Regulation of Serum Tumor Necrosis Factor in Glucocorticoid-Sensitive and -Resistant Rodent Endotoxin Shock Models

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Bolus injection of lethal or sublethal doses of endotoxin or lipopolysaccharide (LPS) results in the rapid and transient rise in tumor necrosis factor (TNF) levels in serum in mammals. TNF levels peak between 1 and 2 h after LPS injection in mice and guinea pigs and approach basal levels by 6 h. Although the kinetics of TNF in serum appear similar between these two species, guinea pigs respond to a lethal dose of LPS of 20 mg/kg by producing approximately 10-fold more TNF than mice do. These two endotoxin shock models also differ in their sensitivity to glucocorticoids. TNF levels in serum are not reduced in the lethal endotoxin shock model in guinea pigs after treatment with dexamethasone at 25 mg/kg. In contrast, TNF levels in mouse serum are inhibited by more than 90% after treatment with dexamethasone at 3 mg/kg. Coincident with the TNF peak in serum is a leukopenia which approaches control levels by 6 h in dexamethasone-treated mice, while remaining depressed in dexamethasone-treated guinea pigs. Treatment with dexamethasone at 25 mg/kg did not save guinea pigs from endotoxin lethality, whereas long-term survival of mice under identical conditions was apparent. These results suggest that the relative glucocorticoid resistance observed in guinea pigs is also apparent in a lethal endotoxin shock model in which dexamethasone does not modulate TNF levels or result in increased survival as occurs in mice. The lack of clear efficacy for steroid therapy in human clinical septic shock trials would suggest that the guinea pig endotoxin model may be a more predictive system than the mouse model for the identification of novel agents useful in the treatment of endotoxin shock.

Tumor necrosis factor (TNF) has been implicated as playing a critical role in endotoxin shock. The appearance of TNF in the serum of mice, rabbits, and baboons following infusion of Escherichia coli or endotoxin and the ability of anti-TNF antibodies to protect these animals from death support the hypothesis that TNF has a role in septic shock (3, 16, 24, 25). In addition, much of the pathophysiology of septic shock can be duplicated by administration of recombinant TNF. These changes include leukopenia, thrombocytopenia, reduction in mean arterial blood pressure, and histologic changes including pulmonary edema, adrenal atrophy, and intestinal necrosis (17, 21, 23).

The beneficial effects of exogenous glucocorticoids in conjunction with antibiotics have been found in mouse, rat, baboon, and canine models of septic shock (1, 9–14). The importance of endogenous glucocorticoids in adrenalectomized rodents has been demonstrated: doses of endotoxin, TNF, and interleukin 1 which are sublethal in normal mice are lethal in adrenalectomized mice, and both the endotoxin-mediated lethality and increase in TNF levels are abrogated by administration of exogenous glucocorticoids (2, 31; L. D. Butler, N. Layman, P. E. Riedl, R. L. Cain, J. Shellhaas, G. F. Evans, and S. H. Zuckerman, J. Neuroimmunol., in press; G. F. Evans, Y. M. Snyder, L. D. Butler, and S. H. Zuckerman, Circ. Shock, in press). In contrast, the results of recent clinical trials designed to reevaluate the therapeutic efficacy of exogenous glucocorticoids demonstrated that high-dose methylprednisolone treatment did not reduce mortality rates or prevent shock in patients with gram-negative or gram-positive sepsis (5, 15). These results demonstrate the necessity of developing small-animal glucocorticoid-resistant septic shock models. The relative resistance of lymphoid cells to exogenous glucocorticoids has been reported for both humans and guinea pigs, in contrast to the sensitivity of monkeys, mice, rabbits, and rats (6). In the present study, an endotoxin model in guinea pigs is described in which dexamethasone at 25 mg/kg failed to reduce TNF levels in serum or save animals from death, whereas dexamethasone at 1 to 3 mg/kg reduced TNF levels in serum in mice and protected them from endotoxin-mediated death. The guinea pigs also produced 10- to 20-fold more TNF than the mice did at comparable endotoxin concentrations.

MATERIALS AND METHODS

Endotoxin shock models. Female BALB/c mice were obtained from Charles River Breeding Laboratories, Inc., Portage, Mich., and were used between 8 and 12 weeks of age. Male Hartley albino outbred guinea pigs were also obtained from Charles River and weighed between 400 and 500 g each. The animals were given intraperitoneal (i.p.) doses of dexamethasone acetate (Sigma Chemical Co., St. Louis, Mo.) at concentrations between 1 and 25 mg/kg, suspended in carboxymethyl cellulose (CMC), 1 h prior to lipopolysaccharide (LPS) injection. Control animals received CMC alone. Lethal (20-mg/kg) doses of endotoxin (Lipopolysaccharide W, Escherichia coli O55:B5; Difco Laboratories, Detroit, Mich.) were then injected i.p., and the animals were bled 1 h later from the retro-orbital plexus (guinea pigs) or by cardiac puncture (mice) for TNF quantitation. Mortality in all experimental groups was evaluated daily. All animal experiments were performed within the National Institutes of Health guidelines for use of experimental animals.

TNF bioassay. TNF levels in serum were assayed by using the murine L929 fibroblast toxicity assay (8). L929 cells were plated in flat-bottom microdilution plates at 5 × 10⁴ cells per well in 100 μl of RPMI 1640 medium with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) and 1 μg

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FIG. 1. TNF levels in serum in rodent endotoxin shock models. LPS at 20 mg/kg was administered i.p. to mice (A) or guinea pigs (B), and animals were bled at the appropriate time points. Three mice per group were sacrificed at the indicated intervals, and each serum sample was assayed in triplicate for TNF. Six guinea pigs were bled at the indicated intervals from the retro-orbital plexus, 0.5 ml per point, and each serum sample was assayed in triplicate. Changes in optical density were converted to nanograms per milliliter by using the linear portion of a recombinant murine TNF standard curve. These curves are from a representative of four experiments.

of actinomycin D (Sigma) per ml. Sera were assayed at dilutions between 1:50 and 1:200 for mice and between 1:200 and 1:5,000 for guinea pigs. After overnight incubation at 37°C, the plates were stained for 5 min in crystal violet (0.5% in 25% methanol) and rinsed, and the A570 was read on a Dynatech MR600 microplate reader. Recombinant murine TNF (provided by Lee Bobbitt, Lilly Research Laboratories) was used for a standard curve.

**Leukocyte profiles.** Blood samples at 1.5 and 6 h after LPS injection were obtained from the retro-orbital plexus or by cardiac puncture and were evaluated for total leukocyte counts, differentials, and platelet counts. All samples were coded before leukocyte profiles were determined.

**RESULTS**

The injection of LPS at 20 mg/kg into both guinea pigs and mice resulted in a rapid rise in TNF levels in serum, which peaked at 1 h in mice (Fig. 1A) and by 2 h in guinea pigs (Fig. 1B). TNF levels rapidly decreased after 2 h and approached basal values by 6 h. Although the kinetics of TNF in serum were similar for mice and guinea pigs, it was apparent that guinea pigs produced 10- to 20-fold more TNF than mice did.

In both species, 20 mg/kg was chosen, because this represented a lethal dose.

In addition to the quantitative differences in TNF, fundamental differences were apparent in the ability of dexamethasone to reduce TNF levels in serum following a challenge with a lethal dose of endotoxin. Pretreatment of mice with dexamethasone at 3, 10, or 25 mg/kg i.p. 1 h prior to LPS injection resulted in a reduction in TNF levels of more than 80% (Fig. 2A). In additional experiments, a dexamethasone concentration of 0.1 mg/kg still reduced TNF levels in serum by 50%. In contrast, pretreatment of guinea pigs with dexamethasone at up to 25 mg/kg did not result in any significant reduction in TNF levels (Fig. 2B).

The sensitivity of mice to glucocorticoid inhibition of LPS-induced TNF in serum was also apparent when the lethal dose of LPS was increased 10-fold, to 200 mg/kg.

**TABLE 1. Effect of LPS concentration on dexamethasone inhibition of TNF in mouse serum**

<table>
<thead>
<tr>
<th>LPS concn (mg/kg)</th>
<th>Dexamethasone concn (mg/kg)</th>
<th>Mean TNF concn (pg/ml) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>252 ± 31</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>4,869 ± 2,446</td>
</tr>
<tr>
<td>20</td>
<td>25</td>
<td>537 ± 66</td>
</tr>
<tr>
<td>200</td>
<td>0</td>
<td>5,970 ± 1,560</td>
</tr>
<tr>
<td>200</td>
<td>25</td>
<td>476 ± 125</td>
</tr>
</tbody>
</table>
endotoxin lethality. Mortality as a result of LPS administration at 20 mg/kg was apparent by 16 h, and by 24 h most mice (Fig. 3A) and guinea pigs (Fig. 3B) had died. Dexamethasone at 3, 10, or 25 mg/kg protected mice against endotoxin lethality, and these mice were long-term survivors, with no deaths occurring beyond 96 h. In contrast, similar concentrations of dexamethasone were ineffective in protecting guinea pigs, and these animals died. With kinetics similar to those observed for the non-dexamethasone-treated controls (Fig. 3B). Therefore, as evidenced by both TNF levels in serum and mortality, glucocorticoids fail to modify the progression of events in the guinea pig endotoxin shock model which result in the death of the host.

Treatment of both mice (Table 2) and guinea pigs (Table 3) with LPS at 20 mg/kg induced a profound leukopenia at 1.5 h posttreatment. In both species, this was primarily a result of lymphopenia and neutropenia. Dexamethasone treatment had no beneficial effect on amelioration of leukocyte counts in either species at this time point. Mice at 6 h after LPS injection had leukopenia characterized by marked lymphopenia and a reduction in the relative lymphocyte/neutrophil ratio. Dexamethasone treatment normalized the total leukocyte count at this time point; however, this was in conjunction with marked lymphopenia and neutrophil leukopenia. Eosinophils were not seen in dexamethasone-treated mice at any time point or in LPS-treated mice at 6 h. Thrombocyte counts were significantly decreased at 6 h after LPS injection (P < 0.05), and this was not reversed by dexamethasone treatment.

Total leukocyte counts in dexamethasone-treated and -untreated (CMC control) guinea pigs at 6 h after LPS injection were decreased compared with control values, but the difference was not significant. Both CMC control and dexamethasone-treated guinea pigs did have significant lymphopenia; however, neutrophil counts were similar in both groups. Hence, there was no apparent effect of dexamethasone treatment. The relative leukocyte profiles were not significantly different from that of the control animals. In contrast to results for dexamethasone-treated mice, eosinophil counts were not altered in guinea pigs. These results indicate that although dexamethasone treatment in LPS-injected mice resulted in a return of total leukocyte counts to normal in 6 h, this effect was a result of the reversal of the lymphocyte/neutrophil ratio. Dexamethasone treatment of guinea pigs, in contrast, had minimal effects on the LPS-induced changes in the leukocyte profiles.

**DISCUSSION**

The guinea pig has served as a useful animal model for anaphylactic, burn, endotoxin, and septic shock (18, 22, 29, 30).

**FIG. 3.** Effect of dexamethasone on LPS lethality. LPS at 20 mg/kg was injected i.p. into mice (A) or guinea pigs (B) 1 h after administration of dexamethasone at 3, 10, or 25 mg/kg. Mortality was assessed at the indicated intervals with 7 mice and 10 guinea pigs per group. Animals which received no dexamethasone were injected with a comparable volume of CMC. No deaths were observed beyond 48 h. These curves are from a representative of five experiments. Symbols: B, LPS; ●, dexamethasone at 3 mg/kg; ■, dexamethasone at 10 mg/kg; ○, dexamethasone at 25 mg/kg.

(Table 1). These results were in contrast to the lack of effect on LPS-induced TNF in the sera of guinea pigs. The observation that TNF levels were comparable in mice injected with 20 or with 200 mg of LPS per kg suggested that the 5 to 20 ng of TNF per ml detected in mouse sera represented maximal achievable levels.

The effects of dexamethasone on TNF levels in serum in mice and guinea pigs were consistent with the results for

**TABLE 2.** Leukocyte and platelet counts in mouse endotoxin shock

<table>
<thead>
<tr>
<th>Group*</th>
<th>Mean counts ± SEM for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total leukocytes</td>
</tr>
<tr>
<td>Control</td>
<td>4.82 ± 0.37</td>
</tr>
<tr>
<td>CMC (1.5 h)</td>
<td>2.36 ± 0.23</td>
</tr>
<tr>
<td>Dexamethasone (1.5 h)</td>
<td>1.70 ± 0.09</td>
</tr>
<tr>
<td>CMC (6 h)</td>
<td>1.64 ± 0.22</td>
</tr>
<tr>
<td>Dexamethasone (6 h)</td>
<td>4.20 ± 1.2</td>
</tr>
</tbody>
</table>

*Animals were injected with LPS at 20 mg/kg 1 h after i.p. injection of CMC vehicle or dexamethasone at 25 mg/kg. Five animals per group were bled, and leukocyte and platelet counts are expressed as the mean for the five animals.

Total leukocyte counts, differentials, and thrombocytes are expressed as x 10⁶ per milliliter.

Control animals received saline and represent the only animals which were not LPS stimulated.
Pathologic changes associated with endotoxin shock in guinea pigs include increased levels of lactate in the lungs and blood, bowel hemorrhage, and lung edema (22, 29). Additional studies have shown early and progressive intrinsic myocardial dysfunction during endotoxin shock (18). In the present investigation, the effect of dexamethasone on LPS-induced TNF levels in serum and lethality in guinea pigs was evaluated and compared with a mouse endotoxin shock model. Although LPS increased TNF levels and resulted in the death of the host, dexamethasone was able to reduce TNF levels in mouse serum and save animals, but had little effect in guinea pigs. Dexamethasone treatment of mice resulted in a significant shift in the lymphocyte/neutrophil ratio at the 6-h time point. The glucocorticoid-induced alteration in this ratio was not detected in guinea pigs.

In addition to differences in glucocorticoid effects on modulating TNF levels and endotoxin lethality, it was apparent that guinea pigs produced significantly more TNF than mice did in response to lethal doses of endotoxin. The levels of TNF observed were at least 10-fold higher than those observed in mice (see above) and higher than those detected in rats and rabbits (S. H. Zuckerman, unpublished observations). Although the mechanism of the greater LPS increase in TNF levels of serum in guinea pigs remains unclear, the possibilities that cytokine synergy and subclinical infections contribute to in vivo macrophage activation must be considered.

The inability of dexamethasone to reduce TNF levels in serum or effect survival in guinea pigs challenged with a lethal dose of endotoxin was surprising, in view of its effects on these parameters in the mouse endotoxin model. In additional experiments, glucocorticoid effects on TNF levels in serum in guinea pigs were evaluated by using sublethal doses of endotoxin. However, in these experiments, the variability in the amount of TNF produced by LPS at 2 mg/kg (<200 to 180,000 pg/ml) precluded any statistical significance between the different experimental groups when five animals per group were used. This was not true with sublethal doses of LPS in mice (S. H. Zuckerman, unpublished observations), suggesting that the non-glucocorticoid-sensitive endotoxin shock model in guinea pigs is primarily useful for monitoring TNF levels when lethal doses of LPS are used.

Glucocorticoids have been demonstrated to have anti-inflammatory effects on guinea pig macrophages both in vitro and in vivo (4, 7, 19, 20, 26–28). Glucocorticoid-pretreated guinea pig macrophages show reduced secretion of elastase (28), prostaglandin, and collagenase (26, 27), decreased synthesis of C2 and C4 complement components, and decreased synthesis of lysozyme (20) without affecting Fc-mediated erythrophagocytosis (7) or Listeria ingestion (4). In vivo administration of cortisone at doses between 100 and 200 mg/kg per day resulted in a suppression of guinea pig pulmonary host defense both in a Listeria model (4) and in evaluating the chemotactic response of alveolar macrophages to fMet-Leu-Phe (19). Clearly, the beneficial effects of glucocorticoids in guinea pig models may require a less severe or acute inflammatory model and may necessitate a longer glucocorticoid administration period prior to the observation of therapeutic effects.

Glucocorticoid plus antibiotic therapy has been demonstrated to increase survival in septic shock models in mice, rats, dogs, and baboons (1, 9–14; Evans et al., in press). However, the conclusions from the most recent multicenter studies demonstrated no clear benefit in the use of methylprednisolone for the treatment of sepsis and septic shock (5, 15). Accordingly, the development of glucocorticoid-resistant animal shock models may provide a useful approach to the understanding of human septic shock. The use of a rodent model will permit more rapid screening of compounds and be less expensive than shock models involving larger mammalian species. Therefore, the characterization and implementation of in vivo glucocorticoid-resistant animal shock models may contribute to the development of novel non-glucocorticoid-related therapeutic modalities for the treatment of septic shock.

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LITERATURE CITED


