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Endogenously opsonized particles divert prostanoid action from lethal to protective in models of experimental endotoxemia

(sepsis/liposomes/prostaglandin/leukocytes)

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ABSTRACT **We report that, in rats, the lethal consequences of high-dose endotoxin challenge are exacerbated by the intravascular administration of prostaglandin E_1 but attenuated by the intravascular administration of endocytosable particles. This protection is mediated by opsonins. Nonopsonizable particles were unable to provide protection unless first pseudoopsonized with antibody directed against the CR3 (CD11b/CD18) phagocyte receptor. We show that endogenously opsonized particles can act in concert with prostaglandin E_1 (putatively by elevation of neutrophil intracellular cAMP and the resultant downregulation of CR3) to completely rescue animals from the lethal late-stage sequelae of experimental endotoxemia. These data illustrate that the interaction of particles with cellular receptors can transform the overall systemic response to prostaglandin E_1 from pro- to antiinflammatory. This suggests a role for multiple receptor engagement events in defining the systemic prostaglandin response and offers a rationale for developing new therapeutic modalities in the treatment of sepsis and other inflammatory diseases.**

Opinion concerning the role of prostaglandins in the inflammatory cascade is divided. Proinflammatory prostaglandin activities such as vasodilation and hyperalgesia have been utilized to explain the mechanism by which nonsteroidal antiinflammatory drugs exert their effects (1–3). In contrast, the release of inflammatory mediators from leukocyte lysosomes is reduced by prostaglandin E_1 (PGE_1) through elevation of intracellular adenosine cyclic-3′,5′-monophosphate (cAMP) (4–6) and the subsequent inhibition of degranulation. This PGE_1-mediated elevation of cAMP is augmented by leukocyte phagocytosis of both digestible opsonized sheep erythrocytes (5) as well as undigestible particles—e.g., zymosan (6). PGE_1 can inhibit the chemotaxis and adhesion of neutrophils to endothelial cells both *in vitro* and *in vivo* (7). Since neutrophil extravasation with release of inflammatory mediators is critical in the development of systemic inflammatory disease, these findings have suggested new paradigms for the development of rational therapies, but clinically relevant studies have not been accomplished (8–14). Here we define a role for surface adhesive proteins and their ligands in establishing new therapeutic uses for prostaglandins.

MATERIALS AND METHODS

Cell Isolation and Incubation. Whole human blood was collected from normal volunteers into Vacutainers containing tripotassium EDTA (Becton Dickinson). For whole blood studies the samples were used as obtained. Neutrophils and monocytes were obtained by sedimentation of erythrocytes on dextran (15). Remaining erythrocytes were hypotonically lysed for 30 sec. The cell suspension was then layered on Histopaque 1077 (Sigma) and centrifuged to separate neutrophils from monocytes, lymphocytes, and platelets. The neutrophils were washed twice with Krebs–Ringer glucose solution and incubated in RPMI 1640 medium (Fisher Scientific). Monocytes were washed twice in ice-cold 0.02% EDTA in phosphate-buffered saline (Versene; GIBCO) and then separated from lymphocytes by panning on polystyrene. Monocytes were incubated in RPMI 1640.

Animals. Male Sprague–Dawley rats (125 g; Charles River Breeding Laboratories) were acclimated for 4 days upon receipt in the animal facility. A standard 12-hr light/dark cycle was utilized with feeding and watering ad libitum. All protocols were approved by the Institution Animal Care and Use Committee.

Reagents. Lipopolysaccharide (LPS) of *Escherichia coli* serotype 011:B5 was obtained from Sigma and solubilized in phosphate-buffered saline prior to injection. Egg phosphatidylcholine was obtained from Lipoid (Ludwigshafen, Germany). PGE_1 was obtained from Chinoin Chemical (Budapest) and solubilized in absolute ethanol. Anti-rat CR3 (CD11b) monoclonal antibody CL042A (clone OX42) was obtained from Cedarlane Laboratories. Anti-rat major histocompatibility complex class I monoclonal antibody MAS101 (clone OX18) was obtained from Sera-Lab (Sussex, U.K.). Anti-rat IgG1 (MARG1-1) was obtained from Accurate Chemicals. Pansorbin was obtained from Calbiochem. Latex microspheres (100-nm diameter) were obtained from Polyscience. Fluorescein-labeled IB4 monoclonal antibody (IgG2a) directed against human CD18 was a gift of D. Chambers (San Diego Regional Cancer Center, La Jolla, CA). Platelet-activating factor was obtained from Sigma.

Liposomes. Liposomes consisting of 40 mg of egg phosphatidylcholine and 100 μg of PGE_1 in buffer containing 1 g of the cryoprotectant maltose monohydrate (16) and 0.3 mg of the antioxidant butylated hydroxytoluene (*Chemical Abstracts* no. 128-31-0) were prepared by the extrusion of liquid crystalline dispersions of phospholipid (smectic mesophases) through 100-nm polycarbonate filters (17) and lyophilized. The lyophilized liposomes were reconstituted prior to use in 10 ml of acetate buffer (acetic acid at 0.609 mg/ml, pH 4.2) and were determined to be 100 nm in diameter by freeze–fracture electron microscopy (18). In this buffer system PGE_1 is protonated and associates with the liposome membrane as detected by using isotopically labeled material. At physiological pH, PGE_1 rapidly disassociates from the liposome with a

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Abbreviations: diI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; LPS, lipopolysaccharide; PGE_1, prostaglandin E_1.

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half-time of < 2 min. Alternatively, liposomes were made as above without the inclusion of PGE_1. The possibility that endotoxin adsorbed to the liposomes and was therefore inactivated was ruled out. Liposomes were incubated with endotoxin at a ratio of 1.6×10^{12} liposome particles to 25 mg of endotoxin for ≥4 hr. After this incubation the endotoxin binding to the liposomes was determined with a *Limulus* amoebocyte lysate assay (Endosafe, Charleston, SC). No liposome-associated endotoxin was detected. Fluorescent liposomes were made as above except that the fluorescent lipophilic label 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (diI; Molecular Probes) was included at 0.5 mol %.

cAMP Assays. Human neutrophils (10^7 cells per ml in RPMI 1640 with 10% human AB serum) were either assayed directly for cAMP or stimulated for 15 min at 37°C with either PGE_1 (10 μM), liposomes (3×10^{10} per ml), or PGE_1 plus liposomes. The reactions were terminated by addition of ice-cold ethanol (final concentration, 65% by volume). The samples were allowed to settle for 1 hr at −20°C and the cAMP-containing supernatants were recovered and centrifuged at $2000 \times g$ for 15 min at 4°C. The remaining supernatants were evaporated under nitrogen and assayed with a commercial kit (Amersham).

Flow Cytometry. Determinations were made with a Profile I flow cytometer (Coulter) equipped with an argon ion laser emitting 15 mW at 488 nm. Leukocyte populations were gated on characteristic forward vs. 90° light scatter. Data from the FL1 channel in linear amplification mode were collected and printed in histogram form. Fluorescence distributions approximated a Gaussian distribution and the mean of each distribution was noted. The linearity of the flow cytometer was verified with prestained fluorescent microbead standards (Quantum, Durham, NC).

Preparation of Antibody-Coated Particles. A 100-μl aliquot of the original 10% (wt/vol) Pansorbin cell suspension [protein A-coated hardened and heat-killed *Staphylococcus aureus* Cowan I strain (19); Calbiochem] was pelleted and the cells were suspended in 100 μl of phosphate-buffered saline containing either 200 μg of antibody or 20 μl of heat-inactivated rat serum (final IgG concentration, 2–4 mg/ml). After incubation for 1 hr at room temperature with constant shaking, the cells were washed twice and suspended in 1 ml of 0.2 M sodium borate (pH 9.0). The antibodies were coupled to the particles by the addition of dimethylpimelimidate (20 mM) followed by 2 hr of incubation in 0.2 M ethanolamine (pH 8.0). The reaction was terminated by washing once and incubating the particles for 2 hr in 0.2 M ethanolamine (pH 8.0). The particles were pelleted, suspended in phosphate-buffered saline containing 0.1% (wt/vol) sodium azide, and stored at 4°C.

RESULTS AND DISCUSSION

Liposomes Target to Activated Phagocytes and, Together with PGE_1, Increase Cellular cAMP and Downregulate CR3. Due to the cooperative effect of PGE_1 and particles on raising neutrophil cAMP concentrations (4–6) and the direct effect of PGE_1 itself on preventing neutrophil adhesion to capillary endothelium (7), we were led to investigate whether large unilamellar liposome vesicles—i.e., biodegradable 100-nm egg phosphatidylcholine particles plus PGE_1—might be effective in reducing mortality in lethal models of experimental endotoxemia. In serum, such vesicles adsorb fibrinogen and fibronectin and fix C3bi and are thus endocytosed as a consequence of engagement of the phagocytic receptor CR3 (CD11b/CD18) (20). In initial *in vitro* experiments we found that liposomes labeled with 0.5 mol % nonexchangeable fluorescent lipophilic probe diI (21) targeted to human neutrophils and monocytes but not lymphocytes in whole blood when these cells were exposed to endotoxin (Fig. 1). In separate *in vitro* experiments utilizing human phagocytes and endothelial cells, we found that this targeting could be blocked by antibodies to the opsonins fibrinogen, fibronectin, and C3bi or to their respective cellular $\alpha_5\beta_1$, $\alpha_v\beta_3$, or CR3 integrin

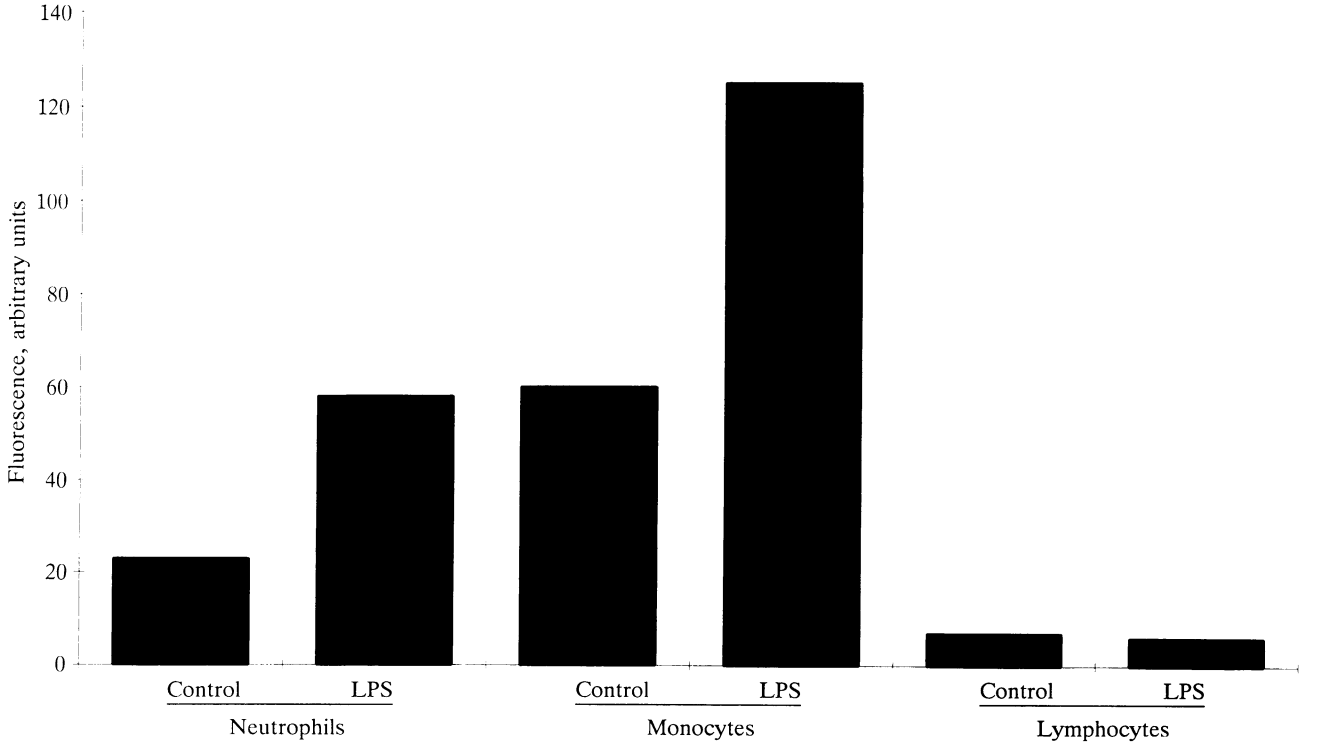

FIG. 1. One-hundred-nanometer liposomes target to endotoxin-stimulated phagocytes in whole blood. Whole human blood, collected as described, was incubated with fluorescent diI-labeled 100-nm liposomes in the presence or absence of endotoxin (LPS at 1 μg/ml) at 37°C with constant rocking. After a 30-min incubation, the blood was supplemented with disodium EDTA (10 mM) and analyzed by flow cytometry.

Table 1. PGE_1 and liposomes cooperatively increase neutrophil cAMP

Treatment	cAMP, pmol per 10^7 cells
Control	0.62 ± 0.31
PGE_1	3.58 ± 0.73
Liposomes	4.86 ± 0.44
PGE_1 + liposomes	8.42 ± 0.49

Human neutrophils were incubated in RPMI 1640 medium supplemented with 10% human AB serum. Neutrophils at 10^7 per ml were either assayed directly or stimulated for 15 min at 37°C by exposure to either PGE_1 (10 μM), liposomes (3×10^{10} per ml), or PGE_1 plus liposomes, harvested, and then assayed for cAMP. Shown are the means and standard deviations from three separate experiments.

receptors (data not shown). Human neutrophil cAMP levels were increased by either PGE_1 or liposomes and were cooperatively increased by liposomes plus PGE_1 (Table 1), presumably through concomitant engagement of CR3 and EP2 receptors. In our hands this cooperative increase in cAMP was accompanied by a decrease in the expression of neutrophil CD18 (Fig. 2), a likely consequence of the inhibition of Ca^{2+} signaling transients (22) and subsequent inhibition of mitogen-activated protein kinase (23, 24) and other Ca^{2+}-dependent protein kinases (25). Since the interaction of CD18 with endothelial intercellular adhesion molecule 1 permits neutrophil extravasation from the vasculature into tissue and thus mediates the systemic inflammatory response, these data suggested that PGE_1 and liposomes might attenuate this response *in vivo*.

Liposomes Divert the Systemic Response to PGE_1 from Pro- to Antiinflammatory. Our initial *in vivo* experiments were designed to assess the efficacy of PGE_1 and liposomes in rat models of endotoxemia. The results (Fig. 3) indicate that when administered alone, PGE_1 was proinflammatory; it increased both the rate and the extent of endotoxin-induced mortality. In contrast, liposomes reduced mortality. Remarkably, PGE_1 administered in combination with liposomes became antiinflammatory and afforded complete protection against endotoxin-induced death. These data mirrored those describing the effect of opsonized particles on the downregulation of CD18 expression (Fig. 2). This allowed us to confirm a pivotal role

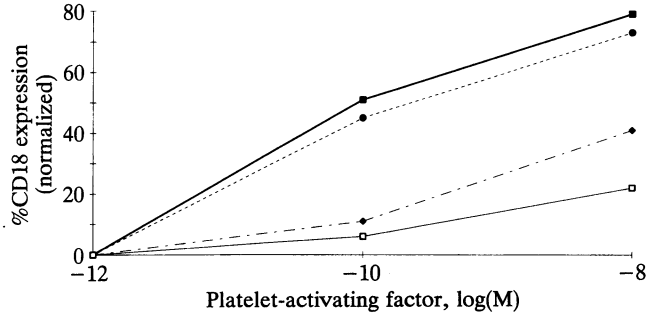

FIG. 2. PGE_1 and liposomes cooperate to lower available neutrophil CD18. Human neutrophils (3×10^6 cells per ml in phosphate-buffered saline) were stimulated with platelet-activating factor at the indicated concentrations at 37°C with constant rocking. Neutrophils were either untreated (■) or treated with PGE_1 (10 μM) (●), liposomes (3×10^{10} per ml) (◆), or PGE_1 plus liposomes (□). Duplicate samples were removed before stimulation and at the indicated times thereafter and added to 0.05-ml aliquots of ice-cold phosphate-buffered saline containing fluorescein-conjugated IB4 (5 μg/ml), a monoclonal antibody directed against human CD18. Rapid reduction in temperature to 0°C prevented further cellular response. To ensure consistent antibody binding, samples were analyzed by flow cytometry after 30 min of incubation on ice. Because the data were collected with the fluorescence amplifier set to the linear mode, changes in mean channel number were directly proportional to changes in surface CD18 expression.

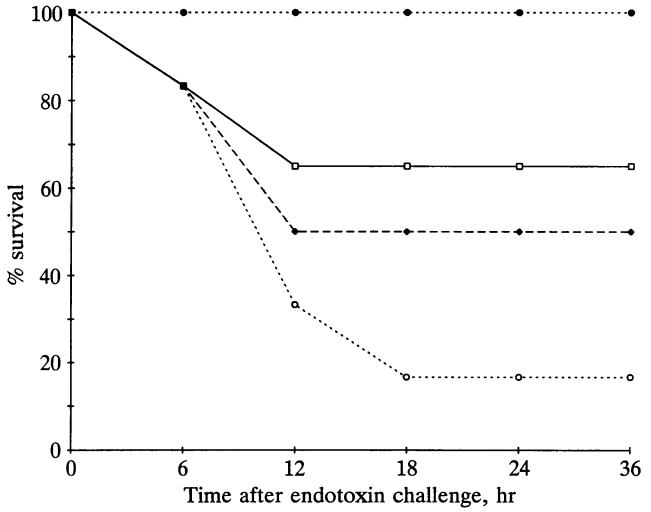

FIG. 3. Liposomes attenuate endotoxin-induced mortality and, together with PGE_1, increase survival. At time 0, rats were restrained and injected in the tail vein with LPS at 50 mg/kg (◆) and either PGE_1 at 40 μg/kg (○), 1.6×10^{12} 100 nm liposome particles per kg (□), or a combination of liposomes plus PGE_1 (●). Survival was assessed at the indicated times. All rats that received saline instead of LPS survived (data not shown). For each group, $n = 12$. Liposomes were assessed for endotoxin absorption by *Limulus* amoebocyte lysate assay. There was no detectable sequestration of endotoxin.

for particles in permitting a systemic antiinflammatory response to PGE_1. In the absence of particles, neutrophil receptor engagement and intracellular signaling were most likely diminished, allowing the inflammatory activities of PGE_1 to predominate.

Liposomes Influence the Systemic Antiinflammatory Response to PGE_1 in a Dose-Dependent Fashion. As mentioned, the increased survival obtained by administering liposomes indicated an important role for particles in the outcome of the PGE_1/liposome treatment modality and suggested a therapeutic particle effect. Accordingly, we assessed survival by treating animals with a constant dose of PGE_1 but increased the particle number (Fig. 4). Increasing the particle number resulted in increased survival. The maximal response was obtained with $\geq 1.2 \times 10^{12}$ particles per kg. Virtually identical responses were obtained with both liposomes and 100-nm latex microspheres. We next assessed survival after treating with increasing doses of PGE_1 either in the absence or presence of

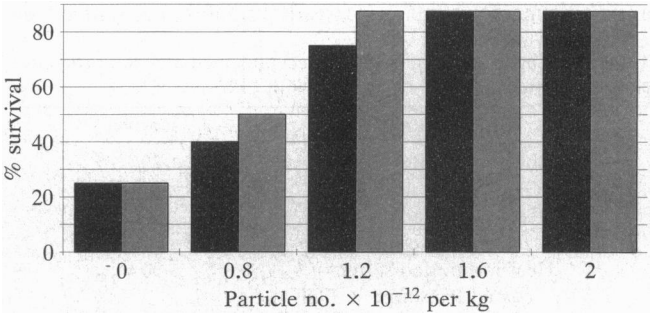

FIG. 4. Systemic antiinflammatory activity of PGE_1 is particle dependent. At time 0, rats were injected intravenously with LPS (50 mg/kg). Particles as either 100-nm liposomes (black bars) or 100-nm latex microspheres plus PGE_1 (40 μg/kg) (gray bars) were intravenously injected simultaneously with PGE_1 (40 μg/kg) at the indicated particle number. Survival was assessed at 24 hr. Saline and endotoxin controls are not shown; survival for these groups was 100% and 50%, respectively. For each group, $n = 16$. Liposomes and latex microspheres were assessed for endotoxin adsorption by *Limulus* amoebocyte lysate assay. There was no detectable sequestration of endotoxin.

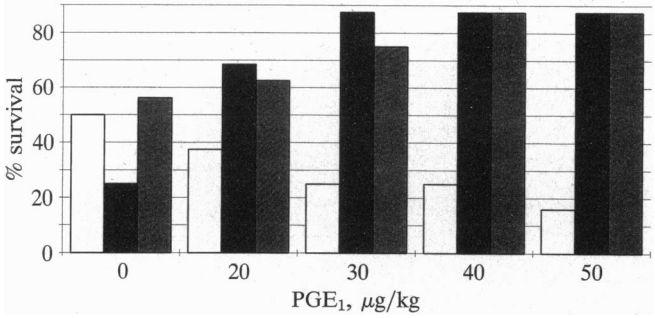

FIG. 5. Systemic activity of PGE_1 is shifted from pro- to antiinflammatory by particles. At time 0, rats were injected intravenously with LPS at 50 mg/kg. PGE_1 at the indicated dose was simultaneously injected via the tail vein in the absence (open bars) or presence of either 1.6×10^{12} 100-nm liposomes per kg (black bars) or 1.6×10^{12} 100-nm latex microspheres per kg (gray bars). Survival was assessed at 24 h. Saline controls are not shown; survival for this group was 100%. For each group, $n = 16$.

1.6×10^{12} particles per kg. Increasing the dosage of PGE_1 in the absence of particles resulted in a dose-dependent increase in mortality (Fig. 5). However, increasing the PGE_1 dose in the presence of particles resulted in a dose-dependent increase in survival. As both liposomes and nonmetabolizable latex microspheres afforded similar responses, we can rule out an effect inherent to organized assemblies of lipids or to other components of liposome systems.

Inherent Protection of Particles in Endotoxemia Is Mediated by Engagement of the CR3 Receptor. To evaluate whether particle interaction with CR3 was obligate for the protective effect observed, we utilized particles which are not endogenously opsonized. In these experiments Pansorbin particles (hardened *S. aureus* cells coated with protein A) incubated in heat-denatured rat serum, and thus nonopsonizable and nonendocytosable, were not protective (Table 2). However, when these particles were first pseudoopsonized by conjugation with antibodies to the CR3 (CD18/CD11b) phagocyte receptor, a procedure known to refresh interaction of the particles with CR3 (26), the protective effect was also refreshed. Neither anti-CR3 alone nor Pansorbin conjugated with antibodies directed against either major histocompatibility class I antigen or IgG1 was protective (data not shown). These data show that the systemic activity of PGE_1 can be changed from inflammatory to antiinflammatory by particle-directed engagement of CR3. Whether the protective effect obtained by pseudoopsonization of Pansorbin is mediated by cAMP and the resultant antiinflammatory signaling cascade remains speculative. Immobilization of CD18 antibodies on Pansorbin has been demonstrated *in vitro* to induce neutrophil intracellular signaling as evidenced by activation of phospholipase D and increased mobilization of intracellular Ca^{2+} (26), but the effect on cAMP has not been defined (27, 28).

Table 2. Engagement of the CR3 receptor mediates the protective effect of particles in rats challenged with LPS

Treatment	% survival
None (LPS only)	30
PGE_1	20
Heat-inactivated serum-coated Pansorbin	30
Heat-inactivated serum-coated Pansorbin + PGE_1	30
Anti-CR3 Pansorbin	50
Anti-CR3 Pansorbin + PGE_1	100

Rats were injected intravenously at time 0 with LPS at 75 mg/kg. The animals were simultaneously injected intravenously with a single bolus of either PGE_1 (40 μg/kg) or heat-inactivated serum-treated Pansorbin (1.6×10^{12} particles per kg), heat-inactivated serum-treated Pansorbin plus PGE_1, anti-CR3-presenting Pansorbin particles, or anti-CR3-presenting Pansorbin particles plus PGE_1. Survival was assessed at 24 hr. Saline controls are not shown; survival for this group was 100%. For each group, $n = 16$.

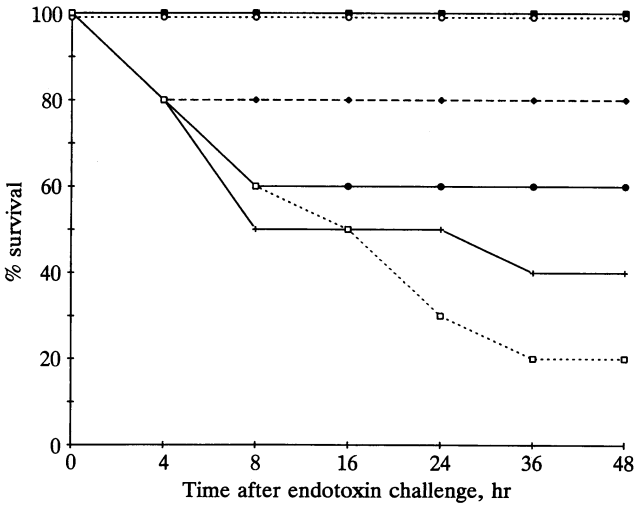

FIG. 6. PGE_1 plus liposomes given after LPS rescues rats from endotoxic shock and subsequent mortality. At time 0, rats were injected intravenously with LPS at 75 mg/kg (□). One-hundred-nanometer liposomes at 1.6×10^{12} particles per kg plus PGE_1 at 40 μg/kg were administered as a single bolus via the tail vein to groups of animals beginning at 0 hr (■), 2 hr (○), 4 hr (◆), 8 hr (●), or 16 hr (+) after LPS. Survival was assessed at the indicated times. Saline controls are not shown; survival for this group was 100%. For each group, $n = 18$.

Endogenously Opsonized Liposomes and PGE_1 Rescue Animals from Endotoxemia When Given After Endotoxin Challenge. The extraordinary protection afforded to rats by PGE_1 plus liposomes led us to further assess this treatment modality. We therefore induced endotoxemia and administered single bolus intravenous injections of PGE_1 plus liposomes at various times after challenge. The results (Fig. 6) indicate that rescue of animals from lethal endotoxic shock can be achieved even when treatment is withheld for significant periods of times after endotoxin challenge. While some mortality occurred prior to treatment, all rats alive at up to 8 hr after challenge survived. Even rats treated up to 16 hr after challenge showed a significant increase in survival as compared with controls. We are unaware of any experimental or clinical data other than these that suggest successful intervention is possible in the later stages of the systemic inflammatory response (29). Current effective experimental treatment modalities for sepsis require either prophylactic treatment (30, 31) or administration at the time of endotoxin challenge (32–34). Unlike these sepsis therapies, treatment with PGE_1 plus liposomes takes advantage of the activation of professional phagocytes and thus exploits the natural cellular immune response to septic insult. Regardless of the actual target cells or mechanisms, our data indicate a clinical potential for PGE_1 and liposomes in ameliorating sepsis and other inflammatory diseases.

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