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LEUKOCYTE ALTERATIONS DO NOT ACCOUNT FOR HEPATITIS INDUCED BY ENDOTOXIN OR TNFα IN GALACTOSAMINE-SENSITIZED MICE

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Abstract—Subtoxic doses of endotoxin (salmonella abortus equi lipopolysaccharide, LPS) (5 μg/kg i.p.) or tumor necrosis factor α (TNFα) (15 μg/kg i.v.) induced fulminant hepatitis within 8 hr, when mice had been sensitized by a subtoxic dose of D-galactosamine (700 mg/kg i.p.). LPS-treatment led to the release of TNF into the circulation, independently of the presence of D-galactosamine. The TNF-dependent development of hepatitis was accompanied by a severe lymphopenia and neutrophilia as assessed by leukocyte differential count. The total leukocyte count was not significantly affected. Lymphopenia and neutrophilia were induced by LPS or TNFα alone; however, the differential count was not influenced by D-galactosamine. A quantity of 260 μg/kg phorbol myristate acetate (PMA) i.p. or 5 μg/kg platelet activating factor (PAF) i.v. or 3.3 mg/kg *N*-formyl-methionyl-leucyl-phenylalanine methylester (FMLP) i.v. or 167 mg/kg zymosan i.v. also caused lymphopenia and neutrophilia in mice. However, none of these agents induced the production of systemic TNF and therefore failed to induce hepatitis in D-galactosamine-sensitized mice. In LPS-insensitive C3H/HeJ mice administration of LPS produced neither differential count changes nor hepatitis while both events were observed when TNFα was given. This shows that TNFα alone gives rise to lymphopenia/neutrophilia as well as hepatitis independent of LPS. When the action of TNFα was blocked by anti TNFα antiserum pretreatment of LPS-sensitive mice, the animals were protected against LPS-induced hepatitis. However, lymphopenia and neutrophilia still occurred to a similar extent. The involvement of a putative additional mediator of LPS-induced leukocyte alterations was checked. The findings suggest that this mediator, if present, is different from IL-1, IL-2, eicosanoids or superoxide. We conclude from our findings that changes in leukocyte numbers and composition following D-galactosamine/LPS or D-galactosamine/TNFα administration is an epiphenomenon rather than a causal event of leukocyte stimulation in the process of inducing a fulminant hepatitis in mice.

Sepsis due to infections by gram-negative bacteria is a major clinical problem associated with fever, shock and multi-organ failure. These reactions have been shown to be due to a cascade of stimuli initiated by bacterial lipopolysaccharides, i.e. endotoxin. In man, endotoxin administration resulted in a pronounced reduction of peripheral lymphocytes [1] and a concurrent increase in granulocytes [2]. Subsequent studies revealed that after a challenge with endotoxin, macrophages synthesize and secrete the cytokine TNFα† [3] which *per se* produced a lymphopenia and neutrophilia in rodents [4] as well as in man following endotoxin administration. It seems therefore straightforward to assume that stimulation of granulocytes is a central mechanism of endotoxin-induced organ failure.

Several *in vitro* observations lend support to this view: in a neutrophil/endothelial cell co-culture system, the ability of endotoxin-stimulated neutrophils to produce endothelial cell injury was demonstrated and shown to be associated with the release of substantial amounts of neutrophil elastase [5]. Moreover, TNFα was found to enhance the adherence of human PMNs to endothelial cell monolayers *in vitro* due to very quick stimulatory effects on PNNs followed by later effects on the endothelial cells [6]. While the slow effect requires the synthesis of endothelial proteins, the immediate activation of neutrophils to enhanced adhesiveness seems to be directly mediated by TNFα and possibly further cytokines [7]. Thus, it appears that cytokines participate in the mobilization of PMNs by stimulation of their adhesiveness to endothelium.

In the context of our previously reported *in vivo* studies on the mechanism of endotoxin or TNFα-induced fulminant hepatitis in GalN-sensitized mice, we were interested in elucidating the role of leukocyte mobilization in this animal model of acute inflammation. We tried to approach this question by examining the influence of specific, known mediators of hepatitis, i.e. endotoxin or TNFα, on changes in the leukocyte differential count in circulating blood in comparison with other known means of stimulating leukocytes. These means include administration of phorbol esters such as PMA [8], leukotactic peptides such as FMLP [9], lipid mediators such as PAF [10], or yeast components such as zymosan. TNFα antiserum was used as a tool to discriminate cause-and-effect relationships between lympho-

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† Abbreviations: PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leukocyte; PBS, phosphate-buffered saline; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine methylester; PAF, platelet activating factor; TNFα, tumor necrosis factor α; GalN, D-galactosamine HCl; LPS, lipopolysaccharide; IL-1, IL-2, interleukin 1, 2.

penia/neutrophilia and pathophysiological consequences on the liver. Investigations were carried out in order to determine whether a further known mediator is required for the appearance of leukocyte alterations upon an originally endotoxic stimulus. The final aim of the study, however, was to find out whether general stimulation of leukocyte alterations is sufficient to generate hepatitis in GalN-sensitized mice, or whether a single and specific activation route mediates LPS hepatotoxicity.

MATERIALS AND METHODS

Male NMRI albino mice were purchased from Thomae (Biberach, F.R.G.), male C3H/HeN and C3H/HeJ mice were from the Hannover'sches Zentralinstitut (Hannover, F.R.G.). They were kept at least 1 week at 22° and 55% relative moisture in a 12-hr day/night rhythm at 100 lux illumination with free access to food and water. At 8 a.m. the animals received a dose of 700 mg/kg D-galactosamine · HCl (GalN) (Serva, Heidelberg, F.R.G.) intraperitoneally together with 5 μg/kg salmonella abortus equi lipopolysaccharide (LPS) or 260 μg/kg phorbol 12-myristate 13-acetate (PMA) (solution in vegetable oil, both from the Sigma Chemical Co., St Louis, MO, U.S.A.).

Alternatively, the following substances were administered intravenously instead of LPS: 15 μg/kg murine recombinant TNFα (specific activity 4×10^7 I.U./mg; Genzyme, Boston, MA, U.S.A.), 5 μg/kg platelet activating factor (PAF) (solution in PBS/0.25% BSA), 3.3 mg/kg *N*-Formyl-L-Methionyl-L-Leucyl-L-Phenylalanine methyl ester (FMLP) (solution in PBS/3.3% DMSO) or 170 mg/kg zymosan (all from the Sigma Chemical Co.). Rabbit antiserum to mouse TNFα (Genzyme) was administered intravenously 15 min before GalN/LPS in a dose of 30.000 neutralizing units per mouse.

Monoclonal anti-human IL-1 or monoclonal anti-human IL-2 (Genzyme) were i.v. injected together with TNFα antiserum 15 min before GalN/LPS in a dosage of 60 μg per mouse, respectively. Each human monoclonal antibody cross-reacted with its corresponding murine antigen. Twenty mg/kg indomethacin suspended in 1% tylose was given p.o. 1 hr before GalN/LPS. Intravenous administration of 3.3×10^4 I.U./kg superoxide dismutase (the Sigma Chemical Co.) was carried out 1 hr before GalN/LPS. Solutions were prepared in pyrogen-free phosphate buffered saline (PBS). TNFα was a generous gift from Dr Martin Schönharting (Hoechst AG, Werk Albert, Wiesbaden, F.R.G.).

TNF in serum of mice was determined essentially as described in [11]. Fibrosarcoma cells (WEHI 164, clone 13, kindly provided by Dr T. Espevik, Trondheim, Norway) at a concentration of 2×10^4 cells/100 μL were incubated with serially diluted test samples in 96-well, flat bottomed microtiter plates (37°, 5% CO_2, 18 hr). The supernatants were removed and MTT (5 mg/mL, 1/10 v/v) was added for determination of cytotoxicity. After incubation for 4 hr at 37° the dye was removed, cells were lysed by addition of 100 μM isopropanol/5% formic acid. Plates were read on a SLT EAR 400 microplate reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm. The titer of TNF is expressed in units/mL and is defined as the reciprocal of the dilution necessary to cause death of 50% of the cells.

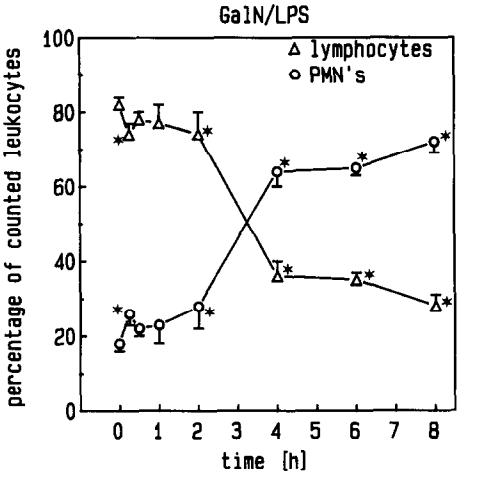

Fig. 1. Time course of the leukocyte differential count after intraperitoneal administration of 700 mg/kg galactosamine and 5 μg/kg LPS to mice. * $P \leq 0.05$, for further controls, *cf* Table 1.

Eight hr after intoxication mice were killed by cervical dislocation. Blood was withdrawn by heart puncture into 2.5% heparin. Liver injury was assessed by measurement of serum aspartate aminotransferase (SGOT), serum alanine amino transferase (SGPT) and sorbitol dehydrogenase (SDH) activities. Blood sampling for differential count and determination of the whole leukocyte count was carried out by eye puncture or by heart puncture during killing. When blood was to be withdrawn several times (Figs 1 and 2), four groups of animals were necessary to determine the time course of lymphopenia and neutrophilia. For determination of differential count, blood smears were stained for 4 min with Wright's staining solution (Merck, Darmstadt, F.R.G.); smears were performed in duplicate. Total leukocyte count was assessed by staining with Türk's solution (Merck). The results were analysed according to Student's *t*-test. Data of SGPT are expressed as mean values ± SE, data of differential count are expressed as mean values ± SD, $P = 0.05$ was considered to be significant.

RESULTS

As reported previously, the combined administration of 700 mg/kg GalN and 33 μg/kg LPS [12] or 15 μg/kg TNFα [13] induced a fulminant hepatic injury in mice within 8 hr. In contrast, administration of either mediator alone did not result in a detectable degree of hepatitis in mice within this time. Therefore, we first checked the influence of the single components in our model on the modulation of the leukocyte differential count. Treatment with GalN alone failed to induce any significant changes in this parameter. However, administration of either LPS or TNFα alone resulted in the known white blood cell

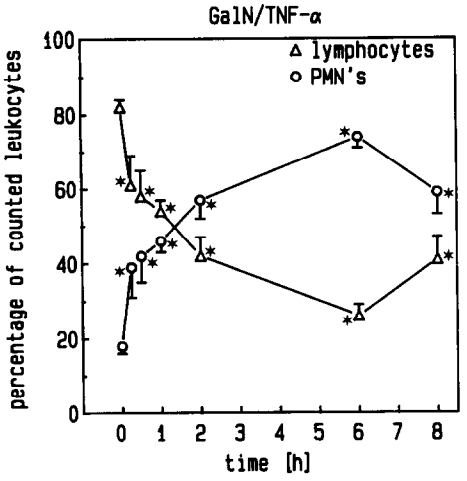

Fig. 2. Leukocyte differential count in mice treated with 700 mg/kg galactosamine i.p. and 15 μg/kg TNFα i.v. (1 hr after GalN). * $P \leq 0.01$.

Table 1. Differential count of leukocytes in mouse blood 6 hr after administration of various stimulators of leukocytes

Treatment	Lymphocytes	PMNs
PBS	79 ± 7	21 ± 7
GalN	81 ± 6	19 ± 6
LPS	33 ± 9†	67 ± 9†
TNFα	22 ± 12†	78 ± 12†
PMA	29 ± 6†	71 ± 6†
PAF	62 ± 4*	38 ± 4*
FMLP	61 ± 3*	39 ± 3*
Zymosan	57 ± 5*	43 ± 5*

* $P \leq 0.05$, † $P \leq 0.001$, vs PBS treated group. N = 6 per group. Blood smears were stained with Wright's solution. Doses and application routes: 700 mg/kg GalN i.p., 5 μg/kg LPS i.p., 15 μg/kg TNFα i.v., 260 μg/kg PMA i.p., 5 μg/kg PAF i.v., 3.3 mg/kg FMLP i.v., 170 mg/kg zymosan i.v. Data: mean values ± SD.

changes, i.e. a relative lymphopenia accompanied by a relative neutrophilia within 6 hr (Table 1). We then investigated the influence of the hepatotoxic combination of GalN/LPS on the leukocyte pattern in mice. The complete time course of the development of the differential leukocyte count after administration of GalN/LPS is shown in Fig. 1. It illustrates a profound decrease of circulating lymphocytes after the third hour of intervention and a mirror-image time course of an increase in neutrophils. As also noticed by others [14], however, the total number of leukocytes showed no corresponding alteration following GalN/LPS administration (data not shown), i.e. the lymphopenia is likely to be matched by the accompanying neutrophilia.

Since TNFα was reported to be a final mediator of GalN/LPS-induced hepatitis [13, 15], we next investigated the influence of this cytokine in combination with GalN on white blood cell changes. Figure 2 demonstrates that an essentially similar time

Table 2. Lack of induction of hepatitis in GalN-sensitized mice by the PMN-activating agents PMA, PAF, FMLP or zymosan, and control experiments

Pretreatment	SGPT [units/L]
None	40 ± 4
GalN	90 ± 49
LPS	60 ± 4
GalN/LPS	4.810 ± 1.880*
TNFα	40 ± 6
GalN/TNFα	4.745 ± 1.170*
PMA	40 ± 1
GalN/PMA	30 ± 7
PAF	30 ± 14
GalN/PAF	50 ± 8
FMLP	50 ± 14
GalN/FMLP	6 ± 2
Zymosan	50 ± 17
GalN/zymosan	40 ± 20

* $P \leq 0.05$ vs untreated control. N = 6 per group. Doses and application routes: 700 mg/kg GalN i.p., 5 μg/kg LPS i.p., 260 μg/kg PMA i.p. GalN/LPS or GalN/PMA were administered simultaneously. 15 μg/kg TNFα or 5 μg/kg PAF or 3.3 mg/kg FMLP or 170 mg/kg zymosan were administered intravenously 1 hr after GalN. SGPT was determined 8 hr after GalN, analogous data were obtained by measurement of SGOT and SDH. Data are expressed as mean values ± SE.

course and degree of modulation is observed in this case as with GalN/LPS. However, the appearance of these alterations was shifted for about 1.5 hr on the time axis in favor of an earlier development. We wondered whether any other means of leukocyte stimulation would be able to bring about the observed changes in white blood cell composition *in vivo*. Data in Table 1 demonstrate that administration of 260 μg/kg of the protein kinase C activator PMA induced leukocyte alterations similar to LPS or TNFα. Likewise, treatment of mice with either 5 μg/kg PAF or 3.3 mg/kg FMLP or 170 mg/kg zymosan resulted also in white blood cell modulations, albeit, to different degrees.

After these results, the central question remained whether leukocyte stimulation as reflected by differential count changes accounts for hepatitis in GalN-sensitized mice. The results in Table 2 show that none of the leukocyte stimulating agents studied, i.e. neither PMA nor PAF nor FMLP nor zymosan, could substitute LPS or TNFα in its property of inducing hepatitis in GalN-sensitized mice. In order to dissect the role of TNFα in liver injury and peripheral blood changes we measured serum TNF-levels 1 hr after application of either agent: serum TNF was detectable (965 ± 165 units/mL) solely after administration of LPS compared to non-detectable amounts of TNF (detection limit: 20 units/mL) after treatment with either PMA or PAF or FMLP or zymosan.

In order to strengthen the experimental evidence for the compulsory involvement of TNFα in inducing hepatitis as well as leukocyte differential count changes, we pretreated NMRI mice with a rabbit anti mouse TNFα antiserum and 15 min later with

Table 3. Protection by rabbit-anti-mouse TNFα antiserum (RAM TNFα AS) against GalN/LPS-induced hepatitis and lack of influence of this treatment on leukocyte differential count alterations in mice

Treatment	SGPT [units/L]	Differential count	
		Lymphocytes	PMNs
PBS Control	40 ± 6	73 ± 9	27 ± 9
RAM TNFα AS	38 ± 7	76 ± 3	24 ± 3
GalN/LPS	2.830 ± 972	35 ± 2†	65 ± 2
GalN/LPS + RAM TNFα AS	62 ± 12*	49 ± 8†	51 ± 8†
GalN/TNFα	3.710 ± 856	21 ± 6†	79 ± 6†
GalN/TNFα + RAM TNFα AS	33 ± 4*	78 ± 8	22 ± 8

* $P \leq 0.01$, † $P \leq 0.001$ vs disease control concerning SGPT or vs PBS control with respect to differential count. N = 6 per group. Doses and application routes: GalN: 700 mg/kg i.p., LPS: 5 μg/kg i.p., TNFα: 15 μg/kg i.v. 1 hr after GalN, RAM TNFα AS: ≈30.000 neutr. units per mouse i.v. (15 min prior to GalN/LPS). Determination of SGPT: 8 hr after GalN/LPS, SGOT and SDH yielded analogous data. Determination of differential count changes: 6 hr after LPS or TNFα. Data: mean values ± SE for SGPT, mean values ± SD for differential counts.

GalN/LPS. As shown in Table 3 the TNFα antiserum significantly protected against GalN/LPS-induced hepatitis, yielding further evidence for the view that TNFα is a terminal mediator of hepatic injury [13, 15]. However, TNFα antiserum did not inhibit leukocyte changes induced by LPS. Therefore, several further control experiments were carried out: (1) even a 5-fold higher neutralizing activity with an antiserum from a different animal species (sheep anti mouse TNFα antiserum) did not abolish LPS-induced leukocyte changes in mice; (2) blank rabbit serum had no effect on the parameters studied; (3) rabbit anti mouse TNFα antiserum alone did not induce leukocyte alterations (Table 3); (4) animals pretreated with rabbit anti mouse TNFα antiserum were protected against GalN/TNFα-induced hepatitis as well as TNFα-induced lymphopenia and neutrophilia (Table 3). These findings clearly show that the pathogenesis of hepatitis in GalN-sensitized mice requires the production of TNF whereas leukocyte differential count changes are inducible by a wide variety of stimulators without affecting the integrity of the liver.

A convenient experimental means of dissecting TNFα from LPS-mediated effects is the use of C3H/HeJ mice which are known to be LPS-insensitive [16]. These animals were not only resistant to LPS-induced hepatitis but also failed to exhibit leukocyte differential count changes after LPS administration compared to a congenic control group of C3H/HeN mice (data not shown, N = 6 per group). This means that both events require intact LPS receptors. However, in C3H/HeJ as well as in C3H/HeN mice, administration of TNFα led to hepatic injury as well as to leukocyte changes. This finding proves that induction of lymphopenia and neutrophilia is possible with TNFα alone without participation of LPS.

Since the anti-TNFα antiserum abrogated the hepatitis without preventing the leukocyte alterations, we checked the possible involvement of a putative second mediator of LPS. Therefore, we administered various agents counteracting known LPS-inducible mediators under conditions where TNFα-inducible leukocyte modulations were blocked. For this purpose, NMRI mice were pretreated with the TNFα antiserum together with an antibody or inhibitor and then challenged with GalN/LPS. None of these agents tested, i.e. neither IL-1 nor IL-2 antibodies, nor indomethacin (dose sufficient to inhibit phospholipase A_2 [17]), nor superoxide dismutase had any significant influence on the differential count (no-effect data, not shown). It therefore seems that LPS-inducible lymphopenia and neutrophilia occurs independently of IL-1, IL-2, eicosanoids or superoxide.

DISCUSSION

Inflammatory reactions are usually characterized by metabolic stimulation of leukocytes, morphological changes of these cells, enhanced adhesiveness, adhesion and infiltration into tissue. Indeed, there is a large body of evidence that leukocytes are essential in the mediation of endotoxic shock [18, 19]. It was unclear, however, whether leukocytes once stimulated would infiltrate and deteriorate organs, or whether specific routes would propagate a primary stimulus induced by LPS to defined target tissues which might be accompanied by reactions of the leukocyte pattern. With the reservation that in this study only GalN-sensitized animals were treated and that further analogies between this hepatitis model and lethal shock have to be proven, our findings confirm earlier observations [4], i.e. that a major acute *in vivo* effect of TNFα consists in the induction of absolute and relative lymphopenia and absolute and relative neutrophilia.

Neither PMA nor PAF nor FMLP or zymosan could substitute for or potentiate the LPS component under *in vivo* conditions with respect to the capability

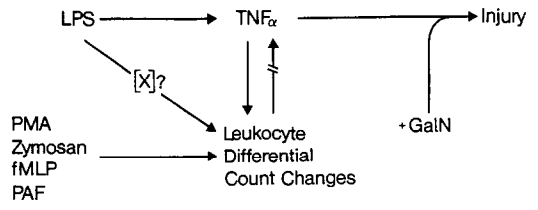

Fig. 3. Possible interrelationships between LPS- or TNF α-induced liver injury, and leukocyte alterations inducible by various stimulators.

to induce hepatitis when given to GalN-sensitized mice in doses sufficient to induce leukocyte differential count alterations. The inability of these agents to stimulate TNF-release offers the rational for this observation.

On the other hand, the use of the LPS-insensitive mouse strain C3H/HeJ allowed to show that TNFα *per se* induces leukocyte differential count changes independent of LPS. Conversely, the anti TNFα antiserum experiments showed that lymphopenia and neutrophilia are LPS-inducible without TNFα. Nevertheless, in terms of the pathophysiological consequences the presence of TNFα is the compulsory step in the sequence leading from LPS to hepatitis.

Since peptidoleukotrienes are known to stimulate endothelial cells to synthesize PAF and bind neutrophils [10], PAF has to be considered as a possible LTD_4-triggered pathogenic candidate in our model. In mice, LTD_4 has been identified as a causally involved endogenous mediator of GalN/LPS-induced hepatitis which clearly precedes the release and action of TNFα [20]. Although an involvement of PAF in endotoxin-induced hypotension has been observed [21], our findings indicate that PAF, even when given near the acute toxicity threshold, plays a minor role in GalN/LPS-induced hepatitis. Also, no potentiation of effects was observed when PAF was given additionally to GalN-sensitized animals which received a subtoxic LPS dose (0.5 μg/kg, data not shown).

The primary aim of this study was to find out whether stimulation of leukocytes would lead to infiltration into the liver, i.e. an acute inflammatory reaction followed by necrosis of hepatic cells, or whether additional humoral signals are needed to reach this final pathophysiological state. Our results clearly indicate that a variety of stimuli lead tc lymphopenia and neutrophilia. The diagram in Fig. 3 as the current interpretation of our findings stresses the pivotal role of TNFα as the pathogenic mediator and characterizes the stimulation of leukocyte changes as a phenomenon inducible by various other means unrelated to hepatotoxic properties. If LPS might stimulate leukocytes not directly but rather via an additional mediator X, our results indicate that—if at all existent—this product is different from IL-1, IL-2, eicosanoids or superoxide. The anti TNFα antiserum experiments confirm our previous claim [13] of a pathogenic sequence from LPS to TNFα in mediator-induced hepatotoxicity in mice.

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