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Defective Prostaglandin Synthesis by C3H/HeJ Mouse Macrophages Stimulated with Endotoxin Preparations

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Macrophages obtained from C3H/HeN mice produced significant amounts of prostaglandin E when exposed to phenol-extracted lipopolysaccharides (LPS), whereas macrophages from C3H/HeJ mice were unresponsive. The lipid A fraction from phenol-extracted LPS was an effective inducer of prostaglandin synthesis by macrophages from C3H/HeN mice. The polysaccharide portion of the LPS molecule had no effect. In contrast, the C3H/HeJ macrophages did not produce prostaglandin E in response to the lipid A moiety of phenol-extracted LPS. LPS prepared by butanol extraction stimulated the production of prostaglandin E by macrophages from both C3H/HeN and C3H/HeJ mice. The component of butanol-extracted LPS that stimulated the C3H/HeJ macrophages was shown to be a lipid A-associated protein. Further studies demonstrated a correlation between prostaglandin production by the macrophages of these two strains of mice in response to butanol- and phenol-extracted LPS and the lethal effects of the endotoxin preparations.

Suggestive evidence exists for prostaglandin mediation of some of the biological consequences of endotoxin administration. Thus, inhibitors of prostaglandin synthesis protect animals against some of the toxic effects of endotoxin (4). Furthermore, endotoxin causes increased prostaglandin levels in vivo (19), and some of the activities of endotoxin are similar to those produced by prostaglandins (19). Since the C3H/HeJ mouse strain has been shown to be resistant to the in vitro and in vivo effects of endotoxin, including lethality (8, 22), induction of antibody formation (25), adjuvanticity (3), and in vitro mitogenicity (3, 9, 15, 20, 23, 25, 26), it was of interest to determine whether this strain was also defective in its response to endotoxin-stimulated prostaglandin synthesis.

Resistance appears to be expressed in the lymphoid cells of C3H/HeJ mice, since their B-lymphocytes (3, 9, 25), T-lymphocytes (10), and macrophages (2, 7, 17) are all unresponsive to endotoxin. Macrophages possess the capacity to synthesize prostaglandins when cultured with endotoxin (16, 24). Therefore, these cells from the nonresponsive C3H/HeJ strain and the closely related responsive C3H/HeN strain were examined for their ability to synthesize prostaglandin E (PGE) when stimulated with endotoxin preparations. In addition, we have investigated the relationship between the in vitro production of prostaglandins by these macrophages and endotoxin-induced lethality in vivo.

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MATERIALS AND METHODS

Animals. Normal 6- to 12-week-old C3H/HeJ female mice were obtained from Jackson Laboratories, Bar Harbor, Me., and 6- to 12-week-old C3H/HeN female mice were obtained from the Division of Research Services, National Institutes of Health.

Cell collection and culture. Peritoneal exudate cells were induced by injecting C3H/HeN and C3H/HeJ mice intraperitoneally with 3 ml of 3% thioglycolate broth (National Institutes of Health Media Unit). Six days later the peritoneal cavities of the mice were lavaged with 10 ml of RPMI 1640 (Grand Island Biological, Grand Island, N.Y.). The cells obtained were suspended in RPMI 1640 at a concentration of 5 $\times 10^6$ cells per ml, and 2-ml samples were placed in 35-mm petri dishes. After incubation at 37°C for 3 h, the petri dishes were washed twice with RPMI 1640 to remove the nonadherent cells. The remaining adherent population contained greater than 95% phagocytic cells and were cultured in serum-free RPMI 1640 containing glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). These macrophage cultures were exposed to varying concentrations of lipopolysaccharide (LPS) from *Escherichia coli* K235 prepared by phenol-water (Ph) extraction according to the method of McIntire et al. (11) or by the butanol-water (Bu) extraction method of Morrison and Leive (13). Lipid A was prepared by acid hydrolysis of LPS

(Ph) (17). Lipid A-associated protein (LAP) was prepared by phenol extraction of LPS (Bu) (12) and was the generous gift of David Morrison. Polysaccharide was prepared from *Salmonella typhimurium* by the method of Freeman (5) and was the generous gift of Constantin Bona.

Prostaglandin assay. The harvested culture media were acidified with 0.5 volume of 1 M sodium citrate (pH 3.5) and extracted in 15 volumes of chloroform. The aqueous phase was removed by aspiration, and the chloroform was evaporated under a stream of air. Prostaglandin concentrations in the chloroform extract were determined either by direct assay or after separation of the individual prostaglandin groups by passage through silicic acid columns (1). Radioimmunoassays were performed utilizing specific antibodies to PGE (Miles Laboratory, Elkhart, Ind.). These immunoglobulins bind equally to PGE_1 and PGE_2. The results are presented as PGE_2 equivalents, since this prostaglandin was utilized to derive the standard curve, and the data obtained are representative of several experiments.

Endotoxin-induced mouse lethality. Eight-week-old female C3H/HeJ or C3H/HeN mice were injected intraperitoneally with varying doses of LPS in pyrogen-free saline (five mice per dose). Mice were conventionally housed and maintained on food and water ad libitum. Deaths were recorded daily for 96 h, and the percentage of mortality at each dose was determined.

RESULTS

Prostaglandin production by macrophages exposed to LPS. Macrophages from C3H/HeN mice exposed to LPS (Ph) released significant amounts of PGE_2 into the media, whereas macrophages from C3H/HeJ mice did not (Fig. 1). A threefold increment in prostaglandin production by C3H/HeN macrophages was detected at 1 μg of LPS (Ph) per ml. A 12- and 16-fold enhancement of PGE_2 concentration in the media occurred when 10 and 30 μg, respectively, of LPS (Ph) were added per ml. When these concentrations of LPS (Ph) were added to macrophages from C3H/HeJ mice, no increase in PGE_2 production was detected. Concentrations above 30 μg/ml were not tested because of their cytotoxic effects on C3H/HeN macrophages (7).

Time-course studies revealed that increases in PGE levels of 8- and 40-fold occurred at 24 and 48 h, respectively, after the addition of LPS (Ph) to macrophage cultures (Fig. 2). Media from C3H/HeJ macrophages exposed to LPS (Ph) did not contain increased amounts of PGE_2 within the first 24 h. However, a slight increase was noted at 48 h.

In assessing the stimulatory effect of different LPS preparations, it was noted that the addition of either LPS (Ph) or LPS (Bu) to macrophages from C3H/HeN mice resulted in a similar stimulation of prostaglandin production (Table 1). As noted previously, LPS (Ph) did not increase prostaglandin synthesis by C3H/HeJ macrophages. However, exposure of these cells to LPS (Bu) caused a significant increase in PGE_2, demonstrating that C3H/HeJ macrophages were capable of enhanced prostaglandin production when appropriately stimulated. In both the C3H/HeN and C3H/HeJ macrophage cultures the increase in PGE_2 in response to LPS could be inhibited by indomethacin, an inhibitor of

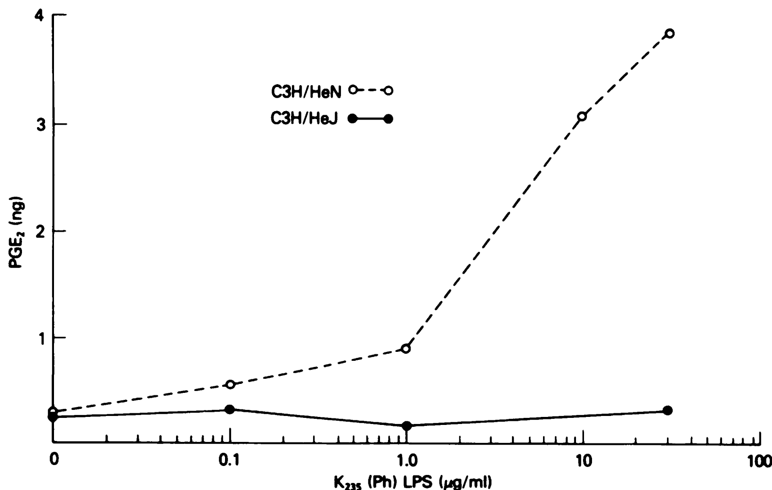

FIG. 1. *Effect of varying concentrations of K235 LPS (Ph) on prostaglandin production by macrophages from C3H/HeN and C3H/HeJ mice. Peritoneal exudate cells were plated at 10^7 cells per 35-mm petri dish in 2 ml of medium. The culture media were harvested 48 h after exposure to endotoxin and assayed for PGE_2. The data are expressed as nanograms of PGE_2 per culture and represent the mean of two determinations from each of the duplicate cultures.*

prostaglandin synthesis.

Stimulation of macrophage prostaglandin synthesis by endotoxin-derived or related components. There are at least three moieties contained within the LPS (Ph) and LPS (Bu) preparations that possess some biological activity. The lipid A moiety is responsible for most of the effects of LPS (16), while the *O*-polysaccharide is responsible for antigenicity but can also protect mice from the effect of lethal X-irradiation (14). In addition, LPS (Bu) contains an LAP that is a B-cell mitogen for murine spleen cells, including those obtained from C3H/HeJ mice (12, 21). These components of the LPS molecule were examined for their ability to induce prostaglandin synthesis by macrophages. Five preparations were tested on C3H/HeN macrophages: LPS (Ph); LPS (Bu); lipid A derived from LPS (Ph); LAP derived from LPS (Bu); and a polysaccharide derived from *S. typhimurium* that has been found to confer protection against lethal irradiation (C. Bona, personal communication). Less prostaglandins were produced by C3H/HeN macrophages in response to lipid A than to the parent LPS (Ph) molecule, especially at lower concentrations (Fig. 3A). However, lipid A was clearly able to stimulate prostaglandin production by macrophages at concentrations above 0.1 μg/ml. In contrast, the polysaccharide fraction was inactive when added at concentrations as high as 500 μg/ml, strongly suggesting that the lipid A moiety of LPS was responsible for macrophage prostaglandin production. The LAP was also a strong stimulator of prostaglandin production (Fig. 3B). However, it was significantly less active than the parent LPS (Bu).

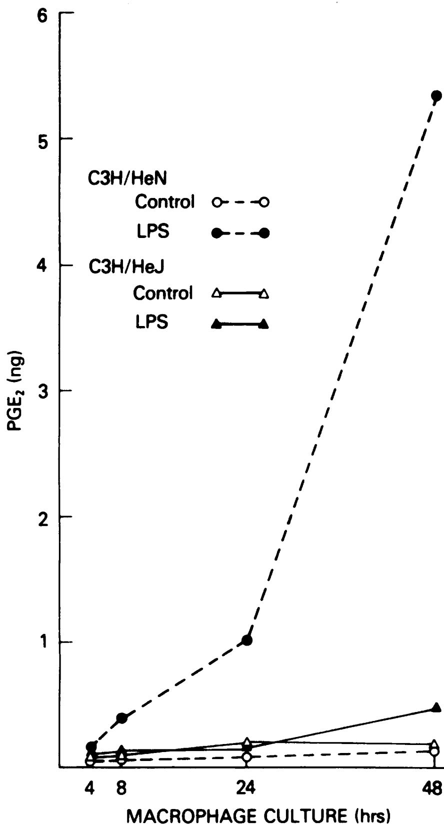

FIG. 2. *Time course of macrophage prostaglandin production in response to K235 LPS (Ph) (30 μg/ml). Peritoneal exudate cells from C3H/HeN and C3H/HeJ mice were plated at 10^7 cells per 35-mm petri dish in 2 ml of medium. The culture media were harvested at the indicated times and assayed for PGE_2. The data are expressed as nanograms of PGE_2 per culture and represent the mean of two determinations from each of duplicate samples.*

These preparations were also added to C3H/HeJ macrophages (Fig. 3C). Neither the LPS (Ph), lipid A, or polysaccharide stimulated C3H/HeJ macrophages to produce prostaglandins. However, both the LPS (Bu) and the LAP were able to stimulate C3H/HeJ macrophages to produce small but significant amounts of prostaglandin. Although much less prostaglandin was produced in these cultures, and higher concentrations of stimulants were required to elicit an effect, these compounds were clearly active on

TABLE 1. *Comparison of endotoxin preparations for their ability to stimulate PGE_2 synthesis in C3H/HeJ and C3H/HeN mice*

Stimulant[a]	Prostaglandin synthesis[b]			
	C3H/HeN	C3H/HeN + indomethacin[c]	C3H/HeJ	C3H/HeJ + indomethacin[c]
None	131 ± 25	210 ± 22	99 ± 49	49 ± 19
K235 LPS (Ph)	1,137 ± 404	144 ± 18	190 ± 21	191 ± 45
K235 LPS (Bu)	964 ± 132	167 ± 47	1,401 ± 73	178 ± 19

[a] LPS at 30 μg/ml of culture media.

[b] Samples of 10^7 macrophages in 2 ml of culture media were plated in 35-mm petri dishes. Culture media were harvested 24 h after exposure to LPS. Results are expressed as picograms of PGE_2 (± standard error) per 10^7 macrophages.

[c] Indomethacin at 10^{-5} M.

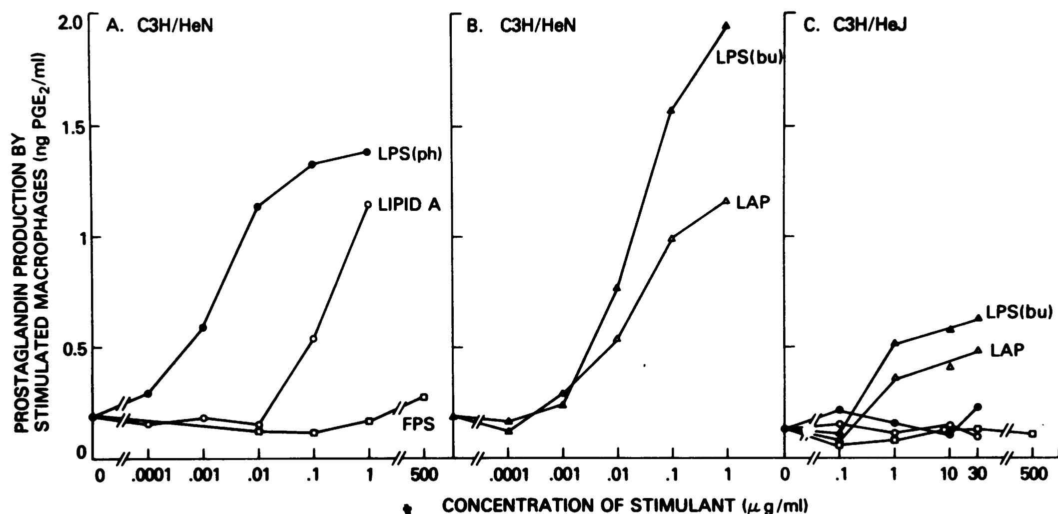

FIG. 3. *Effect of various components of endotoxin on prostaglandin production by C3H/HeN and C3H/HeJ mice. (A) C3H/HeN macrophages were cultured with the indicated concentrations (from 1 to 500 μg/ml) of LPS (Ph), the lipid A derived from LPS (Ph), or PFS. (B) C3H/HeN macrophages were cultured with the indicated concentrations of LPS (Bu) or the LAP derived from it. (C) C3H/HeJ macrophages were tested with all five compounds. Data for all experiments represent the mean of two determinations from duplicate samples. LPS (Ph)* (●); *lipid A* (○); *FPS* (□); *LPS (Bu)* (▲); *LAP* (△).

C3H/HeJ cells.

Induction of mouse lethality by different LPS preparations. If prostaglandin release by macrophages were related to the toxic effects of LPS, LPS (Bu) should be more toxic to C3H/HeJ mice than LPS (Ph). We therefore determined the toxicity of each preparation on C3H/HeJ and C3H/HeN mice. C3H/HeJ mice were not killed by up to 8 mg of LPS (Ph) (Fig. 4). In contrast, 2 mg of LPS (Bu) killed 70% of C3H/HeJ mice, and 100% of these mice were killed by a dose of 4 mg. Thus these findings suggest a possible correlation between macrophage prostaglandin production and endotoxin-induced lethality in vivo.

When these LPS preparations were tested in C3H/HeN mice, LPS (Ph) was more toxic than LPS (Bu) (Fig. 4). One hundred percent of C3H/HeN mice were killed by 250 μg of LPS (Ph), whereas 500 μg of LPS (Bu) was required for a comparable degree of lethality.

DISCUSSION

Murine macrophages are sensitive to a variety of effects of endotoxin in vitro, including direct cytotoxicity (7, 18) and the induction of lymphocyte-activating factor (6). We have demonstrated that endotoxin will also stimulate macrophages to release prostaglandins in vitro. As with most endotoxin-mediated effects, prostaglandin production appears to be due to the lipid A moiety. In addition, macrophages will also release prostaglandins in response to another molecule, LAP, indicating that stimulation of macrophages to produce prostaglandins is not limited to LPS.

We have also found that macrophages derived from endotoxin-unresponsive C3H/HeJ mice do not release prostaglandins in vitro when treated with LPS (Ph). C3H/HeJ macrophages will release increased amounts of prostaglandins in response to LPS (Bu) or LAP, demonstrating that they are capable of being stimulated in vitro. In our hands, prostaglandin production by C3H/HeJ cells has been much more variable than production by C3H/HeN cells (see Table 1, and Fig. 3).

There is considerable evidence linking the release of prostaglandins in vivo to the pathogenesis of endotoxicity. First, prostaglandins are extremely potent vasoactive compounds and can mimic several endotoxin-like activities such as abortion (19). Second, elevated levels of prostaglandins can be measured in endotoxin-treated animals (19). Finally, treatment of experimental animals with inhibitors of prostaglandin synthesis such as indomethacin will protect them against some toxic effects of endotoxin (4). We have recently demonstrated that endotoxin-induced mouse lethality is mediated at least in part by the interaction of endotoxin with lymphoid cells, since C3H/HeJ mice can be rendered sensitive to this effect by the adoptive transfer of C3H/HeN spleen cells (8). The finding that

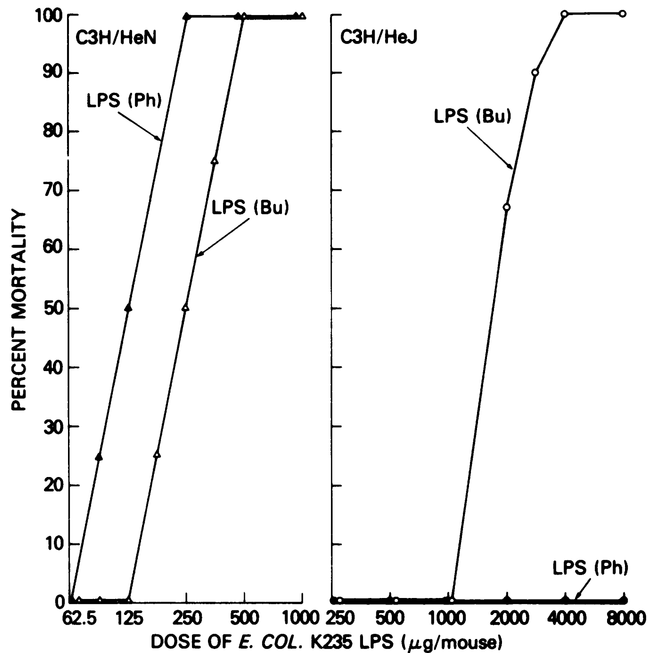

FIG. 4. *Lethal effects of LPS (Ph) and LPS (Bu) in C3H/HeN and C3H/HeJ mice. Mice were injected with the indicated amounts of endotoxin, and mortality was determined daily for 96 h. Results represent the pooled data from three individual experiments with a total of 16 mice of each strain tested at each endotoxin concentration.*

endotoxin will induce prostaglandin synthesis by C3H/HeN macrophages but not by C3H/HeJ macrophages therefore suggests one possible mechanism for the induction of endotoxin-induced lethality. That is, endotoxin activates macrophages and induces prostaglandin synthesis. The released prostaglandins, in turn, induce hypotension, hypothermia, and dehydration, all of which may contribute to the process that ultimately results in death.

The finding that LPS (Bu), which is capable of inducing prostaglandin release from C3H/HeJ macrophages, is also significantly more toxic to this mouse strain than LPS (Ph) is consistent with this hypothesis. Thus, although it is unlikely that macrophage prostaglandins are the only mediators of endotoxicity, the findings presented in this report suggest that they play a significant role in this biological effect.

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