LETHAL TOXICITY OF LIPOPOLYSACCHARIDE AND TUMOR NECROSIS FACTOR IN NORMAL AND D-GALACTOSAMINE-TREATED MICE

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Endotoxins (lipopolysaccharides, LPS) are the main factors of pathogenicity of Gram-negative bacteria. In experimental animals, they induce a broad spectrum of pathophysiological reactions, many of them being similar to those manifested during infection, and which may lead to shock and ultimately death.

Different mammalian species show large differences in their susceptibility to the lethal effects of endotoxin. The natural sensitivity to endotoxin may be increased in a variety of experimental models. One of these, treatment with D-galactosamine, increases the sensitivity of mice to the lethal effects of endotoxin more than 100,000-fold (1). D-Galactosamine is a hepatotoxic agent, its effects being confined to hepatocytes. The early biochemical effects on hepatocytes, which are necessary for the development of sensitization to endotoxin, are depletion of UTP and changes in uracyl nucleotides that result in an impaired biosynthesis of macromolecular cell constituents (RNA, membrane glycoproteins, glycogen, etc.) (2).

The above metabolic changes in hepatocytes also occur in endotoxin-resistant C3H/HeJ mice after treatment with D-galactosamine; however, sensitization to endotoxin is not demonstrable due to the absence of endotoxin sensitive macrophages. Transfer of a relatively small number (2×10^7) of macrophages obtained in culture from bone marrow precursor cells of endotoxin-sensitive C3H/HeN mice rendered D-galactosamine-treated C3H/HeJ mice sensitive to the lethal effect of as little as 1 μg LPS (3). This provided direct evidence for the central role of macrophages in mediating endotoxin reactions. It further demonstrated that D-galactosamine sensitization was not due to an enhancement of the actual mechanisms of endotoxicity (e.g., hyperreactivity of macrophages), but to the lowering of the threshold of susceptibility to the toxic products of macrophages. Although the actual mechanisms of endotoxicity are not known, there exists general agreement today that toxicity is caused by endogenous mediators, which are released on interaction of endotoxin with target cells.

In the past, a number of mediators mainly of macrophage origin have been discussed in relation to endotoxic reactions. Recently, Beutler et al. (4) have provided evidence that cachectin, which is identical with tumor necrosis factor

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(TNF)[1] (5), plays a prominent role in mediating the lethal effects of lipopolysaccharides. This protein is produced in vivo and in vitro by macrophages in response to LPS (6, 7), and binds to high affinity receptors present on a number of cell types such as mouse liver cells, muscle cells, and adipocytes and endothelial cells (8, 9). When mice were passively immunized with a specific polyclonal rabbit antiserum directed against murine cachectin/TNF they were at least partially protected against the lethal effects of LPS (4).

In the present study using human recombinant TNF, we are showing that LPS and TNF are identical in their lethal effects in D-galactosamine-treated mice, and that TNF represents therefore a mediator responsible for the initiation of the lethal toxicity of endotoxin.

Materials and Methods

Animals. 10–12-wk-old male and female C3H/Tif, C57BL/6, BALB/c, and endotoxin-resistant C3H/HeJ (10) mice were obtained from the breeding stock of the Max-Planck Institute. Endotoxin-resistant C57BL/10 ScCr (11, 12) mice at the same age were obtained from Bomholgard, Ry, Denmark.

Materials. LPS from *Salmonella abortus equi* (S-form) in the uniform triethylamine salt was prepared as described earlier (13). D-galactosamine hydrochloride was purchased from C. Roth, Karlsruhe, Federal Republic of Germany; and uridine was from E. Merck AG, Darmstadt, Federal Republic of Germany. Human recombinant TNF was provided by BASF, Ludwigshafen. The specific activity of the preparation was 5×10^7 U/mg, and was homogeneous in SDS-PAGE (14). For administration in mice, all materials were dissolved in pyrogen-free PBS.

Results

Lethal Toxicity of TNF in D-Galactosamine-treated C3H/TifF and C3H/HeJ Mice. The lethal toxicity of TNF was evaluated in D-galactosamine-sensitized mice and compared to that of LPS.

Groups of mice received 18 mg D-galactosamine and different amounts of TNF or LPS as a mixture. Animals receiving D-galactosamine, TNF or LPS alone served as controls. Table I shows that, in accordance with earlier findings, after D-galactosamine treatment a high sensitivity to LPS is established in endotoxin-sensitive C3H/TifF but not in endotoxin-resistant C3H/HeJ mice. In the former mice, 0.01 μg sufficed to cause death in all the mice. In contrast, treatment with D-galactosamine rendered C3H/TifF and also C3H/HeJ mice highly sensitive to TNF. Both strains were identical in their response, resulting in death of all mice with 5 and 1 μg TNF, and in half the mice with 0.1 μg (Table I). There was no significant difference in toxicity between intravenously and intraperitoneally administered TNF. In the control groups receiving D-galactosamine alone, lethality was absent. In the absence of D-galactosamine, 150 μg TNF was without lethal effect in either mouse strain. With 500 μg, TNF caused about 80% lethality in C3H/TifF mice and 75% in C3H/HeJ mice.

The time course of lethality induced with LPS and TNF in D-galactosamine-treated mice was about the same, the majority of the animals receiving LPS died 6–9 h after injection, those receiving TNF died in 6–7 h. The first signs of illness (immobility, rough fur) became apparent only 10–20 min before death occurred.

[1] *Abbreviation used in this paper:* TNF, tumor necrosis factor.

TABLE I

Lethal Toxicity of TNF or LPS in D-Galactosamine-treated Endotoxin-sensitive C3H/TifF and -resistant C3H/HeJ Mice

D-GalN	TNF or LPS	Lethality (dead/total	
		C3H/TifF	C3H/HeJ
mg	*μg*		
	TNF		
18	5.0	6/6	6/6
18	1.0	6/6	6/6
18	0.1	3/6	4/6
18	0.01	0/6	1/6
18	—	0/6	0/6
—	150	0/6	0/6
—	250	1/4	1/4
—	500	5/6	3/4
	LPS		
18	0.01	6/6	0/6
18	500.0	ND	0/6
—	100.0	2/6	0/6

Groups of mice received D-galactosamine and TNF or LPS as mixture intraperitoneally in 0.5 ml PBS. Controls received D-galactosamine, TNF, or LPS alone.

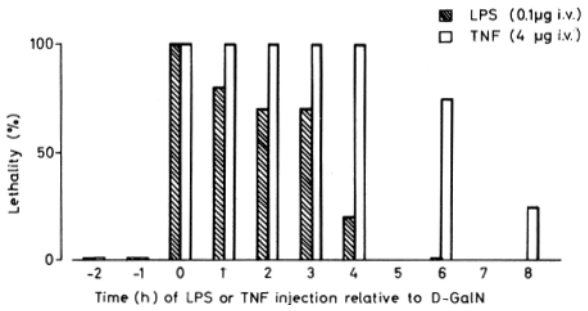

FIGURE 1. Groups of eight mice received D-galactosamine (18 mg) intraperitoneally in 0.4 ml PBS. At the different times before and after (as indicated) the animals received 0.01 μg LPS or 1 μg TNF intravenously in 0.2 ml PBS.

This behavior is characteristic for endotoxin lethality in D-galactosamine-sensitized animals (1). In the absence of D-galactosamine, the time course of lethality obtained with a lethal dose of TNF was very similar to that seen in normal mice receiving LPS only. Deterioration in the condition of the animals began 2–5 h after TNF injection, and death occurred 24–48 h later.

Results similar to the above were obtained in several experiments carried out independently. A comparable sensitization to TNF by D-galactosamine was also seen in C57BL/6, BALB/c, and in the endotoxin-resistant C57BL/10 ScCr mice.

Duration of D-Galactosamine-induced Sensitization to the Lethal Effects of TNF and LPS. Groups of mice received 18 mg D-galactosamine intraperitoneally. At different times before or thereafter the animals received 0.01 μg LPS or 1 μg TNF intravenously. Fig. 1 shows that lethality was absent when LPS or TNF

TABLE II
Inhibition of D-Galactosamine Sensitization to TNF by Uridine in C57/BL6 and C3H/HeJ Mice

Uridine (20 mg) at time:	Lethality (dead/total)	
	C57/BL6 given TNF (2 μg)	C3H/HeJ given TNF (2 μg)
h		
−2	6/10	3/6
−1	0/10	0/6
0	0/10	0/6
1	0/10	ND
2	0/10	0/6
4	10/10	6/6
None	10/10	6/6

Groups of mice received 18 mg D-galactosamine and 2 μg TNF administered intraperitoneally as a mixture in 0.5 ml of Pi/NaCl at 0 h. Immediately and at different times before or thereafter, 20 mg of uridine was administered intraperitoneally in 0.5 ml of PBS. Groups receiving 18 mg of D-galactosamine and TNF but no uridine served as controls.

were administered 1 or 2 h before D-galactosamine. Sensitization to LPS was maximum when LPS and D-galactosamine were administered together, and decreased when LPS was injected 1, 2, 3, and 4 h after D-galactosamine, after which time lethality was no longer obtained. In contrast, sensitization to TNF lasted longer, resulting in 100% lethality when TNF was administered up to 4 h after D-galactosamine. Injection of TNF thereafter revealed the presence of a lower degree of sensitization, which however was still detectable 8 h later.

Inhibition of D-Galactosamine-induced Sensitization to TNF by Uridine in C57BL/6 and C3H/HeJ Mice. The D-galactosamine-induced depletion of liver UTP is a prerequisite for the development of hypersensitivity to LPS. The depletion of UTP pool can be restored by uridine (2). Administration of uridine up to 2 h after injection of otherwise lethal amounts of D-galactosamine/LPS protects mice from death (1, 3).

In the present experiment, the effect of uridine on D-galactosamine sensitization to TNF in C57BL/6 and C3H/HeJ mice was studied. Groups of mice received uridine intraperitoneally at different times before or after administration of D-galactosamine together with lethal amounts (2 μg) of TNF.

The results in Table II show that sensitization to TNF by D-galactosamine was completely inhibited when uridine was administered between 1 h before and 2 h after D-galactosamine/TNF injection. Partial inhibition was obtained when uridine was injected 2 h before, while its administration 4 h after D-galactosamine/TNF had no effect (see Table II).

Discussion

Using recombinant human TNF in direct toxicity tests, we investigated whether TNF possesses lethal activity and may thereby represent a mediator of the lethal toxicity of endotoxin. For this purpose we used mice sensitized to endotoxin by D-galactosamine, in which lethality is obtained by minute amounts

of LPS (1). In the D-galactosamine model, the sensitivity of the host to the toxic products of macrophages is increased without altering the actual mechanisms of toxicity e.g. the reactivity of macrophages (3). Other known macrophage-activating substances (zymosan, muramyl dipeptide, *Propionibacterium acnes*, Bacillus Calmette-Guerin, *Coxiella burnetii*) exhibit no lethal effects in combination with D-galactosamine, showing that the D-galactosamine sensitization is specific for LPS-induced macrophage products (Freudenberg and Galanos, manuscript in preparation). Further, D-galactosamine does not enhance the toxicity of other hepatotoxins such as α-amanitin or CCl_4 (Freudenberg and Galanos, manuscript in preparation). The model is therefore suitable for testing the activity of substances that come in question as mediators of endotoxin reactions.

As shown in the present study, TNF is highly toxic in D-galactosamine-treated mice, inducing 100 and 50% lethality with 1.0 and 0.1 μg, respectively. In the absence of D-galactosamine, TNF caused ~80% lethality in 500 μg amounts.

The results show that the sensitization by D-galactosamine to LPS is also a sensitization towards TNF.

As in the case of LPS, with TNF the early biochemical alterations (depletion of UTP and subsequent inhibition of RNA synthesis) induced by D-galactosamine in hepatocytes are prerequisite for sensitization, since their reversion by administration of uridine inhibited sensitization to TNF.

The state of D-galactosamine-induced hypersensitivity towards TNF lasted longer (up to 8 h) than towards LPS (up to 4 h). This difference probably reflects the time required for LPS to elicit production of lethal amounts of TNF. Accordingly, injection of LPS 4 h or later after D-galactosamine results in the appearance of TNF at a time when the state of hypersensitivity is no longer present. As with LPS (1), with TNF no lethality was seen when this was injected before D-galactosamine.

There is another striking similarity between the above LPS- and TNF-induced lethality. We showed earlier (1) that mice receiving D-galactosamine and a lethal dose of LPS appear completely normal at all times thereafter, and that the first signs of illness become suddenly apparent only 10–20 min before death. Identical behavior was found for D-galactosamine/TNF-induced lethality.

Unlike LPS, TNF was lethal in endotoxin-resistant C3H/HeJ mice treated with D-galactosamine, their susceptibility to TNF being identical to that of endotoxin-sensitive mice. Also in the absence of D-galactosamine, the sensitivity to TNF of normal C3H/HeJ mice (lethal dose ~500 μg) was comparable to that of endotoxin-sensitive C3H/TifF mice. The sensitivity of C3H/HeJ mice to TNF becomes understandable and is to be expected if TNF were the mediator of LPS toxicity for the following reasons. It has been shown (2) that the resistance of D-galactosamine-treated C3H/HeJ mice to LPS is due to the inability of their macrophages to be stimulated by LPS. A high sensitivity was achieved by the mere transfer of 2×10^7 pure endotoxin-sensitive macrophages (3). This demonstrated that D-galactosamine-treated C3H/HeJ mice, although resistant to LPS, are highly sensitive to the toxic effects of macrophage-endogenous mediators, one of which is TNF. Thus, Kawakami and Cerami (15) showed that serum and culture supernatants containing cachectin, which had been induced by LPS in endotoxin-sensitive mice, decreased lipoprotein lipase activity when administered

in C3H/HeJ mice. Dinarello et al. (16) showed that human recombinant TNF induces fever responses in C3H/HeJ mice and in rabbits.

It is not known at present whether TNF exhibits its lethal toxicity directly, or indirectly through other endogenous mediators that it induces. Although pure recombinant TNF was shown here to be lethal in sensitized and normal mice, in view of the enormous complexity of the phenomenon of endotoxicity, in LPS lethality, a direct or indirect participation of other endogenous mediators produced independently of TNF cannot be excluded. Consequently the amounts of exogenous TNF shown to be lethal in this study (~500 μg) must not necessarily correspond to those produced endogenously by lethal amounts of LPS, which in such cases could be considerably lower.

In conclusion, the present study provides direct evidence of the lethal toxicity of TNF and shows that, as with LPS, its activity may be enhanced in animals with impaired liver metabolism. The results support the hypothesis (4) that TNF is a mediator of endotoxin-induced lethality.

Summary

The toxic properties of human recombinant tumor necrosis factor (TNF) were investigated in mice made hypersensitive to endotoxin by treatment with D-galactosamine. C3H/TifF mice treated with D-galactosamine were rendered sensitive to the lethal effects of submicrogram amounts of TNF. In the absence of D-galactosamine, TNF caused ~80% lethality with 500 μg. The duration of sensitization to TNF lasted up to 8 h after D-galactosamine administration, that towards LPS, up to 4 h. As with LPS, with TNF sensitization could be inhibited by uridine administered up to 2 h after D-galactosamine/TNF, showing that the early biochemical alterations in the liver known to be necessary for sensitization to LPS are also necessary for sensitization to TNF.

In contrast to LPS, the toxicity of TNF was expressed also in D-galactosamine-treated endotoxin-resistant C3H/HeJ mice. The susceptibility of these mice to TNF was identical to that of endotoxin sensitive mice. In the absence of D-galactosamine the toxicity of TNF in C3H/HeJ mice was comparable to that obtained in C3H/TifF mice, being lethal with amounts of the order of 500 μg. The present results support the hypothesis that TNF is a mediator of lethal toxicity of endotoxin.

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