Chimerism 4:4, 119–125; October/November/December 2013; © 2013 Landes Bioscience

Chimerism in transfusion medicine

The grandmother effect revisited

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Keywords: transfusion, alloimmunization, Rh, tolerance, pregnancy

Transfusion therapy is complicated by the production of alloantibodies to antigens present in the donor and lacking in the recipient through the poorly-understood but likely multifactorial process of alloimmunization. The low prevalence of alloimmunization in transfused patients (6.1%)[1] suggests that processes central to immunologic tolerance may be operating in the vast majority of transfused patients who do not produce alloantibodies. Using RhD as a prototype, evidence is reviewed that the ability to make antibodies to red blood cell (RBC) antigens may result in part from immunologic tolerance acquired in utero. These ideas are extended to other examples of maternal microchimerism (MMc) of other non-inherited maternal antigens (NIMA). An evolutionary argument is offered that multi-generational immunity supports the hypothesis that MMc may partly explain the "non-responder" phenotype in RBC alloimmunization.

Diversity of Red Cell Antigens

Blood transfusion is the intravascular transfer of blood (or a component thereof) into a recipient. Thanks to advances in the storage and anticoagulation of blood and its components extra corpus, the donor can also be the recipient (i.e., autologous transfusion), but much more commonly the donor and recipient are not the same person (i.e., allogeneic transfusion). Consequently, the immunologic paradigm of self/non-self suggests that most transfusions should constitute an immunologic challenge and elicit some type of response akin to compatibility considerations that are central to transplantation immunology. The 2012 International Society of Blood Transfusion (ISBT) working party on Red Cell Immunogenetics and Blood Group Terminology has recognized 34 blood group systems,[2-5] comprised of 284 disparate antigens.[3] Another 44 antigens have been identified in blood "collections" based on serological data without a characterized molecular basis. Although not as diverse as the HLA system, incompatibility at one of the 328 erythrocyte antigens found either in blood groups or collections is expected and is common in everyday transfusion medicine.

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Submitted: 04/23/2013; Revised: 10/09/2013; Accepted: 10/22/2013
http://dx.doi.org/10.4161/chim.26912

In practice; however, transfusions are routinely and safely performed with universal compatibility consideration given only to two blood groups: The carbohydrate ABO system due to preformed isohemagglutinins and the RhD protein. Ignoring the other 32 blood groups in this way leads to an immunologically-based adverse reaction report to US hospital transfusion services in only about 1 in 6000 transfusions,[6] suggesting that most recipients are extremely tolerant to allogeneic transfusion. When alloimmunization does occur, it can not only cause clinical hemolysis, but also may delay or even prevent timely transfusion. The recipient factors that cause these reactions are poorly understood. This review re-examines the evidence that non-inherited maternal antigens (NIMAs) on erythrocytes may play a role in processes of tolerance or sensitization of offspring to the RhD protein. This phenomenon is known as "the grandmother effect" and if substantiated, it may offer new insight into the genetic factors and mechanisms of erythrocyte alloimmunization.

An Immunologic Complication of Transfusion: The Problem of Alloimmunization

Alloimmunization (also known as a delayed serologic transfusion reaction, or DSTR) is the production of an antibody in response to antigens foreign to the host but derived of the same species and arises through prior pregnancy, transfusion, or transplant. DSTR is observed in approximately 6% of post-transfusion patients, but may be as high as 35–40% in highly transfused populations such as sickle cell anemia patients.[7] There may also be some as yet uncharacterized processes that give rise to so-called "naturally-occurring" alloantibodies as seen in < 1% of healthy blood donors.[1] Other possible stimuli causing alloimmunization could be an unrecognized pregnancy or mucosal exposure to semen.[8] To understand the biological mechanisms that cause alloimmunization, we must remember that the iatrogenic introduction of blood in the form of allogeneic transfusion is only a very recent cultural adaptation of humans such that at most 4–5 generations in Western cultures have been exposed to it, but humans have coevolved with and because of gestation and fetal delivery processes for millions of years. Thus, analysis of pregnancy-induced alloimmunization may provide valuable insights into the genetic factors that contribute to transfusion-associated alloimmunization.

The stimulus to make an anti-RBC antibody may differ in the setting of pregnancy, which lasts much longer than a single transfusion event, does not involve infusion of an anticoagulated

and stored blood component, and which is influenced by many physiological factors such as previous pregnancies, uterine health, or growth and invasion of the placenta. ABO-compatibility between mother and fetus is an important factor in alloimmunization since isohemagglutinin-mediated destruction of fetal cells dramatically decreases the antigen burden and thus the rate of antibody formation: While 16% of RhD-negative women who were ABO-compatible with their fetus made an allo-anti-D in the era before therapeutic Rh-immunoglobulin (RhIG), only 2% of RhD-negative women who were ABO-incompatible were sensitized.[9]

Despite these differences, pregnancy-induced alloimmunization is similar to DSTR in that alloantibody production in both situations is the exception rather than the rule, despite at least some antigenic exposure in both circumstances. Some of this individual variation may have a genetic basis. Although alloimmunization could be described as a quantitative trait (e.g., by the number of alloantibodies an individual forms, the titer of any given antibody, or the count or timing of exposures until the first antibody is detected), the trait is typically assigned dichotomously according to the "responder" vs. "non-responder" paradigm. Labeling patients in this way suggests that some innate property of the patient, such as a genetic predisposition, underpins the alloimmunization phenotype. Once a patient has made one alloantibody, she or he is considered a "responder."[10] The most clinically significant alloantibody was the anti-RhD (or anti-D) antibody for most of the history of transfusion until the licensure of RhIG in 1968 in North America and Europe.[11] The hemolytic disease it could cause in newborns was the topic of important early work in immunologic tolerance and it remains the prototype for antigenic challenge in transfusion medicine.

RhD Sensitization or Tolerance: A Role for the Maternal Grandmother?

In the early 20th century, hemolytic disease of the fetus and newborn, or "Rh Disease," was a major public health problem in North America: It was responsible for 10% of all perinatal deaths (by erythroblastosis fetalis or kernicterus) in Canada in the early 1940s and was still an important cause of morbidity and mortality after the introduction of exchange transfusion in the 1950s.[11] The occurrence of Rh Disease in primigravid women without a history of blood transfusion, blood injection, or abortion was rarely observed, but the fact that it could occur even in scattered case reports ignited debate about the nature of the fetal immune system.[12] At the 1950 Scandanavian Congress of Pathologists, Nevanlinna suggested that "in these cases, the immunized patient herself [may have] received her first antigen stimulus *via* the placenta from her own mother, in utero, or in connection with delivery."[13] After the publication of 2 more cases of Rh Disease in primigravida in the British Medical Journal (BMJ) during May of 1953, Dr. Rosemary Beasley remarked to the BMJ editors that "it would be interesting to hear if anyone has investigated the Rh-grouping of the maternal grandmothers of first-born infants affected by Rh-incompatibility where no known primary sensitization of the mother has occurred."[14]

Just one month later, a group from the National Blood Service (UK) lead by Dr PB Booth submitted data on 113 mothers of Rh-negative mothers (i.e., "grandmothers") showing no evidence that the likelihood of anti-RhD antibody production in the mother proband depended on the RhD-type of the grandmother—the proportions of RhD+ and RhD- grandmothers were almost exactly what would have been predicted based on RhD allele frequency alone.[15]

Possible Tolerance In Utero to RhD

These early investigators sought a specific human example of what had been called "Burnet's 'marker' concept" of the generation of tolerance in chick embryos using human RBC as antigen[16] and extended work from the late 1940s. Ray Owen's observation of mutual tolerance to erythrocyte antigens in dizygotic calf twins in 1945[17] was a seminal contribution to the emerging study of immunologic tolerance and to the allograft experiments that were ultimately awarded the Nobel Prize in 1960.[18] In 1953, Owen extended his work to human erythrocyte antigens, specifically the immunologically-active and clinically-problematic Rh antigens in a pivotal analysis of RhD-negative women and the RhD status of their mothers.[19]

Owen and his colleagues studied RhD- mothers of RhD+ children followed by the Pasadena Rh Testing Laboratory and the Los Angeles Children's Hospital. They classified these women as "tolerant" to RhD if there was no serological evidence of Rh sensitization within three Rh+ pregnancies and as "intolerant" if the subject developed an antibody during or before her third Rh+ pregnancy. They also had clinical correlations with erythoblastosis, making this an excellent study of both an immunologic findings and an important clinical outcome. Analyzing approximately 100 mothers, they found laboratory and clinical results that were difficult to reconcile: RhD- mothers were more likely to be immunologically tolerant as defined by absence of an alloantibody of her RhD+ fetus if her own mother were RhD+ than if she were RhD- (P = 0.01), but the occurrence of clinical erythroblastosis was not associated with the grandmother's phenotype.[19]

Owen himself stated that he was "fortunate [to have] missed the earlier reference"[20] letter by Booth and colleagues reporting no association, because "the negative result of the earlier work would doubtless have discouraged us from conducting a study that in fact gave most provocative results."[20] The apparent discrepancy between an increased probability of tolerance to RhD if the grandmother were RhD+ but yet without decreased risk of erythroblastosis fetalis was provocative indeed. After all, the clinical intuition articulated in Nevanlinna and Beasley's early papers were in fact the opposite effect: That an RhD+ grandmother may in fact sensitize—not tolerize—her RhD- daughters such that a fetus afflicted with erythroblastosis may occur even in primigravida.

Owen offered a "tentative" interpretation of this paradox. He speculated that the "kind or amount" of antibody produced in an Rh-incompatible, non-tolerized gestation allowed most infants to escape without diagnosable erythroblastosis and that the benefit

Table 1. Rh types of grandmothers of affected and non-affected children whose mothers had not been transfused and who were ABO compatible with their mothers from Taylor, 1967

	Rh-positive maternal grandmothers (%)	Rh-negative maternal grandmothers (%)	Total
Mothers with ABO-compatible affected child, any pregnancy	70 (63%)	41 (37%)	111
Mothers with no affected children in 3 or more Rh-positive, ABO-compatible pregnancies	19 (41%)	27 (59%)	46
Total	89 (57%)	68 (43%)	157

of no alloantibody production in tolerized gestations with RhD+ grandmothers was "overridden" by a more extended exposure to the antigen or other vague antibody characteristics.[19,20] There was (and still is) no experimental evidence for this idea, and so the debate continues.

A Critique of Owen (1953)

After Owen's key paper supporting the hypothesis that exposure to RhD antigen in utero was tolerogenic for RhD- fetuses, several reports have either found no association between grandmaternal RhD status[21,22] and future alloantibody formation[23,24] or in fact have found contradictory results suggesting that the in utero experience had been an immune stimulus.[25-28] There is no additional evidence beyond the initial report by Owen showing tolerance after intrauterine exposure to RhD, and although there are a few reports of sensitization to RhD in utero reported and discussed below, the weight of the current evidence suggests this is a rare event.

An important critique of Owen's report is that ABO incompatibility was not considered as a possible explanation for the "tolerant" phenotype in his subjects, despite Levine's discovery and description of this interaction in 1943[29]—many years prior to Owen's paper. ABO incompatibility is very important in HDFN since ABO-incompatible fetal red cells that mix with the maternal circulation at birth will be destroyed by pre-existing isohemagglutinins, thus reducing their potential to sensitize the mother. Given Owen's somewhat modest sample size, accounting for this factor may in fact have changed the inferences of his results, but we cannot know.

Two studies since Owen's report found no association with grandmaternal Rh status, but only one of these took ABO mating type into consideration. The older report from 1957 studied 173 RhD- mothers in Sydney, Australia who had made anti-D antibody, finding an excess of RhD- mothers as consistent with Owen's hypothesis (47.4% vs. their expectation of 41%), but the p-value for a chi-square test was between 0.05–0.1 and ABO compatibility was not determined.[21] In the second study showing no association, Mayeda did consider the importance of ABO mating type and still no difference in the likelihood of producing anti-D was found between RhD- vs. RhD+ grandmothers in 97 mothers ($\chi^2[2] = 0.829$, $P = 0.30$).[22]

Evidence of Sensitization: A Summary and Critique of Taylor (1967)

Contrary to Owen's hypothesis of tolerance, sensitization to RhD in some RhD- daughters of RhD+ women has been reported in four observational studies reported between 1967–1981. The largest study was reported by Taylor, who evaluated grandmothers of 236 families in Ohio in 1967, 157 of whom were ABO-compatible with only 46 families in the "no affected children" group. Sources of selection bias in this study include the fact that the group with erythroblastosis was drawn from a wide referral area, as the samples came from the only hospital in the area to treat these children; however, the comparison group of unaffected children was limited to only families in closer proximity to the prenatal Rh testing service at that hospital. Unlike Owen, she only examined the clinical outcome of erythroblastosis and did not determine alloimmunization status of women without erythroblastic children by an antibody screen. Women with anti-D who did not have erythroblastic children would not be detected in her study. "Affected" erythroblastic families were identified using gestational criteria similar to Owen (women with a child affected by HDFN with anti-D on or before their third pregnancy) and non-affected families (women with at least 3 RhD+ and unaffected pregnancies). Taylor's results in ABO-compatible pregnancies are summarized in **Table 1**.

Importantly, this study suffers from a misinterpretation of the expected proportions of RhD+ and RhD- grandmothers. In 1967, the genetic basis of the Rh blood group was not known. The genetics of Rh have since been extensively investigated and the molecular basis of most RhD- individuals of Caucasian descent has been identified as a deletion of the *RHD* gene that is inherited in an autosomal recessive manner.[30] The chi-square test that Taylor applied to the data in **Table 1** tests the null hypothesis that the proportions in each cell do not differ according to affected status, not that they depart from expectations determined by allele frequencies in the populations tested. Consequently, the reported P value of $P = 0.02$ for the χ^2 test of goodness of fit does indeed support the conclusion that the proportion of RhD+ grandmothers of affected children differs from unaffected children; but it does not evaluate whether that proportion (63%) differs from the expectation based on the allele frequency of the deletion allele in the studied population. In fact, if the Caucasian families in Ohio in 1967 showed a similar allele frequency to modern American Caucasians, then the expected proportion of RhD+ mothers is 61.3%, which is

not significantly different from Taylor's reported value of 63.1% (P = 0.78).

Taylor reported these findings as evidence of "sensitization of Rh-negative daughters by their Rh-positive mothers;" but the analysis above suggests that this is a misinterpretation of the data. In fact, the group of unaffected mothers showed lower proportions of RhD+ Caucasian grandmothers than expected (41% observed vs. 61% expected), which would actually support Owen's hypothesis of tolerance in utero, but with only 46 families, it is not surprising that these proportions are not statistically-significantly different (P = 0.06). This misinterpretation was clarified in 1980 by Jarl Eklund in his argument against a national program in Sweden to prophylactically administer RhIG to all Rh-negative female infants of Rh-positive mothers due to lack of compelling evidence of common in utero sensitization to RhD.[31]

The presence of erythroblastosis in primigravida motivated the remaining studies of sensitization to RhD by grandmothers. A small study in Portugal found no difference in the rates of erythroblastosis according to grandmaternal RhD status (n = 60, P = 0.22). Nonetheless, based on a "striking similarity" between proportions of first-affected erythroblastic children according to birth order (i.e., 20% of erythroblastic children with D+ grandmothers were first in birth order and 20% of erythroblastic children with D- mothers were second in birth order), these authors still advocated for RhIG administration to all RhD- newborn females, although a statistically-significant difference in proportions was not determined.[26] This stance was also promoted in a report of a series of 12 mother-infant pairs in the United Kingdom,[27] but due to a low prevalence (2%) and transient nature of antibodies, RhIG prophylaxis was not endorsed by a study in 96 Swedish mother-child pairs in 1981.[28] With routine antepartum RhIG administration at 28 weeks gestation or following fetomaternal hemorrhage, so-called "RhIG failures" now occur in only 0.1% of RhD-negative mothers.[23,32]

Should we Revisit the Grandmother Hypothesis Today?

No population-based study that adjusted for ABO-incompatibility found any statistically-significant effect of grandmaternal Rh status on the development of erythroblastosis of her grandchild or anti-D in her daughter. In addition to the studies of sensitization in primigravidae, the finding of so-called "naturally-occurring Rh antibodies" may also be interpreted as evidence of possible sensitization in fetal life.[24] Despite these many reports scattered across the decades that have failed to replicate either acquired tolerance or sensitization to RhD in utero, Owen's contribution remains an attractive concept as we still search for mechanisms of tolerance to any red cell antigen in alloantibody non-responders.

The evidence presented above includes reports that suffer from methodological problems, such as failure to exclude ABO-incompatible mother-child pairs to isolate the RhD-incompatibility, and data derived from serological methods that do not consider the molecular diversity of *RHD* and *RHCE* that modern investigations could explore. Quantification of maternal microchimerism in study subjects is lacking in these historical reports, but could be achieved today. Due to these technical and methodological limitations of earlier studies, modern investigators are better equipped to test Owen's hypothesis of tolerance than his own studies. The current awareness of the importance of non-inherited maternal antigens (NIMAs) in the field of transplantation, which was not as well-characterized in Owen's time, motivates modern reconsideration of the grandmother as a possible erythroid antigen source to sensitize her offspring.

A Role for NIMAs in Alloimmunization

Since a fetus is only haploidentical to its mother, non-inherited maternal antigens (NIMA) could exist for any of the numerous pleomorphic proteins arising from maternal heterozygosity.[33] Although NIMAs are typically considered in the context of HLA, this concept could apply to other proteins on the surfaces of any cells that may participate in maternal-fetal cell trafficking, particularly any maternal cells that may transfer to the fetus and establish a maternal microchimera.[34] In particular, NIMAs beyond HLA that are found on maternal peripheral blood cells, such as the blood group proteins found on mature erythrocytes, could gain access to the privileged fetal immune system during the critical window when tolerance may be established.[35] The mechanics and anatomy of placental trafficking are topics of intense investigation,[36,37] but maternal cell transfer appears to be a common event, as maternal microchimerism (MMc) has been observed in 39% of healthy adults in one study[38] and up to 70% of umbilical cord blood samples in another.[39] While the mechanisms of fetal tolerance to NIMAs are not completely understood, fetal regulatory T cells appear to play a role.[40]

Could tolerance to NIMAs from RBCs be transferred without concomitant transfer of mature RBCs (e.g., without frank fetomaternal hemorrhage) capable of sensitizing the fetus instead? The human placenta has recently become recognized as a potential hematopoietic organ,[41] including immature erythroid-lineage precursor cells (which may lack expression of mature alloantigens on blood group proteins)[42] that may be able to traffic into the fetus with minimal associated transfer of mature blood components. Such maternal stem cell traffic across the placenta could confer tolerance in the face of a co-incident transfer of mature blood cells that alone might constitute a likely allogeneic stimulus. These many possibilities imply additional research avenues including a close integration of placental biology and pathophysiology into the basic problem of tolerance to alloantigens from peripheral blood.

Tolerance in Transfusion vs. Transplantation

Do differences between transfusion and transplantation account for the excellent tolerance of most humans to allogeneic transfusion while transplant is constrained by many layers of histocompatibility? Unlike transplants, transfusions expose recipients to most donor cells for only a limited time. The peripheral blood is largely composed of mature cells that

have limited regenerative capacity, and unlike tissues selected for transplantation that must self-renew to achieve the desired post-transplant engraftment, over 99% of the cellular constituents of peripheral blood (i.e., erythrocytes and platelets) perform their desired functions in the recipient without dividing. In fact, to reduce important transfusion-related immune reactions such as transfusion-associated graft-vs.-host disease,[43] components (except peripheral blood-derived stem cells) can be irradiated. Transfusion is more similar to transplant when cells that do still retain a regenerative capacity are included in the blood transfer. Transfusion-associated microchimerism (TA-MC) is thought to occur in this setting, when each transfusion can be considered "blood transplantation."[44]

However, even without the establishment of TA-MC, the duration of exposure for allogeneic RBC antigens when transfused in therapeutic volumes can be as long as 110–120 days[1]—certainly sufficient exposure to expect some antigenic stimulation in most immunocompetent blood recipients. Although a proinflammatory cytokine response to murine[45] and canine[46] models of transfusion have been described, none was seen in a recent study of healthy human volunteers.[47] The mechanisms underlying this so-called "missing human inflammatory cytokine response to transfusion"[48] are not well-characterized, but reproductive physiological differences between humans and mouse and dog models of transfusion could play a role.

Evolutionary Importance of Multigenerational Immunity Involving Erythrocytes

Reproductive differences between species that may contribute to this missing inflammatory response include the differential importance of multigenerational immunity. Maternal microchimerism in blood antigens may provide a mechanism of immune tolerance that allows the features of one generation to have an important influence on the immune development of its progeny and could underpin a portion of the diversity observed in an individual's potential to generate alloantibodies to blood cells.

Mothers exert the greatest influence on her offspring early in development, but her impact declines as offspring age.[49] Maternal contributions to the immune system of her fetus include the transplacental transfer of antibodies, which, together with lactation and even the transfer of gut flora, provide an important trans-generational immune network.[50] The physical incorporation of successful responses to local pathogens through passive immunization has great adaptive value,[51] but trans-generational cell-mediated immunity is not well-characterized. Maternal and feto-maternal microchimerism may contribute to these strategies, but are poorly characterized.[52] RBC alloimmunization could fit into this multi-generational construct as part of some other process or perhaps as a balance with infectious challenges that are species-specific as part of a more complex ecology.

The immune response to allogeneic blood has been assumed to occur via the same processes as infectious agents or environmental antigens;[53] however, unlike exposures to pathogens, the mixing of allogeneic blood is an expected and necessary process in the reproductive process for eutherian mammals. According to the maternal layers retained in the placenta that separate maternal and fetal circulations, this organ is described as epitheliochorial (with a thick endometrial epithelium, connective tissue and uterine endothelium), endotheiochorial (which retain only uterine endothelium), or hemochorial (which retain no maternal barriers, such as the human condition) (http://placentation.ucsd.edu/homefs.html). Placentation is diverse among species[54,55] and it is clear from the anatomical differences in the fetal-maternal barrier that allogeneic mixing is expected to depend in a lineage-specific way. If a meaningful immune response were mounted during each gestation, in the same way a significant immune response should be mounted for each pathogen encounter, then one would predict this challenge to be a major selective force, resulting in decreased fertility and eventual extinction.

Future Directions

The current literature does not definitively end the debate over any putative or actual effect of maternal erythrocyte antigen exposure and tolerance or sensitization to those antigens in her offspring as required by the "grandmother hypothesis." Fetal blood can be detected in greater than 99% of human pregnancies,[56] although these fetomaternal hemorrhages occur without obvious clinical ramifications in most cases.[57] Although the mechanisms that control the immune response to allogeneic red blood cells are presently poorly-defined, they must include sufficient tolerance to permit such highly-prevalent blood mixing during gestation and parturition of eutherian mammals in a way that balances out the fitness advantages of placentation. Taking an evolutionary perspective focuses our search for the meaningful mechanisms that operate in alloimmunization to peripheral blood: The biological processes that manifest as "responder" vs. "non-responder" phenotypes to clinical allogeneic transfusion of stored blood products have been at work much longer than we have had blood on our shelves in the blood bank. Much future work needs to be done to fill this void and extend the early results and hypotheses Ray Owen and colleagues advanced about tolerance to RBC alloantigens, including comprehensive family studies of grandmaternal, maternal and fetomaternal microchimerism. Although family studies can be more labor-intensive than mass population-based strategies, the ability to comprehensively approach these questions of balance between reproductive and immunological systems are well worth the resources and well overdue.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Brunker PAR is supported by the National Heart, Lung, and Blood Institute of the National Institutes of Health under Award Number 2 K12 HL087169-06 Clinical Hematology Research Development Program at Johns Hopkins. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

I thank the editors for the opportunity to contribute to this special edition and acknowledge the ongoing mentorship of Dr Paul Ness. I value the suggestions of the reviewers and particularly acknowledge the contribution of Dr Karina Yazdanbakhsh in discussions of the immunological mechanisms of alloimmunization to blood and her critical review of the manuscript.

Authorship Contribution

Brunker PAR performed the literature review and wrote the manuscript.

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