contrast to previously published data concerning the effects of LPS on human neutrophil expression of CD11b (53). Despite these differences, these data together indicate that cytokines generated by local bacterial infection may alter leukocyte recruitment response, not only at the site of infection, but also at distal tissue sites, since circulating neutrophils that lack L-selectin, and perhaps other selectin ligands, would be less able to interact with the endothelium and therefore less likely to be recruited out of the circulation.

As noted above, the apparent rapidity with which these cytokines are generated and influence leukocyte-endothelial interactions is of significance. All three mediators had significant effects on leukocyte rolling and adhesion within the 2-h time course, although there were definite differences in time course of expression and function of each mediator. For instance, administration of TNFR:Fc was effective in inhibiting LPS-induced leukocyte rolling and adhesion by the earliest time point (30 min), while effects of IL-1R were not significant until 90 min. Whether IL-1 and TNF are being rapidly synthesized or released from preformed stores in our model system has not yet been determined; however, data from previous in vivo studies indicate that LPS can induce rapid and differential cytokine production (13–15). For example, Chensue et al. (15), utilizing a mouse model of endotoxemia in which LPS (80 $\mu$g) was given i.p., demonstrated by both immunohistochemistry and by biological assay that TNF and IL-1 were rapidly produced by mononuclear-type cells in the liver and released into the circulation. TNF levels were maximal at one h after introduction of LPS and rapidly decreased after this time point, while induction of IL-1$\beta$ generation was delayed, not reaching maximal levels until 6 h after introduction of LPS, although present by 1 h. Intravenous infusion of LPS resulted in similar findings in man (13, 14). The fact that simultaneous blockade of both cytokines was necessary to maximally inhibit leukocyte rolling and adhesion highlights the possible requirement for antagonism of multiple mediators to achieve the greatest antiinflammatory effect.

The time course of CINC-1 production observed in our model is also consistent with previous data. Dolecki et al. (18), reported that mRNA for CINC-1 is detectable within 15 min of cell stimulation with IL-1, TNF, and LPS in vitro, and protein is released within 1–2 h. Although all three stimuli resulted in some increase in CINC-1 production, IL-1 was the most potent stimulus for CINC-1, with LPS being the second most potent and TNF the least potent. In our studies, CINC-1 was not found to play a significant role in early leukocyte rolling and adhesion (30 min) but was important at all later time points. This delayed time course for CINC-1 function may indicate that its production is downstream of, and thus mediated by, cytokine production in our model. Similarly, the fact that administration of the anti-CINC Ab was just as effective as administration of both cytokine antagonists in inhibiting leukocyte rolling and adhesion at 60, 90 and 120 min indicates that one of the primary mechanisms by which cytokines may induce leukocyte endothelial interactions is through induction of this chemokine.

Interestingly, the data presented herein demonstrating the ability of the anti-CINC-1 Ab to block leukocyte rolling is the first direct evidence that the chemokine CINC-1 may play a role in mediating leukocyte rolling as well as adhesion. The CINC family of chemokines (i.e., CINC-1, CINC-2a, CINC-2b, CINC-3), which are most closely homologous to human or murine gro proteins, are
similar in function to IL-8 in that they appear to function as neutrophil-specific chemoattractants (18–20). Recombinant CINC-1 has been demonstrated to induce neutrophil recruitment and to increase leukocyte adhesion and transmigration in vivo (54), but a role for CINC-1 in leukocyte rolling has not been established. In the present studies, an anti-CINC-1 Ab blocked both rolling and adhesion in response to LPS, indicating that CINC-1 may induce leukocyte rolling responses. This is supported by data from Harris et al. (55), in which they show a role for the selectins, primarily P-selectin and L-selectin, in CINC-1-induced neutrophil recruitment. These authors contend that P-selectin expression in their model is the result of CINC-1-induced histamine release (55); however, based on studies with histamine (H1) antagonists, we have been unable to demonstrate a role for histamine in our model (our unpublished observations).

In conclusion, the data presented demonstrate that the glucocorticoid DEX inhibits LPS-induced leukocyte recruitment, by inhibiting the earliest phases of leukocyte recruitment, leukocyte rolling and adhesion, and that glucocorticoids also inhibit changes in the adhesion phenotype of circulating neutrophils. One mechanism by which DEX may mediate these effects is through inhibition of inflammatory mediator release, especially the IL-1, TNF, and CINC-1, since all three of these cytokines are implicated in LPS-induced leukocyte rolling and adhesion. These data provide new insight into the mechanisms by which glucocorticoid therapy alters neutrophil recruitment responses to LPS.

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