

# 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  $79\pm 9\%$  (mean $\pm$ SEM) rise in pulmonary arterial pressure,  $30\pm 7\%$  decline in cardiac output,  $11\pm 2\%$  increase in heart rate,  $236\pm 54\%$  increase in pulmonary vascular resistance,  $71\pm 27\%$  increase in systemic vascular resistance, and up to a 100-fold increase in plasma thromboxane  $B_2$ . These changes peaked between 1 and 5 minutes after injection, subsided within 10 to 20 minutes, were lipid dose-dependent ( $ED_{50}=4.5\pm 1.4$  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  $B_2$  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). (*Circulation*. 1999;99:2302-2309.)

**Key Words:** thromboxane ■ hemodynamics ■ hypertension, pulmonary ■ immune system ■ 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.<sup>1</sup> Nevertheless, some of the studies<sup>2-9</sup> 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.”<sup>9</sup> The frequency of such reactions among 705 patients treated with Doxil was 6.8%,<sup>8</sup> which is comparable to the incidence rate reported in other liposome trials.<sup>3,4,6,7,9</sup> 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

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patients, precluding further treatment with the liposomal formulation.<sup>8</sup>

It is known that certain liposomes can activate the complement system<sup>10</sup> and that complement activation can lead to cardiovascular and pulmonary adverse responses very similar to those described above.<sup>11–13</sup> Nevertheless, complement activation has not been implicated previously in the above-described clinical reactions.<sup>2–9</sup> 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.<sup>14</sup> 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)<sup>15,16</sup> 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.<sup>12,13</sup> 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.<sup>17</sup> 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.<sup>17</sup> Blood pressure and leads II and V<sub>5</sub> of the ECG were recorded continually.

### Preparation of Liposomes and LEH

The preparation and characteristics of large multilamellar liposomes consisting of dimyristoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol, and cholesterol (50:5:45 mole ratios) with 0.5%  $\alpha$ -tocopherol and 40 mg/mL heat-sterilized, diaspirin-crosslinked human hemoglobin (in LEH) were described previously.<sup>18</sup> Liposomes were suspended in normal saline or PBS at 1 to 100 mg/mL lipid ( $\approx$ 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<sub>2</sub> (TXB<sub>2</sub>) measure-

ments, as described previously.<sup>19</sup> Blood was also collected for hemoglobin, arterial O<sub>2</sub>, mixed venous O<sub>2</sub>, pH, and plasma HCO<sub>3</sub> 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<sub>50</sub> of 3  $\mu$ g/mL and to significantly inhibit polymorphonuclear leukocyte (PMN) chemotaxis at a dose of 17  $\mu$ g/mL.<sup>20</sup> Administration in pigs at 1.6 mg/kg maintains plasma GS1 levels >40  $\mu$ g/mL for at least 3 hours.<sup>20a</sup>

Recombinant soluble complement receptor type 1 (sCR1)<sup>21</sup> was obtained from T Cell Sciences, Inc. Its plasma clearance in pigs was reported to have  $\alpha$ - and  $\beta$ -phase  $t_{1/2}$  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  $\mu$ g/mL sCR1 to effectively suppress LEH-induced complement activation in human serum *in vitro*.<sup>18</sup>

### 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<sup>2+</sup> for 10 minutes at 37°C with shaking, and after centrifugal separation of vesicles, C5a was measured in serum by a chemotaxis assay.<sup>22</sup> 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')<sub>2</sub> (Jackson) directed against anti-swine antibodies. Fluorescence-activated cell sorting (FACS) analysis was performed in a FACSflow cytometer with live gating set on the forward scatter parameter.

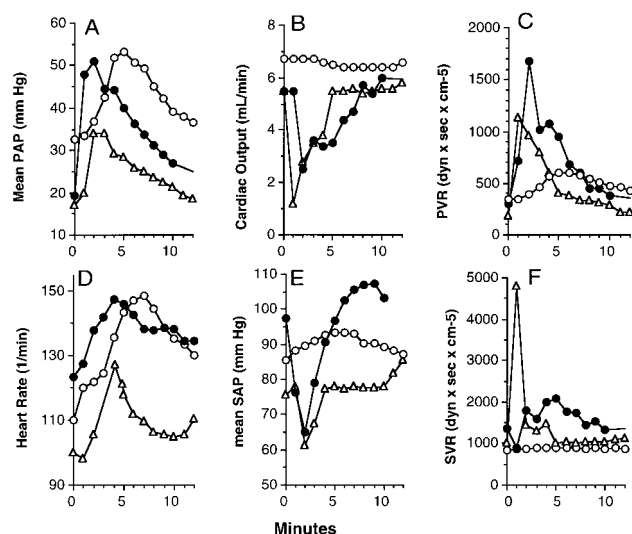
### Statistical Methods

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

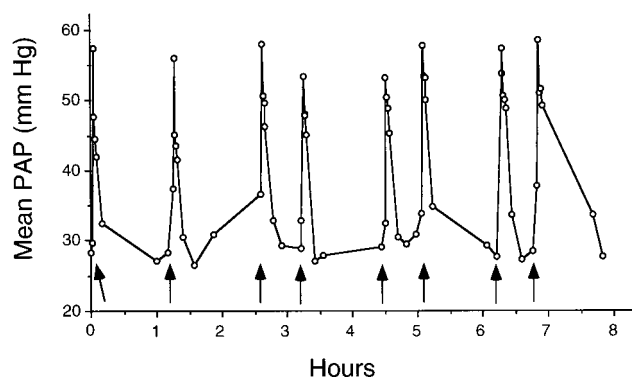


**Figure 1.** Hemodynamic changes induced by intravenous injection of 5-mg liposome boluses in pigs. Representative curves obtained in 3 pigs. Different symbols designate different pigs.

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



**Figure 2.** PAP responses to repetitive liposome injections. A pig was injected intravenously with standard, 5-mg lipid-containing liposome boluses 8 times at indicated time points, and changes in PAP were recorded. Arrows indicate time points of injections.

greater variabilities in PAP and PVR in the case of LEH, suggesting some influence of surface-exposed hemoglobin.<sup>18</sup>

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  $ED_{50}$  of  $4.5 \pm 1.4$  mg lipid. This provided the rationale for using 5-mg lipid boluses as standard test dose. With boluses containing  $\geq 20$  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  $\leq 10$ -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 ( $65 \pm 16\%$ ,  $n=5$  pigs), whereas untreated serum or serum that had been heat-inactivated at  $55^\circ\text{C}$  for 30 minutes before incubation with liposomes caused no or significantly less pulmonary hypertension ( $10 \pm 2\%$  and  $21 \pm 9\%$ , 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**

	% Change Relative to Baseline					
	PAP, mm Hg	CO, L/min	PVR, dyne · s <sup>-1</sup> · cm <sup>-5</sup>	HR, bpm	SAP, mm Hg	SVR, dyne · s <sup>-1</sup> · cm <sup>-5</sup>
<b>Liposomes</b>						
Mean	79	-30	236	11	-1	71
SEM	9	7	54	2	4	27
No. of pigs (injections)	18 (51)	17 (48)	17 (48)	17 (48)	18 (51)	17 (48)
<i>P</i>	8×10 <sup>-8</sup>	8×10 <sup>-4</sup>	10 <sup>-4</sup>	6×10 <sup>-4</sup>	0.81	2×10 <sup>-6</sup>
<b>LEH</b>						
Mean	103	-38.4	466	13	-10	84
SEM	23	15.4	205	5	12	38
No. of pigs (injections)	6 (8)	6 (8)	6 (8)	6 (8)	6 (8)	6 (8)
<i>P</i>	0.3	0.6	0.1	0.6	0.4	0.8

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).

plement/mL values were 147±28, n=6 pigs; 162±26, n=5; and 137±26, n=3, respectively).

### Liposome-Induced Changes in Plasma TXA<sub>2</sub>

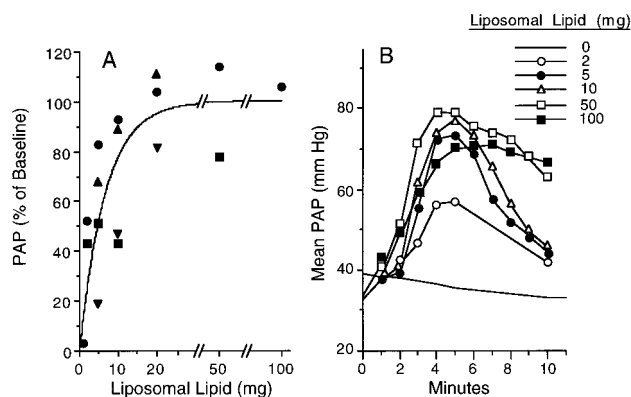
Figure 5A shows that injection of the standard bolus in a pig caused massive (30-fold) increase in plasma TXB<sub>2</sub> levels with a time course that exactly mirrored the rise of PAP. A second injection 30 minutes later, as well as several injections over the course of hours (Figure 5B), produced essentially identical, parallel rises of PAP and TXB<sub>2</sub>.

Figure 6 plots PAP peak responses versus plasma TXB<sub>2</sub>, 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 TXB<sub>2</sub> below ≈1 ng/mL TXB<sub>2</sub> but a strong, linear correlation above these values until the pulmonary response reaches saturation

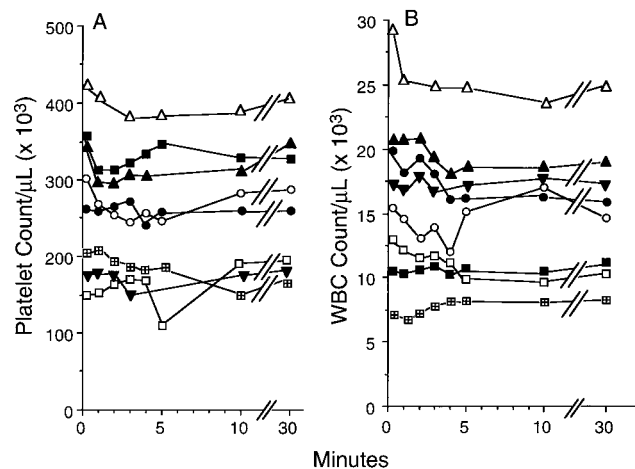
around 80 mm Hg. These data suggest that TXA<sub>2</sub>-induced vasoconstriction is likely to be a major mechanism of pulmonary hypertension.

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

Direct evidence for causal roles of both complement activation and TXA<sub>2</sub> 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

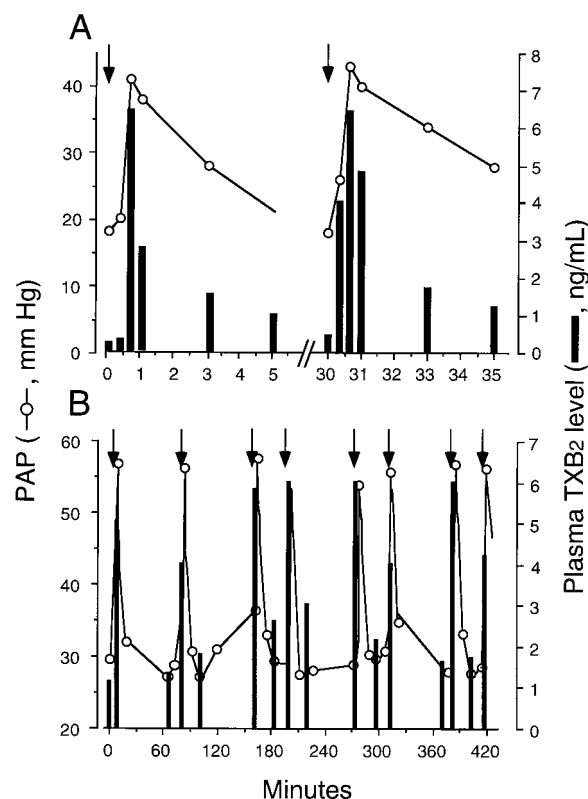


**Figure 3.** A, Dose-response relationship between liposomal lipid and PAP. Data compiled from 4 different pigs, indicated by different symbols. Fitted is a monoexponential curve in the following form:  $PAP = 100.2 \times (1 - e^{-0.15 \times L})$ , where *L* is lipid dose in mg.  $ED_{50} = 4.5 \pm 1.4$  mg;  $R^2 = 0.78$ ; Durban-Watson value = 0.85; and error variance constant (heteroscedasticity)  $P = 0.6$ .<sup>23</sup> B, Dose-response relationship between liposomal lipid and PAP in a representative pig, demonstrating extended response to ≥50 mg lipid.



**Figure 4.** Liposome-induced changes in platelet (A) and white blood cell (WBC, B) counts. Cell counts were determined as described in Methods, before injection of a standard liposome bolus (zero minutes) and at different times thereafter, as indicated. Different symbols designate individual pigs.





**Figure 5.** Time course of liposome-induced changes in plasma TXB<sub>2</sub> and PAP. Pigs were repetitively injected with standard liposome boluses, and changes in PAP (circles) and plasma TXB<sub>2</sub> (bars) were plotted as a function of time for first 2 injections in one pig (A) or over hours in another pig (B).

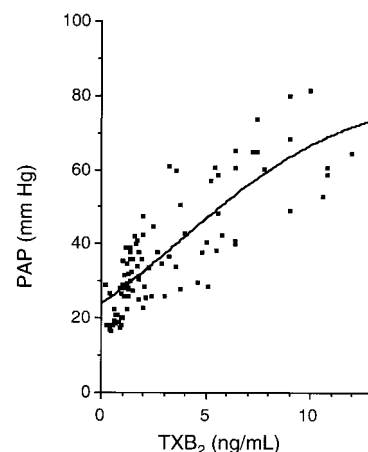
effects of these agents could be overcome by increasing the liposome doses (illustrated for sCR1). Figure 7B demonstrates the time points and extent of maximal inhibition that we observed in 4 pigs treated with each of the above inhibitors. We found that 5 mg/kg indomethacin completely blunted the pulmonary 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 preinhibitor 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  $53 \pm 13\%$  increase in PAP ( $n = 7$  injections in 4 pigs) with a time course that was indistinguishable from that observed with the standard liposome injections.

### In Vitro Studies on the Mechanism of Liposome-Induced Complement Activation

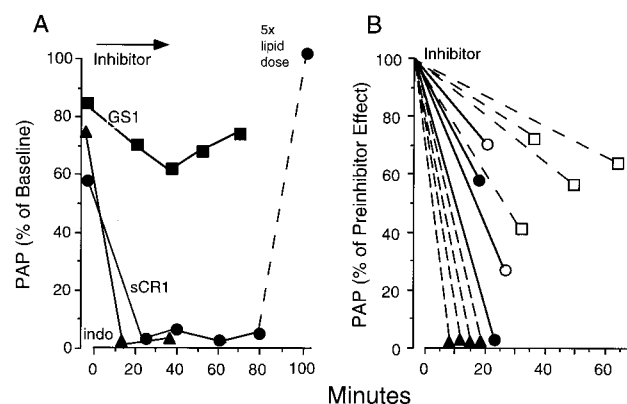
Table 2 shows that (1) incubation of pig serum with liposomes in vitro increased the leukocyte chemotactic activity of serum, (2) this increase was inhibited in the presence of GS1, and (3) the chemotaxis-promoting effect of liposomes was inhibited by EGTA/Mg<sup>2+</sup>. The first 2 observations provide evidence that liposomes can trigger complement activation in



**Figure 6.** Dose-response relationship for PAP as a function of plasma TXB<sub>2</sub> before and at different times after injection of various liposomes. Data ( $n = 96$ ) compiled from 7 pigs. Best fit is a logistic curve in the form  $\ln[(PAP/PAP_{\max})/(1 - PAP/PAP_{\max})] = -0.89 + 0.24 \times [TXB_2]$ , where  $PAP_{\max}$  is 82 mm Hg and  $[TXB_2]$  is plasma TXB<sub>2</sub> level in ng/mL.  $R^2 = 0.67$ ,  $P$  (of coefficients)  $< 10^{-4}$ , Durbin-Watson value = 2.2, and Wilk-Shapiro error variance constant = 0.96.<sup>23</sup> Latter parameters indicate appropriate model specification, ie, that TXB<sub>2</sub> was main if not sole predictor variable of PAP.<sup>23</sup> The 33% unexplained variability of PAP ( $R^2 = 0.67$ ) may be due to interanimal variation plus measurement errors.<sup>23</sup>

pig blood with resultant production of C5a, whereas the inhibitory effect of EGTA/Mg<sup>2+</sup> on this process shows that this activation was Ca<sup>2+</sup>-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 incubation 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.



**Figure 7.** Effects of complement inhibitors and indomethacin on liposome-induced pulmonary hypertension. A, Standard liposome-induced changes in PAP before and at different times after administration of indicated inhibitors, each in a different animal. B, Postinhibitor PAP responses, expressed as % of preinhibitor response, at time of maximal inhibition in 4 pigs in each inhibitor group. Inhibitors were injected into the jugular vein in 4 to 8 mL PBS at following doses: GS1, 1.6 mg/kg ( $\square$ ); sCR1, 0.2 mg/kg ( $\circ$ ) and 2 mg/kg ( $\bullet$ ); and indomethacin, 5 mg/kg ( $\blacktriangle$ ).

**TABLE 2. Chemotaxis of Porcine Neutrophils to Liposome-Activated Porcine Sera**

Experiments	Chemotaxis ( $\times 0.1$ mm)	
	1	2
Serum	$3.44 \pm 0.47$	
Serum + GS1	$1.67 \pm 0.20$ (8)*	
Serum + EGTA/Mg <sup>2+</sup>		$0.71 \pm 0.04$ (8)
Serum + liposomes	$4.90 \pm 0.78$ (8)*	$0.83 \pm 0.03$ (8)†
Serum + liposomes + GS1	$1.71 \pm 0.22$ (8)*‡	
Serum + EGTA/Mg <sup>2+</sup> + liposomes		$0.70 \pm 0.02$ (8)

Liposomes were incubated with two pigs' sera (1 and 2), and the chemotaxis assay was performed as described in the Methods. Phospholipid concentrations in experiments 1 and 2 were 20 and 5 mmol/L, respectively. GS1 was applied at 50  $\mu$ g/mL, and EGTA/Mg<sup>2+</sup> at 10/2.5 mmol/L.

\* $P < 0.05$  vs serum; † $P < 0.05$  vs serum + EGTA/Mg<sup>2+</sup> and serum + EGTA/Mg<sup>2+</sup> + liposomes; ‡ $P < 0.05$  vs serum + liposomes. Statistics were 1-way repeated-measures ANOVA (Newman-Keuls test).

## 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.<sup>3-9</sup> 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.<sup>3-5,8</sup> The phenomenon has been called pseudoallergy<sup>9</sup>; 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.<sup>14</sup> 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 TXB<sub>2</sub> 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.<sup>14</sup> 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.

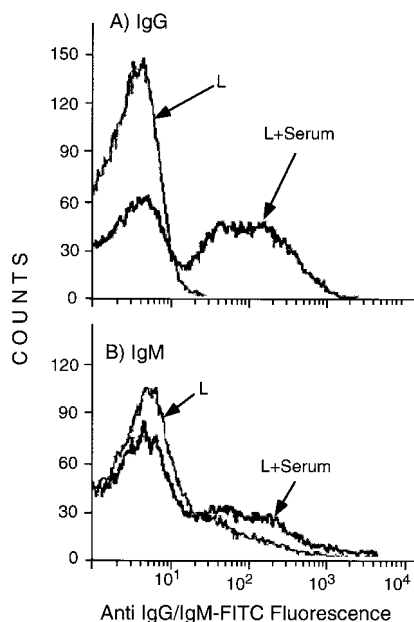
### Evidence for a Causal Role of Complement Activation

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.<sup>14</sup> 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 TXB<sub>2</sub>, one of the secondary mediators produced in response to anaphylatoxin binding to responsive cells,<sup>11</sup> 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.<sup>18,24</sup> 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,<sup>10</sup> 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.<sup>11,25,26</sup> Among the secondary mediators, we focused here on TXA<sub>2</sub>, a potent vasoconstrictor eico-



**Figure 8.** Binding of natural antibodies to liposomes in vitro. After incubation with pig serum or PBS, liposome-bound IgG and IgM antibodies were measured by flow cytometry as described in Methods. For each liposome sample, 10 000 data points were acquired. Vesicles stained with an unrelated polyclonal antibody showed no greater fluorescence than those incubated with PBS (data not shown). An independent experiment gave essentially identical results.



13. Moore FD. Therapeutic regulation of the complement system in acute injury states. *Adv Immunol.* 1994;56:267–299.
14. Wassef NM, Johnson SH, Graeber GM, Swartz GM, Schultz CL, Hailey JR, Johnson AJ, Taylor DG, Ridgway RL, Alving CR. Anaphylactoid reactions mediated by autoantibodies to cholesterol in miniature pigs. *J Immunol.* 1989;143:2990–2995.
15. Rudolph AS. Encapsulation of hemoglobin in liposomes. In: Winslow RM, Vandegriff KD, Intaglietta M, eds. *Blood Substitutes. Physiological Basis of Efficacy.* Boston, Mass: Birkhauser; 1995:98–104.
16. Rabinovici R, Phillips WT, Feuerstein G, Rudolph AS. Encapsulation of hemoglobin in liposomes. In: Rabinovici R, Feuerstein G, Rudolph AS, eds. *Red Blood Cell Substitutes: Fundamental Principles and Clinical Applications.* New York, NY: Marcel Dekker; 1998:263–287.
17. McLoughlin TM, Fontana JL, Alving B, Mongan PD, Bünger R. Profound normovolemic hemodilution: hemostatic effects in patients and in a porcine model. *Anesth Analg.* 1996;83:459–465.
18. Szebeni J, Wassef NM, Hartman KR, Rudolph AS, Alving CR. Complement activation in vitro by the red blood cell substitute, liposome-encapsulated hemoglobin. *Transfusion.* 1997;37:150–159.
19. Szebeni J, Wassef NM, Spielberg H, Rudolph AS, Alving CR. Complement activation in rats by liposomes and liposome-encapsulated hemoglobin. *Biochem Biophys Res Commun.* 1994;205:255–263.
20. Stahl GL, Behrooz F, Morrissey M. Monoclonal antibody, GS1, functionally inhibits porcine C5a induced neutrophil activation, but not C5b-9 formation. *FASEB J.* 1997;11:A331. Abstract.
- 20a. Tofukuji M, Stahl GL, Agah A, Metais C, Simons M, Sellke FW. Anti-C5a monoclonal antibody reduces cardiopulmonary bypass and cardioplegia-induced coronary endothelial dysfunction. *J Thorac Cardiovasc Surg.* 1998;116:1060–1068.
21. Weisman HF, Bartow T, Leppo MK, Marsh HCJ, Carson GR, Concino MF, Boyle MP, Roux KH, Weisfeldt ML, Fearon DT. Soluble complement receptor type 1: in vivo inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. *Science.* 1990;249:146–151.
22. Vakeva AP, Agah A, Rollins SA, Matis LA, Li L, Stahl GL. Myocardial infarction and apoptosis following myocardial ischemia and reperfusion: role of the terminal complement components and inhibition by anti-C5 therapy. *Circulation.* 1998;97:2259–2267.
23. Kang YH, Mallet RT, Bünger R. Coronary autoregulation and purine release in normoxic heart at various cytoplasmic phosphorylation potentials: disparate effects of adenosine. *Pflugers Arch.* 1992;421:188–199.
24. Szebeni J, Wassef NM, Rudolph AS, Alving CR. Complement activation in human serum by liposome-encapsulated hemoglobin: the role of natural anti-phospholipid antibodies. *Biochim Biophys Acta.* 1996;1285:127–130.
25. Stahl G, Morse DS, Martin SL. Eicosanoid production from porcine neutrophils and platelets: differential production with various agonists. *Am J Physiol.* 1997;272:C1821–C1828.
26. Hansch GM, Seitz M, Martinotti G, Betz M, Rauterberg EW, Gerns D. Macrophages release arachidonic acid, prostaglandin E<sub>2</sub>, and thromboxane in response to late complement components. *J Immunol.* 1984;133:2145–2151.
27. Martin SE, Chenoweth DE, Engler RL, Roth DM, Longhurst JC. C5a decreases regional coronary blood flow and myocardial function in pigs: implications for a granulocyte mechanism. *Circ Res.* 1988;63:483–491.
28. Fletcher MP, Stahl GL, Longhurst JC. C5a-induced myocardial ischemia: role for CD18-dependent PMN localization and PMN-platelet interactions. *Am J Physiol.* 1993;265:H1750–H1761.
29. Amsterdam EA, Stahl GL, Pan HL, Rendig SV, Fletcher MP, Longhurst JC. Limitation of reperfusion injury by a monoclonal antibody to C5a during myocardial infarction in pigs. *Am J Physiol.* 1995;268:H448–H457.
30. Stahl GL, Amsterdam EA, Symons JD, Longhurst JC. Role of thromboxane A<sub>2</sub> in cardiovascular response to intracoronary C5a. *Circ Res.* 1990;66:1103–1111.
31. Szebeni J, Muggia FM, Alving CR. Complement activation by cremophor EL as a possible contributor to hypersensitivity to paclitaxel: an in vitro study. *J Natl Cancer Inst.* 1998;90:300–306.