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Lipid A-Induced Tolerance and Hyperreactivity to Hypothermia in Mice

G. GORDON GREER AND ERNST TH. RIETSCHEL
Max-Planck-Institut fuer Immunbiologie, D-78 Freiburg, West Germany

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Mice responded to lipopolysaccharide (LPS) with a dose-dependent, monophasic hypothermia reaching a maximum at 2 h postinjection. Degraded polysaccharide was not active; free lipid A, however, induced a similar pattern of hypothermia, indicating that the hypothermic principle of LPS was embedded within the lipid A component. The hypothermic response of mice to LPS was modified by prior exposure of the host to LPS. This altered reactivity was manifested by refractory periods (early and late tolerance), in which animals no longer responded with hypothermia, or a hyperreactive phase (hypersensitivity), in which hypothermic responses were greatly augmented upon LPS challenge. Thus, tolerance observed 24 h after a single injection of LPS (early tolerance) was followed, on further LPS challenge, by an enhanced hypothermic response reaching a maximum on day 4. Further daily exposure of the animals to LPS eliminated hyperreactivity and led to the establishment of a late tolerance maximally expressed on day 8. Hyperreactivity could also be evoked on day 4 after a single injection of LPS. Mice pretreated with *Salmonella* S- and R-form LPS or free lipid A (*Salmonella*) demonstrated tolerance and hyperreactivity to both homologous and heterologous challenge. In addition, complete cross-tolerance was observed with S-form LPS derived from *Shigella*. It was concluded that the differential effects of LPS on host responses (tolerance and hyperreactivity) were due to lipid A.

Bacterial lipopolysaccharides (LPS, endotoxin) produce numerous, diverse pathophysiological effects in susceptible experimental animals and humans. The degree of host susceptibility, however, can be altered by prior exposure to LPS. Thus, LPS pretreatment renders the host under certain experimental conditions either refractory (1, 2, 6, 8–10, 12, 13, 19, 25, 26, 30–32, 34, 35, 37, 39, 41, 42, 45, 47, 50) or more sensitive (6, 7, 8, 17, 18, 29, 33) to subsequent LPS challenge. These opposing effects have been commonly referred to as endotoxin tolerance and hypersensitivity (8), respectively.

Tolerance has been established by either single (1, 12, 26, 32, 34, 37, 45, 50) or multiple (2, 8, 9, 10, 13, 30, 31, 39, 42, 47) injections of LPS. Tolerance induced by a single LPS injection is demonstrable 24 h postinjection and has been termed early tolerance. It has been observed between 0-antigenically distinct LPS (lack of "interendotoxin specificity"), cannot be transferred with serum from early-tolerant animals, and thereby appears not to be mediated by antibodies (26).

Tolerance induced by a series of daily injections of LPS or free lipid A appears to be mediated by antibodies, since this form of tolerance (here referred to as late tolerance) can be transferred by antiserum (4, 5, 8, 11, 14–16, 27, 30, 34, 40, 44). The question as to the specificity of these immunoglobulins, however, remains controversial. Although late tolerance induced by multiple injections of LPS is usually maximally expressed against homologous challenge (25, 28), cross-tolerance has also been demonstrated in many relevant studies (1, 10, 12, 19, 31, 35, 40, 42, 47). In this regard, antibodies specific for each of the three main regions of the LPS molecule, the 0-specific chain (25, 27), the core oligosaccharide (4, 5, 6, 11), and lipid A (26, 30, 31, 39, 40, 47) have been postulated as mediators of late tolerance.

In contrast to tolerance, other investigators have shown that prior injection of sublethal quantities of LPS resulted in enhanced susceptibility (hypersensitivity) to subsequent LPS challenge (4, 4, 8, 17, 18, 29). Immunological hypersensitivity has been implicated as a major mechanism for LPS hyperreactivity (4, 8, 29). Again, the three main regions of LPS (4, 29, 33), as well as LPS-associated protein (17, 18), have been implicated as the determinants relating to sensitization.

The present study was undertaken to develop

a model in mice that would define the LPS determinant(s) required for the induction of tolerance and hyperreactivity. In the present paper, using LPS (lipid A) to induce hypothermia in mice as a test system, it will be shown that all three phases of altered host reactivity, early tolerance, hyperreactivity, and late tolerance, can be provoked by and are due to lipid A.

MATERIALS AND METHODS

LPS, degraded polysaccharide, and free lipid A. LPS from the *Salmonella minnesota* Re mutant R595 was isolated by the phenol-chloroform-petroleum ether method (22) and from S-form strains (*Shigella flexneri* 5b, *Salmonella typhi*) by the phenol-water procedure (49). Electrodialyzed (21) free lipid A (triethylamine salt) from the LPS of *S. minnesota* R345, which was free of 2-keto-3-deoxyoctulosonic acid and protein (<0.1%), and degraded polysaccharide from *Salmonella typhimurium* (S form) were gifts kindly furnished by C. Galanos.

Mice and injection procedure. The majority of experiments used female mice of the outbred NMRI strain (Hannover). For comparative purposes, female mice of the inbred strains C57/Bl, DBA, and Balb/c and male mice of the inbred strain C3H/HeJ (Jackson Labs) were also used. All mice were 6 to 8 weeks of age (25 to 30 g) when injected and were housed in groups of five per cage under a controlled environmental temperature (21 ± 0.5°C).

LPS and free lipid A were dissolved in pyrogen-free distilled water by heating and ultrasonic treatment. LPS solutions were diluted with phosphate-buffered saline (PBS, pH 7.2) to the desired concentration. Injections were made via the tail vein in a total volume of 0.2 ml, if not otherwise stated.

Temperature measurements. Rectal temperatures were measured after insertion of a thermocouple (2-mm diameter, Atmos, Lenzkirch, West Germany) to a depth of 1 cm. Before LPS (or PBS) injection, temperatures were recorded and further measurements were made at hourly intervals up to 5 h postinjection. The change in temperature (ΔT, °C), relative to preinjection temperature, was computed, and the results were expressed as ΔT(h), plus or minus one standard deviation. The number in brackets (h) refers to the time (hours) of maximal temperature change postinjection.

Statistical analyses. Student's *t* test was used to determine whether hypothermic responses in tolerant and hyperreactive mice were significantly different from those of normal mice.

RESULTS

Induction of hypothermia by LPS and free lipid A. Groups of NMRI mice (normal rectal temperature, 37.2 ± 0.5°C) were injected intravenously (i.v.) with graded doses of *S. minnesota* Re (R595) LPS. The animals responded as shown in Fig. 1 with a dose-dependent, monophasic hypothermia reaching a maximum at 2 h postinjection [ΔT(2) = −1.5 ± 0.6°C after 10 μg of LPS]. When the 2-h response was plotted against the log of the LPS dose (0.1 to 100 μg), a linear dose-response relationship was observed (Fig. 1). Hypothermia was also seen after intraperitoneal administration of LPS. Here, however, doses greater than 10 μg had to be applied to elicit a hypothermic response. Intraperitoneal application of 100 μg of LPS led to a monophasic hypothermia with a maximum at 2 h postinjection [ΔT(2) = −1.9 ± 0.9°C]. A similar pattern of hypothermia was obtained with i.v. applied graded doses of S-form LPS (*S. typhi, S. flexneri*) and free lipid A (*Salmonella*). Thus, with 10 μg of these preparations, the hypothermic responses were ΔT(2) = −1.2 ± 0.6°C (*S. typhi* LPS), ΔT(2) = −1.7 ± 0.6°C (*S. flexneri* LPS), and ΔT(2) = −1.4 ± 0.5°C (free lipid A [*Salmonella*]). Degraded polysaccharide (derived from LPS of *S. typhimurium*), however, was unable to induce hypothermia [100 μg; ΔT(2) = 0.0 ± 0.3°C].

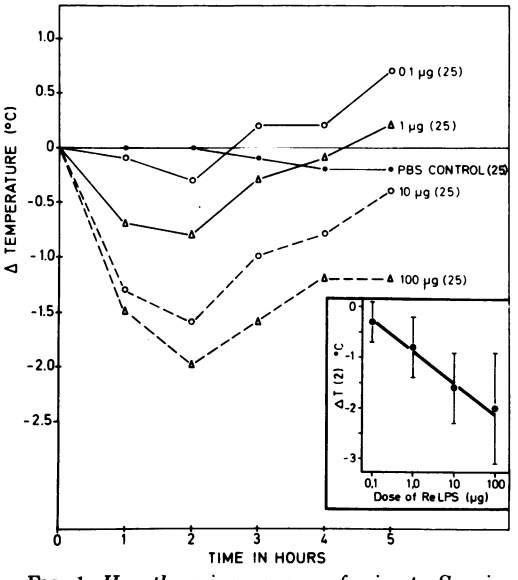

FIG. 1. *Hypothermic response of mice to S. minnesota Re LPS. Mice were injected i.v. at zero time with the indicated doses of LPS, and the change in temperature relative to preinjection (ΔT°C ± 1 standard deviation) were recorded. Numbers in parentheses refer to the number of mice used.*

Induction of early and late tolerance and hyperreactivity to hypothermia. Groups of mice were injected (i.v.) daily on days 0 to 8 with Re LPS (10 μg/day), and the hypothermic responses were recorded (Fig. 2). After the first injection [day 0, ΔT(2) = −1.5 ± 0.5°C], the animals were refractory to a second LPS challenge the following day [day 1; ΔT(2) = +0.3 ± 0.4°C; $P < 0.0005$ compared to day 0 response].

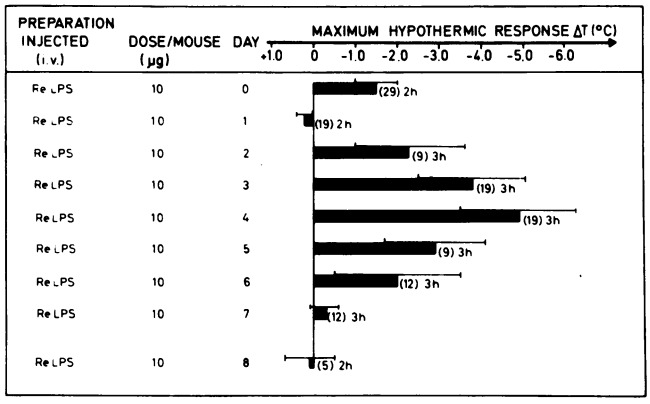

FIG. 2. *Hypothermic response of mice to daily injections of S. minnesota Re LPS. Mice were given repeated injections (i.v.) of LPS (10 μg) at daily intervals, and the maximum hypothermic response (ΔT°C ± 1 standard deviation) was recorded each day at the indicated times. The numbers in parentheses refer to the number of mice used.*

On further LPS challenge, this early tolerance was followed by an enhanced hypothermic response (hyperreactivity) appearing on day 3 and reaching a maximum on day 4 [$\Delta T(3) = -5.0 \pm 1.6°C$; $P < 0.0005$, compared to day 0 response]. As compared to the 2-h maximum of hypothermia observed on day 0, the maximum hypothermic response on day 4 occurred at 3 h postinjection. Further daily exposure of the animals to LPS eliminated hyperreactivity and led to the establishment of a late tolerance, which was seen on day 7 and which was maximal in this experiment on day 8 [$\Delta T(2) = +0.1 \pm 0.7°C$; $P < 0.0005$, compared to day 0 response]. A similar pattern of altered host reactivity was observed after intraperitoneal injection of LPS (100 μg/day, data not shown).

Also, the hypothermic response of mice after two spaced injections of Re LPS was investigated. Groups of mice were injected on day 0 with Re LPS (10 μg) and given a second injection (10 μg) on days 1 through 10. Early tolerance, observed on day 1, was found to wane by day 2 and be lost by day 3 (Fig. 3). This transient early tolerance was followed by a period of increased responsiveness beginning on day 3 and being maximally expressed on day 4 ($P < 0.0005$, as compared to day 0 response). In contrast to the 2-h maximum of hypothermia observed on day 0, the maximum response on day 4 was at 3 h postinjection. This period of hyperreactivity could be demonstrated until day 6. By day 9, mice responded to challenge like normal, nonpretreated animals.

Early tolerance. (i) General. Mice were pretreated on day 0 by an i.v. injection of PBS or Re LPS (10 μg). Twenty-four hours after this pretreatment, these mice were challenged i.v. with Re LPS (10 μg). The temperature responses observed are shown in Fig. 4. While PBS pretreated (day 0) control animals responded with an expected hypothermia to LPS challege on day 1 [$\Delta T(2) = -1.5 \pm 0.9°C$], mice pretreated (day 0) with LPS were refractory to challenge the following day (day 1) [$\Delta T(2) = +0.3 \pm 0.3°C$] . It should be stressed that although tolerant mice were refractory to hypothermia, they did respond, in fact, with a certain degree of fever (Fig. 4 and 5). This was particularly evident in animals pretreated (day 0) with a 100-μg dose of Re LPS, which responded to a 10-μg dose of LPS (day 1) with a significant fever [$\Delta T(2) = +1.4 \pm 0.8°C$] (Fig. 5).

(ii) Dose dependency. To investigate the effect of dose on the degree of early tolerance, the pretreatment dose (day 0) of LPS was altered from 0.01 to 100 μg, keeping the challenge dose (day 1) constant (10 μg). In this experiment a direct relationship was observed between the pretreatment dose and the extent of early tolerance (Fig. 5). This effect was linear in the range of the log of pretreatment doses from 0.01 to 100 μg (Fig. 5).

In another experiment the challenge dose (day 1) was altered from 1 to 100 μg, while keeping the pretreatment dose (day 0) constant at 0.1 μg (Fig. 6). Although complete tolerance to 1 μg was obtained, it was overcome by increasing the challenge dose to 10 and 100 μg, respectively. This effect was linear in the range of the log of challenge doses from 1 to 100 μg (Fig. 6). Pretreated (0.1 μg) mice challenged (day 1) with a 100-μg dose of LPS showed some tolerance 2 h postinjection; their rectal temperature, however, decreased to control values [$\Delta T(4) = -1.7 \pm 0.4°C$] at 4 h postinjection. It is noteworthy that

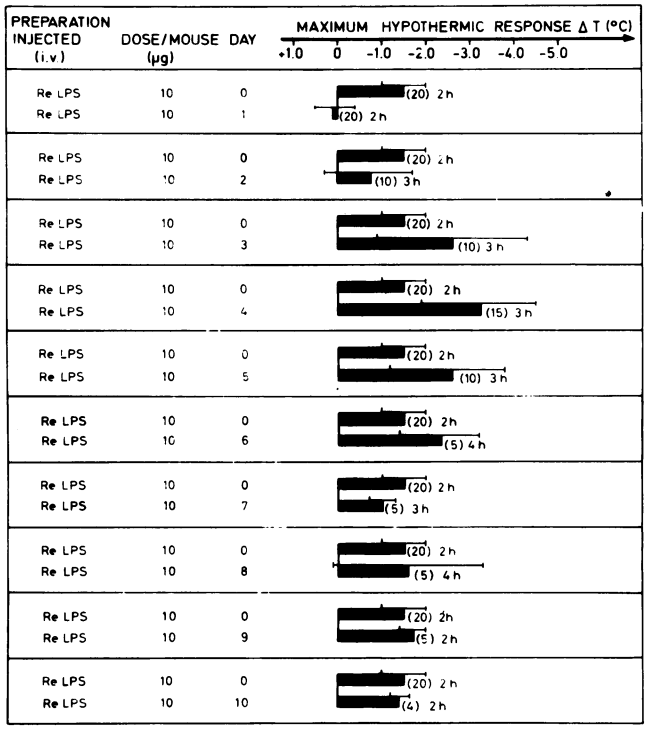

FIG. 3. *Hypothermic response of mice after two spaced injections of S. minnesota Re LPS. Groups of mice pretreated (day 0) with LPS (10 μg) were given a second LPS dose (10 μg) on days 1 to 10, each group receiving two injections (i.v.) only. Results are expressed as the maximum hypothermic response (ΔT°C ± 1 standard deviation) at the indicated times. Numbers in parentheses refer to the number of mice used.*

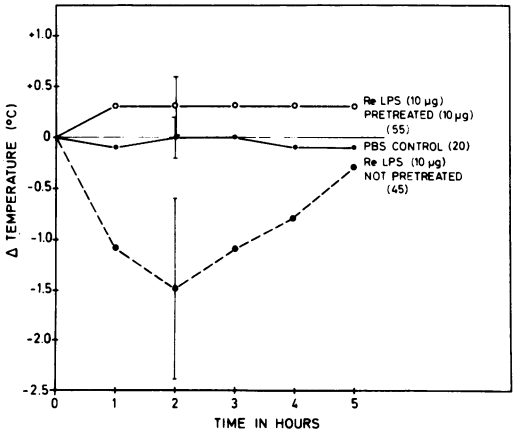

FIG. 4. *Induction of early tolerance after a single injection of S. minnesota Re LPS. Mice were injected i.v. on day 0 with either PBS (not pretreated) or 10 μg of Re LPS (pretreated) and challenged 24 h later (day 1) with 10 μg of Re LPS. The change in temperature relative to preinjection (ΔT°C ± 1 standard deviation) was recorded on day 1 after challenge. The responses of mice injected with PBS only were also included as a control. Numbers in parentheses refer to the number of mice used.*

a LPS dose of 0.1 μg did not produce a significant hypothermic response (day 0), but did induce early tolerance (day 1) (Fig. 6).

(iii) Specificity. In the previously described experiments, LPS from *S. minnesota* Re mutant R595 was used exclusively. To define the determinant of LPS required for the induction of early tolerance, reciprocal cross-tests were performed using free lipid A (*Salmonella*) and Re LPS. It was found (Fig. 7) that free lipid A (10 μg, day 0) induced complete tolerance to free lipid A (10 μg) as well as Re LPS (10 μg) administered on day 1. Also, mice pretreated (10 μg) with Re LPS (day 0) were completely refractory to the hypothermic effect of free lipid A (10 μg) injected on day 1. Additional experiments showed that degraded polysaccharide, which was not hypothermic (100 μg), was also unable to induce early tolerance to Re LPS challenge (10 μg) [ΔT(2) = −1.0 ± 0.6°C in polysaccharide pretreated mice, ΔT(2) = −1.3 ± 0.9°C in PBS pretreated controls].

Complete reciprocal cross-tolerance was also obtained with free lipid A (*Salmonella*) and S-form LPS of *S. flexneri* (Fig. 7). As expected, early hypothermic tolerance could be achieved

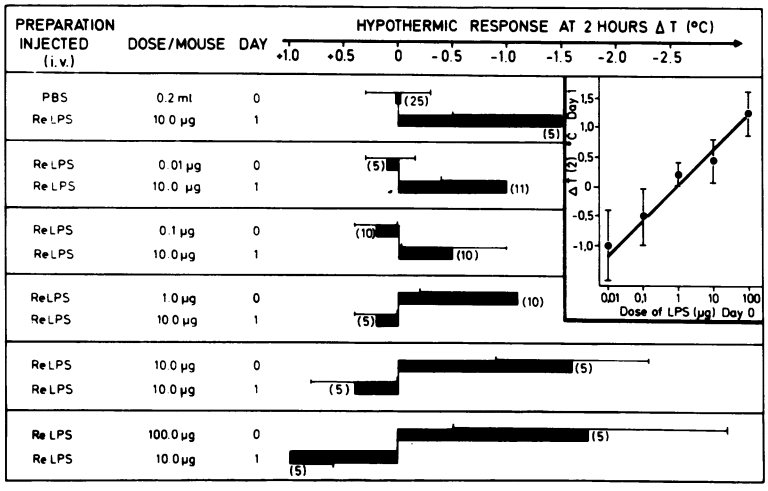

FIG. 5. *Dependency of early hypothermic tolerance to S. minnesota Re LPS on the pretreatment dose (day 0). Mice pretreated (day 0) with PBS or the indicated doses of LPS (i.v.) were challenged (day 1) with LPS (10 µg), and the temperature responses were recorded 2 h postchallenge (ΔT(2)°C ± 1 standard deviation). Numbers in parentheses refer to the number of mice used.*

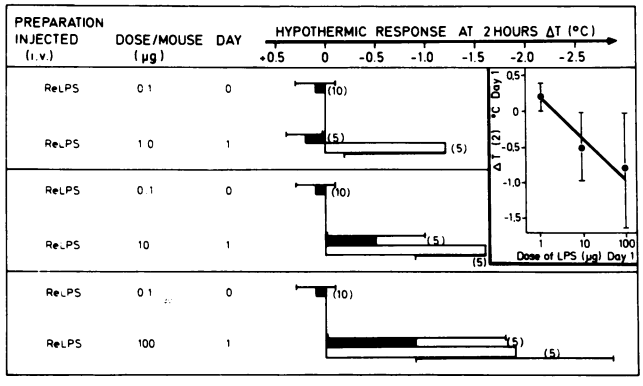

FIG. 6. *Dependency of early hypothermic tolerance to S. minnesota Re LPS on the challenge dose (day 1). Mice pretreated (day 0) with LPS (0.1 µg) were challenged (day 1) with the indicated doses of LPS (i.v.). The response on day 1 of pretreated mice (■) was compared to that of non-pretreated mice (□) 2 h postchallenge (ΔT(2)°C ± 1 standard deviation). Numbers in parentheses refer to the number of mice used.*

with *Shigella* S-form LPS to homologous and heterologous challenge (Fig. 7).

In total, these findings illustrate that lipid A is responsible for the induction of early tolerance to hypothermia.

Hyperreactivity. (i) General. It has been shown above (Fig. 2 and 3) that a hyperreactive state to LPS-induced hypothermia, being maximally expressed on day 4, can be achieved by either a series of daily injections (day 0 to 3) or, alternatively, by a single injection of LPS (day 0). In both cases, the hypothermic response on day 4 was significantly greater than on day 0 ($P < 0.0005$). In addition, there was a shift in the hypothermic maximum from 2 (day 0) to 3 h (day 4) postinjection.

Although hyperreactivity could be induced by single or multiple LPS injections, it was more pronounced after daily LPS administration. In the following experiments, therefore, hyperreactivity was induced by multiple injections (from days 0 to 3).

(ii) Dose dependency. Hyperreactivity in mice sensitized by four successive injections of LPS (Re LPS, days 0 to 3, 10 µg/day) was quantitated by challenging on day 4 with graded doses (0.01 to 100 µg) of Re LPS (Fig. 8). Sensitized animals responded to challenge by all doses with an enhanced hypothermia as compared to non-pretreated controls. Thus, the hypothermic response in sensitized mice to 1 µg of LPS was ΔT(3) = −3.6 ± 1.6°C [compared to ΔT(2) =

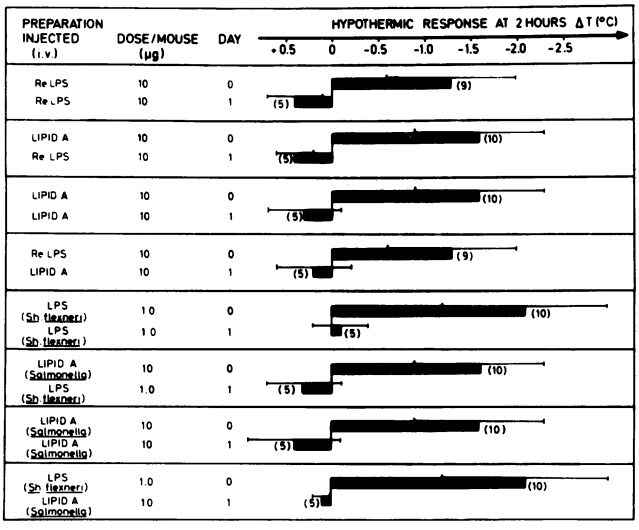

FIG. 7. *Early reciprocal cross-tolerance to hypothermia induced by a single injection of S. minnesota Re LPS, Salmonella free lipid A, or S. flexneri S-form LPS. Mice pretreated i.v. (day 0) with S. minnesota Re LPS (10 μg), Salmonella free lipid A (10 μg), or S. flexneri S-form LPS (1 μg) were subjected to homologous or heterologous challenge with the same doses of LPS or free lipid A (i.v.) on day 1. Results are expressed as ΔT(2)°C ± 1 standard deviation, 2 h postchallenge. Numbers in parentheses refer to the number of mice used.*

−0.8 ± 0.5°C in normal mice]. Further, the hypersensitive response in pretreated (Re LPS, 10 μg) mice was linear with respect to the log of challenge dose in the range of LPS doses from 0.01 to 10 μg (Fig. 8). Increasing the challenge dose to 100 μg did not result in any further increase in responsiveness (Fig. 8).

(iii) Specificity. To establish the specificity of hyperreactivity, reciprocal cross-tests were conducted, using LPS from *S. minnesota* Re and *S. flexneri* as well as free lipid A (*Salmonella*). Mice were sensitized by four daily injections of LPS or free lipid A and subjected to homologous or heterologous challenge on day 4 (Fig. 9). In all cases a significant hyperreactivity was observed, as compared to the response of non-sensitized normal mice (Fig. 9).

Groups of mice were also sensitized by daily injections (days 0 to 3) of S-form LPS (*S. typhi*, 10 μg; *S. flexneri*, 10 μg) and tested on day 4 for their hypothermic response on LPS challenge. The results illustrated in Fig. 10 show that the pretreated mice were hyperreactive to homologous and heterologous challenge.

Collectively, these findings show that lipid A represents the active principle of endotoxin in inducing hyperreactivity to LPS-induced hypothermia.

Late tolerance. (i) Dose dependency. It was shown above (Fig. 2) that daily injections of Re LPS (10 μg) resulted in the development of late tolerance, first appearing on day 7. In some experiments, late tolerance could be demonstrated as early as day 6 or as late as day 9.

The degree of late tolerance was determined by challenging a group of 10 mice pretreated with 13 daily injections of Re LPS (10 μg/day; days 0 to 12) on day 15 with a large dose (1,000 μg) of Re LPS. The tolerant mice responded with a hypothermia comparable to that observed with only 10 μg in non-pretreated mice [ΔT(2) = −1.8 ± 0.6°C in both cases], and their temperatures returned to normal 6 h postinjection. All tolerant animals survived. Non-pretreated control mice did not recover after 1,000 μg of Re LPS, and temperature changes as low as −12°C were recorded before death (24 h postinjection).

(ii) Duration. The group of mice resistant to 1,000 μg of Re LPS on day 15 (see above) was divided into two groups (five mice per group) and challenged with Re LPS (10 μg) on days 18 or 30, respectively. Both groups were found to be completely refractory to hypothermia [ΔT(2) = +0.1 ± 0.3°C), showing that late tolerance persisted for at least 2 weeks after its induction. Thus, late tolerance, produced by repeated LPS injections, is more persistent than early tolerance.

(iii) Specificity. The specificity of late tolerance to hypothermia was examined by injecting mice at daily intervals (day 0 to day 10 or 12) with Re LPS (10 μg/day) or *Salmonella* free lipid A (20 μg/day). Two days after the last

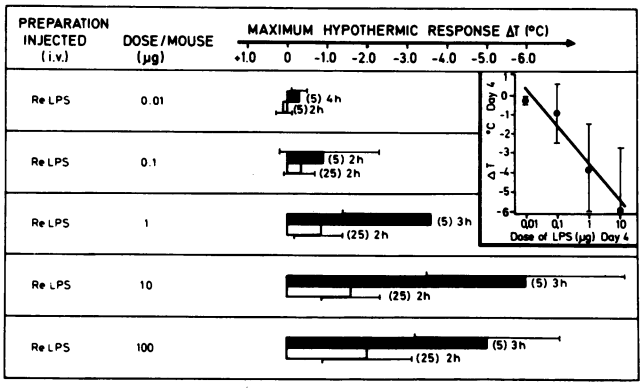

FIG. 8. *Dependency of hyperreactivity to S. minnesota Re LPS-induced hypothermia on the challenge dose. Mice sensitized by four injections (i.v.) of LPS (10 μg/day) at daily intervals were challenged (day 4) with the indicated amounts of Re LPS. The hypothermic response of pretreated animals (■) is compared with that of non-pretreated mice (□). Maximum hypothermic responses (ΔT°C ± 1 standard deviation) were observed at the times indicated. The numbers in parentheses refer to the number of mice used.*

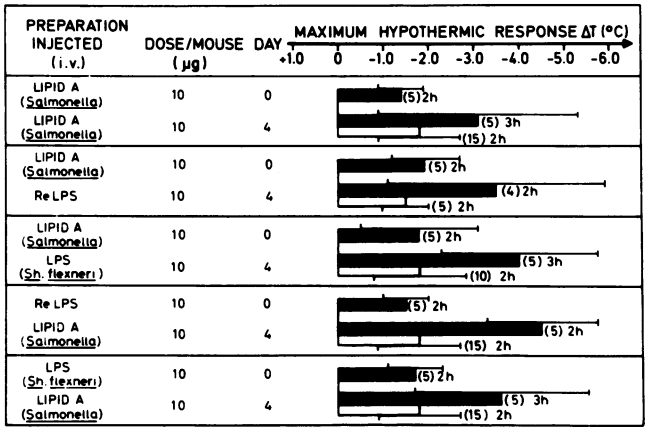

FIG. 9. *Reciprocal cross-hyperreactivity to hypothermia induced by daily injections of S. minnesota Re LPS, free lipid A (Salmonella), and S. flexneri S-form LPS. Groups of mice sensitized by four injections (i.v.) at daily intervals with LPS or free lipid A (10 μg) were subjected to homologous or heterologous challenge (day 4). The response of sensitized animals (■) is compared with that of non-pretreated mice (□). Maximum hypothermic responses (ΔT°C ± 1 standard deviation) postchallenge were observed at the times indicated. Numbers in parentheses refer to the number of mice used.*

injection, the pretreated mice were challenged with Re LPS (10 μg), *Salmonella* free lipid A (20 or 50 μg) or *S. flexneri* S-form LPS (10 μg). Both the free lipid A and Re LPS pretreated mice were tolerant to homologous and heterologous challenge (Fig. 11). Thus, late tolerance induced by repeated injections of LPS is also due to lipid A.

Studies with inbred mouse strains. All experiments described so far had been carried out with outbred NMRI mice. For comparison, inbred strains (C57/Bl, Balb/c, DBA, and C3H/HeJ) were tested for their ability to develop LPS-induced hypothermia, hypothermic early tolerance, and hyperreactivity.

The animals of all strains tested responded similarly in that they developed a dose-dependent hypothermia after i.v. injection of Re LPS. Mice (five per group) of the C57/Bl strain exhibited the following responses to graded doses of LPS 2 h postinjection: 1 μg, $\Delta T(2) = -1.4 \pm 1.2°C$; 10 μg, $\Delta T(2) = -2.2 \pm 2.1°C$; and 100 μg, $\Delta T(2) = -3.8 \pm 0.7°C$.

Five C57/Bl mice were treated with LPS (1 μg) on day 0 and challenged the following day (day 1) with LPS (1 μg). They were found to be refractory to hypothermia ($\Delta T(2) = 0.0 \pm 0.5°C$). On additional challenge (1 μg) on day 2, the pretreated animals responded with an enhanced hypothermia [$\Delta T(3) = -8.0 \pm 0.1°C$]. On day

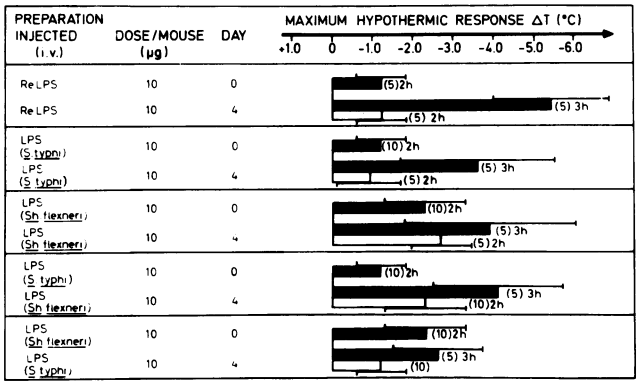

FIG. 10. *Reciprocal cross-hyperreactivity to hypothermia induced by daily injections of S-form LPS (S. typhi and S. flexneri). Groups of mice sensitized by four injections (i.v.), at daily intervals, with S-form LPS (10 μg) were subjected to homologous or heterologous (i.v.) challenge (10 μg) on day 4. The responses of sensitized animals on day 4 (■) is compared with that of non-pretreated mice (□). Homologous hyperreactivity induced with Re LPS was included as a control. Maximum hypothermic responses (ΔT°C ± 1 standard deviation) postchallenge were observed at the times indicated. Numbers in parentheses refer to numbers of mice used.*

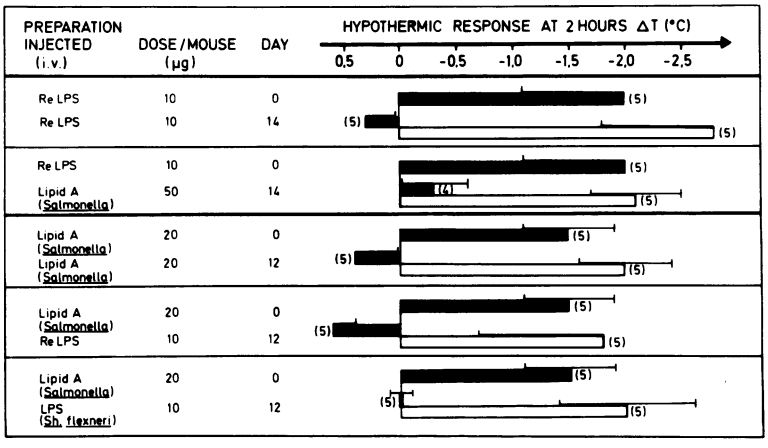

FIG. 11. *Late reciprocal cross-tolerance to hypothermia induced by daily injections of S. minnesota Re LPS or free lipid A (Salmonella). Mice were injected i.v. at daily intervals with S. minnesota Re LPS (10 μg/day) or Salmonella free lipid A (20 μg/day). Two days after the final injection (day 12 or 14), mice were challenged i.v. with Re LPS (10 μg), Salmonella free lipid A (20 or 50 μg), or S. flexneri S-form LPS (10 μg). Results are expressed as ΔT(2)°C ± 1 standard deviation 2 h postchallenge. Numbers in parentheses refer to the number of mice used.*

3, a marked hyperreactivity to 1 μg of LPS was seen [ΔT(3) = −9.0 ± 0.4°C]. The development of late tolerance was not tested in this strain.

A similar pattern of altered host reactivity after LPS treatment (early tolerance and hyperreactivity) was observed also in BALB/c and DBA mice (data not shown). Therefore, the three inbred mouse strains tested responded in a manner similar to that of NMRI mice in that they developed hypothermia, early tolerance, and hyperreactivity.

In contrast, the LPS-resistant mouse strain C3H/HeJ (24) did not respond with hypothermia after Re LPS challenge (10 and 100 μg; ΔT(2) = +0.3 ± 0.3°C in both cases). Further, these animals did not respond with hypothermia after daily injections (days 0 to 4) with Re LPS (10 and 100 μg/day), indicating that they were also refractory with regard to the development of hypothermic hyperreactivity.

DISCUSSION

The results presented here have confirmed the findings of earlier investigators (3, 36, 46) in that parenteral administration of bacterial LPS at normal environmental temperatures (21°C)

induced a dose-dependent hypothermia in mice. These observations were extended to show that lipid A represents the active principle of LPS in this reaction.

The present study revealed that the hypothermic response of mice to LPS could be modified by prior exposure of the host to LPS. This altered reactivity was manifested by two distinct states of refractoriness (early and late tolerance), in which animals no longer responded with hypothermia, as well as with a hyperreactive state, in which hypothermia was greatly augmented. The LPS determinant relating to the induction of these opposing effects was recognized as lipid A.

Two phases of tolerance to hypothermia could be demonstrated: an initial phase (early tolerance), demonstrable 24 h after a single bolus of LPS and a second phase, the induction of which was achieved by a series of daily LPS injections (late tolerance). (The term late tolerance should not be confused with the term late-phase tolerance, which refers to a state of pyrogenic resistance demonstrable in rabbits 6 days after a single injection of immunogenic [trichloroacetic acid-extracted] endotoxin [26].)

The terms refractoriness and tolerance are used to describe the absence of a hypothermic response by pretreated animals to LPS challenge. The terms are not meant, however, to indicate a general refractoriness of pretreated mice. This is emphasized, since it was a consistent finding that animals in the state of early tolerance, i.e., those that had been pretreated with larger doses of LPS (10 to 100 μg, day 0), reacted (on day 1) to LPS with a significant pyrogenic response. It is worthwhile to note that rats, which usually react, like mice, to LPS with hypothermia, respond, when pretreated with LPS, with a fever on days 1 and 2 to a second LPS challenge (43; R. Lützenhoff, H. Fischer, and E. Th. Rietschel, unpublished data).

LPS-induced early tolerance to hypothermia was found to be transient, waning 48 h after a single LPS injection. The degree of early tolerance (day 1) was clearly dependent upon both the pretreatment (day 0) and challenge (day 1) dose, higher doses of LPS (day 0) yielding a higher level of early tolerance (day 1). It is noteworthy, however, that LPS doses (e.g., 0.1 μg) that did not produce a hypothermic response (day 0) nevertheless caused marked early tolerance (day 1, Fig. 6). This indicates that hypothermia is not a necessary prerequisite for the induction of tolerance.

Complete reciprocal cross-tolerance could be shown for early tolerance with different S- and R-form LPS and free lipid A. These results show that the active principle of LPS in inducing early tolerance is embedded within their lipid A component.

The phenomenon of early tolerance has been most intensively studied by Greisman et al. (25, 26) in rabbits, using LPS-induced fever as a test system. These authors showed, that early-phase tolerance is transient, that it exhibits no inter-endotoxin specificity, and that its level is proportional to the LPS dose administered (day 0) for its induction. These conclusions, drawn from experiments on LPS-induced fever in rabbits, were completely confirmed by the present studies on LPS-induced hypothermia in mice.

Little is known of the mechanism of LPS-induced early tolerance. In the rabbit fever system, it seems to be based on an (LPS-induced) inability of hepatic macrophages to release endogenous pyrogen on LPS contact (25). Preliminary experiments in our laboratory show that hypothermia in mice can be induced by prostaglandins E_1, E_2, and $F_{2\alpha}$ and that LPS-induced hypothermia can be (partly) suppressed by indomethacin (G. G. Greer and E. Th. Rietschel, unpublished data). Furthermore, macrophages have been shown to release prostaglandins on LPS and free lipid A contact in vitro (H. Fischer, M. L. Lohmann-Matthes, B. Peskar, D. Suter, E. Th. Rietschel, and M. Weidemann, Eur. Surg. Res., 9:286–287, 1977). Thus, early-phase tolerance could possibly be based on a refractoriness of macrophages to the prostaglandin-provoking effect of LPS and free lipid A. This hypothesis is currently under investigation.

In addition to early tolerance, a later state of hypothermic tolerance was demonstrated in mice that had received a series of daily injections of *S. minnesota* Re LPS or free lipid A (*Salmonella*). It differed from early tolerance in that it was more persistent, being still evident 2 weeks after its induction. Also, in contrast to early-tolerant mice (data not shown), late-tolerant animals were refractory to hypothermia and lethality induced by relatively large doses of Re LPS (1,000 μg). Thus, in late-tolerant mice, a greater level of refractoriness was observed.

Evidence for the specificity of late tolerance was obtained by showing that mice rendered refractory with Re LPS or free lipid A (*Salmonella*) were also resistant to the hypothermic effects of homologous and heterologous challenge with free lipid A, Re LPS, or S-form LPS (*Shigella*). These results support the observations of other investigators, who have reported late cross-tolerance in repeatedly injected animals, and is consistent with the concept that lipid A represents a major determinant of LPS involved in late tolerance induction (31, 39, 40, 47).

In an attempt to define the mechanism of late

tolerance after multiple LPS injections, several workers have noted homologous and a certain degree of heterologous cross-protection transferable by tolerant donor serum (4, 14–16, 27, 30, 40, 44). Thus, heterologous protection has been successively demonstrated by passively transferring tolerance against Shwartzman reactivity (5), intravascular coagulation (6), and lethality (11, 14, 15, 44).

These observations led some workers to conclude that actively induced heterologous tolerance was due to the presence of factors directed against the core-lipid A region of the LPS molecule (4, 5, 6, 28, 30, 44, 47). Support for an immunological mechanism of cross-tolerance was provided by the recent demonstration that passive protection against pyrogenicity and the Shwartzman reaction could be mediated, in part, by lipid A antiserum (40). Whether late tolerance to hypothermia in mice is based on similar mechanisms remains to be elucidated.

Perhaps the most unexpected finding in the current work was the appearance of a phase of marked hyperreactivity to hypothermia being maximally expressed 4 days after multiple injections of LPS or free lipid A. Mice sensitized by a single injection (day 0) also demonstrated a maximal hyperreactivity on day 4, but in contrast to repeatedly injected animals, they exhibited normal hypothermic responses by day 9.

In relation to the specificity of hyperreactivity, the present investigation showed that mice sensitized by four daily injections of S-form LPS (*Salmonella, Shigella*), R-form LPS, and lipid A from *Salmonella* responded to both homologous and heterologous challenge (day 4) with an augmented hypothermic reaction.

These results provide evidence that hyperreactivity to LPS hypothermia is due to the lipid A component. This latter finding extends the earlier proposal that accelerated skin reactivity to LPS in pretreated rabbits was due to factors directed against the toxic component of endotoxins (33).

There are, however, some opposing reports, which have provided evidence for the role of LPS-associated protein (17, 18), the core-oligosaccharide (29), and the 0-specific chain (4) in eliciting hypersensitivity to LPS.

Freedman et al. (17, 18), using S-form LPS, found that a single injection of LPS induced hyperreactivity to LPS (measured by water uptake of mice). This hyperreactivity was seen, however, only if the preparation used for pretreatment contained protein. Since the LPS and free lipid A preparations used in the present study were free of protein, the protein-dependent hypersensitivity observed by Freedman et al. and the lipid A-induced hyperreactivity described here are probably not related.

Subsequent investigations by Kawakami et al. (29) indicated that mice sensitized by infection with S- or R-form bacteria (*Salmonella*) responded with enhanced reactivity (lethality) to challenge with S- and R (Ra)-form LPS. Lethality, however, was not enhanced on challenge with Re LPS. Thus, hypersensitivity in this case appeared to be specific for determinants in the core-oligosaccharide and, therefore, is distinct from the lipid A-provoked hyperreactivity described in this report.

Finally, Davies et al. (11) showed that hypersensitivity to LPS-induced lethality induced by a series of injections of S-form LPS could be passively transferred with homologous antisera and, therefore, appeared to be due to 0-specific antibodies. Since these authors used a different immunization scheme and tested for hypersensitivity to lethality, it is difficult at the present time to compare their results with those presented here. The present finding, however, that marked cross-hyperreactivity to hypothermia in mice can be induced by S- and R-form LPS and free lipid A seems to rule out a possible role of 0-specific humoral factors in this system.

The current observation relative to the lipid A specificity of both tolerance and hyperreactivity to hypothermia is most likely due to the similar structure of the lipid A's of the LPS preparations used. In all cases, lipid A consists of a β1, 6-linked D-glucosamine-disaccharide, which carries (partly substituted) phosphate groups in positions 1 and 4′ and long-chain hydroxylated and nonhydroxylated fatty acids in amide and ester linkage (38). Antibodies against this structure have been induced in a series of experimental animals, including rabbits (23) and mice (20). In addition, lipid A antiserum has been shown to possess the potency of modifying host responses to LPS or free lipid A in that, depending on the experimental conditions and the test system used, it can enhance (20, 48) or suppress (40) endotoxic activities. Whether lipid A-specific humoral factors are involved in the mediation of LPS-induced tolerance and hyperreactivity to hypothermia in mice is not known at the present time and remains to be elucidated.

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This paper is dedicated to Otto Westphal on the occasion of his 65th birthday.

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