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Negligible Role of Antibodies and C5 in Pregnancy Loss Associated Exclusively with C3-Dependent Mechanisms through Complement Alternative Pathway

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Summary

Factors involved in pregnancy failure due to abnormal fetomaternal tolerance are poorly understood. Here we describe distinct defects in placenta formation and subsequent pregnancy loss solely dependent on the activation of the complement alternative pathway and the effector mechanisms provided by the maternal C3. Surprisingly, this effect is independent of other complement activation pathways and of the effector mechanisms provided by other complement components. These findings provide significant insight into the role of the innate immune system in human pregnancy failure, a frequent clinical outcome.

Introduction

The mammalian embryo has been compared to a tissue transplant received from a genetically different individual (Medawar, 1953). The reasons why the maternal immune system does not view the paternal component of the embryo as foreign and rejects it has perplexed immunologists for more than fifty years. Fetal survival during pregnancy depends on the ability of embryonic tissue to avoid rejection by the maternal immune system (Billington, 1992). The maintenance of this fetomaternal tolerance is still poorly understood. Recent experimental observations suggest that inappropriate complement activation during pregnancy impairs this type of immunoregulation (Rooney et al., 1993; Holmes and Simpson, 1992; Holers et al., 2002; Xu et al., 2000).

The complement system consists of a series of serum proteins involved in the regulation of the immune response and the inflammatory process by generating fragments that promote chemotaxis, phagocytosis, cell activation, and cell lysis (Schmidt and Colten, 2000; Abbas et al., 2000). This complex system provides one of the early defense mechanisms against pathogens. The activation of complement, however, is not inconsequential for the host. Activated complement fragments have the capacity to bind to tissues, trigger inflammation, and cause injury. Autologous bystander cells are protected from the deleterious effects of complement by expressing proteins that regulate its activation (Liszewski et al., 1996; Hourcade et al., 1989; Lublin and Atkinson, 1989). Membrane cofactor protein (MCP), decay accelerating factor (DAF), and their mouse functional homolog, Crry, are ubiquitous membrane-bound proteins that regulate the activation of the third component of complement, C3 (Holers et al., 1992; Molina et al., 1992; Kim et al., 1995).

The need for these complement inhibitory proteins to control complement-mediated tissue damage is evident during pregnancy. The placenta expresses, throughout gestation, high levels of complement regulators (Holmes and Simpson, 1992). In addition, up to 20% of first trimester pregnancy losses can be characterized by the onset of hypocomplementemia and, in some conditions, with complement deposition in the placenta (Cunningham and Tichenor, 1995). In some cases this hypocomplementemia is associated with reduced DAF expression on the placenta and increased complement consumption at the fetomaternal interface. Complement activation is also required in a mouse model of recurrent fetal loss associated with anti-phospholipid antibodies, a condition characterized by increased miscarriages and spontaneous abortion (Holers et al., 2002). Furthermore, deficiency in the mouse complement regulator Crry leads to embryonic death (Xu et al., 2000). *Crry*$^{-/-}$ embryos suffer from increased C3 deposition and concomitant inflammation within the developing placenta. C3 involvement in the fetal death has been demonstrated by the complete rescue of this abnormal phenotype in *C3*$^{-/-}$ mice (Xu et al., 2000).

Several mechanisms may be involved in complement-dependent pregnancy loss (Frank and Fries, 1991; Wetsel, 1995; Gerard and Gerard, 1994; Carroll, 1998; Rus et al., 2001). Defining the relative contribution of these mechanisms to fetal loss will facilitate the understanding of the innate immune response during pregnancy and provide potential therapeutic options in the treatment of recurrent miscarriages. To this purpose, we have used the *Crry*$^{-/-}$ mouse model to further investigate the role of complement and complement regulators in recurrent fetal loss. Here, we identify the pathways related to the activation of complement in the *Crry*$^{-/-}$ embryos, the complement components needed to cause the fetal demise, and the pathological consequences of this activation. Surprisingly, activation of the maternal third component of complement through the alternative pathway, and the resulting neutrophil inflammation, is enough to mediate defects in placenta formation and subsequent pregnancy loss. This effect is independent of other complement activation pathways and complement components, or the presence of B cells and antibodies. Thus, abnormal fetomaternal tolerance may be established using exclusively C3 as both the inductive and effector element without the contribution of downstream complement components or mobilization of antibody-mediated immune response components.

Results

Inconsequential Effect of the Classical Pathway

Complement activation occurs through three pathways (Volanakis, 1998). The classical pathway is initiated by

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Table 1. Genotype Analysis of 3-Week-Old Pups Derived from $Crry^{+/-}$ Matings Using Mice with Deficiencies in Specific Complement Components or Antibody-Deficient Mice

	$Crry^{+/+}$	$Crry^{+/-}$	$Crry^{-/-}$	Total
Expected	25%	50%	25%	
Ratio	[1]	[2]	[1]	
$Crry^{+/-}$ mating	150 (47%) [1]	168 (53%) [1.1]*	0 (0%)	318
$C4^{-/-}Crry^{+/-}$ mating	16 (29%)	39 (71%)	0 (0%)	55
$\mu MTCrry^{+/-}$ mating	22 (46%)	26 (54%)	0 (0%)	48
$fB^{-/-}Crry^{+/-}$ mating	12 (24%)	24 (49%)	13 (27%)	49
$C5^{-/-}Crry^{+/-}$ mating	22 (35%)	37 (60%)	3 (5%)	62

Numbers in brackets represent genotype ratios of embryos.
*$p < 0.0001$ as calculated by the χ^2 goodness of fit test.

antigen-antibody complexes and the sequential activation of C1, C4, and C2. Fragments derived from the activation of C4 and C2 form an enzyme known as the classical pathway C3 convertase responsible for the activation of the third component of protein, C3. The lectin pathway is initiated by plasma proteins, known as mannan binding lectins, which attach to carbohydrate moieties on the surface of pathogens. Once bound, these lectins interact with mannan binding lectin-associated serine esterases and induce the activation of C4, C3, and C2. The alternative pathway is initiated directly by the slow and spontaneous deposition of C3 on the target surface, allowing binding and activation of factor B. In the absence of complement regulation, the activated C3/factor B bimolecular complex forms an enzyme directly responsible for further amplification of C3 activation and the formation of enzymes involved in C5 activation.

Of the three pathways for complement activation, we considered the classical pathway as the most likely pathogenic mechanism causing tissue damage to the $Crry^{-/-}$ embryos since this route to complement acitvation is the primary effector mechanism in hyperacute and acute vascular rejection of transplanted tissue and for several hypersensitivity reactions (Geha et al., 2001). Since the Crry mutation was initially present in a mixed genetic background, maternal allospecific antibodies reacting against fetal alloantigens could induce the activation of this pathway. To test this possibility, we initially examined the effect of the Crry mutation in B cell-deficient mice (μMT) (Kitamura et al., 1991). The μMT mouse strain was generated by creating a null mutation in the transmembrane exon of the immunoglobulin M (IgM) heavy chain resulting in the absence of serum antibodies. Compound $\mu MT^{-/-}Crry^{+/-}$ mutant animals were mated. Surprisingly, no $\mu MT^{-/-}Crry^{-/-}$ pups were generated (Table 1). This observation ruled out an important contribution of the classical pathway in this phenotype and provided evidence for a dispensable role of antibodies and Fc receptors, not only as primary precipitating events, but also as effector mechanisms in the $Crry^{-/-}$ embryonic lethality.

To further define the contribution of the classical pathway in the $Crry^{-/-}$ phenotype, we examined the effect of the Crry mutation in mice deficient in C4 (Fischer et al., 1996). We generated compound mutant mice that were $Crry^{+/-}$ and $C4^{-/-}$, and subsequently crossed them to generate mutants that were C4 deficient and either Crry sufficient or Crry deficient. No $C4^{-/-}Crry^{-/-}$ mice could be recovered from a total of 55 births, indicating that the classical pathway is nonessential for the fetal rejection process affecting the Crry-deficient mice (Table 1). In agreement with this observation, 7.5 day postcoitus (dpc) $C4^{-/-}Crry^{-/-}$ embryos exhibited increased C3 deposition similar to $Crry^{-/-}$ embryos (Figures 1B and 1C). Minimal C3 deposition was noted in Crry-sufficient embryos (Figure 1A). Associated with the increased C3 deposition was also increased tissue inflammation in $Crry^{-/-}$ and $C4^{-/-}Crry^{-/-}$ embryos as determined by Gr-1 staining, a marker for neutrophils (Figures 2B and 2C).

Effect of the Alternative Pathway

The alternative pathway promotes complement activation mainly on microbial surfaces and serves as an amplification system for the classical and lectin pathway (Jelezarova et al., 2000). It is unique in that no specific molecular recognition motifs on the target are required for its activation. Due to the unexpected inconsequential role of the classical pathway in the $Crry^{-/-}$ phenotype, we tested the hypothesis that the alternative pathway provided the main mechanism of complement activation and fetal rejection. We bred the mice with the Crry mutation to factor B-deficient mice ($fB^{-/-}$) (Matsumoto et al., 1997). Genotype analysis revealed that 27% (13/49) of the resulting 3-week-old pups were $fB^{-/-}Crry^{-/-}$. In agreement with this observation, 7.5 dpc $fB^{-/-}Crry^{-/-}$ embryos exhibited no increased C3 deposition (Figure 1D) and no neutrophil inflammation as determined by Gr-1 staining (Figure 2D). These results indicate that complement components derived from the activation of the alternative pathway mediate the impaired fetomaternal tolerance related with the Crry deficiency.

Inconsequential Role of C5 Activation

The genetic analysis presented above excluded the role of classical pathway components C1, C2, and C4 in the effector phase of tissue damage. Another mechanism of complement-mediated tissue damage involves the activation of the fifth component of complement, C5 (Abbas et al., 2000). Fragments derived from the activation of C5 serve as potent chemoattractants and bind to target surfaces, initiating the assembly of the membrane attack complex responsible for inducing abnormalities in cell membrane. To determine the role of C5 in the death of Crry-deficient embryos, we examined the effect of the Crry mutation in a C5-deficient background. Sur-

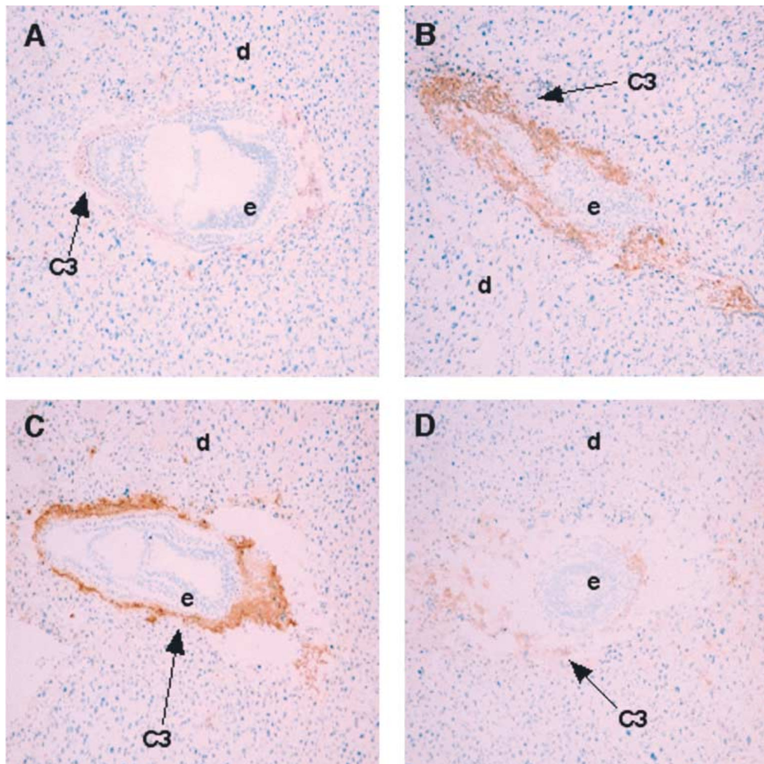

Figure 1. C3 Deposition in 7.5 dpc Fetal Tissues

Tissue sections derived from decidua of wild-type (A), *Crry*$^{-/-}$ (B), *C4*$^{-/-}$*Crry*$^{-/-}$ (C), and *fB*$^{-/-}$*Crry*$^{-/-}$ (D) embryos were stained with an antibody against mouse C3. Extensive C3 deposition (arrows) is noted in the trophoectoderm area of *Crry*$^{-/-}$ and *C4*$^{-/-}$*Crry*$^{-/-}$ fetuses. The embryo proper is spared. d, maternal decidua; e, embryo.

prisingly, lack of C5 failed to rescue the *Crry*$^{-/-}$ embryos from embryonic death, indicating an expendable role for C5 activation in the *Crry*$^{-/-}$ embryonic lethality (Table 1). Taken together, the above observations indicate that the indispensable effector complement component involved in the *Crry*$^{-/-}$ embryonic lethality was C3 itself.

Effect of Maternal C3

Either the fetal or maternal environment could contribute C3 with the capacity to cause the embryonic death. To investigate the role of fetal C3 in this phenotype, we initially examined expression of C3 in normal embryonic tissues. Northern analysis demonstrated the absence of

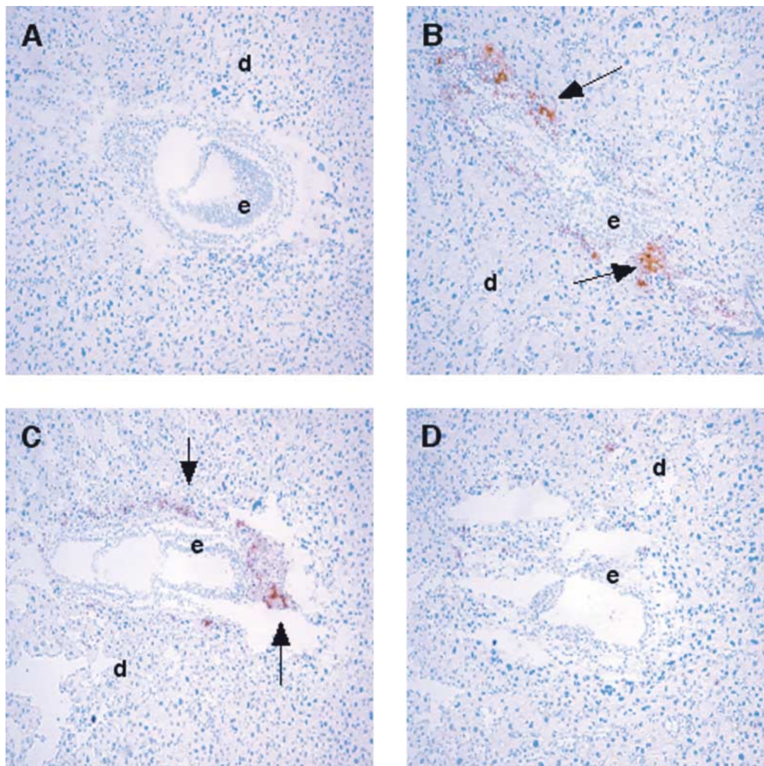

Figure 2. Neutrophil Tissue Infiltration in 7.5 dpc Fetal Tissues

Tissue sections derived from decidua of wild-type (A), *Crry*$^{-/-}$ (B), *C4*$^{-/-}$*Crry*$^{-/-}$ (C), and *fB*$^{-/-}$*Crry*$^{-/-}$ (D) were stained with an antibody against Gr-1, a molecule that serves as a marker for neutrophils. Gr-1 staining is localized in the trophoectoderm area of *Crry*$^{-/-}$ and *C4*$^{-/-}$*Crry*$^{-/-}$ fetuses, but not wild-type or *fB*$^{-/-}$*Crry*$^{-/-}$ deficient mice. d, maternal decidua; e, embryo.

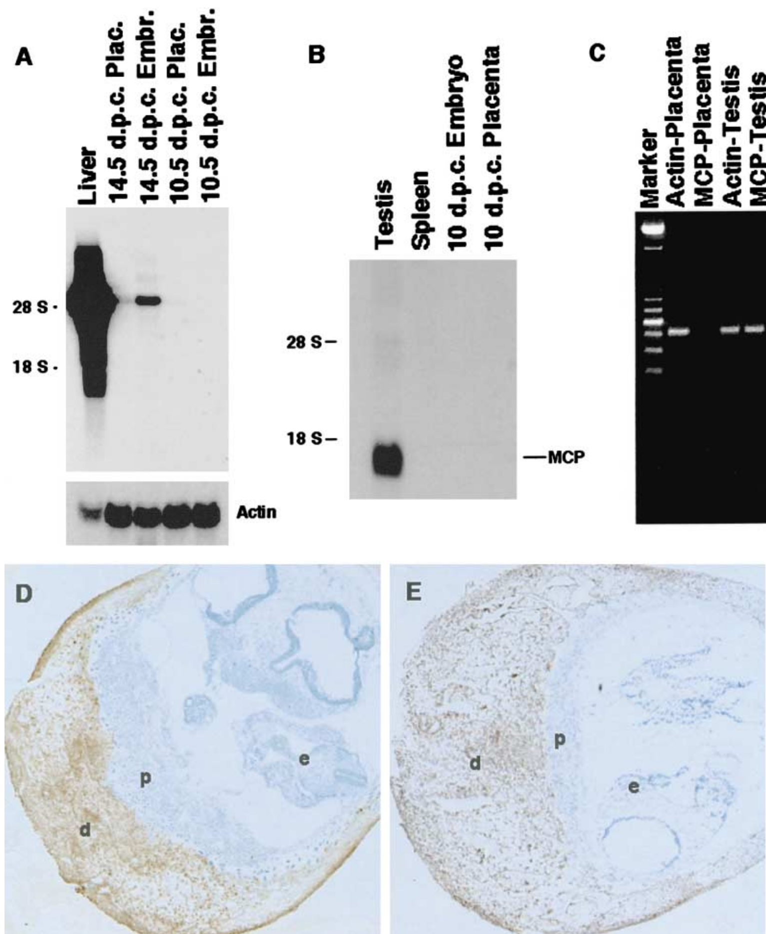

Figure 3. C3 and C3 Regulator Expression in Fetal Tissues

(A) C3 expression in 10.5 and 14.5 dpc placenta and embryo proper as determined by Northern blot analysis. RNA extracted from liver was used as positive control. Actin is used as a control for relative amounts of RNA.

(B and C) Northern blot analysis (B) and RT-PCR analysis (C) for mouse MCP expression in 10.5 dpc embryonic tissue.

(D) Staining of 10.5 dpc wild-type embryos with anti-mouse DAF antibodies.

(E) Staining of 10.5 dpc $Crry^{-/-}$ embryos with anti-mouse DAF antibodies. d, maternal decidua; e, embryo proper; p, placenta.

C3 expression by the placenta and the fetus on 10.5 dpc embryos (Figure 3). Interestingly, at 14.5 dpc C3 mRNA was detected in the embryo but not in the placenta, yet the relative amount of C3 mRNA was lower than in liver of wild-type adult mice. Importantly, this gestational age in our model is beyond the time of fetal rejection (Xu et al., 2000). Thus, the relative absence of detectable fetal C3 mRNA at the time of fetal demise suggested that fetal C3 was not involved in the $Crry^{-/-}$ phenotype.

To examine whether maternal C3 was primarily responsible for the $Crry^{-/-}$ phenotype, we initiated studies in which the main experimental variable was the presence or absence of maternal C3. We bred $C3^{+/+}Crry^{+/-}$ mice to $C3^{-/-}Crry^{-/-}$ mice. According to Mendelian genetics, the expected genotypes of pups derived from this mating combination should be $C3^{+/-}Crry^{+/-}$ (50%) and $C3^{+/-}Crry^{-/-}$ (50%) (Table 2). Mating $C3^{+/+}Crry^{+/-}$ males to $C3^{-/-}Crry^{-/-}$ females did not result in significant fetal rejection. Mating $C3^{-/-}Crry^{-/-}$ males to $C3^{+/+}Crry^{+/-}$ females, however, resulted in only 8% of the pups (4 out of 51 pups) with a $C3^{+/-}Crry^{-/-}$ genotype ($p < 0.001$). Thus, these results indicate that maternal C3 is responsible for the $Crry^{-/-}$ embryonic death.

Histological analysis revealed that maximal C3 deposition and inflammation occurred at 7.5 dpc (Xu et al., 2000). Increased C3 deposition and inflammation was also noted at 8.5 dpc (data not shown). Surprisingly, at 9.5 and 10.5 dpc similar C3 deposition was noted in wild-type and Crry-deficient extraembryonic tissues (Figures 4A and 4B), and inflammation was absent in Crry-deficient embryos at these gestational ages (Figures 4F–4H, 5B, and 5D).

As noted above, the maximal deposition of C3 at 7.5 dpc in $Crry^{-/-}$ mice preceded by 3 days the embryonic loss evident at 10.5 dpc, suggesting that C3 activation did not immediately cause fetal death. This delay in the embryonic loss could be explained by the expression of

Table 2. Role of Maternal C3 in the Lethality Found in $Crry^{-/-}$ Embryos

	$C3^{+/-}Crry^{+/-}$	$C3^{+/-}Crry^{-/-}$	Total
$C3^{-/-}Crry^{-/-} \times C3^{+/+}Crry^{+/-}$ (expected)	50%	50%	
$C3^{+/+}Crry^{+/-}$ (male) $\times$ $C3^{-/-}Crry^{-/-}$ (female)	30 (60%)	20 (40%)	50
*$C3^{-/-}Crry^{-/-}$ (male) $\times$ $C3^{+/+}Crry^{+/-}$ (female)	47 (92%)	4 (8%)	51

* $p < 0.0001$.

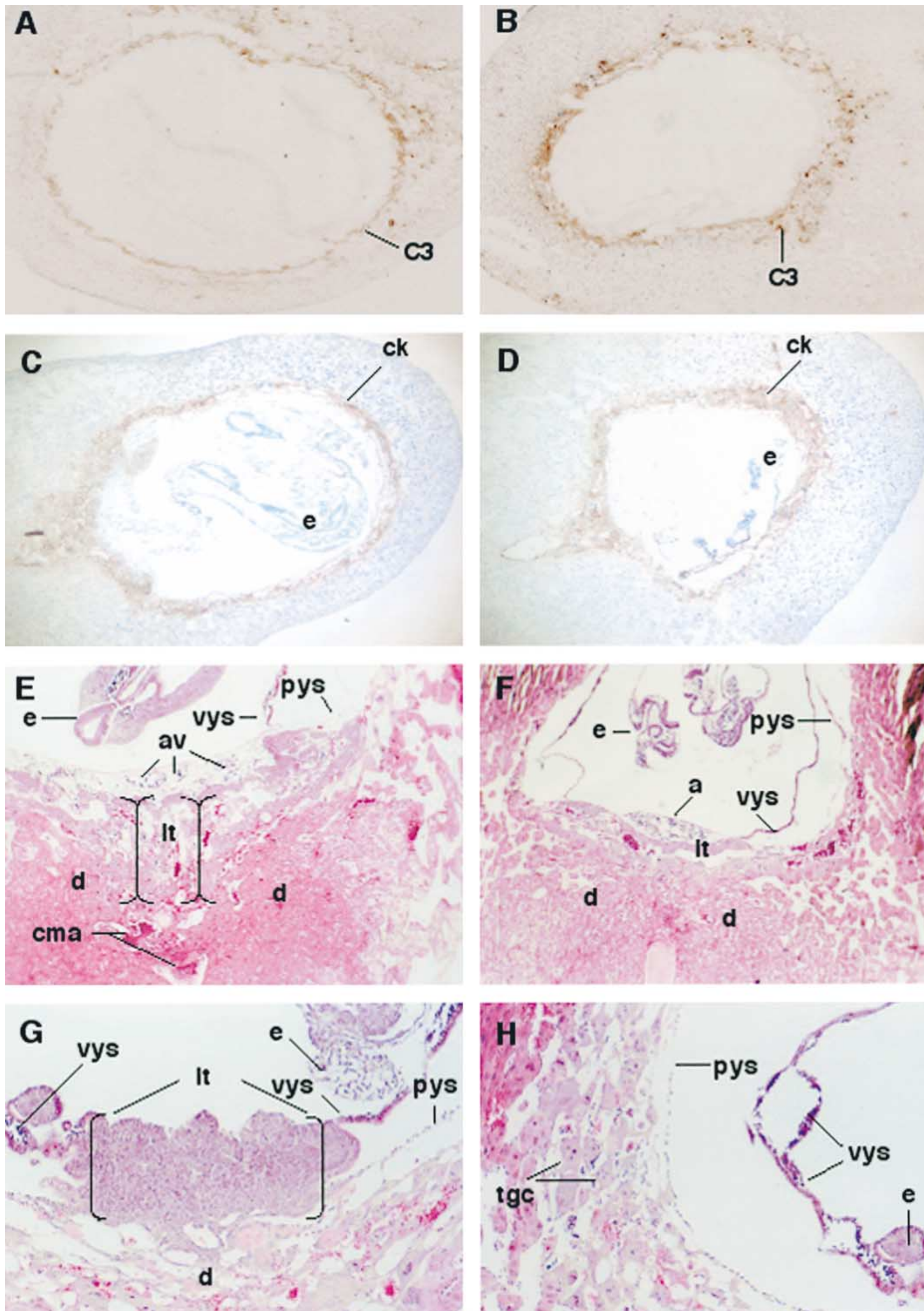

Figure 4. Abnormal Placenta Development Induced by C3 Deposition in *Crry*$^{-/-}$ Fetal Tissue

(A) C3 deposition in 9.5 dpc *Crry*$^{+/+}$ mice. 45×, magnification.

(B) C3 deposition in 9.5 dpc *Crry*$^{-/-}$ mice. 45×, magnification.

(C and D) Anti-cytokeratin staining of (C) *Crry*$^{+/+}$ and (D) *Crry*$^{-/-}$ embryos. 25×, magnification.

(E) Hematoxylin and eosin staining of 9.5 dpc *Crry*$^{+/+}$ placenta. 100×, magnification. Note the discrete area of labyrinthine trophoblast cells in the placenta (lt), adjacent to the allantoic mesenchyme, that contains developing fetal blood vessels (av).

(F) Hematoxylin and eosin staining of 9.5 dpc *Crry*$^{-/-}$ placenta. 100×, magnification.

(G and H) Hematoxylin and eosin staining of 9.5 dpc *Crry*$^{-/-}$ placenta. 200×, magnification. a, allantoic mesenchyme; av, allantoic vessels; cma, central maternal artery; d, maternal decidua; e, embryo proper; lt, labyrinthine trophoblast cells; pys, parietal yolk sac; tgc, trophoblast giant cells; vys, visceral yolk sac.

additional complement regulatory molecules by mouse trophoblats. In previous work we described the absence of DAF expression in murine 7.5 dpc wild-type trophoblasts (Xu et al., 2000). However, it is known that trophoblast DAF expression increases with advancing human gestation (Hsi et al., 1991; Holmes et al., 1992). Upregulation of this protein in response to complement attack has also been observed in vitro (Shibata et al., 1991). In addition, MCP and DAF are present in human trophoblasts (Hsi et al., 1991; Holmes et al., 1992). Expression of MCP or DAF with advancing gestation, possibly induced by complement deposition, is a plausible regulator in our model. To rule out this possibility, we examined for the presence of mouse MCP and DAF in the placenta. By Northern blot and RT-PCR analysis, we did not detect expression of MCP mRNA in 10.5 dpc wild-type embryos and placentas (Figures 3B and 3C). As expected, MCP mRNA was detected in mouse testis (Tsujimura et al., 1998). In addition, although maternally derived decidua does express DAF, there is no DAF expression in 10.5 dpc placenta and embryos of wild-type or *Crry*$^{-/-}$ fetuses as determined by immunohistochemistry (Figures 3D and 3E). Thus, the time difference between maximal complement activation at 7.5 dpc and embryonic loss at 10.5 dpc is not related to the presence of additional membrane-bound complement regulators in either the fetus or placenta.

Defects in Placental Development

In agreement with the selective deposition of complement and the inflammation noted on 7.5 dpc ectoplacental tissue, the main pathological changes were seen in the *Crry*$^{-/-}$ placentas. The visceral yolk sac placenta is the key source for fetal nutrients in the mouse until day 9.5 when a transition to the labyrinthine placenta provides the primary location for maternal-fetal exchange (Vince and Johnson, 1995). We therefore focused on the histology of the mouse labyrinth during this gestational period, comparing *Crry*$^{+/+}$ with *Crry*$^{-/-}$ mice. The presence of trophoblast in both *Crry*$^{-/-}$ and *Crry*$^{+/+}$ placentas was confirmed by the characteristic cell morphology (Kaufman, 1998) and the positive cytokeratin staining (Figures 4C and 4D), a marker for this cell population (Adamson et al., 2002). The labyrinthine of *Crry*$^{+/+}$ mice at 9.5 dpc had a discrete area of trophoblasts adjacent to the allantoic mesenchyme that contained developing fetal blood vessels (Figure 4E). Fetal vessels originating from the *Crry*$^{+/+}$ allantoic mesenchyme penetrated the connective tissue of the labyrinthine columns that were themselves covered with the trilayer of trophoblast bathed in maternal blood derived from the decidual central artery. Thus, dual perfusion of the labyrinthine placenta in the *Crry*$^{+/+}$ mice was clearly apparent by 9.5 dpc.

The placental development at 9.5 dpc in the *Crry*$^{-/-}$ mice was strikingly different from wild-type mice. There was a marked deficiency of vessels in the *Crry*$^{-/-}$ allantoic mesenchyme at 9.5 dpc, yet an aggregate of labyrinthine trophoblast cells was present at this stage (Figures 4F and 4G). Importantly, there were no detectable allantoic vessels penetrating the labyrinthine trophoblast to yield the usual fetal vascular spaces at either 9.5 dpc (Figures 4F and 4G) or 10.5 dpc (Figure 5). The spongiotrophoblast area, the central artery of the maternal decidua, and the trophoblastic giant cells at the periphery of the labyrinthine plate (Figure 4H) were present without differences between *Crry*$^{-/-}$ and *Crry*$^{+/+}$ placentas. There were additionally no observable differences in the histology of the amnion, the visceral yolk sac placenta, or the parietal yolk sac between *Crry*$^{-/-}$ and *Crry*$^{+/+}$ mice (Figures 4F and 4H). Thus, histological findings at 9.5 and 10.5 dpc demonstrated a failure in the development of the allantoic vasculature in the component of the placenta.

Discussion

Recurrent pregnancy loss affects 1% to 3% of all couples, and about half of these cases have no identifiable cause (Hill, 1992). Importantly, recent studies associate some of these pregnancy complications with an abnormal maternal immune response (Billington, 1992; Vince and Johnson, 1995; McIntyre, 1992). The fetus expresses many paternally derived cell surface molecules foreign to the immune system of the mother. Fetal survival during pregnancy greatly depends on the ability of fetal tissue to avoid recognition and rejection by the maternal immune system. Thus, abnormalities in the regulation of the maternal immune response against the fetus are potentially an identifiable cause for pregnancy failure (Billington, 1992).

Accumulating data suggest a role for the complement system as a causative and perpetuating element in recurrent fetal loss (Rooney et al., 1993; Holmes and Simpson, 1992; Holers et al., 2002; Xu et al., 2000). Nevertheless, these studies have not identified the relative contribution of the activation and effector mechanisms that may be involved in this complement-dependent pregnancy loss. Formation of pathogenic antibodies with activation of the classical pathway may have a role (Geha et al., 2001), but this mechanism fails to characterize the majority of cases with unexplained multiple spontaneous miscarriages (Hill, 1992; Vince and Johnson, 1995). Other possibilities include increased complement activation through lectin-mediated recognition of placental carbohydrates or hydrolytic activation of C3 during tickover of the alternative pathway (Pangburn and Muller-Eberhard, 1980). Despite these theoretical mechanisms, the initial trigger for increased activation of the alternative or lectin pathways in complicated pregnancies, compared to normal pregnancies, remains elusive.

The *Crry*$^{-/-}$ mouse is an excellent model for examining the role of complement in pregnancy failure. This model has elucidated a key role of fetal complement regulation in the control of maternal-mediated tissue damage. The analysis presented in this paper indicates that primary genetic abnormalities in the placental expression of complement regulators act as an initial triggering factor in fetal loss by causing increased complement activation through the alternative pathway. Surprisingly, experimental observations derived from the use of B cell-deficient and C4-deficient mice indicate that pathogenic antibodies activating the classical pathway are not precipitating stimuli. In the case of the lectin pathway, in vitro analyses have demonstrated that activation of C3

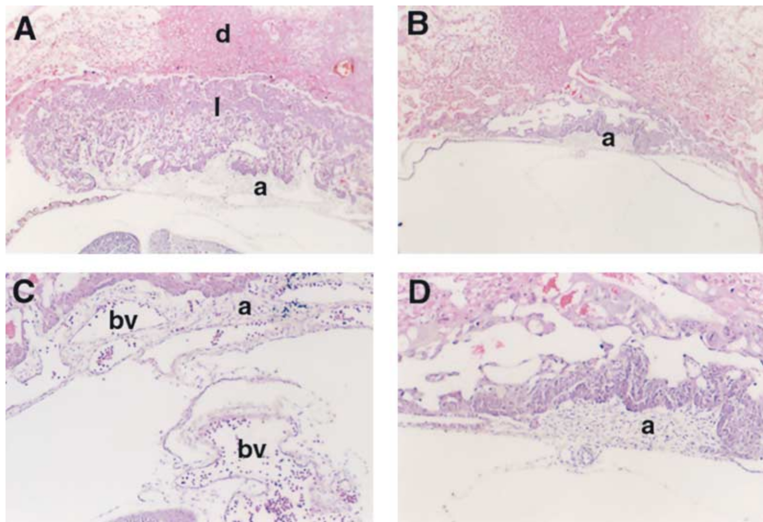

Figure 5. Abnormal Vascular Development of the Placenta Induced by C3 in *Crry*$^{-/-}$ Fetal Tissue

Crry$^{+/+}$ (left panel) and *Crry*$^{-/-}$ (right panel) embryos.

(A and B) Decidua dissection to expose the 10.5 dpc placenta. 100×, magnification.

(C and D) 10.5 dpc placenta. 200×, magnification. Note the lack of detectable allantoic vessels penetrating the labyrinthine trophoblast to yield the usual fetal vascular spaces. a, allantois; d, maternal decidua; bv, blood vessel; e, embryo proper; l, vascular labyrinthine placenta.

through this pathway may occur in the absence of C4 (Matsushita et al., 2000). There is no conclusive evidence, however, that mammalian mannose binding proteins have the ability to recognize self-glycoproteins and spontaneously initiate this pathway in vivo in the absence of pathogens (Hoffmann et al., 1999). Consequently, in this experimental model of abnormal fetomaternal tolerance, the alternative pathway of complement activation is a primary contributor to fetal loss and not just an amplification system surrogated to the classical and lectin pathways (Jelezarova et al., 2000). Complement components derived from the activation of the maternal alternative pathway provide sole factors that cause tissue destruction.

The above outcome provides insight into the complement components needed to effect the fetal demise. On the basis of the above observations we conclude that the indispensable effector complement component involved in the *Crry*$^{-/-}$ embryonic lethality is C3 itself. The experimental observations derived from the genetic analysis exclude the role of classical pathway components C1, C2, and C4 in the effector phase of tissue damage. Although complement-mediated tissue damage can drive from the activation of the fifth component of complement, C5, lack of C5 did not rescue the *Crry*$^{-/-}$ embryos from embryonic death (Table 1) (Gerard and Gerard, 1994; Nicholson-Weller and Halperin, 1993). This suggests an expendable role for the potent soluble inflammatory anaphylatoxic and chemotactic molecule C5a and the C5b-9 membrane attack complex.

A unique finding is the exclusive role and effectiveness of fragments derived from C3 activation in the induction of the observed pregnancy failure. C3a is a potent soluble inflammatory anaphylatoxic molecule that increases vascular permeability and promotes the recruitment of inflammatory cells. C3a also mediates endothelial cell activation and induction of adhesion molecules through its receptor (C3aR, CD88). C3b and iC3b enhance phagocytosis through interaction with complement receptor 1 and 3 on the surface of inflammatory cells.

A small amount of C3 staining was detected in 9.5 and 10.5 dpc wild-type extraembryonic tissue confirming previous observations concerning the presence of complement activation even in normal pregnancies (Holers et al., 2002) (Figure 4A). This complement deposition is devoid of an inflammatory reaction and apparently does not affect the pregnancy. The reason why C3 deposition is present in the wild-type fetus is not known. In addition, it is not known whether the comparable amount of noninflammatory C3 activation noted in the 9.5 dpc *Crry*$^{-/-}$ embryos is still harmful to the fetus, or why, in the absence of membrane-bound C3 regulators, the degree of complement deposition is not increased as compared to wild-type mice (Figure 4B).

The inflammatory reaction in this model of fetal loss is composed mainly of neutrophils. Staining with antibodies against mouse Ig, B220, CD3, Thy-1, CD11b, F4/80 has yielded negative results suggesting a paucity of B cells, T cells, and macrophages in this type of inflammation (data not shown). In addition, no mast cells have been detected as assessed by staining with 0.1% methylene blue staining.

We postulate that the C3 inflammation identified at 7.5 dpc ultimately leads to fetal demise by injuring components destined to become the allantoic vessels of the labyrinthine chorioallantoic placenta (Sherer and Abulafia, 2001; Reynolds and Redmer, 2001). The inadequate development of the allantoic vessels, apparent by 9.5 dpc in the *Crry*$^{-/-}$ mice, yields an insufficient vascular supply between the fetus and the labyrinthine placenta. This is the time when the fetus normally transitions from dependence for nutrition on the visceral yolk sac placenta at $<$9.5 dpc to the labyrinthine placenta at $>$9.5 dpc The dual hemochorial blood supply to the labyrinthine placenta, which is required for ongoing maternal-fetal exchange, is thus not available to support nutritional development of the fetus beyond the observed embryonic lethal gestation of 10.5 dpc, as the visceral yolk sac placenta becomes an inadequate nutritional source. This finding suggests that the complement system plays a key role in vascular development of the labyrinthine placenta. We do not know whether complement dysfunction might contribute to early pregnancy losses through a similar mechanism in human gestations. Surprisingly, at the 10.5 dpc time of embryonic loss, comparable C3 deposition is noted in the fetuses

of *Crry*$^{-/-}$ and wild-type mice, and no inflammation is present. Thus, unlike the allantoic vasculature, the vessels in the fetus do not show effects from the complement deposition.

Our data also imply the existence of potential therapeutic targeting areas based on complement regulation. At least in our model, complement regulation at the level of C5, or distal to C5, is not effective in controlling this phenotype. Targeting C3 directly may not be desirable due to the important contribution of this molecule to the priming of adaptive immune responses and its role in immune complex handling (Abbas et al., 2000). Alternatively, specific regulation of the alternative pathway may provide a practical, less noxious way of controlling tissue inflammation.

Another interesting feature in our model of fetal loss is the inconsequential contribution of humoral immune responses to the development of this phenotype. Antibody-mediated mechanisms of tissue damage, including the role of immunoglobulin Fc receptors, have a negligible role. We have not ruled out directly a role for cellular immune responses. It is noteworthy to point out, however, that we have bred Crry heterozygous mice six generations into the C57BL/6 background. Mating of the resultant *Crry*$^{+/-}$ mice has yielded no *Crry*$^{-/-}$ mice out of 54 pups analyzed, except in a concurrent C3-deficient background. Thus, the occurrence of *Crry*$^{-/-}$ fetal loss in syngeneic mice suggests that cellular immune responses may have a limited effect. Experiments using T cell-deficient mouse models are underway to substantiate the above observation.

Mice used in these experiments are in a F1(129svJ x C57BL/6) mixed genetic background except the *C5*$^{-/-}$ and *C4*$^{-/-}$ and the mice that are in a B10.D2-*H2*^{d} and C57BL/6 background, respectively. It can be argued that this genetic heterogeneity complicates data interpretation since one can attribute the mitigating effects of particular complement deficiencies on genetic background differences that are independent of complement itself. As pointed out above, however, the absolute embryonic lethality related to F1(129svJ x C57BL/6) Crry mice persists in a C57BL/6 mouse strain, suggesting that genetic differences provided by the mouse strains used in these experiments are not a major reason for the *Crry*$^{-/-}$ fetal loss. Moreover, this embryonic lethality can be rescued in C57BL/6 C3-deficient mice, but not in C57BL/6 C4-deficient mice, validating the inconsequential role of the classical pathway in this phenotype. In addition, the experiments analyzing the role of C3, factor B, and B cells in the Crry-related fetal loss are performed in a F1(129svJ x C57BL/6) genetic environment. The F1(129svJ x C57BL/6) mouse strain does not provide a genetic background in which recurrent increased spontaneous abortions occur, facilitating the comparison between F1(129svJ x C57BL/6) mice with different targeted genes, including *Crry*$^{-/-}$ mice, *fB*$^{-/-}$*Crry*$^{-/-}$ mice, *C3*$^{-/-}$*Crry*$^{-/-}$ mice, and μ*MT*$^{-/-}$*Crry*$^{-/-}$ mice, since the mouse strain is not a variable. Furthermore, a genetic heterogeneous background increases the significance of our studies as applied to genetically heterogeneous human populations. Further supporting evidence of the role of different complement components in the Crry-induced phenotype will come from future experiments using specific murine complement inhibitory reagents as they become available.

Although a complete Crry deficiency gives the most dramatic phenotype, our results demonstrate that even a partial decrease in the expression and/or function of these regulators is sufficient to increase the rate of fetal loss (Table 1). Analysis of the *Crry*$^{+/-}$ mating revealed a deviation from the expected Mendelian ratio between the *Crry*$^{+/+}$ and the *Crry*$^{+/-}$ of 1:2 to 1:1, indicating that 45% of *Crry*$^{+/-}$ embryos were also lost (Table 1). These results may also apply to human pregnancy loss in which a decrease, and not necessarily a complete deficiency, in complement regulators within the placenta could increase the rate of fetal miscarriages. Therefore, our studies indicate that further evaluation of this pathogenic mechanism of fetomaternal intolerance is now warranted in human pregnancy loss.

Experimental Procedures

Mice

F1(129Sv/J x C57BL/6) *Crry*$^{+/-}$ mice were generated using standard gene targeting techniques as previously described (Xu et al., 2000). C57BL/6 C4-deficient mice were obtained from Dr. Michael Carroll of Harvard University (Fischer et al., 1996). (129Sv/J x C57BL/6) B cell-deficient mice (μ*MT*$^{-/-}$) were originally obtained from The Jackson Laboratory (Bar Harbor, ME) (Kitamura et al., 1991). F1(129Sv/J x C57BL/6) factor B and C3-deficient animals were obtained from Dr. Harvey Colten (Matsumoto et al., 1997; Circolo et al., 1999). Detection of the mutant alleles was performed by PCR analysis of tail DNA as previously described. B10.D2-*H2*^{d} mice deficient in C5 were obtained from The Jackson Laboratory. They were maintained under specific pathogen-free conditions.

Immunohistochemistry

Deciduas were removed and frozen quickly in OCT compound (Miles, Elkhart, IN), and 10 μm thick sections were cut. Endogenous peroxidase was quenched with 0.2% H_2O_2 in methanol. Sections were first incubated with goat anti-mouse C3 (Cappel, Aurora, OH) followed by rabbit anti-goat IgG conjugated to HRP (Southern Biotechnology), rat anti-mouse Gr-1 (Ly-6G) (Pharmigen, San Diego, CA) followed by rabbit anti-rat IgG conjugated to HRP (Southern Biotechnology). Some sections were stained for cytokeratin, a marker for trophoblast cells (Adamson et al., 2002), using a rabbit anti-cytokeratin antibody (DAKO Corporation, Carpinteria, CA) followed by goat anti-rabbit IgG conjugated to HRP (Southern Biotechnology). In some experiments 10.5 dpc deciduas were stained with goat anti-rat/mouse DAF (Research Diagnostics, Inc., Flanders, NJ) followed by rabbit anti-goat IgG conjugated to HRP (Southern Biotechnology). Bound HRP was detected with diaminobenzidine. Sections were counterstained with 1% methylgreen and covered with crystal-mount (Biomeda, Foster City, CA). Some deciduas were fixed with 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for pathological analysis.

Northern Blot

Total RNA was isolated by homogenizing tissue samples in TRIzol reagent (Gibco BRL, Rockville, MA) using a power homogenizer according to the manufacturer's instructions. Electrophoresis was performed in 0.9% agarose and 0.6% formaldehyde in a buffer containing 0.02 M morpholinopropanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA. RNA was transferred to a nylon membrane and probed with a C3 cDNA fragment or an MCP cDNA fragment (Tsujimura et al., 2001; Circolo et al., 1999). Hybridization and washing were performed using the same conditions as above except that 50% formamide was added to the hybridization buffer and performed at 42°C and washing at 56°C.

RT-PCR Analysis

Total RNA was isolated from testis or placenta and subjected to RT-PCR using the Access RT-PCR system (Promega, Madison, WI).

The following oligonucleotides were used for MCP: 5′-CACGGC CATTTGAAGCTATGGAACTCAAGGG-3′ and 5′-AATATCATGCTTG TTCCAACA-3′ (Tsujimura et al., 2001). Mouse β-actin was used as positive control. Oligonucleotides for mouse β-actin were 5′-TGTGATGGTGGGAATGGGTCAG-3′ and 5′-TTTGATGTCACG CACGATTTCC-3′ (Stratagene, La Jolla, CA). PCR amplification was performed at 94°C for 2 min, followed by 23 cycles of 94°C for 0.5 min, 55°C for 1 min, 68°C for 1 min, and ending with 72°C for 10 min. The PCR products were analyzed in a 1.5% agarose gel. The predicted size of the RT-PCR products for both the β-actin and MCP reactions is around 500 base pairs.

Statistical Analysis

Levels of statistical significance were determined using the χ^2 goodness of fit test.

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References

Abbas, A.K., Lichtman, A.H., and Pober, J.S. (2000). Effector mechanisms of humoral immunity. In Cellular and Molecular Immunology, Fourth Edition (Philadelphia: W.B. Sauders Company), pp. 316–334.

Adamson, S.L., Lu, Y., Whiteley, K.J., Holmyard, D., Hemberger, M., Pfarrer, C., and Cross, J.C. (2002). Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta. Dev. Biol. *250*, 358–373.

Billington, W.D. (1992). The normal fetomaternal immune relationship. Baillieres Clin. Obstet. Gynaecol. *6*, 417–438.

Carroll, M.C. (1998). The role of complement and complement receptors in induction and regulation of immunity. Annu. Rev. Immunol. *16*, 545–568.

Circolo, A., Garnier, G., Fukuda, W., Wang, X., Hidvegi, T., Szalai, A.S., Briles, D.E., Volanakis, J.E., Wetsel, R.A., and Colten, H.R. (1999). Genetic disruption of the murine complement C3 promoter region generates deficient mice with extrahepatic expression of C3 mRNA. Immunopharmacology *42*, 135–149.

Cunningham, D.S., and Tichenor, J.R., Jr. (1995). Decay-accelerating factor protects human trophoblast from complement-mediated attack. Clin. Immunol. Immunopathol. *74*, 156–161.

Fischer, M.B., Ma, M., Goerg, S., Zhou, X., Xia, J., Finco, O., Han, S., Kelsoe, G., Howard, R.G., Rothstein, T.L., Kremmer, E., Rosen, F.S., and Carroll, M.C. (1996). Regulation of the B cell response to T-dependent antigens by classical pathway complement. J. Immunol. *157*, 549–556.

Frank, M.M., and Fries, L.F. (1991). The role of complement in inflammation and phagocytosis. Immunol. Today *12*, 322–326.

Geha, R.S., Sampson, H.A., Askenase, P.W., and Ravetch, J. (2001). Allergy and hypersensitivity. In Immunobiology: The Immune System in Health and Disease, Fifth Edition. C.A. Janeway Jr., P. Travers, M. Walport, and M.J. Shlomchik, eds. (New York: Garland Publishing), pp. 491–500.

Gerard, C., and Gerard, N.P. (1994). C5a anaphylatocin and its seven transmembrane-segment receptor. Annu. Rev. Immunol. *12*, 775–808.

Hill, J.A. (1992). Immunological contributions to recurrent pregnancy loss. Baillieres Clin. Obstet. Gynaecol. *6*, 489–505.

Hoffmann, J.A., Kafatos, F.T., Janeway, C.A., Jr., and Ezekowitz, R.A.B. (1999). Phylogenetic perspectives in innate immunity. Science *284*, 1313–1318.

Holers, V.M., Kinoshita, T., and Molina, H. (1992). The evolution of mouse and human complement C3-binding proteins: divergence of form but conservation of function. Immunol. Today *13*, 231–236.

Holers, V.M., Girardi, G., Mo, L., Guthridge, J.M., Molina, H., Pierangeli, S.S., Espinola, R., Xiaowei, L.E., Mao, D., Vialpando, C.G., and Salmon, J. (2002). Complement C3 activation is required for the antiphospholipid antibody-induced fetal loss. J. Exp. Med. *195*, 211–220.

Holmes, C.H., and Simpson, K.L. (1992). Complement and pregnancy: new insights into the immunobiology of the fetomaternal relationship. Baillieres Clin. Obstet. Gynaecol. *6*, 439–460.

Holmes, C.H., Simpson, K.L., Okada, H., Okada, N., Wainwright, S.D., Purcell, D.F.J., and Houlihan, J.M. (1992). Complement regulatory proteins at the feto-maternal interface during human placental development: Distribution of CD59 by comparison with membrane cofactor protein (CD46) and decay accelerating factor (CD55). Eur. J. Immunol. *22*, 1579–1585.

Hourcade, D., Holers, V.M., and Atkinson, J.P. (1989). The regulators of complement activation (RCA) gene cluster. Adv. Immunol. *45*, 381–416.

Hsi, B.L., Hunt, J.S., and Atkinson, J.J. (1991). Differential expression of complement regulatory proteins on subpopulations of human trophoblast cells. J. Reprod. Immunol. *19*, 209–223.

Jelezarova, E., Vogt, A., and Lutz, H.U. (2000). Interaction of C3b2-IgG complexes with complement proteins properdin, factor B, and factor H: implications for amplification. Biochem. J. *349*, 217–223.

Kaufman, M.H. (1998). The Atlas of Mouse Development (San Diego: Academic Press), pp. 469–477).

Kim, Y.-U., Kinoshita, T., Molina, H., Hourcade, D., Tsukasa, S., Wagner, L.M., and Holers, V.M. (1995). Mouse complement regulatory protein Crry/p65 uses the specific mechanisms of both human decay-accelerating factor and membrane cofactor protein. J. Exp. Med. *181*, 151–159.

Kitamura, D., Roes, J., Kühn, R., and Rajewsky, K. (1991). A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. Nature *350*, 423–426.

Liszewski, M.K., Farries, T.C., Lublin, D.M., Rooney, I.A., and Atkinson, J.P. (1996). Control of the complement system. Adv. Immunol. *61*, 201–283.

Lublin, D.M., and Atkinson, J.P. (1989). Decay-accelerating factor and membrane cofactor protein. Curr. Top. Microbiol. Immunol. *153*, 123–145.

Matsumoto, M., Fukuda, W., Circolo, A., Goellner, J., Strauss-Schoenberger, J., Wang, X., Fujita, S., Hidvegi, T., Chaplin, D.D., and Colten, H.R. (1997). Abrogation of the alternative complement pathway by targeted deletion of murine factor B. Proc. Natl. Acad. Sci. USA *94*, 8720–8725.

Matsushita, M., Thiel, S., Jensenius, J.C., Terai, I., and Fujita, T. (2000). Proteolytic activities of two types of mannose-binding lectin-associated serine proteases. J. Immunol. *165*, 2637–2642.

McIntyre, J.A. (1992). Immune recognition at the maternal-fetal interface: overview. Am. J. Reprod. Immunol. *28*, 127–131.

Medawar, P.B. (1953). Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. Symp. Soc. Exp. Biol. Med. *7*, 320–338.

Molina, H., Wong, W., Kinoshita, T., Brenner, C., Foley, S., and Holers, V.M. (1992). Distinct receptor and regulatory properties of recombinant mouse complement receptor 1 (CR1) and Crry, the two genetic homologues of human CR1. J. Exp. Med. *175*, 121–129.

Nicholson-Weller, A., and Halperin, J.A. (1993). Membrane signaling by complement C5b-9, the membrane attack complex. Immunol. Res. *12*, 244–257.

Pangburn, M.K., and Muller-Eberhard, H.J. (1980). Relation of putative thioester bond in C3 to activation of the alternative pathway and the binding of C3b to biological targets of complement. J. Exp. Med. *152*, 1102–1114.

Reynolds, L.P., and Redmer, D.A. (2001). Angiogenesis in the placenta. Biol. Reprod. *64*, 1033–1040.

Rooney, I.A., Oglesby, T.J., and Atkinson, J.P. (1993). Complement in human reproduction: activation and control. Immunol. Res. *12*, 276–294.

Rus, H.G., Niculescu, F.I., and Shin, M.L. (2001). Role of the C5b-9 complement complex in cell cycle and apoptosis. Immunol. Rev. *180*, 49–55.

Schmidt, B.Z., and Colten, H.R. (2000). Complement: a critical test of its biological importance. Immunol. Rev. *178*, 166–176.

Sherer, D.M., and Abulafia, O. (2001). Angiogenesis during implantation, and placenta and early embryonic development. Placenta *22*, 1–13.

Shibata, T., Cosio, F.G., and Birmingham, D.J. (1991). Complement activation induces the expression of decay accelerating on human mesangial cells. J. Immunol. *147*, 3901–3908.

Tsujimura, A., Kitamura, M., Nomura, M., and Seya, T. (1998). Molecular cloning of a murine homologue of membrane cofactor protein (CD46): preferential expression in testicular germ cells. Biochem. J. *330*, 163–168.

Tsujimura, A., Nunoue, K., Inoue, N., Shida, K., Kurita-Taniguchi, M., Matsumoto, M., Takeya, T., and Seya, T. (2001). Three soluble form messages of murine CD46 are produced through alternative mRNA splicing. J. Biochem. *130*, 841–848.

Vince, G.S., and Johnson, P.M. (1995). Materno-fetal immunobiology in normal pregnancy and its possible failure in recurrent spontaneous abortion? Hum. Reprod. *10*, 107–113.

Volanakis, J.E. (1998). Overview of the complement system. In The Human Complement System in Health and Disease, J.E. Volanakis and M.M. Frank, eds. (New York: Marcel Decker), 9–32.

Wetsel, R.A. (1995). Structure, function, and cellular expression of complement anaphylatoxin receptors. Curr. Opin. Immunol. *7*, 48–53.

Xu, C., Mao, D., Holers, M.V., Palanca, B., Cheng, A.M., and Molina, H. (2000). A critical role for murine complement regulator Crry in fetomaternal tolerance. Science *287*, 498–501.