Endotoxin-Resistant Mice Are Protected from PAF-Induced Bowel Injury and Death: Role of TNF, Complement Activation, and Endogenous PAF Production

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C3H/HeJ (endotoxin-resistant) mice have been used widely in biological research. However, the mechanism of endotoxin (LPS) resistance is only partially understood. In this study, we first investigated the differences in response to PAF, a mediator of endotoxin shock, between normal and C3H/HeJ mice. We found that in control mice, PAF (2.5 μg/kg) caused shock, hemoconcentration, complement activation, intestinal hypoperfusion, and necrosis with 75% mortality, whereas all C3H/HeJ mice survived, without complement activation or intestinal injury, and manifesting only mild hypotension and hemoconcentration. PAF also caused elevated serum TNF-α in some control mice but not in C3H/HeJ mice. We also observed that PAF induces endogenous PAF production and intestinal phospholipase A2 activation in normal mice, whereas PAF production and phospholipase A2 are suppressed in C3H/HeJ mice. The low endogenous PAF production may account, at least in part, for the resistance to LPS and PAF of C3H/HeJ mice.

KEY WORDS: PAF (platelet-activating factor, paf-acether), intestine, endotoxin, TNF (tumor necrosis factor).

Endotoxin shock is a serious clinical condition of grave prognosis. Several pivotal mediators may be responsible for the adverse response to endotoxin, among which PAF (platelet-activating factor, PAF-acether) (1) and tumor necrosis factor-α (TNF) (2–4) have become the focus of considerable interest. Both PAF and TNF have been detected in the sera of patients with septic shock (5, 6) and in animals injected with lipopolysaccharide (LPS) (4, 7). Administration of PAF (8) or TNF (9) induces symptoms and signs of septic shock in animals. Pretreatment with PAF antagonists (10) or anti-TNF (11) prevents LPS-induced shock and improves survival. We have previously shown that PAF (12, 13) or TNF with LPS (14, 15) induce intestinal injury in rats and mice and that bowel injury induced by LPS (16) or TNF with LPS (13, 14) is probably mediated by endogenous PAF formation. Furthermore, the adverse effects of TNF and LPS were largely prevented by pretreatment with PAF antagonists (13, 14), suggesting that endogenous PAF production may be the final common pathway in endotoxin shock.

Phospholipase A2 is one of the key enzymes involved in PAF synthesis (17). It catalyzes deacylation of the phospholipid, 1-alkyl-2-acyl-phosphocholine to form the intermediate, lyso-PAF, which is subsequently acetylated by an acetyl-CoA-dependent acetyltransferase to form PAF (17). We have shown that PAF induces its own production in vivo (18), and...
the endogenous PAF production seems to involve, at least in part, activation of phospholipase A₂ (19).

The LPS-resistant C3H/HeJ mice are hypersensitive to LPS in vivo (20) and at the cellular level (21). Despite years of usage of C3H/HeJ mice in biological research, the mechanism of their resistance to LPS remains elusive. It has been shown that LPS fails to elicit synthesis of interleukin-1 (22) in C3H/HeJ macrophages and that C3H/HeJ mice do not produce interferon (23) or colony-stimulating factor (24) following endotoxin administration. More importantly, C3H/HeJ macrophages have recently been shown to be incapable of producing TNF-α (25), a pivotal mediator of endotoxin shock (reviewed in references 2–4). Given the close interaction of PAF and TNF, it is important to examine the role of PAF in LPS resistance in these mice. In the first part of this study, we demonstrated that C3H/HeJ mice are also hypersensitive to PAF. Since one of the mechanisms for the PAF effect in vivo was mediated by endogenously produced PAF (18, 19), we then examined the PAF levels in C3H/HeJ mice in response to PAF. Lastly, we compared the activity of intestinal PAF-forming phospholipase A₂ between LPS-resistant mice and normal mice.

**MATERIALS AND METHODS**

**Materials.** Stock solution of PAF (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine, Sigma Chemical Co., St. Louis, Missouri) (2 mg/ml) was stored in aliquots. Working solutions were made fresh daily. 1-O-Hexadecyl-2-[³H]arachidonoyl-3-phosphorylcholine (74.3 Ci/mmol) was purchased from New England Nuclear, Boston, Massachusetts. 1-O-Hexadecyl-2-arachidonoyl-3-phosphocholine was obtained from Biomol. Res. Lab. Plymouth Meeting, Pennsylvania. SRI-63-441, a PAF antagonist, was a gift from Dr. D. Handley, Sandoz Research Institute, East Hanover, New Jersey. Total complement (CH100) test kits were purchased from Kallestad Laboratories, Austin, Texas. Human rTNF was a gift from Genentech, South San Francisco, California. Six- to 10-week-old male C3H/HeN (normal control) mice were obtained from Harlan Sprague Dawley, Indianapolis, Indiana. LPS-resistant (C3H/HeJ) mice and C3H/HeOUJ (control) mice of the same age were purchased from Jackson Laboratory, Bar Harbor, Maine.

**Animal Experiments.** Paired control (C3H/HeN or C3H/HeOUJ) and LPS-resistant (C3H/HeJ) mice were anesthetized with Nembutal (65 μg/g, intraperitoneally) and placed on warm pads. The carotid artery was catheterized with PE10 tubing for continuous blood pressure recording, blood sampling, and injection. The first part of the study was to examine the effect of a lethal dose of PAF (2.5 μg/kg). Two hours following PAF injection or immediately before death, blood was drawn from the catheter, and 0.25 ml of Evans blue (2%) was injected intravenously, and the percentage of small intestine perfusion was determined as previously described (13). Sections from unperfused areas of the intestine were fixed in formalin for subsequent histological examination. Hematocrit (Hct) was determined and serum samples were stored at −70°C for TNF and CH100 assays.

The second part of the study aimed to examine endogenous PAF production and intestinal phospholipase A₂ activity. Mice were divided into three groups: sham-operated, PAF (2 μg/kg, intraarterially), and PAF (1.5 μg/kg). At the end of experiment (1.5 hr after first injection), plasma was collected for PAF assay, and the small intestine was removed, washed with cold saline, weighed, and homogenized in 10 vol of HEPES buffer. The homogenate was centrifuged at 1000 rpm for 10 min, and the supernatant was stored in −70°C for phospholipase A₂ study. The protocol for the animal study was approved by the Institutional Animal Care and Use Committee.

**Total Hemolytic Complement Activity.** Serum CH100 was determined by using a radial diffusion quantiplate method using a total complement (CH100) test kit as previously described (13). Briefly, 5 μl serum was added to each well surrounded by agarose gel containing standardized sheep RBCs sensitized with hemolysin. After incubation at 37°C for 6 hr, the diameters of the clear zone surrounding each sample were measured. Each assay also contains reference samples and control serum. The unknown concentrations were determined by locating the value on the reference curve.

**TNF Assay.** Serum and tissue TNF were examined by using a cytolytic assay on TNF sensitivity cell line 1534 RE. 3.5 cell cloned from UV-induced fibrosarcoma cells as previously described (26). Cells (5 × 10⁶ cells/well) were plated on 96-well flat-bottom plates, 100 μl of serially diluted serum or tissue homogenate samples were added, and incubated at 37°C, 7.5% CO₂ for 48 hr. After removing 100 μl of medium from each well, 25 μl of MTT (5 mg/ml PBS) was added to stain the live cells for 4 hr. Then 100 μl of 10% SDS containing 0.01 N HCl was added to solubilize the cells overnight. The degree of cell lysis was quantitated by using an ELISA spectrophotometrical counter (610 nm). The standard curve was constructed with human rTNF (a gift from Genentech). Anti-murine TNF antibody (a gift from Genentech) was used in some samples for the verification of TNF.

**Phospholipase A₂ Assay.** PAF-forming phospholipase A₂ was assayed by using PAF precursor 1-O-hexadecyl-2-arachidonoyl-3-phosphocholine as substrate, as previously described (19). The substrate 1-O-hexadecyl-2-[³H]arachidonoyl-3-phosphocholine (2 × 10⁴ cpm), combined with 0.15 μg of cold carrier, was sonicated for 5 min in 0.1 M Tris buffer (pH 7.4) containing 2 mM CaCl₂, 5 mg/ml BSA, and 0.1 mg/ml sodium deoxycholate. The reaction was started by mixing 100 μl medium from each well. 25 μl of MTT (5 mg/ml PBS) was added to stain the live cells for 4 hr. Then 100 μl of 10% SDS containing 0.01 N HCl was added to solubilize the cells overnight. The degree of cell lysis was quantitated by using an ELISA spectrophotometrical counter (610 nm). The standard curve was constructed with human rTNF (a gift from Genentech). Anti-murine TNF antibody (a gift from Genentech) was used in some samples for the verification of TNF.