Genotyping Applications for Transplantation and Transfusion Management

The Emory Experience

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• Current genotyping methodologies for transplantation and transfusion management employ multiplex systems that allow for simultaneous detection of multiple HLA antigens, human platelet antigens, and red blood cell (RBC) antigens. The development of high-resolution, molecular HLA typing has led to improved outcomes in unrelated hematopoietic stem cell transplants by better identifying compatible alleles of the HLA-A, B, C, DRB1, and DQB1 antigens. In solid organ transplantation, the combination of high-resolution HLA typing with solid-phase antibody identification has proven of value for highly sensitized patients and has significantly reduced incompatible crossmatches at the time of organ allocation. This database-driven, combined HLA antigen/antibody testing has enabled routine implementation of "virtual crossmatching" and may even obviate the need for physical crossmatching. In addition, DNA-based testing for RBC antigens provides an alternative typing method that mitigates many of the limitations of hemagglutination-based phenotyping. Although RBC genotyping has utility in various transfusion settings, it has arguably been most useful for minimizing alloimmunization in the management of transfusion-dependent patients with sickle cell disease or thalassemia. The availability of high-throughput RBC genotyping for both individuals and large populations of donors, along with coordinated informatics systems to compare patients' antigen profiles with available antigen-negative and/or rare blood-typed donors, holds promise for improving the efficiency, reliability, and extent of RBC matching for this population.

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In the era of modern molecular pathology, patients can have their entire HLA antigen genome sequenced from a single swipe of buccal mucosa or an individual hair root. This technologic feat represents a huge advance in a field that emerged in the 1950s, when the first successful, live-donor, human kidney transplant was performed between identical twin brothers.[1] Another major milestone occurred in 1957, when a patient with leukemia was successfully treated with radiotherapy and subsequent bone marrow transplant from an identical twin.[2] Although these identical-twin transplants were successful and represented a paradigm shift in the field of transplantation, transplant procedures between nonidentical donor-recipient pairs proved more problematic for reasons that were not entirely understood at the time.[3,4] The breakthrough in unlocking this mystery came in 1958, when Jean Dausset discovered the human major histocompatibility complex (henceforth, referred to as the *HLA complex*), which shed much needed light on transplant biology. This key discovery was paramount, and ultimately, the concept of immunologic donor-recipient compatibility (ie, histocompatibility) was born.[5]

Following the work of Dausset,[5] the clinical utility of HLA histocompatibility testing was solidified in 1969 by the seminal observations of Patel and Teraski.[6] They showed that, similar to the use of routine red blood cell (RBC) crossmatches to detect recipient antibodies that could cause posttransfusion hemolysis, a positive pretransplant crossmatch, using recipient serum and donor lymphocytes, frequently predicted hyperacute renal allograft rejection.[6] The white blood cell antibodies detected by this test were later confirmed to be HLA specific. This study concluded that proceeding to kidney transplantation without performing a pretransplant HLA crossmatch was malpractice.[6] To this day, one of the main functions of an HLA laboratory supporting solid organ-transplant programs is to identify recipient HLA antibodies that can cause early allograft rejection. To meet this challenge, the laboratory is equipped with an array of

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new technologies. Beginning with a description of early HLA testing methods, the next sections will describe the evolution of histocompatibility testing and how those tests are currently used to improve patient outcomes.

IN THE BEGINNING

Before DNA-based HLA typing and solid-phase antibody-detection methodology became the standard of practice, there was serology. Serologic testing was critical for discovery of HLA antibodies and the antigens to which they reacted. Reactivity between unknown antisera and cells of known phenotype or between well-characterized antisera and cells of unknown phenotype were assessed in early crossmatch assays to determine the specificity of sera and their cognate antigens. A novel antigen would be defined when antisera exhibited a new pattern of reactivity not previously characterized. For example, given serum 1, which reacted with 5 distinct cells, and serum 2 that reacted with 5 different cells, a new antigen would be defined when a third serum was found that reacted with a subset of cells from each of the previous 2 groups. In time, antisera were identified that would react with only a subset of cells expressing a previously described specific HLA antigen. This selective reactivity divided the previously known HLA antigen into to new antigen subgroups or *splits* of the original "parent" antigen.[7] Similarly, investigators would identify sera reactive with multiple cells each expressing a previously established, but unique, HLA antigen. For instance, 5 cells containing distinct HLA antigens (eg, B7, B13, B42, B55, B81) can nonetheless react with a single antiserum because these antigens share a common amino acid sequence. Consequently, any cell containing the same sequence could be used to adsorb an antibody that recognizes that sequence. So, if a B7 cell was used as the adsorber to remove the antibody, it would eliminate reactivity to the other cells as well (B13, B42, B55, B81). Recognition of such antibody cross-reactivity with distinct antigens gave rise to the term *cross-reactive groups*. The cross-reactivity was present due to shared or "public" epitopes present among different HLA antigens. Thus, early serology relied largely on the interdependent and sometimes circular relationship between antibodies and antigens, each necessary to characterize the other.

Clearly, the early pioneers of HLA testing required ingenuity and creativity because neither HLA antigens nor their corresponding antibodies were well defined, and the appearance of the aforementioned splits and cross-reactive groups only added to the confusion. Moreover, the serologic techniques used to assess reactivity, which included relatively crude testing, such as agglutination and cytotoxic assays, were of limited value. First, both agglutination and cytotoxic assays were reliant on cell viability, requiring careful collection, handling, and processing. Second, interpretation of data from those methods was subjective and relied heavily on the expertise and experience of the individual reading the reactions. Finally, those early serologic methods suffered from both poor sensitivity, resulting in false-negative reactions, and poor specificity, leading to false-positive reactions. Clearly, more-sensitive and more-specific testing methods were needed.

TWO ROADS DIVERGE

Starting from the serologic foundation of HLA testing just described, the journey to the modern HLA laboratory testing took 2 distinct roads: one led to the identification and characterization of HLA antigens using techniques of molecular biology, whereas the other led to the identification and classification of HLA antibodies via more sophisticated serologic methods. Importantly, the directions these 2 camps took evolved from the different clinical needs of stem cell and solid organ transplantation. Bone marrow/stem cell transplantation depended on accurate, reliable antigen testing to identify HLA-matched recipient-donor pairs, whereas solid organ transplantation (particularly renal transplantation) required identification of HLA antibodies, particularly donor-specific antibodies, to prevent hyperacute and accelerated antibody-mediated rejection.[8] Each camp travelled its own path to develop tests that addressed its patients' needs.

HLA ANTIGEN TYPING: HEMATOPOIETIC PROGENITOR CELL TRANSPLANTS

Bone marrow transplants from non-HLA–identical donors are associated with delayed engraftment, increased incidence of rejection, transplant-related mortality, and other complications.[9] The HLA laboratories historically performed mixed lymphocyte cultures (MLCs) and serologic HLA typing to identify compatible donor-recipient pairs. This approach was cumbersome, tedious, and time consuming (eg, 7 day turnaround times). To overcome these challenges, high-resolution molecular HLA typing was developed and eventually supplanted MLC testing.[10]

In the mid-1980s, molecular-based HLA genotyping was first performed by hybridizing genomic restriction-enzyme fragments with radiolabeled HLA probes to identify restriction fragment length polymorphisms (RFLPs) associated with specific HLA genotypes.[11] Unfortunately, RFLP analysis was cumbersome, required the use of radioactive ^{32}P probes, and took weeks to complete. Consequently, the RFLP method was not widely adopted in clinical HLA laboratories. By 1987, the discovery of polymerase chain reaction (PCR) revolutionized molecular genotyping. Using PCR, a clinician could amplify and analyze sizeable quantities of target DNA.[12] Moreover, PCRs permitted high-resolution HLA typing that could distinguish as little as a single nucleotide difference between 2 alleles, a feat not easily attainable with serologic testing. Since its inception, many different PCR-based assays have been developed to identify and characterize HLA antigens, including: (1) sequence-specific priming (SSP), (2) sequence-specific oligonucleotide probing (SSOP), and (3) DNA sequence-based typing (SBT) (Figure 1). In addition, the development of real-time PCR, with simultaneous amplification and quantification of amplicons, has been extremely useful because of how fast results can be generated. However, this technique is still not routinely used for screening purposes because it is more costly than the other methods described.

Sequence-Specific Priming

In 1991, SSP started gaining traction when it was employed to subtype HLA-DRB1*01, which had not previously been possible using either serology or RFLP approaches.[13] Sequence-specific priming makes use of sequence-specific primers to bind and amplify a target DNA sequence.[14] If the target sequence is present, an amplicon will be generated, representing a positive reaction. In contrast, if the target sequence is absent, no amplicon is

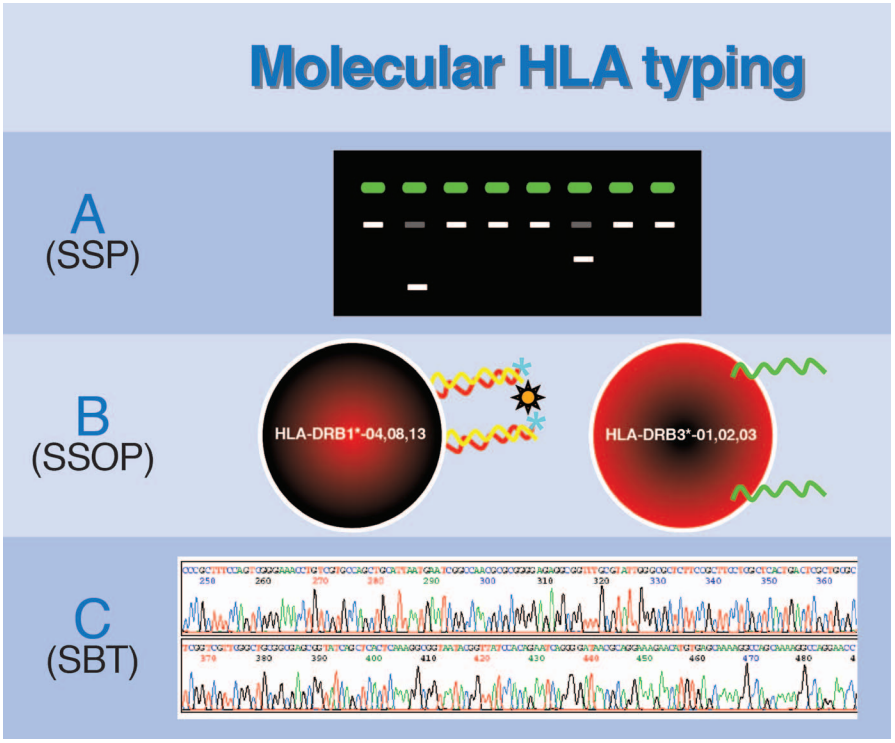

Figure 1. *The different methods used for molecular HLA typing. A, Sequence-specific priming (SSP)-polymerase chain reaction (PCR) products, amplified using sequence-specific primers, are examined by agarose gel electrophoresis, which displays a pattern of positive and negative reactions to determine the presence or absence of HLA alleles. Each lane identifies a unique allele or set of alleles, and the reaction patterns are analyzed using HLA genotyping software. B, Sequence-specific oligonucleotide probing (SSOP)-PCR-amplified products are added to specific oligonucleotide probes affixed to solid-phase matrices (eg, a microparticle or microwell plate), and binding is assessed colorimetrically or via multiplex flow cytometry. C, Sequence-based testing (SBT)-nucleic acid sequencing of the HLA alleles is performed by Sanger or next-generation (massively parallel) sequencing to determine the nucleotide sequence of HLA genotypes.*

produced. The resulting PCR products are then examined via agarose gel electrophoresis, and the pattern of positive and negative reactions is used to establish the HLA alleles. Multiple PCR reactions can be targeted to specific alleles or groups of alleles. Although SSP was found to be more sensitive, more specific, and faster than RFLP, SSP typing is expensive, labor intensive, and requires many primers to achieve high-resolution HLA typing.

Sequence-Specific Oligonucleotide Probes

Although a form of SSOP had been used in the identification of HLA antigens as early as 1986,[15] because of its complexity, several years elapsed before SSOP became routine in clinical HLA laboratories. The development of the reverse dot blot, which immobilized probes, as opposed to targeting DNA, allowed widespread adoption of SSOP. Today, SSOPs employ microarrays of specific oligonucleotide probes, affixed to solid-phase matrices (eg, a microparticle or microwell plate). A generic PCR reaction amplifies all of an individual's HLA genes, and the resulting amplicons are added to the matrix, where they bind to the complimentary probes. Hybridization between amplicons and immobilized probes is typically assessed colorimetrically or via multiplex flow cytometry. Microbead array assays have become the most popular because they provide high-throughput testing with less sample-handling time and can deliver high-resolution typing of HLA class I and class II antigens.[16] Similar to SSP, many primers are required to obtain high-resolution results.

Sequence-Based Typing

Sequence-based testing entails nucleic acid sequencing of HLA alleles and is thus considered the gold standard for molecular HLA typing. Given the degree of allelic interrogation, SBT not only resolves discrepancies in HLA antigens typed by other methods but also allows the discovery of novel HLA alleles. Methods as diverse as standard Sanger sequencing and next-generation sequencing can be used for SBT typing. Even though SBT has advantages over other methods, it is not without its pitfalls. It is an expensive and labor-intensive procedure with longer turnaround times than required for SSP or SSOP. Further, SBT has ambiguities that cannot resolve to which haplotype (maternal or paternal) a given base-pair should be assigned.

Regardless of the actual method employed, molecular HLA typing has been used to accurately and reproducibly assess both prospective hematopoietic progenitor cell donors and recipients, consequently producing steadily better clinical outcomes. The advent of this testing approach have allowed registries, such as the National Marrow Donor Program (Minneapolis, Minnesota) and Bone Marrow Donors Worldwide (Leiden, the Netherlands), to accrue the HLA types of more than 27 million volunteer donors, which provides a rich pool of potential, nonrelated, HLA-matched donors for patients who require lifesaving allogeneic stem cell transplants. Potential donors and recipients are currently typed molecularly to the allele level for HLA-A, B, C, DRB1, and DQB1 (and more recently, HLA-DP) antigens with resulting improved transplant outcomes.[17]

ANTIBODY TESTING: SOLID ORGAN TRANSPLANT

Although ABO compatibility is considered the primary immunologic barrier to successful kidney and other solid organ transplants, HLA compatibility (as opposed to HLA matching) is also critical, which is why recipients and donors are typically typed for HLA-A, B, C, DRB1, and DQB1 in addition to being ABO typed. However, solid organ graft allocations are not based on donor-recipient HLA matching like their hematopoietic progenitor cell counterparts are. Instead, for solid organ transplantation, the detection of donor-specific antibodies is paramount; an analogy would be the typical RBC transfusion recipient, who receives RBC

units compatible with their antibody profile rather than transfusions that are phenotypically identical to their RBCs. The importance of HLA antibodies has been best studied in renal transplants, in which recipient antibodies directed against mismatched donor HLA antigens are associated with antibody-mediated rejection and/or graft loss.[18] Moreover, many studies have detailed the negative effect of HLA antibodies in other solid organ transplants, including heart,[19] lungs,[20] pancreatic islets cells,[21] and recently, liver.[22] It was this clinical importance of HLA antibodies in solid organ transplantation that drove the evolution of testing methods from basic serology to modern solid-phase detection.

Serology

The Centers for Medicare & Medicaid Services stipulate that an HLA crossmatch be performed before solid organ transplantation.[23] Historically, this requirement was met with MLC assays, in which recipient serum was tested against lymphocytes from prospective donors to detect either agglutination or cytotoxicity as signs of incompatibilities. As previously stated, these methods suffer from low sensitivity (possibility of missing clinically relevant antibodies of low titer or low avidity) and from low specificity, wherein a positive crossmatch may be the result of non-HLA antibodies and/or nonspecific reactivities that are clinically irrelevant.[8]

The MLC testing characteristics were subsequently improved, initially through the addition of antihuman globulin, which enhanced the detection of low-level HLA antibodies, and later, with the introduction of flow cytometric crossmatches.[24] Nonetheless, even with these advances, these tests have limited specificity. Thus, a positive crossmatch may not necessarily be a contraindication to transplantation.[8]

Another commonly used serologic assay (still in place today) is the panel-reactive antibody (PRA) test. This assay was designed to assess the degree of HLA alloimmunization. Historically, cytotoxicity was assessed against a panel of HLA typed cells from healthy volunteer donors after incubation with recipient serum. Cell death represented a positive result, and the percentage of positive reactions corresponded to the breadth of HLA sensitization. Cytotoxic PRA suffers from the same shortcomings as the MLC crossmatches. However, similar to the flow cytometric crossmatches, the sensitivity of the PRA test was greatly improved with the introduction of the flow cytometric PRA.

Solid Phase

A significant step forward in HLA antibody testing occurred with the advent of solid-phase approaches that relied on a plastic matrix coated with purified clusters (phenotypes) of native class I and class II HLA antigens or recombinant, single, class I or class II HLA antigens.[25] As with HLA typing, the microbead platform dominated antibody-testing methodologies. For example, patient serum is added to microbeads coated with HLA antigens, and corresponding antibodies can be detected by either conventional flow cytometry or the newer Luminex (Austin, Texas) technology. Current methodologies employ multiplex systems that allow for the simultaneous detection of multiple antibodies; some configurations contain 100 distinct microbead populations, each expressing a unique HLA antigen.[24,26,27] Because of the comprehensive HLA coverage, multiplex solid-phase methods have become the most-common approach for antibody detection in HLA laboratories. The ability to identify individual antibodies among complex sera is particularly helpful in determining suitable donors for highly sensitized recipients.

Solid-phase technology has also led to the concept of a calculated PRA (cPRA). With the conventional PRA test, the assignment of PRA values varied, depending on the antigen composition and distribution of the cell types used to compile a given panel. In contrast, the cPRA is based on antigen frequencies, from a population database of more than 12 000 donors, and evaluates the likelihood of compatibility between a sensitized recipient candidate against an entire population of previously typed donors.[28] When an antibody is detected by solid-phase methodology, the corresponding antigen is listed as unacceptable within the United Network for Organ Sharing Web site. The cumulative frequency of all listed unacceptable antigens is then used to derive the cPRA. Clinically, the cPRA is of specific benefit to highly sensitized patients and predicts more than 90% of positive crossmatches that occur at the time of organ allocation.[29] Similarly, this solid-phase methodology is used to evaluate patients for platelet-transfusion refractoriness by determining the presence and specificity of HLA class I antibodies that may affect the survival of transfused platelets. Two recent reviews[30,31] have detailed the practical approaches for the selection of platelet products for patients with alloimmune platelet-transfusion refractoriness.

TWO ROADS CONVERGE

The innovators in HLA testing shaped the field by traveling 2 distinct pathways: one driven by molecular characterization of HLA antigens, and the other driven by advances in antibody identification. Ultimately, antigen testing led to high-resolution HLA typing, which identifies individual HLA alleles. In contrast, antibody identification has progressed through development of solid-phase methods that can identify epitopes restricted to distinct HLA alleles.

Importantly, these simultaneous, yet separate, advancements have converged, and once again (as in the early days of HLA typing), modern molecular antigen-typing methods are used in conjunction with solid-phase antibody testing to optimize patient outcomes. The convergence of the 2 paths is seen in HLA mismatched stem cell transplants, wherein donors expressing an HLA allele to which the recipient has a corresponding antibody must be considered before moving forward with a transplant because such antibodies can result in failed engraftments. This requires that "high-resolution" antibody testing be performed. Similarly, because allele-specific HLA antibodies can be identified in solid organ transplant recipient, high-resolution HLA typing of donors is a requirement to determine compatibility (Figure 2).

The best example of the convergence of state-of-the-art, high-resolution antigen typing and solid-phase antibody identification is the virtual crossmatch. In brief, by having a detailed list of unacceptable and acceptable donor antigens (based on the recipient's HLA antibody profile) and the high-resolution HLA type of potential donors, an electronic crossmatch (paper crossmatch or crossmatch in silico) can be performed, perhaps one day obviating the need for a physical crossmatch. Virtual crossmatch has not only a high positive predictive value of compatible physical crossmatch-

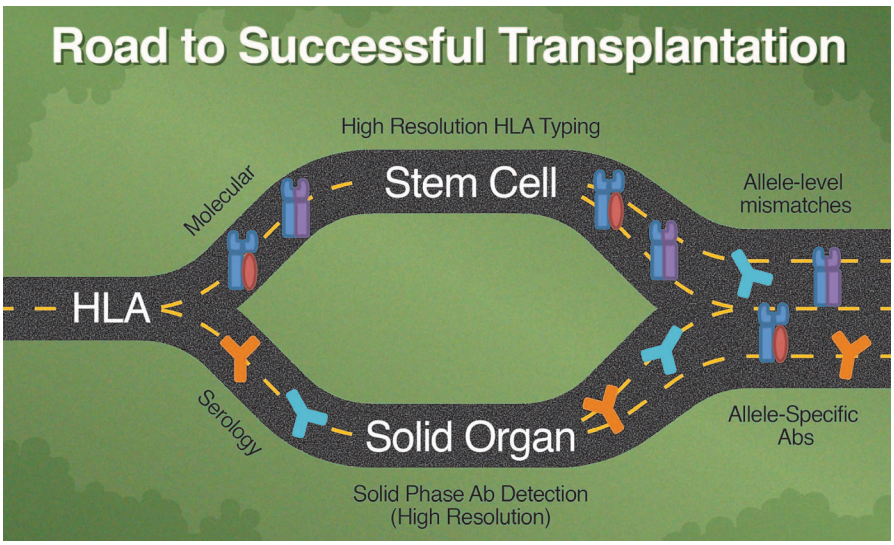

Figure 2. *In the beginning, the foundation of HLA testing was serologic, with an interdependence of antibodies to identify antigens and antigens to identify antibodies. However, as time progressed, HLA laboratory testing took 2 distinct roads: one driven by the need for high-resolution molecular characterization of HLA alleles, and the other driven by the need to identify and classify HLA antibodies via serology. The directions these 2 paths took evolved from the clinical needs in stem cell and solid organ transplantation, respectively: Bone marrow/stem cell transplantation depends on accurate, reliable allele resolution to identify HLA-matched recipient-donor pairs, whereas solid organ transplantation requires identification of HLA antibodies, specifically donor-specific antibodies. Antigen testing led to high-resolution HLA typing, which identifies individual HLA alleles, whereas antibody identification has progressed using solid-phase methods that now have the ability to identify allele-specific antibodies and to characterize unique epitopes restricted to distinct HLA alleles. Eventually, the 2 roads of histocompatibility testing converged, with antigen and antibody testing coming full circle and once again each relying on the other. This convergence is exemplified in stem cell transplants, in which donors expressing an HLA allele to which the recipient has a corresponding antibody must be considered before moving forward with a transplant. This requires high-resolution antigen typing of the donor as well as high-resolution antibody testing of the recipient. Similarly, because allele-specific HLA antibodies can be identified in solid organ transplant recipients, high-resolution HLA typing of donors will be needed to determine compatibility.*

es[32] but also assisted solid organ transplantation by shortening the time to transplant and providing increased access to organs with an increasing geographic radius of potential donors.[33–35] However, the virtual crossmatch is critically reliant on the correct HLA typing of donors and antibody assessment of candidate recipients. To that end, there are concerns in both directions. For example, numerous studies have reported that not all antibodies detected by the solid-phase methods are deleterious (or even "real") but may be directed against denatured HLA proteins or contaminated proteins on the microparticles.[36–38] Conversely, the error rate for HLA typing exceeds 1%, which, when considered in a pool of 6000 to 7000 annual deceased donors, is not a trivial occurrence. Clearly, although new technologies have moved the field of HLA forward significantly, there are still issues to be resolved.

THE EMORY PHILOSOPHY AND EXPERIENCE

Undoubtedly, the science of HLA testing has progressed substantially since its inception with basic serologic testing. However, from a cost and workflow perspective, it may not be practical for a laboratory to perform all the cutting-edge tests. Laboratories and their directors have to take into consideration the resources and technical staff at their disposal, the patient populations served, and the volume of transplants being performed. Furthermore, the advantages of different methodologies should be weighed against their drawbacks. For example, historically the Emory University School of Medicine HLA laboratory (Atlanta, Georgia) performed SBT in-house for the stem cell transplant program and to resolve discrepancies that arose with SSP and/or SSOP. However, the resources needed to perform SBT were extensive, including time, technical expertise, maintenance of competency, and participation in proficiency exercises. Thus, the decision was made to cease in-house SBT. By sending these samples to a reference laboratory whose sole business was HLA SBT, we obtained more-reliable test results at a lower cost and freed up staff to concentrate on assays that had to be performed in-house.

Currently at Emory, we perform molecular HLA antigen typing (SSP, SSOP, and real-time PCR) and high-sensitivity antibody testing (flow cytometric PRA, solid-phase antibody testing, and flow cytometric crossmatch). Specimens follow an algorithm in which sera from candidate recipients are first screened for HLA antibodies. Sera that test positive are then analyzed by solid-phase testing to determine antibody specificity or specificities. The resulting HLA specificities are used to calculate the cPRA, and the corresponding antigens are listed on the United Network for Organ Sharing Web site (https://www.unos.org) as unacceptable. Once a donor organ becomes available, the unacceptable antigen profile helps determine which candidate recipients have a high likelihood of a negative crossmatch. A flow cytometric crossmatch is then performed for sensitized patients and, when negative, the patient is eligible to proceed to transplantation.[39]

THE ROAD AHEAD

The 2 roads of innovation have led to the development of highly sensitive and specific testing and have allowed for the identification of antigens and antibodies down to the allele, and even the epitope, level. Given these advancements, it has been proposed that high-resolution typing may eventually be applied to nonsensitized candidates by adopting allele-based compatibility to avoid posttransplant sensitization.[40] In fact, recent studies suggest HLA allele

matching, and more specifically epitope matching, results in better long-term graft survival through decreased production of donor-specific antibodies compared with established HLA matching.[41] Moreover, with antigen typing able to identify specificity at the allele level, alleles sharing epitopes to which a recipient has antibodies may be avoided. Otherwise a positive crossmatch would not have been predicted because the candidate had never been exposed to the allele in question.[40]

Nevertheless, it is important to underscore that with new technologies comes new problems and shortcomings. Solid-phase antibody testing, for example, has inherent technical characteristics that can lead to discrepant results. Among these potential problematic attributes are variable antigen sources,[42] antigen configurations,[36–38] antigen densities on the microbeads,[43] varying median fluorescence intensity cutoffs for antibody detection,[44–46] and varying testing platforms[27] among others. These drawbacks arise primarily from the lack of standardization among manufacturers of assays and reagents. Although studies have shown that standardization of protocols is attainable,[47] it remains to be seen whether standardization will be implemented. Furthermore, despite advances in molecular technology that have allowed for high-resolution typing, discrepancies in donor typing continue to be reported to United Network for Organ Sharing because of sample mix-ups and transcriptional errors.[35] These errors demonstrate that, no matter how advanced the technology, erroneous results can occur. Wherever the road leads, we must remain vigilant of drawbacks and potential pitfalls.

USE OF RBC GENOTYPING TO AID IN TRANSFUSION MANAGEMENT OF PATIENTS WITH HEMOGLOBINOPATHIES

Introduction

Compared with the genotyping diagnostics for hematopoietic stem cell and solid organ transplantation described earlier in this review, the application of genotyping to transfusion medicine has been more recent. In this area, genotyping has been used to improve the safety and efficacy of both platelet and RBC transfusions. In the former, genotyping can help identify compatible products for patients who are platelet refractory because of antibodies against allogeneic HLA and/or platelet-specific glycoprotein antigens. In addition, HPA genotyping is critical for determining fetal risk of neonatal alloimmune thrombocytopenia in mothers with HPA alloantibodies when the father is found to be heterozygous for the putative HPA.[48] In the latter application to RBC transfusions, genotyping can be used to infer the phenotype of the recipient and/or donor RBCs. Predicted RBC phenotypes are useful for fully characterizing donors with unique/useful blood types, for identifying the safest components for transfusion of patients with RBC autoantibodies, and for solving unusual blood-typing problems, such as the presence of an anti-Rh(D) antibody in a D-positive patient. However, the application of RBC genotyping has arguably been most useful in the management of transfusion-dependent patients with hemoglobinopathies, especially sickle cell disease (SCD).

Current guidelines support transfusion of adults and children with SCD to raise hemoglobin to 10 g/dL before surgical procedures involving general anesthesia (strong recommendation); to treat symptomatic, severe, acute chest syndrome, as defined by an oxygen saturation of less than 90% despite supplemental oxygen (strong recommendation); to address acute splenic sequestration with accompanying severe anemia (strong recommendation); to treat acute stroke (moderate recommendation); and to manage patients with symptomatic acute chest syndrome who have concomitant reductions in hemoglobin of at least 1 g/dL below baseline (weak recommendation). Further, chronic transfusion therapy is endorsed for primary stroke prevention in children with SCD based on transcranial Doppler stroke screening (strong recommendation) and as secondary stroke prophylaxis in adults and children with a previous clinically overt stroke. In addition, transfusion may also be appropriate in other clinical settings in consultation with an expert on the transfusion support of patients with SCD or other hemoglobinopathies.[49]

Guidelines also warn against the risks of adverse transfusion events in these patients, including iron overload, hemolysis, hyperviscosity, and most important for this discussion, the risk of alloimmunization to foreign RBC antigens. Alloimmunization occurs when the transfusion recipient's immune system produces antibodies against variant (nonself) forms of RBC antigens encountered on transfused RBCs. Once RBC alloantibodies are produced, future transfusions must be limited to the use of donor RBCs that do not express the corresponding antigens. Although such blood units are relatively easily identified for patients with a few alloantibodies, providing some patients with hemoglobinopathies with compatible RBC units for transfusion can prove difficult, even with nationwide searches, because of their development of either many RBC alloantibodies or complex antibodies, such as anti-Rh alloantibodies from the high prevalence of *RH* variants in patients with SCD.

Because there are few good therapeutic options for management of highly alloimmunized patients with SCD and thalassemia presenting with severe anemia, most experts advise using approaches that reduce the risks of alloimmunization. These include eliminating unnecessary transfusions and prophylactic matching of RBC antigens between donors and recipients.

PROPHYLACTIC RBC PHENOTYPE MATCHING

In an effort to reduce RBC alloimmunization, many transfusion services have implemented (at a minimum) prophylactic-phenotype matching for Rh (C/c, E/e) and K antigens in patients with SCD and thalassemia. Specifically, in patients with SCD, this transfusion strategy has been associated with a reduction in the prevalence of alloimmunization from 18% to 66% (rate, 1.7–3.9 antibodies/100 units transfused) for ABO/Rh(D)-compatible–only transfusions,[50–64] to between 5% and 24% (0.26–0.50 antibodies/100 units transfused).[56,59,61,65,66] Alloimmunization can be further reduced to between 0% and 7% ($\leq$0.10 antibodies/100 units transfused) when preemptive, extended RBC antigen-matching (beyond C/c, E/e and K antigens) is employed (see Table).[67,68] Further efforts to minimize other non-Rh, non-K donor-recipient RBC antigen discrepancies have aimed at providing RBC units from ethnically matched donors because of reports of lower alloimmunization rates in countries with ethnically similar donor and recipient populations.[68,69] Although identification of donor units with extended matches to the recipients was historically based on RBC phenotyping that used commercial antibody reagents, the availability of genotyping approaches to predict RBC

Red Blood Cell Antigen Matching and Alloimmunization Worldwide						
Source, y	Patients, No.	Patients, Adult/Child[a]	Country	Total Units Transfused	Matching	Patients With Alloantibodies, %
Orlina et al[51] 1978	50	Adult	United States	N/A	ABO, D	36
Coles et al,[50] 1981	72	Child, adult	United States	N/A	ABO, D	23.6
Sarnaik et al,[52] 1986	245	Child, adult	United States	N/A	ABO, D	7.8
Ambruso et al,[53] 1987	85[b]	Child, adult	United States	1941	ABO, D	33
Cox et al,[54] 1988	73	Adult	United States	N/A	ABO, D	30
Rosse et al,[55] 1990	1044	Child, adult	United States	N/A	ABO, D	27
Vichinsky et al,[56] 1990	107	Child, adult	United States	1711	ABO, D	30
Moreira et al,[64] 1996	85	Child, adult	Brazil	1300	ABO, D	12.9
Aygun et al,[57] 2002	140	Child, adult	United States	3239	ABO, D	37
Castro et al,[58] 2002	351	Adult	United States	8939	ABO, D	29
Sakhalkar et al,[59] 2005	387	Child, adult	United States	14 263	ABO, D	31
Bashawri,[60] 2007	350	Child, adult	Saudi Arabia	N/A	ABO, D	13.7
Ameen et al,[61] 2009	110	Child, adult	Kuwait	N/A	ABO, D	65.5
Natukunda et al,[69] 2010	428	Child, adult	Uganda	3366	ABO, D	6.1
Aly et al,[63] 2012	42	Child, adult	Egypt	N/A	ABO, D	21.4
Vichinsky et al,[93] 2001	61	Child	United States	1830	Limited (C, E, K)	11
Sakhalkar et al,[59] 2005	113	Child, adult	United States	2354	Limited (C, E, K)	5
Ameen et al,[61] 2009	123	Child, adult	Kuwait	N/A	Limited (C, E, K)	23.6
O'Suoji et al,[65] 2013	180	Child	United States	N/A	Limited (C, E, K)	14
Chou et al,[70] 2013	182	Child, adult	United States	44 482	Limited (C, E, K)[c]	44
DeBaun et al,[94] 2014	90	Child	United States	3236	Limited (C, E, K)	4.5
Tahhan et al,[67] 1994	40	Child, adult	United States	608	Extended matching[d]	0
Lasalle-Williams et al,[68] 2011	99	Child, adult	United States	6946	Extended matching[e]	7

Abbreviation: N/A, not available.

[a] *Child* is any patient younger than 18 years.
[b] Eight of the 12 patients (75%) followed prospectively in the study were alloimmunized before entry, and 3 (25%) developed new antibodies after receiving blood matched for Rh, Kell, Kidd, and Duffy antigens.
[c] Limited matched units from African American donors.
[d] C/c, E/e, K, Fya, Fyb, S.
[e] C/c, E/e, K, Fya, Jka, Jkb.

phenotypes in patients and donors has significantly improved the speed, reliability, and extent of matching.

Despite receiving Rh phenotype-matched RBCs, many patients with SCD still produce Rh antibodies, which are often considered autoantibodies because the patient's own RBC type is serologically positive to the corresponding Rh antigen. *RH* genotyping has revealed that many patients with SCD carry alleles encoding partial D, C, and/or e antigens and that most of these "autoantibodies" are, in fact, alloantibodies against D, C and e antigens.[70,71] A report of patients with SCD transfused at the Children's Hospital of Philadelphia who had received prophylactic C/c, E/e, and K phenotypically matched RBCs from African American donors demonstrated a high prevalence of antibodies to Rh antigens (91 of 146; >60% of all antibodies reported), with no cases of anti-K and a low rate of anti-Jk, Fy, and S antibodies. *RH* genotyping revealed variant alleles in 87% of individuals, and one-third of the Rh antibodies were associated with laboratory evidence of delayed hemolytic transfusion reactions. Altered *RH* alleles in both the patients and the African American donors were believed to contribute to Rh alloimmunization because Rh antibodies occurred in patients whose RBCs were phenotypically positive for the corresponding Rh antigen and in patients who were transfused RBCs from donors who were serologically negative for the Rh antigen they lacked.[70] Similarly, Sippert et al[71] identified variant *RH* alleles in 31 of 48 (65%) of the Brazilian patients with SCD and Rh antibodies, and 42% of the anti-Rh antibodies produced by patients with *RH* variants were either involved in delayed hemolytic transfusion reactions or demonstrated decreased survival of transfused RBCs.

Complexity of the Rh System: Implications on Alloimmunization and Genotyping

The Rh blood group system consists of numerous antigens in addition to the most-common, clinically significant D, and C, c, E, e antigens. There have been more than 50 Rh antigens identified, all encoded by *RHD* and *RHCE*, 2 genes, each with 10 exons in opposite orientation (5′-RHD-3′; 3′-RHCE-5′). *RHD* and *RHCE* share 92% nucleotide sequence homology and 96% translated amino acid sequence similarity because the 2 genes were evolutionarily created by a duplication event from one gene. The conventional *RHD* and *RHCE* genes encode the D antigen and the CE antigens in various combinations (Dce, DcE, DCe, DCE, ce, cE, Ce, or CE), which are found in all ethnic groups, although with different frequencies.[72–74] In addition, more than 200 *RHD* and approximately 100 *RHCE* alleles encoding partial and weak D and other altered or partial Rh antigens have been described. *RH* variant alleles may encode Rh proteins with amino acid changes that cannot be distinguished with common serologic reagents (eg, V, VS, Goa) but can result in allosensitization upon exposure in an individual lacking the variant allele. Conversely, individuals with *RH* variants in homozygous or compound heterozygous forms can have RBCs that lack high-prevalence Rh antigens (eg, hr^B or hr^S) and may make alloantibodies (which often appear to be autoantibodies) to these antigens upon exposure to conventional Rh antigens through transfusion. *RHD* and *RHCE* variants are found in less than 1% to 2% of Europeans; however, the frequency in individuals of African descent is much higher, with certain *RH* variants being more common in patients with SCD of

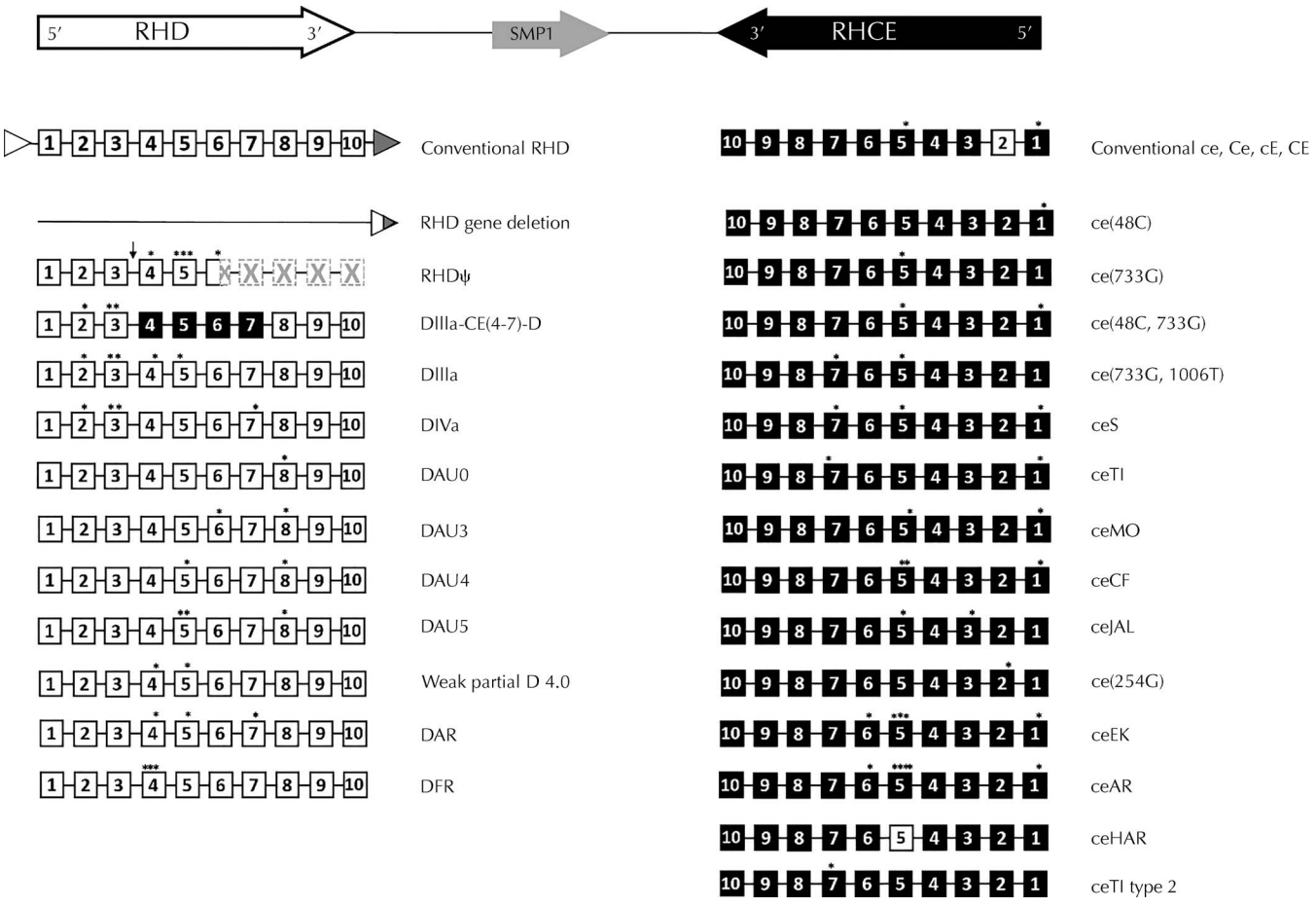

Figure 3. *Inverted orientation of the* RHD *and* RHCE *genes (top), RHD and RHCE locus structures of conventional* RHD *and* RHCE *genes, and the most frequently occurring variant haplotypes in individuals of African descent, which complicate transfusion in patients with sickle cell disease. The 10 coding exons of* RHD *and* RHCE *are shown as white and black boxes, respectively. The location of nucleotide changes are designated with an asterisk (*). Rhesus boxes are shown as white and gray triangles, with a resulting hybrid Rhesus box in individuals with the* RHD *deletion. The arrow (↓) indicates a 37-base pair duplication at the intron 3–exon 4 junction, and the hatched boxes represent exons encoding the untranslated region of the inactive* RHD *pseudogene (*RHDψ*) because the nonsense mutation in exon 6 leads to a premature translation stop codon. Data derived from Chou et al,[70] Chou et al,[73] and Noizat-Pirenne and Tournamille.[74]*

African ancestry. Figure 3 illustrates the most common *RH* variants identified in patients with SCD.

RBC GENOTYPING: CURRENT METHODOLOGIES

Since the discovery of the ABO blood group in the early 20th century, more than 300 authenticated blood-group antigens have been placed into 35 blood-group systems. Moreover, the molecular basis for almost all the genes responsible for the differences in blood-group antigens has been determined, and often this difference is caused by a single-nucleotide polymorphism (SNP), a single change in the DNA sequence of the gene.[75] Identification of these SNPs has led to the development of several blood-group molecular platforms. Most of them currently available for high-throughput platforms are DNA microarray–based assays, of which, only one platform is approved for use by the United States Food and Drug Administration.

These assays start with PCR amplification of a number of genetic regions encoding various blood-group antigens. Target regions amplified by PCR are then hybridized to RBC antigen-allele–specific oligonucleotide probes, which have been linked to either glass slides (BLOODchip ID CORE XT, Progenika Biopharma, Biscay, Spain), colored silica beads assembled on silicon wafers (PreciseType HEA; Immucor, Norcross, Georgia), or fluidic bead suspensions (xMAP, Luminex). Hybridization signals are then (usually) analyzed by measuring fluorescence intensities, and a RBC phenotype is predicted based on the genotype result.[76–78] Predicting the phenotype from a genotype is relatively straightforward for many RBC antigens encoded by SNPs. However, there still remain some blood-group systems for which genotyping remains challenging because the genes encode enzymes involved in the modification of carbohydrate chains (eg, ABO, H, I, GLOB), or blood-group antigens are determined by large insertions, deletions, or hybrid genes from recombination of homologous genes (eg, *RHD*, *RHCE*, *GYPA*, *GYPB*).[78]

The platform used at Emory University Center of Transfusion and Cellular Therapies is Immucor's PreciseType HEA, which uses a proprietary, elongation-mediated, multiplexed analysis of polymorphisms technology to identify the presence or absence of the selected alleles associated with a given phenotype. PreciseType HEA includes 24 polymorphisms associated with 35 human erythrocyte antigens of the Rh (C/c, E/e, V, VS), Kell (K/k, Jsa/Jsb, Kpa/Kpb), Duffy (Fya/Fyb, Fy_{null} because of *GATA*

mutation, Fyb_{weak}), Kidd (Jka/Jkb), MNS (M/N, S/s, U), Lutheran (Lua/Lub), Dombrock (Doa/Dob, Hy, Joa), Landsteiner-Wiener (LWa/LWb), Diego (Dia/Dib), Coltan (Coa/Cob), and Scianna (Sca/Scb) blood-group systems, and detection of the hemoglobin S mutation in the β-globin gene (which is not intended for making a diagnosis of SCD). PreciseType HEA demonstrates an overall greater than 99.4% agreement to serology and 99.8% concordance to DNA sequencing, and it gained United States Food and Drug Administration approval in May 2014.[79] RhD and ABO are not determined by this platform; however, we use *RHD* and *RHCE* variant research use only BeadChips (Immucor, Norcross, Georgia), which identify 75 *RHD* and 35 *RHCE* variants for elucidation of *RH* variants in select clinical circumstances (see "Indications for RBC Genotyping").

Other commercially available high-throughput genotyping platforms have also been described, such as the BLOODchip ID CORE XT types ABO (33 haplotypes; Progenika), *RHD* (91 haplotypes, including various alleles that cause D-negative, partial D, weak D, and Del phenotypes), *RHCE* (9 alleles), KEL (8 alleles), JK (4 alleles, including 2 JK_{null} alleles), FY (4 alleles), MNS (9 haplotypes), DI, DO, and CO. Validation of this platform demonstrated a global accuracy of 99.8%, with the exception of ABO. BLOODchip has been CE-marked in the European Union; however, it is not United States Food and Drug Administration–approved for diagnostic purposes in the United States.[80] Other technologies that have been used for high-throughput genotyping of a few RBC antigens include Luminex xMAP, GenomeLab SNPstream (Beckman Coulter, Fullerton, California), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.[81] In addition, other customizable, high-throughput, genotyping platforms, such as TaqMan Open Array (Applied Biosystems, Grand Island, New York), have also been used.[76]

There are limitations to all current molecular techniques because these platforms have been designed to target only known polymorphisms. As a result, novel alleles that might alter an antigen's expression are unlikely to be detected. Additional SNPs within primer sites may lead to false-negative results because of allele dropout; known (or unknown) mutations not covered by the assay may also influence expression of an antigen, such as the case with the myriad of *RH* variants not covered in many of the current platforms. In difficult cases, DNA sequencing of the blood group gene(s) can mitigate many of these limitations; however, sequencing is an expensive and laborious process.[77] Target-enrichment next-generation sequencing is an emerging technology that can detect both known and de novo SNPs, insertions and deletions, and structural variations, focusing on specified genomic regions. Target enrichment next-generation sequencing for blood group typing may become more widely available in the future as the cost and time required for its sequencing assays continue to decrease.[76,77]

INDICATIONS FOR RBC GENOTYPING

Based on published literature,[82–84] commonly accepted transfusion practices, and our own institutional experience, we routinely perform molecular typing for the following types of patients:

- All patients with congenital hemolytic anemias or blood dyscrasias that affect the erythroid lineage (eg, SCD, thalassemia, Diamond-Blackfan anemia, congenital dyserythropoietic anemias, among others) and who are likely to receive multiple RBC transfusions have RBC genotyping performed within the first year of life or on their initial visit if transferring from another institution (unless already performed). Because PreciseType HEA is United States Food and Drug Administration approved as the "test of record," the RBC phenotype determined by molecular blood group testing does not require confirmation by serologic methods.
- Patients with autoantibodies or other serologic reactivities (eg, multiple antibodies, antibodies to high-prevalence antigens, and antibodies of undetermined specificity) that obscure detection of clinically significant RBC alloantibodies.
- Patients with a suspected antibody against an antigen for which typing antisera are not readily available. Examples include antibodies to: Doa/b, Jsa/b, Kpa/b, V, or VS.
- Patients with a serologic typing discrepancy or weak/inconclusive antigen typing (eg, RhD serologic-typing discrepancy).
- Patients with an apparent autoantibody with antigenic specificity (eg, an anti-C in a C+ patient).
- Patients with unexplained Rh antibodies detected despite antigen matching for RhD, E/e, and C/c, who have RH genotyping performed to determine the presence of and to characterize the RH variant(s) in the individual.

Our institution uses the PreciseType HEA, which detects the RHCE*ce(733G,1006T) allele. This RHCE variant allele is commonly linked to the hybrid RHD*DIIIa-CE(4-7)-D gene, and encodes a partial C antigen. Although individuals with this RH haplotype will commonly serologically type as C+, they are at risk for alloimmunization to the C antigen (if they are lacking a RHCE gene encoding a conventional C antigen in trans) and, therefore, receive C–negative blood at our institution.[72,85] We also use RHD and RHCE variant research-use–only beadchips for elucidation of RH variants in patients with SCD who have complex anti-Rh antibodies because the PreciseType HEA detects only 2 SNPs (733G and 1006T), which account for only a few *RHCE* variant haplotypes, and it does not type for RhD. The *GATA* silencing mutation in the Duffy gene, which prevents the transcription of the Fyb antigen on erythrocytes but not tissue cells, is also detected in this array. When present in Fyb-negative patients, we permit transfusion of Fyb-positive blood because there is no risk for alloimmunization to the Fyb antigen.[86,87]

RBC GENOTYPING AS AN AID TO PROVIDING EXTENDED MATCHED BLOOD FOR PATIENTS WITH HEMOGLOBINOPATHIES

Although many transfusion services provide RBCs phenotypically matched for Rh (C/c, E/e) and K (limited match), some also endorse prophylactic, extended antigen matching to include the Duffy (Fy^a/Fy^b), Kidd (Jk^a/Jk^b), and/or S antigens, citing further reduced alloimmunization rates with this strategy.[68] Even further, some advocate for the recruitment of racially matched, molecularly typed donors to provide extended antigen–matched RBC units for the chronically transfused SCD population. These strategies require the ability to perform high-throughput, DNA-based, extended-antigen phenotyping in both patients and large (ethnically similar) donor populations. Because of the availability of multiple commercially available high-throughput blood-group genotyping systems, the potential

to provide extended antigen–matched RBC units for large populations of chronically transfused patients with SCD may now be considered through expanded use of RBC genotyping in hospitals and blood donor testing centers.

In a feasibility study at BloodWorks (formerly the Puget Sound BloodCenter), Wilkinson et al[88] enumerated the number of extended, matched RBC components available from their inventory of molecularly or serologically typed donors to meet the transfusion needs of molecularly typed patients with SCD. From an inventory that had an average of 335 RBC components typed for 11 or more antigens (most molecularly typed), 37.4 extended, matched (matched at E/e, C/c, K, Fya/Fyb, Jka/Jkb, S/s) RBC components per patient were available for 70 patients with SCD (28.6% with alloantibodies, 15.7% with warm autoantibodies) when allowing Fyb+ components for patients with FY *GATA* mutation. Although 6 (8.6%) of the patients had no extended, matched components available, the blood donor base was predominantly white. The authors concluded that recruitment of racially matched, molecularly typed donors may have allowed for a greater ability to provide extended, matched components in their patient population. Using DNA array analysis (HEA Beadchip, now PreciseType HEA), Ribeiro et al[89] were able to predict compatible donors for a group of multiply transfused patients with SCD (29% with alloantibodies) from a pool of 948 donors. They were able to find units matched for ABO, Rh (C/c, E/e), Kell, Fya/Fyb, Jka/Jkb, Ss, in addition to Dombrock and Diego for 134 of 144 patients with SCD (93%). The 10 patients for which they were unable to find compatible units from their donor pool had unique phenotypic combinations or rare phenotypes, such as R_2R_Z (DcE/DCE), R_2R_2 (DcE/DcE) and U–, which likely would have been able to be matched with a larger donor pool.

A multi-institutional, prospective, observational study was conducted at 4 hospitals to determine the feasibility for hospital transfusion services to maintain an inventory of molecularly typed units to facilitate identification of blood for transfusion at increasing levels of antigen matching for 3 groups of potential transfusion recipients: patients with SCD (alloimmunized and nonalloimmunized), patients who had cardiac surgery without previous alloimmunization and alloimmunized (non-SCD) hematology, and patients who had cardiac surgery who required antigen-negative units. Approximately 730 donor and 128 patient samples from each of the institutions were molecularly typed using HEA Beadchip. This study demonstrated that, by selecting an existing inventory of donor units for genotyping, a substantial fraction of RBC requests could be fulfilled (fill fraction) at lower antigen-matching levels in most patient groups. However, the hospital with the most SCD patients included the lowest fill fractions for C/c, E/e, and K matched units (62%) and extended-matched units (31%) for the SCD patient population (which had a mean of 4.4 alloantibodies per patient).[90] These results show that supplementing the blood inventory with specially ordered RBC units is necessary for supplying limited or extended-matched units in institutions with a lot of heavily alloimmunized patients with SCD.

It is well established that antigen frequencies differ between African Americans and whites and that alloimmunization to specific antigens can be reduced by using RBCs from racially similar donors in patients with SCD. Karafin et al[91] evaluated the RBC antigen frequencies in both a cohort of 54 patients from the adult sickle cell transfusion program and 6066 genotyped, African American donors. They found that the genotype-derived, predicted-antigen frequencies of the donors were similar to the patients with SCD supported by these units. Despite demonstrating that African American sickle-negative donors could support the antigen requirements of their SCD population, they found that most patients received a mix of white and African American donor units. They reported an overall alloantibody prevalence of 22% in this cohort of chronically transfused patients with SCD, and about half of those formed new alloantibodies (9.3%) during their 3-year study interval, despite the availability of the genotyped African American donor units. Similar to other reports,[70,71] a proportion of the patients developed Rh antibodies because of undetected Rh variants and alloantibodies to antigens prevalent among African American donors.

RH genotyping has thus been used to identify altered *RH* alleles and to predict whether Rh antibodies are autoantibodies or alloantibodies, and it is beginning to have an important role in improving transfusion therapy in patients with SCD by expanding the ability to provide true Rh antigen–matched RBCs for those patients with *RH* variant haplotypes who lack a conventional Rh antigen. This is only possible with expansion of large-scale donor molecular screening to identify donors with *RH* variants for genotype matching. Systematic *RHD* and *RHCE* molecular analysis performed on African blood donors in France (established by Fy[a–b–] phenotype) provided indirect evidence that the transfusion needs of patients with SCD and anti-Rh antibodies may potentially be met by screening very large populations of donors for *RH*-variant phenotypes.[92] Therefore, incorporating *RH* genotyping into select patient and donor testing may improve transfusion therapy of patients with SCD by allowing better donor and recipient matching at the Rh level.

GAPS IN KNOWLEDGE AND FURTHER RESEARCH

The prerequisite for providing extended antigen–negative RBCs to patients with SCD who commonly are negative for the C, E, K, Fya, and Jkb antigens is creating and maintaining large inventories of African-American donors typed for conventional blood group antigens. Automated DNA extraction and the ability to test both patients and large groups of donors on high-throughput RBC genotyping platforms are now readily accessible. Combined with database-driven RBC matching, blood group molecular phenotyping may facilitate the identification of antigen-matched RBCs and improve transfusion support of patients with SCD in the future. Although mass screening for antigen-negative and rare phenotype blood donors is now possible, large-scale donor genotyping implementation and future feasibility studies are necessary to confirm the positive effect that RBC genotyping may have on patients with hemoglobinopathies. Although high-resolution *RH* genotyping is currently largely limited to reference molecular immunohematology laboratories, it is increasingly being employed for discerning alloantibodies from autoantibodies, and identification of patients at risk for producing Rh antibodies despite phenotypically matching for Rh antigens. However, future studies are needed to address whether providing *RH* genotype-matched RBCs for patients with SCD (1) is feasible, (2) can prevent the Rh alloimmunization, and (3) will improve transfusion safety in a cost-effective manner. Lastly, the advantages over the current

blood-group molecular-typing platforms make target enrichment next-generation sequencing an attractive prospect in the future for advancing our knowledge of blood-group genetics and for increasing our ability to provide extended antigen–matched RBCs to patients with hemoglobinopathy if costs continue to decrease to a more-competitive level.

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