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Block 20 - Abstract

neutropenia, thrombocytopenia, respiratory distress, cyanosis, pulmonary and systemic hypertension, and decreased cardiac output. Plasma levels of thromboxane B_2 and 6-keto-prostaglandin F_{1a} increased 1300% and 200%, respectively, and leukocyte and platelet counts decreased by 36% and 38% respectively. Injection of cholesterol-free liposomes did not induce the reaction. These results show that naturally-occurring autoantibodies to cholesterol can initiate complement activation and can be associated with an anaphylactoid reaction to exogenously administered cholesterol in pigs.

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ANAPHYLACTOID REACTIONS MEDIATED BY AUTOANTIBODIES TO CHOLESTEROL IN MINIATURE PIGS

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Antoantibodies to cholesterol were detected and purified from normal (nonimmunized) pig serum. The antibodies were assayed by ELISA with crystalline cholesterol as an Ag and by C-dependent damage to cholesterol-laden liposomes. Intravenous injection of liposomes containing cholesterol into anesthetized animals caused decreased hemolytic complement titers, and induced a reaction consisting of transient neutropenia, thrombocytopenia, respiratory distress, cyanosis, pulmonary and systemic hypertension, and decreased cardiac output. Plasma levels of thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ increased 1300 and 200%, respectively, and leukocyte and platelet counts decreased by 36 and 38%, respectively. Injection of cholesterol-free liposomes did not induce the reaction. These results show that naturally occurring autoantibodies to cholesterol can initiate C activation and can be associated with an anaphylactoid reaction to exogenously administered cholesterol in pigs.

In 1925 Sachs and Klopstock (1) used cholesterol dissolved in pig serum as an antigen to induce humoral immunity to cholesterol in rabbits. In the course of producing such antibodies they made the following observation: "The cholesterol (cholesterol-pig serum) injections also often produced shock-type side effects so that with this form of pretreatment animal losses sometimes have to be expected" (translated from German) (1). The observation of shock in rabbits associated with cholesterol-pig serum mixtures was confirmed by Berger and Scholer (2) who found that 8 of 17 rabbits died after injection of the cholesterol-pig serum Ag. The shock phenomenon described by Sachs and Klopstock (1) was observed incidentally in the course of immunologic research and has remained unexplained for 64 yr until now.

Recently in the course of a series of studies on induction of antibodies to cholesterol (3) we made the incidental but as yet unpublished observation that injection of liposomal cholesterol into miniature pigs invariably induced an anaphylactoid reaction. Upon further investigation we discovered, as shown in this report, that all of the pigs that we examined had high levels of naturally occurring IgM and IgG autoantibodies to cholesterol. Based on this finding, the shock syndrome observed earlier in rabbits injected with cholesterol-pig serum mixtures (1, 2) can now be explained as most probably having been caused by massive C activation and mediator secretion due to anticholesterol-cholesterol immune complexes. Although C activation was found to occur after therapeutic injection of a liposome-drug complex in humans it was not associated with any clinical symptoms (4). Humans are much less sensitive than pigs to the spasmogenic effects of C anaphylatoxins (see *Discussion*), and the pig therefore serves as a model in which the anaphylactoid effects of C activation are amplified.

MATERIALS AND METHODS

Injection of liposomes and cardiovascular monitoring. Fifteen male Hanford miniature pigs, with body weights ranging from 35 to 50 kg (mean 46 ± 5.7) were anesthesized and examined for cardiovascular responses. The pigs were immobilized with an intramuscular injection of a solution containing xylazine (3 mg/kg), atropine sulfate (0.01 mg/kg), and ketamine (20 mg/kg); anesthetized with an i.v. injection of sodium pentobarbital (24 mg/kg); and endotracheally intubated and ventilated at FiO_2 0.4, rate 14, and tidal volume 500 ml using a microprocessor ventilator (model 7200, Puritan-Bennett Corp. Los Angeles, CA). The carotid arteries and the internal and external jugular veins were exposed with incisions. A carotid artery was cannulated with no. 18 gauge polyethylene tubing for arterial blood pressure and arterial blood gases measurement, and the external jugular vein on the opposite side of the neck was cannulated with no. 14 gauge polyethylene tubing for the infusion of liposomes, saline, indomethacin, and additional anesthetic. The liposomes were infused over an interval of approximately 3 to 5 min. The internal jugular vein was cannulated with a balloon-tipped, flow-directed, Swan-Ganz thermodilution catheter (model 93A-131-7F, Edwards Laboratories AMS Del Caribe, Inc., Anasco, Puerto Rico) and advanced through the superior vena cava and right atrium into the pulmonary artery outflow tract for CVP[2], pulmonary artery pressure, pulmonary capillary wedge, and CO determinations. Position was assured by monitoring the transduced wave form and the catheter was secured in place with heavy suture. Vascular pressures were continually displayed and recorded on a multichannel, polygraphic recorder (model 7758, Hewlett-Packard Co., Palo Alto, CA).

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[2] Abbreviations used in this paper: ABG, arterial blood gases; ABL_{50}, amount of antibody (or antiserum) required to induce half-maximal complement-dependent immune damage to liposomes; ABP, arterial blood pressure; CO, cardiac output; CVP, central venous pressure; DMPC, dimyristoyl phosphatidylcholine; DMPG, dimyristoyl phosphatidylglycerol; LTC_4, leukotriene C_4; PAP, pulmonary artery pressure; PCW, pulmonary capillary wedge; PGI_2, prostacyclin; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; PVR, pulmonary vascular resistance; SVR, systemic vascular resistance; TXB_2, thromboxane B_2; TXA_2, thromboxane A_2.

Baseline parameters were obtained after a 20-min stabilization period and included CO, arterial blood pressure, arterial blood gases, pulmonary artery pressure, pulmonary capillary wedge, and CVP determinations. Systemic vascular resistance and PVR were calculated using standard formulae (5). All catheters were flushed regularly with heparin solution. Experimentation was begun after a second 20-min stabilization period.

Liposome preparation. Liposomes (hand-shaken multilamellar or sonicated small unilamellar vesicles), which were used either for infusion or for detecting antibodies to liposomes, were composed of dimyristoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol, (Avanti Polar Lipids, Birmingham, AL), and cholesterol (Sigma Chemical Co., St. Louis, MO) in a molar ratio of 1.8:0.2:1.5 (i.e., the liposomes contained 43% cholesterol). Similar liposomes lacking cholesterol were used as controls where indicated. In certain instances, to purify the antibodies from pig serum by affinity binding to liposomes or to detect the purified antibodies, the liposome constituents were in a molar ratio of 1.8:0.2:5.0 (i.e., the liposomes contained 71% cholesterol). The liposomes were prepared essentially according to previously described methods (6). Briefly, chloroform solutions of the appropriate lipids were mixed in a pear-shaped flask. The lipid mixture was dried by removing all of the chloroform first under mild negative pressure and then in a dessicator under very high vacuum (<50 μm Hg) for 1 h. The dried lipids were dispersed and suspended in aqueous medium in an appropriate volume of 0.154 M NaCl. The saline volume was always chosen such that the final phospholipid concentration in the aqueous liposomal dispersion was 50 mM for the liposomes used for infusion and 10 mM for the liposomes used for C-dependent immune assays. The flask was then tightly closed and vortexed vigorously to remove all the dried lipids from the flask wall. The liposome preparation was tested for sterility and pyrogenicity before use. In most cases 0.5 ml of liposomes/kg were injected i.v. in the pig at any one time. In one pig small unilamellar liposomes that had been produced by sonication were substituted for multilamellar liposomes. To produce unilamellar liposomes, multilamellar vesicles at a concentration of 50 mM were sonicated under a stream of nitrogen using a Heat Systems-Ultrasonics Inc. Plainview, NY), model W-220F cell disruptor. The sonication was performed at setting 10 in approximately 10-min intervals separated by 2-min resting periods for a total time of 40 min. The preparation was centrifuged at 20,000 × *g* for 10 min and the supernatant was injected.

Anticholesterol antibodies. The presence of anticholesterol antibodies in pig serum was assayed by two methods: a solid-phase ELISA using crystalline cholesterol as an Ag (3), and by C-dependent immune damage to liposomes containing cholesterol (6). ELISA were performed by coating highly purified crystalline cholesterol (1 μg/well) as an Ag on the bottoms of the wells of microtiter plates (Immulon II, "U" bottom, Dynatech Laboratories, Alexandria, VA). Plates were blocked by washing the wells three times, for 20 min each, with PBS (137 mM NaCl/2.7 mM KCl/9.6 mM phosphate, pH 7.2) containing 10% heat-inactivated fetal bovine serum (M.A. Bioproducts, Walkersville, MD). A total of 50 μl of the serum to be tested, diluted in PBS containing 10% FBS, was added to the wells and incubated 3 h at room temperature. Plates were then washed three times for 5 min each with PBS. Fifty μl of goat antiswine IgM (μ-chain) or IgG (H + L) alkaline phosphase conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) at 0.1 and 0.5 μg/ml, respectively, in PBS containing 10% FBS was added to the wells and incubated 1 h at room temperature. Plates were again washed three times for 5 min each with PBS. A total of 50 μl of the substrate, *p*-nitrophenyl phosphate at 2 mg/ml in diethanolamine buffer (Kirkegaard and Perry Laboratories), was added to the wells and incubated 1 h at room temperature. Plates were scanned for optical activity at 405 nm using a Titertek Multiscan (Flow Laboratories, McLean, VA). Values reported were adjusted by subtracting values in blank wells that lacked Ag.

In the C-dependent immune damage glucose release assay (6) miniature pig serum served simultaneously as the source of antibodies and C for measuring immune damage to liposomes.

Anticholesterol antibodies were purified from pig serum by the technique of affinity binding to liposomes (6). The liposomes used to purify the antibodies contained 71% cholesterol instead of 43% cholesterol (i.e., the liposomal dimyristoyl phosphatidylcholine/cholesterol/dimyristoyl phosphatidylglycerol were in molar ratios of 1.8/5.0/0.2 instead of 1.8/1.5/0.2). The purified anticholesterol antibodies were tested for activities by ELISA and C-dependent immune damage to liposomes containing different amounts of cholesterol and using guinea pig serum as a C source.

C levels in pig serum. Hemolytic C levels in pig serum before and after injecting liposomes were assayed by standard hemolytic C titration using sheep E (7).

PG and thromboxanes. PGE_2, TXB_2, 6-keto-$PGF_{1\alpha}$ and leukotriene C_4 were assayed by RIA using kits obtained from New England Nuclear, Boston, MA.

RESULTS

Antibodies to cholesterol in pig serum. Each of nine pig sera tested by ELISA contained varying levels of both IgG and IgM antibodies to cholesterol (Table I). C-dependent immune damage to liposomes containing cholesterol was assayed using sera from 10 pigs before injection of liposomes (Fig. 1). Antibody activity with liposomal Ag is often influenced by temperature (6, 8), and the antiliposome activity was increased for each pig serum when assayed at 37°C compared to 24°C. Preheating of the pig serum (56°C, 30 min) to inactivate C eliminated all activity at 37°C (Fig. 1). There was no nonspecific activation of C as shown by controls in which incubation of fresh guinea pig serum with liposomes failed to cause spontaneous activation of the guinea pig C (Fig. 1). As shown in Figure 2, the antibody activity of pig serum decreased significantly after injection of liposomes containing cholesterol in each of eight pigs tested. Separate controls demonstrated that no antibody activity was detected in pig serum against cholesterol-free liposomes.

The data for antibody activities derived from the two techniques, ELISA and C-dependent immune damage to liposomes leading to release of trapped glucose, were compared to determine if correlations could be made between them. When the liposomal antibody titers (ABL_{50}) measured by glucose release (9) were compared with the IgM ELISA activities shown in Table I, using the Minitab statistical program (Minitab Inc., State College, PA), a high correlation coefficient (0.97) was obtained with five selected sera of seven total sera examined. Two of the sera did not fit as well in the correlation analysis and when IgM ELISA data from all seven sera were compared with the ABL_{50} data the correlation coefficient was lower (0.62). When the IgG data from Table I were substituted for IgM for comparison with C-dependent glucose release ABL_{50} data, there was no positive correlation. We conclude that C-dependent damage leading to glucose release from liposomes that was mediated by naturally occurring porcine antibodies to cholesterol was caused mainly, but not necessarily exclusively, by IgM

TABLE I

Detection of anti-cholesterol antibodies in normal pig serum by ELISA[a]

Pig	Type of Antibody (Absorbance at 405 nm)	
	IgG (H + L)	IgM (μ)
1	0.121	0.587
2	0.187	0.547
3	0.095	0.506
4	0.088	0.468
5	0.091	0.394
6	0.040	0.372
7	0.129	0.336
8	0.105	0.308
9	0.063	0.257

[a] Absorbance values for the nine pig sera tested are arranged according to decreasing values for IgM. ELISA results for each serum were corrected for background and nonspecific binding by subtracting the mean of absorbance values that occurred in triplicate control wells that contained pig serum but lacked cholesterol. The means of all of the control values (± SD) were 0.038 ± 0.022 and 0.134 ± 0.083 for IgG and IgM, respectively. Absorbance values obtained for IgG and IgM cannot be compared on an absolute basis because a different secondary antiserum was used for detection. In each case the pig serum was diluted 1/100 with PBS. The values shown are averages of three separate experiments.

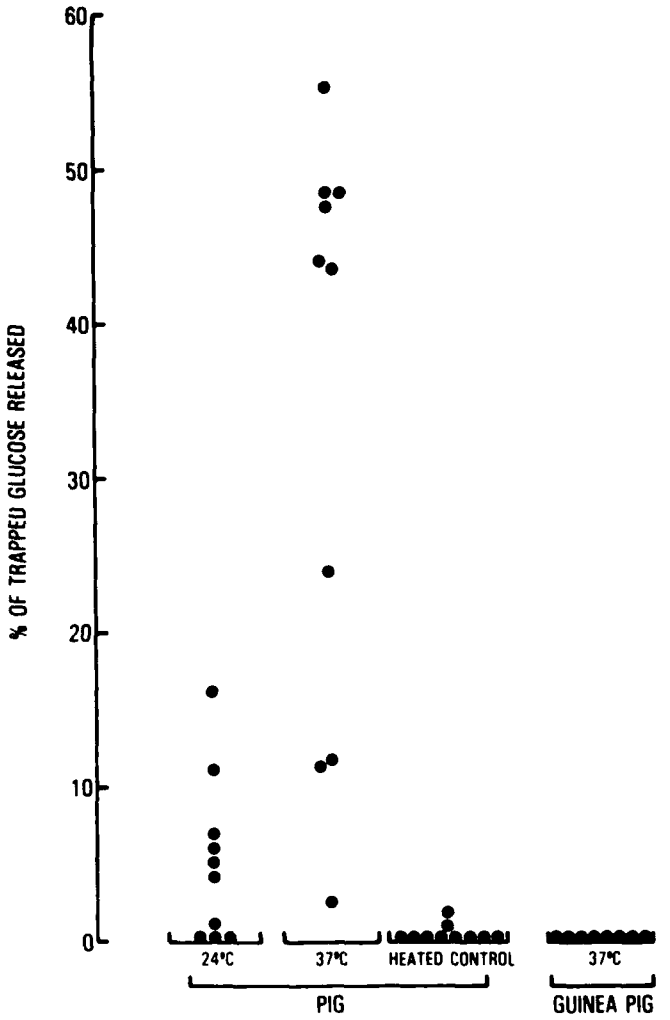

Figure 1. C-dependent immune damage to liposomes containing cholesterol in miniature pig serum. Miniature pig serum (200 μl) was used as source of both the antibody and the C in the glucose release assay. At room temperature (24°C) only 5 of the 10 sera showed clearcut C activation (>5% glucose released due to C damage to liposomes containing cholesterol), whereas at 37°C 9 of the 10 sera gave positive results (with 12 to 55% glucose released from the same liposomes tested at 24°C). The only negative serum was further investigated and it also became positive both when tested by ELISA, and in independent glucose release assays that were supplemented with human serum as source of C. The data therefore indicated that the C in the single unreactive serum had decayed probably because of freezing and thawing. The heated (56°C, 30 min) controls were assayed at 37°C. With each pig serum, C-dependent immune damage did not occur in control experiments performed with cholesterol-free liposomes at either temperature (not shown).

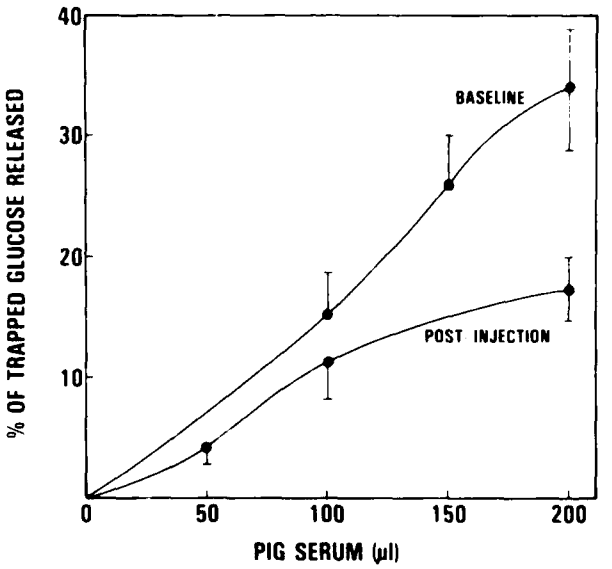

Figure 2. Anticholesterol antibodies in pig sera before and after liposome injection. Miniature pig serum was used as source of both the antibody and the complement in the assay for C-dependent immune damage to liposomes containing cholesterol. The assay was carried out at 37°C and the data represent mean (±SD) of eight pigs.

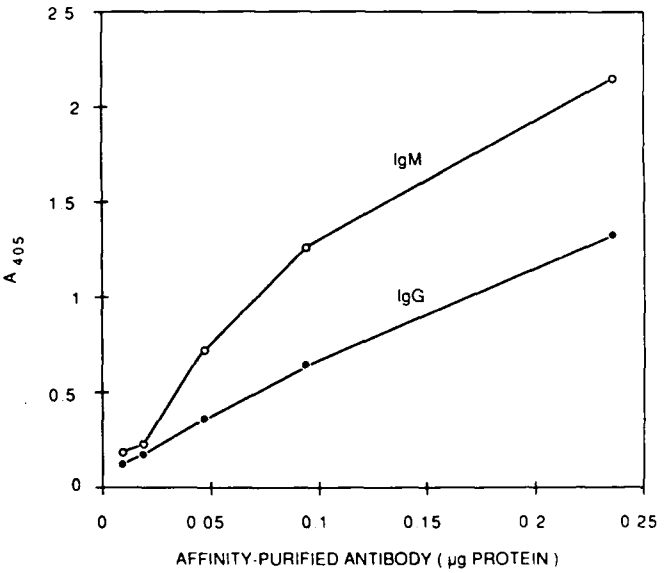

Figure 3. Activities of affinity-purified antibodies to cholesterol from pig serum as determined by ELISA.

rather than IgG antibodies.

Activities of purified naturally occurring antibodies to cholesterol. Affinity-purified antibodies were prepared from a sample of pig serum, and both IgG and IgM antibodies to cholesterol were obtained as detected by ELISA (Fig. 3). The purified porcine anticholesterol antibodies were also tested by C-dependent immune damage to liposomes with guinea pig serum as a C source at 37°C (Fig. 4). The antibodies were purified by elution from liposomes containing 71% cholesterol (see *Materials and Methods*), and as previously observed with murine mAb to cholesterol (3), the purified porcine antibodies had relatively low C-fixing activities against liposomes containing low concentrations (43%) of cholesterol. However, just as with murine mAb to cholesterol (3), the fraction of the porcine antibodies that was purified reacted unequivocally with liposomes containing high levels (71%) of cholesterol (Fig. 4).

Anaphylactoid reactions induced by cholesterol. Infusion of liposomes containing cholesterol into pigs resulted in rapid occurrence of respiratory stridor, disseminated cyanosis, and profound disturbances in cardiovascular hemodynamics. The timing and sequence of events in anesthesized pigs is shown in Figure 5 and was similar in pattern in a total of 15 pigs. After injection of liposomes there was a rapid rise in blood pressure, an increase in both PVR and systemic vascular resistance, and a decrease in CO. Heart rate was not severely affected and a marked tapering of the hemodynamic reaction occurred over a period of 30 min, with all measured and derived parameters returning to baseline values after 60 min. A control experiment to determine whether liposomes size played a role showed that the same adverse reaction was observed with small unilamellar liposomes that were pro-

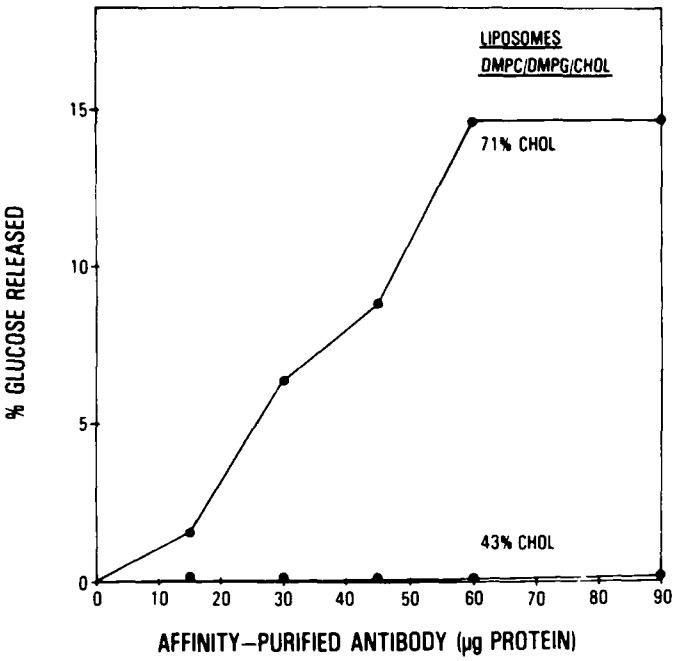

Figure 4. Activities of affinity-purified antibodies from pig serum. The antibodies were tested for the ability to induce C damage to liposomes containing 71% or 43% cholesterol at 37 and 24°C. No activity was detected at 24°C (not shown). Fresh guinea pig serum (120 µl) was used as source of C.

duced by sonication.

Four animals received a second liposome infusion after a 75-min stabilization period. Three of these animals developed severe refractory hypotension and subsequently died. In the animals that died the pulmonary and systemic vascular hypertension observed during the first liposome infusion was not observed after the second liposome infusion.

C activation, eicosanoid secretion, leucopenia, and thrombocytopenia. Hemolytic C titers, and leucocyte and platelet counts were measured before and after infusion of liposomes. Five min after infusion of liposomes, hemolytic C was reduced by 35% compared to preinfusion levels. As shown in Table II, 6-keto-$PGF_{1\alpha}$ and TXB_2 increased 207 and 1300%, respectively, after liposome injection. Two other eicosanoids, PGE_2 and leukotriene C_4, did not change in concentration after liposome infusion (not shown). TXB_2 and 6-keto-$PGF_{1\alpha}$ are the stable metabolites of TXA_2 and PGI_2, respectively. TXA_2 is a potent vasoconstrictor (10) and its increase could have been responsible for the pulmonary hypertension observed. Consequently, it was of interest to determine whether a cyclooxygenase inhibitor, indomethacin, would suppress the anaphylactoid reaction. Pretreatment with indomethacin prevented the precipitous rise in PVR after liposome infusion in all animals (Table II). There was still a rise in CVP (although reduced) and a concomitant decrease in CO, and the leucopenia and thrombocytopenia observed after liposome injection still occurred (Table II).

DISCUSSION

The results in this study indicate that IgG and IgM antibodies to cholesterol were present in serum from each of 10 pigs examined for such antibodies. Infusion of liposomes containing cholesterol into pigs caused C activation and this was associated with an anaphylactoid reaction, probably due to a generation of anaphylatoxins C5a and C3a. A similar anaphylactoid reaction was recently observed after infusion of liposomes containing cholesterol in sheep (11). The latter reaction in sheep was attributed to thromboxane secretion due to an undefined interaction of liposomes with marginated intravascular pulmonary macrophages (11). However, it seems possible, based on our findings, that the phenomenon reported in sheep could also have been caused by C activation.

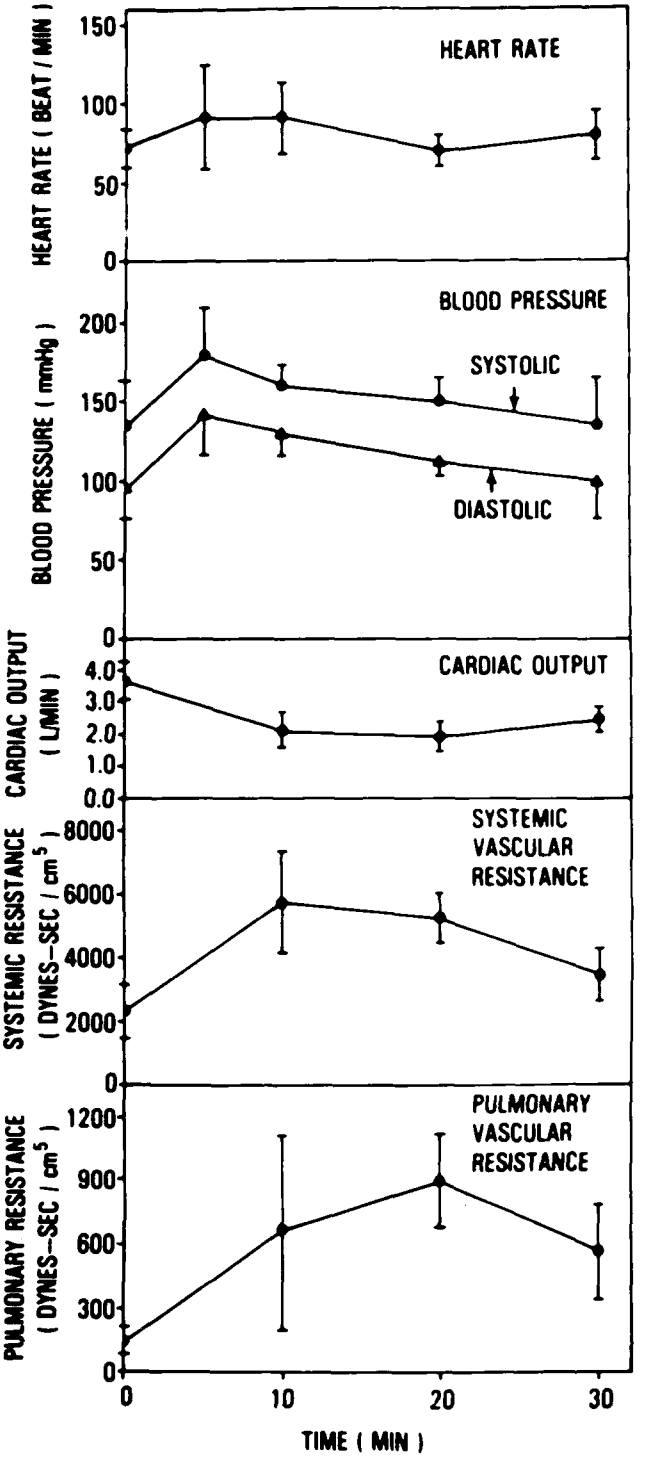

Figure 5. Effects of liposome injection on heart rate, blood pressure, pulmonary vascular resistance, systemic vascular resistance, and cardiac output in miniature pigs. Data represent mean (±SD) of 15 pigs.

TABLE II

Effects of indomethacin[a]

Measured Parameters	No indomethacin			Indomethacin		
	Time After Start of Liposome Injection (min)			Time After Start of Liposome Injection (min)		
	0	5	% Change	0	5	% Change
Hemolytic C titers (CH_{50}/ml)	7.7	5.0	−35			
TXB_2 (ng/ml plasma)	0.55	7.70	+1300	0.45	0.49	+9
6-keto-$PGF_{1\alpha}$ (ng/ml plasma)	0.14	0.43	+207	0.11	0.05	−55
Mean blood pressure (mm Hg)	115	160	+39	116	134	+16
PVR (dynes-s/cm^5)	146	609	+317	141	169	+20
Leucocyte counts (10^3)	10.6	6.8	−36	7.9	4.8	−39
Platelet counts (10^3)	312	193	−38	333	233	−30

[a] Indomethacin was infused at 5 mg/kg before liposome injection. Liposomes were injected at 0.5 ml/kg and 50 mM phospholipid concentration. The results shown are the means of six pigs (five pigs for platelet counts) that received no indomethacin and two pigs that received indomethacin.

Production of C3a, C5a, or active degradation products such as the so-called classical anaphylatoxin, C5a des Arg (12), are known to cause secretion of mediators, including eicosanoids (among which are TXA_2 and PGI_2), histamine, and catecholamines (13–16). Therefore, the high levels of TXB_2 and 6-keto-$PGF_{1\alpha}$ (the stable metabolites of TXA_2 and PGI_2, respectively) that we observed after liposome infusion are compatible with the occurrence of the anaphylactoid reaction as a consequence of immune C activation. TXA_2 and PGI_2 have opposing pulmonic physiologic effects, causing pulmonary hypertension and hypotension, respectively. Based on the ability of indomethacin to block the pulmonary, but not systemic, hypertension it seems likely that the pulmonary hypertension was caused by the disproportionately high secretion of TXA_2, and it is possible that the systemic hypertension was caused by other mediators associated with C activation and anaphylatoxin production. Transient neutropenia, which we also observed, is a known consequence of C activation (17–19). Neutropenia is due to temporary margination and retention of neutrophils in the lung (17–19). It is interesting that transient thrombocytopenia has recently been described following infusion of liposomes into rats (20).

Activation of C by cholesterol and by liposomes containing cholesterol has been reported by many laboratories (21–26). Furthermore, antibodies to a variety of liposomes and lipids, including phospholipids and cholesterol, can be induced in animals and even occur naturally in humans (3, 27). In fact, recent experiments in our laboratory have demonstrated the presence of autoantibodies to cholesterol in more than a hundred normal human sera screened by ELISA with crystalline cholesterol as an Ag (28). Although further investigation is needed to define the role, if any, of antibodies to cholesterol in the pathogenesis of cardiovascular disease, the presence of such antibodies raises the possibility that injection of liposomes as drug carriers in humans could cause C activation in some individuals. In one reported instance in a human, C activation was indeed noted after liposome injection but was not associated with any clinical symptoms (4).

It is unlikely that C activation induced by antibodies will pose any practical impedement to the therapeutic use of cholesterol-containing liposomes in humans. C activation occurs in nearly all patients undergoing hemodialysis with certain types of dialyzer membranes, and profound transient neutropenia is almost always a prominent observation (29, 30). In contrast to the anaphylactoid response usually seen in certain animals such as pigs or sheep, C activation in humans is usually associated only with mild asymptomatic pulmonary dysfunction (30). This is consistent with observations from in vitro models that have shown that human anaphylatoxin is much less spasmogenic than porcine anaphylatoxin (12, 16). Although humans are generally asymptomatic after C activation (30), in the presence of severe preexisting pulmonary disease, examination of human serum for autoantibodies to liposomal lipids might be a useful precaution before i.v. injection of liposomes.

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