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ENDOTOXIN-INDUCIBLE CYTOTOXICITY IN LIVER CELL CULTURES—I

T. HARTUNG and A. WENDEL*
Biochemical Pharmacology, University of Konstanz, POB 5560, D-7750 Konstanz, Germany

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Abstract—It is known that rodents challenged with a combination of galactosamine and endotoxin develop a fulminant hepatitis within several hours. Until now, no *in-vitro* correlate for this organ-specific lesion has been described. Here, *in-vitro* conditions have been developed which allow examination of lipopolysaccharide (endotoxin)-inducible cell injury to hepatocytes. Under these *in-vitro* conditions (RPMI 1640 supplemented with 10% calf serum, 40% oxygen tension) which require the presence of functionally intact Kupffer cells, a concentration-dependent lactate dehydrogenase release is inducible by different lipopolysaccharides in hepatocyte cultures from Fischer rats. It can be abrogated by polymyxin B. These co-cultures secreted tumor necrosis factor-α into the medium upon a lipopolysaccharide stimulus. The presence of a tumor necrosis factor-α antiserum reduced the major part of the endotoxin-inducible cytotoxicity. Similarities *in vitro* and *in vivo* of the cytotoxic potency of various endotoxin species and the different responsiveness of hepatocytes from two different rat strains support that this co-culture system might be useful for studying endotoxin-inducible lesions *in vitro*.

Lipopolysaccharides (LPS†) from gram negative bacteria are known to be causally involved in the pathogenesis of septic shock and multiorgan failure [1]. Moreover, endotoxins have been implicated in fulminant hepatic failure in patients with cirrhosis of the liver [2, 3] as well as various other diseases (reviewed in Ref. 4). The acutely toxic effects of LPS can also be observed in rodents and several *in-vivo* models for LPS-induced lesions exist [5]. In animals sensitized by pretreatment with inhibitors of transcription [6] or by previous infection with bacille calmette guérin [7], the further administration of minute amounts of LPS causes acute hepatitis. Until now, this organotropic effect has not been successfully reproduced in a comparable *in-vitro* model, i.e. in a system where cytotoxicity towards isolated liver cells is caused by the presence of LPS. Neither LPS nor any of its known *in-vivo* mediators [8] including tumor necrosis factor (TNF), the cytotoxic monokine released following administration of LPS [9], showed significant hepatotoxicity *in vitro* [10].

We were interested in finding out which cellular or humoral components are required for an *in-vitro* system of LPS-induced toxicity. The major aim was to work out similarities between *in-vitro* findings and *in-vivo* observations, and to define possible dissimilarities compared to the available knowledge about the *in-vivo* situation. This report provides evidence that the co-culture of hepatocytes with resident macrophages of the liver, the Kupffer cells (KC), is a suitable cellular model for the study of potential agonists and antagonists of LPS-induced cytotoxicity.

* To whom correspondence should be addressed.

† Abbreviations: LPS, lipopolysaccharide, endotoxin; TNF, tumor necrosis factor; KC, Kupffer cells; PC, hepatocytes, parenchymal cells; GalN, galactosamine; LDH, lactate dehydrogenase (EC1.1.1.27).

MATERIALS AND METHODS

Substances. Lipopolysaccharides, polymyxin B, latex and methyl palmitate were purchased from Sigma; gadolinium chloride was obtained from Aldrich. The polyclonal sheep anti-mouse TNF-neutralizing serum (2.2×10^6 neutralizing units/mL) was a gift of M. Niehörster from this laboratory.

Murine recombinant TNF-α was a generous gift of Dr Adolf, Boehringer Institut, Vienna, Austria.

Tissue culture. Hepatocytes (PC) were prepared from male Fischer rats (F344, Charles River, body weight about 250 to 300 g, or alternatively Wistar chbb, Thomae, or Rowett nude rat, Han:RNU-rnu/rnu or Han:RNU-rnu/+ as wildtype control, Hannoversches Zentralinstitut) by the collagenase perfusion method according to Seglen [11]. Cell preparations had a viability of more than 80% as assessed by Trypan blue exclusion. Hepatocyte preparations contained less than 1% Kupffer cells (KC) as judged by staining of unspecific esterase [12]. The proportion of non-parenchymal cells to parenchymal cells was increased by altering the centrifugation scheme (100 *g* instead of the usual 50 *g* [13]).

Cells were allowed to adhere for 2 hr on collagen-coated (rat tail collagen, Serva, Heidelberg) six-well plates with 1.25×10^6 PC per mL (1.3×10^5 cm^{-2}, respectively). RPMI 1640 (Biochrom, Berlin) supplemented with penicilline/streptomycin (100 E/100 μg per mL, final concentration) (Sigma) and 10% calf serum (Serva, Heidelberg) was used as culture medium. Further supplementations were not necessary because during this short period of culture differentiation of the cells was maintained sufficiently. Culture conditions were 37°, 40% oxygen, 55% nitrogen and 5% carbon dioxide.

KC were isolated from the supernatant of the PC after centrifugation at 100 *g*; debris was removed by four additional centrifugation steps (400 *g*, 5 min). KC were separated from other non-parenchymal

Table 1. LDH release of liver cell cultures within 16 hr in the presence of 10 μg/mL *Salmonella abortus equi* LPS under various preparation and incubation conditions

Oxygen (%)	Centrifugation force (*g*)	LPS-inducible LDH release (%) −LPS	+LPS
20	50 (PC)	27 ± 2	33 ± 1
20	100 (PC+KC)	26 ± 1	39 ± 1
40	50 (PC)	19 ± 1	36 ± 1
40	100 (PC+KC)	17 ± 1	52 ± 2

cells by plastic adhesion (10^6 cells in 500 μL medium per well of a Greiner 24-well culture plate) for 2 hr at 37° in the usual culture medium. Of the attached cells, 80–90% were KC. These cells were cultured and incubated as described for hepatocytes. The supernatant was taken 3 hr after addition of LPS, immediately frozen and stored at −20° until TNF measurement.

When lymphocytes were added to hepatocytes in culture, both cell types were prepared from the same animal essentially as follows. First the spleen or thymus was disrupted using a 100 μm nylon gauze. In the spleen preparation, erythrocytes were lysed by 0.17 M ammonium chloride solution. Cells were washed and purified by differential centrifugation (400 *g*, 5 min) using tissue culture medium.

Elutriation. PC were separated from non-parenchymal cells by counterflow elutriation. A protocol similar to the one of LeRumeur *et al.* [14] was used: a Beckmann JE-6 elutriator chamber was filled at 840 rpm with cells (10 mL/min); PC were washed (25 mL/min) and eluted (46 mL/min). This procedure recovers about 30% of the PC with a viability of more than 95%.

Assessment of in-vitro *hepatocytotoxicity.* Incubations were started by replacing the supernatant with fresh medium. Putative effectors or inhibitors were added simultaneously. After a preincubation period of 30 min 1% of a stock solution of 1 mg/mL LPS in PBS, i.e. 10 μg/mL, LPS was added. After 15.5 hr of further incubation the supernatant was removed and the remaining cells were lysed with 1 mL of 0.1% Triton X-100 (Serva, Heidelberg).

LDH release was taken as an indicator of cell death. The activity of LDH was determined separately in the medium as well as in the Triton lysate. Cytotoxicity was expressed as the proportion of LDH released into medium compared to the total amount of LDH present in the cells, i.e. the sum of both determinations. Inhibition of LPS-inducible cytotoxicity was calculated as:

$$\text{inhibition}\,[\%] = \frac{\text{release}_{\text{LPS}} - \text{release}_{\text{LPS + inhibitor}}}{\text{release}_{\text{LPS}} - \text{release}_{\text{control}}}.$$

Cells were monitored by phase contrast microscopy. After 2 hr of adherence cells began to flatten and the nucleus became visible. During cultivation without further additions the cells spread out and regained their morphological organization, e.g. in forming bile canaliculi. At the end of the incubation period cultures exposed to LPS showed reduced cell density, blebbing and increased number of granula.

Assessment of hepatotoxicity in vivo. Male rats (either Fischer F-344, Charles River, or Wistar chbb, Thomae, 250–350 g body weight) were kept at least one week on a standard diet (Altromin 1310) *ad lib.* The animals were injected intraperitoneally with galactosamine (300 mg/kg) plus LPS (300 μg/kg). Alternatively, 300 mg GalN was given 30 min prior to intravenous injection of recombinant murine TNF-α (5 μg per animal).

Blood samples were taken by cardiac puncture under ether anaesthesia 8 hr after intoxication (LPS, i.p.), and with Wistar rats a second time after 16 hr. Liver injury was assessed using serum alanine aminotransferase (GPT), serum aspartate aminotransferase (GOT) and sorbitol dehydrogenase (SDH) as parameters [15].

TNF assay. TNF was measured using a bioassay performed with the fibrosarcoma cell line WEHI 164 clone 13 according to Espevik and Nissen-Meyer [16].

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

With conventionally prepared hepatocytes from Fischer rats incubated in 20% O_2, no significant LPS-induced cytotoxicity was observed following incubation for 16 hr (Table 1, first line). Under these conditions, even high concentrations of LPS (i.e. up to 100 μg/mL) failed to lead to significant LDH release compared to control incubations. However, when the cells were prepared at higher centrifugation speed, a preparation was obtained which released significant activity of LDH in the presence of LPS (Table 1, second line). When the O_2 concentration was raised to 40%, with the conventionally centrifuged cells this LPS-inducible LDH release was further increased compared to control incubations (Table 1, third line). If prepared with the higher centrifugation speed, a cell population was obtained which released 205% more LDH compared to controls without LPS under the chosen conditions. Therefore, all further *in vitro* experiments were performed under these conditions.

The observation that cells which were prepared at higher centrifugation force were more sensitive to LPS-induced cytotoxicity suggested that the preparation contained non-parenchymal cells of lower density which might be responsible for the

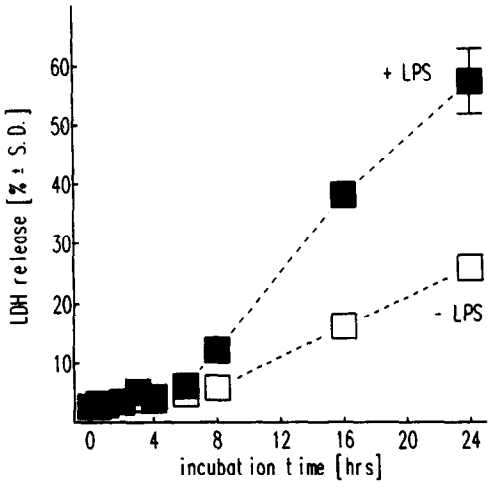

Fig. 1. Time course of LPS-induced cytotoxicity in culture during the continuous presence of 10 μg/mL LPS. Liver cells (1.25 million per mL) of a high speed cell preparation (as described in Materials and Methods) were used; data: mean ± SD, N = 3 separate experiments. Bars represent SD if greater than symbol (all figures)

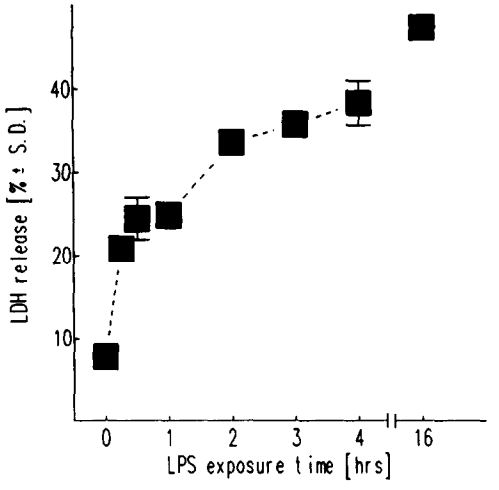

Fig. 2. Effect of different exposure times to LPS on LPS-inducible cytotoxicity of liver cells after 16 hr. Cells were exposed to 10 μg/mL LPS for the times shown by squares on the graph; after this time medium was replaced by supernatants of parallel incubations not exposed to LPS and then further incubated until 16 hr from the beginning.

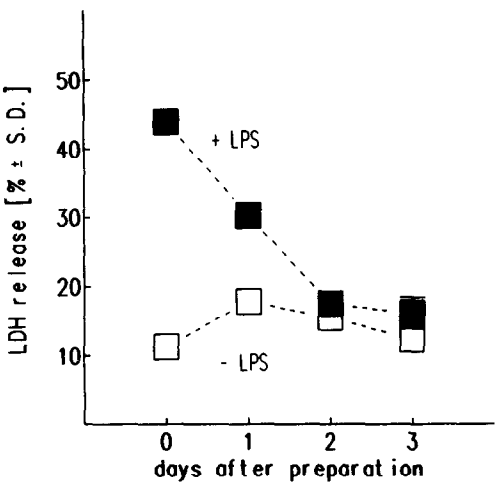

Fig. 3. Influence of culture time after isolation on cell viability after 16 hr of incubation in the absence or presence of LPS. 0 stands for the day of isolation where cells were allowed to adhere to coated plastic dishes for 2 hr; medium was replaced every 24 hr by fresh medium; 10 μg/mL LPS was added at the days represented by the symbols and viability was examined 16 hr later. During the experiment the LDH activity per mg protein did not change significantly (1200 mUnits/mg protein).

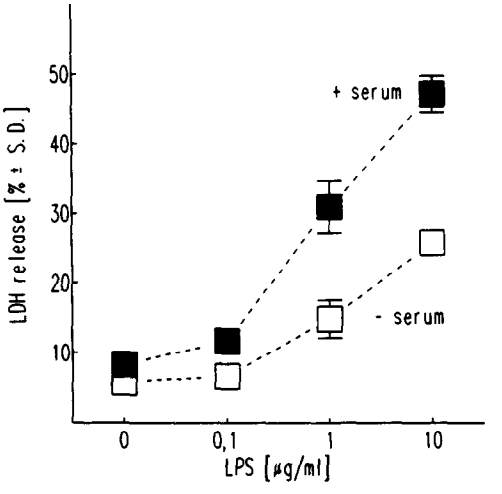

Fig. 4. Dose dependence and decrease by calf serum omission of LPS-inducible cytotoxicity. +serum = 10% newborn calf serum; incubation for 16 hr.

phenomenon. Indeed, using the esterase staining technique, it became evident that the high speed preparation contained 3% KC compared to less than 1% in the low speed preparation. Figure 1 shows the time course of the enhancement of the LDH release from such a high speed preparation in the presence and absence of LPS. The graph shows that after an incubation period longer than 6 hr, a sustained and steady loss of viability was observed in the cells exposed to LPS.

Next we checked how long the exposure period of the cells towards LPS needs to be in order to result in a maximum cytotoxicity. The results in Fig. 2 show that exposure to LPS for periods of 15 min to 4 hr resulted in significant cytotoxicity as measured after 16 hr. The susceptibility of the cells towards LPS depended on the age of the cell preparations. At the day of the isolation, after 2 hr of adhesion to a collagen matrix, the cells turned out to be maximally sensitive to LPS compared to controls in the absence of LPS (Fig. 3). On the next day, the difference between the LPS-treated and the control cells became much less. The LPS sensitivity was even more diminished on days 2 and 3 and was not restored by addition of freshly prepared KC. Consequently all further experiments were performed with cells on the day of isolation, i.e. 2 hr after adhesion. In addition, results in Fig. 4 show

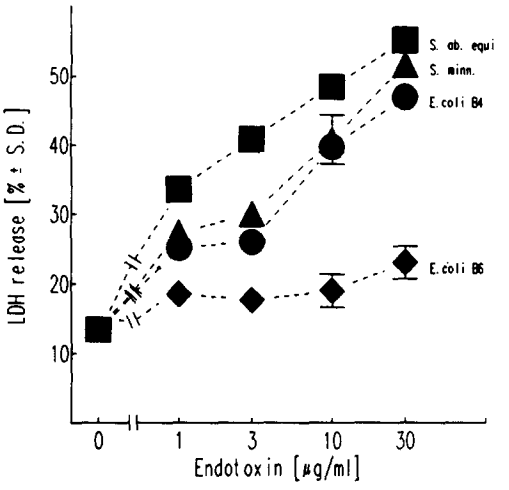

Fig. 5. Potency of different LPS species to induce cytotoxicity in liver cell cultures. The different endotoxins were added from identical stock solutions (1 mg/mL) to yield the final concentrations indicated and incubated for 16 hr.

that at different LPS concentrations omission of serum reduces the LPS-inducible cytotoxicity. In order to check the specificity of this cytotoxic effect for LPS, 1 µg/mL polymyxin B as a known specific LPS binding agent [17] was added. Under these conditions LDH release during the incubation period was identical to controls not exposed to LPS (data not shown).

Since a wide variety of endotoxins from different bacterial sources is known to be toxic *in vivo*, we studied various species of bacterial LPS in our system. Data in Fig. 5 demonstrate that any of the four endotoxins studied led to significant cytotoxicity in the incubation system, however, with different individual potency. The graph shows also that the *Salmonella abortus equi* endotoxin resulted in the greatest cell injury. Therefore, the experiments were continued with this endotoxin species.

In order to assess the contribution of non-parenchymal cells to the measured LPS-inducible LDH release, we performed several experiments where KC function was impaired by various chemical or physical means. Inactivation of KC by either latex particles [18] (0.5 µL/mL), gadolinium chloride [19] (0.75 mg/mL) or methyl palmitate [20–22] (1 mg/mL) led to significant reduction of the LPS-inducible LDH release from the cells by 33 ± 8%, 81 ± 5% and 41 ± 12%, respectively. When the amount of KC was diminished by plastic adhesion (1 hr) or centrifugal elutriation prior to plating, LPS-induced cytotoxicity was also inhibited by 52 ± 16% and 70 ± 13%, respectively. Upon elutriation and readdition of non-parenchymal cells, the LPS-induced cytotoxicity was partially restored (73% of the control).

When lymphocytes were added in a similarly designed experiment in varying proportions up to 30%, they had no influence on LPS-induced hepatocytotoxicity (data not shown). When nude rats which are known to lack functionally intact T-lymphocytes were used as donors for liver cell preparations, the co-cultures obtained from these animals showed a similar sensitivity towards LPS as co-cultures from the wildtype control (data not shown). Taken together these observations allow the conjectural conclusion that KC independent of lymphocyte activation play a pivotal role in mediating LPS-derived cytotoxic effects, presumably directed towards parenchymal cells.

Since evidence is available that *in vivo* one of the major pathogenic mediators of LPS might be represented by TNF, we determined whether TNF is detectable in the supernatant following stimulation of cells with LPS. Figure 6A shows that this is actually the case in LPS-stimulated KC. We were further interested to know whether the co-culture system when exposed to LPS releases similar amounts of TNF. The data in Fig. 6B demonstrate that TNF is also produced under these conditions. The amounts of TNF found in the supernatants of the complete liver cell culture (i.e. containing about 3% KC) was higher than the one released by the pure KC system, i.e. about 30 kUnits TNF per 10^6 KC 3 hr after addition of LPS. Further data in Fig. 6C demonstrate that exogenously supplied TNF-α activity is rapidly removed from the co-cultures. These results suggest that exogenous TNF-α is rapidly degraded by hepatic cells in culture. Since the measured levels of TNF-α are the net result of production minus degradation, the data indicate that the rate of production after LPS is greater than the rate of degradation. In addition to increasing TNF-α production, it is possible that LPS might also decrease the rate of TNF-α degradation.

Under the assumption that it is the TNF produced by KC that kills the PC, we tried to block this sequence by immunological means. KC are known to produce TNF-α in contrast to PC and endothelial liver cells [8]. Experiments shown in Fig. 7 demonstrate that in the KC/PC, i.e. the complete liver cell system, addition of sheep-anti-mTNF-antiserum significantly inhibited the LPS-induced cytotoxicity. On the other hand, when 0.1 µg/mL mTNF-α as such was added instead of LPS no significant cytotoxicity resulted. However, when this amount of TNF was added in addition to 3 µg/mL LPS, LDH release found after 16 hr was 25 ± 10% higher than in control incubations containing only LPS. In a similar experiment using 0.1 µg/mL TNF *plus* 10 µg/mL LPS no further synergism between the two agents was observed. These findings are consistent with the view that LPS stimulates KC to release TNF which in turn contributes to deleterious effects on PC but is not the only factor responsible for hepatocytotoxicity.

The aim of the final part of this study was to investigate to which extent the *in-vitro* model reflects the *in-vivo* situation; in other words, criteria as to the relevance of the model were collected. A comparison of the sensitivity of two different rat strains was made between the galactosamine/LPS model *in vivo* and the responsiveness of cells prepared from these animals to LPS *in vitro*. Fischer rats treated with 300 mg/kg GalN plus 300 µg/kg *Salmonella arbortus equi* LPS developed a fulminant

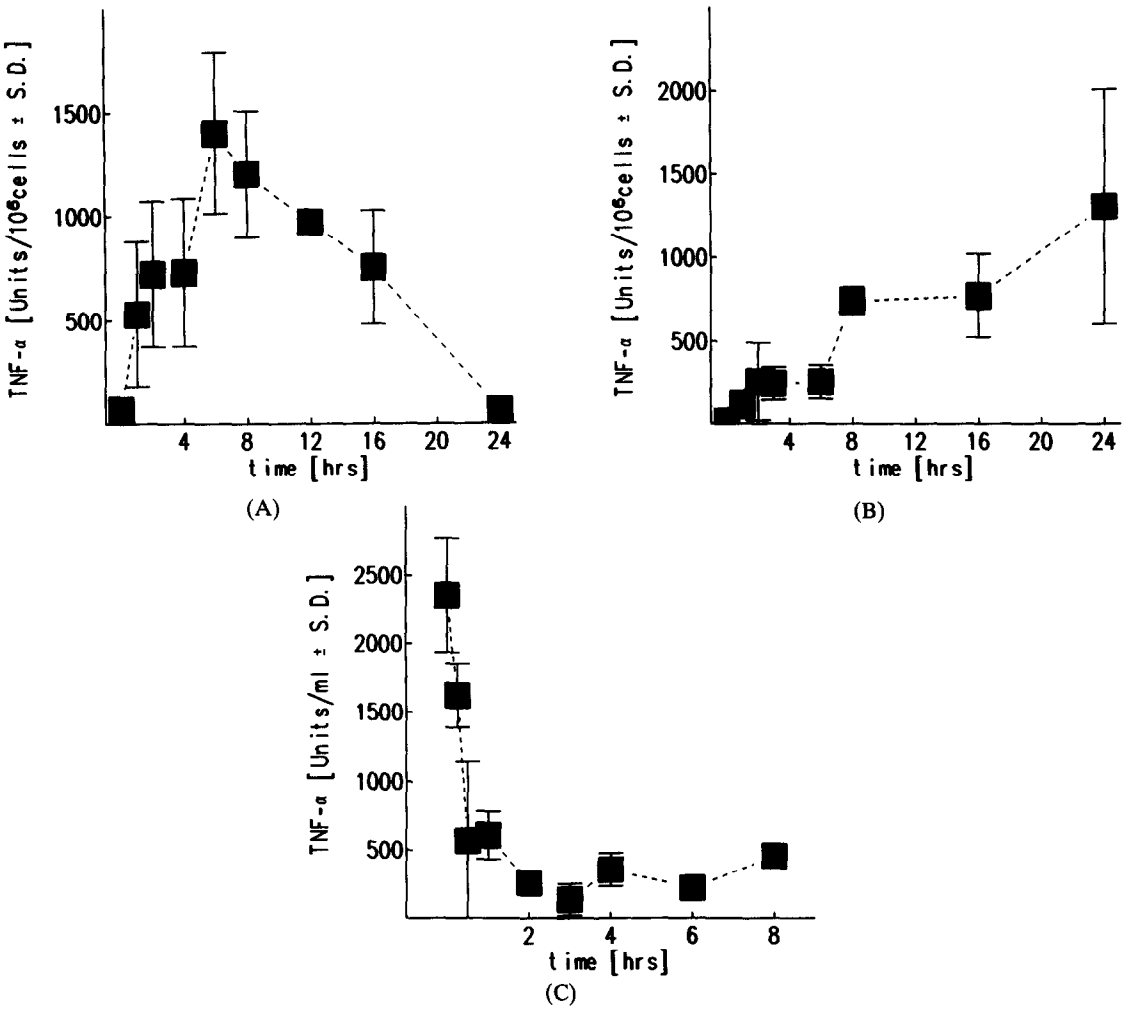

Fig. 6. Kinetics of TNF-α when added to or induced by LPS in liver cell cultures. (A) Time course of LPS-induced production of TNF-α in isolated Kupffer cells. TNF-α was determined by the WEHI bioassay in supernatants from three individual incubations of 10^6 Kupffer cells/mL for the times indicated. (B) Time course of LPS-induced production of TNF-α in liver cell cultures. TNF-α was measured according to (A) in the supernatant of liver cell cultures incubated as described in the case of the cytotoxicity assay. (C) Time course of elimination of murine rTNF-α in liver cell cultures. Murine recombinant TNF-α was added in a final concentration of 0.01 μg/mL to the liver cells incubated as in the case of the cytotoxicity assay. Parallel cell-free incubations of rmuTNF-α did not result in any significant loss of TNF bioactivity.

hepatitis within 8 hr (SGPT = 6090 ± 2740, N = 3) while Wistar rats after a similar treatment had no significant increase in SGPT at this time (SGPT = 215 ± 140, N = 4) and needed 16 hr after the challenge to develop hepatitis (SGPT = 2070 ± 175, N = 4). This shows that Wistar rats are less sensitive to GalN/LPS-induced hepatitis than Fischer rats. When liver cells were prepared under identical conditions from both rat strains and then exposed to LPS (conditions cf. Table 1) much less LDH release was found with the Wistar cells (31 ± 3% compared to 74 ± 10%, cf. Table 1). These findings demonstrate a parallelism of the hepatic *in-vivo* susceptibility and the *in-vitro* responsiveness to LPS in these two animal strains. These findings indicate an additional similarity between the *in-vivo* and the *in-vitro* situation which might allow us to conclude that this liver cell culture reflects endotoxic hepatic injury.

DISCUSSION

Previous work [23] demonstrated that LPS when injected into galactosamine-sensitized animals induces a fulminant hepatitis in rodents at doses which are several orders of magnitude less than the ones that lead to shock and multiorgan failure in the same animal species. A similar sensitization by galactosamine was observed in mice challenged with TNF-α[24]. This implies that the distinct organotropy under these conditions is due to a liver-specific effect of galactosamine. Our first attempts to induce

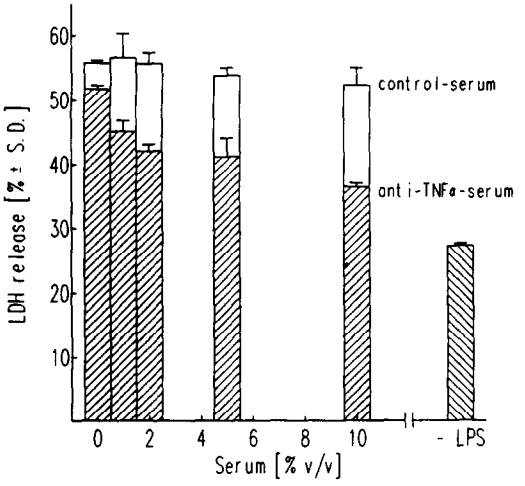

Fig. 7. Inhibition by anti-TNF-α-antiserum of LPS-inducible cytotoxicity in liver cell cultures. Antiserum was added to the cultures when medium was replaced after 2 hr of adherence 30 min before challenge by 10 μg/mL LPS; the volume was kept constant by removing the corresponding amount of medium prior to the addition of antiserum at the percentages indicated. Control serum refers to a preimmune sheep serum.

cytotoxicity by LPS plus varying concentrations of GalN in pure hepatocyte cultures failed to show any significant effect of LPS beyond the basal cytotoxicity of GalN alone. Since macrophages clear systemic LPS from the circulation [25, 26] and are able to secrete TNF [27] a known final candidate of the mediator sequence triggered by LPS, it seemed likely that these cells are needed for the constitution of a destructively working cellular system. Supporting evidence for this reasoning came from the finding that the *in vivo* sensitivity towards LPS is correlated with the number of KC present [28].

The present study demonstrates that hepatocytes are injured when incubated with LPS provided that functionally intact KC are also present. This cytotoxic effect is antagonized by polymyxin B, i.e. specific for endotoxin. An excellent reproducibility of the results was observed within a given cell preparation (SD less than ± 5%). It is noted however, that from preparation to preparation variations with respect to basal as well as LPS-inducible LDH release were observed in the range ± 10% LDH release. Among various endotoxins tested, different cytotoxic potencies were found, a fact that closely reflects the different *in-vivo* toxicities of these LPS species [29].

The requirement of the functional intactness of the KC implies that these cells are able to secrete mediators upon a LPS stimulus. This condition was experimentally verified by showing that TNF is produced by KC alone as well as by hepatocyte/KC co-cultures. It is unclear why TNF release from KC declines after 6 hr while a sustained increase in co-cultures takes place (even if the contribution of elimination is neglected). It is also interesting to note that in the co-culture about seven times as much TNF was found per LPS-stimulated cell in the medium within the first 8 hr after stimulation compared to the KC preparation alone. These findings suggest that autocrine loops between the different liver cell types may exist which enhance TNF secretion.

The antiserum experiment supports the theory that the major part of the cytotoxic activity is due to TNF-α. On the other hand, these results show clearly that TNF-α alone does not fully account for hepatocyte killing. This means that the presence of TNF is obligatory but not a sufficient condition in this process. It seems therefore reasonable to assume that LPS leads also to the secretion of enhancers of the cytotoxic potential of TNF-α. TNF-α is known to mediate most effects of LPS. Since it obviously fails to replace LPS in this system its formation is a necessary but not sufficient condition.

Two details, i.e. the O_2 concentration and the role of serum, in our *in-vitro* system need separate comment.

1. The enhancement of LPS hepatotoxicity under hyperoxic conditions was due to two effects: the basal release in control cells was reduced, i.e. the cells were more viable, while LPS exposed cells released more LDH under this condition. In line with this finding is the fact that hyperoxia was reported to result in enhanced spontaneous formation of reactive oxygen species [30] which is associated *in vivo* and *in vitro* with increased TNF-α formation [31].

2. Serum contains a LPS binding protein [32] which is present in normal serum and supports the interaction of LPS and the macrophage via the CD14 receptor [33]. This protein-bound LPS may represent the physiological form to which cells are exposed. The shortage of this protein might explain why large amounts of LPS are necessary to induce cytotoxicity under culture conditions.

A comment might be useful in order to understand the role of GalN *in vivo* and the reason for omitting it *in vitro*. As stated, treatment with galactosamine *in vivo* does not only greatly sensitize the animals for the lethal effect of LPS but also directs the organ injury towards the liver [34]. An accepted rationale of this fact is that the selective hepatic metabolism of GalN depletes UTP leading to inhibition of transcription and consequently blocking protein biosynthesis. On the other hand, our *in-vitro* system required no GalN in order to produce cytotoxicity on the first day of the preparation (cf. Fig. 3). This might be due to the fact that freshly isolated hepatocytes are subject to a "protein biosynthesis shock" for a period of about 24 hr before they recover [35]. The preferential susceptibility of the co-culture towards LPS during the first day of culture supports this assumption. In other words, the galactosamine part of the *in-vivo* situation may be contributed under *in-vitro* conditions by reduced protein synthesis due to the isolation procedure.

In conclusion, this co-culture system exhibits properties closely related to observations made *in vivo* and therefore provides a promising experimental approach to study potentially interesting compounds directed against endotoxins or some of their mediators in the course of primary drug screening.

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