

## In Vivo Effects of the Antiglucocorticoid RU 486 on Glucocorticoid and Cytokine Responses to *Escherichia coli* Endotoxin

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The endogenous adrenocortical response to sepsis is critical for host survival. The in vivo interactions among the endogenous glucocorticoid response, the induction of cytokines, and host survival during endotoxemia were explored in this study by use of the glucocorticoid receptor antagonist RU 486. Male Lewis rats underwent sterile insertion of a right jugular venous catheter. After a 72-h recovery period, animals received a 50% lethal dose of *Escherichia coli* endotoxin (2.5 mg/kg) via the catheter after pretreatment for 30 min prior to lipopolysaccharide (LPS) treatment with (i) vehicle alone intravenously (i.v.) (–corticosterone [–Cort]/–RU 486/+LPS) ( $n = 10$ ), (ii) the antiglucocorticoid RU 486 (10 mg/kg) i.v. (–Cort/+RU 486/+LPS) ( $n = 11$ ), or (iii) RU 486 (10 mg/kg) i.v. in animals that had undergone subcutaneous implantation of a corticosterone pellet at the time of catheter insertion (+Cort/+RU 486/+LPS) ( $n = 10$ ). Except in animals receiving corticosterone pretreatment, baseline plasma corticosterone levels were low in all groups. Plasma corticosterone levels increased significantly ( $P < 0.001$ ) above the baseline following LPS administration. Animals in the –Cort/+RU 486/+LPS-treated group exhibited significantly increased mortality ( $P < 0.001$ ), with only 9% of the animals surviving at 72 h, as well as significantly increased plasma interleukin-6 levels, compared with animals receiving the vehicle alone (–Cort/–RU 486/+LPS), which showed 50% mortality. Pretreatment with corticosterone and RU 486 (+Cort/+RU 486/+LPS) significantly ( $P < 0.001$ ) reversed the mortality observed with RU 486 pretreatment alone (–Cort/+RU 486/+LPS), with 70% of the animals surviving at 72 h, and significantly attenuated the peak plasma tumor necrosis factor and interleukin-6 responses to LPS, compared with those in the animals treated with vehicle alone. These data demonstrate that the blockade of glucocorticoid binding by RU 486 increases LPS-induced mortality. The reversal of this effect by the induction of hypercorticosteronemia prior to RU 486 and LPS exposure (+Cort/+RU 486/+LPS) improves survival and is further associated with significant attenuation of cytokine production. Therefore, these data suggest that the protective effect of the endogenous glucocorticoid response to acute endotoxemia may result from the down-regulation of a potentially lethal cytokine response.

Bacterial endotoxins have been implicated as a major factor in the pathogenesis of gram-negative septic shock, a clinical problem with significant morbidity and mortality (24). The induction of cytokine synthesis in response to endotoxin exposure in vivo undoubtedly plays a critical role in the host response to septicemia. Recently, two cytokines, tumor necrosis factor (TNF) and interleukin-6 (IL-6), were implicated as key mediators in this response. Infusion of TNF into laboratory animals elicited physiologic changes similar to those observed in animals with gram-negative septicemia (19, 35, 37). Furthermore, a role for TNF in the early response to gram-negative sepsis has been suggested by its detection in the circulation of laboratory animals and humans shortly after the administration of endotoxin (12, 22). Passive immunization of endotoxin-sensitive mice with antiserum to TNF substantially reduced the lethal effects of endotoxin (6), and pretreatment of baboons (34) and rabbits (21) with a monoclonal antibody to TNF prevented their deaths after the administration of a 100% lethal dose of live *Escherichia coli*. Similarly, the detection of circulating IL-6 in both animals and humans shortly after endotoxin administration has also suggested a role for IL-6 in the early

response to gram-negative sepsis (10, 13). IL-6 has been shown to induce an exuberant acute-phase protein response in vivo after endotoxin exposure (2). Finally, recent studies have demonstrated that pretreatment of animals with a monoclonal antibody directed against IL-6 is protective against a lethal *E. coli* infection and a lethal TNF challenge (32).

The acute protective influence of glucocorticoids against the effects of bacterial endotoxin (lipopolysaccharide [LPS]) is well established (3, 4, 29, 30). In experimental models, the protective effect of glucocorticoids against bacterial endotoxin-induced lethality has been well documented (4, 30), although exogenously administered glucocorticoids in the clinical setting of septicemia and/or shock have produced disappointing results (17, 33). An early report by Berry and Smythe demonstrated that the ability of glucocorticoids to protect mice against the lethal effects of LPS was critically dependent on the timing of glucocorticoid administration relative to that of endotoxin challenge (3). When given either prior to or at the time of LPS administration, glucocorticoids were found to be protective; however, they had no protective effect when given after LPS administration. These findings have been confirmed and extended by others who have demonstrated that adrenalectomy or hypophysectomy markedly sensitizes laboratory animals to the lethal effects

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of endotoxin (4, 30). Furthermore, the deleterious effect of adrenalectomy or hypophysectomy on LPS-induced mortality can be abrogated by the administration of exogenous glucocorticoids (4, 30).

During acute endotoxemia, an early endogenous glucocorticoid response ensues and is generally regarded as essential for host survival. Glucocorticoids have been demonstrated to acutely modulate the production of some cytokines *in vitro* (5), whereas the *in vivo* interaction between glucocorticoid and cytokine host responses to an infectious stimulus (e.g., endotoxin) has not been fully elucidated. A new pharmacologic agent, mifepristone (RU 486), acts as a potent glucocorticoid receptor antagonist (25a), in addition to having a well-described role as an antiprogesterational agent (28, 31). The RU 486 molecule is a synthetic steroid analog whose effects are mediated through competitive binding to the cytoplasmic glucocorticoid receptor. RU 486 acts as a potent antiglucocorticoid both *in vitro* and *in vivo*, with a binding affinity equal to or exceeding that of the naturally occurring glucocorticoid agonist in rats and humans, but does not exhibit any agonist activity (14, 20). In the present study, RU 486 was used to investigate further the dynamic interaction between the endogenous glucocorticoid and cytokine responses to endotoxemia in an intact, unstressed host.

## MATERIALS AND METHODS

**Reagents.** Mifepristone (RU 486) was generously provided by Roussel-Uclaf Inc. (Paris, France). RU 486 was dissolved in 95% ethanol to obtain a stock solution concentration of 55 mg/ml. Aliquots of this stock solution were added to sterile physiologic saline to obtain a final concentration of 10 mg/ml, and 0.1 N HCl was added to achieve a pH of 5.5 to 6.0. The vehicle was prepared by the same procedure as that described above but without the addition of RU 486. *E. coli* serotype O55:B5 LPS (lot 47F-4046; Sigma Chemical Co., St. Louis, Mo.) was diluted in sterile physiologic saline to a final concentration of 4 mg/ml. The same lot and dilution of LPS were used for all experiments.

**Corticosterone pellet preparation.** Corticosterone pellets were prepared in advance by gently heating purified corticosterone (Sigma) until molten. Aliquots of 100 mg were molded into rounded pellets approximately 6 to 7 mm in diameter and allowed to cool and solidify in a nitrogen atmosphere. Prior to recovery of rats from anesthesia, the pellets were implanted in a small subcutaneous pocket in the dorsal surface of the neck. This method has been reported to produce reliable pathophysiologic elevations of plasma corticosterone levels for up to 6 days after implantation (16). Corticosterone was used in this experiment since it is the principal bioactive endogenous glucocorticoid secreted by the rat adrenal gland.

**Treatment of animals.** Male Lewis rats (mean weight, 245  $\pm$  4 g; Charles River Laboratories, Tarrytown, N. Y.) underwent insertion of a right jugular venous catheter by a sterile technique. Immediately following placement of the jugular venous catheter, a corticosterone pellet was inserted subcutaneously (see above) in one group of animals. The animals were then housed individually in metabolic cages in a temperature (22°C)- and light (12-hour light-dark cycle)-controlled environment and allowed access to food and water *ad libitum* throughout the experiment. Following the operative procedure, the animals were allowed a 72-h period of recovery. Previous observations have demonstrated this amount of time to be sufficient to allow for the restoration of

a normal growth curve and a return of endogenous stress hormones to baseline levels. After the recovery period, the animals were divided into four groups, and three groups received a 50% lethal dose (LD<sub>50</sub>) of *E. coli* endotoxin (2.5 mg/kg) via the catheter following pretreatment for 30 min prior to LPS treatment with (i) vehicle alone intravenously (i.v.) (-corticosterone [-Cort]/-RU 486/+LPS) ( $n = 10$ ), (ii) the antiglucocorticoid RU 486 (10 mg/kg) i.v. (-Cort/+RU 486/+LPS) ( $n = 11$ ), or (iii) RU 486 (10 mg/kg) i.v. in animals that had undergone subcutaneous implantation of a corticosterone pellet at the time of catheter insertion (+Cort/+RU 486/+LPS) ( $n = 10$ ). The fourth group of animals served as controls ( $n = 10$ ) and received RU 486 pretreatment followed in 30 min by the administration of saline instead of LPS (-Cort/+RU 486/-LPS). Blood samples were collected from the control group at the same time points as those described below.

Previous dose-response experiments with this model identified the LD<sub>50</sub> of the LPS used in this experiment to be 2.5 mg/kg i.v. (data not shown). Aliquots of RU 486 and LPS were administered via the catheter in 0.2-ml volumes so as to minimize hemodilution. Blood samples were obtained via the catheter prior to the administration of antagonist or vehicle and at 90 min, 3 h, and 4 h after LPS administration. Blood sample volumes (0.2 ml per time point) obtained via the catheter were replaced with an equivalent volume of physiologic saline. Samples were collected in EDTA-coated Microtainer tubes (Becton-Dickinson, Rutherford, N.J.) and immediately placed on ice. Plasma was isolated by centrifugation of whole blood and stored at -70°C until assayed. The animals were neither handled nor manipulated following insertion of the jugular venous catheters for 72 h prior to the initiation of the experiment so as to ensure a relatively "unstressed" state. This "no-touch" model was chosen for this experiment, since previous experiments in our laboratory demonstrated that the mere handling of a rat or the moving of its cage results in significant acute elevations of plasma glucocorticoid levels. The experiment was conducted through four separate runs. The same animals as those used for the measurement of glucocorticoid and cytokine levels as described above were observed for a period of 72 h following endotoxin administration for survival data.

**Assays.** (i) **Plasma corticosterone.** Early-morning baseline samples were collected immediately prior to the administration of either RU 486 or LPS. Corticosterone determinations were performed by a direct radioimmunoassay by the method of Keith et al. (18). This assay had a sensitivity of 16 ng/ml. Plasma samples from adrenalectomized rats served as controls and consistently yielded results below the limit of detection. All samples were assayed together as a group in a single radioimmunoassay.

(ii) **Plasma TNF.** A WEHI subclone cytotoxicity bioassay was performed to determine plasma TNF activity by the method of Eskandari et al. (8). In brief, WEHI cells at a concentration of 50,000 cells per well were added to the wells of a 96-well, flat-bottom microtiter plate and allowed to proliferate and adhere over 72 h. At that time, actinomycin D (0.5  $\mu$ g/ml) was added to each well to arrest cell proliferation. Diluted plasma samples to be assayed along with serial dilutions of standard recombinant human TNF (Chiron, Emeryville, Calif.) were added to the wells in triplicate. After incubation overnight, the extent of WEHI cell killing (TNF activity) was quantified by the addition of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (300  $\mu$ g/ml; Sigma) to each well; 4 to 6 h later, the supernatant was removed and the cells were lysed with isopropanol-

0.004 N HCl. The absorbance was determined colorimetrically (570,690 nm) on a microplate reader (model EL312; Bio-Tek Instruments, Winooski, Vt.). Logarithmic linear regression analysis was performed by plotting the log of standard recombinant human TNF concentrations against absorbance values. The activity of the unknown samples was calculated on the basis of the standard curve. Previous studies in our laboratory with neutralizing antibody to TNF have consistently confirmed the specificity of this assay. The sensitivity of this assay was found to be 1 pg/ml.

(iii) **Plasma IL-6.** A previously described B.9 hybridoma cell proliferation bioassay was used to measure plasma IL-6 activity (1). In brief, 0.1-ml aliquots of the diluted plasma samples were added to flat-bottom, 96-well microtiter plates. Samples were run in duplicate, and three serial dilutions were performed. Purified murine IL-6 (a gift from P. B. Sehgal, Rockefeller University, New York, N.Y.) was used as a standard on each plate. B.9 hybridoma cells at a concentration of 3,500 cells per well were added to the wells and allowed to incubate for 96 h. Proliferation was assessed by a standard colorimetric assay as described above. A standard curve was generated by plotting IL-6 standard concentrations against absorbance values. By linear regression analysis, the activity of the unknown samples was calculated on the basis of the standard curve. The amount of IL-6 capable of inducing one-half maximal proliferation of the B.9 hybridoma cells was defined as 1 U of IL-6. The sensitivity of this assay was found to be 500 U/ml.

**Hematocrit and leukocyte determinations.** (i) **Differential leukocyte counts.** Fifty microliters of whole blood was divided into aliquots, and the erythrocytes were lysed by the addition of 950  $\mu$ l of bicarbonate-buffered ammonium chloride solution (Ortho Diagnostic Systems, Raritan, N.J.). This suspension was directly aspirated into a Spectrum III flow cytometer (Becton-Dickinson Immunocytometry Systems, Brea, Mass.) for quantitative differential cell counting by light scatter analysis. Since rat lymphocytes and monocytes are unable to be differentiated from one another on the basis of light scatter analysis, the results are reported as a two-part differential leukocyte count, mononuclear cells and granulocytes.

(ii) **Hematocrit values.** Hematocrit values were obtained by the standard technique of 3 min of centrifugation in a microhematocrit centrifuge (Damon/IEC, Needham Heights, Mass.) and reading on a microhematocrit tube reader (Clay Adams, Parsippany, N.J.).

**Data analysis.** Cytokine and glucocorticoid data are expressed as mean  $\pm$  standard error of the mean. Statistical analyses of these data were performed by analysis of variance. Comparisons between groups were made by use of the Newman-Keuls multiple-range test. Survival data were analyzed by the log rank test for comparison of survival curves. Statistical significance was designated at the 95% confidence level.

## RESULTS

The goal of the present study was to investigate the interaction of the endogenous glucocorticoid response following endotoxemia with cytokine release (i.e., TNF and IL-6) and survival in an intact, unstressed animal model. To accomplish this goal, we chose a model involving minimal handling of the animals so as to ensure accurate and reliable measurements of endogenous glucocorticoid and cytokine responses to endotoxin.

Compared with baseline levels, plasma corticosterone

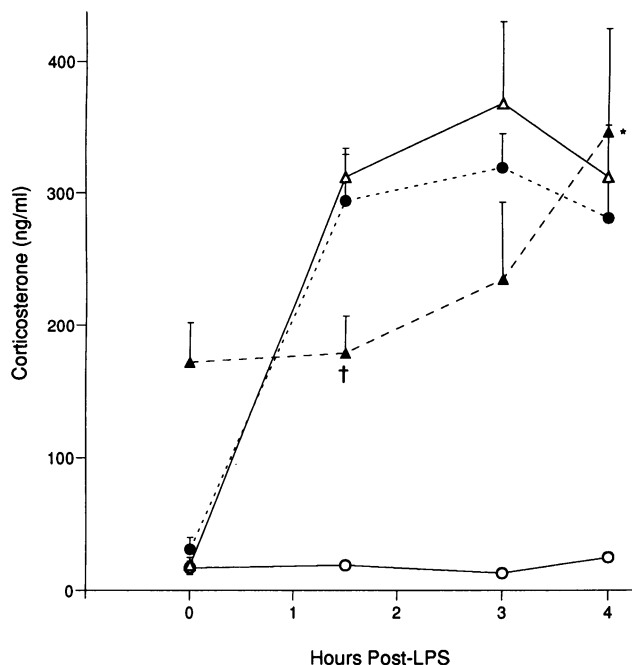


FIG. 1. Plasma corticosterone response to pretreatment with RU 486 alone without subsequent LPS administration ( $-Cort/+RU\ 486/-LPS$ ) ( $n = 10$ ) (○) or after LPS  $LD_{50}$  administration (2.5 mg/kg i.v.) following pretreatment with vehicle alone ( $-Cort/-RU\ 486/+LPS$ ) ( $n = 10$ ) (●), pretreatment with RU 486 alone ( $-Cort/+RU\ 486/+LPS$ ) ( $n = 11$ ) (△), or subcutaneous implantation of a corticosterone pellet 72 h prior to RU 486 and LPS treatment ( $+Cort/+RU\ 486/+LPS$ ) ( $n = 10$ ) (▲). † $P < 0.01$  versus  $-Cort/-RU\ 486/+LPS$ - or  $-Cort/+RU\ 486/+LPS$ -treated group; \*,  $P < 0.01$  versus time zero in  $+Cort/+RU\ 486/+LPS$ -treated group.

levels were elevated more than 10-fold ( $P < 0.01$ ) by 90 min in both the  $-Cort/-RU\ 486/+LPS$ - and the  $-Cort/+RU\ 486/+LPS$ -treated groups and remained significantly elevated throughout the course of the experiment (Fig. 1). As expected, animals receiving corticosterone-RU 486 pretreatment ( $+Cort/+RU\ 486/+LPS$ ) had significantly elevated baseline plasma corticosterone levels (172 ng/ml) prior to LPS exposure, and these levels increased further by 3 to 4 h after LPS exposure ( $P < 0.01$  at the 4-h time point). Corticosterone levels were significantly lower at 90 min after LPS-exposure in this group than in either of the non-corticosterone-pretreated groups ( $-Cort/-RU\ 486/+LPS$  or  $-Cort/+RU\ 486/+LPS$ ). In animals not receiving LPS ( $-Cort/+RU\ 486/-LPS$ ), plasma corticosterone levels did not change significantly from baseline levels during the course of the experiment (Fig. 1).

Compared with baseline levels, plasma TNF levels in all three groups receiving an  $LD_{50}$  of LPS reached peak elevations by 1.5 h (Fig. 2A). In contrast, while plasma IL-6 levels were detectable in all experimental groups by 1.5 h after LPS infusion, these levels continued to rise at 3 to 4 h after LPS infusion (Fig. 2B). A significant attenuation of circulating TNF levels was observed at 1.5 h in animals pretreated with corticosterone and RU 486 ( $+Cort/+RU\ 486/+LPS$ ) compared with those not pretreated with corticosterone and RU 486 ( $-Cort/-RU\ 486/+LPS$ ). Similarly, pretreatment with corticosterone and RU 486 ( $+Cort/+RU\ 486/+LPS$ ) resulted in a significant attenuation of circulating IL-6 levels, to less than one-third those measured in animals pretreated with

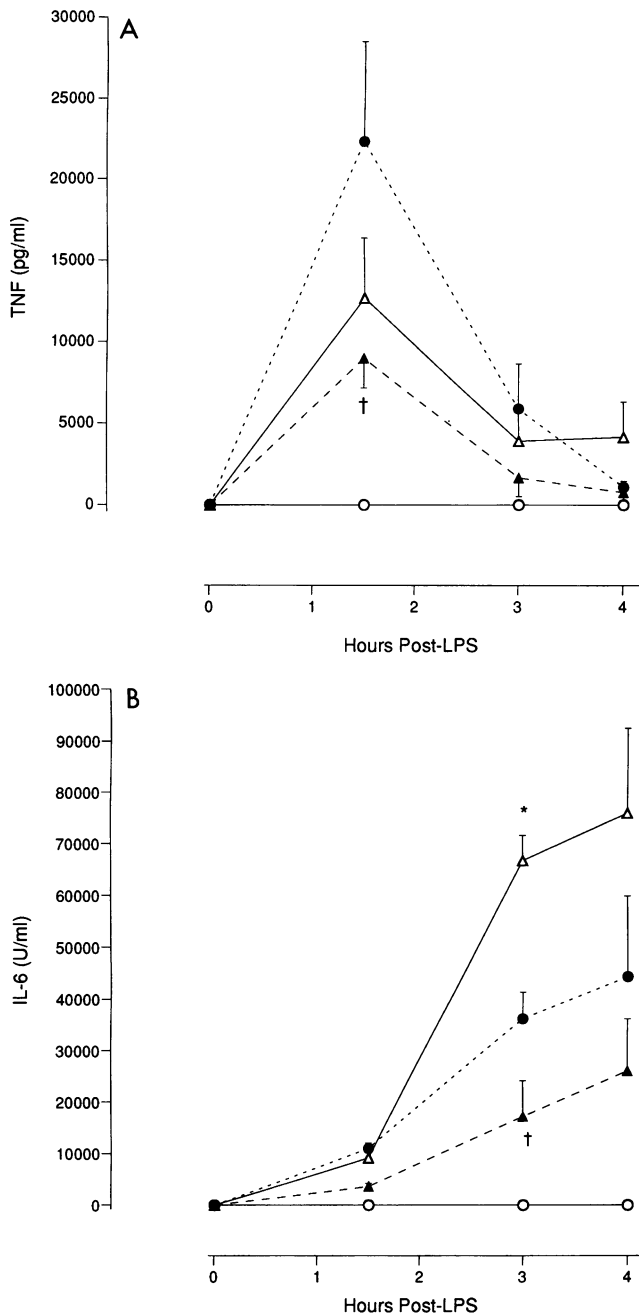


FIG. 2. (A) Plasma TNF response to pretreatment with RU 486 alone without subsequent LPS administration ( $-Cort/+RU\ 486/-LPS$ ) ( $n = 10$ ) ( $\circ$ ) or after LPS  $LD_{50}$  administration ( $2.5\ mg/kg$  i.v.) following pretreatment with vehicle alone ( $-Cort/+RU\ 486/+LPS$ ) ( $n = 10$ ) ( $\bullet$ ), pretreatment with RU 486 alone ( $-Cort/+RU\ 486/+LPS$ ) ( $n = 11$ ) ( $\Delta$ ), or subcutaneous implantation of a corticosterone pellet 72 h prior to RU 486 and LPS treatment ( $+Cort/+RU\ 486/+LPS$ ) ( $n = 10$ ) ( $\blacktriangle$ ).  $\dagger P < 0.01$  versus the  $-Cort/+RU\ 486/+LPS$ -treated group. (B) Plasma IL-6 response to pretreatment with RU 486 alone without subsequent LPS administration ( $-Cort/+RU\ 486/-LPS$ ) ( $n = 10$ ) ( $\circ$ ) or after LPS  $LD_{50}$  administration ( $2.5\ mg/kg$  i.v.) after pretreatment with vehicle alone ( $-Cort/+RU\ 486/+LPS$ ) ( $n = 10$ ) ( $\bullet$ ), pretreatment with RU 486 alone ( $-Cort/+RU\ 486/+LPS$ ) ( $n = 11$ ) ( $\Delta$ ), or subcutaneous implantation of a corticosterone pellet 72 h prior to RU 486 and LPS treatment ( $+Cort/+RU\ 486/+LPS$ ) ( $n = 10$ ) ( $\blacktriangle$ ).  $\dagger P < 0.01$  versus  $-Cort/+RU\ 486/+LPS$ - or  $-Cort/+RU\ 486/+LPS$ -treated group;  $*P < 0.01$  versus the  $-Cort/+RU\ 486/+LPS$ -treated group.

RU 486 alone ( $-Cort/+RU\ 486/+LPS$ ). A significant increase in circulating IL-6 levels was observed in the group treated with RU 486 alone ( $-Cort/+RU\ 486/+LPS$ ) compared with the group treated with either vehicle alone ( $-Cort/+RU\ 486/+LPS$ ) or corticosterone and RU 486 ( $+Cort/+RU\ 486/+LPS$ ) (Fig. 2B) at 3 h after LPS infusion; however, no significant changes in the TNF profile were observed in the group treated with the RU 486 alone ( $-Cort/+RU\ 486/+LPS$ ) (Fig. 2A). Plasma IL-6 concentrations in the  $-Cort/+RU\ 486/+LPS$ -treated group were intermediate between those in the other two groups (Fig. 2B). By 3 h after LPS infusion, although still elevated, TNF levels had returned to baseline levels in all three groups (Fig. 2A). Plasma TNF or IL-6 was not detectable at any time point (Fig. 2A and B) throughout the experiment in animals not receiving LPS ( $-Cort/+RU\ 486/-LPS$ ). These results indicate that glucocorticoid receptor blockade by RU 486 has a differential effect on the magnitude of plasma TNF and IL-6 responses to LPS, while the administration of exogenous glucocorticoid uniformly attenuates the response of these cytokines to LPS.

Endotoxemia induced acute leukopenia characterized by a significant reduction in the absolute circulating leukocyte count to one-fifth the baseline (time-zero) value by 1.5 h. This reduction in the leukocyte count persisted throughout the experiment in all three treatment groups (data not shown). No significant differences among experimental groups were observed, except at the baseline (time zero) in the group pretreated with corticosterone and RU 486 ( $+Cort/+RU\ 486/+LPS$ ), in which a significant decline in the total number of leukocytes, accounted for almost entirely by a decline in mononuclear leukocytes, was observed compared with the number in the  $-Cort/+RU\ 486/+LPS$ - or  $-Cort/+RU\ 486/+LPS$ -treated group; however, significant leukopenia was still observed in the latter two groups following LPS administration. Except in the corticosterone-pretreated group ( $+Cort/+RU\ 486/+LPS$ ), baseline leukocyte counts were consistent with those previously observed in normal, unstressed rats of this strain (23).

Following LPS administration, hematocrit values increased slightly, with a peak occurring at 3 to 4 h in all experimental groups; however, these changes were statistically significant only in the  $-Cort/+RU\ 486/+LPS$ - and  $+Cort/+RU\ 486/+LPS$ -treated groups (data not shown). Rats in the corticosterone-pretreated group ( $+Cort/+RU\ 486/+LPS$ ) exhibited significantly higher (by 10%) baseline hematocrit values than any of the other groups. Animals receiving RU 486 alone without LPS ( $-Cort/+RU\ 486/-LPS$ ) did not exhibit any significant changes in hematocrit values during the experiment (data not shown).

Animals pretreated with RU 486 prior to LPS exposure ( $-Cort/+RU\ 486/+LPS$ ) exhibited significantly decreased survival (9%) compared with rats receiving the vehicle alone ( $-Cort/+RU\ 486/+LPS$ ), which showed 50% survival at 72 h after LPS administration (Fig. 3). In contrast, animals pretreated with corticosterone ( $+Cort/+RU\ 486/+LPS$ ) exhibited significantly improved survival compared with animals in either of the non-corticosterone-pretreated groups ( $-Cort/+RU\ 486/+LPS$  or  $-Cort/+RU\ 486/+LPS$ ), with 70% surviving at 72 h (Fig. 3). The mortality observed in all groups occurred relatively early in the course of endotoxemia, usually within 12 h following LPS administration. All of the animals in the control group, receiving only RU 486 without subsequent LPS administration ( $-Cort/+RU\ 486/-LPS$ ), survived (Fig. 3).

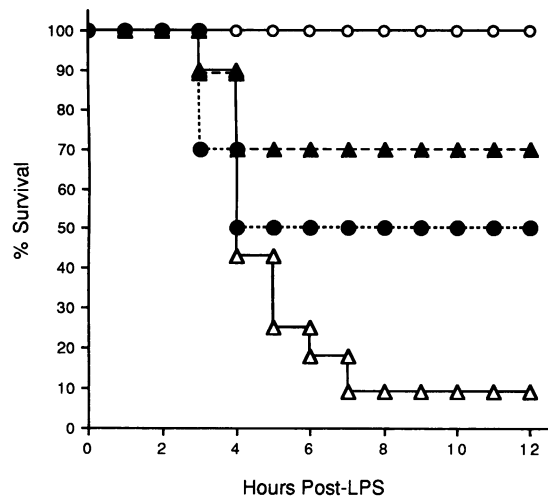


FIG. 3. Survival after pretreatment with RU 486 alone without subsequent LPS administration ( $-Cort/+RU\ 486/-LPS$ ) ( $n = 10$ ) ( $\circ$ ) or after LPS  $LD_{50}$  administration (2.5 mg/kg i.v.) following pretreatment with vehicle alone ( $-Cort/-RU\ 486/+LPS$ ) ( $n = 10$ ) ( $\bullet$ ), RU 486 alone ( $-Cort/+RU\ 486/+LPS$ ) ( $n = 11$ ) ( $\triangle$ ), or subcutaneous implantation of a corticosterone pellet 72 h prior to RU 486 and LPS treatment ( $+Cort/+RU\ 486/+LPS$ ) ( $n = 10$ ) ( $\blacktriangle$ ). All groups were significantly ( $P < 0.01$ ; log rank statistic) different from one another.

## DISCUSSION

In the present investigation, we demonstrated that the blockade of glucocorticoid receptors increased LPS-induced mortality. Additionally, mortality in the RU 486-pretreated animals ( $-Cort/+RU\ 486/+LPS$ ) was of significantly greater magnitude than mortality in the animals pretreated with either the vehicle alone ( $-Cort/-RU\ 486/+LPS$ ) or corticosterone ( $+Cort/+RU\ 486/+LPS$ ). Also, elevation of plasma corticosterone to levels comparable to those measured following severe injury ( $+Cort/+RU\ 486/+LPS$ ) reversed the increased mortality observed with RU 486 pretreatment ( $-Cort/+RU\ 486/+LPS$ ), a finding consistent with previous studies in which glucocorticoid pretreatment could reverse, at least in part, the increased LPS-induced mortality in adrenalectomized or hypophysectomized animals (4, 30).

No effect on the endogenous glucocorticoid response to endotoxemia was seen as a result of RU 486 pretreatment in this model. In addition, despite pretreatment with corticosterone for 72 h prior to LPS administration, a significant rise in the plasma corticosterone level (4 h) after LPS administration was observed, albeit with a delayed time course. This delayed glucocorticoid response to LPS challenge in the corticosterone-pretreated animals likely resulted from negative feedback inhibition by the exogenously administered corticosterone. However, the ability to mount a significant endogenous glucocorticoid response to endotoxemia above the levels achieved by exogenous pretreatment with corticosterone suggests (i) the preservation of adrenal glucocorticoid secretory capacity in response to an appropriate challenge and (ii) the ability of LPS and/or LPS-induced mediators to acutely overcome any feedback inhibition of adrenocorticotropin secretion, perhaps through a direct effect on the hypothalamus. The failure of RU 486 pretreatment to overcome the protective glucocorticoid effect in the corticosterone-pretreated animals ( $+Cort/+RU\ 486/+LPS$ ) suggested temporally advanced glucocorticoid "priming" of

glucocorticoid-responsive cells, rendering them insensitive to subsequent RU 486 exposure.

Although protection against LPS-induced mortality through a glucocorticoid-related mechanism has been demonstrated in this model and others (3, 4, 30, 38), the action of the glucocorticoids as mediators in this event remains unclear. This study demonstrated that the induction of hypercortosteronemia prior to LPS exposure not only improved survival but also significantly attenuated both TNF and IL-6 responses. In addition, pretreatment with RU 486 alone ( $-Cort/+RU\ 486/+LPS$ ) resulted in a significant increase in circulating IL-6 levels. These data suggest that the endogenous glucocorticoid response to endotoxemia may act, in part, to down-regulate the appearance of some cytokines.

Recent *in vivo* studies have provided further evidence for a potential modulatory role of glucocorticoids in cytokine production. Bertini et al. demonstrated that adrenalectomized mice were sensitized to the lethal effects of both IL-1 and TNF *in vivo* (4). Similar findings were obtained in a recent study by Silverstein et al., who demonstrated that pretreatment of animals with hydrazine sulfate induced a significant glucocorticoid response that protected D-galactosamine-sensitized mice against endotoxin- and TNF-induced lethality (30). Finally, it has also been shown that the infusion of pharmacological doses of cortisol into normal human volunteers prior to LPS administration results in a diminished appearance of TNF in the circulation as well as an attenuation of the clinical signs and symptoms of endotoxemia (1a).

In this study, glucocorticoid pretreatment ( $+Cort/+RU\ 486/+LPS$ ) significantly attenuated the release of both IL-6 and TNF in response to LPS challenge, while pretreatment with the antiglucocorticoid RU 486 ( $-Cort/+RU\ 486/+LPS$ ) resulted in an increase in the levels of circulating IL-6 but not TNF. This disparity in the circulating IL-6 and TNF responses to LPS is intriguing and may be due, in part, to a relative lack of glucocorticoid influence on the initial TNF response to LPS. As previously shown, elevated plasma TNF levels are correlated with increased mortality (35). In the present study, plasma TNF levels peaked at 1.5 h and returned to baseline levels by 3 h after LPS administration, while plasma IL-6 levels peaked at 4 h after LPS administration. Differences in mortality between groups, however, did not begin to become apparent until at least 4 h after LPS administration. The temporal association between peak plasma TNF levels at 1.5 h and differences in mortality between groups at 4 h after LPS administration suggests that TNF may have a critical role in the observed differences in mortality, at least in the corticosterone-pretreated group ( $+Cort/+RU\ 486/+LPS$ ). However, the increased mortality observed in the RU 486-pretreated group ( $-Cort/+RU\ 486/+LPS$ ) and the lack of a significant change in plasma TNF levels following endotoxin administration in this group suggest that perhaps other mediators, such as IL-6, which was elevated to the greatest extent in this group, may be involved in the observed increased mortality. Although increased plasma IL-6 levels are consistently observed to correlate with mortality in clinical studies and animal models (15, 32, 36), such a correlation does not provide evidence that this cytokine is the ultimate mediator of LPS-induced tissue injury or mortality. In fact, exogenous IL-6 administration does not replicate the tissue injury and mortality observed with LPS administration in experimental animals (26). These findings suggest that a complex synergy is necessary to manifest the full expression of cytokine-related injury and shock.

Following LPS administration, leukocyte counts were significantly decreased within 90 min in all experimental groups and remained significantly diminished throughout the course of the experiment. This relative leukopenia is consistent with previous studies done with both animals and humans and demonstrating that endotoxemia results in a dramatic, acute decline in leukocyte numbers (25, 27). The baseline leukocyte count following pretreatment with corticosterone (+Cort/+RU 486/+LPS) was significantly lower than the baseline leukocyte counts in the other groups. This low baseline count can be accounted for almost entirely by a decrease in the numbers of mononuclear leukocytes (i.e., lymphocytes and monocytes), whose numbers have been shown previously to decline in the presence of elevated circulating glucocorticoids (9).

Hemoconcentration is a well-known consequence of acute endotoxemia and is thought to be secondary to increased capillary endothelial vascular permeability allowing for egress of intravascular fluid into the interstitial or extravascular space. In our model, after LPS administration, hematocrit values increased slightly in all experimental groups. Since neither RU 486 pretreatment (-Cort/+RU 486/+LPS) nor corticosterone pretreatment (+Cort/+RU 486/+LPS) had an effect on the observed hemoconcentration, acute elevations in glucocorticoid levels may have a minimal effect on LPS-induced hemoconcentration. This finding is supported by that of others, who have demonstrated that dexamethasone pretreatment prior to LPS administration has no significant effect on subsequent hemoconcentration (11).

Although the present study and other studies (3, 4, 30) point to a protective role of glucocorticoids in acute, lethal endotoxemia, such may not be the case in chronic gram-negative infections. In the latter situation, a sustained elevation of glucocorticoid levels may suppress the development of specific immunity (7) to the invading microorganism(s), resulting in delayed bacterial clearance or actual progression of an otherwise controllable infection.

These findings and those of others suggest a critical role for an intact endogenous glucocorticoid response to endotoxemia in the protection of the host from LPS-induced mortality. It was further demonstrated that RU 486 pretreatment augments IL-6 production, while corticosterone pretreatment significantly attenuates the appearance of both IL-6 and TNF in the circulation in response to endotoxemia. Therefore, it appears that the protective effect of the endogenous glucocorticoid response to endotoxemia may result from the down-regulation of a potentially lethal cytokine response to LPS. The data further suggest a complex, dynamic interaction between glucocorticoid activity and regulation of the cytokine cascade.

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