Hemodynamic Changes Induced by Liposomes and Liposome-Encapsulated Hemoglobin in Pigs

A Model for Pseudoallergic Cardiopulmonary Reactions to Liposomes: Role of Complement and Inhibition by Soluble CR1 and Anti-C5a Antibody

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Background—Intravenous administration of some liposomal drugs can trigger immediate hypersensitivity reactions that include symptoms of cardiopulmonary distress. The mechanism underlying the cardiovascular changes has not been clarified.

Methods and Results—Anesthetized pigs (n = 18) were injected intravenously with 5-mg boluses of large multilamellar liposomes, and the ensuing hemodynamic, hematologic, and laboratory changes were recorded. The significant (P < 0.01) alterations included 79±9% (mean±SEM) rise in pulmonary arterial pressure, 30±7% decline in cardiac output, 11±2% increase in heart rate, 236±54% increase in pulmonary vascular resistance, 71±27% increase in systemic vascular resistance, and up to a 100-fold increase in plasma thromboxane B2. These changes peaked between 1 and 5 minutes after injection, subsided within 10 to 20 minutes, were lipid dose-dependent (ED50 = 4.5 ± 1.4 mg), and were quantitatively reproducible in the same animal several times over 7 hours. The liposome-induced rises of pulmonary arterial pressure showed close quantitative and temporal correlation with elevations of plasma thromboxane B2 and were inhibited by an anti-C5a monoclonal antibody (GS1), by sCR1, or by indomethacin. Liposomes caused C5a production in pig serum in vitro through classic pathway activation and bound IgG and IgM natural antibodies. Zymosan- and hemoglobin-containing liposomes and empty liposomes caused essentially identical pulmonary changes.

Conclusions—The intense, nontachyphylactic, highly reproducible, complement-mediated pulmonary hypertensive effect of minute amounts of intravenous liposomes in pigs represents a unique, unexplored phenomenon in circulation physiology. The model provides highly sensitive detection and study of cardiopulmonary side effects of liposomal drugs and many other pharmaceutical products due to “complement activation–related pseudoallergy” (CARPA).

Key Words: thromboxane n hemodynamics n hypertension, pulmonary n immune system n blood cells

During the past few years, numerous clinical studies have been performed with liposomal formulations of anticancer drugs and other therapeutic agents. These studies attest to the general safety of intravenous liposomes, as 4 liposomal drugs entrapping doxorubicin (Doxil), daunorubicin (DaunoXome), and amphotericin B (Abelcet and Ambisome) are already licensed in several countries and many others are in advanced clinical trials. Nevertheless, some of the studies have also revealed a hypersensitivity reaction to liposomes that develops immediately after the start of infusion and includes symptoms of cardiopulmonary distress, such as dyspnea, tachypnea, tachycardia, hypotension and hypertension, chest pain, and back pain. Unlike IgE-mediated (type I) allergy, the reaction to liposomes arises at first exposure to the drug without prior sensitization, and the symptoms usually lessen or disappear on later treatments. Because of these unusual features, the reaction has recently been called “pseudoallergic.”

The frequency of such reactions among 705 patients treated with Doxil was 6.8%, which is comparable to the incidence rate reported in other liposome trials. With the underlying cause not being understood, at present it is impossible to anticipate or specifically treat these reactions, which are severe, life-threatening in some 0.9% of
patients, precluding further treatment with the liposomal formulation.\textsuperscript{8} 

It is known that certain liposomes can activate the complement system\textsuperscript{9,10} and that complement activation can lead to cardiovascular and pulmonary adverse responses very similar to those described above.\textsuperscript{11–13} Nevertheless, complement activation has not been implicated previously in the above-described clinical reactions.\textsuperscript{2–9} In an effort to test the hypothesis that complement activation plays a causal role in the cardiopulmonary reaction to intravenous liposomes, we extended here an earlier report from our laboratory on liposome-induced anaphylactoid reaction in miniature pigs.\textsuperscript{14} It was suggested in that study that the reaction was due to complement activation; however, direct, conclusive evidence regarding the causal role of complement was not available.

Another goal of the present study was to examine the acute physiological effects of the oxygen-carrying blood substitute liposome-encapsulated hemoglobin (LEH)\textsuperscript{15,16} in pigs. One of the potential applications of LEH is substitution of shed blood in trauma patients, who are prone to develop adult respiratory distress syndrome partly as a consequence of injury-related complement activation.\textsuperscript{12,13} Liposome-induced complement activation with additional cardiopulmonary distress therefore represents a critical safety issue that could be usefully addressed in a model sensitive to complement-mediated vasoactivity.

**Methods**

**Closed-Chest Instrumented Pig**

Experiments were performed in accordance with guidelines of the Committee on Animal Care of the Uniformed Services University of the Health Sciences. Female Yorkshire swine (32 to 48 kg) were sedated with intramuscular ketamine, anesthetized with halothane (1%), and instrumented as described previously in detail.\textsuperscript{17} In brief, a catheter was advanced via the right internal jugular vein into the pulmonary artery to measure pulmonary artery pressure (PAP), central venous pressure (CVP), and cardiac output (CO); another was advanced through the right femoral artery into the proximal aorta to measure systemic arterial pressure (SAP) and for blood sampling; and a third catheter was placed into the left ventricle through the left femoral artery to monitor left ventricular end-diastolic pressure (LVEDP). Systemic vascular resistance (SVR) and pulmonary vascular resistance (PVR) were calculated from SAP, PAP, CO, CVP, and LVEDP by standard formulas.\textsuperscript{17} Blood pressure and leads II and V_{1} of the ECG were recorded continually.

**Preparation of Liposomes and LEH**

The preparation and characteristics of large unilamellar liposomes consisting of dimyristoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol, and cholesterol (50:5:45 mole ratios) with 0.5% α-tocopherol and 40 mg/mL heat-sterilized, diaspircin-crosslinked human hemoglobin (in LEH) were described previously.\textsuperscript{18} Liposomes were suspended in normal saline or PBS at 1 to 100 mg/mL lipid (≈1 to 100 mmol/L phospholipid).

**Experimental Protocol**

Liposomes were injected into the jugular vein in 1-mL boluses containing 5 mg lipid (3.4 mg phospholipid and 1.6 mg cholesterol). These injections were repeated several times at 20- to 60-minute intervals. Before each injection, at 1-minute intervals up to 10 to 15 minutes, and finally at 30 minutes, hemodynamic parameters were recorded and arterial samples (2 to 3 mL) were withdrawn into EDTA-containing vials for white blood cell, platelet, total serum hemolytic complement/mL, and thromboxane B\textsubscript{2} (TXB\textsubscript{2}) measurement.

ments, as described previously.\textsuperscript{19} Blood was also collected for hemoglobin, arterial \textit{O\textsubscript{2}}, mixed venous \textit{O\textsubscript{2}}, pH, and plasma HCO\textsubscript{3} measurements, which remained in the normal range throughout the studies.

In further experiments, 2-mL aliquots from freshly prepared pig serum were incubated with liposomes (10 mg/mL lipid) at 37°C for 10 minutes with shaking. After addition of 4 volumes of PBS, vesicles were pelleted and the supernatant was immediately injected into pigs as described above for liposomes.

**Complement Antagonists**

Murine anti-porcine C5a (GS1, Chemicon) was prepared from tissue culture or ascites, purified by protein G affinity chromatography, and dialyzed against PBS (purity, >95%). It was shown previously to inhibit C5a-induced porcine neutrophil aggregation with an IC\textsubscript{50} of 3 µg/mL and to significantly inhibit polymorphonuclear leukocyte (PMN) chemotaxis at a dose of 17 µg/mL.\textsuperscript{20} Administration in pigs at 1.6 mg/kg maintained plasma GS1 levels >40 µg/mL for at least 3 hours.\textsuperscript{20a}

Recombinant soluble complement receptor type 1 (sCR1)\textsuperscript{21} was obtained from T Cell Sciences, Inc. Its plasma clearance in pigs was reported to have α- and β-phase \textit{t}_{1/2} values of 8.3 and 363 minutes, respectively, with 31% of drug clearing slowly (US patent 5,456,909). Previous studies showed 0.8 to 20 µg/mL sCR1 to effectively suppress LEH-induced complement activation in human serum in vitro.\textsuperscript{18}

**C5a Production and Immunoglobulin Binding by Liposomes In Vitro**

Liposomes were incubated with pig serum with or without 10 mmol/L EGTA/2.5 mmol/L Mg\textsuperscript{2+} for 10 minutes at 37°C with shaking, and after centrifugal separation of vesicles, C5a was measured in serum by a chemotaxis assay.\textsuperscript{22} To measure liposome-bound IgG and IgM, vesicles were fixed in 1% paraformaldehyde (30 minutes, 4°C), washed with PBS, and stained with class-specific anti-swine antibodies (Kirkegaard). Fluorescence labeling was done with FITC-conjugated F(ab\textsuperscript{′})\textsubscript{2} (Jackson) directed against anti-swine antibodies. Fluorescence-activated cell sorting (FACS) analysis was performed in a FACSort flow cytometer with live gating set on the forward scatter parameter.

**Statistical Methods**

Data are presented as single values or mean±SEM. Differences were analyzed by Student’s \textit{t} tests or by ANOVA followed by Newman-Keuls correction. Fitting of nonlinear equations was done as described previously\textsuperscript{23} by use of maximum-likelihood algorithms of Gauss System 3.02 and Gauss 3.5 (Aptech Systems). Confidence limits and standard errors of coefficients were obtained by computations of multiple regression coefficient (\textit{R}²), residual-sum squares, and Durban-Watson statistics for serial errors.\textsuperscript{23} Randomness of residuals and error variance were examined by Wilk-Shapiro statistics (Statistix 4.1, NH Analytical Software) and the heteroscedasticity routines of Gauss 3.5, respectively.

**Results**

**Effects of Liposomes and LEH on Pulmonary and Systemic Circulation**

Figure 1 demonstrates typical hemodynamic changes caused by injections of liposome boluses containing 5 mg lipid, defined as “standard” injection. These changes were transient and included a 50% to 250% increase in PAP (A), a 0% to 80% decline in CO (B), a 2- to 6-fold increase in PVR (C), a 5% to 10% increase in heart rate (D), a 20% to 40% fall or rise of biphasic changes in SAP (E), and a 0% to 400% rise of systemic vascular resistance (F). The reactions started within 1 to 2 minutes after injection, reached peaks within 5
to 6 minutes, and returned to respective baselines within 10 to 15 minutes.

Figure 2 illustrates that 8 repetitive injections of standard liposome boluses into a pig at 30- to 60-minute intervals produced virtually identical rises in PAP, implying a lack of tachyphylaxis and remarkable quantitative reproducibility of hypertensive response. The latter properties were verified for most animals examined by injection of the standard boluses 2 to 3 times at the beginning and toward the end of the 6- to 8-hour experiments.

Table 1 summarizes all hemodynamic data obtained in 18 pigs injected several times with the standard liposome boluses or an equivalent amount of LEH. The changes, expressed as percentage of baseline for peak responses, were significantly different from baseline (P<0.01, paired t test), except for SAP. Furthermore, the hemodynamic effects of empty liposomes were not significantly different from those caused by LEH, indicating that the changes were accounted for primarily by the phospholipid bilayer of liposomes. Nevertheless, there was a tendency for larger increases and greater variabilities in PAP and PVR in the case of LEH, suggesting some influence of surface-exposed hemoglobin.

In addition to the above changes, we also observed that the most intense reactions were associated with transient ST-segment depression and T-wave changes on the ECG (data not shown), implying cardiac ischemia. Furthermore, the most marked elevations of PAP and declines of CO were associated with an initial decline of SAP (Figure 1E), with increased CVP and decreased LVEDP (data not shown); these observations point to increased PVR as the primary effect of liposomes. The observation that the increase in heart rate (Figure 1D) occurred independently of the changes in CO and/or SAP (Figure 1B and E) raises the possible involvement of mechanisms other than baroreflex response; for example, transient blockage of coronary circulation and/or direct humoral effects (complement split products, catecholamines) on the heart.

Dose-Response Relationship

Figure 3A shows that the pulmonary hypertensive effect of liposomes displayed linear dependence on lipid dose in the 0- to 20-mg range, with an estimated ED50 of 4.5±1.4 mg lipid. This provided the rationale for using 5-mg lipid boluses as standard test dose. With boluses containing ≥20 mg lipid, the dose-response curve reached its plateau, indicating saturation of response. We also observed a dose-dependent change in the kinetics of PAP response, with readily reversible (within 10 minutes), symmetrical peaks after the injection of ≥10-mg liposome boluses and slowly reversing, asymmetrical waves after administration of 50 and 100 mg lipid (Figure 3B).

Involvement of Serum in Mediating the Hemodynamic Effects of Liposomes

Intravenous injection of the pigs’ own serum after in vitro incubation with liposomes caused significant increases in PAP (65±16%, n=5 pigs), whereas untreated serum or serum that had been heat-inactivated at 55°C for 30 minutes before incubation with liposomes caused no or significantly less pulmonary hypertension (10±2% and 21±9%, respectively, n=4 pigs). These observations suggest that the pulmonary hypertensive effect of liposomes was linked primarily to an interaction of the vesicles with serum rather than to physical obstruction of pulmonary microcirculation or direct effects on tissue or blood cells. The heat-sensitivity of serum elements that are involved in this interaction points to a key role of complement proteins.

Effects of Liposomes on Platelets, White Blood Cells, and Serum Complement Levels

Figure 4 shows early (2 to 5 minutes), minor (5% to 18%) decreases in platelet counts in 4 of 8 tested animals (A), with a parallel, minor decline of white blood cell count in 1 pig (B). Similar measurements after 2 to 3 subsequent liposome injections produced essentially identical results, with transient, <20% drops in cell counts. Measurements of hemolytic complement levels in pig plasma before and at 5 and 30 minutes after injection of standard boluses indicated no significant complement consumption (total hemolytic com-
TABLE 1. Descriptive Statistics of Liposome- and Liposome-Encapsulated Hemoglobin–Induced Hemodynamic Changes in Pigs

<table>
<thead>
<tr>
<th></th>
<th>% Change Relative to Baseline</th>
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<tbody>
<tr>
<td></td>
<td>PAP, mm Hg</td>
</tr>
<tr>
<td><strong>Liposomes</strong></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>79</td>
</tr>
<tr>
<td>SEM</td>
<td>9</td>
</tr>
<tr>
<td>No. of pigs (injections)</td>
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</tr>
<tr>
<td>P</td>
<td>8x10⁻⁴</td>
</tr>
<tr>
<td><strong>LEH</strong></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>103</td>
</tr>
<tr>
<td>SEM</td>
<td>23</td>
</tr>
<tr>
<td>No. of pigs (injections)</td>
<td>6 (8)</td>
</tr>
<tr>
<td>P</td>
<td>0.3</td>
</tr>
</tbody>
</table>

The hemodynamic parameters were recorded before the injection of 5-mg liposome boluses and at the peak of reaction, and the percentage changes were obtained from the peak-baseline/baseline ratio. P values for liposomes compare the maximal changes with baseline and were obtained with Student's paired t test (2-tailed). For LEH, P values compare LEH vs liposomes by use of Student's 2-sample t test (2-tailed).

Liposome-Induced Changes in Plasma TXA₂

Figure 5A shows that injection of the standard bolus in a pig caused massive (30-fold) increase in plasma TXB₂ levels with a time course that exactly mirrored the rise of PAP. A second injection 30 minutes later, as well as several injections over the course of hours (Figure 5B), produced essentially identical, parallel rises of PAP and TXB₂.

Figure 6 plots PAP peak responses versus plasma TXB₂, using all matched preinjection and postinjection readings from 7 pigs. The best fit is a sigmoidal dose-response curve that shows no correlation between PAP and TXB₂ below ~1 ng/mL TXB₂ but a strong, linear correlation above these values until the pulmonary response reaches saturation around 80 mm Hg. These data suggest that TXA₂-induced vasoconstriction is likely to be a major mechanism of pulmonary hypertension.

Effects of Complement Inhibitors and Indomethacin on Liposome-Induced Pulmonary Hypertension

Direct evidence for causal roles of both complement activation and TXA₂ release in liposome-induced pulmonary hypertension came from experiments using the specific complement inhibitors GS1 and sCR1 and the cyclooxygenase inhibitor indomethacin. These blockers inhibited the liposome-induced rises in PAP relative to preinhibitor (baseline) response (Figure 7A), most efficiently indomethacin. The suppression of hypertensive response was not due to nonspecific toxicity or tachyphylaxis, because the inhibitory
effects of these agents could be overcome by increasing the
liposome doses (illustrated for sCR1). Figure 7B demon-
strates the time points and extent of maximal inhibition that
we observed in 4 pigs treated with each of the above
inhibitors. We found that 5 mg/kg indomethacin completely
blunted the pulmonary reaction to 5-mg liposome boluses in
all pigs tested, whereas 1.6 mg/kg GS1 exerted 25% to 60%
inhibition, and 0.2 and 2 mg/kg sCR1 (in 2 to 2 pigs) caused
30% to 100% inhibition. These differences between preinhib-
itor and postinhibitor rises in PAP were significant (P < 0.01)
by Student’s paired t test.

Pulmonary Vascular Effects of Zymosan
Injection of the (alternative pathway) complement activator
zymosan in pigs in a fashion and at a dose level (5 mg) that
simulated the administration of liposomes caused a 53 ± 13% increase in PAP (n = 7 injections in 4 pigs) with a time course
that was indistinguishable from that observed with the stan-
dard liposome injections.

In Vitro Studies on the Mechanism
of Liposome-Induced Complement Activation
Table 2 shows that (1) incubation of pig serum with lipo-
somes in vitro increased the leukocyte chemotactic activity of
serum, (2) this increase was inhibited in the presence of GS1,
and (3) the chemotaxis-promoting effect of liposomes was
inhibited by EGTA/Mg2+. The first 2 observations provide
evidence that liposomes can trigger complement activation in
pig blood with resultant production of C5a, whereas the
inhibitory effect of EGTA/Mg2+ on this process shows that
this activation was Ca2+-dependent, a characteristic of classic
pathway activation.

One possibility for complement activation via the classic
pathway is the binding of natural anti-lipid antibodies to
liposomes. To test this possibility, we measured the amount
of immunoglobulins on the surface of liposomes after incu-
bation with pig serum in vitro. The FACS analysis shown in
Figure 8 indicated binding of both IgG (A) and IgM (B)
antibodies to liposomes, implying that preconditions for
classic pathway activation exist in pigs.
The present experiments extended a previous study from our laboratory reporting that intravenous injection of liposomes in miniature pigs triggered a dramatic anaphylactoid reaction. The liposomes were the same as applied here but were injected slowly (3 to 5 minutes) at a 500-fold higher dose. The reaction was associated with hemodynamic and TXB2 changes similar to those described here, except that they were prolonged for 30 to 60 minutes and were associated with significant (36% to 38%) leukopenia and thrombocytopenia. Furthermore, unlike in the present study, repeat injections of liposomes caused death in 3 of 4 animals. Thus, the reduction of liposome dose and a change in administration protocol resulted in improved reproducibility and control of hemodynamic changes, providing a sensitive animal model for liposome-induced cardiopulmonary distress.

## Discussion

### A Novel Porcine Model of Pseudoallergic Cardiopulmonary Distress

The advance of liposomal drugs into clinical trials brought attention to an unusual hypersensitivity reaction that included hemodynamic changes with cardiopulmonary distress. Although in most cases the symptoms are manageable with corticosteroids and antihistamines, the reaction can be life-threatening in an occasional patient with a history of allergy and cardiopulmonary disease. The phenomenon has been called pseudoallergy; however, its mechanism has not been clarified and, to the best of our knowledge, has never been studied in an animal model.

The present experiments extended a previous study from our laboratory reporting that intravenous injection of liposomes in miniature pigs triggered a dramatic anaphylactoid reaction. The liposomes were the same as applied here but were injected slowly (3 to 5 minutes) at a 500-fold higher dose. The reaction was associated with hemodynamic and TXB2 changes similar to those described here, except that they were prolonged for 30 to 60 minutes and were associated with significant (36% to 38%) leukopenia and thrombocytopenia. Furthermore, unlike in the present study, repeat injections of liposomes caused death in 3 of 4 animals. Thus, the reduction of liposome dose and a change in administration protocol resulted in improved reproducibility and control of hemodynamic changes, providing a sensitive animal model for liposome-induced cardiopulmonary distress.

### Evidence for a Causal Role of Complement Activation

Based on the symptoms, laboratory changes, and evidence of serum-induced, anti-cholesterol antibody– and complement-dependent immune damage to liposomes in vitro, we proposed previously that the liposome-induced anaphylactoid reaction in pigs could be due to complement activation. The present work obtained the following new, more direct support for this concept: (1) the pulmonary response to liposomes was mediated by a heat-sensitive serum component; (2) the hemodynamic effects of liposomes were mimicked by the complement activator zymosan; (3) incubation of pig serum with liposomes in vitro led to the formation of C5a; (4) the liposome-induced rises of plasma TXB2, one of the secondary mediators produced in response to anaphylatoxin binding to responsive cells, closely paralleled the rises of PAP; and (5) the specific complement inhibitors GS1 and sCR1 caused significant inhibition of liposome-induced pulmonary hypertension. This latter observation was of particular importance because it provided direct evidence for a causal role of complement activation in the hemodynamic changes.

### Reaction Sequence

Figure 9 provides a hypothetical reaction scheme for liposomal complement activation and subsequent cellular and molecular interactions that may underlie the hemodynamic response. With regard to the mechanism of complement activation, our data are consistent with natural antibody–mediated classic pathway activation, as described previously for liposome and LEH-induced complement activation in human serum. However, the involvement of other mechanisms, such as the alternative pathway amplification loop or direct binding of C1q and/or C3 to the phospholipid bilayer, cannot be excluded.

The efficient coupling of relatively weak complement signal to massive hemodynamic changes was most likely achieved through the actions of vasoactive mediators from PMNs, platelets, macrophages, basophils, and mast cells released in response to the binding of anaphylatoxins and C5b-9 to these cells. Among the secondary mediators, we focused here on TXA2, a potent vasoconstrictor eico-
nary circulatory systems, whereby a minimal exposure of foreign particles to blood leads to substantial circulatory derangements. The reaction is a major manifestation of pseudoallergy, a poorly understood immediate hypersensitivity syndrome. Our evidence that complement activation is causally involved in the phenomenon provides a rationale to tentatively define it as "complement activation-related pseudoallergy," or "CARPA," and to use complement and cyclooxygenase inhibitors for the prevention or alleviation of symptoms.

Our finding that LEH causes cardiopulmonary distress in pigs suggests that the formulation tested may aggravate the clinical state of trauma patients. Therefore, it seems critical to reduce or eliminate the complement-activating potency of this or similar blood substitutes. The porcine model presented affords a uniquely sensitive bioassay for this purpose, as well as for the screening of liposomal drugs for potential cardiopulmonary side effects. The model could also be used for the biocompatibility testing of colloidal dispersions, particulate biomaterials, oil-based drug vehicles (such as Cremophor EL31), and many other pharmaceutical products that may cause unexplained hypersensitivity reactions.

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References


Implications of the Porcine Liposome-Induced Cardiopulmonary Distress Model

The present experiments highlight a little-known yet clinically important interaction between the immune and pulmo-


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