Requirement for Lipopolysaccharide-Responsive Macrophages in Galactosamine-Induced Sensitization to Endotoxin

M. A. FREUDENBERG, D. KEPLER, AND C. GALANOS

Max-Planck-Institut für Immunbiologie, 1 Biochemisches Institut der Albert-Ludwigs-Universität, 2 D-7800 Freiburg, Federal Republic of Germany

Received 9 August 1985/Accepted 18 November 1985

Injection of D-galactosamine sensitizes mice many thousand-fold to the lethal action of endotoxin (lipopolysaccharide [LPS]). Comparable sensitization was practically absent in LPS-resistant C3H/HeJ mice, which after D-galactosamine treatment were about 500,000 times less sensitive to LPS lethality than histocompatible LPS-sensitive C3H/HeN mice. D-Galactosamine induces changes in the hepatocytes of treated animals, such as depletion of UTP and alterations in the pattern of UDP sugars. These early biochemical changes, which are necessary for development of sensitization, were similar in both mouse strains which we examined. High sensitivity to the lethal effects of LPS was achieved in C3H/HeJ mice after D-galactosamine treatment by transfer of C3H/HeN macrophages obtained in culture from bone marrow precursor cells.

In a previous study Galanos et al. showed that administration of D-galactosamine to different animals leads to an increase in the susceptibility of the animals to the lethal effects of endotoxin (lipopolysaccharide [LPS]) (5). In mice, depending on the strain and age, administration of 2 to 4 mmol of D-galactosamine per kg of body weight increased the sensitivity of the animals to endotoxin up to 100,000-fold. The sensitization was only of short duration, lasting up to 3 h after galactosamine treatment. Since galactosamine is a known hepatotoxic agent (3) and its primary effects are confined to hepatocytes, the liver is assumed to play a central role in the development of sensitization. The early biochemical changes induced by D-galactosamine in hepatocytes are characterized by a marked decrease in the UTP level to less than 10% of the control level. This decrease impairs biosynthesis of RNA and other macromolecular cell constituents, resulting in damage to, and ultimately in the death of, hepatocytes (3). A comparable effect of D-galactosamine on Kupffer cells has not been observed (8). The decrease in UTP level may be reversed by uridine (10), which also reverses the inhibition of RNA synthesis and prevents development of hepatotoxic effects when it is administered within 3 h after D-galactosamine (3). The biochemical changes described above are also considered to play an important role in the development of endotoxin hyperreactivity, because the reversal of galactosamine-induced decreases in UTP levels by uridine also prevents sensitization to endotoxin (5). It is interesting that other, less cell-specific inhibitors of RNA synthesis also sensitize animals against the lethal action of endotoxins (24).

During our studies on D-galactosamine-induced sensitization to endotoxin, we found that such sensitization is virtually absent in the endotoxin-resistant C3H/HeJ mice. This was particularly interesting since the susceptibility of these mice to the lethal action of endotoxin could be increased by a number of other treatments, such as treatment with Mycobacterium bovis BCG (27), Coxiella burnetii (23), or Propionibacterium acnes, or an adrenalectomy (unpublished data). C3H/HeJ mice are resistant to almost all known biological effects of LPS, and it is believed that this resistance is due to genetically controlled hyporeactivity of the lymphoreticular cells of these animals (13, 15, 19, 21, 25, 26, 29).

In the present study we compared the effects of D-galactosamine on the susceptibility to the lethal effects of endotoxin in endotoxin-resistant C3H/HeJ mice and histocompatible endotoxin-sensitive C3H/HeN mice. We found that, although D-galactosamine induces very similar alterations in hepatic uracil nucleotides in both strains, sensitization to endotoxin is demonstrable only in C3H/HeN mice. A high level of susceptibility to endotoxin could be achieved in D-galactosamine-treated C3H/HeJ mice by transferring LPS-responsive C3H/HeN macrophages to them.

MATERIALS AND METHODS

Animals. Male and female C3H/HeJ and histocompatible (18) C3H/HeN mice were obtained from the breeding stock of the Max-Planck-Institute; 6-week-old mice served as bone marrow donors for macrophage cultures. For lethality tests and liver nucleotide analyses, 10- to 14-week-old mice with an average body weight of 25 g were used.

Materials. LPS from Salmonella abortus-equus in triethyl-amine salt was prepared as described previously (6). D-Galactosamine hydrochloride was purchased from C. Roth, Karlsruhe, Federal Republic of Germany, and uridine was obtained from E. Merck AG, Darmstadt, Federal Republic of Germany.

For injection, all materials were dissolved in pyrogen-free phosphate-buffered saline (PBS).

Cultivation of bone marrow-derived macrophages. Bone marrow cells were flushed from mouse femora and cultivated at a concentration of 5 × 10⁶ cells per ml in hydrophobic Teflon film bags (Heraeus, Hanau, Federal Republic of Germany) as described previously (4). The culture medium contained 70% high-glucose formulation of Dulbecco modified Eagle medium (GIBCO BRL GmbH, Karlsruhe, Federal Republic of Germany), 10% fetal calf serum, 5% horse serum, 0.01 mM sodium pyruvate (GIBCO), 50 nM 2-mercaptoethanol (Roth), 50 U of penicillin, 50 µg of streptomycin (Seromed, Berlin, Federal Republic of Germany) per ml, and 30% L-cell-conditioned medium (12) containing

* Corresponding author.
TABLE 1. Lethality of LPS in normal and d-galactosamine-
treated C3H/HeN and C3H/HeJ micea

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>d-Galactosamine dose (mg/mouse)</th>
<th>LPS dose (µg/mouse)</th>
<th>Lethality (no. of deaths/ total no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H/HeN</td>
<td>20</td>
<td>0.05</td>
<td>10/10</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>20</td>
<td>0.01</td>
<td>7/10</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.002</td>
<td>2/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>9/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>3/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3,000</td>
<td>0/10</td>
</tr>
</tbody>
</table>

a Groups of animals received intravenously different amounts of LPS alone or LPS and d-galactosamine as a mixture in 0.2 ml of PBS. Similar groups which received d-galactosamine alone served as controls. Lethality was recorded up to 72 h.

RESULTS

Effect of d-galactosamine on the susceptibility of C3H/HeN and C3H/HeJ mice to LPS. d-Galactosamine (20 mg per mouse, corresponding to 3.8 mmol/kg of body weight) and different amounts of LPS from S. abortus subsp. equi were administered intravenously as a mixture to groups of mice in 0.2 ml of PBS. Animals receiving d-galactosamine or LPS alone served as controls.

In C3H/HeN mice, 400 µg of LPS alone per mouse caused 90% lethality, and 200 µg of LPS per mouse caused 30% lethality (Table 1). Administration of d-galactosamine increased the sensitivity of mice to LPS by a factor of about 100,000, resulting in 70% lethality with 0.01 µg of LPS per mouse and 20% lethality with 0.002 µg of LPS per mouse. d-Galactosamine alone had no lethal effect. Deaths occurred earlier in the d-galactosamine-sensitized animals (6 to 15 h after injection) than in animals injected with a lethal dose of LPS alone (15 to 72 h after injection). In C3H/HeJ mice, administration of up to 3 mg of LPS alone per mouse intravenously and up to 10 mg of LPS per mouse intraperitoneally caused no lethality. Treatment with d-galactosamine established a low degree of susceptibility to LPS (Table 1), with 60% of the animals dying after administration of 2 mg of LPS per mouse and 20% dying after administration of 1 mg of LPS per mouse. Therefore, d-galactosamine-sensitized C3H/HeJ mice were more resistant to LPS than d-galactosamine-treated C3H/HeN mice by a factor of about 500,000.

Effect of d-galactosamine on hepatic nucleotides. The inability of d-galactosamine to sensitize C3H/HeJ mice to LPS to a degree anywhere comparable to that seen with endotoxin-responsive mice might have been due to a higher resistance of the livers to the effects of d-galactosamine. For this reason the effects of d-galactosamine on the hepatic nucleotides were investigated in C3H/HeN and C3H/HeJ mice.

In both mouse strains d-galactosamine induced significant decreases in the hepatic UTP levels, which were less than 10% of the control levels (Table 2). The decrease in UTP level was accompanied in both strains by a decrease in the UDP-glucose and UDP-galactose contents to about 20% of the control levels. The levels of UDP-N-acetyl-d-glucosamine and UDP-N-acetyl-d-galactosamine, which are metabolites of d-galactosamine (3), increased 4.2- and 4.4-fold in livers of d-galactosamine-treated C3H/HeN and C3H/HeJ mice, respectively. UDP-d-glucosamine and UDP-d-galactosamine, which are nonphysiological nucleotide sugars that are derived exclusively from d-galactosamine (3), accumulated in the livers of both mouse strains in comparable amounts. Apart from the typical d-galactosamine-induced changes in the levels of liver pyrimidine nucleotides, in both strains of mice (Table 2) no significant alterations were detected in other nucleotide contents, including adenine and guanine nucleotide contents (data not shown).

From these results we conclude that the effects of d-galactosamine on hepatic UTP and UDP sugar levels are indistinguishable in endotoxin-sensitive and endotoxin-resistant mice, and therefore the absence of susceptibility in C3H/HeJ mice to LPS is not due to an absence of d-galactosamine in effects on livers.

Effect of macrophage on the susceptibility of d-galactosamine-treated C3H/HeJ mice to LPS. Macrophages and other lymphoreticular cells are believed to play a central role in endotox reactions (13, 15, 16, 20). In contrast to the

TABLE 2. d-Galactosamine-induced changes in liver uracil nucleotides in C3H/HeN and C3H/HeJ micea

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Treatment</th>
<th>UTP (mmol/kg of liver)</th>
<th>UDP-glucose + UDP-galactose (mmol/kg of liver)</th>
<th>UDP-GlcNAc + UDP-GalNAc (mmol/kg of liver)</th>
<th>UDP-GlcN + UDP-GalN (mmol/kg of liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H/HeN</td>
<td>PBS</td>
<td>0.22 ± 0.03</td>
<td>0.36 ± 0.03</td>
<td>0.24 ± 0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>C3H/HeN</td>
<td>d-Galactosamine</td>
<td>0.02 ± 0.002</td>
<td>0.08 ± 0.02</td>
<td>1.01 ± 0.10</td>
<td>1.17 ± 0.15</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>PBS</td>
<td>0.21 ± 0.04</td>
<td>0.43 ± 0.07</td>
<td>0.24 ± 0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>d-Galactosamine</td>
<td>0.02 ± 0.003</td>
<td>0.07 ± 0.03</td>
<td>1.06 ± 0.15</td>
<td>0.87 ± 0.19</td>
</tr>
</tbody>
</table>

a D-Galactosamine was injected intraperitoneally at a dose of 2.8 mmol/kg of body weight 4 h prior to freeze-clamping of the livers. The results represent the means ± standard deviations for groups containing four or five animals. UDP-GlcNAc, UDP-N-acetyl-d-glucosamine; UDP-GalNAc, UDP-N-acetyl-d-galactosamine; UDP-GlcN, UDP-d-glucosamine; UDP-GalN, UDP-d-galactosamine.
macrophages of histocompatible C3H/HeN mice and other mouse strains, C3H/HeJ macrophages are hypersensitive to LPS (9, 18, 23). Therefore, we investigated whether in D-galactosamine-treated mice expression of hypersensitivity to endotoxin required the presence of LPS-responsive macrophages. Pure macrophages derived from the bone marrows of C3H/HeN and C3H/HeJ mice were obtained from 10-day cultures as described in Materials and Methods. These macrophages (2 × 10^6 cells per mouse) were injected intravenously in 0.25 ml of PBS into C3H/HeJ mice, and 2 h later the animals were challenged with D-galactosamine and LPS. Table 3 shows that in D-galactosamine-treated C3H/HeJ mice receiving C3H/HeN macrophages, a dose of 1 μg of LPS per mouse caused 74% lethality. Thus, transfer of LPS-responsive macrophages lowered the lethal dose of LPS by a factor of about 2,000. The onset of lethality was fast, commencing 6 h after injection of D-galactosamine and LPS. Surprisingly, transfer of macrophages derived from C3H/HeN mice, also lowered the lethal dose of LPS, even though compared with C3H/HeN macrophages, they were about 200 times less effective. In this case 100% lethality was achieved with a dose of 1,000 μg of LPS per mouse, and 40% lethality was achieved with a dose of 100 μg of LPS per mouse.

**Inhibition of D-galactosamine-induced sensitization to LPS by uridine in C3H/HeJ mice which received C3H/HeN macrophages.** The early metabolic alterations in livers induced by D-galactosamine may be reversed by uridine (10). Injection of uridine (4 mmol/kg) into LPS-sensitive mice up to 3 h after injection of D-galactosamine and LPS prevents the development of LPS hypersensitivity (5). The effect of uridine on the development of D-galactosamine-induced sensitization to LPS was studied in C3H/HeJ mice which received 2 × 10^7 C3H/HeN macrophages and then 2 h later received 20 mg of D-galactosamine per mouse and 2 or 20 μg of LPS per mouse as a mixture. Uridine (20 mg per mouse) was injected intraperitoneally 1 h after injection of D-galactosamine and LPS. Table 4 shows that treatment with uridine resulted in complete protection from the lethal effects of D-galactosamine and LPS. A protective effect by uridine was also observed in D-galactosamine-treated C3H/HeJ mice which received homologous cultured macrophages (2 × 10^7 cells per mouse) and a lethal amount of LPS. Administration of 20 mg of uridine intraperitoneally 1 h after injection of D-galactosamine (20 mg) and LPS (1 mg) completely protected these animals (data not shown).

**TABLE 4. Inhibition of D-galactosamine–LPS-induced lethality by uridine in C3H/HeN macrophages.**

<table>
<thead>
<tr>
<th>D-galactosamine dose (mg/kg)</th>
<th>LPS dose (μg/mouse)</th>
<th>Uridine dose after 1 h (mg/mouse)</th>
<th>Lethality (no. of deaths/total no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>2</td>
<td>10/10</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>0/10</td>
<td></td>
</tr>
</tbody>
</table>

*Groups of C3H/HeJ mice received 2 × 10^7 C3H/HeN macrophages in 0.25 ml of PBS intravenously, and 2 h later the animals received 0.2 mg of LPS intravenously. This was followed 1 h later by intraperitoneal injection of uridine in 0.1 ml of PBS.

**DISCUSSION**

D-Galactosamine induces a high degree of sensitization to the lethal effects of LPS in mice and other experimental animals (5). In this study we found that in endotoxin-resistant C3H/HeJ mice treatment with D-galactosamine may establish low sensitivity to endotoxin (50% lethal dose, approximately 2 mg of LPS). However, compared with histocompatible endotoxin-sensitive C3H/HeN mice (50% lethal dose, less than 0.01 μg of LPS), after galactosamine treatment C3H/HeJ mice remain less sensitive to LPS by a factor of about 500,000. The early biochemical effects of D-galactosamine on livers (UTP depletion) are necessary for the development of sensitivity to endotoxin (see above). A comparison of the uracil nucleotide contents in the livers of C3H/HeJ and C3H/HeN mice 4 h after D-galactosamine injection revealed a large decrease in UTP levels, which would have been sufficient to inhibit hepatocellular RNA synthesis. In both mouse strains the alterations were identical. Therefore, the resistance of D-galactosamine-treated C3H/HeJ mice to the action of LPS is not due to resistance of hepatocytes to the effects of D-galactosamine.

The difference in LPS sensitivity between D-galactosamine-treated C3H/HeN and C3H/HeJ mice is much greater than the difference observed in a number of other sensitization models. The levels of sensitization obtained with *C. burnetii* (23), *M. bovis* BCG (27), and *P. acnes* (unpublished data) in C3H/HeJ mice were only 10, 30, and 100 times lower than the levels obtained in C3H/HeN mice, respectively. Of the above, *M. bovis* BCG sensitization has been studied most thoroughly (28), and it has been shown that in C3H/HeJ mice the higher level of sensitivity of the animals to LPS was paralleled by a T-cell-mediated enhanced LPS responsiveness of the macrophages. Macrophages are thought to play a central role in the host response to endotoxin (20). It has been shown repeatedly that macrophages of C3H/HeJ mice are hypersensitive to LPS (1, 2, 11, 13, 19, 21, 22; C. Bianco and P. J. Edelson, Fed. Proc. 36:1263, 1977).

In this study C3H/HeJ mice that received 2 × 10^7 C3H/HeN macrophages became susceptible to 1 μg of LPS (74% lethality) after D-galactosamine treatment. Compared with controls which received no macrophages, 2,000 times less LPS was sufficient to cause lethality. In the absence of D-galactosamine, transfer of C3H/HeN macrophages did not render C3H/HeJ mice sensitive to LPS (tested at concentrations up to 1 mg per mouse). The LPS susceptibility of D-galactosamine-treated mice supplied with macrophages lasted for at least 24 h after macrophage transfer (data not available).
shown). The time between injection of D-galactosamine and LPS and occurrence of lethality was short (6 to 15 h), which is typical for the D-galactosamine–LPS model (5). Sensitization of C3H/HeJ mice was inhibited by administration of uridine. Therefore, reversion of D-galactosamine-induced UTP depletion and the resulting inhibition of the early biochemical changes in hepatocytes interrupted the development of sensitization of C3H/HeJ mice to LPS in the same way as has been shown for LPS-responsive mice (5).

It is interesting that the transfer of cultured C3H/HeJ macrophages to homologous C3H/HeJ mice also had a significant sensitizing effect in the D-galactosamine model (50% lethal dose, approximately 100 μg), even though it was 200 times lower than the effect achieved with the same number of C3H/HeN macrophages. This shows that the unresponsiveness of C3H/HeJ macrophages to endotoxin may be at least partially reversed by in vitro culturing, as carried out in this study. It is not known at present which factor(s) is responsible for this effect. It was reported previously that it is possible to increase the sensitivity of C3H/HeJ macrophages to LPS in vitro (20, 21, 27, 28, 30).

In our case one likely candidate to do this is the colony-stimulating factor, which is present in cultures during the propagation of macrophages and has been shown previously (14) to enhance LPS-induced interferon production of at least responder (C3H/HeN) macrophages. Our results allow the following conclusions to be drawn. In D-galactosamine-sensitized mice, the lethal toxicity of LPS is expressed only in the presence of LPS-responsive macrophages. This supports the hypothesis that the macrophages are the central effector cells in endotoxin-mediated host responses. The almost complete absence of endotoxin susceptibility in D-galactosamine-treated C3H/HeJ mice and the high susceptibility achieved by the transfer of a relatively small number of LPS-responsive macrophages indicate that the macrophages of C3H/HeJ mice remain hypo-responsive after D-galactosamine treatment. Thus, in contrast to M. bovis BCG, D-galactosamine does not exert its sensitizing effect by enhancing the response of macrophages to LPS, but most likely exerts its effect by increasing the reactivity of the host to macrophage products or by impairing the liver in the clearance of such products. Thus, in the galactosamine model sensitization to endotoxin and triggering of endotoxicity are based on different mechanisms.

The present model in which C3H/HeJ mice which receive responder macrophages are made highly sensitive to LPS by D-galactosamine treatment makes possible a distinction between development of sensitization and triggering of the endotoxic process and allows the underlying mechanisms to be studied independently.

ACKNOWLEDGMENTS

The expert technical assistance of H. Stübig is gratefully acknowledged. We thank W. Hagmann and C. Schulz-Hostege for their help in the analysis of hepatic nucleotides.

This work was supported by the Deutsche Forschungsgemeinschaft through Sonderforschungsbereich 154 to C.G. and D.K.

LITERATURE CITED


ROLE OF MACROPHAGES IN ENDOTOXIN LETHALITY