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PII: S0887-7963(16)30011-6
DOI: doi: 10.1016/j.tmrv.2016.05.008
Reference: YTMRV 50466

To appear in: *Transfusion Medicine Reviews*

Received date: 20 February 2016
Accepted date: 23 May 2016

Please cite this article as: Hendrickson Jeanne E., Delaney Meghan, Hemolytic Disease of the Fetus and Newborn: Modern Practice and Future Investigations, *Transfusion Medicine Reviews* (2016), doi: 10.1016/j.tmrv.2016.05.008

Hemolytic Disease of the Fetus and Newborn: Modern Practice and Future Investigations

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Keywords: hemolytic disease of the fetus and newborn, red blood cell, alloimmunization

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Abstract:

Red blood cell (RBC) sensitization occurs in some women in response to exposure to paternally derived RBC antigens during pregnancy or to non-self antigens on transfused RBCs during their lifetime. Once sensitized, future pregnancies may be at risk for hemolytic disease of the fetus and newborn (HDFN). Although great strides have been made over the past few decades in terms of identifying blood group antigens and in predicting fetal anemia through the use of non-invasive monitoring, many questions remain in terms of understanding RBC alloimmunization risk factors, preventative therapies, and treatment strategies. At the present time, there is room for improvement in these areas in both developed and developing countries. Evidence based, universal guidelines describing recommended RBC antigen matching transfusion strategies for girls or women, prior to pregnancy or during intrauterine transfusions (IUTs), would be welcomed. A better understanding of the mechanism(s) of action of RhIg, first introduced over half of a century ago and one of the most successful immunoprophylaxis therapies in existence today, would also be a large step forward. For example, answers to questions of the role(s) that fetal RBC clearance, antigen masking, antigen modulation, and immune suppression play in the effectiveness of RhIg may help to guide the development of novel preventative therapies during pregnancy for immunization to RhD and non-RhD antigens. Further, a better understanding of the importance of anti-RhD or other alloantibody glycosylation patterns may be beneficial not only in developing such novel immunoprophylaxis therapies, but also in predicting the clinical significance of existing maternal alloantibodies. One other area of need includes the development of therapies beyond IUTs to mitigate the dangers of maternal alloantibodies to developing fetuses. We challenge physicians, scientists, and funding agencies to prioritize studies of RBC alloimmunization and HDFN, and to invest in the children of our future.

Introduction

Maternal alloimmunization to blood group antigens occurs through exposure to non-self antigens on RBCs via transfusion or prior pregnancy. RBC alloantibodies may be detrimental in transfusion or pregnancy settings, depending on the specificity and depending on the presence or absence of the cognate antigen(s) on transfused or fetal RBCs. In a transfusion setting, incompatible RBCs may be hemolyzed. In a pregnancy setting, fetal RBCs expressing a paternally derived antigen against which a mother is alloimmunized may be hemolyzed or may be altered such that erythropoiesis is suppressed, either resulting in hemolytic disease of the fetus and newborn (HDFN).

Alloantibodies against more than 50 non-ABO blood group antigens have been implicated in HDFN, with many blood group antigens historically first identified after the birth of a hydropic infant[1]. In addition to antibodies against non-ABO blood group antigens, naturally occurring maternal isohemagglutinins, often in group O mothers, are capable of leading to anemia in fetuses expressing the A or B antigens. This anemia, though relatively common, is usually mild and rarely requires intervention. The majority of clinically significant HDFN cases are due to alloantibodies against antigens in the Rh, Kell, Duffy, Kidd, and MNS families[2-4], with 1/300-1/600 live births being affected by maternal RBC alloimmunization[5]. Of note, maternal alloantibodies against Rh antigens are significantly less likely to develop when fetal cells are ABO incompatible with maternal plasma, presumably due to the rapid clearance of fetal RBCs by maternal isohemagglutinins. Although alloantibodies to antigens in the Rh family remain a leading cause of severe HDFN world-wide, antibodies against the antigens in the Kell family are emerging to be a leading cause of HDFN in parts of the world where RhIg is widely used for immunoprophylaxis[6].

The outcomes of antigen positive fetuses developing in utero of alloimmunized women vary, depending on characteristics of the maternal antibody and the RBC antigen. Fetuses affected by maternal anti-D alloantibodies may have anemia and such severe hyperbilirubinemia that they develop kernicterus.

Fetuses affected by maternal anti-Kell antibodies, however, are more likely to have anemia and reticulocytopenia but rarely have significant hyperbilirubinemia. Because many different maternal antibodies are capable of leading to hydrops fetalis and intrauterine fetal demise, the early diagnosis of at risk pregnancies is critically important. Maternal antibody titers against antigens such as RhD are commonly reported by US transfusion medicine services. Bioassays such as chemiluminescence or monocyte monolayer assays have a higher specificity to predict fetal outcomes than maternal antibody titers[7, 8], though they are not routinely used in the US. Recent studies suggest that other antibody characteristics, including glycosylation patterns, may associate even more closely with antibody dependent cellular cytotoxicity and fetal outcome than titer[9, 10].

A better understanding of characteristics of maternal antibodies and fetal RBC antigens that result in adverse fetal outcomes would be helpful in the development of novel/targeted therapeutic interventions, as discussed in more detail below. For example, it is unclear why maternal antibodies against antigens on the KEL glycoprotein so efficiently lead to reticulocytopenia. Is has been hypothesized that the expression of the Kell antigen on early fetal RBC precursors plays an important role, with direct suppression of erythropoiesis by maternal anti-Kell reproduced in cultures *in vitro*[11]. However, phagocytosis of RBC precursors expressing the Kell antigen has also been observed *in vitro*[12], making it plausible that the lack of hyperbilirubinemia observed in fetuses affected by maternal anti-Kell antibodies is due in part to immune mediated clearance of very early RBC precursors which don't contain hemoglobin. In this review, we summarize the latest clinical approaches to HDFN and describe research that is underway as well as the topics in need of further investigation and development to continue to improve management of this disorder.

Diagnosis and Treatment

At the first prenatal visit, all pregnant women should be tested for RBC alloantibodies using the indirect antibody test (IAT). After the woman is found the have RBC antibodies, the risk of clinically significant

HDFN is determined by several techniques. Using the maternal sample, serial (usually monthly) RBC antibody titration is done to assess if the fetal red blood cells are acting as an immunizing stimulus. If available for testing, the paternal blood type provides inheritance information; homozygous fathers have 100% chance of passing the implicated antigen to the fetus; heterozygous fathers have a 50% chance of having an offspring with the offending blood group antigen. In the case of anti-D sensitization, serological testing cannot determine zygosity and molecular testing for the RHD gene copy number are needed [13]. Fetal DNA can also be obtained by amniocentesis for blood group genotyping to directly determine the fetal blood type using cultured amniocytes. Due to the risk of fetal loss with the procedure, newer techniques have been developed that isolate fetal DNA from a maternal peripheral blood sample for RBC genotyping[14, 15].

To determine the clinical significance of HDFN, the fetus should be monitored for well-being by ultrasound to look for evidence of ascites (hydrops), heart rate monitoring and for anemia. Fetal anemia assessment is carried out non-invasively using ultrasonic measurement of fetal blood flow through the large cerebral vessels, usually the middle cerebral artery. The velocity of the blood flow indicates the degree of anemia.

Although maternal alloantibody titers are used to predict the risk to the fetus, some fetuses have severe anemia despite low titers and others have no anemia despite high titers. Pan-IgG reagents are typically used for measuring titers in the US, but evaluating IgG subtypes may be informative for certain antibodies[16]. In one study, for example, fetal anemia correlated positively with the amount of maternal IgG1 anti-D, and negatively with the amount of IgG3 anti-D bound to fetal RBCs[17]. In addition to antibody titers, some countries also use *in vitro* antibody dependent cellular cytotoxicity (ADCC) biological assays to predict alloantibody activity[18, 19]

The importance of antibody glycosylation patterns on FcγR binding avidity on clinical outcomes is increasingly being appreciated, in multiple biologic systems[20]. Recent studies have described a significant association between such antibody glycosylation patterns and fetal outcomes. Maternal anti-D alloantibodies with the lowest degrees of fucosylation (as measured by mass spectroscopy), for example,

have been reported to be associated with more severe fetal anemia[9]. Similarly, maternal anti-platelet glycoprotein alloantibodies with low fucosylation patterns have been shown to be more clinically significant than those with higher fucosylation patterns[21]. One potential explanation for the increase in clinical significance is high binding avidity to FcγRIIIa monocytes and FcγIIIb polymorphonuclear cells.

The treatment of a fetus affected by HDFN is focused on monitoring and support until delivery. Fetuses at gestation >16-24 weeks and are found to have blood flow velocities 1.5 times the multiple of the mean (MoM) is indicative of moderate to severe anemia. Periumbilical blood testing is indicated to directly sample the fetal circulation [2, 22]. This is often directly followed by intrauterine red blood cell transfusion (IUT) if the fetus is not of acceptable gestational age for delivery. These RBC transfusions are selected as Group O, negative for the offending antigen(s), leukocyte reduced, irradiated, and concentrated to ensure maximal delivery and to ensure the hematocrit of the fetus is ≥30%. Extended RBC antigen matching is done at some centers. IUT is able to preserve neurologic outcome in many children, though those with severe hydrops may develop cerebral palsy, severe developmental delay, and deafness[23]. Potential alternatives or adjunctive maternal therapies during pregnancy to decrease the severity of fetal anemia include IVIG and plasma exchange (PE), though the evidence to support the efficacy of these therapies is limited. PE has been reported in women with high titer RBC antibodies and antecedent HDFN[24]; it is given a Category II by the American Society for Apheresis, meaning it is considered second line therapy[25].

After delivery, infants are closely monitored for hemoglobin and bilirubin levels as their own physiological systems must function independently from the mother. Infants affected by HDFN must be intensively observed with laboratory monitoring of hemoglobin and bilirubin to determine if phototherapy, simple transfusion, or exchange transfusion are necessary[26, 27]. Although exchange transfusion is a critical therapy for prevention of kernicterus in neonates with rapidly rising bilirubin, it does have complications[28]. IVIg has been shown to reduce the need for exchange transfusion in some studies[29, 30]. However, it does not affect anemia significantly, and top off transfusions may still be needed

[27]. In addition, IVIg contains anti-A and anti-B antibodies which may potentially worsen HDFN among group A or B newborns.

The majority of maternal alloantibody transfer into the fetal circulation ends at birth. However, neonatal anemia may persist beyond the natural half-life of the antibodies in some circumstances, and may resolve more quickly than predicted in other circumstances. Anemia may persist regardless of the presence of circulating alloantibodies in neonates who undergo multiple intrauterine transfusions, due to the suppression of erythropoiesis. Further, it is possible that maternally derived alloantibodies in neonates who have undergone intrauterine transfusions or post-natal exchange transfusions may persist longer than one may predict, due to a lack of antigen positive RBCs to bind the antibodies. As the infant ages, signs of poor feeding and increased sleep can be signs of persistent hypoproliferative anemia. Continued monitoring with regular checks of hematocrit and reticulocytes provides critical information for marrow recovery or the need for red cell transfusion. Of note, recent studies have introduced the idea that maternal antibodies against antigens on platelets or RBCs may be transferred to neonates via breast milk. A study describing the transfer of anti-platelet IgA antibodies in breast milk from mother to neonate and resulting in prolonged neonatal thrombocytopenia[31] led to murine studies evaluating the possibility of RBC alloantibody transfer in breast milk. Anti-KEL glycoprotein RBC alloantibodies (IgG and IgA) were, in fact, shown to be transferred via breast milk from mothers to nursing pups in a murine fostering model[32]. In considering the translation of these murine findings to humans, however, one must remain mindful that murine FcRn receptors transfer IgG significantly more efficiently from intestine to circulation than human receptors[33, 34].

Prevention

The prevention of maternal alloimmunization falls into three different categories; primary, secondary and tertiary (Table 1). Primary prevention focuses on preventing the possibility of maternal alloimmunization through reduced exposure to foreign RBC antigens. In most industrialized nations, the emergency supply of RBCs that is kept sequestered to support sudden and unexpected bleeding

emergencies includes Group O (universal donor) RBC products. These products may be RhD negative or positive. In many institutions, particularly those supporting trauma programs, the RhD positive RBC products are allowed to be given to males so that the rarer RhD negative RBC products can be preferentially provided to females of childbearing potential, such as those <50 years [35]. These widely accepted practices are in place to provide a measure of serological prevention for young women that require life-saving transfusion that does not endanger future pregnancies. Although not as universally practiced, some nations and regions attempt to prevent exposure of other RBC antigens when females of childbearing potential are transfused with RBC products in routine medical and surgical settings. For example, several European nations provide K antigen negative, and/or E, and c antigen matched products to all females less than 45 – 50 years (exact practices vary by region). It is not clear if the approach of extended RBC antigen matching is entirely successful due to other routes of potential exposure, such as through previous pregnancy and receipt of transfusion outside of the region. However, for regions that are able to accomplish it, there may be a measure of prevention added [36].

The administration of Rh immunoglobulin (RhIg) is secondary prevention aimed at removing or masking fetal RhD positive RBCs found in the maternal circulation during pregnancy or after delivery. The product has had an enormous impact on the prevalence of HDFN due to anti-D, decreasing the incidence from 16% to <0.1% [37]. Notably, many low resource countries do not have prenatal screening programs and RhIg availability, putting infants born in these regions at risk.

Appropriately identifying women who need RhIg is an area of study. Partially because of the product's success and also because it was implemented before there was a feasible way to determine the fetal RhD type and precise maternal RhD typing, practice has evolved to administer RhIg to all RhD negative pregnant women prenatally, and often postnatally, even when the infant's RBC type could also be determined at that time. Moreover, practice often includes giving RhIg to all women with weak RhD testing results, referred to as weak D blood type because there has not been acceptance or access to testing to more precisely guide prevention measures. The advent of genetic testing for RHD has led to

improvements in the ability to guide RhIg administration decisions and to stratify risk for women who already have made anti-D. There are some examples of forward thinking national approaches to testing fetal DNA from a sample of peripheral maternal blood to determine which pregnant women should receive RhIg due to pregnancy with an RhD positive fetus[15]. In 2015, a practice recommendation was made by several professional organizations (AABB, CAP, ACOG, Armed Forces Blood Program, ABC, American Red Cross) to begin to phase in the use of RhD genotyping methods to categorize women with initial RhD typing results that are weak instead of strongly reactive in the serological test system [38]. The recommendation suggests that if the woman carries the RHD genetic variants weak D type 1, 2, or 3, then she can safely be managed as RhD positive and does not need RhIg. The testing offers more precise information than can be provided by traditional blood typing approaches and can provide a measure of assurance for women that have been given different blood typing results at different times as well as improve resource utilization of RhD negative blood products and RhIg. In fact, a financial modelling study of the suggested approach found that the genotyping approach is cost neutral or incrementally cost saving than providing RhIg to all women who are weak D by serological methods[39].

Rarely, an RhD negative female that is of childbearing potential and desiring of future pregnancy may receive RhD positive RBC units due to emergency needs, or occasionally, due to errors in the process of blood sample collection and preparation of the blood product(s). In these exceptional circumstances, the use of large doses of RhIg and/or automated red blood cell exchange (RCE) using an apheresis instrument are types of secondary prevention that have been reported. Several case reports have been published that cite the use of approximately 20 ug of RhIg for each mL of RhD positive RBCs transfused, although data are limited and there is no accepted standard[40]. For RhD negative females that have received larger quantities of RhD positive RBC products, an RCE procedure has been used to lower the RhD positive RBCs in circulation [41, 42]. Then the patient is administered RhIg to cover the remaining calculated residual RhD positive RBCs. The ASFA plans to rank this as a category III in the 2016 Seventh Special Issue to be published in the Journal of Clinical Apheresis, suggesting that apheresis should be used on a case-by-case basis due to the paucity of published experience and efficacy.

The use of extended antigen matched RBC units for IUT transfusions is tertiary prevention. In this setting, the pregnant woman already has alloimmunization to RBC antigens and has a fetus that is affected with anemia due to the RBC antibody. Although there is not currently a thorough scientific understanding to pinpoint the factors that make a person's immune system respond to RBC antigens, there is evidence that such "immune responder" women are at risk for further RBC alloimmunization to other RBC antigens that they have not yet formed antibodies against. The broadening of her RBC antibody specificities may impact future pregnancies. Researchers in the Netherlands have led several studies investigating the effect of providing antigen matched RBC units to women undergoing IUT procedures. Supporting the "immune responder" hypothesis, they found that when Rh (C, c, E, e) and K antigen matched RBCs were used for IUT, 25% of women (53/212) formed additional RBC antibodies[43]. In an attempt to abrogate this response, the group attempted to provide IUT transfusions with the Rh, K blood antigens as above, plus the addition of Fy, Jk and Ss blood group antigens for 159 women that needed IUT for HDFN. They found that it was increasingly difficult to find appropriately matched units (48% received full antigen matched, 52% received partially antigen matched), and that 4.3% of women getting high match and 11% getting moderate match formed additional antibodies[44]. Together, these data show the difficulty of prevention of alloimmunization for patients that appear to have "immune responder" characteristics. Further research is needed to define and understand the underlying biological mechanisms.

Research in Focus

RhIg and alternative RhD specific therapies

RhIg, in widespread use in many countries for half of a century now, is one of the most successful immunomodulatory therapies in existence today. Despite its efficacy, the mechanism of action remains poorly understood[45, 46]. The current practice of generating RhIg by immunizing male RhD negative volunteers not only puts the volunteers at risk for hemolytic transfusion reactions should they receive RhD positive RBCs in trauma/emergent situations, however, but it also leads to variability in RhIg

product composition. For example, differences in fucosylation patterns have recently been identified between brands of RhIg, with these patterns theoretically impacting product efficacy by altering Fcγ binding[47].

Investigations of alternative sources of anti-D (including *in vitro* production) have been hampered due in part to a lack of animal models available for reductionist/mechanistic studies; the generation of an animal model of RhD alloimmunization has been difficult[48, 49]. It is logical that RhIg simply prevents a mother's immune system from "seeing" the RhD antigen on fetal RBCs, given observational studies in the pre-RhIg era of the risk of maternal anti-D formation in ABO compatible pregnancies (16% risk of RhD alloimmunization) versus ABO incompatible pregnancies (<2% risk of RhD alloimmunization)[37, 50]. However, some RhD antigen sites on fetal RBCs are thought to remain unbound with the low dose of RhIg given. Further, correlations between RBC clearance and anti-D alloimmunization have not consistently been observed, with some monoclonal anti-D preparations that increase RBC clearance enhancing rather than preventing alloimmunization[51]. A similar finding of increased alloimmunization in situations of rapid RBC clearance has been reported in at least one murine model, suggesting that rapid clearance of a bolus of antigen positive RBCs has the capability, in certain situations or with certain antigens, to enhance RBC alloimmunization[52, 53]. Of note, antigen presentation alone is known to be insufficient to lead to an alloantibody response in some blood group systems[54] as well as in multiple non-RBC systems, with a simultaneous danger signal of sorts also being necessary[55, 56].

Antibody mediated immune suppression

The term "antibody mediated immune suppression" (AMIS) has been applied to models in which passively infused antibodies such as RhIg prevent active alloimmunization. The actual degree of "immune suppression" (versus other mechanism(s) of action) of these antibodies, however, remains unknown. Regardless of mechanism, an antigen specific effect is seen in many systems. For example, passively infused polyclonal antibody ("KELIg") against the KEL glycoprotein antigen expressed on murine RBCs prevents alloimmunization to transfused RBCs expressing the KEL glycoprotein.[57]

The phenomenon of a passively administered antibody altering active RBC alloimmunization has been investigated not only in the KEL murine model but also in murine models with other antigens and in murine models transfused with sheep RBCs.[58] Multiple subtypes of monoclonal antibodies as well as polyclonal antibodies have been investigated, with some but not all being capable of preventing alloimmunization altogether or of decreasing the magnitude of an alloimmune response[59-61]. In at least one model, the efficacy of passively infused antibodies at preventing an alloimmune response to transfused RBCs has been shown to be independent of the inhibitory FcRIIb receptor[60].

Antigen modulation

Antigen modulation, also known as antigen loss or antigen suppression, has been described to occur on RBCs as well as on WBCs in the presence of antibodies, in humans and in animal models alike[62, 63]. At the present time, it is not known whether RhD antigen modulation occurs in the presence of RhIg and, if it occurs, whether it is in part responsible for the immunoprophylaxis effect of RhIg. Of note, modulation of the RhD antigen in an RhD positive patient treated with RhIg for ITP has recently been reported, suggesting that modulation of the RhD antigen on fetal RBCs is plausible[64]. If antigen modulation correlates with immunoprophylaxis effect, then a better understanding of antigen/antibody characteristics as well as recipient cell types that impact this modulation will be informative as novel therapies to prevent maternal alloimmunization are developed.

Reductionist studies in murine models have allowed antigen modulation to be studied in greater detail than human studies permit. Studies completed using transgenic murine RBCs labeled with lipophilic dyes or those that are naturally fluorescent have found that passive administration of KELIg modulates the KEL glycoprotein antigen to the point that it is not detectable by flow cytometric methods within 24-48 hours after transfusion[57]. Although the relationship between antigen modulation and RBC clearance is not well understood, the accelerated clearance of antibody bound RBCs slows in some models once the cognate antigen can no longer be detected[65].

Alternate strategies to prevent pregnancy associated alloimunization

Recent work in murine models, based on the success of RhIg, has focused on using antibodies to prevent alloimmunization. However, there may be alternate approaches beyond the use of antibodies for prevention of alloimmunization in pregnancy. One approach that has been studied in a transfusion setting includes using RBCs themselves to induce non-responsiveness. Murine studies have shown that RBCs expressing the human glycophorin A (hGPA) antigen are not immunogenic in a transfusion setting in the absence of an adjuvant. Exposure to hGPA RBCs in this murine model not only leads to non-responsiveness, but instead results in tolerance: subsequent transfusions, given even in the presence of an adjuvant, are incapable of leading to alloimmunization[54]. If antigen specific non-responsiveness extends beyond transfusion to exposure in a pregnancy setting, then one may potentially envision a strategy of tolerance induction using RBCs themselves.

Conclusions

Although much progress has been made in the past few decades in terms of understanding the molecular basis of blood group antigens and in predicting fetal anemia through the use of non-invasive monitoring, there has been no significant progress in the prevention or treatment of RBC alloimmunization in pregnancy. Practical clinical questions and research challenges remain (Table 2); these include applying advances in immunobiology, molecular medicine, and transgenic technology to the study of HDFN. A better understanding of which patients are at risk of alloantibody formation may allow more personalized transfusion or pregnancy immunoprophylaxis strategies. Though challenging to design and execute, studies in this area may yield revolutionary improvements in preventative approaches. Beyond preventative therapies, the development of targeted strategies to mitigate the dangers of existing maternal antibodies to developing fetuses would also be transformative. We challenge physicians, scientists, and

funding agencies to dedicate resources to RBC alloimmunization and HDFN, and to invest in the children of our future.

Conflict of interest

The authors declare no conflicts of interest related to this manuscript.

Table 1. Prevention of HDFN

Prevention type	Measure
Primary	• Red cell transfusion with antigen matched or antigen negative RBC products to prevent exposure to foreign RBD antigens
Secondary	• RhIg for RhD negative women that have given birth to an RhD positive infant (post-natal dose)
Tertiary	• Antigen matching of RBC products used for intrauterine transfusion (IUT), to prevent broadening of maternal alloantibody specificities

Table 2. Clinical questions and research challenges

Unanswered Questions/Knowledge	Future Research Areas
What defines a "responder" to RBC antigens in transfusion or pregnancy?	• Immunobiology studies of responders and non-responders, in clinical settings and animal models
What RBC antigen matching strategies are ideal to prevent alloimmunization in transfusion prior to or during pregnancy?	• Studies evaluating the efficacy of simple transfusion or IUT RBC antigen matching programs in different countries
How does RhIg work?	• Studies to better understand RBC clearance, antigen modulation, immune suppression, etc.
How can RhIg or other immunoprophylaxis therapies be more readily available world-wide?	• Public health initiatives; work to develop a more affordable, standardized product
How do glycosylation patterns impact RhIg effectiveness and maternal RBC alloantibody clinical significance?	• In vitro generation and characterization of antibodies with modified glycosylation patterns; correlative studies of maternal antibody glycosylation patterns with fetal outcome
How can monoclonal or recombinant antibodies be optimized for immunoprophylaxis against RhD or other RBC antigens?	• Studies to understand the efficacy of monoclonal versus polyclonal antibodies, as well as those with various IgG subtypes or altered Fc regions
What strategies beyond antibody based therapies may prevent alloimmunization in pregnancy?	• Consideration of tolerance induction strategies
How can the dangers of existing maternal alloantibodies to developing fetuses and neonates be mitigated?	• Studies of non-invasive, novel targeted therapies to decrease antibody binding to fetal RBCs; studies considering whether breast milk alloantibody transfer occurs in humans

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Highlights (3-5 bullet points, max 85 characters including spaces)

- RBC alloimmunization and HDFN remain significant problems in all countries
- A better understanding of factors influencing responsiveness to RBC antigens is needed
- Optimized RBC antigen matching transfusion strategies for girls and women are necessary
- Understanding how RhIg works is essential for novel immunoprophylaxis development
- Targeted therapies to mitigate the dangers of alloantibodies to fetuses would be beneficial