Complement C3 Activation Is Required for Antiphospholipid Antibody-induced Fetal Loss

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Abstract

The antiphospholipid syndrome (APS) is characterized by recurrent fetal loss, vascular thrombosis, and thrombocytopenia occurring in the presence of antiphospholipid (aPL) antibodies. The pathogenesis of fetal loss and tissue injury in APS is incompletely understood, but is thought to involve platelet and endothelial cell activation as well as procoagulant effects of aPL antibodies acting directly on clotting pathway components. Recent studies have shown that uncontrolled complement activation in the placenta leads to fetal death in utero. We hypothesized that aPL antibodies activate complement in the placenta, generating split products that mediate placental injury and lead to fetal loss and growth retardation. To test this hypothesis, we used a murine model of APS in which pregnant mice are injected with human IgG containing aPL antibodies. We found that inhibition of the complement cascade in vivo, using the C3 convertase inhibitor complement receptor 1–related gene/protein y (Crry)-Ig, blocks fetal loss and growth retardation. Furthermore, mice deficient in complement C3 were resistant to fetal injury induced by aPL antibodies. While antigenic epitopes recognized by aPL antibodies are important in the pathogenesis of APS, our data show that in vivo complement activation is required for aPL antibody-induced fetal loss and growth retardation.

Key words: complement • anticardiolipin antibodies • pregnancy • thrombosis • lupus

Introduction

The antiphospholipid syndrome (APS)* is characterized by increased risk of vascular thrombosis involving venous, arterial, and placental circulations. When thrombosis occurs in the placenta, it is associated with poor obstetrical outcomes, including fetal death and growth retardation. Patients with systemic lupus erythematosus are particularly prone to APS, though it also occurs in individuals without other manifestations of autoimmune disease.

Although it is clear that the specific antigenic reactivity of antiphospholipid (aPL) antibodies is critical to their effect, the pathogenic mechanisms that result in thrombosis and tissue injury in vivo are incompletely understood. To better understand these mechanisms, several murine models have been developed and studied. In one model, passive transfer of human IgG isolated from aPL antibody-positive sera into pregnant mice induces fetal loss and growth retardation, thereby demonstrating the direct pathogenic role for aPL antibodies (1). Murine and human monoclonal aPL antibodies, which react specifically with the phospholipid-binding protein β2-glycoprotein I (β2GPI) or with anionic phospholipids in the absence of β2GPI, also deposit in the placenta and produce fetal loss, growth retardation, and necrosis (2–4). Recent in vitro studies in human placentas have shown that trophoblast cell membranes behave

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**Abbreviations used in this paper:* aCL, anticardiolipin; aPL, antiphospholipid; aPL-IgG, human IgG containing aPL antibody; APS, antiphospholipid syndrome; β2GPI, β2-glycoprotein I; Crry, complement receptor 1–related gene/protein y; MAC, membrane attach complex.

as targets for β2GPI-dependent and β2GPI-independent aPL antibodies (5), suggesting that these antibodies and their effects are specifically targeted to the placenta.

Although the pathogenic potential of aPL antibodies that recognize specific antigens has been demonstrated, their ability to alter target antigen function has not, in and of itself, been shown to be sufficient to cause deleterious outcomes, such as fetal loss. In addition, while experimental models have emphasized the role of thrombosis in placental tissue, histopathologic findings in placentas from women with APS argue that other proinflammatory factors may contribute to tissue injury (6, 7). Thus, although in vivo and in vitro studies demonstrate that aPL antibodies trigger activation of endothelial cells and platelets, the specific mechanisms that link these events to tissue injury and, in particular, to fetal loss and growth retardation remain unknown (8–10).

Recent experiments in mice have underscored the importance of fetal regulation of complement activation to modulate potentially damaging maternal immune responses. These studies have focused on complement receptor 1–related gene/protein y (Crry), a membrane-bound intrinsic complement regulatory protein whose role is to block C3 and C4 activation on self-membranes in mice (11). That appropriate complement inhibition is an absolute requirement for normal pregnancy has been demonstrated by the finding that Crry deficiency in utero progressively leads to embryonic death (12). $Crry^{-/-}$ embryos, generated by gene targeting, have increased deposition of activated C3 fragments and polymorphonuclear cells in the ectoplacental cone and surrounding trophoectoderm within the developing placenta. Although $Crry^{-/-}$ embryos are present in normal numbers on day 9 of gestation, they show signs of growth retardation and all die by day 15 of gestation. No live births have been found in cohorts of >250 newborns (12). Importantly, when the $Crry^{+/-}$ parents are intercrossed with $C3^{-/-}$ mice to generate $C3^{-/-}$, $Crry^{-/-}$ embryos, there is complete rescue from this 100% lethal outcome and live $C3^{-/-}$, $Crry^{-/-}$ pups are born at a normal Mendelian frequency (12). This outcome provides proof that the $Crry^{-/-}$ embryos die in utero due to their inability to suppress spontaneous complement activation and tissue damage mediated by C3.

In normal pregnancy, the human placenta appears to be subjected to complement-mediated immune attack at the maternal-fetal interface (13, 14). Although there is evidence of activated complement in the placenta, it is likely that in successful pregnancies uncontrolled complement activation is prevented by complement inhibitory proteins, such as decay-accelerating factor, membrane cofactor protein and CD59, which are present at high levels on trophoblast cells (15–20). Taken together, the murine and human findings suggest that there is recognition of fetal tissues by immune mechanisms that trigger complement fixation and that in the presence of excessive complement activation or in the absence of complement inhibitors the fetus is as risk for injury.

We hypothesized that complement activation is a necessary intermediary event in the pathogenesis of fetal loss associated with aPL antibodies. In addition to the rationale outlined above, we have considered this possibility because it is well-established that activated complement fragments themselves have the capacity to bind and activate inflammatory and endothelial cells as well as induce a prothrombotic phenotype, either directly through the membrane attach complex (MAC) or through C5a receptor (CD88)-mediated effects (21, 22). In this setting, the effects of recognition of relevant target antigens by aPL antibodies and the recruitment of the complement activating effector function of the antibody Fc domain of the antibody would both be necessary for pathogenic effects in vivo. We have tested this hypothesis in the studies presented in this report. Our results demonstrate that complement activation is required for the induction of fetal loss in vivo by aPL antibodies and, therefore, that activation of complement is a critical proximal effector mechanism in aPL antibody-induced fetal injury.

Materials and Methods

Preparation of IgG. Human IgG containing aPL antibodies (aPL-IgG) was obtained from a patient with APS (characterized by multiple cerebrovascular accidents, livedo reticularis, and the lupus anticoagulant) with high titers of aPL IgG antibodies (>145 GPL U) identified through the Autoimmune Registry and Repository of the Rheumatic Disease Service at the Hospital for Special Surgery, New York, NY. IgG was purified by affinity chromatography using Protein G sepharose chromatography columns (Amersham Pharmacia Biotech). Control human IgG from a healthy nonautoimmune individual was purified by an identical method. Polyclonal mouse IgG control was obtained from Jackson ImmunoResearch Laboratories. All IgG samples were treated to deplete endotoxin with Centriprep ultrafiltration devices (Millipore) and determined to be free of endotoxin contamination by the limulus amebocyte lysate assay to a sensitivity of 0.06 EU/ml (Associates of Cape Cod).

Detection of aPL Antibodies. Levels of human anticardiolipin (aCL) antibodies were measured by standard ELISA method performed as described previously (23). The cardiolipin solid phase assay was performed with serum samples or purified IgG fractions at 1:50 dilution. GPL U (1 GPL U equals activity of 1 μg affinity purified IgG aCL antibody) were calculated using international standards supplied by the Rayne Institute (24). The purified IgG containing aPL antibodies used herein demonstrated high reactivity in the aCL ELISA (82 GPL U).

To determine the in vivo $t_{1/2}$ of human aPL antibodies in the murine pregnancy model, mice were injected intraperitoneally with aPL-IgG (10 mg) and serial blood samples were collected at time points up to 36 h. Levels of human aCL antibodies in serum of the mouse were measured using the standard ELISA at 1:5 dilution.

Complement Inhibitor Crry-Ig. Crry-Ig was produced and purified using a previously described method (25). In brief, a soluble chimeric protein was created that contains at the amino terminus the extracytoplasmic five short consensus repeat domains of Crry that retain classical and alternative pathway complement C3 convertase inhibitory activity followed by the hinge, CH2 and CH3

domains of the noncomplement fixing mouse IgG1 isotype. This nonimmunogenic protein is produced in NS0 cells to a level of ~50 mg/L, purified using a multicolumn FPLC method and has been shown to effectively block complement activation by both the classical and alternative pathways (11, 25). Previous studies have also demonstrated that Crry-Ig ameliorates complement-dependent in vivo effects in antiglomerular basement membrane–induced glomerular injury (25) and ischemia-reperfusion induced intestinal injury (unpublished data). For these studies, Crry-Ig was purified under LPS-free conditions and had LPS levels <0.4 ng/mg Crry-Ig using the limulus amebocyte lysate assay (BioWhittaker).

Murine Pregnancy Model. Female BALB/c mice (2–3-mo-old) (Taconic) were allowed to mate with previously isolated males. The presence of a vaginal plug was taken as an evidence for pregnancy. On days 8 and 12 of pregnancy female mice were treated with intraperitoneal injections of aPL-IgG (10 mg), control human IgG (10 mg), or saline as described previously (1, 26). Some mice received intraperitoneal injections of Crry-Ig (3 mg) or murine IgG (3 mg) every other day from days 8–12, an appropriate timing as the $t_{1/2}$ of Crry-Ig in vivo is ~40 h and this dose has resulted in protection in other complement-dependent models. Mice were killed on day 15 of pregnancy. Uteri were dissected, fetuses and placentas were weighed, and frequency of fetal resorption was calculated (number of resorptions divided by the total number of formed fetuses and resorptions). Resorption sites are easily identified (see Fig. 2) and result from the loss of a previously viable fetus at that site. In healthy untreated mice, the frequency of fetal resorption was <0.05.

$C3^{-/-}$ mice were generated by intercrossing $C3^{+/-}$ mice at F1 during a backcross to C57BL/6 and then propagating $C3^{-/-}$ progeny (27). The same aPL-IgG (10 mg) and control IgG (10 mg) were injected intraperitoneal on days 8 and 12 of pregnancy into these mice. Control mice for these experiments were B6129F1/Tac hybrid mice obtained from Taconic, which also contain a mixture of C57BL/6 and Sv129 genes. Pregnancy outcome was assessed as described above.

Immunohistochemistry. Deciduas were removed and frozen quickly in O.C.T. compound (Sakura) and 10 μm-thick sections were cut. Endogenous peroxidase was quenched with 0.2% H_2O_2 in methanol. Sections were first incubated with normal rabbit serum to block nonspecific binding (Cappel), then incubated with primary goat anti–mouse C3 (Cappel), followed by rabbit anti–goat IgG conjugated to HRP (Sigma-Aldrich). Bound HRP was detected with diaminobenzidine. Sections were counterstained with 1% methylgreen and covered with mounting medium (Cytoseal). Sections of frozen tissue were also stained with H&E.

Analysis of Thrombus Dynamics. To investigate the effects of Crry-Ig on thrombophilia induced by aPL antibodies, male CD1 mice (25–30 g, 4–8-wk-old) (Charles River Laboratories) were injected intraperitoneally with 500 μg of affinity purified human aPL-IgG antibodies or control human IgG preparations at time 0 and 48 h. Methods for purification of the aPL antibodies has been described previously (28). Half of the mice in each group were treated with Crry-Ig (3 mg intraperitoneally at 0 and 48 h) and the other half were treated with polyclonal murine IgG (3 mg intraperitoneally at 0 and 48 h). Surgical procedures to study thrombus dynamics were performed 72 h after the first aPL-IgG (or control human IgG) injection. ACL antibody titers were also measured at that time.

The mouse model of thrombosis formation employed in this study has been described in detail (28, 29). Briefly, mice were anesthetized, and the right femoral vein was exposed, resulting in a 0.5-cm segment of vein free for manipulation and observation. The vein was pinched with a pressure of 1,500 g/mm^2 to introduce a standardized injury that induced a clot. Clot formation and dissolution in the trans-illuminated vein were visualized with a microscope equipped with a closed-circuit video system (including a color monitor and a recorder). Thrombus size (in square micrometers) was measured 1 min after the pinch injury by freezing the digitized image and tracing the outer margin of the thrombus. 3–5 thrombi were successfully induced in each animal, and mean values were computed.

Data Analysis. Data are expressed as mean ± SD. The Student's unpaired *t* test was used to compare the frequency of fetal resorption, fetal weights, thrombus size, and aPL titers between groups. In all analyses, a probability of <0.05 was used to reject the null hypothesis.

Results

Crry-Ig Ameliorates aPL Antibody-induced Pregnancy Loss in BALB/c Mice. To determine whether complement activation is necessary for aPL-IgG–induced fetal loss, we first used Crry-Ig to block complement activation in vivo. No soluble forms of Crry are found in mice, and treatment of mice with this recombinant protein results in nearly complete complement inhibition in vivo (25). BALB/c mice were treated with aPL-IgG (10 mg), control human IgG (10 mg), or saline intraperitoneally on days 8 and 12 of pregnancy. Half of the mice received Crry-Ig (3 mg intraperitoneally) every other day from days 8–12. On day 15, pregnant mice were killed, and the uterine contents were evaluated.

Treatment with aPL-IgG caused a nearly fourfold increase in the frequency of fetal resorption, while simultaneous treatment with Crry-Ig prevented aPL antibody-induced pregnancy losses (Fig. 1 A). Fig. 2 (top) shows a representative placenta from an aPL antibody–treated mouse. The fetuses are small and two fetal resorptions are seen. In contrast, Fig. 2 (bottom) demonstrates a uterus from an aPL antibody-treated mouse that received Crry-Ig. Here, the fetal sacs are larger and no resorptions are seen.

Crry-Ig also prevented aPL antibody–mediated growth retardation in the surviving fetuses (Fig. 1 B). Treatment with aPL-IgG alone resulted in a 45% decrease in fetal weight, whereas in mice treated with aPL-IgG and Crry-Ig the weights of fetuses were nearly identical to those of control mice (Fig. 1 B). Crry-Ig prevented the aPL-IgG induced growth retardation in the fetus and in the placental elements. Importantly, Crry-Ig had no effect on either the frequency of fetal resorption or on fetal size in the absence of aPL antibody. Therefore, its protective effects are dependent on the presence of aPL antibodies in the injected human IgG.

That Crry-Ig ameliorates these effects of aPL-IgG on fetal resorption and fetal weight by complement inhibition was tested in a second series of experiments. Pregnant BALB/c mice injected with aPL-IgG were treated with 3 mg of either Crry-Ig or control murine polyclonal IgG. In these studies, the capacity of Crry-Ig to specifically prevent aPL antibody-induced fetal resorption was confirmed (Ta-

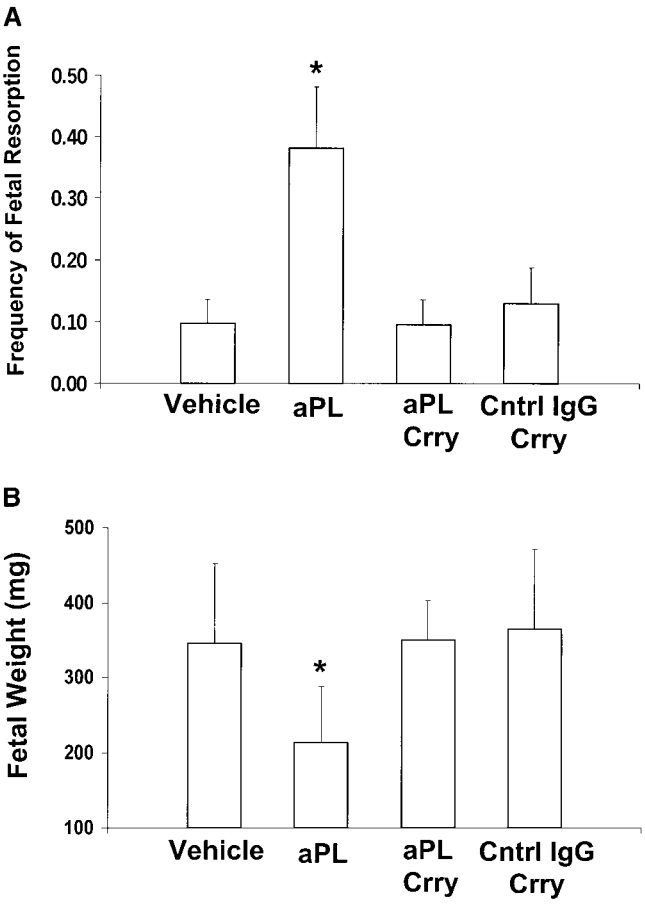

Figure 1. Crry-Ig ameliorates aPL-IgG–induced pregnancy complications. Female BALB/c mice were treated intraperitoneally with IgG (10 mg) from a patient with APS (aPL), normal human IgG (Cntrl IgG), or saline (Vehicle) on days 8 and 12 of pregnancy. Some the mice received Crry-Ig (3 mg intraperitoneal) every other day from days 8–12. Mice were killed on day 15 of pregnancy, uteri were dissected, fetuses were weighed, and frequency of fetal resorption calculated (number of resorptions/number of fetuses plus number of resorptions). There were six mice in each group. (A) Treatment with aPL-IgG caused an increase in fetal resorptions compared with vehicle or control human IgG (*$P < 0.05$) which was prevented by Crry-Ig (*aPL versus aPL plus Crry-Ig; $P < 0.05$). (B) aPL-IgG caused fetal growth retardation (*aPL versus Cntrl IgG; $P < 0.01$) which was also prevented by Crry-Ig (*aPL versus aPL plus Crry-Ig; $P < 0.01$).

ble I). Mice treated with aPL-IgG along with murine IgG showed a threefold increase in fetal resorption as compared with mice treated with control human IgG and murine IgG. Murine IgG had no effect on either induction of fetal loss or growth retardation by aPL-IgG. In contrast, Crry-Ig consistently prevented fetal loss and impaired growth associated with aPL antibodies (Table I). That results from experiments with monoclonal human aPL antibodies are similar to results using polyclonal aPL antibodies (data not shown), indicates that antibodies reactive with aPL, rather than xenoreactive antibodies which may be present in polyclonal human IgG, initiate complement activation, and fetal damage in our model.

To exclude the possibility that Crry-Ig altered the handling of aPL antibodies, we measured levels of human aCL-

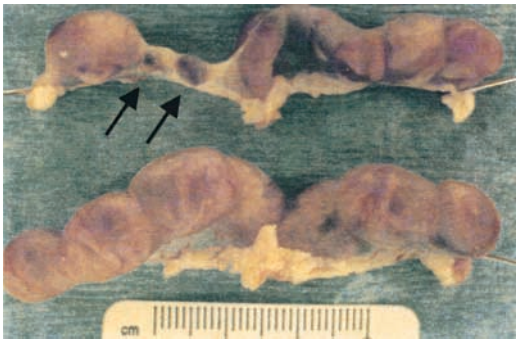

Figure 2. Effects of Crry-Ig on aPL antibody-induced fetal resorption. Representative uteri from BALB/c mice killed at day 15 of pregnancy are shown. The top panel, from a mouse treated with aPL-IgG and murine IgG, shows eight fetuses and deciduas of varying size and two resorptions (arrows). The bottom panel, from a mouse treated with aPL and Crry-Ig, contains larger fetuses with deciduas and no resorptions.

reactive IgG in mice treated with Crry-Ig and control murine IgG. The serum $t_{1/2}$ (3.7 ± 0.9 h vs. 4.1 ± 0.5 h, respectively), and peak levels (65 ± 18 GPL U vs. 68 ± 22 U, respectively) were indistinguishable.

Immunohistological Analysis of aPL Antibody-induced Fetal Loss. Spontaneous maternal complement activation at the fetal-maternal interface presents a danger to the developing fetus. In *Crry*$^{-/-}$ mice complement activation causes fetal death (12). To determine whether excessive complement activation occurs within the placenta in aPL-treated mice, we conducted immunohistological analyses of decidua 90 min after the first treatment (day 8 of pregnancy) with aPL-Ig or control IgG. In preliminary experiments we confirmed that human IgG was deposited in the decidua of mice treated with aPL-IgG or with aPL-IgG and Crry-Ig, whereas there was no evidence of IgG deposition in mice treated with control human IgG (data not shown). In aPL-treated mice, the decidua was abnormal morphologically, showing focal necrosis and apoptosis with neutrophil infiltrates and loss of the fetal membrane elements (Fig. 3). This was in contrast to relatively normal appearing decidua of mice treated with control human IgG or aPL-IgG and Crry-Ig.

Staining with antibodies against the murine complement component C3 revealed extensive complement deposition within the decidua and on the extraembryonic membranes in the mice treated with aPL-IgG (Fig. 4). In the mice treated with control human IgG small amounts of C3 staining were detectable, mostly in the extraembryonic tissues, but the decidua was not inflamed and had normal cellular elements (Fig. 4). We infer that as a consequence of the large amount of activated complement produced when aPL-IgG is present within the decidua, membrane bound complement inhibitors, such as Crry, are unable to prevent complement deposition.

Treatment with Crry-Ig at the time of administration of aPL-Ig completely prevented C3 deposition on the decidua (Fig. 4). In mice treated with aPL-IgG and Crry-Ig, there was no infiltration of neutrophils, and there was normal fe-

Table I. *Crry-Ig Specifically Prevents aPL Antibody-induced Fetal Resorption and Growth Retardation*

Human IgG source:	aPL	aPL	Cntrl IgG	Cntrl IgG
Mouse reagent:	mIgG	Crry-Ig	mIgG	Crry-Ig
Number of mice	11	14	10	11
Frequency of fetal resorption	0.29 ± 0.09[a]	0.08 ± 0.03	0.10 ± 0.05	0.08 ± 0.03
Fetal weight (mg)	171 ± 27[a]	256 ± 38	273 ± 32	281 ± 34

Female BALB/c mice were given aPL-IgG (10 mg) or normal human IgG (Cntrl IgG) (10 mg) intraperitoneally on days 8 and 12 of pregnancy. Mice also received either Crry-Ig (3 mg) or polyclonal murine IgG (mIgG) (3 mg) intraperitoneally every other day from days 8–12. Mice were killed on day 15 of pregnancy, uteri were dissected, fetuses were weighed, and frequency of fetal resorption calculated (number of resorptions/number of fetuses plus number of resorptions). Treatment with aPL-IgG caused an increase in fetal resorptions and a decrease in fetal weight ([a]aPL plus mIgG versus Cntrl IgG plus mIgG; $P < 0.001$) which was prevented by Crry-Ig ([a]aPL plus mIgG versus aPL plus Crry-Ig; $P < 0.001$). Treatment with mIgG did not affect either pregnancy outcome.

tal development (Fig. 3). Our results indicate that placental complement deposition and extensive inflammation is associated with aPL-treatment and that these effectors can be inhibited by Crry-Ig.

C3-deficient Mice Are Protected from aPL-associated Fetal Resorption and Growth Retardation. As an alternate approach to test the hypothesis that complement activation is required for aPL-induced pregnancy complications, we performed studies in mice deficient in complement component C3. First, aPL-IgG was shown to result in poor pregnancy outcomes in the B6129F1 hybrid mice, the control strain for the $C3^{-/-}$ mice at the F1 backcross to C57BL/6. Similar to BALB/c mice, there was a ~4-fold increase in the frequency of fetal resorption (Fig. 5). In addition, Crry-Ig treatment, when compared with the administration of control mouse IgG, specifically prevented this outcome (Fig. 5 A) as well as growth retardation (Fig. 5 B).

These experiments established the validity of using hybrid mice containing ~50% each of C57BL/6 and Sv129 genes. As predicted by experiments with Crry-Ig, aPL-IgG did not increase the frequency of fetal resorption in $C3^{-/-}$ mice on the same genetic background (Fig. 5 A). The protective effects of a complete lack of complement

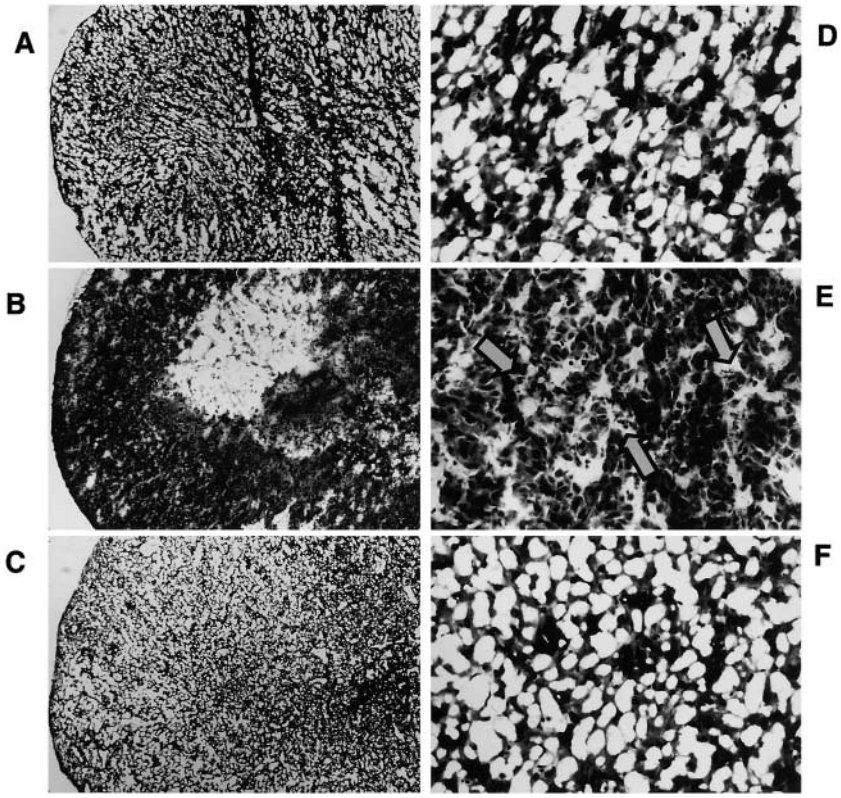

Figure 3. APL antibody-induced necrosis and infiltration of neutrophils in decidual tissue is prevented by Crry-Ig. Pregnant mice (day 8) were injected with control human IgG (A and D), aPL-IgG (B and E), or aPL-IgG and Crry-Ig (C and F), and uteri were removed after 90 min. In mice treated with aPL-IgG there was increased density of cells and focal necrosis within the decidual tissue (B) and clumping, apoptosis, and scattered clusters of neutrophils (arrows) throughout (E). Decidual tissue from mice treated with aPL-IgG and Crry-Ig was similar to mice treated with control Ig, showing uniform, sparse cellularity (A and C) and normal appearing trophoblastic cells (D and F). Sections were stained with H&E. (A–C) Original magnification: ×10; (D–F) original magnification: ×40.

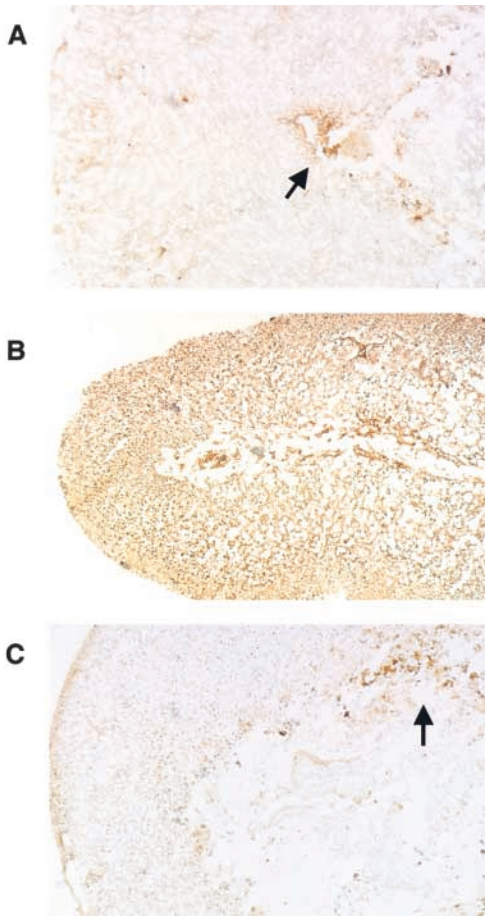

Figure 4. Complement activation in decidual tissue of aPL antibody-treated mice is prevented by Crry-Ig. Sections of decidual tissue from mice injected with control human IgG (A), aPL-IgG (B), or aPL-IgG and Crry-Ig (C) as described in Fig. 3 were stained with antibody to mouse C3. In the aPL-IgG treated mice (B), there was extensive C3 deposition in the decidua and extraembryonic membranes. In contrast, the decidual tissues from mice treated with control IgG (A) or aPL-IgG and Crry-Ig (C) show minimal staining for C3 with more intense staining on the extraembryonic tissues (arrows). Original magnification: ×10.

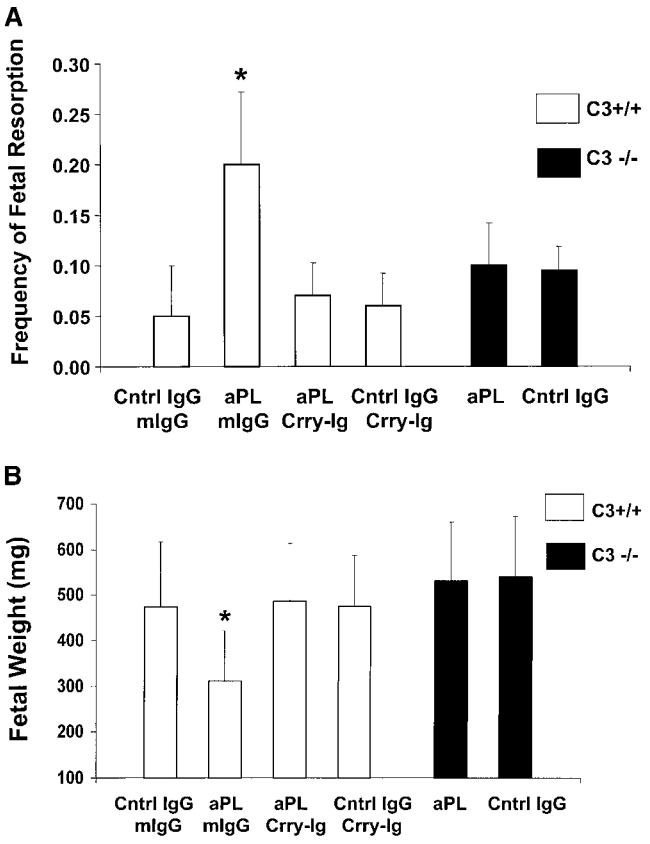

Figure 5. C3-deficient mice are protected from aPL antibody-induced pregnancy complications. $C3^{+/+}$ mice (B6/Sv129F1) were treated with aPL-IgG (10 mg intraperitoneally) (aPL) or normal human IgG (Cntrl IgG) on days 8 and 12 of pregnancy. Half of the mice in each group received Crry-Ig (3 mg intraperitoneally) every other day from days 8–12 and half received control murine IgG (mIgG). $C3^{-/-}$ mice were treated with either aPL-IgG or normal human IgG. Pregnancy outcomes were assessed as described in the legend for Fig. 1. There were 10–14 mice in each experimental group. (A) Analysis of the four groups of $C3^{+/+}$ mice shows that treatment with aPL-IgG caused an increase in frequency of fetal resorptions in this strain (*aPL plus mIgG versus Cntrl IgG plus mIgG; $P < 0.01$), while $C3^{-/-}$ were protected from aPL-induced pregnancy loss (aPL versus Cntrl IgG; P = NS). In the $C3^{+/+}$ mice, Crry-Ig prevented aPL-induced fetal resorption (*aPL plus mIgG versus aPL plus Crry-Ig; $P < 0.01$). (B) Similarly, aPL treatment caused a decrease in fetal weight in $C3^{+/+}$ mice (*aPL plus mIgG versus Cntrl IgG plus mIgG; $P < 0.01$) which was absent in $C3^{-/-}$ mice, and this was ameliorated by Crry-Ig (*aPL plus mIgG versus aPL plus Crry-Ig; $P < 0.01$).

C3 were also found when fetal weights were examined (Fig. 5 B). Serum $t_{1/2}$ and peak levels of human aPL antibodies were comparable in $C3^{+/+}$ and $C3^{-/-}$ mice ($t_{1/2}$ 3.1 ± 0.8 h vs. 3.5 ± 0.7 h, respectively; peak aCL levels 78 ± 12 vs. 83 ± 19 GPL U). These data eliminate the possibility that differences in handling antibodies in the two strains of mice account for differences in aPL-IgG–mediated pathogenicity.

Thus, using three distinct approaches, a specific complement inhibitor (Crry-Ig), genetically deficient mice ($C3^{-/-}$), and immunohistochemical evidence that absence of C3 deposition in Crry-Ig–treated mice correlated with improved outcomes, we have demonstrated that complement activation is required for fetal loss and growth retardation in this murine model of APS.

Crry-Ig Inhibits aPL Antibody-induced Enhancement of Thrombosis. To better understand the mechanism by which Crry-Ig protects against aPL-induced fetal loss, we tested the hypothesis that complement inhibition blocks thrombosis generation in vivo. Because complement fragments (such as C3a or C5a) can directly activate endothelial cells by binding to cell surface receptors or indirectly activate endothelial cells by binding to receptors on neighboring phagocytes or platelets, they may induce a prothrombotic phenotype (30, 31). We predicted that this thrombophilic state would be prevented if complement activation were inhibited. An in vivo microcirculation model had been developed and used to show that aPL-IgG antibodies induce endothelial cell activation and enhance and accelerate thrombus formation in the presence of a vascular injury (28, 29, 32). We employed this experimental model

of APS to examine the effects of complement inhibition on aPL-IgG–induced thrombus formation. CD1 male mice were treated with aPL-IgG or control human IgG, and half the mice in each of the two groups were treated with Crry-Ig and half with polyclonal murine IgG. 72 h after the first injection, the dynamics of surgically induced thrombus formation were measured in the right femoral vein by a standard pinch injury. There was a fivefold increase in the average size of thrombi in mice treated with aPL-IgG compared with those treated with control IgG (Fig. 6). Whereas Crry-Ig did not alter thrombus size in mice treated with control human IgG, administration of Crry-Ig significantly decreased aPL-induced enhancement of thrombosis, resulting in values near those of the controls (Fig. 6). As before, there was no difference in the peak levels of human aCL activity between mice treated with Crry-Ig and mice treated with control murine IgG (25 ± 14 U vs. 31 ± 16 GPL U, respectively), excluding the possibility that the inhibitory action of Crry-Ig was related to alterations in levels of aPL antibodies.

In sum, these results demonstrate that complement activation is a central mechanism contributing to aPL antibody-induced thrombophilia, pregnancy loss, and fetal growth retardation. Although the cause of tissue injury in this disease is likely multifactoral, we have shown that complement activation is an absolute requirement for two of the most deleterious phenotypic outcomes in this condition and, therefore, that this pathway acts upstream of other effector mechanisms.

Discussion

In a murine model of APS induced by passive transfer of human aPL-IgG antibodies, we have made the unanticipated finding that complement blockade at the point of C3 activation prevents fetal loss and growth retardation. In this in vivo model, using Crry-Ig, an exogenously administered inhibitor of C3 activation, and using mice deficient in C3, we have shown that complement activation plays an essential and causative role in fetal loss and tissue injury. Our studies demonstrate that, while the nature of the antigens recognized by aPL antibodies is clearly important, complement activation is the major effector mechanism by which these antibodies mediate tissue injury.

In the first series of experiments, we used Crry-Ig, a C3 convertase inhibitor that blocks activation of C3 by both the classical and alternative pathways. Because Crry-Ig is a chimera between the complement regulatory domains of Crry and the Fc domain of mouse IgG1, we compared its effects with those of polyclonal mouse IgG. That treatment with control murine IgG did not prevent the deleterious effects of aPL antibodies on fetal growth and survival indicates that the ability of Crry-Ig to bind to Fcγ receptors or to mediate other nonspecific effects does not determine this biologic outcome. Furthermore, measurement of aPL titers in Crry-Ig-treated and control mice demonstrated that Crry-Ig did not act simply as a competitive inhibitor of aPL antibody. Rather, our results argue that fetal damage is averted through Crry-Ig–mediated blockade of C3 activation. Indeed, our observations that Crry-Ig prevents C3 deposition within the decidua and that $C3^{-/-}$ mice are protected from APS support our conclusion that the critical in vivo effect of Crry-Ig is related to inhibition of complement activation. In sum, using several strategies, we have shown that complement activation is the mediator of injury in this model of APS.

It is important to note that our results are also consistent with the observation that inactivation of the endogenous gene encoding for Crry is associated with inappropriate complement activation and fetal loss (12). The requirement for an inhibitor of complement suggested that complement activation is present in normal pregnancy. Indeed, we found small amounts of C3 deposition within the placentas of normal pregnant mice treated with control human IgG. However, in mice treated with aPL-IgG, C3 deposition was markedly increased. Thus, it appears that excessive complement activation in the placenta, whether due to a lack of a critical regulatory protein such as Crry or the presence of complement-fixing aPL antibodies, places the fetus at risk for growth retardation or death.

Based on our findings, we propose the following mechanism for the pathogenic effects of aPL antibodies on pregnancy outcome. First, aPL antibodies are preferentially targeted to the placenta. This has been suggested by several previous in vitro studies that showed that placental trophо-

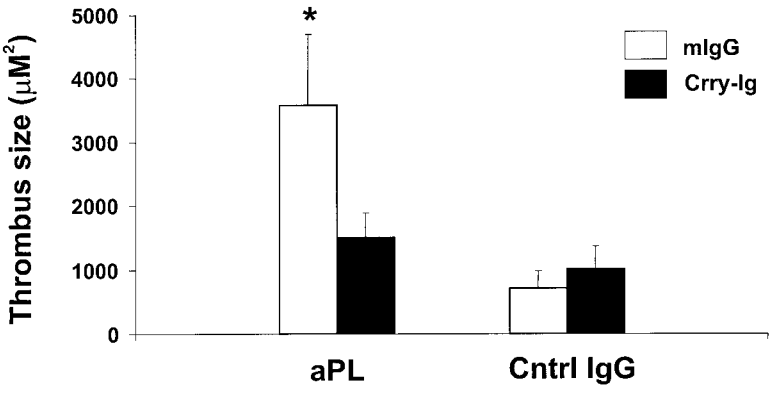

Figure 6. aPL-IgG–induced thrombophilia is inhibited by Crry-Ig. CD-1 mice were injected intraperitoneal with affinity purified aPL-IgG (aPL) or normal human (Cntrl IgG) at 0 h and 48 h. Half the mice in each group received Crry-Ig, and half the mice received control murine IgG (mIgG). At 72 h after the first injection, surgically induced thrombus formation was measured as described in Materials and Methods. There were 11–14 mice in each experimental group. Treatment with aPL-IgG caused an increase in thrombus size (*aPL plus mIgG versus Cntrl IgG plus mIgG; $P < 0.05$), while Crry-Ig prevented aPL-induced enhancement of thrombosis (*aPL plus mIgG versus aPL plus Crry-Ig; $P < 0.05$; Cntrl IgG plus Crry-Ig versus aPL plus Crry-Ig; P = NS).

blast cell membranes bind both β2GPI-dependent and β2GPI-independent aPL antibodies (5, 33). Second, placental aPL antibodies promote platelet and endothelial cell activation and directly induce procoagulant activity through interaction with elements of the coagulation pathway (8, 32, 33–36). This activity, however, does not appear to be sufficient to cause fetal loss or growth retardation, perhaps because of counterregulatory mechanisms. Activation of the complement pathway by aPL-IgG amplifies these effects by stimulating the generation of further potent mediators of platelet and endothelial cell activation, including C3a, C5a, and the C5b-9 MAC. The addition of these complement activation products causes thrombosis, tissue hypoxia, and inflammation within the placenta, and ultimately leads to fetal injury. Depending on the extent of damage, either death in utero or fetal growth retardation results. That Crry-Ig blocks thrombosis initiated by aPL antibodies in an in vivo vascular injury model is consistent with our suggestion that complement activation products play a pivotal role in aPL-induced injury.

Though it is not clear from our results which complement components or receptors are primarily responsible for fetal loss mediated by aPL antibodies, likely candidates include C3a and/or C5a or the C5b-9 MAC. C5a and the C5b-9 MAC have well described effects on platelets and endothelial cells (31, 36). Future studies in mice deficient in specific complement components and receptors may identify the particular complement activation products that promote fetal loss and growth retardation.

Our model of the pathogenesis of fetal wastage in APS is fundamentally different from that previously presented by Merrill et al. (37). These authors proposed that β2GPI and C4-binding protein, a complement inhibitor that also regulates the clotting pathway, compete for binding to protein S. Their hypothesis is focused on the activities of C4-binding protein that are not related to complement. Instead, we propose that pathogenic aPL antibodies, in addition to their direct effects on platelet and endothelial cell targets, generate complement split products and MAC which cause increase tissue injury.

To extend our model of the mechanisms of fetal damage in APS to patients with this syndrome, it is important that our findings are consistent with clinical and laboratory features of patients with APS and recurrent fetal loss. We believe that they are. Histopathologic findings in placentas from women with APS argue that proinflammatory factors contribute to injury (6, 7). Previous studies have provided evidence for complement activation in the serum of pregnant patients with systemic lupus erythematosus (38), and indicate that specific patterns of complement activation are associated with either a flare of disease or preeclampsia (39). In addition, elevated complement split products have been detected in the serum of patients with other manifestations of aPL-associated disease, such as stroke and transient ischemic attack (40). Finally, recent studies using a novel assay in which complement-fixing human aPL can be distinguished from noncomplement-fixing aPL antibodies have shown that complement-fixing antibodies are highly associated with thrombosis and/or recurrent fetal loss (41). Thus, we believe that our results are consistent with the findings in patients with aPL antibody-associated recurrent fetal loss.

Treatment of patients with recurrent aPL antibody-associated fetal loss is difficult and requires the use of several anticoagulation strategies and, in some cases, immunosuppression. Despite these measures, many patients continue to suffer recurrent spontaneous abortions (42). Given the finding that the complement inhibitor Crry-Ig protects mice from the effects of human aPL antibody, it is possible that a similar strategy would provide an effective new therapy for pregnancy loss in patients. Likewise, it is possible that aPL antibody-associated thrombosis in other organs is also mediated by complement activation and may be amenable to this therapeutic approach.

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