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Pretreatment of mice with lipopolysaccharide (LPS) or IL-1β exerts dose-dependent opposite effects on Shiga toxin-2 lethality

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SUMMARY

Haemolytic uraemic syndrome (HUS) has been closely associated with infection with a group of Shiga toxin-producing enterohaemorrhagic *Eschericchia coli* in young children. Shiga toxins (Stx) have been implicated as pathogenic agents of HUS by binding to the surface receptor of endothelial cells. LPS is a central product of the Gram-negative bacteria and several reports have documented that both LPS and Stx are important for disease development. In this study the reciprocal interactions between LPS and Stx2 are analysed in a mouse model. The results demonstrated that LPS was able to reduce or enhance Stx2 toxicity, depending on the dose and the timing of the injection. The involvement of the main early cytokines induced by LPS, tumour necrosis factor alpha (TNF-α) and IL-1β, in those LPS opposite effects on Stx2 toxicity was evaluated. Stx2 toxicity was enhanced by *in vivo* injection of murine TNF-α and low doses of murine IL-1β. However, at higher doses of IL-1β which induced corticosteroid increase in serum, Stx2 lethality was decreased. Considering that dexamethasone and IL-1β reproduce the LPS protective effects, it is suggested that endogenous corticosteroids secondary to the inflammatory response induced by LPS, mediate the protection against Stx2. It can be concluded that the fine equilibrium between proinflammatory and anti-inflammatory activities strongly influences Stx2 toxicity.

Keywords Shiga toxin tumour necrosis factor-alpha IL-1β corticosterone lipopolysaccharide haemolytic uraemic syndrome

INTRODUCTION

The epidemic form of the haemolytic uraemic syndrome (HUS), the most common cause of acute renal failure in childhood, is characterized by thrombocytopenia, haemolytic anaemia and acute renal failure [1]. Typically, HUS develops in young children as a vascular disease several days after the occurrence of diarrhoea and bloody gastroenteritis [2] caused either by *Shighella dysenteriae* type 1 [3,4] or by particular serotypes of enterohaemorrhagic *Escherichia coli* (EHEC) producing significant quantities of Shiga toxins (Stx), also referred to as verocytotoxins or Shiga-like toxins [5]. From the beginning of the 1980s it has become clear that Stx-producing *E. coli* infections are the main cause of HUS [6,7]. A family of three Stx has been described: Stx1, Stx2 and Stx2 variants (Stx2c, Stx2e) [8]. The toxins are heterodimers constituted by one A subunit (31·5 kD) and five B subunits (7·7 kD each), and act as inhibitors of protein synthesis when they bind to a specific cell receptor through one of the B subunits [9]. However, it has been suggested that although Stx is necessary for HUS development, it is not sufficient and the simultaneous action of other pathogenic factors appears to be necessary [10–12]. In fact, HUS appears to be more likely to develop in those with bloody diarrhoea than in those with non-bloody stools [13,14], suggesting that colonic vascular damage by Stx may cause LPS and other bacterial products to gain access to the circulation, thus inducing an inflammatory response and favouring the development of HUS. Moreover, compelling evidence has indicated increased levels of neutrophil products [15] and cytokines such as IL-1, IL-6 and IL-8 in patients with HUS [16].

Different animal models have been used to study the histological alterations and the LPS contributory effects for Stx pathogenicity [17–19]. Karpman *et al*. [20] observed that mice injected with Stx develop neurological and systemic symptoms leading to death even in C3H/HeJ, a LPS non-responder strain of mice, indicating that Stx is responsible for these symptoms. However, several *in vivo* and *in vitro* studies suggested the possibility of co-operation between Stx and LPS in producing the pathologic changes observed in HUS [18–21]. On the other hand, Barret

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et al. [18] observed significant protection in rabbits and mice when LPS was injected 20 h previously to Stx.

The aim of this study was to analyse the factors that contribute to the LPS dual effects on Stx toxicity using the murine model. The understanding of LPS and Stx interactions could contribute to the elucidation of the pathogenesis of HUS, leading to a better handling of this disease.

MATERIALS AND METHODS

Mice

BALB/c mice were bred in the animal facility at the Department of Experimental Medicine, Academia Nacional de Medicina. Female and male mice aged 8–16 weeks and weighing 20–24 g were used throughout. They were maintained under a 12-h light–dark cycle at a temperature of 22 ± 2°C and fed with standard diet and water *ad libitum*.

Stx2 preparation

Stx2 was kindly provided by Dr S. Junichi (Denka Seiken Co., Ltd., Niigata, Japan). Purity was analysed by the supplier, showing only one peak in high performance liquid chromatography (HPLC). Stx2 preparation was checked for endotoxin contamination by the *Limulus* amoebocyte lysate assay, given that 1 IU/ml is equal to 0·1 ng/ml of United States Pharmacopoeia standard *E. coli* endotoxin [22]. Stx2 preparation contained < 40 pg LPS/μg of Shiga toxin protein.

Stx2 was tested for cytotoxic activity on Vero cells, as previously described [7], in the Instituto Nacional de Enfermedades Infecciosas, ANLIS, 'Dr C. G. Malbrán' (Buenos Aires, Argentina). Briefly, Vero cells were grown in Eagle's minimum essential medium with Earle's salts and non-essential amino acids (GIBCO Diagnostics, Madison, WI) supplemented with 7% fetal calf serum (FCS; Sigma Chemical Co., St Louis, MO), 0·03 M glutamine, 50 μg/ml gentamycin and 2·5 μg/ml fungizone in microtitre plates (Nunc, Intermed, Roskilde, Denmark). Aliquots (50 μl) of serial two-fold dilutions of the samples containing Stx2 were added to each well (25 000 Vero cells) and incubated for 3 days at 37°C in 5% CO_2. Vero cells were examined daily for cytotoxicity. The 50% cytotoxic dose (CD_{50}) corresponded to the dilution required to kill 50% of the Vero cells: CD_{50} was approx. 0·063 pg.

Stx2 treatment

The same batch of Stx2 preparation was used throughout the experiments. In preliminary experiments we evaluated the *in vivo* lethality by serial dilutions in pyrogen-free saline. We chose a dose of 600 pg/mouse (approx. 3 ng/kg mouse) which induced a mortality > 50% between 3 and 4 days after injection.

Endotoxin preparation

To modulate Stx2 pathogenicity we used commercial LPS from *E. coli* O111:B4 (Sigma), as previously reported [18,19]. The LPS O111:B4 lot used throughout the experiments had a LD_{50} of 150 μg/mouse. It was diluted in sterile saline prepared with pyrogen-free distilled water immediately before use at the desired concentration.

Stx2 and LPS treatments

Mice were given LPS by i.p. injection at different time intervals before and after i.v. injection of 600 pg/mouse of Stx2. Each experiment was repeated more than five times, using 8–12 mice per group. Mice were examined for gastrointestinal (changes in faecal colour and consistency), neurologic (ataxia, rigidity, convulsions and coma) and/or systemic (lethargy, anuria, ruffled fur, tachypnoea, restlessness, body swelling) symptoms every 4 h after inoculation. Mice were killed by cervical dislocation when signs of terminal disease were evident or 10 days after inoculation.

Corticosterone assay

To minimize stress induced by handling, the day before the experiments animals were housed one per cage. Mice were injected intravenously with saline, LPS, recombinant murine IL-1β and tumour necrosis factor-alpha (TNF-α) (Sigma). Blood samples were collected 2 h post-treatment in another room; controls were always killed last in the sequence. Trunk blood was collected into iced EDTA plastic centrifuge tubes after decapitation. Plasma was immediately separated and stored at −20°C until hormonal assay. Plasma corticosterone concentrations were determined by radioimmunoassay (RIA) using a commercially available [^{3}H]kit (ICN Biomedicals, Costa Mesa, CA). The detection limit was 30 ng/ml. Intra- and interassay coefficients of variation were 7% and 12%, respectively.

Biochemical studies

Blood was obtained by puncture of the retro-orbital plexus and urine samples were collected simultaneously. Biochemical determinations of urea, creatinine, glucose, total proteins and albumin, in serum and urine were done in an autoanalyser CCX Spectrum (Abbott Diagnostics System, Buenos Aires, Argentina) following standard instructions. Specific gravity in urine was measured by Test strips *in vitro* diagnostic (Combur^{10} Test; Boehringer Mannheim, Buenos Aires, Argentina).

Statistical analysis

The χ^2 test, Fisher's exact test, was used for class variables in two independent samples. Intergroup contrasts of dimensional variables were appraised with Student's *t*-test. All statistical tests were interpreted in a two-tailed fashion to estimate *P* values.

RESULTS

LPS action on Stx2 toxicity

Mice inoculated with 600 pg of Stx2 developed severe neurological symptoms consisting of ataxia, intense tremor, spastic paralysis, convulsions, coma and death. These symptoms were observed in 60–80% of treated mice after 3–4 days.

LPS from *E. coli* O111:B4 (5 μg/mouse) was injected intraperitoneally at different times before and after i.v. administration of Stx2. This dose of LPS induces macrophage stimulation measured as serum TNF-α levels 1 h after injection (LPS 5 μg = 256 ± 55 U/ml, $n = 6$; saline = 4 ± 2 U/ml, $n = 6$; quantified by using a standard L929 cytotoxicity assay), but it is far less than the LD_{50} dose (LPS LD_{50} 150 μg/mouse). Lethality, renal histopathology and biochemical parameters were evaluated.

As shown in Fig. 1, Stx2 mortality was dramatically enhanced by LPS injected 1 h before the toxin. However, when LPS was injected 18 h before Stx2, mice were protected against Stx2 toxicity. LPS injected simultaneously or after Stx2 did not significantly modify Stx2 mortality.

Since LPS modulation of Stx2 toxicity has not been carefully investigated, a wide range of LPS doses and times of injection was evaluated. Relatively high doses of 25 μg/mouse and very low ones such as 0·5 μg/mouse were included. These doses of LPS were

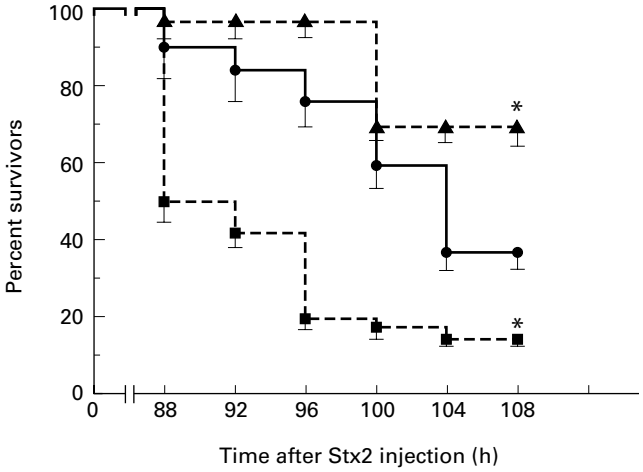

Fig. 1. LPS modulatory effects upon Shiga toxin 2 (Stx2)-induced toxicity. Groups of mice were treated with LPS (5 μg/mouse) at −18 h (▲) or LPS at −1 h (■), followed by Stx2 (600 pg/mouse) at time 0. Controls of Stx2 alone were assessed in parallel (●). Lethal effects of Stx2 were evaluated at regular periods of 4 h. The figure represents the percentage of survivors at each point time from a total of 35 mice per group, examined in three independent experiments. This treatment schedule was repeated twice, giving the same results. *$P < 0{\cdot}001$, significantly different from Stx2-induced mortality by Fisher's exact test.

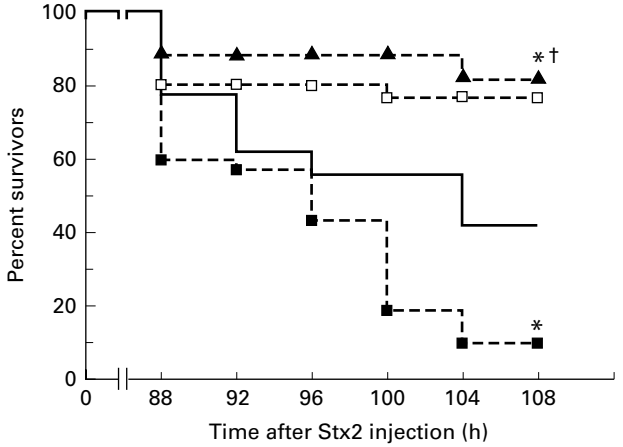

Fig. 3. Effect of protective treatments upon LPS and Shiga toxin 2 (Stx2) synergism. Mice were injected with dexamethasone (2·5 mg/kg) (▲) or LPS (5 μg/mouse) (□) 18 h before Stx2. One hour before Stx2, mice were also injected with LPS (5 μg/mouse).Groups of mice treated with Stx2 alone (solid line) and controls of LPS synergism (LPS, 5 μg/mouse, at −1 h) (■) were also assayed in parallel. Data represent the percentage of survivors at each point time of groups of 12 mice. *$P < 0{\cdot}01$, significantly different from Stx2-induced mortality; †$P < 0{\cdot}001$, significantly different from LPS (−1 h) plus Stx2-induced mortality by Fisher's exact test.

injected from 4 days to 1 h before Stx2. All animals received the same dose of Stx2 at time 0. The percentage of survivors at 90 h post-Stx2 inoculation is presented in Fig. 2. The results indicate that each dose is able to either enhance or reduce Stx2 lethality depending on the time elapsed up to Stx2 injection. Contrary to previous reports [18], it can be concluded that pretreatment of mice with LPS does not always induce a protective effect on Stx

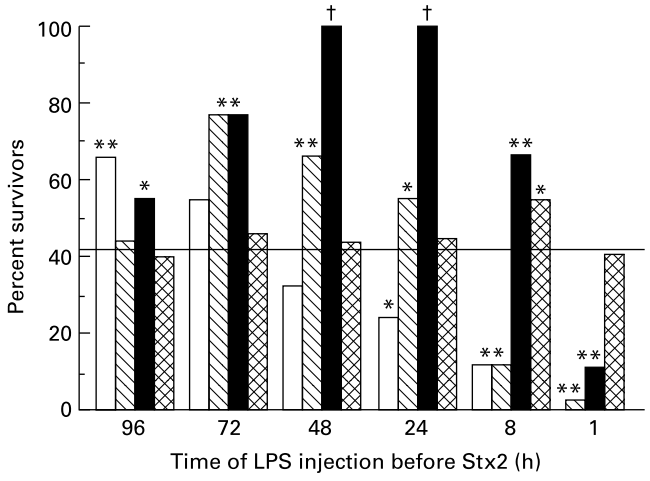

Fig. 2. LPS modulatory effects upon Shiga toxin 2 (Stx2)-induced toxicity. Effect of doses and timing of LPS injection. Bars represent the percentage of survivors (15 mice per group) at 90 h post-Stx2 inoculation. Mice were injected with different doses of LPS: 25 μg/mouse (□); 5 μg/mouse (hatched); 1 μg/mouse (■); and 0·5 μg/mouse (cross-hatched). Each dose was injected intraperitoneally at the time indicated on the abscissa before Stx2. The results are representative of two independent experiments. The percentage of survivors injected with Stx2 alone is depicted as the horizontal line. †$P < 0{\cdot}001$; **$P < 0{\cdot}001$; *$P < 0{\cdot}05$, significantly different from Stx2-induced mortality by Fisher's exact test.

toxicity. In fact, different doses of LPS administered at different times, according to the above described protocols, are capable of either decreasing or enhancing Stx2 lethality.

It has been reported that pretreatment with dexamethasone protects mice from Stx2 toxicity [23]. Therefore, it was decided to evaluate whether the protective treatments described (LPS or dexamethasone injected 18 h before Stx2) could counteract the potentiation of Stx2 toxicity by LPS administered 1 h before Stx2. For this purpose, mice were pretreated with either saline, or 5 μg i.p. LPS, or 2·5 mg/kg i.v. dexamethasone. After 18 h, all animals received a dose of 5 μg of LPS, and 1 h later Stx2 by the i.v. route. Results presented in Fig. 3 indicate that both protective treatments, LPS and dexamethasone (−18 h), were able to abrogate LPS synergism on Stx2 toxicity observed when LPS was injected −1 h. Moreover, an additional protection against Stx2 lethality could be seen.

Cytokines involved in LPS action

Further studies were directed to understanding the possible mechanisms of LPS action. It is known that among several cytokines released after LPS injection, IL-1β and TNF-α play a central role as proinflammatory mediators [24,25]. Taking this into account, these cytokines were evaluated as possible mediators of LPS modulation on Stx2 toxicity. Both murine recombinant TNF-α and IL-1β were injected intravenously 1 h before Stx2. The results depicted in Fig. 4 indicate that TNF-α has a synergistic toxic effect on Stx2 mortality. This was true for all doses and times assayed (from 10 pg to 10 ng/mouse and 18 h to 1 h before Stx2). On the other hand, IL-1β injected 1 h before Stx2 showed a dual effect on Stx2 toxicity. While low doses of 20 ng/mouse potentiated Stx2 pathogenicity, 100 ng/mouse significantly counteracted it (Fig. 4). This effect was also observed when IL-1β was injected 18 h before Stx2. There is convincing evidence that LPS stimulates the hypothalamic–pituitary–adrenal axis mainly through IL-1β action [26]. As a consequence of this, LPS and IL-1β increase

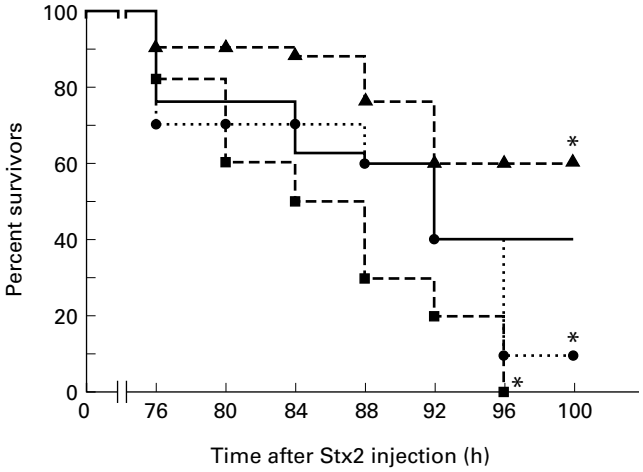

Fig. 4. Cytokine modulatory effects upon Shiga toxin 2 (Stx2) toxicity. Groups of mice were injected intravenously with tumour necrosis factor-alpha (TNF-α; 1 ng/mouse) (–■–) or IL-1β (100 ng/mouse; –▲–) or IL-1β (20 ng/mouse; ··●···) 1 h before Stx2. Controls of Stx2 alone were assessed in parallel (solid line). Lethal effects of Stx2 were evaluated at regular periods of 4 h. The figure represents the percentage of survivors at each point time from a total of 10 mice per group. This schedule was repeated twice, giving the same results. *$P < 0{\cdot}05$, significantly different from Stx2-induced mortality by Fisher's exact test.

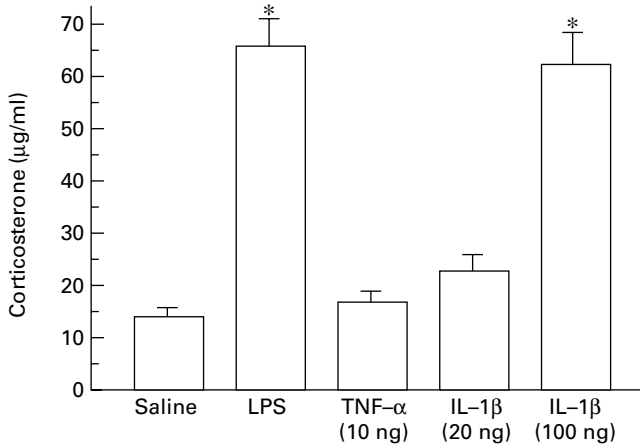

Fig. 5. Levels of corticosterone in plasma. Mice were injected intravenously with saline, LPS (5 μg/mouse), tumour necrosis factor-alpha (TNF-α) (10 ng/mouse) and IL-1β (20 or 100 ng/mouse). Blood samples were obtained 2 h post-treatment and plasma corticosterone concentration was determined by radioimmunoassay as detailed in Materials and Methods. Bars represents the medium ± s.e.m. of six mice assayed in duplicate. *$P < 0{\cdot}001$ significantly different from saline-treated mice by Student's *t*-test.

the plasma concentration of corticosteroids [27]. Therefore, plasma corticosterone levels after LPS, TNF-α and IL-1β injections were checked. It was found that only LPS and IL-1β (100 ng/mouse) were able to increase significantly serum levels of corticosterone (Fig. 5).

Biochemical and histological studies

In order to characterize further this murine model and better define the cause of death and the reciprocal interactions between LPS and Stx2, biochemical and histological parameters were evaluated in mice treated with Stx2 alone or in combination with LPS. Protocols of potentiation of Stx2 toxicity (5 μg of LPS injected at −1 h) and protection (5 μg of LPS or dexamethasone, at −18 h) were analysed. Controls of LPS alone and saline-treated mice were studied in parallel. All animals were bled daily and urine samples were simultaneously collected. The biochemical parameters determined in blood and urine samples 24 h post-Stx2 inoculation were within normal ranges for all groups. However, 48 h later mice treated with LPS 1 h before Stx2, which potentiates Stx2 mortality, showed marked elevations of urea values in serum, suggesting an accelerated renal damage as a consequence of LPS potentiation (uraemia mg/%: control = 58 ± 4; Stx2 = 79 ± 4; LPS-1 h + Stx2 = 108 ± 9; LPS-1 h + Stx2 *versus* Stx2 $P < 0{\cdot}05$; $n = 5$ mice/group). On the other hand, 72 h later all mice injected with Stx2 had abnormal renal function, evaluated as increased urea and creatinine in serum (Table 1). However, mice receiving protective treatments (LPS and dexamethasone at −18 h) had intermediate urea values between controls and Stx2-treated mice, showing statistical differences with both groups (Table 1). It can be concluded that urea and creatinine values in serum directly correlate with mortality. Moreover, when animals belonging to the same group were compared, individual determinations corroborated that the highest urea values corresponded to the mice that died the earliest (data not shown). Haematocrit, serum glucose, total proteins and albumin were

Table 1. Biochemical parameters in serum and urine 72 h after Shiga toxin 2 (Stx2) injection

Treatment	Creatinine (mg/dl ± s.e.m.)	Uraemia (m/dl ± s.e.m.)	Urea (g/*l* ± s.e.m.)	Haematocrit (% ± s.e.m.)	Proteins (mg% ± s.e.m.)	Glucose (mg% ± s.e.m.)
Saline	0·7 ± 0·2	58 ± 3	142 ± 14	43 ± 3	6·3 ± 0·2	68 ± 7
LPS	0·8 ± 0·2	65 ± 4	136 ± 12	45 ± 3	6·4 ± 0·3	70 ± 6
Stx2	3·2 ± 0·6**	210 ± 25**	22 ± 2**	53 ± 2†	8·7 ± 0·2†	119 ± 10†
Stx2 ± LPS − 1 h	3·1 ± 0·8**	208 ± 40**	38 ± 5**	51 ± 3†	8·2 ± 0·3†	183 ± 12†
Stx2 ± LPS − 18 h	2·2 ± 0·3*‡	100 ± 7*‡	84 ± 11*‡	49 ± 2†	6·6 ± 0·2	100 ± 7†
Stx2 ± DEX	1·1 ± 0·2‡	110 ± 7*‡	64 ± 7*‡	48 ± 1	7·7 ± 0·2	120 ± 13*

Groups of 10 mice were daily bled and samples of urine collected after Stx2 injection. Combined treatments with LPS (5 μg/mouse) and dexamethasone (DEX) (2·5 mg/kg) were also assayed. The table includes the control values of saline and LPS (5 μg/mouse). Table shows levels of creatinine, uraemia, haematocrit, glucose and total proteins in serum, and urea in urine obtained 72 h after Stx2. The results are representative of three independent experiments.

†$P < 0{\cdot}05$; *$P < 0{\cdot}01$; **$P < 0{\cdot}001$, significantly different from saline-treated group by Student's *t*-test; ‡$P < 0{\cdot}001$, significantly different from Stx2-treated group by Student's *t*-test.

similarly increased in all mice treated with Stx2 (Table 1). Such increases are probably due to dehydration secondary to the tubular damage induced by the toxin [20]. In fact, daily measurement of urine-specific gravity indicated an early period of polyuria (Stx2 = $\delta < 1{\cdot}010$; control = $\delta = 1{\cdot}025$–$1{\cdot}030$), before final anuria developed in all mice injected with Stx2.

Control mice injected with 5 μg of LPS did not show any alteration in the biochemical studies. Moreover, groups of mice inoculated with significantly higher doses of LPS (100 μg/mouse) showed systemic symptoms such as intense tremor and lethargy during the first hours post-LPS injection, but all animals survived and showed biochemical parameters within normal ranges (data not shown), confirming that Stx2 is responsible for the renal toxicity leading to death.

In another set of experiments, 72 h post-Stx2 injection animals were bled and killed. Their kidneys were excised and histologically examined. The biochemical parameters were compared with the toxic effect of Stx2 on the kidneys, in the different groups. All Stx2-treated mice showed kidney damage. In agreement with previous reports [18,19], major lesions were observed on tubular cortical structures. Tubules showed mild to severe lesions ranging from loss of apical brushes and mild vacuolation to necrosis and cell exfoliation with eosinophilic intratubular casts (not shown). No evidence of thrombi or renal microangiopathy was found. It is important to point out that there were mice with great renal damage in all Stx2 groups; however, there was a close correlation between histological signs and serum urea values, i.e. major tubular lesions always corresponded to mice with maximal urea levels in serum. The control group did not show tubular damage.

DISCUSSION

Shiga toxin exerts its cytotoxic effects only when it can bind to a specific cell receptor [10], a neutral glycolipid known as globotriaosylceramide (Gb3) [28,29]. Gb3 receptors have been identified on renal epithelial, endothelial, and recently, on glomerular mesangial cells [30]. However, compelling evidence has indicated that other pathogenic factors would be necessary for Stx-induced tissue injury [10–12]. Substances thought to be involved in endothelial damage, which are increased in patients with HUS, include different inflammatory cytokines and neutrophil products [15,16,31]. In this context and using a murine model, we studied the contribution of LPS and the induced cytokines on Stx2 toxicity.

Although LPS has been implicated in the pathogenesis of HUS in different animal models [17,18,32], the detailed mechanisms of action are still elusive. LPS has been shown to induce either a protective or synergistic effect on Stx action, depending on whether it is injected before or after Stx administration, respectively [18–20]. In contrast, our results show that LPS pretreatment of mice does not always induce a protective effect on Stx2 action. In fact, the effect was strictly dependent on the dose and time of LPS injection. For example, 25 μg of LPS enhanced Stx2 toxicity when injected 48 h before Stx2, but the same dose of LPS induced protection when it was injected 4 days before Stx2. Similar opposite effects could be obtained using 1 μg of LPS. This amount of LPS, which induced a lesser level of inflammation than 25 μg, was able to potentiate Stx2 toxicity only when it was given 1 h before Stx2 (Fig. 2). On the other hand, the maximal protection with 1 μg of LPS was obtained injecting it 24–48 h before Stx2. A particular interesting feature of this model is that LPS could potentiate Stx2 pathogenicity even when injected before Stx2, in contrast with previous data [18–20]. Considering the time course of HUS, where the LPS may precede or accompany Stx blood presence, the modulatory effects of LPS described here could have crucial importance in the development of the disease.

It is well known that LPS is a powerful inflammatory stimulus and, as long as the response is successful and the stimulus is removed, inflammation is counteracted, and homeostasis is restored [33]. The results presented in this study are in agreement with this hypothesis, in that the intensity and duration of the inflammatory response are dependent on the magnitude of the stimulus. Accordingly, LPS enhancing effect of Stx2 toxicity can be interpreted as the coincidence between maximal proinflammatory activity induced by LPS and Stx2 insult. In contrast, LPS protection could be the consequence of antagonistic effects between a late anti-inflammatory response induced by LPS and Stx2 toxicity. A decrease in inflammation not only reflects the disappearance of inflammatory mediators (i.e. IL-1β, TNF-α, IL-6, interferon-gamma (IFN-γ)) but also the appearance of anti-inflammatory ones (i.e. IL-10, IL-4, glucocorticoids). The intensity and/or duration of the anti-inflammatory response would be closely related with the dose of the stimulus. In this regard, preliminary data have shown that while 1 μg of LPS induced a peak of corticosterone only detectable 2 h later, 25 μg of LPS induced high levels of corticosterone even 8 h after its injection (unpublished observations). In addition, the anti-inflammatory effect induced by 5 μg of LPS 18 h before Stx2 was able to counteract the enhancement of Stx2 toxicity induced by LPS injected 1 h before Stx2 (Fig. 3). Such results resemble the LPS desensitization phenomenon [34], and reinforce the concept that LPS-induced protection is an active mechanism and does not merely result from the lack of an inflammatory effect.

LPS inflammatory actions have been often associated with the induction of endogenous cytokines, mainly TNF-α and IL-1β. Accordingly, we sought to determine whether opposite effects on Stx pathogenicity could be ascribed to these cytokines.

We showed that treatment of mice with murine recombinant TNF-α significantly enhanced Stx2 pathogenicity at all doses and times assayed (from 10 pg to 10 ng/mouse and 18 h to 1 h before Stx2). This is in agreement with recent studies [35], and correlated with *in vitro* observations, where endothelial cells incubated with TNF-α expressed an enhanced number (10–100-fold) of Stx2 receptors [3,21]. Similarly, previous studies have shown that IL-1β enhanced Gb3 receptor expression and Stx toxicity in human vascular endothelial cells *in vitro* [36–40]. Collectively, these results led to the conclusion that TNF-α and IL-1β mediate LPS potentiation of Stx toxicity. Our results were coincident with these reports when TNF-α and 20 ng/mouse of IL-1β were assayed. However, 100 ng/mouse of IL-1β, a dose capable of inducing adrenal stimulation, were able to protect mice from Stx2 toxicity. Obviously, this systemic action of adrenal stimulation could not be observed when the IL-1β effect was evaluated on endothelial cells *in vitro* [38]. Taking these data into account, we suggest that endogenous corticosteroids could be responsible for, at least in part, LPS and IL-1β protection. This reasoning is also supported by the protective effects of dexamethasone in the murine model of HUS [1], in spite of its enhancing effect on Gb3 expression on endothelial cells [41]. These data reinforce the concept that Stx toxicity is not always proportional to the Gb3 content [5]. Taking these results into account, it can be concluded that the immune system may play an active role in modulating Stx toxicity through a complex and dynamic process in which the result of the balance

of opposite actions leads to either exacerbation or inhibition of Stx pathogenicity.

Biochemical analysis, histopathology and lethality were highly correlated. Symptoms such as anuria and body swelling have been described, suggesting renal involvement [20]. However, altered biochemical parameters demonstrating renal failure in the mouse model have not been reported before.

The synergism between LPS and Stx was previously interpreted as a sensitization induced by Stx to the lethal effect of LPS, but the opposite, i.e. sensitization of mice to the lethal effect of Stx by LPS, did not occur [19]. However, biochemical and histological data shown here support the opposite interpretation. In fact, no alteration in urea and creatinine serum concentrations was observed even after high doses of LPS (100 μg) per mouse. Therefore, it is more probable that low doses of LPS (5 μg) sensitized mice to the renal toxicity induced by Stx2, and not *vice versa*.

In conclusion, in this study we have demonstrated three important and new features of the LPS and Stx2 interaction: (i) contrary to previous reports, LPS injected before Stx2 is not only able to attenuate, but also to enhance Stx2 toxicity, depending on the dose of LPS injected; (ii) the synergism between LPS and Stx2 was a consequence of the enhancement of Stx2 renal toxicity by LPS, and not *vice versa* as has been previously proposed; (iii) despite the fact that IL-1β has been postulated as one of the proinflammatory cytokines inducing the enhancement of Stx2 toxicity *in vitro*, we found that IL-1β *in vivo* has a protective role against Stx2 toxicity, in association with the induction of endogenous glucocorticoids. This controversy with early studies based on *in vitro* experiments led us to caution against *in vivo* extrapolation from *in vitro* observations.

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