Protective effect of ethyl-3-(3-dimethyl aminopropyl)urea dihydrochloride (EDU) against LPS-induced death in mice

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Evaluation of anti-adhesive gels and bioresorbable films in animal models of intra-abdominal infection has shown that a product of the cross-linking reaction between hyaluronic acid (HA) and CM-cellulose, 1-ethyl-3-(3-dimethyl aminopropyl)urea dihydrochloride (EDU), has immunomodulatory properties. The effects of EDU were evaluated by using an endotoxin-induced shock mouse model. Pre-treatment of mice with EDU (50 mg kg$^{-1}$) in DMSO resulted in a significant reduction in mortality following injection of LPS, compared to vehicle (DMSO) pre-treatment alone. Serum levels of TNF-α, IL1β and IFN-γ in EDU-treated mice were significantly lower than those in vehicle-treated mice. Nitric oxide (NO) concentrations in the sera of mice after inoculation with LPS were significantly lower in the EDU-treated group than in the vehicle-treated group at various time-points. In contrast, EDU pre-treatment was associated with an enhanced IL10 response after LPS injection, compared to vehicle pre-treatment alone. In vitro studies revealed that IL10 production by RAW 264.7 macrophages, elicited by LPS, was increased significantly when EDU was added to the culture medium. These results suggest that the protective effect of EDU during LPS-induced shock in mice is the result of inhibition of proinflammatory cytokines and NO production and an enhanced IL10 response.

INTRODUCTION

Adhesion-reduction membranes, such as Seprafilm and Seprafilm II (Genzyme), are bioresorbable membranes that are composed of hyaluronic acid (HA) and CM-cellulose, which have been modified chemically with 1-(3-dimethyl aminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) to cross-link the two polymers. As these membranes are designed for use within the peritoneal cavity under circumstances in which bacterial contamination may occur, evaluation of these membranes and anti-adhesion gels of similar composition in an animal model for intra-abdominal sepsis was performed. Coincident with this evaluation of anti-adhesion membranes and gels, it was noted that certain HA-based adhesion-reduction compounds, when given prior to bacterial challenge, protected animals against the lethal outcome of experimental infection (Tzianabos et al., 1999). More precisely, it was shown that the mortality rate was related directly to the specific composition of the membranes used. In vehicle-treated control animals, there is increased access of bacteria to the bloodstream during peritonitis and the host response to bacterial endotoxin (LPS) is characterized by the induction of proinflammatory cytokines, vaso-dilation and increased vascular permeability (Tzianabos et al., 1999). However, in our previous study, we found that administration of HA/CM-cellulose gels before bacterial challenge not only did not increase mortality, but protected rats significantly against lethal infection (Ruiz-Perez et al., 2003). The results revealed that protection was conferred not by the HA/CM-cellulose gels themselves, but by ethyl-3-(3-dimethyl aminopropyl)urea (EDU), a small molecule that is released from the gel complex under physiological conditions.
Our results also suggest that the protective effect exhibited by EDU is related to downregulation of T-cell-dependent responses and suppression of the proinflammatory cytokine cascade that is associated with mortality during the early phase of intra-abdominal sepsis. However, mechanisms of downregulation of the proinflammatory cytokine response are not well understood. We therefore examined whether pre-treatment with EDU could alter the course of the murine endotoxin-induced shock model, which is an established, simple model of systemic inflammation with the extent of LPS exposure (Raetz, 1990). The effects of EDU pre-treatment on the production of various mediators that are believed to be crucial for the induction, propagation and resolution of septic shock in this animal model were also evaluated.

METHODS

Animals. Virus-antibody-free, outbred, 5-week-old, male CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). The animals were housed in microisolator cages and received sterile distilled water and food ad libitum.

Reagents. EDU palmitate (Genzyme) was dissolved in DMSO (Sigma) at various final concentrations prior to injection (as indicated). All compounds were tested for the presence of endotoxin prior to use and exhibited levels that were undetectable (< 5 pg ml⁻¹). LPS purified from Escherichia coli strain O111:B4 (Sigma) was used for all experiments.

Murine endotoxin-induced shock model. Mice were injected intravenously (IV) with LPS (100 mg kg⁻¹) that was dissolved in sterile, pyrogen-free saline solution. For experiments in which mortality was the outcome parameter, mice were injected intraperitoneally (IP) with 50 mg EDU kg⁻¹, 2 h prior to LPS injection. Control mice were pretreated with DMSO alone 2 h before LPS inoculation. Mice were observed at least twice per day and mortality was recorded for 7 days after LPS injection.

Preparation of serum samples. For the determination of cytokine and NO levels in serum, mice were challenged (IV) with 100 mg LPS kg⁻¹. Mice were treated with 50 mg EDU kg⁻¹ (IP) 2 h before IV injection of LPS. Mice were killed and blood was obtained by percutaneous transthoracic cardiac puncture at various time-points after LPS challenge. Serum samples were kept at −80 °C until cytokine levels were determined.

Cell culture and reagents. The mouse macrophage cell line RAW 264.7 (ATCC TIB-71) was obtained from the Dainippon Pharmaceutica Company, Osaka, Japan. Cells were grown at 37 °C with 5% CO₂ in minimum essential medium (ICN Biomedicals) that was supplemented with 5% fetal bovine serum (Sigma), L-glutamine (ICN Biomedicals) and antibiotics (50 U penicillin ml⁻¹ and 50 µg streptomycin ml⁻¹; GibcoBRL). Cells were resuspended at 2 × 10⁵ ml⁻¹ and plated onto 24-well plates at 1 ml per well. They were incubated for 24 h in the absence or presence of EDU (1 µg ml⁻¹) and with LPS at a concentration either of 10 or 100 pg ml⁻¹. Supernatants were assayed for IL10 levels as described below.

Cytokine and NO assay. Serum levels of TNF-α, IL1β, IFN-γ and IL10 were determined by using commercially available ELISA kits (PharMingen). Serum NO levels were assessed by measuring the amount of nitrite, a metabolic product of NO, by using a commercially available kit (Griess reagent system; Promega).

Statistical analysis. A χ² test was used to analyse survival data. Differences in cytokine and NO levels were analysed statistically by using the Mann–Whitney U test. P-values < 0.05 were considered to be significant.

RESULTS

Effect of EDU on mouse survival

IV challenge of mice with 100 mg LPS kg⁻¹ was lethal for 90–100% of animals. Treatment with EDU (50 mg kg⁻¹, IP) resulted in 46.7% survival (P < 0.05), compared to a 10% survival rate in DMSO-treated (control) mice (Fig. 1). Treatment with 10 mg EDU kg⁻¹ resulted in survival of 26.7% of the mice, which was not significantly different from controls. Although the period of observation continued for 7 days, no change in mortality was noted beyond 72 h after LPS challenge. Results of preliminary experiments revealed that pre-treatment with 50 mg EDU kg⁻¹, 2 h before LPS challenge, was optimal for our model (data not shown).

Serum levels of proinflammatory cytokines during endotoxin shock

Serum levels of proinflammatory cytokines were evaluated in mice by using the same conditions as described for survival studies. IP treatment with 50 mg EDU kg⁻¹, 2 h before IV challenge with 100 mg LPS kg⁻¹, resulted in significantly lower levels of TNF-α compared to DMSO treatment alone (Fig. 2). Furthermore, 6 h after challenge with LPS, serum levels of both IL1β and IFN-γ in EDU-treated mice were significantly lower than those in DMSO-treated mice (Fig. 3). We did not detect TNF-α, IL1β or IFN-γ in the sera of mice 1-5, 3, 6 or 24 h after IP treatment with the higher dose of EDU (50 mg kg⁻¹) alone.

![Fig. 1. Effect of EDU on survival of mice after endotoxin shock. Each mouse was pre-treated with EDU palmitate [50 (●) or 10 (■) mg kg⁻¹] dissolved in DMSO, or DMSO alone (○), 2 h before challenge with LPS. After administration of 100 mg LPS kg⁻¹, mortality for each group of mice was monitored (n = 20 in DMSO-treated group; n = 15 in EDU-treated group). * P < 0.05.](image-url)
Serum levels of NO during endotoxin shock

Kinetics of serum NO levels after challenge with a lethal dose (100 mg kg⁻¹, IV) of LPS were evaluated 1 h after LPS injection. EDU-treated mice exhibited moderately enhanced levels of NO (60 μM) that gradually rose to 70 μM at 24 h (Fig. 4). In contrast, DMSO-treated mice exhibited significantly higher levels both at 1 h (75 μM) and at 24 h (100 μM).

Serum levels of IL10 during endotoxin shock

Serum levels of IL10 at various time-points after inoculation with LPS (100 mg kg⁻¹, IV) in EDU-treated mice were significantly higher than those observed in DMSO-treated mice (Fig. 5). EDU treatment alone did not affect baseline serum levels of IL10 (data not shown).

Effect of EDU on TNF-α and IL10 production after stimulation with various doses of LPS

To evaluate potential milder case usage, we also studied the influence of EDU on cytokine production after a sublethal dose of LPS. Serum levels of TNF-α from mice that were pre-treated with 50 mg EDU kg⁻¹ before IV inoculation with either 0.5 or 5 mg LPS kg⁻¹ were significantly suppressed (Fig. 6). Furthermore, EDU pre-treatment significantly enhanced IL10 production in mice that were stimulated with various dosages of LPS (0.05–5 mg kg⁻¹) (Fig. 6).

Effect of EDU on IL10 production by mouse macrophages

To further investigate the effect of EDU on IL10 production, we performed a series of experiments using the murine macrophage cell line RAW 264.7. These experiments revealed...
significantly increased IL10 production by RAW 264.7 macrophages on LPS stimulation (10 and 100 pg ml\(^{-1}\)) when EDU was added to the culture medium at 1 \(\mu\)g ml\(^{-1}\) (Fig. 7). Our preliminary experiments revealed that EDU has no direct effect on LPS, as preincubation of LPS with EDU before LPS challenge showed no effect on the cytokine production of the macrophage cell line RAW 264.7 (data not shown).

**DISCUSSION**

Previous studies have documented that certain anti-adhesion compounds that contain cross-linked HA and CM-cellulose increase mortality rates for rats in an intra-abdominal sepsis model, when given at the time of bacterial challenge (Tzianabos \textit{et al.}, 1999). It is important to note that the increase in mortality associated with these compounds following bacterial challenge was not observed when animals were given a therapeutic course of gentamicin and clindamycin concurrently with test compounds. Blood and peritoneal fluid cultures revealed that animals that received anti-adhesive compounds at the time of challenge had significantly more micro-organisms in their blood than control animals, whereas bacterial counts in peritoneal fluid from all groups were similar. These findings suggested that the increased mortality in treated animals was probably due to a greater number of organisms gaining entry to the bloodstream. Although cytokine profiles were not determined in these earlier studies, it is known that proinflammatory cytokines that are induced by bacterial endotoxin facilitate the translocation of bacteria from the peritoneal cavity into the bloodstream (Lundblad \& Giercksky, 1995; Lundblad \textit{et al.}, 1996). It was also shown that pre-treatment of animals with the same anti-adhesion compounds protected against the lethal effects of bacterial challenge when given 4–24 h prior to challenge. HA/CM-cellulose anti-
adhesion compounds are modified with EDC, which releases EDU as a product of hydrolysis and cross-linking of the polymers. In a series of experiments that attempted to identify the protective moiety that was responsible for altering the outcome of experimental intra-abdominal sepsis, EDU was identified as the active factor (Ruiz-Perez et al., 2003).

The present study indicates that mice that are pre-treated with EDU have a markedly higher survival rate in an animal model for endotoxin-induced shock compared to vehicle-treated controls. In addition to survival studies, the effects of EDU on TNF-α production after challenge with a lethal dose of LPS were determined. It was shown that EDU significantly reduced serum levels of TNF-α in this model. Furthermore, it was demonstrated that a significant decrease in serum levels of other proinflammatory cytokines, namely IL1β and IFN-γ, occurred in the EDU-treated group. Notably, EDU administration itself did not affect serum levels of the various cytokines that were studied in the LPS-induced shock model.

Cytokines are key regulators of host responses to infection, immune responses, inflammation and trauma. Specific cytokines, such as TNF-α, IL1β and IFN-γ, promote inflammation and are therefore called proinflammatory cytokines (Dinarello, 1997, 2000; Gogos et al., 2000). These cytokines are elevated in the sera of patients with septic shock and it has been shown that they are related strongly to events that are associated with endotoxin-induced shock (Beutler & Cerami, 1988; Calandra et al., 1990; Pinsky et al., 1993; Waage & Steinshamn, 1993). Most studies indicate an association between TNF-α levels, severity of shock and mortality (Calandra et al., 1990; Pinsky et al., 1993; Waage & Steinshamn, 1993). Recent reports mention that treatment with either tetracycline or fluoroquinolones modulated the immune response to LPS and was associated with markedly reduced mortality in mice (Shapira et al., 1996; Khan et al., 2000). In these studies, the protective effect of the antibiotics was related directly to a reduction in TNF-α production after LPS challenge. Furthermore, inhibition of LPS-stimulated TNF-α production by human peripheral blood mononuclear cells was detected in the presence of various kinds of antimicrobial agent, including fluoroquinolones (Krehmeier et al., 2002).

Along with proinflammatory cytokines, LPS stimulates expression of the inducible NO synthase that, in turn, generates NO. Excessive NO synthesis plays a substantial role in the haemodynamic dysfunction associated with sepsis, leading to the typical phenomenon of multi-organ failure (Thiemermann, 1997; Titheradge, 1999). Therefore, the effects of EDU on induction of NO were evaluated by measuring serum NO levels after LPS challenge. These studies revealed that EDU reduced serum NO levels significantly in this experimental model.

Previously, it was shown that blocking either IFN-γ or TNF-α had no effect on LPS-induced NO release. However, blocking both IFN-γ and TNF-α at the same time prevents NO release almost completely after LPS challenge (ter Steege et al., 1998). These findings suggest that the presence of both TNF-α and IFN-γ is essential for induction of NO by LPS. The results and mechanisms described in these studies also support the hypothesis that suppression of both TNF-α and IFN-γ production after LPS challenge by EDU led to the decrease in NO induction.

IL10 is produced by both T-cells and macrophages and possesses anti-inflammatory properties. IL10 is known to suppress LPS-activated synthesis of several proinflammatory cytokines (Howard et al., 1993; Takakuwa et al., 2000). Furthermore, IL10 protects mice from lethal endotoxaemia (Gerard et al., 1993; Howard et al., 1993; Matsumoto et al., 1998). Other studies (ter Steege et al., 1998) indicated that blocking IL10 with an anti-IL10 mAb resulted in a twofold increase in LPS-induced serum NO levels. In order to examine whether the downregulation of production of proinflammatory cytokines by EDU was in fact mediated by changes in IL10 production, serum IL10 levels were measured after LPS challenge in mice that were pre-treated with either EDU or DMSO. Interestingly, these studies showed significantly higher serum levels of IL10 after LPS inoculation in EDU-treated groups than in controls. Our in vitro study also revealed that EDU significantly enhanced IL10 production by RAW 264.7 macrophages after exposure to LPS. Together with the fact that IL10 levels reached a peak at such an early time-point (1-5 h after LPS inoculation) in the inflammatory cascade, we hypothesize that downregulation of proinflammatory cytokines and NO by EDU may well be a result of early induction of IL10 by this compound, at a time-point prior to the production of significant levels of proinflammatory cytokines.

This is the first study of EDU to use an animal model of endotoxin-induced shock in mice. The protective effects of EDU for LPS-induced shock in mice require further study to determine whether this compound will suppress systemic inflammatory response syndromes in other species, including humans. When considering the use of EDU in patients with endotoxin-induced shock, the potential anti-inflammatory properties of IL10 will have to be weighed carefully against its immunosuppressive properties. In conclusion, the decrease in plasma levels of the proinflammatory mediators TNF-α and NO and increase in circulating IL10 levels by EDU suggest that this compound might offer a new therapeutic approach for inflammatory diseases and septic shock.

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