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Induction of Tolerance to Lipopolysaccharide (LPS)–D-Galactosamine Lethality by Pretreatment with LPS Is Mediated by Macrophages†

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In mice treated with D-galactosamine, lipopolysaccharide (LPS) exhibits enhanced toxicity (C. Galanos, M. A. Freudenberg, and W. Reutter, Proc. Natl. Acad. Sci. USA 76:5939–5943, 1979). Pretreatment of mice with LPS before D-galactosamine rendered them tolerant to the enhanced lethal effect of LPS. Tolerance was established at 1 h after LPS injection and, depending on the dose of LPS used for pretreatment, lasted for up to 48 h. With C3H/HeJ mice with acquired sensitivity to LPS (M. A. Freudenberg, D. Keppler, and C. Galanos, Infect. Immun. 51:891–895, 1986), i.e., mice that had been administered C3H/HeN macrophages, pretreatment with LPS induced tolerance only if the C3H/HeN macrophages were already present at the time of pretreatment. This indicates that, like lethality, induction of tolerance by LPS is a macrophage-mediated phenomenon. Direct interaction of LPS with macrophages is the first step in the initiation of tolerance or toxicity. C3H/HeN macrophages (2×10^7), incubated with minute amounts of LPS (0.5 to 0.02 μg) in vitro and transferred subsequently to C3H/HeJ mice, induced lethality when administered together with or after D-galactosamine and tolerance when injected before D-galactosamine. Macrophages activated in vitro lost their tolerance- and lethality-inducing properties upon further incubation in LPS-free culture medium for 18 h. Such macrophages could be successfully restimulated by a new addition of LPS.

Treatment of different animal species with D-galactosamine leads to a considerable increase of their sensitivity to the lethal effects of endotoxin (lipopolysaccharide [LPS]) (9). According to present knowledge, the sensitization is related to the biochemical alterations induced by D-galactosamine in the liver parenchymal cells (hepatocytes) (3). In mice at 4 h after D-galactosamine treatment, a decrease of liver UTP to below 10% of the normal level is observed (6). This UTP depletion is considered to be responsible for the development of sensitization because its reversal by administration of uridine prevents sensitization to LPS. UTP deficiency inhibits RNA synthesis in the liver, which impairs biosynthesis of other macromolecular cell constituents. It is interesting that two other inhibitors of RNA synthesis, α-amanitin and actinomycin D, also increase the susceptibility of mice to the lethal effects of LPS (23).

D-galactosamine sensitization to LPS is a useful model for studying mechanisms involved in the lethal toxicity of LPS. It was shown that LPS-resistant C3H/HeJ mice, in contrast to LPS responder C3H/HeN mice, do not become susceptible to LPS after D-galactosamine administration, despite induction of a marked UTP deficiency in the liver. However, transfer of LPS-responsive C3H/HeN macrophages into D-galactosamine-treated C3H/HeJ mice rendered these mice very sensitive to the lethal effects of LPS (6). This provided direct evidence that macrophages mediate LPS lethality. It indicated further that sensitization of animals by D-galactosamine is due not to increased sensitivity of macrophages to LPS but to an increase in the susceptibility of the host to LPS-induced toxic macrophage products. Direct evidence for this was provided recently by showing that the susceptibility of mice to the lethal effects of tumor necrosis factor (TNF; cachectin), a monokine produced by macrophages upon stimulation with LPS, is increased several thousandfold after treatment of mice with D-galactosamine (19). Unlike responsiveness to LPS, the enhanced toxicity of TNF occurred in both LPS responder and nonresponder mice. Demonstration of enhanced lethal toxicity of TNF in D-galactosamine-treated mice and toxicity in normal mice at higher concentrations (about 3,000-fold) supported results of Beutler et al. (1) which indicated that TNF participates in mediation of the lethal effects of LPS.

In mice, enhanced susceptibility induced by D-galactosamine is seen only if LPS is injected together with D-galactosamine or a few hours (up to 4 h) later (9). Administration of LPS before D-galactosamine was without lethal effect even when the time interval between the two injections was as short as 1 h. This phenomenon was investigated in more detail in the present study.

It will be shown that administration of LPS renders mice tolerant within 1 h to the lethal effect of LPS–D-galactosamine. Evidence is presented that induction of both lethality and tolerance by LPS is mediated by macrophages and that direct interaction of LPS with macrophages is the first step in the development of both activities.

MATERIALS AND METHODS

Animals. C3H/TifF, C3H/HeN, and C3H/HeJ mice of both sexes were obtained from the breeding stock of our institute. For lethality tests, mice 10 to 14 weeks old were used. Six-week-old mice served as donors of bone marrow for macrophage cultures. Injections in mice were performed intravenously into the lateral tail vein or intraperitoneally. For blood collection, mice were exsanguinated by puncturing the axillary blood vessels after ether anesthesia. Blood was allowed to clot at 37°C for 15 min and centrifuged at 4°C, and the resulting serum was stored at −70°C until use.

Materials. LPS from *Salmonella abortus-equi*, *S. typhimu-*

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† This paper is dedicated to Otto Lüderitz in friendship and appreciation.

rium, and *S. minnesota* (S forms) were obtained from parent bacteria by the phenol-water method (24). *S. abortus-equi* LPS was purified further by the phenol-chloroform-petroleum ether procedure (13). Rough-form LPS from *S. minnesota* R60 (Ra) and R595 (Re) and *Escherichia coli* EH100 (Ra) and F515 (Re) were extracted from bacteria by the phenol-chloroform-petroleum ether procedure (12). All preparations were electrodialyzed, converted to the triethylamine salt (10), and lyophilized. Biosynthetically labeled [^{14}C]LPS of *S. abortus-equi* was obtained from bacteria grown in culture medium containing *N*-acetyl-D-[1-^{14}C]glucosamine (Amersham Buchler, Braunschweig, Federal Republic of Germany). The specific activity of the LPS was 3.33×10^5 cpm/mg.

Free lipid A was obtained by mild acid hydrolysis of *E. coli* F515 LPS and converted to the triethylamine salt form as described earlier (2). Synthetic lipid A (compound 506) (11) was a gift from Daiichi Seiyaku Co. Ltd., Tokyo, Japan.

Completely deacylated LPS from *S. abortus-equi* was prepared by heating the LPS in water-free hydrazine at 103°C for 18 h (15). Stock solutions of the different LPS and lipid A were prepared in pyrogen-free distilled water (10 mg/ml). S-form LPS preparations for injection were diluted further with pyrogen-free phosphate-buffered saline (PBS), and R-form LPS and lipid A were diluted in pyrogen-free distilled water. D-Galactosamine hydrochloride (Hepasamine) was obtained from C. Roth, Karlsruhe, Federal Republic of Germany.

Radioactivity measurements. All measurements were carried out in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Rockville, Md.) after oxidation of dried samples to $^{14}CO_2$ by combustion (18) in a Tri-Carb sample oxidizer (Packard).

Preparation of macrophages. Macrophages of LPS-sensitive C3H/HeN and LPS-resistant C3H/HeJ mice were obtained from bone marrow precursors in mass cultures in the presence of L-cell-conditioned medium as described previously (6). After 10 days of culture, the cells were washed three times with ice-cold, serum-free culture medium (high-glucose formulation of Dulbecco modified Eagle medium), suspended in pyrogen-free PBS, and used immediately for injection (2×10^7 macrophages per 0.25 ml per mouse intravenously). Under these conditions, the purity of macrophages was more than 96% as determined by adherence, staining with α-naphthylbutyrate (nonspecific esterases), Pappenheim staining (morphology), and uptake of colloidal carbon as previously described (6).

Treatment of macrophages with LPS in vitro. Macrophages from 10-day cultures were centrifuged and washed twice as indicated above and suspended in serum-free culture medium at a concentration of 4×10^7 cells per ml. The cells were placed in hydrophilic Teflon bags (Heraeus, Hanau, Federal Republic of Germany) with different amounts of LPS (0.008 to 1.0 μg in 1-μl/ml cell suspension) and incubated at 37°C for 90 min in a humidified atmosphere containing 8% CO_2. Macrophages incubated under identical conditions but without LPS served as a control. After incubation, the cells were washed three times with medium, suspended in PBS, and injected into mice as described above.

RESULTS

Lethal activity of LPS injected at different times in relation to D-galactosamine injection. C3H/TifF mice received different amounts of *S. abortus-equi* LPS, followed at different times (0, 30, 60, and 90 min) by D-galactosamine (18 mg per mouse). Lethality was scored at up to 24 h later. As expected, LPS was highly toxic when administered together with D-galactosamine, inducing 50% lethality with 0.001 μg per mouse and 100% lethality with 0.01 μg per mouse (Table 1). Similar results were obtained when LPS was injected 30 min before D-galactosamine. However, administration of LPS 60 or 90 min before D-galactosamine caused no lethality, even in 10,000-fold-higher amounts (10 μg). To study this phenomenon, we determined the amount and toxicity of LPS which was still circulating 90 min after the LPS injection.

Concentration and toxicity of LPS present in the circulation 90 min after injection. Ten C3H/TifF mice were injected with 10 μg of ^{14}C-radiolabeled LPS (3,300 cpm), and 90 min later they were exsanguinated and serum was prepared. ^{14}C activity in individual serum samples (0.2 ml) was measured separately as described in Materials and Methods, and amounts of LPS were calculated. On average, 5.0 μg of LPS (1,665 cpm)/ml of serum was found, and there were only small variations among individual animals (±0.15 μg/ml). This amount of circulating LPS corresponds to more than 50% of the injected dose. The toxicity of LPS present in serum was tested by pooling 0.2 ml from each sample and injecting different amounts into D-galactosamine-treated C3H/TifF (LPS-sensitive) and C3H/HeJ (LPS-resistant) mice. It was found that the serum expressed high toxicity in LPS-sensitive mice. It induced 100% lethality at 8.0 μl and 33% lethality at 0.8 μl, amounts which, according to radioactivity measurements, contained 0.04 and 0.004 μg of LPS, respectively. Thus, the toxicity of the LPS present in serum at 90 min after injection was comparable to that of the starting LPS.

In D-galactosamine-treated, LPS-resistant C3H/HeJ mice, neither the serum (up to 200 μl) nor the starting LPS (10 μg) was toxic. This indicated that the serum did not contain lethal amounts of toxic mediators such as TNF, which is also known to be lethal for C3H/HeJ mice (19), and that the lethal toxicity of serum obtained from C3H/TifF mice was indeed due to its LPS content. The results show conclusively that mice treated with LPS and 90 min later with D-galactosamine survive although an excess of toxic LPS is still present in the circulation at the time of D-galactosamine injection.

Effect of LPS pretreatment on LPS–D-galactosamine-induced lethality. Groups of C3H/TifF mice were injected with different amounts of LPS (0.0001 to 1.0 μg), and 90 min later they were injected with an otherwise lethal mixture of D-galactosamine (18 mg) and LPS (0.1 μg). This pretreatment of mice with LPS afforded dose-dependent protection. Whereas the lowest amount of LPS used (0.0001 μg) was without effect, pretreatment with 0.001 or 0.01 μg or more protected 50 and 100% of the animals, respectively. In another experiment, pretreatment with 0.1 μg of LPS com-

TABLE 1. Toxicity of LPS administered at different times before D-galactosamine[a]

Time (min) of LPS administration in relation to D-galactosamine injection	% Lethality when the amt (μg/mouse) of LPS administered was:				
	0.0001	0.001	0.01	0.1	10
0	0	50	100	100	100
−30	ND[b]	ND	100	100	100
−60	ND	ND	0	0	ND
−90	ND	0	0	0	0

[a] Groups of 10 C3H/TifF mice received LPS in 0.1 ml of PBS intravenously at different times before an intravenous injection of D-galactosamine (18 mg/0.1 ml).

[b] ND, Not done.

pletely inhibited the lethal effects of up to 10 μg of LPS, i.e., an amount which in D-galactosamine-treated mice is approximately a 10,000-fold lethal dose. There were no significant differences in the results when pretreatment with LPS was carried out by the intravenous or intraperitoneal route.

The duration of protection was tested in C3H/TifF mice pretreated with 0.1 μg of LPS at different times before a lethal dose of LPS–D-galactosamine (1 μg–18 mg) (Fig. 1). Under these conditions, the mice were protected against the challenge from 60 min to 48 h after the pretreatment. Pretreatment with higher amounts of LPS (1 μg) did not significantly prolong the duration of protection. However, with lower amounts of LPS a shorter period of protection was seen. Thus, pretreatment with 0.02 μg of LPS protected completely for up to 24 h, and with 0.004 μg protection lasted for about 6 h (data not shown).

Apart from the LPS of *S. abortus-equi*, several other smooth (*S. minnesota* and *S. typhimurium*) and rough (*S. minnesota* R60 and R595 and *E. coli* EH100 and F515) LPS and free lipid A (natural and synthetic) were tested for protective activity. All preparations were highly active, and 0.1 μg of any preparation administered 90 min to 48 h before the lethal combination of LPS (*S. abortus-equi*; 0.1 μg) and D-galactosamine (18 mg) resulted in 100% protection. In contrast, pretreatment with hydrazine-treated LPS of *S. abortus-equi*, in which the lipid A portion is deacylated, did not induce tolerance when tested at up to 10 μg per mouse. Therefore, we conclude that lipid A is the part of the LPS molecule responsible for induction of this tolerance.

Effect of LPS pretreatment on subsequent lethal challenge with LPS in normal mice. After showing that a single LPS injection evokes tolerance to lethal combinations of LPS and D-galactosamine, we tested whether pretreatment with LPS would also induce tolerance to lethal amounts of LPS in mice not treated with D-galactosamine. Mice were injected with 1 μg of LPS of *S. abortus-equi*, and at different times thereafter (0 to 54 h later) they were challenged with 38 and 100% lethal doses (400 and 800 μg, respectively) of LPS (without D-galactosamine).

In no case could LPS toxicity be inhibited by pretreatment. On the contrary, pretreatment enhanced the sensitivity of mice to LPS, which increased with time and reached a maximum after 18 h. At this time, 400 μg of LPS caused 100% lethality in pretreated mice, compared with 38% in nonpretreated animals. Sensitivity then decreased, but a certain degree of sensitization was still observed at 54 h after pretreatment.

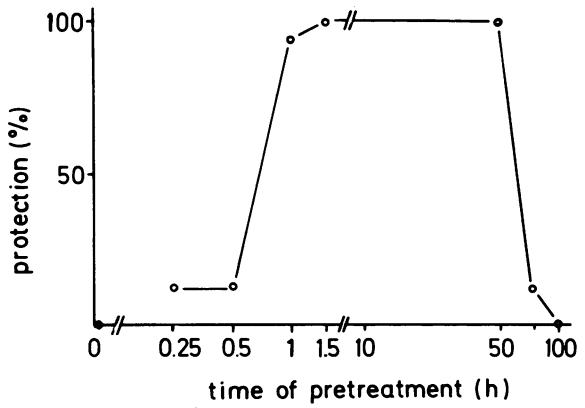

FIG. 1. Duration of LPS-induced tolerance to the lethal effects of LPS and D-galactosamine. Groups of 10 C3H/TifF mice received LPS (0.1 μg) and, at different times thereafter, a lethal combination of D-galactosamine (18 mg) and LPS (1 μg = 1,000 times the 50% lethal dose).

TABLE 2. Induction of lethality and tolerance to LPS in LPS-resistant (C3H/HeJ) mice and requirement for LPS-sensitive (C3H/HeN) macrophages[a]

Intravenous pretreatment		Challenge			% Lethality
Macrophages (C3H/HeN)/ mouse	LPS (μg)	D-Galactosamine (mg)	Macrophages (C3H/HeN)/ mouse	LPS (μg)	
		18	2×10^7	20	100
	20	18	2×10^7	20	100
2×10^7		18		20	100
2×10^7	20	18		20	0
2×10^7	20	18	2×10^7	20	0

[a] C3H/HeJ mice were used. For pretreatment, macrophages (C3H/HeN) were injected intravenously, followed immediately by LPS. For challenge, D-galactosamine was injected intraperitoneally, followed 30 min later by macrophages intravenously and LPS intraperitoneally. The time between pretreatment and challenge was 24 h.

Effect of LPS pretreatment on LPS–D-galactosamine lethality in C3H/HeJ mice. C3H/HeJ mice, because of their lack of LPS-sensitive macrophages, remain resistant to LPS even after D-galactosamine sensitization. For this reason, the effect of LPS pretreatment on sensitivity to LPS cannot be studied in these animals. C3H/HeJ mice, however, acquire a high sensitivity to LPS–D-galactosamine when they receive C3H/HeN macrophages (6). This made it possible to study the effect of LPS pretreatment on endotoxin lethality in these mice. C3H/HeJ mice were injected with LPS (1 to 20 μg) or PBS (controls) and at different times (2 and 24 h) later with D-galactosamine, C3H/HeN macrophages (2×10^7), and lethal amounts of LPS (20 μg). After this treatment, all of the mice died, showing that, in contrast to LPS-sensitive mice, pretreatment with LPS did not induce tolerance in endotoxin-resistant mice.

In the above-described experiment, LPS pretreatment was carried out before the transfer of LPS-sensitive macrophages. To test whether induction of tolerance to LPS–D-galactosamine lethality may proceed in the presence of LPS-responsive macrophages, administration of responsive macrophages was performed before pretreatment with LPS. C3H/HeJ mice were injected with macrophages from C3H/HeN mice (2×10^7 cells per mouse) and treated with LPS (20 μg). At 24 h thereafter, the animals received D-galactosamine and a lethal amount of LPS (20 μg) (Table 2). In controls that received only macrophages, lethality was 100%. In the group pretreated with macrophages and LPS, all animals survived, showing that induction of tolerance by LPS is also a macrophage-dependent phenomenon.

Because LPS-responsive macrophages are required for induction of both tolerance and lethality, it seemed possible that tolerance is due to LPS hyporeactivity of the macrophages which was induced by the first LPS treatment. For this reason, we investigated whether a second transfer of new LPS-responsive macrophages at the time of the lethal challenge would overcome this tolerance. C3H/HeJ mice were made tolerant by injection of C3H/HeN macrophages (2×10^7) and LPS (20 μg). At 24 h later, the mice received new C3H/HeN macrophages (2×10^7) and were challenged immediately with D-galactosamine (20 mg) and LPS (20 μg). Also under these conditions 100% survival was obtained (Table 2), which showed that tolerance could not be overcome by a second macrophage administration.

Induction of lethality and tolerance by macrophages pretreated with LPS in vitro. C3H/HeN macrophages were incubated in vitro with different amounts of LPS (0.004 to 0.5 μg/2 × 10^7 cells) at 37°C for 2 h and extensively washed. For induction of lethality, 2 × 10^7 washed cells were administered to D-galactosamine-treated C3H/HeJ mice. Under these conditions, macrophages preincubated with 0.5, 0.1, 0.02, and 0.004 μg of LPS caused 100, 80, 60, and 0% lethality, respectively (Table 3). In the control group that received macrophages incubated without LPS, no lethality was seen. Absence of lethality was also seen when, instead of LPS-sensitive C3H/HeN, LPS-resistant C3H/HeJ macrophages incubated with LPS (up to 10 μg/2 × 10^7 cells) were administered. When LPS-activated C3H/HeN macrophages (0.5 μg/2 × 10^7 cells) were administered to normal C3H/HeJ mice, they induced tolerance (100% survival) to a lethal challenge with D-galactosamine and LPS-activated C3H/HeN macrophages given 2 to 24 h later. In the control that received unstimulated macrophages, tolerance was not obtained; challenge with D-galactosamine and LPS-activated macrophages at 2 to 24 h later induced 100% lethality. Protection was also not seen when, instead of C3H/HeN, C3H/HeJ macrophages incubated with LPS (up to 10 μg/2 × 10 cells) were used for induction of tolerance.

The actual amount of LPS that was associated with the macrophages and caused lethality or tolerance was measured by using [^{14}C]LPS of *S. abortus-equi*. The macrophages were incubated with radiolabeled LPS and washed as described above. Radioactivity measurements revealed the presence of 0.052 μg of LPS per 2 × 10^7 cells in the macrophages incubated with the highest amount (0.5 μg/2 × 10^7 cells) of LPS (Table 3). This amount was about 200 times lower than that (10 μg) necessary to cause 100% lethality when LPS and macrophages were injected separately into D-galactosamine-treated C3H/HeJ mice.

To see whether macrophages treated once with LPS become tolerant to a second treatment with LPS, cultured macrophages were incubated with LPS (0.5 μg/2 × 10^7 cells) in vitro at 37°C for 2 h and washed. These macrophages caused 100% lethality when transferred into D-galactosamine-treated C3H/HeJ mice (2 × 10^7 per mouse). LPS-activated macrophages lost their ability to induce lethality on further incubation for 18 h without LPS. Such macrophages could be restimulated with LPS (0.5 μg/2 × 10^7 cells) in vitro (37°C for 2 h) and caused lethality in D-galactosamine-treated C3H/HeJ mice.

TABLE 3. Lethal toxicity of C3H/HeN macrophages treated with LPS in vitro in D-galactosamine-sensitized C3H/HeJ mice[a]

LPS (μg)/2 × 10^7 macrophages[b]	% Lethality
0.5 (0.052)	100
0.1 (<0.01)	80
0.02[c]	60
0.004[c]	0
Control macrophages	0

[a] C3H/HeN macrophages were incubated with [^{14}C]LPS as described in Material and Methods and injected intravenously (2 × 10^7 macrophages per mouse) into C3H/HeJ mice treated 1 h before with D-galactosamine (20 mg per mouse intraperitoneally).

[b] The values in parenthesis are micrograms of [^{14}C]LPS associated with macrophages after incubation and washing, determined by radioactivity measurement.

[c] Radioactivity was not detectable.

DISCUSSION

In a previous study, it was shown that D-galactosamine induces a transient state of hypersensitivity to LPS that lasts for about 3 h after its administration (9). Lethality was not seen, however, when the LPS was injected 1 to 2 h before D-galactosamine, even when large amounts of LPS were used. This finding was unexpected because it was found earlier that in rats the LPS preparation used here (*S. abortus-equi*, triethylamine salt) has a long clearance time and that its toxicity in vivo remains unaltered (5, 7). The long persistence of the LPS in the circulation and the absence of detoxification were shown in this study to be true also for mice. It was therefore difficult to understand why injection of, e.g., 10 μg of LPS 90 min before D-galactosamine was without lethal effect although at the time of D-galactosamine treatment toxic LPS in amounts several thousandfold in excess of a lethal dose were present in the circulation.

The present data provide evidence that the above-described absence of lethality is due to the development of tolerance; i.e., LPS administered before D-galactosamine induces tolerance which protects the animals from the enhanced lethal toxicity of the LPS that circulates at the time of D-galactosamine treatment. The tolerance-inducing effect of LPS pretreatment was high enough to protect mice from the lethal effects of a second LPS administration together with D-galactosamine. Tolerance was established at 60 to 90 min after LPS pretreatment and lasted for up to 48 h, depending on the dose used for pretreatment. Thus, the tolerance obtained with 0.004, 0.02, and 0.1 μg of LPS lasted for approximately 6, 24, and 48 h, respectively. Pretreatment with still higher amounts did not prolong the state of tolerance.

In addition to the LPS of *S. abortus-equi*, tolerance was obtained by pretreatment with a number of other S- and R-form LPS and free lipid A. Deacylation of LPS, which is known to abolish its toxic activity (8), abolished its ability to induce tolerance. This is evidence that, like many other activities of LPS (14), induction of tolerance is a function of lipid A.

It has long been recognized that administration of LPS to experimental animals and humans renders them tolerant to a second LPS challenge. Two types of tolerance were recognized: early- and late-phase tolerance (for a review, see reference 17). The tolerance described here has some characteristics of early-phase tolerance, being transient and showing no LPS specificity. In contrast to early tolerance, however, it develops much faster, within 1 h instead of 1 to 4 days. It should be stressed, however, that a direct comparison with classical endotoxin tolerance cannot be made because the lethality which is inhibited here is not due to LPS alone but to a combination of LPS and D-galactosamine. It was shown earlier that the mechanisms of LPS toxicity and D-galactosamine sensitization are distinct and proceed independently. Therefore, suppression of either mechanism would lead to tolerance.

LPS pretreatment induced tolerance in a variety of mouse strains (data not shown). It failed to do so, however, in LPS-resistant C3H/HeJ mice. This was demonstrated in D-galactosamine-treated C3H/HeJ mice administered LPS-responsive macrophages which made them sensitive to the lethal activity of LPS. Pretreatment of such mice with LPS before D-galactosamine and macrophage administration did not protect them to a subsequent lethal challenge with LPS. If LPS-sensitive macrophages were administered at the time of LPS pretreatment, the mice were completely protected.

Therefore, induction of tolerance and induction of lethality by LPS are both mediated by macrophages.

Direct interaction of LPS with macrophages is the first step in the initiation of both activities. C3H/HeN macrophages incubated with LPS in vitro induced lethality in C3H/HeJ mice when injected after D-galactosamine and tolerance when injected before D-galactosamine, in both cases without further in vivo administration of LPS being necessary. From this it is evident that although in vivo LPS interacts with different cells (granulocytes, platelets, hepatocytes, etc.) its interaction with macrophages would alone suffice to cause lethality or tolerance. The fact that tolerance may be established by macrophages treated with LPS in vitro without necessitating the presence of LPS in intact animals suggests strongly that LPS tolerance is effected by macrophage mediators.

The mechanism of protection by LPS pretreatment from the enhanced toxicity of LPS in D-galactosamine-sensitized mice is not known. One possibility is that tolerance is related to an unresponsiveness to the macrophages to LPS induced by the pretreatment. Macrophages obtained from tolerant animals or after incubation with LPS in vitro were repeatedly shown to be hyporesponsive on a second challenge with LPS (4, 20–22, 25). On the other hand, in the present study it was shown that LPS-stimulated macrophages that are lethal for recipient mice lose their toxicity upon further in vitro cultivation (18 h) under LPS-free conditions; such macrophages, after a second LPS addition, again caused lethality when injected into D-galactosamine-treated C3H/HeJ mice, a finding that does not indicate that tolerance is due to unresponsiveness of macrophages. In any case, unresponsiveness of macrophages induced by LPS pretreatment could not alone explain tolerance. This is shown by the finding that administration of new toxic macrophages to tolerant animals did not overcome the tolerance, proving that the enhanced toxicity of macrophage mediators was not expressed in D-galactosamine-treated tolerant mice. Support for this was obtained recently by showing that mice made tolerant to LPS are also tolerant to the lethal effect of TNF and D-galactosamine (C. Galanos and M. A. Freudenberg, manuscript in preparation).

The tolerance obtained by LPS pretreatment may result from suppression of the response of the LPS-mediator targets (true tolerance) or from inhibition of sensitization by D-galactosamine. In the former case, LPS pretreatment should induce tolerance not only in sensitized mice but also in nonsensitized ones. In the latter case, tolerance would be restricted only to the enhanced toxicity of mediators and would be evident only in D-galactosamine-sensitized mice.

This could in fact be the case here. LPS-pretreated mice tolerated amounts of LPS that are lethal only in combination with D-galactosamine (0.01 to 10 μg) but not amounts that are lethal in nonsensitized animals (400 μg). In the latter case, LPS pretreatment somewhat increased the sensitivity of normal mice to endotoxin during the 48-h period after pretreatment, which is in agreement with an earlier finding (16).

In conclusion, induction of tolerance by LPS pretreatment may result from unresponsiveness of the macrophages to LPS, from unresponsiveness of the host to an LPS-induced macrophage mediator(s), or from inhibition of the sensitization by D-galactosamine. Each of these mechanisms or a combination of them may be involved.

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