ORIGINAL REPORT

Modeling alloantibody formation to high-incidence red blood cell antigens in immune responders using genotypic data

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Alloimmunization to red blood cell antigens is unpredictable and poorly understood. Patients who are negative for high-incidence antigens (HIAs) are at risk for developing the corresponding antibodies. Molecular methods can easily predict the lack of an antigen and thus, the risk of an individual to become immunized. We examined the prevalence and risk factors for HIA alloimmunization in patients at risk based on genotyping results. Genotyping using a molecular method (HEA BeadChip™, Immucor, Warren, NJ) was performed on all patient specimens referred for molecular testing over 45 months; serologic and clinical data were analyzed. We used simple and multiple logistic regression to model the risk factors for alloimmunization to an HIA. Of the 2591 patients genotyped, 32 (1.2%) were homozygous for at least one variant predicting absence of an HIA. Of these 32 patients, prior transfusion or pregnancy history was available for 29 (91%). Four susceptible patients made an antibody to an HIA (12.5% of all, 13.8% of those with a documented exposure). Two of these four patients (50%) had made an alloantibody to another antigen. The odds of forming an antibody to an HIA were not related to the total number of transfusions (p = 0.47), the total number of alloantibodies (p = 0.61), or diagnosis of sickle cell disease (p = 0.77) in simple logistic regression. Adjustment for the other two variables in a multiple logistic regression was also not significant for each variable (p = 0.6, p = 0.7, and p = 0.7, respectively). Although they had a known exposure to alloantigens through transfusion or pregnancy, 86.2 percent of patients (25 of 29) at risk for alloantibody formation to an HIA in fact did not mount an immune response to that antigen. Possible risk factors including the number of transfused units or the total number of alloantibodies made were not predictors of making an alloantibody to an HIA in our sampling. Our results suggest that other patient-specific risk factors for alloimmunization exist.
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Before the use of molecular technology, the detection of patients who are negative for high-incidence antigens (HIAs) remained largely impractical until these patients developed an antibody with HIA specificity. The development of multiplexed molecular platforms has uncovered a hidden population of patients at risk for alloimmunization against HIAs before any such antibodies are formed or detected.[1] By definition, high-incidence blood group antigens occur in greater than 99.9 percent of all individuals.[2] The rare individuals who lack these HIAs are at risk for alloimmunization. Serologic detection of "HIA-negative" donors or patients (expected, by definition, to constitute 0.1% of the general population) is not a routine practice due in part to scarce reagents and resources. Obtaining rare donor red blood cell (RBC) units for supportive therapy for patients with an antibody to an HIA can cause serious transfusion delays because of the rarity of suitable donors, making transfusion management for these patients an ongoing challenge.[3,4] If alloimmunization to an HIA does occur, a rare unit request to the local blood supplier and possibly to the American Rare Donor Program (ARDP) may be required. Although programs such as the ARDP have reported to fill 92 percent of requests for an HIA-negative component,[3] the need to request it from such a specialized agency rather than fill the request from units in available hospital inventory necessarily delays transfusion.

Beyond a delay in transfusion, the clinical relevance of alloimmunization to HIAs differs according to the specific antigen. Many alloantibodies to HIAs can cause serious clinical complications such as hemolytic disease of the fetus and newborn and transfusion reactions, as has been reported with antigens included on multiplexed molecular platforms such as k, Kp^b, Js^b, U, Lu^b, Di^b, Co^a, Jo^a, Hy, and Lw^a.[5] Although some in vitro methods have been developed to attempt to predict the clinical significance of a particular alloantibody in vivo (such as the monocyte monolayer assay), it is difficult to reliably predict the clinical importance of a given alloantibody in a given patient.[6] Consequently, avoidance of alloantigens in transfused components has been the mainstay for improving transfusion safety in alloimmunized patients. In the responder/non-responder model of alloimmunization,[7] individuals who have made an alloantibody to an HIA have demonstrated that they are an alloimmune "responder" and thus may be at higher risk of forming antibodies to the other common RBC antigens for which they are antigen-negative. Our institutional policy is to phenotype-match all alloimmunized patients for a full panel of clinically significant antigens for prophylaxis

in addition to their specific antigen-negative requirements; thus, the formation of an antibody to a single HIA renders the rare blood request for that patient even narrower because of the requirement for extended matching to the full panel of antigens.[8]

Alloimmunization to RBC antigens is a complex process that is incompletely understood.[9] Many factors have been suggested that may influence alloimmunization risk, including genetic predisposition,[7] mismatch between donor and recipient ethnicity,[10–12] class of antigen,[13] transfusion intensity,[14,15] clinical immune modulation,[16] and intervals between transfusions.[12] Despite conflicting data on risk factors for alloimmunization, the current paradigm requires, at minimum, that a recipient must be antigen-negative and exposed to an alloantigen for immunization to occur. The probability of exposure to HIA-positive RBCs when transfusing units found in a typical hospital inventory is very high. We exploited this previously hidden HIA-negative transfused patient population to study risk factors for alloimmunization in general, since these patients met the minimum criterion of being antigen-negative, but were likely to have been transfused with antigen-positive blood.

We selected a panel of HIAs that were available on our genotyping platform to model the risk factors for alloimmunization in transfused patients who are negative for at least one HIA and therefore at risk for alloimmunization to that HIA. We examined the clinical and serological data of patients who are negative for HIAs and used simple and multiple logistic regression modeling techniques to estimate the risk of alloimmunization based on diagnosis, number of transfusions, and total number of detected alloantibodies.

Materials and Methods

Study Population

EDTA-preserved blood samples were obtained from July 2009 to March 2013 from 2591 patients who had samples referred for molecular testing at our transfusion medicine reference laboratory. Referral criteria according to institutional standard operating procedures were specimens submitted to the transfusion medicine laboratory that demonstrated any alloantibody (including those of inconclusive specificity) or warm- or cold-reactive autoantibody on immunohematology screening, selected specimens submitted for a suspected delayed serologic or hemolytic transfusion reaction, or specimens from all patients with a diagnosis of sickle cell disease (SCD) or from potential bone marrow transplant donors for a patient with SCD. We have identified patients who were negative for the following HIAs: k, Kp^b, Js^b, U, Lu^b, Di^b, Co^a, Jo^a, Hy, LW^a, or Sc1.

Genotyping and Phenotyping

An automated DNA extractor (QIAcube, QIAGEN, Inc., Germantown, MD) was used to extract the DNA, and genotyping was performed to predict RBC antigens using a molecular method (HEA BeadChip™, BioArray Solutions, Immucor, Warren, NJ) according to manufacturer instructions. Alloimmunization status was performed using a two-cell antibody screen by a solid-phase method (Capture-R Ready Screen 2, Immucor, Norcross, GA) on an automated analyzer (Galileo, Immucor) at the time of sample processing, according to institutional standard operating procedures. Patients were categorized as alloantigen responders if they had ever demonstrated an antibody to any RBC antigen.

Data Extraction

Using a search engine–created database (Phetch, Immucor), we retrospectively chose patients who were negative for an HIA from July 2009 to March 2013. Additional clinical information including transfusion history (continuous variable measured as number of RBC units transfused), alloimmunization status (continuous variable measured as number of alloantibodies), and patient diagnosis (dichotomous variable measured as SCD vs. other diagnosis) were obtained using the internal computerized medical record, and specimens were anonymized for analysis. The outcome was alloimmunization to an HIA (measured as a dichotomous variable).

Statistical Analysis

We performed a retrospective cohort study and used simple and multiple logistic regression and exact methods (STATA, Statacorp, College Station, TX) to model the risk factors for the outcome of HIA alloimmunization status (dichotomously as responder vs. non-responder) in this susceptible and highly transfused group. Logistic regression modeling, which is a common method in risk factor analysis and is particularly useful in genetic epidemiology, was used because we were evaluating a dichotomous outcome (responder vs. non-responder) and considering explanatory variables that were both continuous and categorical. The multiple logistic regression model included diagnosis, number of transfusions, and total number of alloantibodies as covariates (which allow adjustment for variables included in the model), and the simple logistic regression evaluated each of these variables individually.

Results

A total of 2591 patients were genotyped (Table 1). Only 32 (1.2%) of these patients were predicted to be antigen-negative for one HIA because of homozygosity for a low-frequency variant, consistent with the definition of an HIA. Transfusion and pregnancy history was obtained for 29 of the 32 (91%) patients. These data were abstracted from the patients' medical records at our institution, so data may be limited for any patients who received care at other institutions. Eight (25%) patients were negative for more than one HIA, six were negative for both Hy and Jo^a in the Dombrock system, and two were negative for both Jo^a and U in the Dombrock and MNS systems, respectively (Table 2). Four patients made an antibody to an HIA (12.5% of all 32 patients; 13.8% of patients with a documented exposure). Therefore, 25 (86.2%) of the 29 patients who were at risk of HIA alloimmunization did not form an alloantibody, despite significant RBC exposure in this group (range of RBC units transfused was 1–521 units, mean 51.8 units, median 19 units). One of eight Js(b–) patients demonstrated anti-Js^b, and three of seven U– patients developed anti-U (Table 2). Our HIA panel included two antigens encoded by the same gene, specifically Hy and Jo^a, which are single-nucleotide polymorphisms (SNPs) in the ADP-ribosyltransferase 4 (*ART4*), responsible for the Dombrock blood group.

Table 1. Description of the study population tested by HEA BeadChip

Variable	Number of patients (*N* = 2591)	%
Female	1649	63.6
Patients with sickle cell disease	406	15.7
Female	243	
Bone marrow transplant status		
Recipients	20	0.7
Donors	48	1.9
Antibodies		
Alloantibodies	2119	81.8
Warm autoantibodies only	77	3.0
Cold autoantibodies only	13	0.5
None	382	14.7
Race		
Black	1381	53.3
White	1078	41.6
Asian	31	1.2
Hispanic	13	0.5
Other	88	3.4

Table 2. HIA-negative patients with and without HIA alloantibodies

By patient, allowing for individuals to be negative for multiple HIAs

Rare RBC antigen phenotype	Number of patients with rare type	Number of patients with alloantibody to HIA	Number of patients with other alloantibody(ies) (%)
k–	1	0	1 (100)
Js(b–)	8	1	8 (100)
U–	5	2	4 (80)
U–, Jo(a–)	2	1	2 (100)
Co(a–)	2	0	2 (100)
Lu(b–)	1	0	1 (100)
Hy–, Jo(a–)	6	0	5 (83)
Jo(a–)	7	0	5 (71)
Total	32	4	28

By antigen, only for consideration of immunogenicity

Rare RBC antigen phenotype	Number of patients with rare type	Number of patients with alloantibody to HIA
k–	1	0
Js(b–)	8	1
U–	7	3
Co(a–)	2	0
Lu(b–)	1	0
Hy–	6	0
Jo(a–)	15	1

HIA = high-incidence antigen; RBC = red blood cell.

Two other patients were negative for two HIAs that are encoded by separate genes and separate chromosomes: U is encoded by glycophorin B (*GYPB*) on chromosome 4 and Jo^a in *ART4* on chromosome 12. Two of the four patients with antibodies to HIAs (50%) had alloantibodies to other blood group antigens. Two patients had no antibody screen data available. Using simple logistic regression on the 29 patients with complete data, the odds of forming an antibody to an HIA were not related to the total number of transfusions ($p = 0.47$), the total number of alloantibodies found ($p = 0.61$), or diagnosis of SCD ($p = 0.77$). Adjustment for each of these variables in a multiple logistic regression model including all three covariates was not significant for any variable ($p = 0.6$, $p = 0.7$, and $p = 0.7$, respectively).

Discussion

Determining alloantigen exposure to so-called minor RBC antigens in transfused populations is challenging, because blood components are not routinely phenotyped or genotyped beyond ABO and D; similarly, most recipients are

not phenotyped or genotyped for these additional antigens. We therefore selected a group of patients known to be negative for an HIA because any transfusion or pregnancy exposures experienced by this group would, by definition, have a greater than 99 percent chance of exposing them to a foreign antigen, compensating for the fact that minor antigen data were not directly available for each transfused unit or for each fetus. To best model the problem of alloimmunization to RBC antigens, it is important to characterize at-risk patients (e.g., to define an antigen-negative population), gather exposure details, and test for antibodies post-exposure. Our approach has included all of these important variables.

In our study, 25 (86.2%) of the 29 patients with known, documented exposure to foreign antigens from RBC transfusion or pregnancy failed to produce an antibody to an HIA. Because our study population was drawn from those patients who were referred for molecular testing and our indications for referral included identification of any RBC alloantibody, it is not surprising that 27 of 32 (84%) of our HIA-negative group had at least one alloantibody and would be characterized as alloimmune "responders." Although this selection bias is a limitation of our study, even in a population so enriched for alloimmune responders, we still found that 86 percent of HIA-negative patients were actually tolerant to HIA exposure. Details of the four HIA responders (Table 3) reveal that they were female, only one had SCD, and two of them lacked other RBC alloantibodies other than that to the HIA. Unfortunately, because 100 percent of the HIA-alloimmunized patients were all female, we could not include gender in our regression models. Only 63.6 percent of our total sample was female, but these proportions are not statistically significantly different in this sample (χ^2_1, $p = 0.13$), likely attributable to the wide sampling error because of the rarity of HIA alloimmunization in the cohort.

Ten patients who were negative for an HIA had SCD, five of whom also had either previous history of an antibody or developed additional antibodies at our institution. Our regression models did not support SCD as a risk factor for alloimmunization in our cohort. One strength of regression modeling is the ability to adjust for multiple possible covariates or confounders by comparing risk estimates used in a multiple variable approach to those obtained using a simple regression model that only evaluates a single variable at a time. We exploited these features of regression analysis to allow consideration of three important putative explanatory variables: the total number of transfusions, the total number of alloantibodies, and the diagnosis of SCD. We selected these three attributes as possible risk factors based on a combination of biological plausibility or prior epidemiologic evidence. The number of prior transfusions is an appealing potential risk factor for alloimmunization because the notion of repeat immune stimulation to generate strong humoral responses is a well-known phenomenon that is exploited in vaccination, where a schedule of repeated exposures is routine. The total number of alloantibodies may be a risk factor for HIA alloimmunization because it reveals that a patient has the capability to respond to other RBC antigens. Last, we selected SCD diagnosis as a possible HIA alloantibody risk factor because other investigators have reported high rates of alloimmunization in this patient population.[11]

There was no increased risk of alloimmunization to HIAs according to the three important putative risk factors in our study; therefore, these factors do not satisfactorily explain the variation in alloimmunization risk that we observed in our patient cohort. To therefore identify risk factors that may explain this variation, future work includes expanding our study to include additional patient-specific risk factors other than the three we examined here. Additional risk factors for consideration in future modeling could include patient gender (which we could not model explicitly in this small study because all four patients who experienced the HIA alloimmunization event were female, and therefore there was no variation in that attribute), independent indicators of a proinflammatory state at the time of transfusion, markers of immune tolerance such as regulatory T-cell development, polymorphisms in genes related to innate or humoral immunoregulation, age of blood component transfused, or blood donor attributes, among a host of other creative hypotheses.

Table 3. Summary of patients with alloantibodies to HIAs

Patient	Age (years)	Gender	RBC antigen phenotype	Antibody to HIA	Diagnosis	Other antibodies	History of RBC transfusion
1	78	F	Js(b–)	Anti-Js^b	Renal failure	Anti-C, -E, -Jk^a	Multiple
2	28	F	U–	Anti-U	Pregnancy	None	Unknown
3	40	F	U–	Anti-U	Sickle cell disease	Anti-D, -C, -E, -Fy^a, -Jk^b, -Le^a	Multiple
4	30	F	U–, Jo(a–)	Anti-U	Pregnancy	None	None

HIA = high-incidence antigen; RBC = red blood cell.

Seven of the 15 patients who were Jo(a–) had developed an inconclusive reactivity that could suggest the potential to form an antibody to an HIA, but because these antibodies did not demonstrate conclusive reactivity, these patients were scored in our study as non-responders to HIAs. This conversion from an inconclusive to a potential antibody to an HIA could represent early anamnestic response.[11] We also included the number of transfused packed RBC units in our model, but this variable was not associated with alloimmunization to HIAs in our sample in either simple or multiple logistic regression analyses. On further review, one Jo(a–) patient received a total of 521 RBC units and still was not immunized to this antigen, demonstrating the complexity of the immune response despite intense exposure. The extent to which differential immunogenicity across RBC antigens is responsible or contributing to the tolerance to HIAs that we observed is difficult to quantify.

Immunogenicity has been described on a population level by dividing the probability of an antigen-positive donor by the probability that an individual recipient may be antigen-negative, as described by Giblett's equation[17] and modifications thereof, to include phenomena such as evanescence.[13] Attempts to calculate immunogenicity using these methods for HIAs necessarily results in extremely low "potency," because the probability of being antigen-negative (which is near 0) is divided by the probability of exposure (near 1), so the product is near 0. For an individual patient, however, such as any of those who were HIA-negative in our study, the probability of being antigen-negative is 1 (because it was empirically determined) and the probability of exposure to an antigen-positive unit remains at nearly 1, so immunogenicity by Giblett's method would be near 1, which is not consistent with our data. Therefore, we suggest that some other biological processes are likely contributing to immunogenicity of HIAs that are not captured in the population-based estimates developed by Giblett. Further research on membrane biochemistry, including antigen density or processing and presentation of HIAs in antigen-presenting cells, may help clarify some of this unclear biology.

We were unable to identify a risk factor associated with HIA alloimmunization in our study. If the transfusion community could identify specific risk factors associated with an increased risk of alloimmunization to HIAs or other RBC antigens, there would be important implications for clinical transfusion medicine practice, particularly in terms of triaging rare blood. If one could reliably predict the alloimmune responders, then perhaps we could confidently transfuse the remaining patients with HIA-positive blood and reduce transfusion delays without compromising transfusion safety. Until then, there is still an important role for routine donor genotyping to include testing to predict HIAs to provide antigen-negative blood.

An important strength of our approach is that we characterized at-risk, antigen-negative recipients with known exposures to foreign antigens. Alloimmunization to the HIAs defined in this study is uncommon even in patients who have been heavily transfused. Although they had a known exposure to allogeneic blood through transfusion and/or pregnancy, 86.2 percent of patients at risk for alloimmunization to an HIA in fact did not mount an immune response to that antigen. Possible risk factors for alloimmunization, including the number of RBC units transfused, SCD, or the total number of antibodies formed, were not predictors for making an antibody to an HIA in our study. Differential immunogenicity of the HIAs based on mechanisms or properties that have not been characterized may contribute to the high tolerance we observed, but our results more generally support the notion that other patient-specific risk factors for alloimmunization, such as a genetic basis to a "responder" phenotype, exist.

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