Neutralization of Endotoxin by a Phospholipid Emulsion in Healthy Volunteers

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Background. An approach to endotoxin (lipopolysaccharide [LPS]) blockade makes use of the ability of lipoproteins, via surface phospholipids, to bind and neutralize LPS. The aim of the present study was to determine whether the intravenous administration of a protein-free, phospholipid-rich emulsion is an effective method for neutralizing the effects of LPS in healthy persons.

Methods. This was a double-blind, placebo-controlled study in 20 volunteers. Volunteers received Escherichia coli endotoxin (2 ng/kg) intravenously 2 h into a 6-h infusion of either emulsion (210 mg/kg) or placebo (Intralipid diluted 1:64).

Results. The volunteers who received emulsion had a lower mean clinical score ( ), temperature ( ), pulse rate ( ), neutrophil count ( ), tumor necrosis factor–α level ( ), and interleukin-6 level ( ) than did the volunteers who received placebo. Response was related to serum phospholipid level. The greatest effects were observed in the volunteers achieving phospholipid levels of ∼500 mg/dL or higher.

Conclusion. Phospholipid emulsion attenuates the clinical and laboratory effects associated with the administration of LPS in humans, suggesting a novel approach to the treatment of endotoxemia.

More than 700,000 people in the United States develop severe sepsis every year, and >25% of them die of associated complications [1]. Bacteria-derived lipopolysaccharide (LPS) is associated with cardiac depression, organ dysfunction, and death in patients with sepsis and positive blood cultures [2]. Therapies aimed at neutralizing endotoxin via antibodies [3, 4] or at inhibiting the inflammatory response generally have been ineffective [5]. An inhibitor of the coagulation cascade, recombinant human activated protein C, has shown beneficial effects in patients with sepsis, although there are concerns with regard to bleeding complications [6]. Another approach to endotoxin blockade makes use of the natural ability of lipoproteins to bind to endotoxin [7, 8]. LPS interaction with LPS-binding protein, in association with cell-surface CD14 [9], will trigger the Toll-like receptor [10] and initiate the systemic inflammatory response syndrome. In contrast, the binding of LPS to lipoproteins attenuates cytokine release [11]. Lipoprotein neutralization of LPS has been shown to be related to the phospholipid in the lipoprotein particle [12, 13].

Lipid and lipoprotein levels have been linked to clinical outcomes. Low cholesterol levels have been associated with enhanced mortality in a long-term observational study [14] and with the development of infectious disorders in the Kaiser Permanente study in 15,000 healthy men and women [15]. Lipid levels are reduced by 30%–40% during a variety of acute disorders [16, 17], potentially impairing an individual’s response to endotoxin. In support of this concept, hypolipidemia has been associated with poor clinical outcomes and increased risk of infection in critically ill patients [18]. Lower serum lipoprotein levels have been found in patients with systemic inflammatory response syndrome (SIRS) than in patients without SIRS [19], and lower apolipoprotein A-I levels have been observed in patients with increased numbers of SIRS criteria than in patients...
without such an increase [20]. Kitchens et al. [21] have demonstrated that lipoproteins associated with the acute phase of SIRS have better endotoxin-neutralizing and -binding capabilities than do lipoproteins from normal serum. However, this enhanced binding ability may not be sufficient to overcome the marked reduction in lipid and lipoprotein levels found in most critically ill patients.

Lipids and lipoproteins are, therefore, targets for the prevention and treatment of endotoxemia. Transgenic mice made hyperlipidemic with high-density lipoprotein (HDL) transgenes have been shown to be resistant to endotoxin challenge [22]. Infusion of plasma-derived reconstituted HDL suppressed LPS-induced cytokine production in rabbits [23] and also blocked many of the physiological effects seen with infusion of LPS in volunteers [24].

The development of a protein-free, phospholipid-rich emulsion would provide the ability to bind and neutralize bacterial endotoxin while avoiding the potential adverse effects of blood-derived lipoproteins. Preclinical studies using this emulsion have focused on a pig peritonitis model of sepsis [25]. Pigs receiving the emulsion had a survival rate of 80% (16/20), whereas the focused on a pig peritonitis model of sepsis [25].

The effective phospholipid level was determined to be ~300 mg/dL in this pig model. The phospholipid-rich emulsion was studied in a phase 1 human trial in 30 volunteers [27]; peak phospholipid levels of 700 mg/dL were achieved and were well tolerated.

A logical step in the development of an antiendotoxin therapy would be to use a model in which small quantities of standardized endotoxin are administered to healthy volunteers. This endotoxin-infusion model has been used in many studies of the effects of LPS on human physiology [28]. After administration of an intravenous dose of 2–4 ng/kg endotoxin, there is a marked increase in the levels of tumor necrosis factor (TNF)–α and interleukin (IL)–6, with peaks at 2 and 4 h, respectively [29]. Clinical effects are transient flu-like symptoms, including headache, myalgias, low-grade fever, and tachycardia. Therefore, we sought to determine whether the intravenous administration of a protein-free, phospholipid-rich emulsion is an effective method by which to neutralize the physiological effects of bacterial endotoxin in healthy volunteers.

VOLUNTEERS, MATERIALS, AND METHODS

Volunteer population. Twenty healthy male and female volunteers, 21–40 years old, were studied. The volunteers were required to abstain from alcohol and tobacco consumption during the study. All women of childbearing potential could not be pregnant or breast-feeding and had to commit either to using a double-barrier method of contraception or an intrauterine device or to abstaining from sexual intercourse for the duration of the study and for 1 month after completion of the study. The volunteers were screened for drugs of abuse and for human immunodeficiency virus. Exclusion criteria included a low-density lipoprotein (LDL) cholesterol level of >190 mg/dL or a triglyceride level of >300 mg/dL. The protocol was approved by the Committee on Human Rights in Research of the Rockefeller University (New York, NY). All subjects provided informed consent before participating in the study.

Study design. This was an investigator-initiated, double-blind, placebo-controlled study designed to determine whether the pretreatment of healthy volunteers with a protein-free, phospholipid-rich emulsion would attenuate the clinical effects of administration of endotoxin and block the associated cytokine release. Clinical score (see Symptoms, below) was the primary outcome. Secondary outcomes included levels of cytokines, white blood cell (WBC) count, and cardiac index (CI), as measured by echocardiography.

The study included 2 outpatient prestudy screening visits completed within 8 weeks of the administration of endotoxin (day 0), an inpatient admission of ~2 days duration, and an outpatient visit 1 week after the administration of endotoxin. The volunteers were admitted to the Rockefeller General Clinical Research Center the day before the endotoxin challenge. They went home the morning after completion of the study. The volunteers fasted (except for drinking water) from 10 P.M. the evening before receiving endotoxin until 24 h after administration of emulsion or placebo. Volunteers were randomly assigned to receive either emulsion or an equal volume of placebo (Intralipid diluted 1:64). The randomization schedule was prepared by the Rockefeller University hospital statistician and provided to the Rockefeller University hospital pharmacist. Two intravenous catheters were inserted in contralateral arms the morning of treatment. One catheter was used to infuse emulsion or placebo, and the other catheter was used to draw blood, administer endotoxin, and infuse saline. The volunteers received the infusion of emulsion or placebo over a 6-h period, divided into a 2-h prime at 75 mg/kg/h phospholipid and then another 4 h at 15 mg/kg/h phospholipid. Two hours after the initiation of the infusion of emulsion or placebo, endotoxin (2 ng/kg) was given intravenously over 1 min by a physician, followed by a slow flush of 10 mL of sterile normal saline. Before the administration of endotoxin, the volunteers were given normal saline (125 mL/h) for 2 h; after the administration, the volunteers were given 500 mL/h normal saline for 4 h, followed by 5% dextrose and normal saline (100 mL/h) until the next morning, when blood was drawn.

All volunteers were continuously monitored via electrocardiography and pulse oximetry for 6 h after the administration of endotoxin. Vital signs (blood pressure, heart rate, respiratory rate, and temperature) were taken by a nurse every 30 min and then 6, 8, 12, and 22 h after the infusion of endotoxin. Echocardi-
oigraphy was performed immediately before initiation of the infusion of emulsion and 3 h after the administration of endotoxin. Heart rate, left-ventricular outflow track diameter, and velocity time integral across the aortic valve were recorded and used to calculate stroke volume. Cardiac output was calculated by multiplying stroke volume by the simultaneous heart rate.

**Symptoms.** Clinical observations were summarized by a clinical score. Six hours after the administration of endotoxin, a nursing staff member administered a questionnaire, querying each volunteer for complaints related to chills, headaches, myalgias, nausea, and backaches. Scores were calculated for each symptom on the basis of the following values: none, 0; mild, 1; moderate, 2; and severe, 3. The maximum possible clinical score for the 5 symptoms was 15.

**Blood samples.** Venous blood samples were obtained from indwelling catheters. Samples were drawn for determination of TNF-α and IL-6 levels and WBC and platelet counts at −2, 0, 1, 2, 3, 4, 6, 8, 12, and 24 h; for determination of lipid and lipoprotein levels at −2, 0, 2, 4, 6, 12, and 24 h; and for determination of biochemical profiles at −2, 6, and 24 h relative to the administration of endotoxin.

Assays were performed at the Iris and B. Gerald Cantor Clinical Research Laboratory of The Rogosin Institute (New York, NY). TNF-α was measured by use of an ELISA (Amersham Life Science). IL-6 was measured by use of an enzyme immunoassay on the Roche COBAS Core II (Roche Diagnostic Systems). Total cholesterol and triglyceride levels were measured by use of enzymatic methods (Roche Diagnostic Systems). HDL cholesterol levels were measured after precipitation of β lipoproteins, and LDL cholesterol levels were determined by calculation. Sodium cholate levels were measured indirectly as the change in the level of total serum bile acids, by use of an enzymatic kit (Sigma).

**Phospholipid emulsion.** The emulsion used is a protein-free, phospholipid-rich emulsion with endotoxin-neutralizing capabilities. It was manufactured by Fresenius Kabi for The Rogosin Institute as described elsewhere [27]. It is similar to GR270773, the phospholipid emulsion in clinical development of total serum bile acids, by use of an enzymatic kit (SigmaStat for Windows (version 2.03; SPSS).

**RESULTS**

**Patients.** Sixteen men and 4 women were enrolled in the study. Eight men and 2 women received placebo, and 8 men and 2 women received emulsion. There were no dropouts, and all subjects completed the protocol. The male volunteers ranged in age from 21 to 40 years. The female volunteers ranged in age from 22 to 35 years. The ethnicity of the 20 volunteers was as follows: 5 were black, 5 were Hispanic, 8 were Caucasian, and 2 were of other ethnicity.

**Phospholipid levels.** Figure 1 shows the clearance kinetics for serum phospholipid in the emulsion and placebo groups. The shape of the curve for phospholipid emulsion reflects the administration protocol, which used a phospholipid infusion rate of 75 mg/kg/h for 2 h followed by the administration of endotoxin and then an infusion rate of 15 mg/kg/h for 4 h. At the time when endotoxin was administered (0-h time point), phospholipid level had increased in the emulsion group from 165 ± 19 to 415 ± 70 mg/dL. In contrast, there was a tendency toward lower endogenous phospholipid levels in the volunteers who received placebo. A decrease in phospholipid levels after the administration of endotoxin has been previously reported [30].
Figure 1. Change in serum phospholipid level. Emulsion (●) or placebo (○) was administered at a phospholipid infusion rate of 75 mg/kg/h for 2 h, followed by administration of endotoxin (lipopolysaccharide); emulsion or placebo was then administered at an infusion rate of 15 mg/kg/h for 4 h.

Clinical symptoms. The administration of endotoxin produced the typical symptomatic response of headaches, chills, and myalgias in the volunteers who received placebo. A marked attenuation of this response was noted in the volunteers who received emulsion, as is documented by the mean clinical score of 7 for the placebo group, compared with 2 for the emulsion group (P < .01). Significant differences in symptoms were noted for headaches (P < .001), chills (P < .02), and myalgias (P < .02) (figure 2). Among the volunteers who received emulsion, the trends observed for the responses to the endotoxin in the female volunteers were similar to those observed in the male volunteers (data not shown). The small number of women recruited into the study precluded a statistical analysis by sex.

There were 19 complaints or events in 9 volunteers occurring >12 h after the administration of endotoxin and were, by study design, considered to be adverse events. Six of these adverse events were noted in volunteers who received emulsion (diarrhea, 1; headaches, 1; musculoskeletal related, 3; and dermatological related, 1). In contrast, 13 of these adverse events were noted in volunteers who received placebo (related to intravenous access/phlebotomy, 8; headaches, 3; gastrointestinal related, 1; and musculoskeletal related, 1). None of the adverse events required intervention or were considered to be serious.

TNF-α and IL-6 levels. In the placebo group, the administration of endotoxin produced the expected burst in TNF-α level within 1 h, which was followed shortly thereafter by a rapid increase in IL-6 level (figure 3). Infusion of the phospholipid emulsion markedly blunted this cytokine response.

Vital signs. Administration of the phospholipid emulsion significantly inhibited the endotoxin-induced alteration in vital signs (figure 3). The emulsion group had significantly lower temperatures 2.5 h (emulsion group, 37.4°C; placebo group, 38.2°C) and 3 h (emulsion group, 37.7°C; placebo group, 38.5°C) after the administration of endotoxin. No differences were observed with respect to sex. The tachycardia associated with the administration of endotoxin was also attenuated in the emulsion group; significantly lower heart rates were observed at the 2.5-, 3-, and 5-h time points. The maximum difference in heart rate occurred at the 3-h time point (emulsion group, 81 bpm; placebo group, 94 bpm). There were no significant differences in systolic or diastolic blood pressures between the emulsion and control groups (data not shown).

Hemodynamics and oxygen saturation. The CI (expressed in L/min/m²) increases as part of the hyperdynamic state during sepsis. Three hours after the administration of endotoxin, the CI in the placebo group increased by 1.7 L/min/m² (from 2.1 ± 0.3 at baseline to 3.8 ± 0.7), and the CI in the emulsion group increased by 1.3 L/min/m² (from 2.0 ± 0.4 at baseline to 3.3 ± 1.2). This difference in increase between the placebo and emulsion groups (0.4 L/min/m²) was not statistically significant. There was a slight decrease in oxygen saturation after the administration of endotoxin for both groups (data not shown) that trended toward less of a decrease in the emulsion group at all time points. These differences were not statistically significant.

WBC count. The WBC response during experimental endotoxemia consists of an initial brief leukopenia followed by a
Neutralization of Endotoxin by a Lipid Emulsion

**Relationships between clinical score and (1) phospholipid level and (2) bile acid level.** Clinical score was inversely related \( r^2 = 0.557; P < .001 \) to the maximum phospholipid level achieved during the infusion of endotoxin (figure 4A). In the volunteers who achieved a maximum phospholipid level of \( \sim 500 \text{ mg/dL} \) or higher, there was a virtually complete inhibition of clinical response to endotoxin. A similar relationship was found between maximum phospholipid level and maximum TNF-\( \alpha \) level (figure 4B), IL-6 level (figure 4C), WBC count (figure 4D), and pulse rate (figure 4E). There was a significant dose-response relationship \( (P < .05) \) between the change in CI from baseline to the 3-h time point and the maximum phos-

Figure 3. Changes in cytokine levels, vital signs, and white blood cell (WBC) counts over time in the placebo (○) and emulsion (●) groups. IL-6, interleukin-6; TNF-\( \alpha \), tumor necrosis factor-\( \alpha \). * \( P < .05 \).

leukocytosis, with peak WBC counts at 6–8 h [29]. An initial decrease in WBC count was observed in both treatment groups (figure 3), with a marked, statistically significant \( (P < .05) \) leukocytosis in the placebo group from the 4-h time point (emulsion group, \( 7.6 \times 10^3 \) cells/\( \mu L \); placebo group, \( 9.8 \times 10^3 \) cells/\( \mu L \)) to the 22-h time point (emulsion group, \( 4.7 \times 10^3 \) cells/\( \mu L \); placebo group, \( 6.3 \times 10^3 \) cells/\( \mu L \)). This difference was predominately due to neutrophils, in that the absolute neutrophil count from the 4-h time point (emulsion group, \( 6.6 \times 10^3 \) cells/\( \mu L \); placebo group, \( 9.0 \times 10^3 \) cells/\( \mu L \)) to the 22-h time point (emulsion group, \( 2.4 \times 10^3 \) cells/\( \mu L \); placebo group, \( 4.2 \times 10^3 \) cells/\( \mu L \)) was significantly different between the 2 groups \( (P < .05) \).

Figure 4. Effect of phospholipid levels on symptom score, tumor necrosis factor (TNF-\( \alpha \), interleukin (IL)-6, white blood cell (WBC) count, and pulse rate in the placebo (○) and emulsion (●) groups. Max., maximum.
phospholipid level achieved (data not shown). With respect to the relationship between peak bile acid level and clinical outcome, the peak (2-h time point) bile acid level in the emulsion group was 130 ± 46 μmol/L, compared with 41 ± 18 μmol/L in the placebo group. In univariate analysis, peak bile acid level correlated with clinical score and peak phospholipid level. In multivariate analysis, the phospholipid level accounted for all of the relationship between peak bile acid level and clinical score, so that inclusion of peak bile acid level did not add to the ability of the model to predict the primary outcome.

**Lipoprotein levels.** The administration of endotoxin to the volunteers who received placebo produced a slight decrease in cholesterol and lipoprotein levels and a biphasic response (increase then decrease) in triglyceride level (figure 5). The administration of emulsion prevented the decrease in total and LDL cholesterol levels observed in the placebo group without producing a noticeable difference in HDL cholesterol level.

**DISCUSSION**

Unfavorable clinical outcomes have been observed in critically ill patients with hypolipidemia [18]. Our study supports the concept that increasing the low blood lipid levels found in patients with critical illness could have beneficial effects for treatment of endotoxemia. The protein-free, phospholipid-rich emulsion we used in our study was developed to test the hypothesis that increased blood phospholipid levels would promote endotoxin neutralization in vivo. The results of our study support this conclusion and provide additional evidence for the important role that lipids play in the metabolism of endotoxin. That the administration of emulsion provides benefits was shown by the significant difference in the primary study-outcome measure (i.e., clinical score) between the volunteers who received emulsion and those who received placebo. The clinical advantage for the administration of emulsion was associated with a blunting of the typical changes induced by LPS in temperature, pulse rate, cytokine levels, and WBC count. That there were fewer vascular-access complications in the emulsion group than in the placebo group was likely due to the marked vasoconstriction noted in the latter group, which made drawing blood difficult and precipitated a loss of peripheral venous vascular access in several of the volunteers who received placebo.

Blood-derived lipoproteins have been previously used to neutralize endotoxin in animals and humans [24, 31]. The use of a protein-free emulsion eliminates the safety concerns that exist with blood-derived lipoproteins and decreases the costs should substitution of a recombinant protein for the natural apolipoprotein be considered. The emulsion consists of soy-derived phospholipid, soy-derived triglyceride, and sodium cholate, all substances that are readily available and individually approved for parenteral use in humans.

The normal variability in serum phospholipid levels permitted

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**Figure 5.** Changes in lipid and lipoprotein levels in the placebo (○) and emulsion (●) groups. HDLC, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride.
a dose-response analysis despite the administration of a fixed dose of emulsion. There was a dose-response relationship between phospholipid level and (1) clinical score and (2) cytokine levels in the volunteers who received emulsion. In the present study, optimal clinical benefits appeared at a serum phospholipid level of ~500 mg/dL or higher. The volunteers who achieved this phospholipid level had few or no clinical symptoms in response to the administration of endotoxin. The limited number of volunteers in the placebo group precluded a meaningful analysis of the relationship between phospholipid levels and clinical response to endotoxin in these control subjects.

Studies have demonstrated that all lipoprotein species, including very-low-density lipoproteins, have some endotoxin-neutralizing capabilities. Our previous studies demonstrated that the phospholipid level of the lipoprotein most closely correlates with endotoxin-binding ability [12]. Among the lipoproteins, HDL has the highest percentage of phospholipid and has received the most study. The present study provides evidence that phospholipid has endotoxin-neutralizing ability, because the emulsion we used is 92.5% phospholipid. Given that this phospholipid emulsion would be expected to remodel quickly across all lipoprotein species in the circulation, all circulating lipoproteins would be enriched for phospholipid and would have enhanced endotoxin-neutralizing capabilities.

The bile salt sodium cholate, a component of the phospholipid emulsion, could contribute to the emulsion’s antiendotoxin activity. A related bile salt, sodium deoxycholate, was found to disaggregate LPS in vitro [32], but at a level ∼30-fold higher than the blood levels of cholate achieved in the present study. Furthermore, little of the cholate added to the emulsion is free and available in vivo for direct neutralization of LPS. Rather than cholate directly affecting LPS, it is possible that the inclusion of cholate produces a phospholipid particle that neutralizes LPS more effectively in vivo.

The present study used a pretreatment protocol to provide protection against the administration of endotoxin in the volunteers. Because we did not administer the emulsion after the infusion of endotoxin, we cannot make any statements on the ability of the emulsion to inhibit the endotoxin cascade once endotoxin is already present. However, the ability of lipoproteins to remove LPS from the CD14 receptor [33] suggests that some degree of rescue is theoretically possible. Furthermore, a study has demonstrated the repeated and recurrent nature of endotoxin exposure in patients with septic shock in a critical care unit [2].

The present study provides encouraging data supporting further investigation of the use of the emulsion for the treatment of patients at risk for endotoxemia and the sepsis syndrome. It also provides direct evidence that phospholipid plays a role in the neutralization of endotoxin.

Acknowledgments

We acknowledge the assistance of Knut Witkowski, for providing help in designing the study; Daniel Bonville, for participating in the care of the volunteers; the nursing staff of the Rockefeller General Clinical Research Center (New York, NY); and Johanne Andersen, for preparing the lipopolysaccharide, placebo, and phospholipid emulsion for administration.

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