Role of Endogenous Endotoxin on Tumor Necrosis Factor-Hypersensitivity Caused by β-Galactosamine Challenge

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We examined the role of endotoxin in the mechanism of recombinant human tumor necrosis factor (rhTNF)-hypersensitivity caused by β-galactosamine (GalN). We used polymyxin B, an antibiotic with anti-endotoxin activity, to determine the participation of endogenous endotoxin. The glycogen and blood glucose level of rhTNF (1×10^4 units/mouse, i.v.)-injected mice was lower at 7 h post-intoxication than that in the control. Administration of rhTNF to GalN (700 mg/kg, i.p.)-treated mice resulted in lower levels of glycogen and blood glucose than those in animals treated with rhTNF alone. In mice pretreated with polymyxin B (20 mg/kg, i.p.), the level at 7 h after rhTNF/GalN-injection was markedly increased compared to that in mice treated with rhTNF/GalN alone. The injection of a low endotoxin dose (0.1 mg/kg, i.p.) markedly decreased the rectal temperature in mice treated with rhTNF (5×10^4 units/mouse, i.v.) and GalN, and none of these animals survived after treatment for 18 h. These findings suggest that endogenously produced endotoxin may contribute to the extent of rhTNF-hypersensitivity caused by GalN.

Key words endotoxin; tumor necrosis factor; hypersensitivity; galactosamine; polymyxin B

Septic shock may be associated with a toxic state initiated by the stimulation of monocytes by bacterial toxins, such as endotoxin, which is released into the blood-stream. Endotoxin induces shock symptoms in humans and animals which is characterized by fever, hypotension, intravascular coagulation and finally multiple organ failure. On the other hand, tumor necrosis factor (TNF), a macrophage-derived cytokine inducible by endotoxin, has frequently been reported to cause a shock syndrome similar to endotoxin, and has been suggested to be one of the major mediators of shock.1) This mediator is responsible, at least in part, for a number of pathophysiological responses in the liver, including acute phase response, inflammatory cell infiltration, hepatocyte proliferation, hyperlipidemia, free oxygen radical formation, fibrogenesis and cholestasis.1-3)

Spillover-endotoxia from the gastrointestinal tract is important in the relationship between endotoxin and hepatotoxicity. Endotoxin is believed to be initially detoxified in the reticuloendothelial system, particularly in liver Kupffer cells. Sensitization with β-galactosamine (GalN) markedly increases the sensitivity of animals to endotoxin and augments its lethal activity.4,5) GalN has been clearly shown to increase sensitivity to TNF-mediated effects.5) The lethal effects of endotoxin on GalN-sensitized mice are generally considered to be as an experimental model for clinical endotoxic shock or septic shock. It is also well known that the sensitization is related to biochemical alterations induced by GalN in the hepatocytes. Further, Grün et al.6) concluded that endotoxin contributes significantly to the pathogenesis of GalN-hepatitis and its clinical symptoms. We recently reported7) that intracellular Ca^2+ plays a role in lipid peroxide formation due to endotoxin/GalN-induced hepatotoxicity under conditions of macrophage activation, especially of Kupffer cells, by zymosan. We further suggested8) the participation of spillover-endotoxia from the gastrointestinal tract on lipid peroxide formation in mice treated with TNF under depression of the reticuloendothelial system. Also, a very strong synergism between TNF and endotoxin has been observed.8-10) These reports support our conclusion concerning the role of endogenous endotoxin in TNF-induced shock symptoms. Therefore, based on the present knowledge of metabolic responses in endotoxemia, we designed the following experiments to examine the role of endogenously produced endotoxin using polymyxin B, an antibiotic with anti-endotoxin activity, to clarify the mechanism of TNF-hypersensitivity caused by GalN challenge.

MATERIALS AND METHODS

Animals and Treatment Male ddY mice, 4 weeks old and weighing 18 to 20 g, were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and maintained in the Tohoku Pharmaceutical University Experimental Animal Center. Salmonella typhimurium lipopolysaccharide (endotoxin), Difco Laboratories, (Detroit, MI, U.S.A.) was used throughout this study. Recombinant human TNF(1) (rhTNF) was generously provided by Dainippon Pharmaceutical Co., Ltd., Osaka, Japan. The endotoxin content of this cytokine was shown to be less than 0.04 ng/mg protein using a Limulus test kit (Seikagaku Co., Tokyo, Japan). Polymyxin B Sulfate (PMB, Nacalai Tesque, Inc., Kyoto, Japan) was injected into mice at a dose of 20 mg/kg i.p. After 24 h, β-galactosamine hydrochloride (700 mg/kg, i.p.) (GalN, Nacalai Tesque, Inc., Kyoto, Japan) and rhTNF (1×10^4 units/mouse, i.v.) were injected simultaneously into mice treated with PMB. Control mice were injected with 0.2 ml of saline alone.

Measurements of Liver Glycogen and Blood Glucose Estimation of liver glycogen was carried out by a modification of the method of Wilder and Sword.12) Liver homogenate was heated at 100°C for 20 min in 30% KOH solution. Glycogen was isolated by the addition of ethanol, and estimated colorimetrically using an anthrone reagent (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan). The level of blood glucose was estimated by the Glucose B-Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Measurement of Body Temperature Rectal tempera-
ture was measured using a thermometer with a thermister element (Natsume Manufacturing Co., Ltd., Tokyo, Japan). Mice with a body temperature of 37–38°C were used in this experiment after selection by measuring twice after a 15 min interval.

Statistical Analysis  Student’s t-test was used evaluate the statistical significance of differences between groups.

RESULTS

Effects of Administration of PMB on the Liver Glycogen and Blood Glucose Levels in rTNF/GaIN-Injected Mice  From many investigations it is well known that endotoxin-poisoned animals initially exhibit hyperglycemia, which is followed by a prompt fall in blood glucose to a hypoglycemic level with a concomitant decrease in the levels of liver and muscular glycogen, producing profound lactacidaemia. Therefore, in this study, experiments were carried out using PMB to determine whether TNF/GaIN-induced liver glycogen depletion and hypoglycemia can be prevented by the inactivation of endotoxin. As shown in Fig. 1A, the glycogen level in the liver of rTNF (1X10^6 units/mouse, i.v.)-treated mice was lower than that in the control at 7 h post-intoxication, while a more significant decrease was found in the liver glycogen level of rTNF/GaIN-treated mice. However, the rTNF/GaIN-induced liver glycogen depletion was markedly inhibited by pretreatment with PMB. Similarly, a significant increase was found in the blood glucose level of rTNF/GaIN/PMB-treated mice as compared to that in mice treated with rTNF/GaIN (Fig. 1B). These findings suggest the role of endogenous endotoxin in rTNF-induced hypoglycemia and liver glycogen depletion in GaIN-sensitized mice.

Effects of Endotoxin on the Rectal Temperature of rTNF/GaIN-Treated Mice  The rectal temperature in endotoxin-poisoned mice drops in proportion to the severity of symptoms. In the experiments described above, the administration of PMB resulted in a reduction of rTNF-sensitivity by GaIN treatment. Therefore, we designed the following experiments to examine the role of endotoxin in rTNF/GaIN-induced lethality by measuring rectal temperature in the presence of a sublethal dose of endotoxin. Table 1 shows changes in the level of rectal temperature after the administration of low doses of rTNF (5X10^5 units/mouse, i.v.) and endotoxin (0.1 mg/kg, i.p.) in GaIN-treated mice. No effects were observed in the rTNF/GaIN, endotoxin/GaIN or rTNF/endotoxin groups. However, rTNF/GaIN-mice injected with endotoxin showed a markedly reduced rectal temperature as compared to that in the control at 4 h post-intoxication, and there were no surviving animals at 18 h. These results suggest that endotoxin may enhance the rTNF-sensitivity induced by GaIN treatment.

DISCUSSION

The endotoxin lipopolysaccharide elicits various responses in the host involving hemodynamic, cardiovascular, immunologic and metabolic mechanisms. Most administered endotoxins are localized to cells of the reticuloendothelial system in animals, particularly Kupffer cells and splenic macrophages. Macrophages, stimulated by endotoxin release numerous cytokines. TNF administered intravenously with nanogram quantities of the endotoxin has been reported to cause lethal shock, and it appears that TNF and endotoxin act synergistically in activating the complement system which plays an important role in mediating tissue injury and lethality. Previously, we suggested that TNF-induced oxidative stress occurs as a result of bacterial or endotoxin

Table 1. Effects of Endotoxin on Rectal Temperature in rTNF/GaIN-Treated Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rectal temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 h</td>
</tr>
<tr>
<td>Control</td>
<td>37.3 ± 0.09*</td>
</tr>
<tr>
<td>LPS 0.1 mg/kg (i.p.)</td>
<td>37.0 ± 0.11</td>
</tr>
<tr>
<td>rTNF 5X10^5 units/mouse (i.v.)</td>
<td>37.2 ± 0.13</td>
</tr>
<tr>
<td>rTNF/GaIN 700 mg/kg (i.p.)</td>
<td>36.8 ± 0.14</td>
</tr>
<tr>
<td>LPS/GaIN</td>
<td>37.1 ± 0.16</td>
</tr>
<tr>
<td>LPS/rTNF</td>
<td>36.7 ± 0.12</td>
</tr>
<tr>
<td>LPS/rTNF/GaIN</td>
<td>32.2 ± 0.12*</td>
</tr>
</tbody>
</table>

*a) Mean values ± S.E. of 5 mice. *p < 0.05, significantly different from rTNF/GaIN-treated group. Mice with a body temperature of 37–38°C were employed in this experiment after selection by measuring twice at 15 min intervals.
translocation in various disease states in which reticuloendothelial system function is reduced. GalN has been used for many years to sensitize animals to the lethal effects of endotoxin\(^{13}\) and TNF\(^{14}\). GalN has been used to induce alterations in hepatocytes, such as uridine triphosphate depletion,\(^{15}\) which prevents transcription and translation. These changes are thought to be responsible for liver sensitization to endotoxin-induced toxicity. However, the mechanism responsible for this sensitization has not been established. Therefore, the present study was performed to clarify the cause of GalN-induced TNF-hypersensitivity using PMB as a specific endotoxin binding agent.\(^{15}\)

In the present study, we found that the glycogen level was markedly lower in the liver of mice 7 h after rhTNF (\(1 \times 10^4\) units/mouse, i.v.)/GalN (700 mg/kg, i.p.) injection as compared to that in animals treated with rhTNF alone. The level was significantly increased in the liver of mice treated with rhTNF/GalN plus PMB (20 mg/kg, i.p.). Similarly, the glucose level was significantly increased in the blood of rhTNF/GalN-treated mice as compared to that in mice given rhTNF/GalN. In addition, PMB was noted to protect the rhTNF-induced lethality in GalN-hypersensitized mice, and also PMB-pretreated mice were protected against decreases in rectal temperature after rhTNF/GalN administration (data not shown). Therefore, endogenously produced endotoxin may contribute to the extent of rhTNF/GalN-hypersensitivity. Macrophages and other lymphoreticular cells are believed to play an important role in endotoxin reactions, in particular within an acquired state of hypersensitivity.\(^{13,16}\) Freudenberg et al.\(^{17}\) demonstrated that the transfer of macrophages from endotoxin-responsive C3H/HeN mice to unresponsive C3H/HeJ mice rendered them sensitive to the lethal effects of endotoxin by GalN sensitization. In the absence of GalN, the transfer of C3H/HeN macrophages did not render C3H/HeJ mice sensitive to endotoxin, indicating that in the GalN model sensitization to endotoxin and a triggering of endotoxicity are based on different mechanisms. As GalN did not enhance the cytokine formation induced by endotoxin, it is likely that GalN exerts its effect by increasing the reactivity of the host to the products induced by endotoxin, in particular, to TNF, but not by an increase in endotoxin-induced mediator formation.\(^{18}\) This has been confirmed by the administration of rhTNF to GalN-treated animals.\(^{19,20}\)

Bacterial translocation from the gastrointestinal tract, i.e., spillover-endotoxemia, is important in the relationship between endotoxin and hepatotoxicity, since endotoxin clearance may be due to rapid uptake in the reticuloendothelial system (RES), especially by Kupffer cells in the liver. We reported previously\(^{21}\) that the lipid peroxide level was markedly higher in the liver of mice injected with rhTNF and lead acetate (an RES depressor agent) as compared to that in mice treated with rhTNF alone, and that lipid peroxide formation was inhibited by pretreatment with the anti-endotoxin drug PMB. In the present study, the rectal temperature of GalN/rhTNF (\(5 \times 10^5\) units/mouse, i.v.) mice injected with a low dose of endotoxin (0.1 mg/kg, i.p.) was markedly reduced in comparison to that in mice injected with rhTNF/GalN (Table 1). Rush et al.\(^{21}\) reported increased permeability of the gut under shock conditions, with the spreading of endotoxin or bacteria into circulating blood and translocation into other organs, and they emphasized the role of gut barrier failure. The findings described above indicated that the extent of rhTNF-hypersensitivity caused by GalN may be a result of endogenously produced endotoxin under conditions of reduced reticuloendothelial system function.

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**REFERENCES**