**Microbiology.** In the article “Sets of EcoRI fragments containing ribosomal RNA sequences are conserved among different strains of *Listeria monocytogenes*” by James L. Bruce, Romeo J. Hubner, Eileen M. Cole, Channey I. McDowell, and John A. Webster, which appeared in number 11, May 23, 1995, of *Proc. Natl. Acad. Sci. USA* (92, 5229–5233), Fig. 6 was incorrectly reproduced due to a printing error. The corrected Fig. 6 and its legend are shown below.

<table>
<thead>
<tr>
<th>Set Description</th>
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<td>G 6.2, H 9.0, · F</td>
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<td>G 8.1, H 7.1, · F</td>
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<tr>
<td>G 5.8, H 7.1, · F</td>
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</tbody>
</table>

**Medical Sciences.** In the article “Endogenously opsonized particles divert prostanoid action from lethal to protective in models of experimental endotoxemia” by David Eierman, Machiko Yagami, Scott M. Erme, Sharma R. Minchey, Paul A. Harmon, Kerri J. Pratt, and Andrew S. Janoff, which appeared in number 7, March 28, 1995, of *Proc. Natl. Acad. Sci. USA* (92, 2815–2819), the authors call to the reader’s attention that because of an error in calculation, (a) liposome and latex particle numbers in *in vivo* experiments should be multiplied by the factor $1.4 \times 10^2$; (b) the liposome concentration given in Table 2, Fig. 2, and the second paragraph on page 2816 should be corrected to $2.0 \times 10^{13}$ per ml; and (c) the number of liposome particles incubated with 25 mg of endotoxin, serotype 055:B5 (p. 2816) should be corrected to $1.4 \times 10^{14}$. In Table 2 and Fig. 6, $n = 10$ for each group. In Fig. 4, $n = 12$, and at "0.8 $\times 10^{12}$" particles per kg, data from liposomes and latex particles were inadvertently transposed. The authors wish to point out that none of these changes affects the conclusions of the paper.
Endogenously opsonized particles divert prostanoid action from lethal to protective in models of experimental endotoxemia

(sepsis/liposomes/prostaglandin/leukocytes)

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Communicated by Bengt Samuelsson, Karolinska Institutet, Stockholm, Sweden, December 27, 1994

ABSTRACT We report that, in rats, the lethal consequences of high-dose endotoxin challenge are exacerbated by the intravascular administration of prostaglandin E₁ but attenuated by the intravascular administration of endosomizable particles. This protection is mediated by opsonins. Nonopsonizable particles were unable to provide protection unless first pseudopropsonized with antibody directed against the CR3 (CD11b/CD18) phagocyte receptor. We show that endogenously opsonized particles can act in concert with prostaglandin E₁ (putatively by elevation of neutrophil intracellular CAM 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 E₁ 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 E₁ (PGE₁) through elevation of intracellular adenosine cyclic-3',5'-monophosphate (cAMP) (4–6) and the subsequent inhibition of degranulation. This PGE₁-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). PGE₁ 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). PGE₁ was obtained from Chino 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 microparticles (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 PGE₁ in buffer containing 1 g of the cryoprotectant maltose monohydrate (16) and 0.5 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 saline buffer (acetic acid at 0.609 mg/ml, pH 4.2) and were determined to be 100 nm in diameter by freeze–octract electron microscopy (18). In this buffer system PGE₁ is protonated and associates with the liposome membrane as detected by using isotopically labeled material. At physiological pH, PGE₁ rapidly dissociates from the liposome with a 2815

Abbreviations: dil, dil 1:1, diocetyl-gl-3,3',3'-tetramethylendioxy-

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half-time of < 2 min. Alternatively, liposomes were made as above without the inclusion of PGE₁. 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 × 10¹² 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 (dio; Molecular Probes) was included at 0.5 mol %.

**cAMP Assays.** Human neutrophils (10⁷ 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 PGE₁ (10 μM), liposomes (3 × 10¹⁰ per ml), or PGE₁ 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 × 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 heparinized and heat-killed *Staphylococcus aureus* Cowan I strain (19); Calbiochem] was pelleted and the cells were resuspended 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) at 4°C. The antibodies were coupled to the particles by the addition of dimethylpiperazinemide (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 Targeted to Activated Phagocytes and, Together with PGE₁, Increase Cellular cAMP and Downregulate CR3. Due to the cooperative effect of PGE₁ and particles on raising neutrophil cAMP concentrations (4–6) and the direct effect of PGE₁ in 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 PGE₁—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 dio (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 αβ₁, αβ₅, or CR3 integrin.

![Fig. 1](image-url) One-hundred-nanometer liposomes target to endotoxin-stimulated phagocytes in whole blood. Whole human blood, collected as described, was incubated with fluorescent dio-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. PGE₁ and liposomes cooperatively increase neutrophil cAMP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cAMP, pmol per 10⁷ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.62 ± 0.31</td>
</tr>
<tr>
<td>PGE₁</td>
<td>3.58 ± 0.73</td>
</tr>
<tr>
<td>Liposomes</td>
<td>4.86 ± 0.44</td>
</tr>
<tr>
<td>PGE₁ + liposomes</td>
<td>8.42 ± 0.49</td>
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</table>

Human neutrophils were incubated in RPMI 1640 medium supplemented with 10% human AB serum. Neutrophils at 10⁷ per ml were either assayed directly or stimulated for 15 min at 37°C by exposure to either PGE₁ (10 μM), liposomes (3 × 10¹⁰ per ml), or PGE₁ plus liposomes, harvested, and then assayed for cAMP. Shown are the means and standard deviations from three separate experiments.

Liposomes and receptors (data not shown). Human neutrophil cAMP levels were increased by either PGE₁ or liposomes and were cooperatively increased by liposomes plus PGE₁ (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 Ca²⁺ signaling transients (22) and subsequent inhibition of mitogen-activated protein kinase (23, 24) and other Ca²⁺-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 PGE₁ and liposomes might attenuate this response in vivo.

Liposomes divert the systemic response to PGE₁ from proto to antiinflammatory. Our initial in vivo experiments were designed to assess the efficacy of PGE₁ and liposomes in rat models of endotoxemia. The results (Fig. 3) indicate that when administered alone, PGE₁ was proinflammatory; it increased both the rate and the extent of endotoxin-induced mortality. In contrast, liposomes reduced mortality. Remarkably, PGE₁ 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 for particles in permitting a systemic antiinflammatory response to PGE₁. In the absence of particles, neutrophil receptor engagement and intracellular signaling were most likely diminished, allowing the inflammatory activities of PGE₁ to predominate.

Liposomes influence the systemic antiinflammatory response to PGE₁ 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 PGE₁/liposome treatment modality and suggested a therapeutic particle effect. Accordingly, we assessed survival by treating animals with a constant dose of PGE₁ but increased the particle number (Fig. 4). Increasing the particle number resulted in increased survival. The maximal response was obtained with ~1.2 × 10¹² 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 PGE₁ either in the absence or presence of...
1.6 × 10^{12} particles per kg. Increasing the dosage of PGE₁ in the absence of particles resulted in a dose-dependent increase in mortality (Fig. 5). However, increasing the PGE₁ 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 endogously 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 pseudopsonized 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 PGE₁ can be changed from inflammatory to antiinflammatory by particle-directed engagement of CR3. Whether the protective effect obtained by pseudopsonization of Pansorbin is mediated by cAMP and the resultant antiinflammatory signaling cascade remains speculative. Im

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

<table>
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<th>Treatment</th>
<th>% survival</th>
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<tr>
<td>PGE₁</td>
<td>20</td>
</tr>
<tr>
<td>Heat-inactivated serum-coated Pansorbin</td>
<td>30</td>
</tr>
<tr>
<td>Heat-inactivated serum-coated Pansorbin + PGE₁</td>
<td>30</td>
</tr>
<tr>
<td>Anti-CR3 Pansorbin</td>
<td>50</td>
</tr>
<tr>
<td>Anti-CR3 Pansorbin + PGE₁</td>
<td>100</td>
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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 PGE₁ (40 μg/kg) or heat-inactivated serum-treated Pansorbin (1.6 × 10^{12} particles per kg), heat-inactivated serum-treated Pansorbin plus PGE₁, anti-CR3-presenting Pansorbin particles, or anti-CR3-presenting Pansorbin particles plus PGE₁. Survival was assessed at 24 hr. Saline controls are not shown; survival for this group was 100%. For each group, n = 16.

mobilization 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 Ca²⁺ (26), but the effect on cAMP has not been defined (27, 28).

**Endogenously Opsonized Liposomes and PGE₁ Rescue Animals from Endotoxemia When Given After Endotoxin Challenge.** The extraordinary protection afforded to rats by PGE₁ plus liposomes led us to further assess this treatment modality. We therefore induced endotoxemia and administered single bolus intravenous injections of PGE₁ 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 time 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 PGE₁ 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 PGE₁ and liposomes in ameliorating sepsis and other inflammatory diseases.

We thank G. Weissmann, C. Swenson, and E. Mayhew for critical reading of the manuscript and discussion. We thank D. Chambers for technical assistance with flow cytometry.


![Fig. 5](image-url) Systemic activity of PGE₁ is shifted from pro- to anti-inflammatory by particles. At time 0, rats were injected intravenously with LPS at 50 mg/kg. PGE₁ 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 hr. Saline controls are not shown; survival for this group was 100%. For each group, n = 16.

![Fig. 6](image-url) PGE₁ 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 PGE₁ 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.