Published in final edited form as:

Transfusion. 2010 August ; 50(8): 1749–1760. doi:10.1111/j.1537-2995.2010.02589.x.

Identification of Specificities of Antibodies Against Human Leukocyte Antigens in Blood Donors

Robert O. Endres, Steven H. Kleinman, Danielle M. Carrick, Whitney R. Steele, David J. Wright, Philip J. Norris, Darrell Triulzi, Ram Kakaiya, and Michael P. Busch for the National Heart, Lung, and Blood Institute (NHLBI) Retrovirus Epidemiology Donor Study-II
Blood Systems Research Institute, San Francisco, CA HLA Laboratory, Blood Systems Laboratories, Tempe, AZ Westat, Rockville, MD

Abstract

Background—Transfusion-related acute lung injury (TRALI) is the leading cause of transfusion-related mortality. Blood centers are implementing TRALI risk reduction strategies based on screening apheresis donors for antibodies to human leukocyte antigens (HLA).

Study Design and Methods—HLA antibody screening was performed on 7920 blood donors from the Leukocyte Antibody Prevalence Study (LAPS) using Luminex based Normalized Background (NBG) cutoff ratios of 10.8 (class I) and 6.9 (class II). Single antigen bead (SAB) assay cutoffs of 2500 Median Fluorescence Intensity (MFI) units (class I) and 1500 (class II) were established based on results of two subpopulations of LAPS donors. Antibody frequencies against HLA A, B, C, DR, DQ and DP antigens were determined for screen-reactive donors with prior pregnancies.

Results—SAB reactivity for samples above our multi-antigen bead NBG cutoffs was 78% for class I and 79% for class II. The SAB positive rate increased among women with zero to ≥ 4 pregnancies (0.3% - 15.6% class I and 0.4% - 18% class II; $p < 0.00001$). The highest frequency antibodies were DR11 and B15 (4.4% of women with prior pregnancies). The majority of class I positives contained > 5 specificities. For class II, antibody positive women segregated into two groups; a single specificity or > 5 specificities.

Conclusions—Identification of HLA antigen specificities supports pregnancy associations previously found with screening assays. The significance of particular HLA specificities for inducing TRALI is currently being evaluated in a large lookback study of recipients of high plasma volume components from this donor cohort.

Keywords

TRALI; HLA antibody; blood donors; LAPS

Author Responsible for Correspondence Robert O. Endres HLA Laboratory Blood Systems Laboratories 2424 W Erie Dr Tempe, AZ 85282 Phone: 602-343-7092 Fax: 602-343-7115 rendres@bloodsystems.org .
The Retrovirus Epidemiology Donor Study (REDS)- II is presently the responsibility of the following persons: American Red Cross Blood Services, New England Region - R. Cable, J. Rios, R. Benjamin; American Red Cross Blood Services, Southern Region / Department of Pathology and Laboratory Medicine, Emory University School of Medicine - C.D. Hillyer, K.L. Hillyer, J.D. Roback; Blood Center of Wisconsin - J. Gottschall, A. Mast - Hoxworth Blood Center, University of Cincinnati Academic Health Center - R.A. Sacher, S.L. Wilkinson, P.M. Carey; Blood centers of the Pacific, University of California San Francisco, Blood Systems Research Institute - E.L. Murphy, B. Custer, N. Hirschler; The Institute for Transfusion Medicine - D. Triulzi, R. Kakaiya, J. Kiss; Blood Systems Research Institute (Central Laboratory) - M.P. Busch, P. Norris; Westat, Inc. (Coordinating Center) – J. Schulman, M. King; National Heart, Lung, and Blood Institute, NIH - G.J. Nemo, T. Mondoro.

Conflict of Interest There is no conflict of interest for any of the coauthors.

Disclaimers There are no conflicts of interest for any of the authors listed.

INTRODUCTION

As of the end of 2008, the leading adverse event contributing to transfusion mortality was transfusion-related acute lung injury (TRALI)¹⁻². One of the major putative etiologies of TRALI is the presence of antibodies to human leukocyte antigens (HLA) in transfused blood components, particularly those with high plasma volume, reacting with cognate antigens in the recipient³⁻⁶. Therefore, one measure to potentially reduce TRALI risk is to screen appropriate platelet and/or plasma apheresis donors for HLA antibodies and to redirect HLA antibody positive donors away from these high plasma volume donations.

We and others have reported a strong association of HLA antibodies with donor gender and pregnancy history⁷⁻⁸. To date, however, detailed specificities of the HLA antibodies detected with sensitive solid phase assays have not been reported in any large scale study. Such data are important to verify the gender and pregnancy associations found with HLA antibody screening assay results, to provide clues regarding differential immunogenicity of HLA antigens, and to lay the foundation for further studies to potentially establish the relative risk for TRALI of different cognate antigen-antibody pairs.

In a previous study we used a multi-antigen bead-based platform to screen for HLA antibody using six distinct beads for class I and three distinct beads for class II specificities⁸. Each bead was covalently coated with class I antigens from seven cell lines or with class II antigens from eight cell lines. In this study we report the results of further testing on selected samples from the Leukocyte Antibody Prevalence Study (LAPS) using single antigen beads (SAB) that were each coated with a single highly purified HLA antigen. The SAB assay allowed us to determine the antigen specificity of HLA antibodies detected by the screening assay.

The technologies used for detecting HLA antibodies have become highly sensitive to benefit solid organ and hematopoietic stem cell transplant patients. In such patients, even low levels of HLA antibodies can be detrimental⁹⁻¹². However such sensitivity may not be appropriate for blood donor screening. Therefore, we evaluated less sensitive cutoffs for both the screening and SAB assays to avoid unnecessarily assigning TRALI risk to donors with low concentrations of HLA antibodies and to reduce the identification of heterophile antibodies. This was considered an acceptable compromise in the absence of good evidence for threshold antibody concentrations required for TRALI induction.

HLA antibody reactivity in the screening and SAB assays is reported here for males and for females in relation to the number of previous pregnancies. The frequency of antibodies found for each HLA-A, B, C, DR, DQ and DP antigen is reported for females with prior pregnancies.

MATERIALS AND METHODS

Study population

LAPS was a prospective cross-sectional six-center study conducted by the Retrovirus Epidemiology Donor Study – II (REDS-II) program of the National Heart, Lung, and Blood Institute. Enrollment and study design have been previously described in detail⁸. Donors consenting to the study provided a blood sample for HLA class I and II antibody testing and a detailed history of pregnancy and transfusion. A total of 8171 (6011 females, 2160 males) donors were enrolled. Females and transfused males were intentionally oversampled.

HLA antibody screening and antibody identification assays

Screening for anti-HLA class I and II antibodies was performed on plasma samples (or less frequently on serum, see below) using One Lambda (Canoga Park, CA) LabScreen LSM12 (LabScreen Mixed) multi-antigen bead kits according to manufacturer's instructions. Antibody identification assays for individual HLA class I or II antibody specificities were performed with One Lambda (Canoga Park, CA) LS1A04 or LS2A01 SAB kits according to manufacturer's instructions. Both the screening and SAB assays measured the binding of IgG to fluorescently tagged microbeads. Briefly, 5 μ L microbeads were incubated with 20 μ L plasma in a 96-well V-bottomed polystyrene plate (Whatman, Brentford, UK) for 30 minutes in the dark at 25°C, then washed three times. R-Phycoerythrin-conjugated goat anti-human IgG was added for a second 30 minute incubation, followed by two more washes. Negative control serum provided by One Lambda was included in each batch of specimens. Samples were acquired on a luminometer (Luminex Corp., Austin, TX), capable of discriminating up to 100 unique beads in each well. Class I kits contained distinct beads with purified individual antigens for HLA A, B, or C; and class II kits contained distinct beads with purified individual antigens for HLA DR, DQ, or DP. Both kits also contained negative and positive control beads.

Screening test results were reported as the normalized background (NBG) ratio for each bead in the assay, which was calculated according to the formula provided by the manufacturer. SAB results were reported as the normalized median fluorescence intensity (MFI) for each single antigen coated bead.

Screening assay interpretation

The manufacturer's suggested NBG cutoff for a reactive interpretation in the LabScreen Mixed screening test was either a default value of 2.2 or a cutoff value independently established by the user. Prior to analysis of LabScreen Mixed data in the LAPS cohort, it was determined that samples above the 2.2 cutoff should undergo supplemental testing by the SAB assay. It was subsequently decided to set the LabScreen Mixed NBG cutoff by calculating the mean plus three standard deviations (SD) of the natural log transformed distribution of NBG values in the 1138 non-transfused male blood donors tested as part of the LAPS cohort⁸. This approach was chosen based on plausible estimates of the expected frequency of HLA antibodies in a non-alloexposed normal population and conventions for establishing normal ranges for laboratory screening assays. Based on this analysis, samples with NBG >10.8 for any class I multi-antigen bead were considered screen positive for HLA class I antibody and those with any class II multi-antigen bead NBG >6.9 were considered screen positive for HLA class II antibody. Samples with a LabScreen Mixed NBG >2.2 were also tested in the SAB assay according to their screen reactivity with class I or II antigens.

SAB assay interpretation

The One Lambda SAB assay output files from these tests were distributed to three HLA experts for review and interpretation of antibody specificities. A high percentage of these samples were classified as SAB positive by the experts using a rough cutoff of MFI >500, combined with consistency of reaction across beads within cross reactive groups of antigens.

In order to more fully evaluate the performance of the SAB assay, an additional subset of samples with LabScreen Mixed NBG results <2.2 were also tested by SAB. A large proportion of these were SAB positive using an MFI cutoff of 500. Because of the high SAB positive rate in normal donors with negative screening results, it was decided to test a subset of non-alloexposed individuals (i.e., male donors with no transfusion history) with LabScreen Mixed NBG results <2.2 to investigate establishing a new SAB MFI cutoff value for a normal blood donor population as opposed to organ transplant candidates.

Antibody specificity

Based on studies described in the results section, an SAB assay result was classified as positive if any individual bead in the SAB test had an MFI >2500 for class I or >1500 for class II. Antibody specificities were assigned based on the HLA antigen that was coated on each bead exceeding the MFI cutoff. Since the SAB kits contained multiple beads coated with different proteins for some of the HLA antigens, a positive result for any of these beads resulted in a positive interpretation for that serologically equivalent antibody specificity. For example, A2 was represented by three different beads coated with A*0201, A*0203, or A*0206. If any of these beads gave an MFI >2500, the result was scored positive for an A2 antibody.

RESULTS

Preliminary studies to define the SAB assay cutoff

In a previous study, SAB data from 1000 serum samples, which included LabScreen Mixed NBG values above and below 2.2, were examined¹³. SAB results at various class I and class II MFI cutoff values were compared with the LAPS generated screening test cutoffs (NBG >10.8 for class I and >6.9 for class II adjusted upward by a factor of 1.3 based on testing of sera rather than plasma)¹³. Using the class I SAB cutoff of 2500 MFI, 92.7% of serum samples testing positive in the screening assay were positive by the SAB assay and 87.4% that tested negative in the screening assay were negative in the SAB assay. Similarly, using the class II SAB cutoff of 1500 MFI, 93.0% of the serum samples testing positive in the screening assay tested positive on the SAB assay and 95.4% of samples that tested negative in the screening assay were also negative in the SAB assay. Based on these relatively good correlations, it was decided that these SAB MFI cutoffs would provide the optimal balance between sensitivity and specificity when evaluated against the screening NBG test cutoffs.

To determine the correlation of the SAB assay with low NBG values on the screening assay, plasma samples with NBG values <2.2 from 132 male donors with no alloexposure history via transfusion were selected from 1057 males in the LAPS cohort who met this criterion. Budget constraints prevented testing of a larger number of samples. Since the very high percentage of positive results (e.g., 42% for class I) at an MFI cutoff >500 was not likely to truly represent previous exposure to HLA antigens, the impact of using higher MFI cutoffs on the rate of HLA antibody positivity was examined (Table 1). Based on this analysis and the previous study mentioned above, the MFI values of 2500 for class I and 1500 for class II were selected as cutoffs.

Correlation between reactivity in HLA antibody screening and SAB assays

A total of 1739 LAPS donors had screening assay NBG values >2.2 (812 for class I only, 397 for class II only, and 530 for both) resulting in 1342 samples eligible for testing by the class I SAB assay and 927 samples eligible for class II SAB testing. Only samples with NBG >2.2 for both class I and class II received both kinds of SAB testing.

Results of class I and II SAB testing using MFI cutoffs of 2500 and 1500 for class I and class II, respectively, are given in Figure 1, expressed as the percentage of donors tested in each category with the indicated combination of screen and SAB assay reactivities. For the 722 donors with class I NBG ratios between 2.2 and 10.8, 14.5% had SAB reactive results (Panel A, columns labeled All), whereas for the 619 donors with NBG >10.8, 77.9% had SAB reactive results (Panel B, columns labeled All). For the 236 donors with class II NBG ratios between 2.2 and 6.9, 11% had SAB reactive results (Panel C, columns labeled All), whereas for the 690 donors with NBG >6.9, 79% had SAB reactive results (Panel D, columns labeled All).

For the 530 samples that received both class I and II SAB testing, 35.8% (190 out of 530) had both class I and II specificities. This compares to 27.5% (223 out of 812) with class I antibody only and 49.6% (197 out of 397) with class II antibody only.

Roughly 5-6% of males and never pregnant females were reactive in the class I screening assay but nonreactive in the class I SAB assay (white bars in Figure 1A), whereas less than 2% were reactive in the class II screening assay but nonreactive in the class II SAB assay (white bars in Figure 1C). The frequency of donors with detectable antigen-specific HLA class I and class II antibodies by SAB testing increased with the number of pregnancies for women who were reactive above the LAPS screening cutoffs on either the class I or class II assays. The class I results ranged from a minimum of 0.3% SAB reactive for never pregnant women to a maximum of 16% for women with >3 pregnancies; $p < 0.00001$ (Figure 1B, black bars).

Corresponding percentages for class II SAB reactive results were 0.4% to 18% respectively; $p < 0.00001$ (Figure 1D, black bars). A similar pattern of results was obtained for samples that screened >2.2, but ≤ 10.8 for class I. SAB reactivity increased from 0.6% to 3.2% across the pregnancy strata (Figure 1A, black bars). In contrast, class II SAB reactivity in samples that screened >2.2, but ≤ 6.9 showed relatively equal reactivity levels from 1 to 3 pregnancies (0.3-0.5%) and only increased with the fourth pregnancy (1.3%) (Figure 1C, black bars).

Effect of different SAB MFI cutoffs applied to the cohort of samples with NBG >2.2

Figure 2 shows the percentage of class I and class II SAB reactive samples when cutoffs were defined at several different MFI levels. The relationship between the potential MFI cutoff value and the number of positive samples was nearly linear above MFI 1500 for screening results of NBG >10.8 for class I or 6.9 for class II, but not for samples that screened between 2.2 and these NBG cutoff values. These data demonstrate that there was a higher proportion of weakly SAB reactive results (MFI of 500-1500) for samples with an NBG screening test value below our study-defined screening assay cutoffs than for samples above these NBG values.

Specificity of HLA antibodies in female donors with history of pregnancies

SAB assay results were analyzed for females that screened positive to evaluate the frequencies of different antigen specificities. Figures 3A-F present data from ever pregnant females and show antibody specificity data for the A, B, C, DR, DQ and DP loci. Each figure shows the frequency of antibody specificities ranked in decreasing order. Data are shown in two ways. On the left axis, data are given as a percentage of all ever pregnant females in the LAPS population. On the right axis, data are expressed as a percentage of the subgroup that screened positive.

For HLA A antigens, four of the five highest antibody frequencies were for those A locus antigens that share the Bw4 epitope with many HLA B antigens (A23, A24, A25, and A32) (Figure 3A). During the initial data review by our HLA experts, the Bw4 specificity was defined if these specificities were detected in conjunction with antibodies to those HLA B antigens that carry the Bw4 epitope (data not shown). In 41 samples (1.03% of ever pregnant females), all 4 of these specificities were detectable using the 2500 MFI cutoff, and in 32 of these (78%), there were no other HLA A specificities.

For HLA B antigens, seven of the ten antibody specificities with frequencies greater than 2% contained the Bw4 epitope (Figure 3B). For HLA Cw antigens, antibodies to Cw17 and Cw18, which had not been defined by serological means prior to the availability of DNA testing, were among the highest HLA Cw antibody frequencies found. (Figure 3C)

Antibodies to the class II HLA DR (Figure 3D), HLA DQ (Figure 3E), and HLA DP (Figure 3F) antigens were also found. The highest antibody frequency in this study was DR11, just slightly more frequent than B15 and B27. One fourth of female donors with pregnancy histories who screened class II positive demonstrated antibodies to DR11. This is equivalent to 4.4% of all females with pregnancy histories in the study. Antibodies against DQ antigens were found in 6-16% of ever pregnant females who screened positive.

Number of antigen specificities in individual samples

Figure 4A shows the number of samples with increasing numbers of class I specificities among the HLA A, B and/or C loci. The majority of samples contained >5 specificities. Donors with only 1, 2 or 3 specificities had roughly equal proportions of A and B antigen specificities, but as the number of specificities grew larger, HLA B antigens became predominant.

Figure 4B shows the same type of results for class II specificities at the HLA DR, DQ and DP loci. Donors segregated into two major groups. One group had only a single specificity (predominantly at DR), and the second group had >5 specificities (predominantly at DR and DQ). As the number of specificities increased between 1 and >5, the proportion of DR specificities declined and the proportion of DQ specificities rose.

Antibody detection in male donors at an MFI cutoff of 1000

We analyzed data from 94 non-transfused male donors whose serum samples underwent SAB testing as part of our previous serum-plasma comparison¹³. Figure 5 compares our detailed HLA antibody specificity data to those extracted from a recently published study of 424 healthy, non-transfused Mexican male sera that used the SAB MFI cutoff of 100015. Using an MFI cutoff value of 1000, 29% of our donors had only class I antibody, 5% had only class II antibody, and 5% had both class I and II antibody. These percentages are lower than percentages in the recent publication of Morales-Buenrostro et al.¹⁵: 42% had only class I antibody, 11% had only class II antibody, and 12% had both class I and II ($p<0.001$). We found only one bead (as opposed to 29 beads in the Mexican study) with a reactivity rate >4.3%. This was A3002, (12.8% in our study, 18.9% in the Mexican study). Comparison of individual bead reactivities yielded statistically different results between the two studies for only two beads (A3101 and DPA1*0201/DPB1*0101). The small sample size, however, gave this study insufficient power for comparison of other beads.

DISCUSSION

HLA antibody reactivity in bead array based screening and SAB assays is reported here for LAPS donors. The frequency of antibodies detected by Luminex technology is reported for females with prior pregnancies. As previously reported, antibodies identified by the screening assay correlate well with the number of pregnancies, demonstrating the value of this testing platform⁸.

One of the first challenges faced in analysis of the LAPS SAB results was to define appropriate cutoffs that could be used to assign antibody specificities by computer in high volume testing. Initially, a panel of 3 HLA experts was recruited to manually interpret several hundred SAB results. After completion of their analysis, it was apparent that human interpreters were identifying HLA antibody specificities at very high rates including some that were considered unacceptably weak for the purpose of confirming an antibody screening result that would be used to defer platelet or plasma donors from making future donations. Such weakly reactive antibodies are unlikely to be associated with TRALI cases,

although little is known about what antibody titer is clinically relevant and it may vary depending on cell surface density or tissue distribution of the relevant antigen.

In proficiency test surveys of clinical histocompatibility laboratories, there is frequently low concordance between participating laboratories in identifying antibody specificities that are weak. Concordance between at least 90% of laboratories is required for grading purposes and is usually achieved for strong antibody specificities. Until evidence is obtained regarding the antibody concentration required for induction of TRALI, we decided to define MFI cutoffs for the SAB assay that might exclude some weak antibodies but that would provide a reliable estimate of the occurrence of stronger antibody specificities potentially more relevant to TRALI. This was achieved by analysis of reactivity in non-alloexposed males.

The antibody strength, as assessed by signal to cutoff values, required for a positive result in either the screening or SAB assay in this study is acknowledged to be higher when compared to how these tests are used in the transplantation setting. In both the multi-antigen bead screening assay and the SAB assay, default cutoffs have been determined for testing patients awaiting transplant who could potentially produce more antibody in the absence of immunosuppressive drugs, and were therefore designed to be very sensitive so the risk of antibody-mediated acute rejection is minimized. Antibody identification tests could be used by transplant laboratories to confirm the presence of antibodies when screening results were suspected to be falsely positive. In this study we decided to determine new cutoffs for reactivity in both the screening assay as previously reported⁸, and the SAB assay, reasoning that HLA antibodies should only occur at low rates in a population with no known alloexposure.

We adopted this approach even though there had been a publication reporting that apparently non-alloexposed male subjects had a high rate of HLA antibody reactivity when SAB assays were interpreted using lower assay cutoffs¹⁵. Although we detected HLA antibody reactivity when we analyzed our data in a similar fashion, the frequencies we found were 37% lower than the published data for class I and 57% lower for class II antibodies; both of these differences were statistically significant. Such antibodies in non-alloexposed persons may represent an immune system response to environmental antigen exposure leading to the formation of HLA cross-reactive antibodies (termed “natural” antibodies by Morales-Buenrostro et al¹⁵). If this is the case, then perhaps such environmental exposure is higher in Mexico than in the US. On the other hand, it is our opinion that the data are not yet strong enough to exclude other explanations such as assay variability and non-specificity as possible explanations for some of the findings. We believe that further studies of this issue are warranted.

In our study, some discrepancies between the screening and SAB assays were noted. 21% of samples that had positive class I screening results had negative SAB results; (1.7% of all LAPS donors); and 22% of samples that had positive class II screening results had negative SAB results (1.8% of all LAPS donors). This reactivity could be due to multiple low-titer antibody specificities binding to the same individual screening beads that were coated with antigens from multiple cell lines or due to false positive screening test results.

We also observed the opposite type of discrepancy. A small percentage of samples that screened negative by our cutoff but with an NBG >2.2 were positive in the SAB assay, possibly due to higher antigen density on the SAB beads. In the screening assay, class I antigens from seven cell lines or class II antigens from eight cell lines were attached to each bead in the lot used. In contrast, only one purified antigen is attached to each bead in the SAB assay, yielding a much higher density of antigens on each bead. On a population-wide

basis, these discrepant results represent only 1.3% of tested donors for class I and 0.3% for class II. Although these might potentially be donors whose blood components present some risk for inducing TRALI in recipients with cognate antigens, more work on critical antibody concentrations must be done before an acceptable rate of false negatives in a screening assay can be determined.

At this point, we do not believe that the SAB assay should be used as a donor screening test. This assay is more complex and costly than the multibead screening assay and there is insufficient evidence to indicate that this assay is better able to detect donors who pose a TRALI risk. The purpose of using the SAB assay in this study was to identify the specificities of antibodies detected by the screening assay and secondarily to provide supporting evidence that the majority of antibodies detected by the screening assay could be corroborated by another test configuration.

The observation that males and never pregnant females demonstrated higher rates of screen positive, but SAB negative results for class I as opposed to class II antibodies may indicate either that there are more low-level antibodies against class I than against class II antigens, or that there is a greater false positive rate for class I than for class II in the screening assay. If this is the result of low-level antibodies that bind to HLA antigens in these nonalloimmunized donors, they may be heterophile antibodies against environmental antigens that happen to crossreact with HLA antigens at low avidity. Why this is predominantly restricted to HLA class I reactivity remains to be determined.

The high frequencies for antibodies against A23, A24, A25, and A32 suggested that the presence of the Bw4 epitope on these four HLA A antigens may explain a portion of their individual frequencies. If a donor had been alloimmunized against a Bw4-containing HLA B antigen, even in the absence of any of these four HLA A antigens, antibodies could be induced that would also react with all four of these antigens. Not all samples with antibodies against a Bw4-bearing HLA B antigen are expected to contain antibodies against that epitope, but the probability of such antibodies is always present if the alloimmunized individual lacks the Bw4 epitope. In a series of 16 TRALI cases, 3 of 16 donors had antibodies directed against one or more of these four antigens supporting clinical importance of these antibodies¹⁶. The most frequently observed HLA B specificity was the B15 antigen. This serological specificity was designated if any of seven beads was positive. These can be divided into seven serologically distinct antigen groups, but due to the high degree of crossreactivity between these antigens, it was decided to present the aggregate data. The beads were coated with B*1501 (B62), B*1516 (B63), B*1510 (B71), B*1503 (B72), B*1502 (B75), B*1512 (B75), or B*1513 (B77). In Caucasians, the most common of these alleles is B*1501 (B62). The frequencies of all the other six B15 antigens added together (0.006) is less than 10% of the frequency of B*1501 (0.067) in European North Americans according to tables published by the National Marrow Donor Program (http://bioinformatics.nmdp.org/HLA/Haplotype_Frequencies/index.html). B62 and B63 antibodies have been described in one TRALI case for each, giving clinical support to the importance of B15 antibodies¹⁶. Antibody specificities against the uncommon antigens HLA Cw17 and Cw18 were seen nearly as frequently as against the much more common Cw3 antigen (Figure 3C). This may result from the observation that Cw17 and Cw18 share epitopes with more common Cw antigens, and also with A or B antigens¹⁷.

A recent summary of 10 years of investigations in the UK indicated that the most frequent cognate recipient antigen-donor antibody pairs identified in TRALI cases were DR52 (18%) and DR4 (16%), either alone or in association with other cognate matches¹⁸. It is of interest that DR4 antibody was the second most common DR antibody identified in our previously pregnant female donors (3.4%) but DR52 was the least frequent (<1%). The interpretation of

the relationship of our findings to that of the UK investigators is currently unclear but may be better elucidated as clinical studies are performed (see below). The kits used in SAB assays for this study did not include a full array of known DP antigens, but with the limited group of relatively common DP antigens, anti-DP antibodies were found in females with pregnancy histories. Much less is known about antibodies against HLA DP compared to other HLA loci. Both alpha and beta chains are polymorphic and could each potentially bear alloimmunizing epitopes. Although our data show that previously pregnant donors may show HLA DP antibodies, we are not aware of any published reports of HLA DP antibodies involved in TRALI cases.

With data on the frequency of occurrence of HLA antibody against each specificity, it will be possible to calculate the risk of receiving potentially reactive antibodies against cognate antigens if a recipient's HLA phenotype is known before transfusion. This issue will be addressed in a subsequent manuscript.

Donors with class I antibody specificities predominantly demonstrated >5 different HLA A, B and/or C specificities. Assuming the majority of these contained specificities for at least some common HLA antigens, these donors would be expected to pose a risk for TRALI induction in a very large percentage of recipients who could have cognate antigens matching their antibody specificities. Donors with class II antibody specificities largely segregated into two groups: those with only one HLA DR, DQ or DP specificity and those with >5 specificities. Donors with only a single class II specificity would be expected to pose a limited risk for TRALI induction, but those with >5 specificities would pose a risk for a larger percentage of recipients. Of note, preliminary analyses of our data indicate that there is a strong relationship between NBG ratios on the screening assay (for those NBG values above our empirically determined cutoffs) and the number of HLA specificities observed by SAB testing, suggesting blood components from donors with higher NBG ratios may pose a greater risk for TRALI (data not shown, Carrick D., 2009 AABB Annual Meeting oral presentation).

While the pathophysiology of TRALI remains uncertain, the leading hypothesis is a two-stage model in which the patient's underlying condition results in neutrophil priming and sequestration in the lungs and a second factor such as the transfusion of HLA antibodies recognizing a cognate recipient HLA antigen resulting in neutrophil activation, release of neutrophil contents, and subsequent events leading to non-cardiogenic pulmonary edema. Consistent with this proposed mechanism, the risk that a specific HLA antibody-containing component could lead to TRALI may be expected to vary with the cumulative frequency of cognate antigens in the population. If TRALI requires a critical threshold of infused antibody in the associated blood component to activate neutrophils sequestered in the lung, then the effective concentration of infused antibody in the lung may be influenced by the distribution of cognate HLA antigens on different cell types in the recipient.

Neutrophils normally express only HLA class I antigens, but γ -interferon can induce expression of class II antigens as well¹⁹. Monocytes may also be involved in TRALI, and they express both class I and II antigens. Leukocytes, platelets, endothelial cells, and cells in other tissues that come into contact with the bloodstream of the recipient express the class I antigens HLA A and HLA B. Endothelial cells and leukocytes also express the HLA C class I antigen, but platelets express much less HLA C²⁰⁻²¹. Only certain subsets of leukocytes normally express the class II antigens HLA DR, HLA DQ and HLA DP, although γ - interferon can induce their expression on other cell types, including vascular endothelial cells²². Such induction of HLA antigen expression on lung endothelium and other cells could contribute to the "first hit" in TRALI, with infusion of cognate HLA antibodies representing the "second hit".

Antibodies against cognate HLA A or HLA B antigens will therefore bind to a wider array of cells after transfusion than antibodies against HLA C, and antibodies against HLA C should bind to a wider array of cells than antibodies against HLA DR, HLA DQ, or HLA DP. If there is a critical threshold concentration of antibody required to trigger clinically significant TRALI in a recipient, the starting concentration in a transfused blood product of a certain volume might need to be higher for antibodies against HLA A and HLA B than for other HLA antigens, providing that sufficient binding of these antibodies to other cell types occurred prior to passage of the transfused plasma through the pulmonary circulation. Non-neutrophil leukocytes in the blood, and possibly endothelial cells could also play an active secondary role in the pathogenesis of TRALI. HLA antibodies could have a wider potential for exacerbating the condition by binding to non-neutrophil leukocytes or other cells and causing release of cytokines or other mediators that affect neutrophils or endothelial cells in the lungs or by binding to parenchymal lung tissues and causing them to be more susceptible to neutrophil adhesion or damage.

Thus, the exact specificities and the total amount (concentration and plasma volume) of antibodies in blood components associated with cases of TRALI needs to be understood better. REDS-II is currently conducting a large-scale lookback study based on detailed chart-reviews to ascertain the rates of TRALI occurrence in recipients of high plasma volume components from a percentage of HLA antibody positive donors in this current study. The identification of antibody specificities by SAB testing in this cohort of donors, when combined with the clinical data from the lookback study, may allow us to increase our understanding of the importance of certain specificities in TRALI.

Acknowledgments

The authors gratefully acknowledge Mila Lebedeva and Deborah Bunch for technical assistance, Yu Sun for data analysis assistance, and Jarhow Lee and Donna Phelan for expert manual interpretation of Luminex antibody results. Dr. Jarhow Lee is an employee of One Lambda Corporation, the manufacturer of the test kits used in this study. The authors thank staff at all six participating blood centers. Without their help, this study would not have been possible.

Sources of Support This work was supported by NHLBI contracts N01-HB-47168, -47169, -47170, -47171, -47172, - 47175 and -57181.

REFERENCES

1. Holness L, Knippen MA, Simmons L, Lachenbruch PA. Fatalities caused by TRALI. *Transfus Med Rev.* 2004; 18:184–8. [PubMed: 15248168]
2. Vamvakas EC, Blajchman MA. Transfusion-related mortality: the ongoing risks of allogeneic blood transfusion and the available strategies for their prevention. *Blood.* 2009; 113:3406–17. [PubMed: 19188662]
3. Cooling L. Transfusion-related acute lung injury. *JAMA.* 2002; 288:315–6. author reply 6. [PubMed: 12117393]
4. Nicolle AL, Chapman CE, Carter V, Wallis JP. Transfusion-related acute lung injury caused by two donors with anti-human leucocyte antigen class II antibodies: a look-back investigation. *Transfus Med.* 2004; 14:225–30. [PubMed: 15180814]
5. Popovsky MA, Moore SB. Diagnostic and pathogenetic considerations in transfusion-related acute lung injury. *Transfusion.* 1985; 25:573–7. [PubMed: 4071603]
6. Palfi M, Berg S, Ernerudh J, Berlin G. A randomized controlled trial of transfusion-related acute lung injury: is plasma from multiparous blood donors dangerous? *Transfusion.* 2001; 41:317–22. [PubMed: 11274583]
7. Sachs UJ, Link E, Hofmann C, Wasel W, Bein G. Screening of multiparous women to avoid transfusion-related acute lung injury: a single centre experience. *Transfus Med.* 2008; 18:348–54. [PubMed: 19140817]

8. Triulzi DJ, Kleinman S, Kakaiya RM, Busch MP, Norris PJ, Steele WR, Glynn SA, Hillyer CD, Carey P, Gottschall JL, Murphy EL, Rios JA, Ness PM, Wright DJ, Carrick D, Schreiber GB. The effect of previous pregnancy and transfusion on HLA alloimmunization in blood donors: implications for a transfusion-related acute lung injury risk reduction strategy. *Transfusion*. 2009
9. Terasaki PI, Cai J. Human leukocyte antigen antibodies and chronic rejection: from association to causation. *Transplantation*. 2008; 86:377–83. [PubMed: 18698239]
10. Takemoto SK, Zeevi A, Feng S, Colvin RB, Jordan S, Kobashigawa J, Kupiec-Weglinski J, Matas A, Montgomery RA, Nickerson P, Platt JL, Rabb H, Thistlethwaite R, Tyan D, Delmonico FL. National conference to assess antibody-mediated rejection in solid organ transplantation. *Am J Transplant*. 2004; 4:1033–41. [PubMed: 15196059]
11. Bray RA, Harris SB, Josephson CD, Hillyer CD, Gebel HM. Unappreciated risk factors for transplant patients: HLA antibodies in blood components. *Hum Immunol*. 2004; 65:240–4. [PubMed: 15041162]
12. Zeevi A, Lunz JG 3rd, Shapiro R, Randhawa P, Mazariegos G, Webber S, Ginita A. The Emerging Role of Donor-Specific Anti-HLA Antibody Determination for Clinical Management after Solid Organ Transplantation. *Hum Immunol*. 2009
13. Norris PJ, Lee JH, Carrick DM, Gottschall JL, Lebedeva M, de Castro BR, Kleinman SH, Busch MP. Long-term in vitro reactivity for human leukocyte antigen antibodies and comparison of detection using serum versus plasma. *Transfusion*. 2009; 49:243–51. [PubMed: 18980615]
14. Marsh, SGE.; Parham, P.; Barber, LD. *The HLA Facts Book*. Academic Press; 2000.
15. Morales-Buenrostro LE, Terasaki PI, Marino-Vazquez LA, Lee JH, El-Awar N, Alberu J. “Natural” human leukocyte antigen antibodies found in nonalloimmunized healthy males. *Transplantation*. 2008; 86:1111–5. [PubMed: 18946350]
16. Kopko PM, Paglieroni TG, Popovsky MA, Muto KN, MacKenzie MR, Holland PV. TRALI: correlation of antigen-antibody and monocyte activation in donor-recipient pairs. *Transfusion*. 2003; 43:177–84. [PubMed: 12559013]
17. El-Awar NR, Akaza T, Terasaki PI, Nguyen A. Human leukocyte antigen class I epitopes: update to 103 total epitopes, including the C locus. *Transplantation*. 2007; 84:532–40. [PubMed: 17713439]
18. Chapman CE, Stainsby D, Jones H, Love E, Massey E, Win N, Navarrete C, Lucas G, Soni N, Morgan C, Choo L, Cohen H, Williamson LM. Ten years of hemovigilance reports of transfusion-related acute lung injury in the United Kingdom and the impact of preferential use of male donor plasma. *Transfusion*. 2009; 49:440–52. [PubMed: 18980623]
19. Gosselin EJ, Wardwell K, Rigby WF, Guyre PM. Induction of MHC class II on human polymorphonuclear neutrophils by granulocyte/macrophage colony-stimulating factor, IFN-gamma, and IL-3. *J Immunol*. 1993; 151:1482–90. [PubMed: 8335942]
20. Mueller-Eckhardt G, Hauck M, Kayser W, Mueller-Eckhardt C. HLA--C antigens on platelets. *Tissue Antigens*. 1980; 16:91–4. [PubMed: 6970427]
21. Datema G, Stein S, Eijssink C, Mulder A, Claas FH, Doxiadis II. HLA-C expression on platelets: studies with an HLA-Cw1-specific human monoclonal antibody. *Vox Sang*. 2000; 79:108–11. [PubMed: 11054049]
22. Geppert TD, Lipsky PE. Antigen presentation by interferon-gamma-treated endothelial cells and fibroblasts: differential ability to function as antigen-presenting cells despite comparable Ia expression. *J Immunol*. 1985; 135:3750–62. [PubMed: 3934267]

Figure 1. Correlation between reactivity in the class I or class II antibody screen assay and reactivity in the SAB assay

HLA antibody screening was performed on 7920 subjects (2086 males, 1816 never pregnant females, 634 females with 1 pregnancy, 1307 females with 2 pregnancies, 1058 females with 3 pregnancies, 993 females with 4 or more pregnancies, and 26 females without reported pregnancy histories). Panel A shows class I test results for donors with class I screen NBG > 2.2, but ≤ 10.8. Panel B shows class I test results for donors with class I screen NBG > 10.8. Panel C shows class II LAPS test results for donors with class II screen NBG > 2.2, but ≤ 6.9. Panel D shows class II LAPS test results for donors with class II screen NBG > 6.9. Open bars indicate samples that were Screened Reactive at the indicated NBG level; Black bars indicate samples that were reactive on both the Screening and SAB assays.

Figure 2. Impact of SAB assay cutoff on SAB reactivity

Panel A shows the impact of various class I SAB assay cutoffs on SAB reactivity in LAPS samples. Squares indicate the percentage of donors who screened above NBG 10.8 (N=619 donors) and were SAB reactive (one or more beads above the designated MFI value) at the given cutoff. Circles indicate the percentage of donors who screened between NBG 2.2 and 10.8 (N=722 donors) and were SAB reactive at the given cutoff. Thick vertical line represents the class I MFI cutoff (MFI > 2500) selected. Panel B shows the impact of various class II SAB cutoffs on SAB reactivity in LAPS samples. Squares indicate the percentage of donors who screened above NBG 6.9 (N=690 donors) and were SAB reactive at the given cutoff. Circles indicate the percentage of donors who screened between NBG 2.2 and 6.9 (N=236 donors) and were SAB reactive at the given cutoff. Thin vertical line represents the class II MFI cutoff (MFI > 1500) selected.

A. HLA-A

D. HLA-DR

Figure 3. Antibodies observed against specific HLA antigens in ever pregnant females

The left Y-axis shows the percentage of antibodies against each specificity in all ever pregnant LAPS females (N = 3992). The right Y-axis shows the percentage of antibodies against each specificity in ever pregnant LAPS females who screened positive for HLA class I antibodies (N = 585) in Panels A-C and class II antibodies (N = 664) in Panels D-F. Panel A. HLA-A specificities; Panel B. HLA-B specificities; Panel C. HLA-C specificities; Panel D. HLA-DR specificities; Panel E. HLA-DQ specificities; and Panel F. HLA-DP specificities. In Panel B, the black bars show antigens with the Bw4 specificity¹⁴. The B15 bar is shown in grey, since only two of the seven B15 beads (B63 and B77) have the Bw4 epitope and their combined frequency is much lower than the combined frequencies of the other antigens in the B15 group, which have the Bw6 epitope.

Figure 4. Total number of SAB specificities observed in LAPS donors who screened reactive (i.e. CLI NBG > 10.8; CLII NBG > 6.9) and were SAB reactive (i.e., CLI MFI > 2500; CLII MFI > 1500)

Panel A. Class I antibody specificities: Solid bars, total number of donors with the number of reactive HLA-A specificities as indicated on the x-axis; striped bars, total number of donors with the number of reactive HLA-B specificities as indicated on the x-axis; open bars, total number of reactive HLA-C specificities as indicated on the x-axis; gray bars, total number of class I specificities as indicated on the x-axis. Panel B. Class II antibody specificities: Solid bars, total number of donors with the number of reactive DR specificities as indicated on the x-axis; striped bars, total number of donors with the number of reactive DQ specificities as indicated on the x-axis;; open bars, total number of donors with the number of reactive DP specificities as indicated on the x-axis; gray bars, total number of class II specificities as indicated on the x-axis.

Figure 5. Comparison of CLI and CLII SAB results in sera from 94 male donors in LAPS (open circles) with those reported by Morales-Buenrostro et al in 424 Mexican male subjects (closed diamonds)

Subjects from each study underwent SAB testing without regard to NBG ratios on the multibead screening assay. Panel A: Class I A locus; Panel B: Class I B locus; Panel C: Class I C locus; Panel D: Class II loci.

Data from all individual beads reported in the Mexican study are included in the Figure. Both studies used One Lambda SAB reagent lots that were almost identical in the antigen specificities bound to individual beads. Slight differences in antigen composition between the comparable class II beads in the two studies are indicated in the footnotes.

In order to compare LAPS data to that from Morales-Buenrostro¹⁵, the LAPS data are expressed differently than in the previous figures. The y axis shows the percentage of sera giving an MFI value of >1000, rather than the study defined cutoffs of 2500 and 1500 MFI for class I and II respectively; and the x axis shows data for each individual bead, rather than aggregating serologically equivalent beads under a single specificity; e.g., data are plotted separately for A3001 and A3002 beads rather than combining the two under the designation A30.

indicates the individual bead frequencies that were statistically significantly different between the two studies using Fishers Exact Test ($p < 0.05$ after applying the Bonferroni correction for 53 individual comparisons).

§ The Mexican study included individual beads for DQA1*0303/DQB1*0301 and DQA1*0505/DQB1*0301. In LAPS, these antigens were both present on a single bead and hence the LAPS data for this bead has been used to plot results for each of the two individual beads reported in the Mexican study.

¶ The LAPS SAB assay lacked the individual bead DQA1*0503/DQB1*0301 which was present in the Mexican study.

Table 1

SAB assay results from 132 non-transfused males with LabScreen NBG values ≤ 2.2

SAB Test Type	Number (percentage) positive on the SAB test at various MFI cutoff values *				
	500	1000	1500	2500	5000
Class I	55 (41.7%)	24 (18.2%)	16 (12.1%)	7 (5.3%)	2 (1.5%)
Class II	19 (14.4%)	3 (2.3%)	0 (0%)	0 (0%)	0 (0%)

* A value above the designated normalized MFI cutoff value for one or more beads leads to a positive test interpretation for class I or class II antibodies.