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Invited Review

Neonatal Hemochromatosis: Is It an Alloimmune Disease?

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Neonatal hemochromatosis (NH) has been defined clinically as severe neonatal liver disease in association with extrahepatic siderosis in a distribution similar to that seen in HFE-associated hereditary hemochromatosis (1). Though it is generally considered a rare disease, it is one of the most commonly recognized causes of liver failure in the neonate and a frequent indication for liver transplantation in the first 3 months of life (2–4). Its etiology and pathogenesis are as yet unknown. Indeed, it has been considered to be a syndrome in which a number of primary etiologies, such as infection, genetic-metabolic disease and toxic insult, lead to a common phenotype. We have hypothesized that much, if not all, NH is a consequence of gestational alloimmune disease. This review presents the clinical evidence leading to this hypothesis, the data we have collected in its support, and the direction our investigations are taking to ultimately prove or disprove it.

NEONATAL HEMOCHROMATOSIS IS A FETAL LIVER DISEASE

Considerable evidence exists to suggest that NH is a gestational disease in which fetal liver injury is the dominant feature. Liver histology of affected newborns is characterized in most cases by marked structural damage with intense fibrosis and cirrhosis (1,5,6). Most affected live-born babies show evidence of fetal insult (i.e., intrauterine growth restriction (IUGR) and oligohydramnios) and exhibit liver failure within the first few days of life (7,8). It seems impossible that cirrhosis and its complications could develop unless the process began before birth. In support of this supposition, several affected babies with NH have also had renal dysgenesis (9–12). Correlation with the process of normal renal development dates the arrest of renal development to about 24-weeks gestation. It is believed that this final stage of renal development is dependant upon liver function, and therefore, the presence of renal dysgenesis dates the associated liver dysfunction to the late second and early third trimester. Finally, we have diagnosed NH in necropsies of stillborns as early as 22-weeks gestation (unpublished observations).

THE SOURCE OF THE THEORY OF ALLOIMMUNE ETIOLOGY

At first the theory that NH is an immune-mediated gestational disease was based entirely on clinical observations made in families with more than one affected child. NH has an unusual pattern and high rate of recurrence in the progeny of affected women; after the index case there is an approximately 80% probability that each subsequent baby born to that mother will be affected (1,6,13). At present, there is no test to determine if a pregnancy will be affected, and there is no effective approach to prenatal diagnosis. Counseling related to future child bearing has been based on making a firm diagnosis in the index case and expecting a high recurrence rate (14).

That NH recurs in families has suggested to some investigators that NH is a genetic disease. However, the observed pattern of recurrence is exceedingly difficult to explain on a genetic basis. The recurrence rate is much too high to consider autosomal recessive inheritance (the most common pattern of inheritance in metabolic disease), and consanguinity has not been reported. Alternate inheritance patterns have been considered. It is possible that NH represents a marked variability in penetrance of a dominant gene. Against this possibility are the following observations: the rate of recurrence in sibships exceeds 50%; no parent of an affected child has been found to have a disorder resembling NH with subdued penetrance; and no sibling of a parent of an affected child has been reported to have had an affected baby. Another interesting observation is that no man has ever been reported to have fathered infants with NH with different women, while there are several documented instances of a woman giving birth to affected babies with different male parentage (13,15). Explanations for this observation include: possible gonadal mosaicism for new and dominant mutations lethal in spermatogenesis but not in

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oogenesis; mitochondrial disease inherited through the mother; and maternal transmission of an imprinted gene (1). In studies to date no gene locus has been identified (13,16). We have concluded that the balance of evidence is against a genetic etiology.

Similarly, we have found no evidence that an infectious disease can produce recurrent disease that mimics NH. *Cytomegalovirus* infection and non-A, non-B hepatitis have rarely been associated with a near NH phenotype (17,18). These infections rarely if ever cause neonatal liver failure; their phenotype never completely mimicks the extrahepatic siderosis seen in NH; and they have never been found to cause recurrent cases. Perinatal enterovirus infection, particularly echovirus, has been associated with neonatal liver failure, but histopathology shows multi-organ hemorrhagic necrosis not at all resembling NH (19–22).

The pattern of recurrence in NH most resembles that of gestational alloimmune diseases such as hydrops fetalis and alloimmune thrombocytopenia. This and lack of evidence for another etiology have led to our hypothesis that NH is an alloimmune disease.

ETIOLOGIES AND MECHANISMS OF IMMUNE-MEDIATED FETAL DISEASES

Maternal antibodies of the immunoglobulin G (IgG) class and only IgG are actively transported across the placenta to the fetus from about the 18^{th} week of gestation (23,24). The principle function of this process is to provide humoral immunity for the fetus and newborn against a broad array of microbiologic antigens to which there has been no exposure. Maternally-derived IgG constitutes the main humoral defense against infections during gestation and for the first few months of life. Maternal immune cells do not traverse the placenta, and the placenta (an allograft) is protected from cell-mediated rejection by very complex mechanisms (25,26). Thus, the humoral limb of the maternal immune system is uniquely important as it relates to fetal immunity and to immune-mediated phenomena in the fetus.

In unusual circumstances, a mother can become sensitized to a fetal antigen and develop an IgG antibody response to that antigen. For maternal sensitization to occur, brief access of the fetal target antigen to the maternal circulation is required, as well as lack of the antigen in the maternal repertoire of ''self-antigens''. As the IgG is directed against a human antigen, this is a humoral alloimmune response. When alloimmune IgG of sufficient titer is transported to the fetus, it binds to the target of alloimmunity in the fetus. It is not known how often this occurs without producing a physical effect or fetal injury, but in some circumstances fetal injury does result from maternal humoral immunity.

An immune mechanism is well established in several fetal diseases. In the most commonly occurring gestational alloimmune diseases, the fetus expresses a dominant paternal allele for blood group antigens expressed on erythrocytes (hydrops fetalis) or platelets (alloimmune thrombocytopenia) that the normal mother lacks (27,28). A rare gestational alloimmune disease occurs when the mother has a homozygous genetic deficiency that results in an inability to synthesize a common, species-related protein and therefore develops an immune response to a fetus expressing that protein from the normal paternal allele. This alloimmune condition has been demonstrated in cases of antenatal membranous glomerulonephritis (29,30). Another mechanism of fetal alloimmune disease involves sensitization of the mother to a fetal antigen that significantly differs from the isoform expressed in mature individuals as seen in cases of recurrent arthrogryposis multiplex congenita (31,32). This condition represents a lapse of memory by the immune system of what was ''self'' during fetal life. Finally, maternal autoimmunity resulting in passive fetal autoimmunity has been implicated as a cause of open neural tube defects (33) and fetal myocarditis with congenital heart block (34,35).

There are 4 possible mechanisms by which maternal IgG can produce injury to fetal cells and tissues. Antibodies may bind to cell surface antigens and fix compliment. This can result in direct cell lysis and/or activation of the reticulo endothelial (RE) system with increased clearance of cells. This mechanism of injury applies mainly to cells circulating in the fetal blood stream and is the mechanism of fetal injury in Rh disease (hydrops fetalis) and alloimmune thrombocytopenia (27,28). Second, antibodies may incite a cell-mediated immune reaction by the T cells of the fetus. This mechanism may be important in congenital heart block associated with maternal lupus autoantibodies against SSB/La and SSA/Ro (34,35). The cardiac histopathology in cases of neonatal lupus demonstrates inflammation and myocardial fibrosis. The proposed mechanism involves apoptosis of myocardiocytes in the rapidly developing heart, which leads to exposure of otherwise inaccessible ribonucleoproteins to circulating maternally-derived IgG (34). Opsonized ribonucleoproteins are taken up by resident macrophages, which are thereby activated to secrete proinflammatory and profibrotic cytokines. Third, maternal antibodies may form antigen-antibody complexes with circulating antigens that can be deposited or with in situ fetal antigens that interfere with organ function. This mechanism has been demonstrated in cases of antenatal membranous glomerulonephritis (29,30). Mothers who are genetically deficient in neutral endopeptidase, a protein normally expressed in the kidney and present in the circulation, are sensitized to fetal neutral endopeptidase and develop IgG antibodies that are transported to the circulation of the fetuses of subsequent gestations. Deposition of antigen-antibody complexes in the glomerular basement membrane results in congenital nephrotic syndrome in these babies. Fourth,

maternal antibodies may bind to the antigen target and interfere with some important function. This mechanism has been described as a cause of recurrent arthrogryposis multiplex congenita (31). Mothers of affected babies have been found to have antibodies against the fetal acetylcholine receptor (an isoform that significantly differs from the isoform expressed in mature individuals), but not against the adult receptor. These mothers did not have myasthenia gravis themselves, but passage of antibodies to the fetus resulted in fetal myasthenia, muscle weakness, reduced fetal movement and contractures. Similarly, maternal autoimmunity has recently been implicated as a cause of fetal open-neural-tube defects due to interference with the function of a folate receptor that is important in fetal development (33). Furthermore, anti-Ro/La antibodies interfering with cardiac repolarization may partially explain the mechanism of congenital heart block in fetal lupus (36).

THE ALLOIMMUNE MECHANISM OF FETAL LIVER INJURY IN NH

Based on several observations we favor the possibility that alloimmunity against a common fetal antigen is the cause of liver injury in NH. NH is not common enough to be the result of discordant maternal-fetal expression of common genetic alleles. Yet, it is too common to be the result of maternal genetic deficiency. Furthermore, women may have several normal children before having one with NH, and no offspring of female siblings of affected women have been shown to be affected with NH. Both of these occurrences seem unlikely if the condition is a result of maternal genetic deficiency. Finally, finding autoimmune disease or autoantibodies in affected women has been inconsistent when examined, perhaps no greater than in the general female population (37).

THE TRIAL OF GESTATIONAL TREATMENT TO PREVENT RECURRENT LETHAL NH

Some alloimmune gestational diseases have been treated by administering intravenous immunoglobulin to the mothers (38). Treatment with exogenous pooled adult IgG is thought to alter the natural course of gestational alloimmune disease by 1 or more of 3 mechanisms: blunting the maternal immune response to fetal antigens; flooding the placental IgG transport mechanism with non-reactive antibodies; and nonspecific antibody binding that limits the binding of reactive alloantibodies to target antigens (28). Even though the specific mechanism of action is not known, experience with this treatment in alloimmune hematologic disorders demonstrates its effectiveness in reducing the severity of fetal disease.

More than 8 years ago we initiated a protocol for treating women during gestation to prevent recurrent lethal NH based on the hypothesis that it is an alloimmune disease. The treatment consists of intravenous immunoglobulin derived from pooled serum of multiple donors (IVIG) administered weekly at a dose of one g/kg body weight from the 18^{th} week until the end of gestation. The use of IVIG was purely empiric; we tried it because it was effective in Rh incompatibility and to a lesser degree in alloimmune thrombocytopenia (27,38,39). The dose was derived from the literature on treatment of Rh incompatibility and the starting point was chosen because of the generally held belief that active IgG placental transport starts around 18 weeks (23,24). We have chosen for treatment women whose most recent gestation was affected with proven NH in lieu of any other marker for high risk of recurrence. These studies were approved by the IRB of Children's Memorial Hospital.

Eighteen women have completed gestational treatment to date. Three women have been treated through two pregnancies each. Twenty two babies have been born (including one set of twins), all of whom have survived with medical therapy or no therapy. No IUGR, fetal liver disease or other evidence of fetal distress was detected in any case. This is in stark contrast to the typical progression of NH, where severe IUGR and oligohydramnios are essentially universal. Only six babies showed clinically significant evidence of liver disease. However, there was biochemical evidence that the majority of babies were affected with NH, including 17 babies with substantially elevated serum α-fetoprotein (AFP) (range 100,820–670,000 ng/ml) of whom most also had elevated serum ferritin (range 1,250–15,948 ng/ml). Liver biopsies were obtained from four babies: two showed severe hepatitis with extensive necrosis and intense iron deposition; and two demonstrated well-preserved hepatic architecture and modest numbers of giant cells with siderosis. We have reported the results of 15 women treated through 16 pregnancies (6). When analyzed on a per-mother basis comparing outcomes of treated gestations to randomly selected previous affected gestations, gestational IV-Ig therapy was associated with improved infant survival ($p = 0.0009$). We conclude from this experience that treatment with high-dose IV-Ig during gestation appears to have modified recurrent NH so that it was not lethal to the fetus or newborn. These results provide additional strong evidence for an alloimmune mechanism for recurrent NH.

WHY THE FETAL LIVER AND WHY HEMOCHROMATOSIS?

There is considerable evidence that the fetal liver is the primary site of injury in NH and that liver injury precedes extrahepatic siderosis (18). Therefore, we hypothesize that the target antigen in NH is a fetal liver protein involved in iron homeostasis. Our summary hypothesis is depicted in Figure 1. In this theory, there is maternal exposure during pregnancy to a common human liver

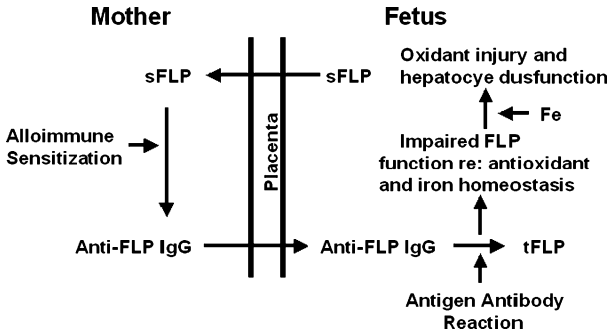

FIG. 1. Proposed mechanism for alloimmune causation of NH. Soluble fetal liver protein (sFLP) crosses the placenta where it sensitizes the mother to produce specific IgG antibody, which is transported across the placenta to the fetal circulation. In the fetus, the antibody is free to react with in situ fetal liver protein (tFLP) and to interfere with vital functions of the protein that involve iron homeostasis and protection from iron-induced oxidant injury. The normally large amount of iron fluxing through the fetal liver then produces oxidant injury and hepatocyte dysfunction, which in turn results in NH.

antigen that is expressed mainly in fetal life. Maternal exposure is assumed to occur frequently if not in all gestations. Failure of the maternal immune system to recognize the fetal antigen as ''self'' (loss of memory) is probably a very rare event. Antigens that are typically expressed at high levels in the fetus (ie alpha-fetoprotein) often have low level expression in adults, but are still present in sufficient quantities to stimulate deletion of recognition epitopes and maintain memory. However, if memory has been lost, exposure can result in sensitization and production of a specific immune response, including immunoglobulin of the IgG class capable of recognizing and binding to the antigen. Transplacental passage of maternal IgG to the fetus (in this and subsequent pregnancies) is accompanied by movement of anti-fetal-liver-antigen IgG to the fetal circulation, where it binds the in situ liver antigen and either interferes with a vital function or results in immune injury of the fetal liver.

A potential target of alloimmunity in NH should be a fetally expressed protein (i.e. is many-fold over expressed in fetal liver relative to adult) to be potentially sensitizing to mothers during gestation. It should have an essential role in overall iron homeostasis. The liver is the central organ in fetal iron distribution and homeostasis as reviewed elsewhere (1). It is thought that all iron traversing the placenta is processed by the liver and distributed to other tissues such as the bone marrow that require iron. Iron is a potent oxidant. Our theory suggests that immunologic blockade of function renders the liver, specifically hepatocytes, susceptible to oxidant injury from the large quantities of iron normally processed there. The oxidant injury causes impaired hepatocyte function and even cell death. Among the liver functions impaired are those related to maintaining iron homeostasis and protection from oxidative injury. The liver's inability to manage the flux of iron permits spillover to other tissues capable of iron uptake (40,41), mainly tissues of epithelial origin and to a lesser degree myocardium, which produces the multi-organ disease that characterizes NH.

THE FETAL LIVER TARGET OF NH ALLOIMMUNITY

We formulated a strategy to use serum samples from women enrolled in our gestational treatment protocol to identify the fetal antigen target of alloimmunity. Briefly, we isolated proteins from various human fetal tissues, subjected them to SDS-PAGE and immunoblotted with IgG isolated from affected women's sera as primary antibody and monoclonal anti-human IgG as the secondary. Using this approach, we found no protein in placentas and cord blood samples from premature and full-term babies that was recognized by any of the sera from affected women we tested. We concluded that the proposed antigen was not present in those tissues. However, we found a single protein band with approximate molecular weight of 32kD in whole liver homogenate of fetal liver (20-week gestation) that was consistently recognized by the sera of affected women at high titer ($\geq$1:500 serum dilution). These sera did not recognize any proteins in homogenates of child and adult liver specimens. We also tested fetal (10 day gestation) and adult mouse liver (6-weeks), and found that the sera of affected women strongly detect a ~32kD protein in the fetal mouse liver but not the adult. Sera from unaffected individuals do not detect the human or mouse ~32kD fetal liver protein. These data suggest that the target of alloimmunity in NH is a fetal liver protein.

This candidate protein must be fully identified and its functions characterized to prove its involvement as a target of alloimmunity. We propose that blocking the function of this fetal liver protein by alloantibodies, in particular its functions in iron homeostasis and as an antioxidant may result in increased susceptibility to oxidant injury induced by iron naturally fluxing through the liver. Oxidant injury results in specific or global hepatocyte injury, impairing many liver functions including those needed for maintaining iron homeostasis. Liver failure results from accelerating oxidative injury and events secondary to it, whereas iron deposition in extrahepatic tissues results from the inability of the liver to process iron being delivered to it.

FUTURE DIRECTIONS

The long-term goal of our research is to improve the diagnosis, prevention and treatment of NH. To do that, it is imperative that we clearly understand the pathophysiologic mechanisms of the disease. The first step in this

understanding is to identify the fetal antigen target of alloimmunity. Once the antigen is identified we should be able to devise an efficient serologic test for alloimmune NH, which would be a huge advance in diagnosis and management. NH is a disease of women in which their fetuses and newborns are affected. Thus, it is important that a test be developed to diagnose the condition in the mother rather than in the baby. Some potential outcomes of an effective serologic test would be the ability to determine if all cases of NH represent a single disease – alloimmune NH – or whether some cases are secondary to another, perhaps sporadic fetal liver injury. Such a finding would explain the 20% lack of recurrence and would free women not having specific NH antibody to have another baby without treatment and with minimal risk of recurrence. If all or most NH is alloimmune, measuring maternal antibody could become a powerful diagnostic tool when dealing with a sick newborn in which liver failure is a component. Being able to measure antibody titer would allow preventative treatment trials aimed at streamlining and reducing the costs of the current IVIG protocol (6). It could also allow an improvement in the care of affected newborns. The presence of high-titer antibody would be cause to remove maternal IgG from the baby by exchange transfusion or plasmapheresis. Moreover, developing an animal model for the disease, which is dependent upon identifying the antigen target, would lead to improved understanding of the disease and its many ramifications and perhaps improved preventative therapy and improved therapy of affected babies.

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