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ENDOTOXIN-INDUCIBLE CYTOTOXICITY IN LIVER CELL CULTURES—II

DEMONSTRATION OF ENDOTOXIN-TOLERANCE

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Abstract—Endotoxins from gram negative bacteria, central mediators of septic shock, share the characteristic property of inducing tolerance against their own action. This work investigates whether a corresponding *ex-vivo* tolerance can be observed in a cellular system with endotoxin-inducible hepatocytotoxicity. The following experimental approaches were chosen in order to induce an endotoxin-unresponsive state prior to cell preparation: (1) pretreatment of rats with endotoxin, (2) partial hepatectomy, (3) use of neonatal rats and (4) pretreatment of rats with silica. An *in-vivo* protection against endotoxin-induced liver injury was obtained by all of these four measures: cells prepared from these groups of animals showed greatly diminished sensitivity towards endotoxin-induced hepatocytotoxicity *in vitro*. The suppressed endotoxin sensitivity after silica pretreatment was partially restored *in vitro* by the addition of native Kupffer cells (KC). Isolated KC of all but the endotoxin-pretreated animals secreted tumor necrosis factor-α in response to endotoxin. It is concluded that different types of tolerance can be distinguished: (a) impairment of macrophage functions (silica pretreatment), (b) hepatocyte unresponsiveness (neonatal rats and hepatectomy) and (c) impaired macrophage function combined with hepatocyte unresponsiveness (endotoxin-pretreated rats).

Endotoxins from gram negative bacteria are potent immunomodulators *in vivo* as well as *in vitro*. These lipopolysaccharides (LPS†) induce a broad spectrum of defense mechanisms of the host organism [1]. If present in excess they cause endotoxic shock. The frequently observed subsequent organ failure [2] includes the liver. Endotoxemia is found in patients suffering from different types of liver disease and has been implicated in the pathogenesis of hepatic injury [3, 4]. Experimentally, LPS-induced liver damage can be caused in rodents when they are sensitized by pretreatment with inhibitors of transcription [5], e.g. GalN). Under these conditions a challenge by minute amounts of LPS causes acute hepatitis in the sensitized animals.

We have described recently an *in-vitro* model [6] where cytotoxicity towards isolated liver cells is induced by LPS. It was demonstrated that this cellular system requires the presence of functionally intact KC, the resident liver macrophages. Evidence was obtained, that TNF-α is likely to participate in mediating this hepatocytotoxicity.

A common observation concerning LPS sensitivity is a pronounced tolerance, i.e. the response towards a second stronger stimulus is reduced or even absent within a certain time frame after a first challenge [7, 8]. Thus, *in-vivo* pretreatment of mice with subtoxic amounts of LPS protects them from an otherwise toxic administration of GalN plus LPS (GalN/LPS) [9].

Previous investigations showed that animals which have received an intravenous injection of particles like silica, latex or carbon are resistant to GalN/LPS-induced hepatitis [10–12]. These results were interpreted in terms of impaired function of the KC. Alternatively, partial hepatectomy led to an LPS-insensitive state in rodents for at least 2 weeks [13]. Neonatal rats are completely insensitive towards liver injury induced by GalN/LPS [14].

The objective of this study was to examine the existence of a possible *ex-vivo* tolerance against LPS and to disclose analogies between the *in vivo* and *in vitro* situations.

MATERIALS AND METHODS

Substances. LPS from *Salmonella abortus equi* was purchased from the Sigma Chemical Co. (Deisenhofen, F.R.G.). H_2O_2 was obtained from Merck (Darmstadt, F.R.G.). The polyclonal sheep anti-mouse TNF-neutralizing serum (2.2×10^6 neutralizing units/mL) was from this laboratory. Murine recombinant TNF-α was a generous gift of Dr Adolf, Boehringer Institut (Vienna, Austria). Silica (Steinkohlen-Bergbau-Verein, Essen, F.R.G.); particles of 0.5–5 μm) was kindly provided by Dr V. Kolb-Bachofen (Düsseldorf).

Tissue culture. PC and KC preparation was carried out as described previously [6]. Briefly, cells were prepared from male Fischer rats (F344, Charles River, body wt $\approx$ 250–300 g) by the collagenase perfusion method according to Seglen [15]. The

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† Abbreviations: LPS, lipopolysaccharide, endotoxin; TNF-α, tumor necrosis factor-α; KC, Kupffer cells; PC, hepatocytes, parenchymal cells; NPC, non-parenchymal cells; GalN, galactosamine; LDH, lactate dehydrogenase (EC 1.1.1.27); ALT, alanine aminotransferase (EC 2.6.1.1); IL, interleukin.

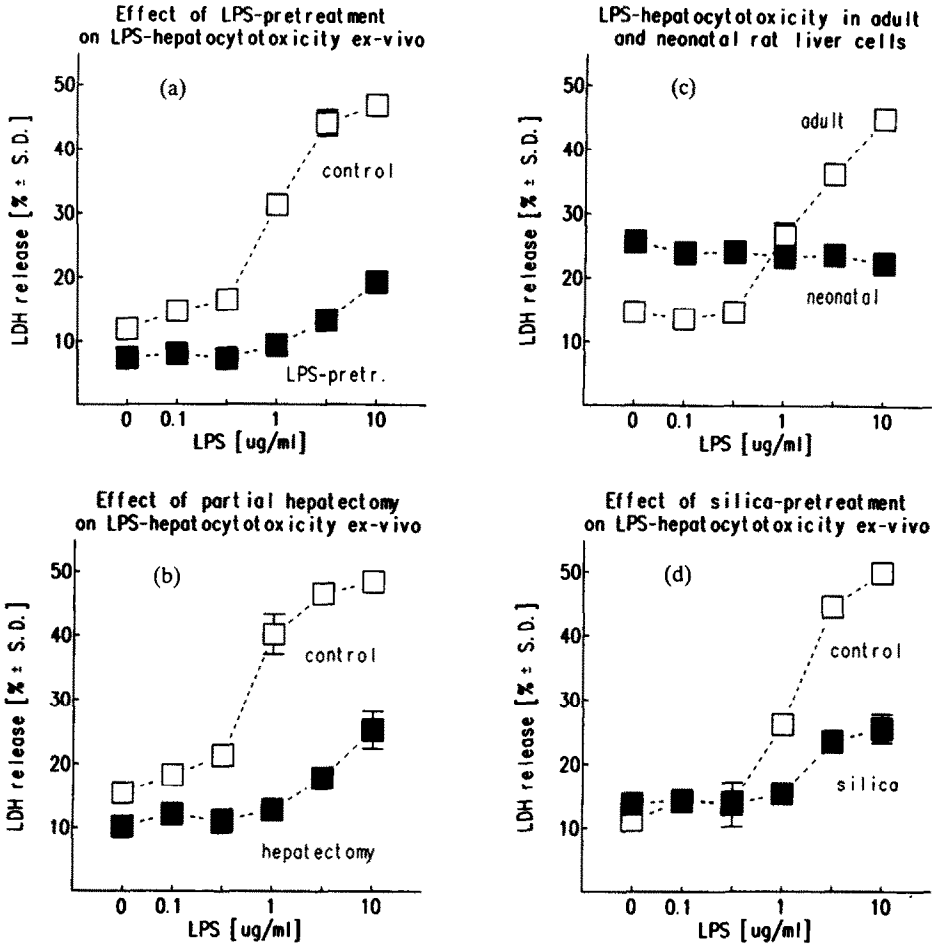

Fig. 1. Influence of different types of *in-vivo* pretreatment of rats on LPS-inducible cytotoxicity in liver cell cultures *ex-vivo*. (a) Influence of *in-vivo* pretreatment of rats with LPS on the cytotoxicity of LPS in liver cell cultures prepared from these animals. Rats were treated with 300 μg/kg LPS 8 days, with 1 mg/kg LPS 6 days, with 3 mg/kg 4 days, and with 10 mg/kg 2 days prior to cell preparation, respectively. Liver cell cocultures prepared from these animals were incubated after 2 hr of collagen adhesion for 16 hr with LPS from *S. abortus equi* at the concentrations indicated. Error bars in all figures are given if greater than symbol and represent SD of three incubations. (b) Influence of partial hepatectomy of rats on LPS-induced cytotoxicity in liver cell cultures prepared from these animals. Two thirds of the liver of the rat were removed 10 days before cell preparation; cocultures prepared from this animal were incubated after 2 hours of collagen adhesion for 16 hr with LPS from *S. abortus equi* at the concentrations indicated. (c) Comparison of LPS-induced hepatocytotoxicity in liver cell cultures from neonatal and adult rats. Liver cells were prepared from neonatal (10-day-old) and adult rats. Cocultures from these animals were incubated after 2 hr of collagen adhesion for 16 hr with LPS from *S. abortus equi* at the concentrations indicated. (d) Inhibition of LPS-induced hepatocytotoxicity after pretreatment of the donor rat with silica. Silica at 25 mg/kg was injected i.p. and 25 mg/kg were given i.v. (v. iliaca) under light anaesthesia 17 hr before cell preparation. Cocultures prepared from these animals were challenged after 2 hr of collagen adhesion for 16 hr with LPS from *S. abortus equi* at the concentrations indicated.

proportion of non-parenchymal cells to parenchymal cells was increased to more than 3% by altering the centrifugation scheme (100 *g* instead of 50 *g*, as usually [16]); further increases in KC number had no effect on the model.

In the case of the neonatal rats a non-recirculating *in situ* perfusion was chosen using essentially the same procedure as in the case of the adult rats. The cell preparation obtained was 94% viable and contained 6×10^6 hepatocytes. Sticking of the cells to each other, however, was much more pronounced when compared to the cell preparation derived from adult rats.

The incubations were started 2 hr after plating of PC by renewing the culture medium (RPMI 1640, 10% calf serum). Putative effectors were added at this time point; 10 μg/mL LPS were added 30 min later. Incubations were stopped 16 hr later and cell

death was measured as the proportion of LDH released into the supernatant.

Inhibition of LPS-inducible cytotoxicity was calculated as:

$$\text{inhibition (\%)} = \frac{\text{release}_{\text{LPS}} - \text{release}_{\text{LPS+inhibitor}}}{\text{release}_{\text{LPS}} - \text{release}_{\text{control}}}.$$

Assessment of hepatotoxicity in vivo. Male rats (Fischer F-344, Charles River, 250–350 g body wt or 12-day-old neonates 20 g body wt, as described above) were kept at least one week on a standard diet (Altromin 1310) *ad lib.* The animals were injected i.p. with GalN (300 mg/kg)/LPS (300 μg/kg).

Blood samples were taken 8 hr after intoxication (LPS, i.p.) by cardiac puncture after cervical dislocation. Liver injury was assessed using serum ALT, serum aspartate aminotransferase and sorbitol dehydrogenase as parameters [17].

In-vivo pretreatment. Four types of pretreatment were used: short term LPS, 300 μg/kg LPS i.p. 2 hr before challenge with GalN/LPS or cell preparation. Repeated LPS, 300 μg/kg LPS i.p. at −day 8, 1 mg/kg LPS i.p. at −day 6, 3 mg/kg LPS i.p. at −day 4 and 10 mg/kg LPS i.p. at −day 2. Silica, 25 mg/kg silica were injected i.p. and 25 mg/kg were given i.v. (vena iliaca) 17 hr before treatment. Hepatectomy, two thirds of the liver of an anesthetized animal were removed essentially as described previously [18]. Cells were prepared 10 days after the operation.

KC were isolated by differential centrifugation and plastic adhesion. Incubations were performed as described for the cocultures. TNF was measured in the supernatant after 3 hr using a bioassay with WEHI 164 clone 13 cells [19]. The cytotoxic activity of control supernatants was blocked completely by a sheep antiserum directed against murine recombinant TNF-α. IL-1-like activity was measured using a bioassay performed with D10G4.1 T cell clone according to Beuscher *et al.* [20]. Since no antibody against IL-1 of the rat is available the measured activity is termed "IL-1-like". TNF-α had no activity in this assay (data not shown).

All *in-vitro* experiments were performed in triplicate. Data are expressed as means ± SD; statistical analysis was performed using the Student's *t*-test.

RESULTS

When Fischer rats (F344) were challenged with 300 mg/kg GalN and 300 μg/kg LPS by simultaneous i.p. administration, they developed a severe hepatitis within 8 hr (ALT 5204 ± 2168 U/L, N = 6); those animals treated with GalN showed only a little hepatic damage (ALT 434 ± 171 U/L, N = 3) within this time. Pretreatment of the animals with 300 μg/kg LPS only 2 hr prior to GalN/LPS challenge reduced the extent of liver injury significantly but did not fully protect the animals (ALT 1750 ± 70 U/L, N = 3, $P < 0.05$). In contrast, repeated administration of increasing amounts of LPS (300 μg/kg, 1, 3 and 10 mg/kg at 8, 6, 4 and 2 days before challenge) induced an *in-vivo* state in which the animals were only a little susceptible to a subsequent single GalN/LPS challenge (ALT 420 ± 110 U/L, N = 3, $P < 0.05$).

Pure PC cultures were not sensitive towards LPS. When PC containing at least 3% KC after 2 hr of attachment to collagen-coated plastic dishes were incubated for a further 16 hr in the presence of 10 μg/mL *S. abortus equi* LPS, release of 45 ± 10% of LDH was observed. Under these conditions controls incubated in the absence of LPS released 15 ± 10% of LDH. A range of ± 10% was the variation between independent cell preparations from more than 50 individual rats; the standard deviation of the LDH release within a given preparation was ± 2% in parallel incubations.

When cocultures were prepared from animals pretreated once *in vivo* with LPS a small significant reduction (−35.6 ± 10.1%) of LPS-inducible LDH release was observed. Cells prepared after repeated administration of LPS *in vivo* showed a reduced responsiveness towards LPS *ex vivo* compared to cells from control animals (Fig. 1a).

Experiments were carried out in order to investigate a possible *in-vitro* cross-tolerance between two different LPS species. The data in Table 1 show, first of all, that *in-vitro* cytotoxicity can be dose-dependently induced by *S. abortus equi* as well as by *S. minnesota* LPS (left hand columns). Previous *in-vivo* treatment of the donor rats by *S. abortus equi* LPS made the cells prepared from these animals less vulnerable to either LPS species added *in vitro*. These findings suggest an *in-vitro* cross-tolerance between individual LPS species.

Notably, addition of rat serum to the cultures generally reduced the LPS cytotoxicity. Serum at a concentration of 10% from repeatedly LPS-pretreated animals had no further effect on LPS-induced hepatocytotoxicity in cocultures from control rats.

Following partial hepatectomy rat liver regenerates within weeks after partial remission. During this time, the animals are insensitive towards GalN/LPS-induced liver injury [13]. Therefore, we investigated this issue under our conditions. Cells prepared 10 days after surgery showed essentially no LPS-induced cytotoxicity (Fig. 1b). This *ex-vivo* unresponsiveness indicates a further analogy between our system and the *in-vivo* situation.

Neonatal rats have been reported to be insensitive towards liver injury induced by the combination of GalN and LPS. This was reproduced with 12-day-old Fischer rat neonates (ALT 280 ± 100 U/L, N = 3; GalN/control: ALT 310 ± 200 U/L). When cells were prepared by a slightly modified procedure from a 10-day-old rat, no LPS-induced hepatocytotoxicity at LPS concentrations greater than 1 μg/mL was observed (Fig. 1c). At lower LPS concentrations an opposite effect was seen which was due to the fact that cell survival during culture in the absence of LPS was generally lower.

Silica is known to destroy macrophages, especially KC [21]. *In-vivo* pretreatment of rats with silica protected the animals significantly against hepatitis induced by GalN/LPS (ALT 430 ± 560 U/L, N = 3, $P < 0.05$ compared to GalN/LPS controls: ALT

Table 1. LPS-inducible LDH release (% of total) of liver cell cultures from control rats or rats pretreated with *S. abortus equi* LPS

Donor rat LPS concn (μg/mL)	Control		LPS-pretreated	
	S. abortus equi	*S. minnesota*	*S. abortus equi*	*S. minnesota*
0	12 ± 0.7	12 ± 1.7	7 ± 0.6	8 ± 0.3
0.1	15 ± 1.3	13 ± 1.0	8 ± 1.0	8 ± 0.3
1.0	31 ± 1.5	20 ± 2.0	9 ± 1.5	9 ± 0.2
10	47 ± 1.4	42 ± 0.5	19 ± 1.4	14 ± 0.3

Rats were treated with 300 μg/kg and 1, 3 and 10 mg/kg LPS i.p. 8, 6, 4 and 2 days prior to cell preparation, respectively. Liver cell cocultures prepared from these animals were incubated after 2 hr of collagen adhesion for 16 hr with LPS from *S. abortus equi* or *S. minnesota* at the concentrations indicated. Data represent means ± SD of three incubations.

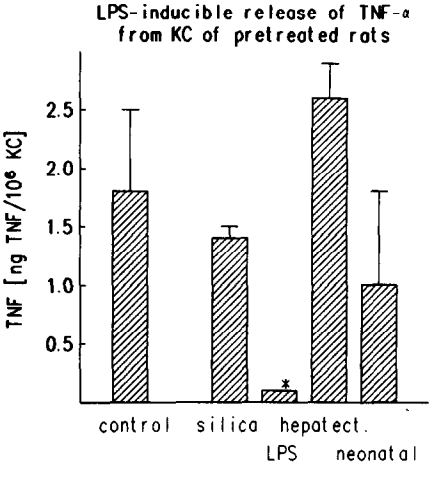

Fig. 2. LPS-induced release of TNF from isolated KC after differential *in-vivo* pretreatment of the donor rat. Rats were pretreated either repeatedly with LPS or once with silica. Alternatively, partial hepatectomy was performed or neonatal rats were used; the details are described in Materials and Methods. KC were incubated with fresh medium in the absence or presence of 10 μg/mL LPS from *S. abortus equi* after 2 hr of plastic adhesion. The supernatant was removed after 3 hr and TNF bioactivity was determined.

5200 ± 2170 U/L, N = 3). Likewise, liver cell cocultures from donors which had been pretreated *in vivo* with silica were significantly less sensitive to LPS-induced cell death at concentrations greater than 0.3 μg/mL LPS compared to cells from untreated controls (Fig. 1d). These findings demonstrate that an *in-vivo* property is recovered in the *in-vitro* system and emphasizes the central role of the macrophages in this system.

One of the major products of macrophages is TNF-α, a cytokine which is known to participate in the mediation of LPS-induced liver injury *in vivo* as well as *in vitro*. In the liver, KC are competent in producing this cytokine. Therefore, KC were isolated from control and pretreated animals by plastic adhesion of the non-parenchymal cells. TNF-α was determined in the supernatant of 10^6 KC/mL after 3 hr of incubation in the presence of 10 μg/mL LPS. Data in Fig. 2 show that all preparations with the exception of those prepared from the repeatedly LPS-pretreated animals produced comparable amounts of TNF-α. KC from rats prepared after short term LPS-treatment produced less TNF-α than controls (0.9 ng/10^6 KC; control: 2.3 ng/10^6 KC).

In order to check whether KC from LPS-pretreated animals were generally unresponsive to LPS, we examined the effect on LPS-induced release of IL-1-like activity in the same cells. LPS pretreatment did not significantly change the amount of IL-1-like activity *ex vivo* (LPS-tolerant; 17.2 ± 4.5 U; control: 13.4 ± 1.6 U). Therefore, impairmant of KC seems to be limited to some of the LPS-induced responses only.

Consequently, we tried to reconstitute LPS sensitivity by the addition of TNF-α in order to check whether the inhibition of TNF-α release alone might be responsible for the observed unresponsiveness. Murine recombinant TNF-α (0.1 μg/mL) was added to the cells alone or in combination with LPS. TNF-α did not reconstitute the LPS-induced cytotoxicity. Therefore, a mere suppression of TNF-α production by KC after LPS pretreatment (Fig. 2) is not sufficient to explain the *ex-vivo* LPS tolerance.

The impairment of LPS-induced hepatocytotoxicity can be due to either altered functioning of effector cells, i.e. NPC, or to changes in target cells. In order to characterize the different types of LPS tolerance we tried to reconstitute the LPS sensitivity by the addition of native KC from control rats to the LPS-tolerant liver cell cultures. Only in the case of silica pretreatment was a partial reconstitution (60% of control) of LPS susceptibility possible. It is concluded that silica pretreatment impairs KC function but leaves PC essentially sensitive towards LPS-induced cell death. In contrast, alterations in the PC might lessen the restitution of sensitivity in the case of neonatal rats, LPS pretreatment or hepatectomy.

DISCUSSION

Before carrying out the *in-vitro* investigations, it was checked by *in-vivo* experiments whether the animals had actually been made tolerant against LPS by the different experimental approaches. This was obviously the case. A species difference became

evident in these *in-vivo* experiments indicating that the refractory state in mice is much more pronounced than in rat.

The first approach carried out was based on the fact that the application of silica has been shown to kill macrophages [22]. When NPC were prepared from the silica-pretreated rat, the total number of NPC in relation to PC was found to be increased 3-fold. The increased number of NPC is likely to be due to infiltration by leukocytes from the blood into the liver. Adherent cells isolated from silica-pretreated rats still produced TNF-α. It seems feasible that infiltrated monocytes substituted for the impaired KC in producing the monokine. Despite this ability, however, the NPC from the silica-pretreated animal did not induce cytotoxicity in the presence of LPS. NPC from a control rat, however, restored this lost cytotoxic property at least partially. It maybe assumed that hepatocytes remain sensitive but lack a factor induced by LPS in NPC. This factor seems to be different from TNF-α because the addition of either effector did not restore the effect.

In the second approach where LPS was used to induce tolerance, KC from LPS-pretreated rats produced only small amounts of TNF-α *ex vivo*. Reduced secretion of TNF-α has been associated with LPS tolerance in rats *in vivo*, previously [23]. In contrast, the cells still secreted IL-1-like activity in response to LPS. It has been reported previously that LPS tolerance does not attenutate IL-1 formation [24]. Because substitution of TNF-α failed to reconstitute sensitivity it seems unlikely that reduced formation of TNF alone is the reason for tolerance. Indeed, various other alterations in LPS-tolerant macrophages have been reported, i.e. depressed eicosanoid [25–27], and IL-6 [8, 28] formation, while the refractory state is independent of acute phase response [29], Interferon γ [24] and phagocytosis [30]. The hepatocytotoxic effect was not reconstitutable by readdition of NPC from control animals. From this observation it becomes evident that tolerance induced by LPS is not only based on impaired NPC function but includes a functional change in the hepatocyte as well. Serum obtained from the repeatedly LPS-treated animal did not alter the LPS-induced cytotoxicity in liver cell cultures from control animals. Therefore, it is unlikely that the refractory state *in vivo* is caused by a condition known as "late-phase tolerance" [31] in which specific antibodies against LPS are thought to be formed.

A third approach, i.e. partial hepatectomy, also resulted in cultures *ex vivo* which were insensitive towards LPS-induced cell death. KC from partially hepatectomized animals were obtained which still secreted TNF-α upon a LPS stimulus. Therefore, KC function seems not to be impaired after hepatectomy as observed after LPS-pretreatment. As NPC from a control animal did not restore LPS susceptibility, hepatocytes of the regenerating liver would seem to be generally insensitive towards LPS or its mediators.

In the fourth approach using cells from neonatal rats cells were obtained which were insensitive towards LPS. Susceptibility was not restored by the addition of adult KC from untreated rats.

These different findings concerning the LPS responsiveness of cells under the various approaches allow three types of LPS tolerance to be distinguished (1) silica pretreatment, where tolerance is induced essentially by the impairment of KC. (2) the use of neonatal liver cells or partial hepatectomy, where tolerance is due to alterations in PC. (3) LPS pretreatment, where tolerance is induced by both impairment of KC resulting in failure to produce TNF-α and alterations in PC.

The obvious association of LPS tolerance and liver cell growth in neonatal or regenerating liver favours the hypothesis that the turning on of cell proliferation may be the general principle behind LPS tolerance. Several findings provide corroboration for this idea: rats free of endogenous, i.e. enteral, LPS show no liver regeneration after hepatectomy [32]. Administration of LPS [33] or TNF-α [34] induces cell division in the liver *in vivo*. *In-vivo* LPS enhances the expression of growth-related genes such as c-*myc* in hepatocytes [35]. Serum from control rats, which is known to stimulate liver cell division in culture [36], reduced also LPS hepatocytotoxicity *in vitro* as shown in this study. Thus, cell proliferation might be a mechanistic link between different types of LPS tolerance.

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