Gestational Alloimmune Liver Disease in Cases of Fetal Death

Peter F. Whitington, MD, Xiaomin Pan, MD, Susan Kelly, RN, Hector Melin-Aldana, MD, and Padmini Malladi, MS

Objective To determine whether alloimmune liver disease can be identified as a cause of fetal death.
Study design This is a retrospective examination of the autopsy tissue of 6 stillborn fetuses and 2 extreme preterm infants (gestational age, 20 to 34 weeks) drawn from families referred for suspected neonatal hemochromatosis. Thirteen appropriate nondisease controls and 8 cases of neonatal acute liver failure with known etiology were also examined. Liver sections were immunostained using anti-human C5b-9 complex.
Results All of the study cases had died with no preceding evidence of fetal distress. Histopathology showed findings of acute liver injury, including global hepatocyte necrosis with minimal reticulum collapse and no fibrosis. Hepatocytes in cases stained strongly positively for C5b-9 complex, suggesting premortem IgG complement–mediated liver injury. Hepatocytes in acute liver failure case controls did not demonstrate a similar mechanism of liver injury.
Conclusions Alloimmune liver disease is sometimes associated with fetal death. *(J Pediatr 2011;159:612-6)*.

Neonatal hemochromatosis (NH), defined as the coexistence of severe liver disease and siderosis of extrahepatic tissue,[1-4] is a phenotype that apparently results from fetal liver injury.[1,2,5] Recent evidence suggests that most cases stem from gestational alloimmune liver disease (GALD).[6,7] Hepatocyte damage in GALD is mediated by fetal complement via classical activation of the terminal complement cascade.[8] The presence in hepatocytes of C5b-9 complex, the terminal complement cascade (TCC) neoantigen formed in assembly of the membrane attack complex (MAC), indicates TCC activation on the plasma membrane. This finding is unique to the cases of NH among cases of severe neonatal liver diseases and thus provides an alternative approach to diagnosing GALD in cases without the extrahepatic siderosis characteristic of NH.

In a small fraction of GALD cases with the NH phenotype, the liver demonstrates acute hepatocyte injury and minimal fibrosis.[8] These findings suggest that in some circumstances, materno-fetal alloimmunity may cause an acute form of fetal liver injury, as opposed to the subacute or chronic disease more commonly associated with NH. They further suggest that tissue siderosis may develop very soon after the onset of fetal liver disease if the liver injury is severe. It follows that materno-fetal alloimmunity could produce severe, acute liver injury in the fetus, possibly resulting in fetal acute liver failure (ALF). If this occurred in a brief time frame, extrahepatic siderosis might not have time to develop, and then the diagnosis of NH could not have been made based on standard criteria. We analyzed autopsy tissue specimens from stillborn fetuses and extremely preterm infants to investigate whether global hepatic necrosis due to GALD was the probable cause of death.

Methods

This study involved a retrospective analysis of autopsy tissue. The cases were an unselected sample of tissues provided by families referred for consultation regarding the need for gestational therapy to prevent recurrent NH.[7] The cases comprise all nonmacerated postmortem liver specimens from stillborn fetuses and live-born infants of gestational age ≤34 weeks (**Table I**). For comparison, we used specimens from autopsies of 11 newborns of 24 to 39 weeks gestational age, as described previously.[8,9] These newborns all died of perinatal asphyxia due to immaturity, meconium aspiration, or other causes. We also reviewed the electronically available records of stillbirths of 18 to 34 weeks' gestational age with postmortem examination performed in our institution between 2008 and 2010. We identified 2 (22 weeks and 28 weeks gestation) with no liver maceration, an unknown family history, and no autopsy evidence of liver disease for inclusion as comparison cases. Finally, we searched the autopsy records of Children's Memorial Hospital for the years 1996 to 2010 and identified 8 case controls in whom ALF was considered the primary cause of death based on clinical and/or autopsy grounds. These were all term infants who died within the first 3 months of life, with all but 1 dying in the first 2 weeks of life (**Table II**). The collection and study of these samples were approved by the Children's Memorial Hospital Institutional Review Board by exemption.

ALF	Acute liver failure
GALD	Gestational alloimmune liver disease
MAC	Membrane attack complex
NH	Neonatal hemochromatosis
TCC	Terminal complement cascade

From the Children's Memorial Research Center, Feinberg School of Medicine, Northwestern University, Chicago, IL

The authors declare no conflicts of interest.

0022-3476/$ - see front matter. 10.1016/j.jpeds.2011.03.048

Table I. Cases: Clinical characteristics and liver immunohistochemistry

Case	Gestational age, weeks	Birth status	Sibling with NH	Extrahepatic siderosis	C5b-9 staining
1	22	Live birth	Yes	No	4+
2	22	Stillbirth	No	No	4+
3	34	Stillbirth	No	Yes	4+
4	30	Live birth	Yes	Yes	4+
5	22	Stillbirth	No	Yes	4+
6	20	Stillbirth	No	Yes	4+
7	20	Stillbirth	No	No	4+
8	21	Stillbirth	No	Yes	4+

Histology and Immunohistochemistry

From paraffin-embedded liver tissue specimens, 5-μM sections were obtained for histology (hematoxylin and eosin, trichrome, reticulum [modified silver], and Perls' Prussian blue staining) and immunohistochemistry analyses. The immunohistochemistry techniques used have been reported previously.[8,9] In brief, sections were treated with monoclonal antibody to human SC5b-9 neoantigen (TCC-neoantigen, monoclonal antibody to human SC5b-9 neoantigen; Quidel, San Diego, California), followed by treatment with an appropriate biotinylated antibody (Vector Laboratories, Burlingame, California), followed by development with VECTASTAIN ABC reagent (Vector Laboratories) according to the manufacturer's instructions. Sections processed without primary antibody were considered controls for nonspecific reaction.

Quantification of TCC-Neoantigen Expression in Hepatocytes

Liver sections stained for TCC-neoantigen were photographed to determine the fraction of hepatocytes displaying MAC. Random nonoverlapping 200× magnification images were obtained of hepatic parenchyma from each liver section stained for TCC-neoantigen; 3 to 5 images per specimen were acquired to ensure adequate sampling. Because the specimens often contained few intact hepatocytes, a semiquantitative scale for positive staining was used rather than the quantitative approach reported previously.[8] The total number of identifiable hepatocytes in the images and then the number either showing or not showing positive staining (depending on which condition was not dominant and thus easier to tally) were tallied. The proportion of positively stained hepatocytes was calculated, and a staining scale of 0 to 4+ was applied, with 0 = no staining, 1+ = <25% positive hepatocytes, 2+ = 25%-50% positive hepatocytes, 3+ = 50%-75% positive hepatocytes, and 4+ = >75% positive hepatocytes.

Results

Eight cases were acquired from centers in the United States (n = 5) and Australia (n = 3) (Table I). The indication for family referral was a positive family history of NH in siblings in 2 cases and suspicion of NH in 6 cases, 3 because of hepatic siderosis detected on autopsy and 3 because of recurrent stillbirths in a maternal sibship. Two of these patients were full siblings (cases 2 and 3), and the remainder were unrelated. In addition to these siblings, 4 cases studied (cases 1, 4, 5, and 8) had 7 stillborn maternal siblings that were not studied because no postmortem examination was performed (n = 4) or the tissues were too macerated to allow examination (n = 3).

No signs or symptoms of fetal distress preceding the terminal event were reported, and in no case was the cause of death discovered on postmortem examination. Postmortem examination revealed anasarca (hydrops fetalis) in 3 cases (2, 3, and 7), one with reported edema detected on ultrasound a few days before fetal death. The mean gestational age of the cases was 23.5 ± 5.2 weeks. Six cases were stillborn, and 2 died within minutes after abrupt spontaneous labor. Our reexamination of the 8 cases found extrahepatic siderosis and an anatomic diagnosis of NH in 5 cases, including 3 of the 4 cases with unexamined stillborn maternal siblings (cases 4, 5, and 8).

Hepatic Histopathology

Most cases of NH have histopathology that is most consistent with subacute or chronic injury.[1,2,5,8,10] In contrast, our cases demonstrated evidence only of acute liver injury. Global,

Table II. ALF case controls: Clinical characteristics and liver immunohistochemistry

Case control	Age at death	Autopsy diagnosis	C5b-9 staining
CC-1	12 days	HSV acute hepatic necrosis	1+
CC-2	9 days	Echovirus liver necrosis	1+
CC-3	3 months	Adenovirus ALF	1+
CC-4	12 days	Enterovirus hepatic necrosis	2+
CC-5	9 days	HSV acute hepatic necrosis	1+
CC-6	13 days	HSV acute hepatic necrosis	1+
CC-7	1 day	Ischemic hepatic necrosis	1+
CC-8	0 days	Maternal HELLP syndrome	1+

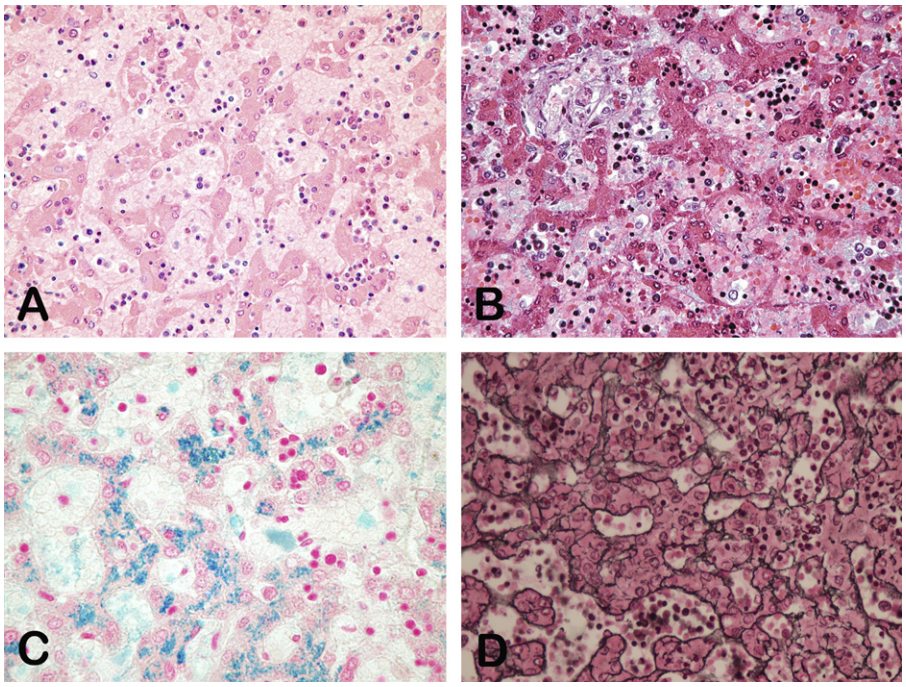

Figure 1. Liver histopathology of a 22-weeks' gestation stillbirth with alloimmune liver injury (case 5). **A,** Hematoxylin and eosin–stained section shows residual hepatic cords, but hepatocytes are globally necrotic with poor cellular definition and uniform pink cytoplasm. Hemopoetic elements are prominent, whereas inflammation is scant. **B,** Masson's trichrome stain reveals minimal lobular fibrosis. **C,** Perls' Prussian blue stain shows excess siderosis of hepatocytes and ghosts of hepatocytes. This infant also had extrahepatic siderosis. **D,** Reticulum stain. Hepatic cords show good definition. The absence of reticulum collapse suggests that an acute process led to hepatic necrosis. Sinusoidal spaces between hepatic cords lined by reticulum are occupied by blood elements. (Original magnification 400×.)

panlobular hepatocyte necrosis was the dominant feature in 5 cases (Figure 1). In 3 cases (2, 3, and 7), no viable hepatocytes could be identified; only "ghosts" remained. These 3 cases showed marked loss of identifiable hepatocytes and absent hepatic cords, a finding not consistent with postmortem hepatocyte autolysis in which cords remain but with loss of cellular definition. The anatomic pathology was similar in the ALF case controls, all of which had confluent hepatocyte necrosis. The major difference was that some case controls had evidence of viral infection, with inclusion bodies and smudged cells. Comparison of reticulum stains between cases and case controls showed little difference in the amount of collapsed reticulum. No significant fibrosis was identified in any case or case control. Most cases demonstrated considerable extramedullary hematopoiesis, in keeping with the gestational age. Inflammation was minimal in both the NH cases and the case controls.

Demonstration of TCC-Neoantigen by Immunohistochemistry

The finding of immunoreactive TCC-neoantigen on or in a cell is considered irrefutable evidence of MAC assembly on its plasma membrane.[11] We previously reported that neonatal asphyxia comparison cases had sparse staining in few hepatocytes, with 0.9% ± 1.3% (range, 0 to 3%) of hepatocytes staining positively.[8] On the semiquantitative scale used in this study, 9 of these 11 cases had 0 staining and 2 had 1+ staining. The 2 stillborn comparison cases had 0 staining for C5b-9 complex. All study cases had 4+ staining for C5b-9 complex in hepatocytes (Table I and Figure 2). Among the case controls with ALF (Table II), the 2 cases with nonviral etiology had little more hepatocyte TCC-neoantigen than the comparison cases (1+ staining). All of the cases of viral-related disease demonstrated some hepatocyte TCC-neoantigen, mainly in cellular debris of unclear origin and mononuclear inflammatory cells (Table II and Figure 2). Some hepatocytes showed significant accumulation of TCC-neoantigen in association with viral inclusions, in contrast to the uniform staining of hepatocytes in the cases studied.

Discussion

We present evidence that materno-fetal alloimmunity can cause severe acute fetal liver disease relatively early in gestation and perhaps even liver failure, resulting in intrauterine fetal death or extreme premature birth. In all of the cases we studied, the liver demonstrated global hepatic necrosis, and the finding of dense expression of TCC-neoantigen in

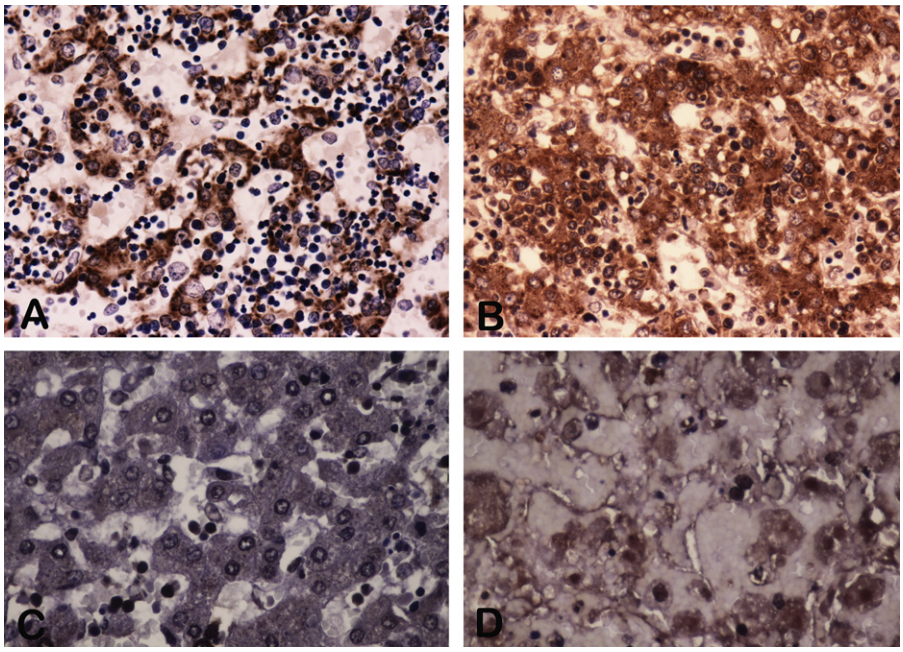

Figure 2. Immunoperoxidase staining for C5b-9 TTC-neoantigen of liver parenchyma. **A,** Case 5 (histopathology shown in Figure 1). Hepatic cords intensely express TCC-neoantigen, as demonstrated by the deep-brown staining. **B,** Case 7, a 20-weeks' gestation stillbirth with neither extrahepatic siderosis nor family history of NH. Residual hepatocytes and ghosts in cords stain intensely for TTC-neoantigen. **C,** Enterovirus hepatic necrosis (CC-4). Hepatic cords remain intact. Individual hepatocytes show minimal brown staining for C5b-9 complex. **D,** Herpes simplex 2 hepatic necrosis (CC-5). Hepatocytes showed marked ballooning and numerous inclusions. The only significant accumulation of MAC in this case was associated with inclusion bodies. (Original magnification 400×.)

residual hepatocytes and hepatocyte ghosts strongly suggests an alloimmune mechanism for the acute injury.[8] Taken together, our findings suggest that GALD may cause liver failure in the fetus and contribute to fetal death.

Our findings are important in regard to autopsy diagnosis in otherwise unexplained cases of stillbirth or premature birth. None of the cases studied had been diagnosed with NH before our examination. In the classical diagnostic paradigm, liver pathology suggestive of NH leads to an evaluation for extrahepatic siderosis and thus to the diagnosis of NH. However, the histopathology of the cases reported here is atypical for NH, with global hepatocyte necrosis in the absence of any evidence of chronic injury, and thus might not have prompted evaluation for siderosis. Furthermore, some of the cases lacked extrahepatic siderosis. Pathologists should be aware of how easily the liver pathology can be overlooked in these cases, as well as the difference in appearance from typical NH. The possibility of GALD might be considered in selected cases of unexplained stillbirth, particularly if the liver pathology suggests necrosis rather than autolysis. Examination for evidence of extrahepatic siderosis may be useful in these cases. Among our cases with extrahepatic siderosis, 3 had preceding stillborn maternal siblings. Fetal tissue examination might have established the diagnosis in some of those cases. However, 3 of our 8 cases did not have extrahepatic siderosis. Even in cases with no pathological siderosis, the possibility of alloimmune acute hepatic necrosis should be kept in mind. Immunostaining for C5b-9 is one approach to identifying IgG complement–mediated alloimmune injury.

Our findings are likewise important for pediatricians and obstetricians to keep in mind. Pediatricians presented with a newborn with coagulopathy or liver failure whose mother has had a previous stillborn baby might want to consider linking the 2 events and immediately evaluate for NH. Newborns with coagulopathy are often treated for sepsis, and even if liver failure is diagnosed, NH often is not the first etiologic consideration, even though it is the most frequent cause of liver failure in newborns.[12] A previous stillbirth suggests congenital disease, and GALD NH is the most common cause for severe congenital liver disease.[6,13] Obstetricians should be aware that alloimmune fetal liver injury is a risk factor for fetal loss. In our large series of women who gave birth to a child with NH diagnosed based on standard criteria, approximately 1 in 8 pregnancies ended in fetal death.[7] We cannot say whether all of the losses involved alloimmune liver injury, but the circumstances, along with the current demonstration of association with GALD, suggest that this high rate of fetal loss is related to gestational alloimmunity. Studies of a large cohort of women with history of intrauterine fetal death and appropriate controls are needed to determine whether the presence of NH-associated

alloantibody is a risk factor for fetal loss in the general population.

The mechanism by which alloimmune injury produces hepatocyte death has not been fully established, although experimentally it appears to involve direct MAC-mediated cell lysis.[14] It is unclear why the immune attack produces acute hepatocyte necrosis in some cases and subacute or chronic injury in others. Alloimmune blood diseases vary in severity, presumably because of variations in the maternal IgG adaptive immune response to sensitization and individual differences in the capacity of fetal complement to promote and sustain injury. Alloimmune liver injury may involve another variable, the ability of nucleated cells, including hepatocytes, to resist complement-mediated injury.[15,16] It is possible that by mid-gestation, development of the proinjury mechanisms might exceed development of hepatic protective capacity, leading to acute overwhelming injury in some cases. In addition, individual sensitivity to alloimmune injury is evident from the fact that twins may differ significantly in the clinical severity of liver disease.[17] All evidence considered, these cases appear to represent acute global hepatocyte necrosis due to alloimmune injury.

The ramifications of establishing a proper diagnosis in cases such as those reported herein are related to the management of subsequent pregnancies of the mothers involved. This is an important concept, because GALD is extremely likely to recur in subsequent pregnancies, and recurrence can be prevented by gestational therapy.[7] By showing that liver injury in the present cases occurred via the same complement-dependent humoral immune mechanism as is seen in typical cases of NH, that the histopathology was entirely consistent with acute injury, and that this liver injury was the only apparent cause of fetal death, our findings support the concept of fetal ALF due to alloimmunity as a potential cause of intrauterine fetal death. Our findings add fetal death associated with GALD as an indication for treatment during subsequent pregnancies. ■

Submitted for publication Dec 1, 2010; last revision received Feb 21, 2011; accepted Mar 22, 2011.

Reprint requests: Peter F. Whitington, MD, Children's Memorial Hospital, 2300 Children's Plaza, Box 57, Chicago, IL 60614. E-mail: p-whitington@northwestern.edu

References

1. Knisely AS, Mieli-Vergani G, Whitington PF. Neonatal hemochromatosis. Gastroenterol Clin North Am 2003;32:877-89.
2. Knisely AS, Narkewicz MR. Iron storage disorders. In: Suchy FJ, Sokol RJ, Balistreri WF, eds. Liver Disease in Children. 3rd ed. New York: Cambridge University Press; 2007. p. 661-76.
3. Whitington PF. Fetal and infantile hemochromatosis. Hepatology 2006; 43:654-60.
4. Whitington PF, Fleming RE. Hemochromatosis. In: Kleinman RE, Goulet O-J, Sherman PM, Sanderson IR, Shneider BL, Walker WA, eds. Walker's Pediatric Gastrointestinal Disease: Pathophysiology, Diagnosis, and Management. 5th ed. Hamilton, ON, Canada: BC Decker; 2008. p. 1049-56.
5. Silver MM, Valberg LS, Cutz E, Lines LD, Phillips MJ. Hepatic morphology and iron quantitation in perinatal hemochromatosis: comparison with a large perinatal control population, including cases with chronic liver disease. Am J Pathol 1993;143:1312-25.
6. Whitington PF. Neonatal hemochromatosis: a congenital alloimmune hepatitis. Semin Liver Dis 2007;27:243-50.
7. Whitington PF, Kelly S. Outcome of pregnancies at risk for neonatal hemochromatosis is improved by treatment with high-dose intravenous immunoglobulin. Pediatrics 2008;121:e1615-21.
8. Pan X, Kelly S, Melin-Aldana H, Malladi P, Whitington PF. Novel mechanism of fetal hepatocyte injury in congenital alloimmune hepatitis involves the terminal complement cascade. Hepatology 2010;51: 2061-8.
9. Bonilla SF, Melin-Aldana H, Whitington PF. Relationship of proximal renal tubular dysgenesis and fetal liver injury in neonatal hemochromatosis. Pediatr Res 2010;67:188-93.
10. Witzleben CL, Uri A. Perinatal hemochromatosis: entity or end result? Hum Pathol 1989;20:335-40.
11. Stoltzner SE, Grenfell TJ, Mori C, Wisniewski KE, Wisniewski TM, Selkoe DJ, et al. Temporal accrual of complement proteins in amyloid plaques in Down's syndrome with Alzheimer's disease. Am J Pathol 2000;156:489-99.
12. Squires RH, Jr., Shneider BL, Bucuvalas J, Alonso E, Sokol RJ, Narkewicz MR, et al. Acute liver failure in children: the first 348 patients in the Pediatric Acute Liver Failure Study group. J Pediatr 2006;148:652-8.
13. Whitington PF, Kelly S, Ekong UD. Neonatal hemochromatosis: fetal liver disease leading to liver failure in the fetus and newborn. Pediatr Transplant 2005;9:640-5.
14. Malladi P, Wang H, Pan X, Kohli R, Whitington PF. The pathogenesis of neonatal hemochromatosis involves maternal IGG alloantibody-mediated complement-dependent hepatocytolysis and necrosis. Hepatology 2006;44:56A.
15. Koch CA, Kanazawa A, Nishitai R, Knudsen BE, Ogata K, Plummer TB, et al. Intrinsic resistance of hepatocytes to complement-mediated injury. J Immunol 2005;174:7302-9.
16. Morgan BP. Complement membrane attack on nucleated cells: resistance, recovery and non-lethal effects. Biochem J 1989;264:1-14.
17. Ekong UD, Kelly S, Whitington PF. Disparate clinical presentation of neonatal hemochromatosis in twins. Pediatrics 2005;116:e880-4.