REPORT

Complement activation as a mediator of antiphospholipid antibody induced pregnancy loss and thrombosis

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The antiphospholipid antibody syndrome (APS) is characterised by increased risk of vascular thrombosis involving venous, arterial, and placental circulations. The last of these is associated with poor obstetrical outcomes, including fetal death and growth retardation. Pregnancy loss is a defining criterion for APS and occurs with particularly high frequency in systemic lupus erythematosus (SLE) patients bearing this antibody. Patients meet the criteria for APS if they have three otherwise unexplained embryonic losses (before 10 weeks gestation) or one otherwise unexplained fetal loss after 10 weeks, with or without placental infarction or fetal growth restriction.^{1–3}

Over the past two decades, APS has emerged as a leading cause of pregnancy loss and pregnancy related morbidity. It is now recognised that recurrent miscarriage occurs in 1% of couples,^{4–7} that up to 20% of women with recurrent miscarriage have antiphospholipid (aPL) antibodies, and that in about 15% of otherwise apparently normal women aPL is the sole explanation for recurrent fetal loss.^{8–9} The primary treatment for these patients, anticoagulation throughout pregnancy, is inconvenient, sometimes painful, expensive, and fraught with potential complications, including haemorrhage and osteoporosis. Moreover, it is often ineffective. Thus, the identification of a novel mechanism for pregnancy loss in women with aPL antibodies holds the promise of new, safer, and better treatments.

Awareness of the association of aPL antibodies with pregnancy complications has increased the frequency with which APS is diagnosed and generated substantial interest in elucidating its pathophysiology. At present, though the association of aPL antibodies with pregnancy loss and pregnancy morbidity is secure, its mechanism remains unknown.¹⁰ Defining this mechanism is crucial for several reasons. Firstly, there is considerable variability in the success rates of the various treatments,¹¹ suggesting that different but still undefined treatments may be optimal in specific subsets of women with aPL. Secondly, current treatment regimens are imperfect and many treated pregnant patients suffer complications such as pre-eclampsia, uteroplacental insufficiency, intrauterine fetal growth restriction, and preterm birth.^{11–12} Thirdly, understanding how aPL antibodies “cause” miscarriage will probably lead to important insights into the mechanisms of miscarriages in general, thereby benefiting women with non-aPL related miscarriages.

ANTIPHOSPHOLIPID ANTIBODIES INDUCE TISSUE DAMAGE

Although it is clear that the specific antigenic reactivity of aPL antibodies is critical to their effect, the pathogenic mechanisms that result in injury *in vivo* are incompletely understood. To elucidate these mechanisms, several murine models have been developed. In one particularly informative model, passive transfer of human IgG isolated from aPL antibody positive sera, or of murine and human monoclonal aPL antibodies, into pregnant mice induces fetal loss and growth

retardation, demonstrating the direct pathogenic role of aPL antibodies.^{13–17} Recent *in vitro* studies in human placentas have shown that trophoblast cell membranes attract both phospholipid binding protein β 2-glycoprotein I (β 2GPI) dependent and β 2GPI independent aPL antibodies,¹⁸ suggesting a mechanism by which these antibodies specifically target the placenta.

Despite appreciation of the importance of antigens recognised by aPL antibodies, it has not been shown that the ability of aPL antibodies to change target antigen functions is in itself sufficient for such highly deleterious *in vivo* outcomes as recurrent fetal loss. In addition, while experimental models have emphasised the role of thrombosis in placental tissue, histopathological findings in placentas from women with APS argue that proinflammatory factors may contribute to injury.^{15–18–20} *In vivo* and *in vitro* studies have demonstrated that aPL antibodies trigger activation of endothelial cells, monocytes, and platelets, which are also capable of causing tissue damage.^{21–23}

We hypothesised that complement activation is a necessary *in vivo* intermediary step critical for the full pathogenic effect of aPL antibodies on platelets and endothelial or trophoblast cells within the placenta and at other sites where aPL antibodies are deposited. We considered this mechanism 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 MAC or through receptor mediated effects (reviewed in Holers²⁴). These *in vivo* pathogenic effects require both recognition of relevant target antigens by aPL antibodies and Fc domain mediated recruitment of complement activating effector functions. The validity of this novel hypothesis has been confirmed in our recently published studies that show that complement activation is required for the induction of fetal loss *in vivo* by aPL antibodies and consequently, that complement activation is a critical proximal effector mechanism in aPL antibody induced fetal injury.²⁵

COMPLEMENT ACTIVATION AND TISSUE INJURY

The complement system, comprised of over 30 proteins that act in concert to protect the host against invading organisms, starts the inflammatory response and tissue injury.^{26–27} The classic and alternative pathways of the complement system are activated by mechanisms relevant to the immunopathogenesis of vascular injury in SLE. The classic pathway is activated when natural or elicited antibodies bind to antigen and unleash effectors associated with humoral responses in immune mediated tissue damage. Alternative pathway activation mechanisms differ in that they are initiated by the binding of spontaneously activated complement components to

Abbreviations: aPL, antiphospholipid; APS, antiphospholipid antibody syndrome; SLE, systemic lupus erythematosus

Figure 1 The complement cascade. *Indicates known biologically active complement fragments with the potential to influence the pathophysiology of disease.

the surface of pathogens and other targets such as self tissues. By means of these recognition and activation mechanisms, as well as the lectin pathway of activation, the complement system identifies and responds to “dangerous” situations presented by foreign antigens, pathogens, tissue injury, ischaemia, apoptosis, and necrosis.²⁸ This capacity places the complement system at the centre of many clinically important responses to foreign pathogens and to fetal injury mediated by cellular or humoral immune mechanisms.

Three complement activation pathways converge on the C3 protein, a nexus to effector functions, which is then cleaved to generate fragments C3a and C3b (fig 1). C3a is an anaphylatoxin that binds to receptors on leucocytes and other cells, resulting in the activation and release of soluble inflammatory mediators.²⁹ C3b and its further sequential cleavage fragments, iC3b and C3d, are ligands for complement receptors 1 and 2 (CR1 and CR2) and for the β 2 integrins, CD11b/CD18 and CD11c/CD18, which are present on a variety of inflammatory and immune accessory cells.^{24–30} C3b attaches covalently to targets, followed by assembly of C5 convertase and the subsequent cleavage of C5 to C5a and C5b. C5a is a potent soluble inflammatory anaphylatoxic and chemotactic molecule that promotes recruitment and activation of neutrophils (PMN) and monocytes and mediates endothelial cell activation through its receptor (C5aR, CD88), a member of the heptahelical seven transmembrane spanning protein family.^{31–32} On endothelial cells, C5a binding results in increased expression of P selectin and markedly increases neutrophil adhesion.³³ C5aR has also been described on vascular smooth muscle cells, renal mesangial cells, and bronchial and alveolar cells. Binding of C5b to the target initiates the non-enzymatic assembly of the C5b-9 MAC. Insertion of C5b-9 MAC can result in erythrocyte lysis. However, the important effect of C5b-9 MAC in disease is on nucleated cells where it activates proinflammatory signalling pathways through the interaction of membrane associated MAC proteins with heterotrimeric G proteins.^{34–35}

In contrast with the somewhat uncertain role of C3 and the more proximal complement components, such as C1q, SAP or C4, in promoting or protecting from lupus-like autoimmune disease, it is well accepted that activation of C5 and the insertion of MAC in the cell membrane results in a profound proinflammatory state without the likelihood of changes in self tolerance.³⁶ For this reason, therapeutic strategies that target C5

and the more distal complement components but leave C3 and the more proximal components unaffected are now considered an especially promising approach to complement inhibition.

ROLE OF COMPLEMENT IN FETAL TOLERANCE

As fetal tissues are semi-allogeneic and alloantibodies commonly develop in the mother, the placenta is potentially subject to complement mediated immune attack at the maternal-fetal interface.³⁷ Though activated complement components are present in normal placentas,^{38–39} in successful pregnancy it seems that uncontrolled complement activation is prevented by three regulatory proteins present on the trophoblast membrane.^{40–41} Several studies have shown that the level of activated complement components present at a given site depends on the relative effects of complement activators and its inhibitors: DAF, MCP, and CD59.⁴² All three proteins are expressed on the trophoblast in contact with maternal blood and tissues, providing a mechanism to protect the fetus from damage due to complement pathway activation by alloantibodies. Indeed, on the basis of their characteristic distribution patterns, it is likely that these proteins are strategically positioned for this purpose.

Recent murine studies have underscored the importance of complement regulation in fetal control of maternal processes that mediate tissue damage. In mice, Crry is a membrane bound intrinsic complement regulatory protein whose role, like that of DAF and MCP, is to block C3 and C4 activation on self membranes.⁴³ As such, Crry acts as an inhibitor of classic and alternative pathway C3 convertases and blocks C3, C5, and subsequent MAC activation. That appropriate complement inhibition is an absolute requirement for normal pregnancy has been demonstrated by the finding that Crry deficiency in utero leads to progressive embryonic lethality.⁴⁴ Crry^{-/-} embryos are surrounded by activated C3 fragments and PMN. Importantly, Crry^{-/-} embryos are completely rescued from this 100% lethality and live pups are born at a normal Mendelian frequency when Crry^{+/-} parents are intercrossed with C3^{-/-} mice to generate C3^{-/-}, Crry^{-/-} embryos.⁴⁴ This outcome is genetic proof that Crry^{-/-} embryos die in utero because of their inability to suppress complement activation and tissue damage mediated by C3. These findings underscore the need for a systematic evaluation of our hypothesis: that appropriate complement regulation is necessary to control

Figure 2 Inhibition of C3 convertase ameliorates aPL-IgG induced pregnancy complications. Female BALB/c mice were treated ip 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 of the mice received an inhibitor of C3 convertase, Crry-Ig (3 mg ip) 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 + 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 v aPL + Crry-Ig $p < 0.05$). (B) aPL-IgG caused fetal growth retardation (*aPL v Cntrl IgG $p < 0.01$), which was also prevented by Crry-Ig (*aPL v aPL + Crry-Ig $p < 0.01$). Reproduced from the *J Exp Med* 2001;195:214 by copyright permission of The Rockefeller Press.

maternal alloreactivity and placental inflammation in humans, and that a local increase in complement activation fragments is highly deleterious to the developing fetus.

COMPLEMENT C3 ACTIVATION IS REQUIRED FOR APL ANTIBODY INDUCED FETAL LOSS AND THROMBOSIS

To test the hypothesis that aPL antibodies activate complement in the placenta, leading to fetal injury, 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 Crry-Ig that blocks activation of C3 by both the classic and alternative pathways, prevents fetal loss and growth restriction (fig 2). Furthermore, mice deficient in complement C3 were resistant to fetal damage induced by aPL antibodies (fig 3). While antigenic epitopes recognised by aPL antibodies are important in the pathogenesis of APS, our data show that in vivo complement activation is required for aPL induced injury. That results from experiments with monoclonal human aPL antibodies were similar to results obtained with polyclonal aPL antibodies 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 discover if excessive complement activation occurs within the placenta in aPL treated mice, we conducted immunohistological analyses of decidua on day 8 of pregnancy, after the first treatment 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. In aPL treated mice, the decidua was abnormal morphologically, showing focal necrosis, apoptosis, and PMN infiltrates. In addition, there was extensive C3 deposition. Human IgG deposition and C3 activation were evident within 30 minutes after aPL treatment. In contrast, in mice treated with control human IgG only small amounts of C3 staining were detectable, mostly in the extraembryonic tissues; the decidua was not inflamed and had normal cellular elements. Treatment with Crry-Ig at the time of administration of aPL-IgG completely prevented inflammation and C3 deposition. Thus, using three distinct approaches, specific complement inhibitor (Crry-Ig), genetically deficient mice (C3^{-/-}), and immunohistochemical evidence that absence of C3 deposition in Crry-Ig treated mice

Figure 3 C3 deficient mice are protected from aPL antibody induced pregnancy complications. C3^{+/+} mice (B6/Sv129F1) were treated with aPL-IgG (10 mg ip) (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 ip) every other day from days 8–12 and half received control murine IgG (mlgG). C3^{-/-} mice were treated with either aPL-IgG or normal human IgG. Pregnancy outcomes were assessed as described in the legend for fig 2. 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 + mlgG v Cntrl IgG + mlgG $p < 0.01$), while C3^{-/-} were protected from aPL induced pregnancy loss (aPL v Cntrl IgG $p = NS$). In the C3^{+/+} mice, Crry-Ig prevented aPL induced fetal resorption (*aPL + mlgG v aPL + Crry-Ig $p < 0.01$). (B) Similarly, aPL treatment caused a decrease in fetal weight in C3^{+/+} mice (*aPL + mlgG v Cntrl IgG + mlgG $p < 0.01$), which was absent in C3^{-/-} mice, and this was ameliorated by Crry-Ig (*aPL + mlgG v aPL + Crry-Ig $p < 0.01$). Reproduced from the *J Exp Med* 2001;195:216 by copyright permission of The Rockefeller Press.

Figure 4 C3 activation is required for aPL induced thrombophilia. (A) aPL-IgG induced thrombophilia is inhibited by Crry-Ig. CD-1 mice were injected ip with affinity purified aPL-IgG (aPL) or normal human (Cntrl IgG) at 0 hours and 48 hours. Half the mice in each group received Crry-Ig, and half the mice received control murine IgG (mlgG). At 72 hours after the first injection, surgically induced thrombus formation was measured as described in the text. There were 11–14 mice in each experimental group. Treatment with aPL-IgG caused an increase in thrombus size (*aPL + mlgG v Cntrl IgG + mlgG $p < 0.05$), while Crry-Ig prevented aPL induced enhancement of thrombosis (*aPL + mlgG v aPL + Crry-Ig $p < 0.05$; Cntrl IgG + Crry-Ig v aPL + Crry-Ig, $p = \text{NS}$). In a separate series of experiments, aPL did not significantly increase thrombosis in C3 $^{-/-}$ mice (aPL v control IgG 1524 (825) μm v 1083 (443), $p = \text{NS}$). There was no difference in the levels of human aPL activity between C3 $^{+/-}$ mice and C3 $^{-/-}$ mice. Reproduced from the *J Exp Med* 2001;195:217 by copyright permission of The Rockefeller Press.

correlates with improved outcomes, we demonstrated that complement activation is required for fetal loss and growth restriction in this murine model of APS.

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. Using an in vivo microcirculation model in which aPL antibodies had been shown to enhance and accelerate thrombus formation in the presence of a vascular injury, we demonstrated that Crry-Ig prevents thrombophilia and that C3 $^{-/-}$ mice were resistant to aPL induced effects (fig 4).

Although it is not clear from our results which complement components or receptors are primarily responsible for fetal loss mediated by aPL antibodies, probable 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.^{45–46} Blockade of C5 may be as effective as blockade of C3, perhaps because the anaphylatoxin C3a is unable to recruit neutrophils (PMN) whereas C5a attracts PMN to inflammatory sites and activates them.⁴⁷ Of note, histological evidence suggests that PMN are important effectors in our model of fetal loss. In addition, inhibition of the complement cascade focused at the level of C5 is attractive from a clinical standpoint because it blocks downstream effectors while preserving important complement derived immunoprotective functions, such as opsonisation and immune complex clearance.

In summary, our findings show that complement activation is a central mechanism contributing to aPL antibody induced thrombophilia, pregnancy loss, and fetal growth restriction. Although the cause of tissue injury in this disease is probably multifactorial, 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 important effector mechanisms.

CONCLUSIONS

Based on our findings, we proposed the following mechanism for the pathogenic effects of aPL antibodies (fig 5). Firstly, aPL antibodies are preferentially targeted to the placenta. Secondly, placental aPL antibodies promote platelet and endothelial cell activation and directly induce procoagulant activity through interaction with elements of the coagulation pathway.^{21 23 48–51} This activity, however, does not seem to be sufficient to cause fetal loss or growth restriction as C3 $^{-/-}$ mice are protected. Activation of the complement pathway amplifies these effects by the generation of further potent mediators of effector cell activation, including C3a, C5a, and C5–9 MAC, causing thrombosis, tissue hypoxia, and inflammation within the placenta, and ultimately leading to fetal injury. Depending on the extent of damage, either death in utero or fetal growth restriction ensues.

Figure 5 Proposed mechanism for the pathogenic effects of aPL antibodies on pregnancy outcome. aPL antibodies are preferentially targeted to the placenta where they may promote platelet and endothelial cell activation and directly induce procoagulant activity through interaction with elements of the coagulation pathway. This activity, however, does not seem to be sufficient to cause fetal loss or growth restriction; C3 $^{-/-}$ are protected. Activation of the complement pathway by aPL-IgG overwhelms the normally adequate inhibitory mechanisms and amplifies these effects by stimulating the generation of further potent mediators of effector 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.

Identification of complement activation as a mechanism that is necessary for aPL induced tissue damage and definition of the complement components necessary to trigger such injury is likely to lead to a better understanding of the pathogenesis of vascular and tissue injury in SLE and to new and improved treatments. Our data generated in the murine model of APS, the availability of newer and more accurate tests of complement activation, and the recent development of effective and specific complement inhibitors argue persuasively that the role of complement in aPL associated pregnancy complications should be further examined. Indeed, our studies show that complement is a new target for treatment in patients with thrombotic and vascular complications of aPL syndrome.

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