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Analysis of a High-Throughput HLA Antibody Screening Assay for Use with Platelet Donors

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Abstract

Background—Passive infusion of HLA antibodies has been implicated in transfusion reactions. A rapid, inexpensive method of screening blood donors for HLA antibodies might reduce the incidence of reactions. A high-throughput microbead-flow analyzer HLA antibody detection technique was compared with an ELISA method.

Materials and Method—96 apheresis platelet donors were tested for antibodies to Class I and II HLA antigens using mixed antigen microbead-flow analyzer and ELISA assays. For both assays, samples reactive in the mixed antigen assay were tested with a panel reactive antibody (PRA) assay. Samples reactive in both the mixed antigen and PRA assays were considered positive.

Results—In the mixed antigen microbead assay 46 (48%) samples were reactive to Class I antigens and 20 (21%) to Class II. Further testing in the microbead PRA assay revealed that 34 (35%) had antibodies to Class I antigens, 18 (19%) to Class II, and 42 (44%) to either Class I or II. Class I antibodies were present in 56% of females and 36% of males. In the mixed antigen ELISA assay 4 samples were reactive with Class I antigens; 4 with Class II antigens, and 5 with Class I or Class II. All 5 reactive samples were also reactive in the ELISA PRA assay and were from females.

Conclusion—The microbead assay was more sensitive than the ELISA assay and detected antibodies in a large proportion of donors. Samples reactive in the mixed antigen microbead assay should be confirmed by a second assay before concluding that antibodies are present.

Introduction

Transfusion-related acute lung injury (TRALI) has been linked to the inadvertent transfusion of antibodies to neutrophil-specific and HLA antigens and is currently the leading cause of transfusion related mortality.[1] In addition the transfusion of leukocyte antibodies can cause less severe reactions.[2] Many centers now limit fresh frozen plasma and other plasma-containing components to those prepared from male donors in order to minimize the risk of transfusing components containing leukocyte antibodies. Transfusion guidelines in the USA and elsewhere now recommend that measures be implemented by all centers to prevent the transfusion of plasma-containing blood components from alloimmunized subjects to reduce the incidence of transfusion reactions such as TRALI.[3]

Platelet concentrates collected by apheresis contain large quantities of plasma and can cause TRALI. However, deferring multiparous women from donating platelet components would

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likely lead to a shortage of these components. An alternative strategy is to test apheresis platelet donors for antibodies to HLA antigens and defer donors found to be alloimmunized.

Many HLA laboratories have adopted high-throughput sequence-specific oligonucleotide probe (SSOP) methods for genotyping HLA Class I and II antigens. One such method uses up to 100 different color-coded microbeads and a modified flow cytometer or flow analyzer.[4,5] This platform also can be used for high-throughput testing of antibodies to HLA Class I and II antigens.

When the microbead-flow analyzer is used for HLA antibody testing, the color-coded microbeads are coated with HLA antigens and after serum or plasma is incubated with the antigen coated microbeads, fluorochrome-labeled antihuman IgG is added and a flow analyzer is used to determine the color-code of the reactive beads and hence the specific antigen(s) to which the antibody is reactive.[6–8] If the microbeads are coated with antigens from individual cells, the assay can be used to determine the percentage of panel reactive antibodies (PRA) and antibody specificities can be identified using microbeads coated with single HLA antigens. The microbead-flow analyzer assay may be useful for screening blood donors for antibodies to HLA antigens.

The purpose of this study was to compare the results of testing apheresis platelet donors for antibodies to HLA Class I and II antigens with the microbead-flow analyzer assay with an ELISA assay. A secondary goal was to assess the feasibility of using the microbead-flow analyzer mixed antigen assay for high-throughput screening of platelet donors for HLA antibodies.

Materials and Methods

Study design

Whole blood samples were collected in 10 mL red top tubes from 96 volunteers donating apheresis platelets at the Platelet Center, Department of Transfusion Medicine (DTM), Clinical Center, National Institutes of Health (NIH) after obtaining informed consent. Serum was separated from the whole blood and stored frozen at −20°C.

The serum samples were analyzed for the presence of IgG antibodies to HLA Class I and Class II antigens using two methods. One method involved testing samples with color-coded microbeads coated with HLA antigen (LABScreen, One Lambda, Inc. Canoga CA) and analysis with a flow analyzer (LABScan 100 flow analyzer, One Lambda). The other was an ELISA-based method (LAT, One Lambda).

When testing samples with the microbead-flow analyzer, all samples were first tested against microbeads coated with a mixture of HLA Class I and Class II antigens (LABScreen Mixed, One Lambda). Samples reactive in the mixed antigen microbead assay were then tested with microbeads coated with HLA antigens from individual cells (LABScreen PRA, One Lambda). All samples reactive in the PRA assay and with equivocal results in the PRA assay were tested with microbeads coated with individual HLA Class I antigens (LABScreen Single Antigen, One Lambda).

For the ELISA assay all samples were tested against a mixture of HLA Class I and II antigens (LAT Mixed, One Lambda). Samples reactive in the mixed antigen ELISA assay were tested in an ELISA assay with 88 antigens isolated from EBV-transformed human B cell lines (LAT 1288, One Lambda).

The samples were tested according to the manufacturer's instructions. For the microbead-flow analyzer, the package insert recommended that undiluted serum be tested. In the mixed antigen ELISA assay (LAT Mixed) serum was diluted 1 to 2, and in the ELISA PRA (LAT 1288) assay 1 to 3 using diluent provided by the test manufacturer (One Lambda).

Microbead-flow analyzer assay for HLA antibody detection

When testing the samples with microbeads coated with a mixture of HLA Class I and II antigens (LABScreen Mixed, One Lambda) 5 μL of microbead suspension was mixed with 20 μL of undiluted serum. The microbead-serum solution was incubated in one well of a 96-well plate in the dark for 30 minutes at 20–25°C with gentle shaking. After incubation, 150 μL of wash buffer (One Lambda) was added to each well and the plate was centrifuged at 1300×g for 5 minutes. The wash buffer was discarded from the wells and the plate washed two more times using 200 μL of wash buffer and centrifuging at 1300×g for five minutes after each wash. Conjugated goat anti-human IgG, 100 μL, was then added to each well and incubated in the dark for 30 minutes at 20–25°C with gentle shaking. After incubation, the plate was centrifuged at 1300×g for 5 minutes. The conjugate was removed by plate flicking and washed 2 more times with 200 μL of wash buffer. Phosphate buffered saline, 80 μL, was then added to each well and the samples were tested with a flow analyzer (LABScan 100, One Lambda).

Determination of cut-offs for reactive samples was based on calculations performed by the manufacturer's software (One Lambda). This calculation is based a normalization of the data called the Normalized Background (NBG) ratio. The NBG makes use of reactions of the test sample and negative control serum (One Lambda) with each bead and with a negative control bead which is included in the kit. The formula is explained in detail in the package insert. The cut-off for positive samples used for this study was the NBG ratio recommended by the manufacturer. The testing of samples in the PRA assay (LABScreen PRA, One Lambda) and the individual Class I antigens assay (LABScreen Single Antigen, One Lambda) was performed using the same method.

In the microbead-flow analyzer mixed antigen assay some reactions had indeterminate results due to high fluorescent values for reactions with negative control beads. Testing of these samples was repeated in the microbead-flow analyzer PRA assay.

ELISA assay

For the mixed antigen ELISA assay (LAT Mixed, One Lambda) 10 uL of serum diluted 1 to 2 in buffer was added to a 96-well Terasaki tray and incubated for one hour at 20–25°C. After washing with wash buffer (One Lambda), 10 μL of alkaline phosphatase conjugated anti-human IgG was added to each well and incubated for 40 minutes at 20–25°C. After washing the conjugate, 10 μL of colorimetric enzyme substrate added to each well and incubated for 10–15 minutes at 37°C. Reaction was stopped by adding 5 μL of stop reagent and read with an ELISA reader adapted for the Terasaki tray format. Cut-offs were calculated as a percentage of the range of the reactivity of the provided positive serum control (One Lambda) tested in the positive HLA wells minus the nonspecific background of the antibody diluent (One Lambda) tested in the blank wells. This value was calculated automatically by the test manufacturer's software (One Lambda). Results were recorded as positive if it exceeded the established cut off.

When samples were tested in the PRA ELISA assay (LAT 1288, One Lambda), the testing was performed as above except that samples were tested at a dilution of 1 to 3. The kit contains an 88 antigen panel. The PRA was calculated by dividing the number of wells with values over the cut-off by the total number of antigen preparations in the test panel, 88.

Statistical analysis

Group comparisons were preformed using t-tests.

Results

Testing for HLA antibodies using a microbead-flow analyzer assay

In the mixed antigen microbead-flow analyzer assay samples from 46 of the 96 donors were reactive with HLA Class I antigens and samples from 20 donors were reactive with Class II antigens. Overall, samples from 51 donors were reactive for either Class I or Class II antigens and samples from 15 donors were reactive for both Class I and Class II antigens (Table 1). Samples from 54% of female donors and 44% of male donors were reactive with Class I antigens and 32% of female donors and 16% of male donors were reactive with Class II antigens (Table 1).

The results of testing some samples in the mixed antigen microbead-flow analyzer assay could not be determined due to a high level of reactivity with the negative control bead. Among the 96 samples, 5% were indeterminate for the antibodies to Class I and 5% were indeterminate for antibodies to Class II antigens. Only one sample was indeterminate for both Class I and II antigens.

To confirm that the samples reactive in the mixed antigen microbead-flow analyzer assays contained antibodies to HLA antigens, all samples which were reactive in the mixed antigen assay were tested in the microbead-flow analyzer PRA assay. In addition the samples with indeterminate results were also tested in the PRA assay. A total of 31 samples were reactive with Class I antigens in the PRA assay and 18 were reactive with Class II antigens in the PRA assay. Results of reactivity for 3 samples in the PRA assay with Class I antigens and 3 samples in the PRA assay with Class II antigens could not be interpreted because the PRA was low and specificity could not be determined. The 3 samples with questionable reactivity to Class I antigens were tested in the single Class I antigen assay. All three showed reactivity to a specific antigen(s) and these samples were considered positive for Class I antibodies. Single Class II antigen assay kits were not available at the time of the study.

Based on the results of testing of the 96 samples in the screening assay, the PRA, and single Class I antigen assay a total of 33 (34%) samples were positive for antibodies to Class I antigens, 18 (19%) to Class II antigens, 42 (44%) to either Class I or II antigens, and 9 (9%) to both Class I and II antigens (Table 2). Females were more likely than males to have antibodies to Class II antigens (32% versus 9%, $p<0.02$), but there was no difference among females and males in the prevalence of Class I antibodies (41% versus 29%, $p>0.05$). There was no difference in the PRA for Class I antibodies among females and males with Class I antibodies ($50 \pm 34\%$ versus $45 \pm 38\%$, $p = 0.71$), but the PRA for Class II antibodies was greater in females than males ($48 \pm 29\%$ versus $6 \pm 5\%$, $p = 0.005$).

Testing for HLA antibodies in an ELISA assay

Testing of the samples from the 96 apheresis platelet donors in the mixed antigen ELISA found samples from 4 donors were reactive with HLA Class I antigen, 4 were reactive with Class II antigen, 5 were reactive with either Class I or Class II antigens, and 3 donors were reactive for both Class I and Class II antigens (Table 3).

All reactive samples were from females. All samples reactive in the mixed antigen ELISA assay were also reactive in the ELISA PRA, the microbead-flow analyzer mixed antigen, and the microbead-flow analyzer PRA assays (Table 4). The PRA results for the 4 samples with Class I antibodies detectable by the ELISA assay were greater than 75% in the microbead-flow

analyzer PRA assay and for the 4 samples reactive with antibodies to Class II antigens detectable in the ELISA assay were greater than 26% in the microbead-flow analyzer PRA assay (Table 4).

When the Class I antigen PRA, determined by the microbead-flow analyzer assay, was compared between the 4 donors with Class I antibodies detected in both the ELISA and microbead-flow analyzer assays and the 30 donors with antibodies detected in only the microbead-flow analyzer assay, the microbead-flow analyzer PRA for antibodies to Class I antigens was greater in the donors with antibodies detected in both assays (85 ± 10% versus 42 ± 35%, $p = 0.02$). The results of the Class II microbead-flow analyzer PRA assay in the 4 donors with antibodies detected in both assays did not differ from those of the 14 donors with antibodies detected only in the microbead-flow analyzer assay (56 ± 22% versus 31± 31%, $p = 0.14$).

Discussion

The microbead-flow analyzer method was more sensitive than the ELISA assay at detecting antibodies to HLA Class I and II antigens. Although the mixed antigen microbead-flow analyzer assay allows for the rapid testing of samples, it's use as a screening tool for blood donors requires careful consideration. The microbead-flow analyzer assay is a highly sensitive assay and the incidence of reactions that were not confirmed with the PRA microbead-flow analyzer assay was high, 18% for antibodies to Class I antigens and 7% for antibodies to Class II antigens, and many samples required repeat testing. We also found that with the mixed antigen assay the results of testing approximately 5% of samples were difficult to interpret. If the mixed antigen microbead-flow analyzer assay is to be used to screen blood donors, it would be worthwhile to test reactive samples in a second assay to confirm that antibodies are present to prevent the inappropriate exclusion of blood donors. The extra testing required to confirm reactive samples and to clarify the result of some tests adds several hours to the testing process and requires the use of additional reagents.

The microbead-flow analyzer PRA assay would likely be better than the microbead-flow analyzer mixed antigen assay to use as a platelet donor screening assay. In the limited number of samples tested in this study there was a very good correlation between the results of PRA assay and the microbead-flow analyzer single antigen assay suggesting that there were be less false positive reactions with the PRA assay than with the mixed antigen assay. In addition, the PRA assay will soon include beads with neutrophil specific antigens HNA-1a, HNA-1b and HNA-2b. Testing of samples in the microbead-flow analyzer PRA assay is similar to testing in the mixed antigen assay in that a single tube is used for both assays and the time to complete the assay is the same. However, the cost of regents for the PRA assay is greater than for the mixed antigen assay.

The prevalence of both Class I and II antibodies detected in the microbead-flow analyzer assay was much greater then in the ELISA assay. Since the results of testing reactive samples in the microbead-flow analyzer assay were confirmed with a PRA assay and sometimes single antigen assay, these likely represent true positive results. However, it is unclear if the antibodies detected in microbead-flow analyzer assay but not the ELISA assay would cause transfusion reactions. Since only 1 in 1,000 to 1 in 10,000 transfusions results in TRALI, the transfusion of most of the components from donors with antibodies in the microbead-flow analyzer assay will not cause TRALI. Furthermore since only approximately 1% to 2% of all transfused platelet components cause a reaction[9], it is likely that the transfusion of most platelet components with HLA antibodies will not cause any type of reaction.

Studies that compare the outcomes of transfusions of components with HLA antibodies and those without are needed to determine the role of HLA-specific antibodies in TRALI. The fact that the incidence of TRALI is much lower than the incidence of HLA antibodies in platelet donors suggests that specific antibody characteristics or other factors may be important to the pathogenesis of TRALI. It may be that antibody titer, specificity, or the expression of the cognate antigen by the recipient are important in the response of the recipient to the transfusion of a blood component with HLA antibodies. Other studies have shown that bioactive lipids,[10] soluble CD40L,[11] and neutrophil-specific antibodies[12] can cause TRALI. These factors may have a more important role than HLA antibodies in the pathogenesis of TRALI.

In some cases the false positive reactions in the microbead-flow analyzer mixed antigen assay may be due to non-specific binding of immunoglobulin to the microbeads. Several samples were tested utilizing a solution of microparticles treated with a blocking solution (Adsorb Out™, One Lambda, Canoga Park, CA). These microparticles did not contain specific antigen coating. High levels of background reactivity, assumed to be nonspecific reactivity which was binding to the negative control beads, were significantly reduced when these beads were utilized.

In contrast, the ELISA assay was not reactive with a number of samples in which antibodies were detected in the microbead-flow analyzer assay. The ELISA assay was more likely to detect Class I antibodies with a high PRA than those with a low PRA. Patients that are refractory to platelet transfusions may benefit from the transfusion of HLA-matched platelets and it would be better to test the serum from these patients for HLA Class I antibodies using the microbead-flow analyzer rather than the ELISA assay since the microbead-flow cytometer assay provides more complete information.

Other investigators have also found that the microbead-flow analyzer assay is more sensitive that traditional antibody detection assays. Gibney and colleagues tested in the microbead flow-analyzer PRA assay samples from 155 kidney transplant patients with a negative anti-human globulin-augmented complement-dependent cytotoxicity (AHG-CDC) crossmatch. They found that the microbead-flow analyzer assay detected donor-specific antibodies in 20 of the 155 patients.[13]

The proportion of female and male donors with HLA antibodies detected by the microbead-flow analyzer assay was very high. It may be that a higher percent of women have been alloimmunized during pregnancy than previously suspected, but it is not certain why so many male donors were alloimmunized. We did not question donors concerning any history of transfusions, but it is unlikely that the high rate of alloimmunization is due to transfusions alone. Gibney and colleagues also detected HLA antibodies with the microbead-flow analyzer assay in several patients without a history of a sensitizing event.[13] Interestingly, heterosexual couples who have unprotected sexual intercourse with the same partner for over a year have significant peripheral alloimmune response to their partner's leukocytes as demonstrated by responses in mixed leukocyte reaction (MLR).[14] This may be due to repeated exposure to HLA antigens expressed by leukocytes and epithelial cells in semen and female genital secretions.[14] It may be that sexual contact can result in HLA alloimmunization of males and females. These results suggest that if the lack of HLA antibodies is used as a donor eligibility criteria, then screening for HLA antibodies should not be limited to women with a history of pregnancy.

The increased sensitivity of the microbead-flow analyzer assay may be due in part to the manufacturers' recommendation to test serum or plasma undiluted, while in the mixed antigen ELISA assay the manufacturer recommends using a diluted sample. However, when samples were tested in the ELISA mixed antigen assay undiluted, diluted 1 to 2 and diluted 1 to 3, there was little difference in the results.

The best cut-off for a reactive sample in the microbead-flow analyzer mixed antigen assay has been a topic of discussion. Rather than using the NBG ratio recommended by the manufacturer some have advocated a higher ratio so that a larger proportion of samples would be non-reactive. The manufacturer indicates that a center may determine its own cut-off NBG ratios and higher or lower "sensitivities" can be seen based on the center's established cut-off ratios. However, the manufacturer cautions that extensive validation is required to determine an appropriate NBG ratio. Our analysis made no adjustments to the manufacturer's recommended NBG ratio for setting the cut-off for a reactive sample.

Gibney and colleagues considered samples with a PRA of greater than 15% in the microbead-flow analyzer assay as positive.[13] However, we found that samples from most donors with a Class I antibody PRA of less than 15% were also reactive in the single Class I antigen microbead-flow analyzer assay indicating that these reactions are due to specific antibodies. Studies are needed which correlate clinical out comes of transfusions with results of the microbead-flow analyzer assays in order to identify antibody parameters that are important in causing transfusion reactions.

In conclusion, the microbead-flow analyzer assay was more sensitive than the ELISA assay and detected antibodies in a large proportion of healthy blood donors, but approximately 20% of samples in the microbead-flow analyzer mixed antigen assay could not be confirmed in the microbead-flow analyzer PRA assay. If the microbead-flow analyzer mixed antigen assay is used to test for HLA antibodies, reactive samples should be tested in another assay to confirm the results. The microbead-flow analyzer assay is useful for assessing patients refractory to platelet transfusions, but it would need to be used with careful consideration if used for screening blood donors to prevent the excess exclusion of donors.

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Table 1

Results of testing samples from 96 apheresis platelet donors in a mixed antigen microbead-flow analyzer assay

	All Donors (n = 96)	Females (n = 41)	Males (n = 55)
Antibodies to Class I antigens			
Positive	46 (48%)	22 (54%)	24 (44%)
Indeterminate	5 (5%)	4 (10%)	1 (4%)
Antibodies to Class II antigens			
Positive	20 (21%)	11 (32%)	9 (16%)
Indeterminate	5 (5%)	4 (10%)	1 (4%)
Antibodies to Class I or II antigens			
Positive	51 (53%)	26 (59%)	25 (45%)
Antibodies to Class I and II antigens			
Positive	15 (16%)	7 (17%)	8 (15%)

Table 2

Number and percent of samples from 96 apheresis platelet donors with HLA Class I and II antibodies*

	Class I n (%)	Class II n (%)	Either Class I or II n (%)	Both Class I and II n (%)
All donors (n = 96)	33 (34)	18 (19)	42 (44)	9 (9)
Females (n = 41)	17 (41)	13 (32)	23 (56)	7 (17)
Males (n = 55)	16 (29)	5 (9)	20 (36)	2 (4)

*Samples were reactive in both the microbead-flow analyzer mixed antigen and microbead-flow cytometer PRA assays and in some cases the Class I single antigen assay.

Table 3

Results of testing samples from 96 apheresis platelet donors using the mixed antigen ELISA assay

	Class I n (%)	Class II n (%)	Either Class I or II n (%)	Both Class I and II n (%)
All donors (n = 96)	4 (4%)	4 (4%)	5 (5%)	3 (3%)
Females (n = 41)	4 (9%)	4 (9%)	5 (12%)	3 (7%)
Males (n = 55)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Table 4

Results of testing the 5 platelet donor samples reactive in the ELISA mixed antigen assay in the ELISA and microbead PRA assays

	Mixed antigen ELISA		PRA ELISA (%)		PRA Microbead (%)	
Donor	**Class I**	**Class II**	**Class I**	**Class II**	**Class I**	**Class II**
852	Negative	Positive	1	59	4	63
869	Positive	Negative	94	6	98	0
871	Positive	Positive	58	6	82	26
891	Positive	Positive	51	34	87	57
892	Positive	Positive	62	46	75	80