Liposome-induced pulmonary hypertension: properties and mechanism of a complement-mediated pseudoallergic reaction

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Szebeni, Janos, Lajos Baranyi, Sandor Savay, Mihaly Bodo, David S. Morse, Milan Basta, Gregory L. Stahl, Rolf Bünger, and Carl R. Alving. Liposome-induced pulmonary hypertension: properties and mechanism of a complement-mediated pseudoallergic reaction. Am J Physiol Heart Circ Physiol 279: H1319–H1328, 2000.—Intravenous injection of liposomes can cause significant pulmonary hypertension in pigs, a vasoconstrictive response that provides a sensitive model for the cardiopulmonary distress in humans caused by some liposomal drugs. The reaction was recently shown to be a manifestation of “complement activation-related pseudoallergy” (CARPA; Szebeni J, Fontana JL, Wassef NM, Mongan PD, Morse DS, Dobbins DE, Stahl GL, Bünger R, and Alving CR. Circulation 99: 2302–2309, 1999). In the present study we demonstrate that the composition, size, and administration method of liposomes have significant influence on pulmonary vasoactivity, which varied between instantaneously lethal (following bolus injection of 5 mg lipid) to nondetectable (despite infusion of a 2,000-fold higher dose). Experimental conditions augmenting the pulmonary hypertensive response included the presence of dimyristoyl phosphatidylglycerol, 71 mol% cholesterol, distearoyl phosphatidylcholine, and hemoglobin in liposomes, increased vesicle size and polydispersity, and bolus injection vs. slow infusion. The vasoactivity of large multilamellar liposomes was reproduced with human C3a, C5a, and xenoreactive immunoglobulins, and it correlated with the complement activating and natural antibody binding potential of vesicles. Unilamellar, monodisperse liposomes with 0.19 ± 0.10 μm mean diameter had no significant vasoactivity. These data indicate that liposome-induced pulmonary hypertension in pigs is multifactorial, it is due to natural antibody-triggered classic pathway complement activation and it can be prevented by appropriate tailoring of the structure and administration method of vesicles.

hypersensitivity reactions; anaphylatoxin; hemoglobin; IgM-enriched intravenous immunoglobulin; hemodynamics

LIPOSOMAL FORMULATIONS of some drugs, most importantly doxorubicin (Doxil) and amphotericin B (Ambi-}

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method influence the pulmonary reaction? 2) What are the roles of C3a, C5a, and C-activating antibodies? and 3) Is there correlation between in vitro antibody binding and C activation by liposomes and their in vivo pulmonary vasoreactivity? Among the various liposome preparations, we studied liposome-encapsulated methemoglobin (LEH) to evaluate the safety of this potential red blood cell substitute (27) and liposomes containing 71 mol% cholesterol (Chol), which were previously used in our laboratory as efficient activators of C (3) and inducers of anticholesterol antibodies in animals (5, 32).

MATERIALS AND METHODS

Reagents. Dimyristoyl and distearoyl phosphatidylcholines (DMPC and DSPC), dimyristoyl phosphatidylglycerol (DMPG), and Chol were purchased from Avanti Polar Lipids (Alabaster, AL). Human C5a and rabbit anti-swine IgG-FITC conjugate were from Sigma Chemical (St. Louis, MO). Goat anti-swine IgM-alkaline phosphatase conjugate and FITC-conjugated F(ab)2, directed against goat Ig, were from Cappel (West Chester, PA), respectively. IgM-enriched intravenous Ig (IVIG, Pentaglobin) was obtained from Biotest Pharma (Dreieich, Germany), and IVIG containing Fc-depleted IgG (Gamma-Venin P) was from Centeon Pharma (Wien, Austria). Human C3a was obtained from Calbiochem (San Diego, CA). Lipopolysaccharide (LPS; Escherichia coli 0111:B4) was from Difco, Chicago, IL. Heat-sterilized, biotinylated (3,5-dibromosalicyl)formate (diasspirin)-cross-linked human hemoglobin (ααHb) was provided by the Blood Research Detachment of the Walter Reed Army Institute of Research. It was prepared as described earlier (41) and contained 12 g Hb/100 ml with <5% methemoglobin.

Measurement of hemodynamic changes. Experiments were performed in accordance with the guidelines of the Committee on Animal Care of the Uniformed Services University of the Health Sciences. Yorkshire swine in the 25–40 kg range (female, except for 2 male pigs when indicated) were sedated with intramuscular ketamine (500 mg) and anesthetized with 1% halothane using an anesthesia machine. Other details of surgery, instrumentation and hemodynamic analysis were described previously (34). In the present study, we focused only on the changes of PAP, as it was shown previously to be the most consistent and reproducible measure of liposome-induced hemodynamic changes (34).

Preparation of liposomes and LEH. LMV and large multilamellar liposome-encapsulated ααHb were prepared by thin-film hydration as follows. Chloroform solutions of the phospholipids and Chol were mixed in appropriate ratios in sterile, pyrogen-free round-bottom flasks, and a film was formed on the wall of the glass by removing the solvent in a rotary evaporator. This was followed by drying under vacuum for >1 h and hydration with PBS or ααHb by vigorous vortex mixing. To remove unencapsulated ααHb from LEH, liposomes were washed in PBS three times for 20 min at 10,000 g. LMV containing only DSPC were intermittently heated (at ∼65°C in a water bath) upon hydration to facilitate bilayer formation. Large unilamellar PBS and ααHb-containing vesicles (LUV and LU-LEH) were prepared by subjecting the respective LMV to high-pressure shear stress in a small-volume microfluidizer (4 cycles at 60 psi; Microfluidics, Boston, MA). Because the washings and microfluidization were not associated with significant loss of lipid, the liposome doses injected and concentrations used for in vitro incubations were calculated on the basis of initial lipid input.

Endotoxin levels in different preparations were tested and sterility verified as described earlier (36). LPS levels in ααHb, LMV, and LEH were 1.2, <0.04, and <15 IU/ml, respectively.

Analysis of vesicle size. The size distribution of microfluidized LUV was measured by quasi-elastic light scattering (QELS), using a Nicomp model 370 (Pacific Scientific, Silver Spring, MD) submicron particle sizer. This method was not sensitive in the supramicron range; therefore, LMV were analyzed in a Cell-DYN 3500 (Abbott Laboratories, Abbott Park, IL) flow cytometer in the platelet window [measuring particle volume distribution in the 2–30 femtoliter range, corresponding to spherical vesicles with diameter (d) 1.6 μm ≤ d ≤ 3.9 μm]. We used the width/height ratio of the descending slope of the distribution curve (at 50% peak height) to quantitate the large liposome content (and, hence, polydispersity) of different LMV preparations.

Experimental protocol. Liposomes and LEH were administered in the jugular vein either as a bolus or by infusion. Bolus injections were 1- to 25-ml aliquots from the 5–40 mg lipid/ml (5–40 mM phospholipid) stock solutions, as specified in the legends to Figs. 1 and 2. They were injected within about 5–15 s and washed into the pulmonary circulation with 5–6 ml PBS. Infusion of 40 mg/ml liposomes (or LEH) at 2–8 ml/min, as specified in the legend to Fig. 3, and on Fig. 4 and Table 1, was performed with an infusion pump.

Measurement of liposome-induced complement consumption in vitro. Liposomes were incubated with undiluted pig serum for 5–30 min at 37°C with shaking, followed by centrifugal separation of vesicles (18,000 rpm for 3 min) and determination of the serum volume causing 50% hemolysis under standardized assay conditions (CH50) per milliliter as described earlier (38).

Flow cytometric analysis of immunoglobulin binding to liposomes. Liposomes were incubated with undiluted pig serum (10 mg/ml) for 30 min at 37°C with gentle shaking. After centrifugal separation, vesicles were fixed in paraformaldehyde, washed, stained with FITC-conjugated antibodies directed against swine IgG and IgM, and analyzed with a FACSort flow cytometer as described previously (34).

Statistical methods. Data are presented as means ± SD, SE, or as individual observations. The efficacy of (liposome) treatments was established by comparisons with baseline using paired t-test; correlations between two variables were analyzed by linear regression, and differences between groups were examined using ANOVA followed by multiple comparisons with the Student-Newman-Keuls test.

RESULTS

Influence of lipid composition on the pulmonary hypertensive effect of LMV. Figure 1A demonstrates typical pulmonary responses to 5 mg DMPC/DMPG/Chol (50:5:45 mol%) LMV (defined as “standard” bolus in Ref. 34). As reported previously (34), this reaction was remarkably reproducible both in different animals and in one animal upon repetitive injection of the same dose, affording quantitative analysis of the contribution of individual liposome components and other experimental conditions.

Regarding the role of lipids, Fig. 1B compares the effects of equivalent doses of LMV differing in lipid composition. It is seen that LMV consisting of only DMPC had a minor (~20%) pulmonary hypertensive effect, significantly less than that caused by the standard bolus. This small vasoactivity was not altered by
coincorporating Chol in the membrane at 45 mol%, whereas coincorporation of 5 mol% DMPG caused significant (P < 0.01) increase in PAP response. Further data in Fig. 1B indicate that inclusion of 71% Chol in the liposome membrane in addition to 5% DMPG caused significant further enhancement of vasoactivity, whereas 20% Chol had no such effect. These 71% Chol-containing LMV (called “high-Chol” LMV) were the most potent liposomal inducers of pulmonary hypertension in pigs, causing circulatory collapse with instant death in two of four tested animals. Finally, Fig. 1B shows that monocomponent LMV prepared from the 18-C-dialkyl phospholipid, DSPC, had significantly greater vasoactivity than the corresponding 14-C-dialkyl (DMPC) preparation. Thus DMPC is not unique in causing pulmonary hypertension but may actually be less potent than DSPC in this regard. Taken together, the above data suggest that the small intrinsic vasoactivity of phospholipid bilayers is greatly enhanced by negative surface charges and 71% Chol in the membrane.

Influence of vesicle size. Another unsolved question regarding the pulmonary hypertensive effect of liposomes was the influence of size and associated homogeneity of vesicles. As shown in Fig. 2A, microfluidized liposomes displaying the lowest mean diameter and narrowest size distribution (DMPC/DMPG/Chol LUV, d = 0.19 ± 0.10 μm) had either no or minimal pulmonary hypertensive effect (%25% increase in PAP) up to 1 g injected lipid. This (lack of) activity fundamentally differed from that of chemically identical LMV containing large, highly heterogeneous vesicles (d in the %1.6–

Table 1. Tolerance of liposomes and LEH administered in infusion

<table>
<thead>
<tr>
<th>Liposome</th>
<th>Mean d, μm ± SD</th>
<th>Infusion Rate</th>
<th>Maximum Rise in PAP</th>
<th>Duration, min</th>
<th>Σ, mg</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC/DMPG/Chol</td>
<td>0.19 ± 0.10</td>
<td>4/240</td>
<td>&lt;5</td>
<td>34</td>
<td>5,440</td>
<td>+</td>
</tr>
<tr>
<td>LUV (50:5:45)</td>
<td>0.35 ± 0.25</td>
<td>4/160</td>
<td>73</td>
<td>60</td>
<td>9,600</td>
<td>+</td>
</tr>
<tr>
<td>DSPC/DMPG/Chol</td>
<td>0.45 ± 0.48</td>
<td>4/80</td>
<td>15</td>
<td>50</td>
<td>4,000</td>
<td>–</td>
</tr>
<tr>
<td>DMPC/DMPG/Chol</td>
<td>0.85 ± 0.84</td>
<td>4/160</td>
<td>19</td>
<td>85</td>
<td>20,000</td>
<td>–</td>
</tr>
<tr>
<td>LUV (24:5:71)</td>
<td>1.07 ± 1.60</td>
<td>4/160</td>
<td>2</td>
<td>118</td>
<td>60</td>
<td>320</td>
</tr>
<tr>
<td>LMV + Indomethacin</td>
<td>1.60 – 3.90*</td>
<td>4/160</td>
<td>22</td>
<td>444</td>
<td>50</td>
<td>8,000</td>
</tr>
<tr>
<td></td>
<td>4/160</td>
<td>13</td>
<td>59</td>
<td>20</td>
<td>3,200</td>
<td></td>
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</tbody>
</table>

Entries are data in individual pigs, infused with the liposome preparation specified in the first and second columns in terms of lipid composition, molar ratios (in parentheses), and diameter (d). The parameters of infusion are given in columns 3 and 4, whereas the start of rise in PAP (minutes after the start of infusion), maximum increase in pulmonary arterial pressure (PAP, as % of baseline), and duration of liposome-induced pulmonary hypertension (minutes) are presented in columns 5–7, respectively. Total amount of infused lipid (Σ, in mg) and the survival of animals (− died, + survived) are shown in columns 8–9. Supercript “M” means male pig. Other experimental conditions are described in the MATERIALS AND METHODS and in the legend for Fig. 3. Indomethacin was applied at 10 mg/kg iv 15 min before the start of large multilamellar vesicle (LMV) infusion. *Range of size distribution. Flow cytometry did not provide mean ± SD reading (see MATERIALS AND METHODS). DMPC, dimyristoyl phosphatidylcholine; DSPC, distearoyl phosphatidylcholine; DMPG, dimyristoyl phosphatidylglycerol; Chol, cholesterol; LUV, large unilamellar vesicle; and LU-LEH, large unilamellar liposome-encapsulated hemoglobin.
3.9 μm range), the hypertensive effect of which was >40% above 2 mg lipid, and reached its plateau (at 100% increase of PAP) at doses ≥20 mg lipid (34).

Figure 2B shows the dose dependence of the pulmonary vasoactivity of other microfluidized liposomes, which had larger mean diameter (in the 0.4–1.1 μm range) and broader size distribution relative to the above-described LUV preparation. These preparations (DSPC-, 71% Chol-, and Hb-containing LUV) caused significant pulmonary hypertension; however, only at 10- to 20-fold higher doses compared with LMV (Figs. 1B and 2A). Another observation shown in Fig. 2B was that the diameters of these liposomes increased in the same order as their hypertensive potency, suggesting a correlation between vesicle size and pulmonary vasoactivity.

Such correlation was also established for the case of LMV, when we plotted the pulmonary hypertensive effects of different preparations (Fig. 1B) against the width-to-height ratio (at 50% height) of the upper, descending part of the distribution curves (Fig. 2C). This latter parameter represents an arbitrary measure of the amount of larger-than-average sized vesicles in the population and can be taken as a measure of liposome heterogeneity. Linear regression analysis indicated highly significant correlation between the hypertensive effect and the amount of large liposomes in these preparations (R² = 0.99, P < 10⁻⁵), providing further evidence for a critical role of vesicle size in the pulmonary vasoactivity of liposomes.

Influence of administration method. Next we addressed the question whether slow infusion vs. rapid bolus administration has an impact on the vasoactivity of liposomes. As shown in Fig. 3, A–C, infusion of the smallest, highly homogeneous LUV caused no changes in PAP (Fig. 3A), whereas intermediate size LUV (Fig. 3B) and LMV (Fig. 3C) led to significant pulmonary hypertension. Thus the pattern of pulmonary response to liposome infusion was the same as seen with bolus injection; i.e., the hypertensive effect correlated with vesicle size above a certain threshold. Figure 3, A–C, and Table 1 also reveal the pigs’ cardiovascular tolerance of various liposomes; we could infuse up to 10 g (0.2 g/kg) DMPC/DMPG/Chol LUV without detectable rise in PAP and up to 20 g intermediate size LUV or LEH with only transient, reversible rises in PAP. Because infusion of LMV led to irreversible circulatory collapse and death within minutes, the question of interest with these large liposomes was how effective indomethacin, the most potent inhibitor of standard LMV bolus-induced hemodynamic changes (34), was in the case of LMV infusion. As shown in Fig. 3C and Table 1, indomethacin allowed the infusion of up to 4.5 g LMV without changes in PAP; however, its protective effect vanished after about 30 min, and a steep rise in PAP caused the death of animals.

Influence of infusion speed. The extent of pulmonary hypertension caused by infusion of intermediate size LUV (e.g., that containing αHb or DSPC) depended on the rate of infusion. This effect is illustrated in Fig. 4...
for the case of microfluidized LEH, where increasing the infusion rate from 4 ml/min to 6 and 8 ml/min caused additional 20–30% elevations in PAP over the values observed at the lower speed.

Correlation between liposome-induced complement activation in vitro and pulmonary hypertension. Table 2 shows that short-term (10 min) incubation of different liposomes with pig serum resulted in C consumption that was proportional with the pulmonary hypertensive effects of vesicles. However, after 30-min incubation, or at a higher serum liposome level (8 vs. 2 mg/ml), C activation was advanced with all preparations, and the proportionality with pulmonary effects tended to disappear. Accordingly, linear regression analysis of PAP (% of baseline) plotted against %C consumption gave statistically significant linear correlation at 10 min ($R^2 = 0.71$, $P < 0.05$), but not at 30 min. These data are consistent with a causal relationship between C activation and pulmonary vasoactivity of liposomes, showing at the same time that the parameters of in vitro C assay are critical in demonstrating such correlation.

Pulmonary effects of human C3a, C5a, and immunoglobulins. Additional yet unresolved questions regarding the involvement of C in the pulmonary reaction to liposomes relate to the roles of C5a vs. C3a and to the exact pathway of C activation. We injected pigs intravenously with human C3a, C5a, and two human Ig preparations: 1) an IgM-enriched mixture of immunoglobulins that reacts with porcine endothelial cells causing massive C activation (Pentaglobin) (9, 29) and 2) an IgG F(ab)_2 product (Gamma-Venin P) that lacks the C activating Fc portion of Ig. Table 3 shows that C3a, C5a, and Pentaglobin caused significant rises in PAP on a time course that was essentially identical (within 1–2 min) with that of the liposome-induced reactions, whereas Gamma-Venin P caused no pulmonary reaction even at a 3,000- to 5,000-fold higher dose. These observations provide evidence that 1) both C3a and C5a can induce pulmonary hypertension, although C5a has significantly greater efficacy, and that 2) the reaction to liposomes has the same basic features as an anaphylactic shock induced by xenoreactive antibodies via classic pathway activation of C.

Correlation between pulmonary vasoactivity and antibody binding to liposomes. To investigate further the possibility that classic pathway activation of C is a major underlying cause of pulmonary reaction, we measured the binding of natural antibodies to different liposomes in vitro. The fluorescence-activated cell sort-
ing (FACS) analysis shown in Fig. 5 indicated abundant binding of IgG and IgM to the highly vasoactive LMV, whereas the chemically identical, vaso-inactive LUV showed no or minimal binding. In further correlation with pulmonary vasoactivity, liposomes with 71% Chol bound significantly more Ig than those containing 45% Chol. These findings, taken together with analogous correlations between in vitro C consumption and liposome size and Chol content (Table 2), imply that the binding of naturally occurring IgG and IgM antibodies to liposomes may be rate limiting to both C activation and subsequent pulmonary changes. Consequently, natural antibody-mediated classic pathway C activation may be the predominant ultimate cause of liposome-induced pulmonary changes.

DISCUSSION

Role of C in liposome-induced pseudoallergy. Since the first description of liposome-induced C activation in 1969 (20), numerous studies have analyzed this phenomenon (reviewed in Ref. 33). The profound hemodynamic actions of anaphylatoxins were also recognized some 30 years ago (6), yet, to our knowledge, the possible causal connection between C activation and acute hemodynamic side effects of liposomal drugs in patients has not been proposed and investigated in a systematic fashion prior to a recent study from our laboratories (34). In the latest report on Doxil-induced hypersensitivity reactions (31), for example, the authors recognized the similarity of clinical symptoms to those caused by hemodialysis, which is known to be mediated by C5a (22). Also, they described transient neutropenia and increased leukocyte adherence, i.e., classic hallmarks of C activation (22), only in patients who displayed hypersensitivity symptoms. Nevertheless, C activation was not considered as an underlying cause (31). One reason for a common oversight of this

Table 2. Complement activation by different liposomes in pig serum in vitro

<table>
<thead>
<tr>
<th>Liposome</th>
<th>Complement Consumption, % Relative to Baseline</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1 mg/ml‡</td>
</tr>
<tr>
<td></td>
<td>10 min§</td>
</tr>
<tr>
<td>DMPC/DMPG/Chol (50:5:45) LMV</td>
<td>18.9 ± 4.2(5)</td>
</tr>
<tr>
<td>DMPC/Chol (100) LMV</td>
<td>8.9 ± 4.9†(5)</td>
</tr>
<tr>
<td>DMPC/DMPG (55:45) LMV</td>
<td>9.4 ± 9.9†(6)</td>
</tr>
<tr>
<td>DMPC/DMPG (95:5) LMV</td>
<td>13.9 ± 7.2(5)</td>
</tr>
<tr>
<td>DMPC/DMPG/Chol (24:5:71) LMV</td>
<td>100 ± 0†(3)</td>
</tr>
<tr>
<td>DMPC/Chol (50:5:45) LUV</td>
<td>−4.1 ± 4.5†(5)</td>
</tr>
</tbody>
</table>

Entries are mean complement (C) consumption ± SE, with n independent tests with different pig sera in parentheses. Complement consumption was calculated by the formula 100 − (CH<sub>50LMV</sub>/CH<sub>50PBS</sub>), where CH<sub>50LMV</sub> denotes CH<sub>50</sub>/ml values measured in the presence of liposomes, and CH<sub>50PBS</sub> denotes CH<sub>50</sub>/ml values measured in PBS-containing controls. Baseline CH<sub>50</sub>/ml at 10 min was 48.8 ± 4 (n = 6). ANOVA of C consumption values at each incubation time, followed by Student-Newman-Keuls test, indicated significant difference relative to DMPC/DMPG/Chol (50:5:45) LMV at *P < 0.05 or †P < 0.01. ¶Final lipid concentration. §Incubation time.

and liposome size and Chol content (Table 2), imply that the binding of naturally occurring IgG and IgM antibodies to liposomes may be rate limiting to both C activation and subsequent pulmonary changes. Consequently, natural antibody-mediated classic pathway C activation may be the predominant ultimate cause of liposome-induced pulmonary changes.
mechanism may lie in the traditional perception of the
C system as being associated with immunology (host
defense) and not with pharmacology (adverse drug
reactions) or physiology (circulatory changes).

Our previous study highlighting the immune back-
ground of liposome-induced cardiopulmonary distress
(34) reported dramatic hemodynamic changes follow-
ing intravenous injection of minute amounts of DMPC/
DMPG/Chol LMV in pigs and presented several lines of
evidence that the reaction was due to C activation (34).
The aim of the present study was to delineate further
details of this newly recognized reaction method. In
particular, we attempted to clarify the roles of vesicle
composition, size and administration method in the
pulmonary reaction, the contributions of C3a and C5a,
and the exact pathway of C activation.

Influence of liposome lipids. The fact that electrically
neutral LMV composed of only DMPC and DSPC were
able to inducing pulmonary hypertension was un-
expected, as earlier in vitro studies showed C activa-
tion only by vesicles supplemented with charged and/or
antigenic (glyco)lipids or other ligands (33). Thus the
present study provides evidence for the first time that
C-mediated vasoactivity may be an intrinsic property
of even the simplest monocomponent phospholipid ves-
icles, provided they exceed a threshold size, as dis-
cussed below. The observation that DMPG augmented
the pulmonary reaction to liposomes is in keeping with
numerous reports on C activation by anionic liposomes
(8, 25, 33). This may occur both through the classic and
the alternative pathways by binding immunoglobulins
and C3, respectively (8, 25, 33). The effect of immuno-
globulins may, however, overshadow activation via the
alternative pathway (24).

Unlike DMPG, the presence of Chol in LMV did not
appear to be critical for pulmonary vasoactivity, at
least at levels $\leq 45$ mol%. In contrast, 71 mol% Chol
increased the C-activating and the pulmonary hyper-
tensive effects of liposomes to such a degree that
minute quantities (5 mg) of these vesicles caused im-
mediate death in $\approx 50\%$ of tested pigs with symptoms of
anaphylactic shock. This high-Chol LMV has been the
most efficient liposomal C activator in our hands so far,
causing significant hemodynamic changes in rats as
well (unpublished data). Liposomes containing 71%
Chol were originally used to maximize Chol-depend-
tent, C-mediated damage to model membranes (3).
They were shown to trap glucose and to provide
barrier to its diffusion with equal efficacy as their
low-Chol (45%) counterparts (3), indicating sealed bi-
layer vesicle structure. High-Chol LMV were also ap-
plied as efficient inducers of anti-Chol antibodies in
mice (32) and rabbits (5). Although these liposomal
vaccines contained lipid A as well, it is possible that the
extra C activation caused by the high Chol content may
have played an important role in the success of this
immunization protocol. It is increasingly recognized
that C activation plays an important role in the devel-
opment of specific immune responses, among others,
through regulation of B cell responses to antigen (7,
12).

Effect of liposome size. In theory, antibody binding
and C activation by liposomes should be proportional
with the overall surface area of vesicles directly ex-
posed to plasma, which (considering equal amounts of
lipid) is smaller with LMV than with small unilamellar
vesicles. Our findings therefore that LMV had signifi-
cantly greater C-activating and pulmonary hyperten-
sive effects than their smaller counterparts, and that
liposomes with $\leq 0.2$ $\mu$m caused no hemodynamic
changes, were counter-intuitive and unexpected. It is
conceivable that natural antibodies have increased
binding affinity to liposomes with flat surface, due to
steric proximity or favorable positioning of epitopes. A
further factor could be the spatial arrangement of
surface-bound antibodies, as the activation of com-
plement protein 1 (C1) requires multiple IgG molecules
to be aligned in a special parallel configuration (22).
Finally, it is not excluded that other plasma proteins
(albumin, C reactive protein, C1q) bound on the sur-
face of LMV accelerate C activation in a cooperative
fashion.

Role of endotoxin. In our previous study (33) we have
shown that the liposome-induced hemodynamic re-
response in pigs can be completely blocked by the specific
C inhibitor, soluble C receptor type 1 (sCR1), which
strongly argues against a major direct role of endotoxin
in vasoactivity. Endotoxin can activate C; neverthe-

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**Fig. 5.** Binding of porcine IgG and IgM to liposomes in vitro. Following incubation with PBS or pig serum, liposomes were fixed, washed, incubated with antib-
dies against porcine IgG or IgM, and counterstained with FITC-conjugated F(ab)$_2$. 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). The PBS control, i.e., vesicles incubated with PBS instead of pig serum and stained with anti-IgG or
anti-IgM, were identically negative for all varieties of liposomes (shown in the first column for 45% Chol liposomes). The presented data were repeated in an
independent experiment with essentially identical re-
results. The PBS control, i.e., vesicles incubated with PBS and stained with anti-IgG or anti-IgM, indicated no antibody binding to any of the studied preparations (shown for 45% Chol LMV).
less, the possibility that the trace amounts coinjected with liposomes played a major role in C activation could also be ruled out on the following basis. First, in control experiments, we observed significant rise of PAP after intravenous injection of 50 μg/kg E. coli LPS, but not 2.5 μg/kg, i.e., only at five to six orders of magnitude higher LPS doses than the maximal amount coinjected with liposomes (data not shown). Consistent with these data, the reported dose where S. enteritidis caused massive hemodynamic changes in pigs was in the 100–250 μg/kg range (16). Second, there was no correlation between LPS contamination and PAP changes, with the purest empty LMV preparation causing maximal rises in PAP. Finally, previous studies in rats provided evidence that LPS contamination was not responsible for C activation by liposomes and LEH in vitro (37).

Mechanism of liposome-induced C activation. At present our hypothesis regarding the mechanisms of C activation by LMV in pig blood and its acceleration by 71% membrane Chol is as follows. The reaction is essentially due to the binding by LMV of naturally occurring, C-activating anti-lipid antibodies that are present in the blood of most mammalian species including pigs (4, 40) and which include anti-phospholipid and anti-Chol antibodies. As mentioned, it is known from the literature that antibody binding to liposomes is accelerated by negative charges on the membrane, and we have shown here that 71 mol% Chol further increases this binding (vs. 45%). One possible explanation for this difference is that the excess Chol in 71% liposomes may become exposed on the membrane surface as patches of microcrystals, becoming increasingly accessible to anti-Chol antibodies. There are several lines of evidence supporting this theory: 1) phosphatidylcholine dispersions enriched in Chol to levels of 2–3 mol Chol per mole phosphatidylcholine are metastable, with formation of Chol monohydrate crystals (10); 2) negatively charged liposomes containing 71% Chol were shown to bind IgM with associated formation of giant, multimicron structures in plasma (15); 3) the recognition of Chol by anti-Chol antibodies in biological membranes and in lipoproteins critically depends on the steric position of β-OH epitopes (4), and 4) crystalline Chol is known to efficiently bind anti-Chol antibodies and to activate C in human blood (19, 30). It seems worthwhile to point out in this context that C activation by microcrystalline Chol may be an important pathogenetic factor in precipitating acute thrombotic or thromboembolic events in arteriosclerosis, at sites of ulcerated arteriosclerotic plaques (17–19, 30).

Pulmonary effects and long-term consequences of liposome and LEH infusion. In addition to composition and size, the third critical factor in liposome-induced pulmonary hypertension was the method and speed of intravenous administration. In particular, administration of intermediary size (0.2 μm ≤ d ≤ 1.1 μm) liposomes by infusion attenuated and delayed the pulmonary reaction relative to that observed with bolus injection, and the speed of infusion was also critical with these vesicles. These observations can most easily be rationalized by blood anaphylatoxin levels being rate limiting to pulmonary vasoactivity. As is known, the steady-state levels of functional anaphylatoxins are set by the relative rates of production from plasma C3 and C5 and clearance by cellular receptors and plasma carboxypeptidases (22). With clearance most likely uninfluenced by the method of administration, anaphylatoxins level in the blood will depend not only on the potency of vesicles for C activation but also on the speed by which liposomes enter in blood. This mechanism provides explanation for the reversal of hypersensitivity reaction to liposomes in some patients by slowing down the rate of infusion (14).

We could infuse at least 10 g of LUV that contained the smallest, most homogeneous liposomes with no significant rise in PAP. Thus such small liposomes appear entirely safe in terms of cardiovascular side effects. Infusion of gram quantities of microfluidized LEH, i.e., a state-of-art preparation in this field (27, 36), was associated with reversible but nevertheless significant elevation of PAP. According to these data, vasoactivity could still be a concern with this type of blood substitute.

Role of anaphylatoxins and pathway of liposome-induced C activation. The observation that human C3a and C5a mimicked the liposome-induced vascular reaction is consistent with the partial efficacy of murine anti-C5a antibody in our previous study (34). It suggests that inhibition of the formation or the action of C5a may not be sufficient to completely suppress the vasoactivity of liposomes. The observed difference between the efficacies of C3a and C5a is in keeping with the relative efficacies of these anaphylatoxins in other spasmogenuity assays (23).

We found that a classic pathway C activation-based reverse xenograft reaction, caused by an IgM-enriched mixture of human immunoglobulins, mimicked the pulmonary effects of liposomes. In contrast, IgG F(ab)2, which was not supposed to activate C, caused no hemodynamic changes. These observations provide further support for classic pathway activation being the predominant underlying mechanism of liposome-induced pulmonary changes.

Clinical and theoretical implications. By highlighting the C mechanism, the present study helps in solving the hypersensitivity riddle observed with Doxil and other liposomal drugs. We demonstrate that small unilamellar, highly homogeneous liposomes, like those supposedly present in liposomal drugs, are not prone to activate C. This suggests that the clinical formulations of reaction-causing liposomal drugs may have unique yet unclarified features that render them C activators in vivo that may need to be explored.

The concept of C activation being causally involved in a drug-induced hypersensitivity reaction appears to have important theoretical implications. At present, textbook examples for C-mediated hypersensitivity reactions are usually limited to radiocontrast dye-induced reactions and serum sickness-related hives (26). Our study expands this list and highlights the need for
reevaluating the physiology and classification of hypersensitivity reactions. Clearly, the mediators and symptoms of C- and IgE-mediated acute reactions overlap but are not identical, and there is mounting evidence that C activation plays a significant role in some common pollen allergies as well (13). These facts may provide the rationale to refer to anaphylatoxins, along with IgE, as “allergomedins” and distinguish C-mediated acute systemic reactions as a novel subcategory of immediate hypersensitivity reactions called C activation-related pseudoallergy (CARPA) (34). In addition to liposomal drugs and radiocontrast dyes, the list of agents causing CARPA appears to include widely used intravenous drugs (e.g., Taxol and cyclosporin A) that are solubilized with particle-forming surfactants (35).

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