Red Blood Cell Alloimmunization in Sickle Cell Disease: Listen to Your Ancestors

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Summary

Red blood cell (RBC) alloimmunization occurs in approximately 30% of transfused sickle cell disease patients compared to 2–5% of all transfusion recipients. Because RBC transfusion is an important part of therapy in sickle cell disease, the need for additional antigen matching once alloimmunization occurs is problematic and leads to therapeutic limitations. Thus, identification of risk factors would benefit this patient population. Genome-wide analyses, in particular, methods which take into account genetic ancestry such as admixture mapping, could identify molecular markers which could be used to identify immune responders to transfusion.

Introduction

We all grow up with the weight of history on us. Our ancestors dwell in the attics of our brains as they do in the spiraling chains of knowledge hidden in every cell of our bodies. – Shirley Abbott (born 1934)

Alloimmunization to red blood cell (RBC) antigens occurs at a rate of 2–5% among all transfusion recipients. Sickle cell disease (SCD) patients receive RBC transfusions for treatment of acute chest syndrome or acute stroke, or are often on chronic transfusion regimens for prevention of stroke recurrence. Among SCD patients transfused with RBCs matched only for ABO and D antigens, the rate of alloimmunization to

non-ABO RBC antigens is much higher than for other transfused patient populations, ranging from 18 to 37% [1–3]. The reasons for this finding include the differences in RBC antigen expression frequencies between the mostly Caucasian donor base and the mostly African-American SCD patients. For example, the RBC antigen expression differences for Duffy are striking: 68% of African-Americans are negative for both Fy^a and Fy^b compared to 34% of Caucasians who are negative only for Fy^a and 17% who lack only Fy^b [4]. However, one of the most common alloantibodies made by SCD patients is against the Rh antigen E, for which the antigen expression frequency between donor and recipient is similar. Immunogenicity of blood group antigens thus must also be taken into account as well as the volume of transfusion which usually increases the risk of exposure to foreign antigens. This last notion may be challenged by the recent finding that among pediatric SCD patients receiving chronic RBC exchange transfusions, when compared to patients receiving simple transfusion, the exchange transfusion patients actually had lower rates of alloimmunization (0.04 antibodies/100 units vs. 0.171 antibodies/100 units; $p = 0.04$) [5]. In addition, in populations of SCD patients who receive blood transfusion from donors of similar racial/ethnic background, the rate of RBC alloimmunization is still greater than expected. A study of the rate of RBC alloimmunization in SCD patients in Uganda showed that the rate of alloimmunization was 6.1% after receiving up to 10 transfusions [6]. In Kuwaiti Arab SCD patients the rate of RBC alloimmunization was retrospectively assessed after assignment to either receipt of only ABO- and RhD-matched transfusion (group 1) or receipt of ABO, RhD and Kell antigen-matched transfusion (group 2). 65% of group 1 patients produced clinically significant RBC alloantibodies, in group 2, 23.6% of patients developed RBC alloantibodies [7].

RBC Alloimmunization in SCD: Cost Benefit of Prophylactic Antigen Matching

In recent years, phenotypic matching protocols have become more widely used for SCD patients in order to prevent RBC alloimmunization. Such protocols include matching for C, E, and K1 even for non-alloimmunized patients, to extended antigen matching for patients who have made one or more alloantibodies. A survey of 1,182 North American transfusion laboratories found that the majority of laboratories (63%) did not provide prophylactically phenotypically matched RBCs for non-alloimmunized patients. The remaining 37% of services did provide prophylactically matched RBCs, most commonly for Rh antigens C and E, and K1 [8]. One concern often raised by transfusion services that do not provide prophylactically antigen-matched RBCs is that it would not be feasible to obtain enough antigen-matched RBCs on a routine basis for this patient population. A nationwide study of pediatric patients receiving RBC transfusion for stroke prevention demonstrated that the provision of C-, E- and K1-matched RBCs was feasible, with only 16% out of 1,632 transfusions inadvertently unmatched for the necessary antigens. The alloimmunization rate during the study was 8% [9]. Such transfusion protocols are not without cost. A Markov model of the financial impact of prospective antigen matching for SCD patients found that matching for C, E, and K1 cost an additional USD 765.56 million over 10 years, but would result in 2,072 fewer alloimmunization episodes. Extended antigen matching beyond Rh and Kell antigens could cost USD 1.86 billion more over 10 years for all transfused SCD patients in the USA, rather than providing extended antigen-matched RBCs after a patient has already made one or more alloantibodies [10].

Rh variants are more commonly identified in populations of African descent, and thus this impacts RBC alloimmunization in SCD patients. *RHD* and *RHCE* are a pair of genes which share 93.8% homology over all introns and coding exons. They are closely linked but in opposite orientation: 5'-*RHD*-3'-3'-*RHCE*-5' [11]. In Rh(D)-negative persons, there is usually a complete absence of the D protein, with the C/c and E/e antigens present. Homozygosity for the complete deletion of the D antigen is commonly found in populations of European descent; however, in 67% of people of African descent who are Rh(D)-negative there is at least one copy of a pseudogene, *RHD** ψ , which encodes a *RHD* gene inactivated by a 37 base pair duplication in exon 4, and a nonsense mutation in exon 6 [12]. Indicative of the burgeoning utilization of molecular methods in the study of RBC antigens, over the past decade over 200 *RHD* and *RHCE* variant alleles have been reported. Partly to evaluate the impact of the identification of these variants, a 15-year retrospective study of patients with SCD transfused with RBCs prospectively matched for D, C, E, and K1 from primarily African-American donors was undertaken. In 182 patients, 58% of chronically and 15% of

occasionally transfused patients had formed RBC alloantibodies. Of 146 alloantibodies identified, 91 were unexplained Rh antibodies – 56 antibodies in patients whose RBCs typed positive for the antigen in question, and 35 in patients whose RBCs were negative for the antigen but who were transfused with only Rh-matched RBCs. High resolution *RH* genotyping found 1 or more variant *RH* alleles in 87% of the patients. 17% of serologically D-positive patients with only variant *RHD* had formed anti-D. 40% of those inheriting only the hybrid *RHD**DIIIa-*CE*(4-7)-D, which encodes partial C, made anti-C. 19% of those with only variant *RHCE**ce produced anti-e [13]. Just as more centers are accepting the idea that it is difficult, yet feasible, to obtain prophylactically C-, E- and K1-matched RBCs for transfusion for SCD patients, we are now faced with the proposition that matching by serologic antigen type may not be good enough for patients with Rh variants. Matching donor and recipient by Rh variants is less likely to be as practicable as serologic antigen matching, although studies of this protocol have not been performed just yet. Such practice will also probably prove to be more expensive than serologic testing, and taking into consideration the already high costs of prophylactic antigen matching, it is prudent to consider other approaches, especially in light of the fact that although approximately 30% of transfused SCD patients have RBC alloantibodies – approximately 70% of them do not. A strategy for determining which patients are likely to derive the greatest benefit of prophylactic antigen matching, i.e. likely high immune responders versus low immune responders, would be beneficial. If a method for risk stratification was available, extended antigen-matched RBCs, even potentially those matched by Rh variant, could be reserved for likely high immune responders, conserving an already rare resource.

SCD: Genetic Background and Ancestral Admixture

SCD is seen in people of Mediterranean, Middle Eastern and Southeast Asian ancestry, but the greatest concentration of individuals carrying the sickle globin (β^S) gene are in central West Africa, where the gene frequency may be as high as 0.14. In the USA, 10% of African-Americans are carriers, with a gene frequency of 0.04 to 0.05 in persons of African descent in North America. Compound heterozygote states, such as sickle cell hemoglobin C (HbSC) disease, HbS β -thalassemia (sickle cell/ β -thalassemia) make up the remainder of cases of SCD in African-Americans [14]. Interestingly, the distribution of these compound heterozygote states differs geographically. In university hospitals in Nigeria and Senegal, the HbSS genotype was seen in 88–96% of patients, and HbSC ranged from 4 to 12%, with HbS β -thalassemia at <1%. This is compared to cohorts from Greece and India, where the predominant genotype is HbS β -thalassemia (81% and 64%, respectively) compared to HbSS cases (19 and 36%, respec-

tively). By contrast, in an urban hospital in Burkina Faso, HbSS occurred in 50% of the patients with SCD, with the other 50% having HbSC. In the USA, HbSS is predominant among those with SCD (75% in the PUSH cohort), with 18% having HbSC, and 4% having HbS β -thalassemia [15].

What the above areas outside the USA all have in common, however, is that within these regions malaria is endemic. The global distribution of not only the *HbS* gene but also other hemoglobinopathies such as E-, α - and β -thalassemias, glucose-6-phosphate dehydrogenase deficiency, and South-east Asian ovalocytosis was strongly impacted by the distribution of endemic malaria [16]. The genes for the above disorders are presumably thought to have arisen because of the significant protection that heterozygotes have against *Plasmodium falciparum*. In the case of *HbS*, this association has been alluded to since 1949; however, it has been more recently confirmed using *HbS* allele frequency data from the Malaria Genomic Epidemiology Network Consortium (MalariaGEN, www.malariagen.net) which was adjusted to exclude non-indigenous populations and mapped versus malarial endemic regions as they were defined prior to attempts to control the parasite. The findings support the malaria hypothesis globally, with a strong relationship in Africa but an unresolved relationship in the Americas and in Asia [17].

It is not a new concept that 'race' may predispose individuals to inherit genes that increase the probability of some diseases. Why then, is the term 'race' problematic? Early definitions by anthropologists and geneticists were centered on skin color, hair texture, head shape and size, but definitions of race have come to include social and cultural characteristics. The ability to acquire genetic data from various populations has demonstrated that there are actually far greater within-group differences than between-group differences. Therefore, this terminology does not actually explain human biological variation, as it is based on socio-cultural meanings and colloquial terms. Additionally, when used as a causal genetic variable this term perpetuates biological determinism. Ancestry, however, is a more useful index for human biological variation. When used as an index for genetic background, it allows for a better investigation of biological risk factors. Haplotype studies of African populations have demonstrated larger numbers of haplotypes, less linkage disequilibrium, and a larger degree of population stratification than non-African populations. These characteristics can be leveraged for studies of genetic diseases, particularly when there has been extreme selection pressure (such as malaria). What about African-Americans, who comprise most SCD patients in the USA and who have a unique population history, often originating in West Africa (but sometimes East Africa) and admixing with European, South American and Asian populations? An emerging popular approach to mapping susceptibility genes in recently admixed populations is called admixture mapping. This novel approach attempts to find genes that underlie ethnic differences in disease risk [18, 19]. Recent gene flow (admixture)

between long separated populations creates linkage disequilibrium (LD) between loci which can extend over large chromosomal regions (exceeding 30 cM). In this approach, genome-spanning ancestry informative markers (AIMs) are typed in the clinical population to infer ancestry at each locus and then test locus ancestry for association with the trait of interest in the population. A greater degree of genetic West African ancestry has been associated with a trend for increasing prediction of prostate cancer diagnosis linked to prostate-specific antigen levels [20]. The role of genetic admixture among African-Americans has also been investigated in breast cancer and diabetic nephropathy [21, 22]. Thus, African-American SCD patients are uniquely situated for evaluation of ancestral admixture and genes that may play a role in alloimmunization.

RBC Alloimmunization: Genome-Wide Analyses and Molecular Markers

Tatari-Calderone et al. [23] hypothesized that the incidence of RBC alloimmunization in SCD patients was influenced by linked inheritance of the hemoglobin beta S allele and a C/T single nucleotide polymorphism (SNP) (rs660) in the nearby *Ro52* gene. 50% of T/T homozygous and 42.9% of C/T heterozygous patients had RBC alloantibodies. Increased expression of *Ro52* was associated with the T/T genotype. The authors believed that these results suggest that rs660 is a marker of efficiency of immune competence development to RBC antigens in SCD patients; rs660 could decrease the expression of TRIM21, an intracellular antibody effector, resulting in a loss of a negative feedback pathway and increased RBC alloimmunization. Recently, this hypothesis was examined in a murine model of platelet and RBC transfusion using TRIM21 KO recipient mice [24]. Significant increases were not seen in the frequency or magnitude of RBC or platelet alloimmunization in TRIM21 KO recipient mice. Decreased expression of TRIM21 is therefore unlikely to independently determine an enhanced immune response to RBC alloimmunization. Other factors involving the cellular regulation of alloimmunization are likely to account for the increased rate of alloimmunization in these patients.

The completion of the mapping of the human genome paved the way for use of this information in genome-wide association studies (GWAS). GWAS are a method of scanning the genome of individuals with a particular disease, and comparing it to that of those without the disease, looking for SNPs that are found significantly more often in one of the two samples. An example of a significant finding made using this technique has been the identification of complement factor H to be a causal factor for age-related macular degeneration [25]. One issue with GWAS studies has been the identification of moderate- or low-effect SNPs that do not reach the statistical significance required for GWAS (generally $p \leq 10^{-8}$) when

complex disease processes, such as the immune response to transfusion, are examined [26]. For example, Dworkis et al. [27] performed a GWAS of alloimmunization responders with SCD. In their study of 570 subjects, 25.3% developed at least one red cell alloantibody. No SNPs reached genome-wide significance although multiple SNPs were nominally associated with alloimmunization response (VAV2, CFH, and chromosome 6 HLA cluster). Although these findings are encouraging, a major limitation of these RBC alloimmunization and other GWAS studies in African descent populations is the limited coverage of African genetic variation by the previous Illumina (San Diego, CA, USA) and Affymetrix (Santa Clara, CA, USA) 6.0 SNP Array GWAS platforms (only ~60% coverage for African populations). Given the much higher genetic variation in individuals of African ancestry, the GWAS array used should provide optimum coverage for African descent populations.

The Sick Cell Center at the University of Illinois Hospital and Health Sciences System treats a large population of pediatric and adult patients with SCD in the Chicago region and includes many patients undergoing chronic as well as episodic RBC transfusion. Until 2007, our center did not perform any prophylactic RBC antigen matching and only provided antigen-negative RBCs when SCD patients had a clinically significant RBC alloantibody identified. Between 2007 and 2014, we began providing extended antigen matching in the Rh (C, c, E, e), Kell (K1), Kidd (Jk^a, Jk^b), and Duffy (Fy^a, Fy^b) blood groups and for S/s antigens, whenever possible, for patients who had a history of alloimmunization to one or more clinically significant RBC antigens. Patients who did not have a history of RBC alloimmunization received ABO- and Rh(D)-matched RBCs. Beginning in 2014, our center started providing prophylactically C-, E- and K1-matched RBCs for SCD patients without a history of RBC alloimmunization. SCD patients with one or more clinically significant RBC alloantibodies continue to receive extended phenotypically matched RBC. These changes were made to prevent continuing high levels of RBC alloimmunization in this population, but have had, as noted earlier, quite an economic impact. Given this important patient population, we set out to determine if, using a SNP array more specific for populations of West African ancestry, we could identify candidate genes that were associated with RBC alloimmunization [28]. We reviewed the transfu-

sion records of 256 SCD patients from whom DNAs were available. 33.6% of the patients had RBC alloantibodies; the most frequent were anti-C, anti-E, and anti-K1. Patients were genotyped for 2,217,402 SNPs using the Axiom™ Genome-Wide PanAFR Array (Affymetrix). This array offers high genomic coverage (>85%) for alleles with frequencies >2% in populations with West African ancestry. SNPs with a call rate <90%, a Hardy Weinberg equilibrium $p < 0.0001$, or having a minor allele frequency <1% were excluded from the analysis. In total, 2,113,177 SNPs were analyzed in 256 individuals. The population structure was examined by principal components analysis. No outliers were identified, and no samples were excluded. We calculated p values, odds ratios, and 95% confidence intervals by logistic regression models using age, gender, and West African ancestry as covariates. To account for multiple testing, a genome-wide significance threshold was set at $p < 2.4 \times 10^{-8}$. Logistic regression analyses revealed that female gender ($p = 0.003$) and percent of African ancestry ($p = 0.03$) were associated with RBC alloimmunization, but interestingly age ($p = 0.90$) and number of transfusions ($p = 0.20$) were not significant in our cohort. None of the SNPs reached genome-wide significance for the association with RBC alloimmunization; however, we are currently continuing investigation with additional samples in order to increase statistical power.

Conclusion

In summary, RBC transfusion is a cornerstone of therapy for SCD. Thus, when RBC alloimmunization occurs, it poses significant problems for these patients. Prevention has centered on provision of phenotypically matched RBCs, but the effectiveness of this is limited by the incidence of RH variants as well as the supply of appropriate phenotypes. The identification of markers for those likely to become alloimmunized would aid in identifying which patients would most benefit from phenotypically matched RBC transfusions.

Disclosure Statement

The authors declare that they have no conflict of interest.

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