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Leukocyte Antigen and Antibody Detection Assays: Tools for Assessing and Preventing Pulmonary Transfusion Reactions

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Abstract

Antibodies to neutrophil and HLA antigens can cause pulmonary transfusion reactions and in some cases acute lung injury. When evaluating cases of pulmonary transfusion reactions it is often necessary to test donors for neutrophil and HLA antibodies and also type the recipient for neutrophil and HLA antigens. A variety of ELISA and flow cytometry based solid phase assays are available to test for HLA class I and class II antibodies, but not neutrophil antibodies. Screening for neutrophil antibodies requires the preparation of panels of fresh neutrophils and testing in agglutination, immunofluorescence, or flow cytometry assays. Genotyping of HLA class I and II antigens is performed with a variety of sequence specific primers, sequenced specific oligonucleotide probe and sequence based typing assays. Neutrophil specific antigens HNA-1a, -1b, -1c, -4a and -5a can be genotyped, but not HNA-2a or -3a. Phenotyping of HNA-2a can be preformed with CD177 monoclonal antibodies, but the gene encoding HNA-3a has not been identified and the genomic basis for the HNA-2a-negative phenotype is not known. In conclusion, patients and donors involved with pulmonary transfusion reactions can be quickly typed for HLA antigens and tested for HLA antibodies but testing for neutrophil antibodies and antigens requires the use of a reference laboratory.

Leukocyte antigens were described more than 50 years ago using leukocyte agglutination assays. Classic leukoagglutinins included antibodies to HLA antigens and neutrophil specific antigens. The HLA antigens, a family of closely related molecules located in the same region of chromosome 6, have been studied extensively and have been found play a critical role in almost all aspects of cellular and humoral immunity. Antibodies to HLA antigens can cause acute rejection of transplanted organs, refractoriness to platelet transfusions, and transfusion reactions. Neutrophil-specific antigens are known as Human Neutrophil Antigens (HNA). HNA antigens are made up of several unrelated molecules expressed predominantly if not exclusively by neutrophils. HNA antigens are a diverse group of molecules with widely different functions. Neutrophil antibodies cause neonatal alloimmune neutropenia, autoimmune neutropenia of childhood, and transfusion reactions including TRALI.

While the HLA and HNA antigen systems are quite different, these two antigen systems are associated by transfusion reactions. Antibodies to both antigen systems can cause both febrile and pulmonary transfusion reactions. The identification of HLA and neutrophil antigens and antibodies to these antigens are important for the evaluation of patients and donors involved in pulmonary transfusion reactions. The methods currently used to type HLA and neutrophil

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antigens and screen for HLA and HNA antibodies as well as strategies to evaluate patients and donors implicated in transfusion reactions are reviewed.

TYPES OF TRANSFUSION REACTIONS CAUSED BY LEUKOCYTE ANTIBODIES

Antibodies to neutrophil and HLA antigens can cause a variety of transfusion reactions. These include febrile transfusion reactions, pulmonary transfusion reactions in recipients of granulocyte concentrates as well as recipients of plasma containing blood components.

Febrile Transfusion Reactions

In the 1950's Brittingham found that the transfusion of whole blood into patients with leucoagglutinins could cause febrile reactions.¹ Both HLA and neutrophil-specific antibodies cause these reactions. Brittingham found that these reactions could be prevented by removing the buffy-coats from the whole blood¹. The risks of such reactions can now be significantly reduced in frequency and severity by transfusing leukocyte-reduced RBC and platelet components.²⁻⁴

Pulmonary Transfusion Reactions

Granulocyte transfusion recipients sometimes experience transfusion reactions characterized by shortness of breath, fever, and hemoglobin oxygen desaturation.⁵⁻⁷ Chest x-rays in patients experiencing these reactions may reveal new or worsening pulmonary infiltrates, but the pulmonary dysfunction is usually mild to moderate in severity and resolves within a few hours. These reactions occur in granulocyte transfusion recipients alloimmunized to HLA class I or neutrophil-specific antigens. Most often they are caused by antibodies to HLA antibodies.⁵⁻⁷ These reactions are likely a result of antibody-antigen interactions that cause the transfused granulocytes to become trapped in the pulmonary capillaries causing ventilation/perfusion mismatching and hypoxia.

Plasma Containing Blood Products can also cause pulmonary transfusion reactions. In 1957 Brittingham found that the transfusion of leucoagglutinins sometimes caused severe pulmonary transfusion reactions.¹ The transfusion of 50 mL of plasma with strong leukocyte agglutinins resulted in immediate faintness followed in about 45 minutes by vomiting, diarrhea, chills, fever, severe hypotension, severe tachypnea, dyspnea, cyanosis, and initial leukopenia followed by leukocytosis. The following day the transfusion recipient was comfortable, but a chest x-ray showed marked bilateral pulmonary infiltrates and a small pleural effusion. Three days later the x-ray abnormalities disappeared.¹ Several case reports of these "hypersensitivity" reactions followed in the 1960's and 1970's.

In the 1980's the term "Transfusion Related Acute Lung Injury" or TRALI was first used by Popovsky and colleagues to describe these types of reactions and the idea that antibodies to HLA class I antigens was an important cause of TRALI became widely accepted.⁸ In 1985 Popovsky and Moore reported 36 cases of TRALI⁹. They defined TRALI as x-ray evidence of pulmonary infiltrates appearing within four hours of a transfusion. Seventy-two percent of the patients required mechanical ventilation. Eighty percent of patients with TRALI had a rapid resolution of the pulmonary infiltrates and return of arterial blood gas values to normal within 96 hours after the initial respiratory insult. However, pulmonary infiltrates persisted for at least 7 days following the transfusion reaction in 17% of the TRALI patients. The mortality associated with TRALI in these patients was 6%.⁸ Lymphocytotoxic antibodies were found in 72% of the donors and antibodies with specificity to HLA antigens were found in 65% of the donors.

In 2005, an NHLBI consensus group defined TRALI as acute lung injury occurring within 6 hours of a transfusion of a plasma-containing or plasma-derived blood product in a patient without pre-existing acute lung injury or alternative acute lung injury risk factors.¹⁰ Acute lung injury is the acute onset of respiratory distress with 1) pulmonary artery occlusion pressure ≤ 18 mmHg or without clinical evidence of left atrial hypertension, 2) bilateral infiltrates on chest x-ray, and 3) hypoxemia with a ratio of $\text{PaO}_2/\text{FIO}_2 \leq 300$ mm Hg or oxygen saturation of $\leq 90\%$ on room air.¹⁰ This definition of TRALI is not dependent on leukocyte antibodies in the blood component.¹⁰

One of the most difficult aspects of diagnosing TRALI is determining if a factor other than the transfusion caused the acute lung injury.¹¹ The consensus group definition also allows for the diagnosis of TRALI when a patient has one or more acute lung injury risk factors. When acute lung injury meets all the criteria for TRALI except that another acute lung injury risk factor is present and the acute lung injury can only be attributed to transfusion, a diagnosis of TRALI can be made.

LEUKOCYTE ANTIGENS

In order to evaluate a transfusion reaction, it's important to determine if leukocyte antibodies are present in the transfused blood component or blood donor. When leukocyte antibodies are detected it is often necessary to determine if the antibody is specific to HLA antigens, neutrophil antigens or both and to identify the specific antigens the antibody is directed. It is also helpful to assess the HLA and neutrophil antigen type of the transfusion recipient in order to determine if the antibody is directed to an antigen expressed by the recipient. The structure of HLA and neutrophil antigens are reviewed as well as methods to type these antigens and detect antibodies to these antigens.

HLA Antigens and Genes

Antibodies to both HLA class I and class II antigens can cause transfusion reactions. In general, HLA class I and II genes have a very similar structure and function.^{12,13,14} Both HLA class I and class II molecules are made up of a heavy and light chain. The genes encoding the HLA class I heavy chain and both the HLA class II heavy and light chains are located in the MHC region of chromosome 6. All HLA class I molecules share the same light chain, β_2 microglobulin, which is encoded outside the MHC on chromosome 15.¹⁵

The class I antigens relevant to transfusion reactions are HLA-A, -B, and -C. Three separate genes encode the class I HLA-A, -B and -C heavy chains, *HLA-A*, *HLA-B*, and *HLA-C*, and all three have a similar structure. Each contains eight exons encoding for the α_1 , α_2 and α_3 extra-cytoplasmic domains, a transmembrane domain, and a cytoplasmic tail. The first exon of the class I α chain encodes a leader sequence. Exons 2 through 4 are highly polymorphic and encode extracellular domains, α_1 , α_2 , and α_3 , that are responsible for peptide binding and T cell receptor (TCR) engagement. Exon 5 encodes the transmembrane region, exons 6 and 7 the cytoplasmic tail, and exon 8 the 3' untranslated region.

The HLA class II antigens include HLA-DR, -DP and -DQ. The structure of class II antigens differs from that of the HLA class I antigens. The HLA class II molecules are heterodimers composed of an α and β chain of approximately the same size. The genes encoding the class II α and β chains have a similar structure, but the α chain has 5 exons and the β chain has 6 exons. For both the α and β chain genes, exon 1 encodes the leader peptide, exons 2 and 3 encode the two extra cellular domains. For the β chain gene, exon 4 encodes the transmembrane domain, exon 5 for the cytoplasmic tail and exon 6 for the 3' untranslated region. For the gene encoding the α chain, exon 4 encodes the transmembrane domain and the cytoplasmic tail and exon 5 the 3' encode untranslated region.

There are 5 isotypes of the class II molecules which are designated as HLA-DM, HLA-DO, HLA-DP, HLA-DQ, and HLA-DR. Within the class II region of the MHC, the α and β genes encoding most of the isotypes are organized as pairs of α and β chains that contribute to the same isotype. The genes encoding the α chains are designated as “A” and those encoding the β chain “B”. The HLA-DM molecules are the product of HLA-DMA and HLA-DMB, HLA-DO of HLA-DOA and HLA-DOB, HLA-DP of HLA-DPA1 and HLA-DPB1 and HLA-DQ of DQA1 and DQB1 genes respectively.

The expression of HLA-DR molecules is more complicated than that of the expression of the other HLA class II molecules. All HLA-DR α chains are all encoded by the same DRA gene, HLA-DRA1. However, there are several functional HLA-DRB genes that encode the HLA-DR β chains and several HLA-DRB pseudo genes. In addition, the number of HLA-DRB genes and pseudogenes present varies among individuals. The different combinations of HLA-DRB genes are known as DRB haplotypes and several different haplotypes have been described. The HLA-DR molecules can use alleles coded by the DRB1, DRB3, DRB4 or DRB5 genes for β chains. HLA-DRB2, HLA-DRB6, HLA-DRB7, HLA-DRB8, and HLA-DRB9 are pseudo genes. The HLA DRB1 gene and DRB9 pseudogene loci are expressed in all HLA haplotypes. At most, one other HLA DRB gene is present in each of the 5 haplotypes. Some haplotypes have no functional DRB genes other than DRB1 (DR1, DR8 and DR10 haplotypes). The other haplotypes have DRB1 plus one of the other three functional DRB genes; DRB3, DRB4 or DRB5.¹⁶

Neutrophil Antigens and Genes

There are several clinically important neutrophil antigen systems referred to as Human Neutrophil Antigens or “HNA”.¹⁷ The antigen systems are designated by integers, and specific antigens within each system are designated alphabetically by date of publication.¹⁷ There are 5 HNA antigen systems, HNA-1, HNA-2, HNA-3, HNA-4 and HNA-5. These antigens are not as closely related as the HLA antigens. In fact, most HNA antigens are located on different molecules and are only related in their importance in neutrophil immunology.

The best characterized neutrophil antigen system is HNA-1, which has three alleles: HNA-1a, HNA-1b, and HNA-1c. The first allele was identified by Lalezari and colleagues in 1966 and was originally called NA1.¹⁸ They identified its allele, NA2, in 1972.¹⁹ These antigens are now known as HNA-1a and HNA-1b. HNA-1 antigens are located on human neutrophil Fc-gamma-receptor IIIb (Fc γ RIIIb) (CD16) and encoded by the *FCGR3B* gene located on chromosome 1.²⁰ HNA-1 antigens are only located on neutrophils and hence are neutrophil specific, however, soluble Fc γ RIIIb expression HNA-1 is present in plasma.²¹

The HNA-2 system has one allele, HNA-2a. HNA-2a is located on the NB1 gp and is encoded by the gene *CD177* which is located on chromosome 19q13.3.^{22,23} *CD177* is a member of the *LY6* gene family. *CD177* gp is a 58 to 64 kDa GPI-anchored protein that is expressed only on neutrophils.²⁴ It recently, has been found to be a ligand for PECAM-1 and to play a role in the adhesion of neutrophils to endothelial cells.²⁵ *CD177* has also been described as *PRV-1* and the expression of *CD177* mRNA is increased in patients with the myeloproliferative disorders polycythemia vera and essential thrombocytosis and in healthy subjects given G-CSF.²⁶

There is only one known allele in each of the other three antigen systems; HNA-3, HNA-4, and HNA-5. HNA-3a is located on a 70–90 kDa neutrophil gp, but the gene encoding this glycoprotein is not yet known.²⁷ HNA-4a and HNA-5a are located on neutrophil β_2 integrins.²⁸ HNA-4a is due to a polymorphism in the α_M chain of complement receptor 3 (CR3) (CD11b) and HNA-5a is due to a polymorphism in the α_L chain of leukocyte function antigen-1 (LFA-1) (CD11a).

TYPING OF LEUKOCYTE ANTIGENS

Traditionally, both HLA and neutrophil-specific antigens were identified and phenotyped using techniques that made use of alloantibodies and isolated leukocytes. Serological typing is being replaced by genotyping for several reasons. Alloantibodies are difficult to obtain and typing with alloantibodies is less precise than molecular typing.

Phenotyping assays also require working with fresh whole blood samples and lymphocytes or granulocytes must be isolated from red cells and other leukocytes. In contrast, molecular genotyping methods do not require that the blood samples tested are fresh or that leukocytes be separated from the other blood cells. Another advantage to working with DNA is that it can be easily stored for many months before testing; however, isolated leukocytes must be tested within a day or two. Most laboratories are switching to higher resolution molecular methods which make use of easier to handle DNA.

Typing of HLA Antigens

Phenotyping of HLA antigens—Classically, HLA typing was performed using serological methods. The serologic assessment of HLA antibodies and antigens took advantage of fetal-maternal sensitization. Since each child carries a full haplotype of paternal origin, pregnant women often develop antibodies against antigens expressed by the paternal haplotype. Maternal sera collected at term were characterized by testing their ability to kill HLA bearing cell lines of known phenotype in the presence of complement (complement-dependent cytotoxicity or CDC).²⁹ After panels of antisera were assembled CDC was then used for HLA phenotyping by exposing circulating cells expressing HLA class I (most cells) and class II (predominantly B cells) antigens from the individual to be typed to the previously characterized alloantisera.³⁰

Phenotyping of HLA antigens with the CDC assay is now seldom used because antibodies are difficult to obtain and serological typing is imprecise. Monoclonal antibodies specific for HLA antigens have been produced, but the resolution of typing provided by even the best serological methods is not sufficient for typing patients enrolled in some vaccine studies nor for the matching of unrelated hematopoietic progenitor cell transplant donors and recipients. Since antibodies identify structural differences on the surface of HLA molecules, variations caused by nucleotide polymorphisms in areas that are not exposed such as the peptide binding groove of the HLA heavy chain are not detectable. However, the differences that are located in the peptide binding groove are of functional significance as they determine the specificity and affinity of peptide binding^{31,32} and T cell recognition of self and allogeneic target cells.^{33,34}

Genotyping of HLA antigens—Molecular based typing methods directly determine the sequence of HLA molecules.³⁵ Several different methods of HLA genotyping are being used. All involve PCR amplification of HLA alleles. These methods include high resolution sequence-based typing and lower resolution sequence-specific primer (SSP)^{35,36} and sequence specific oligonucleotide probe (SSOP)³⁷ based methods.

There is no single standard method for intermediate resolution HLA typing. The method used by each laboratory is dependent on the quantity of tests a laboratory performs and resolution of the typing that is required. For SSP typing a unique PCR oligonucleotide primer pair is used to selectively amplify each allele at each locus. Only the oligonucleotide primer pair with an identical sequence to alleles carried by the patient is amplified. In most SSP assays the amplicons are analyzed by gel electrophoresis. For SSOP typing a single PCR reaction is used for each locus and the amplicon is probed with multiple oligonucleotides. A wide variety of methods have been used to detect reactions of the probes and the amplicons. SSOP genotyping

systems are most often used for the rapid genotyping of samples. For both SSP and SOPP typically 20 to 100 different primer sets or probes are used for each locus. Number is dependent on the level of resolution desired. The greatest number of primer sets and probes are required for HLA-B typing and the least for HLA-C.

Recently, a high throughput PCR-SSOP method of HLA typing using a flow cytometry based detection system has been developed.^{38,39} In this method, oligonucleotide probes are bound to polystyrene microbeads 5.5 μm in diameter.⁴⁰ The microbeads are color-coded with shades of two different dyes that provide a unique color code is given to each of the oligonucleotide probes. These color-codes allow one laser of a flow cytometer to distinguish 100 different color codes.³⁸ Each oligonucleotide probe is bound to a bead with a unique color-code. Amplified DNA from each subject to be typed is labeled with biotin and incubated with a pool of microbeads coated with oligonucleotide probes. Streptavidin-PE is added to the microbeads and binds to biotinylated DNA bound to probes (Figure 1). The microbeads are analyzed with a flow cytometer with two lasers. One laser identifies the beads with bound amplified DNA and the second laser determines the color code of the reactive beads and hence the reactive probe(s) (Figure 2). The microbeads are analyzed by modified flow cytometers with gates and computer software that allow the rapid analysis of pools of the 100 color-coded beads. The modified flow cytometers can identify the reactive probes and determine the HLA type of a subject in a shorter period of time than any other prior method had allowed. They also can automatically analyze samples in 96-well plates which allows for the rapid analysis of multiple samples. This high throughput intermediate resolution HLA typing system is commonly used to inexpensively type people for unrelated marrow donor registries, however, this system is also well suited for typing patients experiencing transfusion reactions.

Although SSP and SSOP methods could theoretically discriminate any known polymorphic site, they have two major limitations. First, SSP methods require a specific PCR reaction for each allele investigated and SSOP methods require a specific probe for each allele. Since each individual has only two alleles for each locus, for SSP typing a disproportionately large number of PCR reactions must be performed to cover all possible polymorphisms in order to identify the two alleles in all individuals and for SSOP typing a large number of probes must be used to identify all polymorphisms. In addition, since both SSP and SSOP oligonucleotide-based methods are based on specific interactions with known oligonucleotide sequences unique to a particular allele. They cannot identify unknown polymorphisms unless, by chance, the variation occurs within the region spanned by one of the oligonucleotide primers or pairs used in the assay. Because of these limitations interest is growing for definitive typing methods that yield conclusive information about the identity of the alleles typed.

The most comprehensive HLA genotyping method is sequence based typing (SBT), however, its utilization has been limited by cost of equipment and reagents, the high level of expertise and time required for the interpretation of each typing. In addition, some of the alleles and allele combinations are difficult to resolve even with sequence based typing.⁴¹ Finally, new methods based on high-density array technology are being developed that may allow extensive typing of known and unknown polymorphisms on micro-chips.⁴²

For some applications very high resolution HLA typing methods are required including the accurate matching of unrelated hematopoietic progenitor cell transplant donors and recipients. In addition, due to the important role that HLA molecules play in antigen presentation and the stringency of the relationship between epitope and associated HLA allele, high-resolution typing is increasingly requested for appropriate enrollment of patients into immunization protocols aimed at the enhancement of T cell responses. However, HLA typing of patients involved with pulmonary transfusion reactions doesn't require high resolution HLA typing. The purpose of the typing of a patient experiencing a severe transfusion reaction is to determine

if the patients experiencing the reaction expresses an antigen(s) to which antibodies in the implicated blood donor are directed. For this purpose promptly available intermediate resolution typing is needed. Because antibodies specific to both HLA class I and class II antigens have been implicated in TRALI, typing for both class I and/or II antigens is required.

Phenotyping and Genotyping of Neutrophil Antigens

Phenotyping—Traditionally, neutrophil antigen phenotyping has been performed using human alloantibodies in the granulocyte agglutination or granulocyte immunofluorescence assays. However, as with HLA alloantisera, alloantisera specific to neutrophil antigens are difficult to obtain. Alloantibodies to HLA-1a, -1b, -2a and -3a are available but antibodies to HNA-1c, -4a and -5a are difficult to obtain.

Monoclonal antibodies specific to HNA-1a, HNA-1b, and HNA-2a have been described, are commercially available, and have been used to phenotype neutrophils using flow cytometry. Granulocyte phenotyping with monoclonal antibodies and flow cytometry is faster and easier than phenotyping with alloantibodies. Phenotyping with alloantibodies requires the isolation of neutrophils while phenotyping with monoclonal antibodies can be done with whole blood instead of isolated neutrophils.

Genotyping—Genotyping assays to HNA-1a, -1b, -1c, -4a and 5a have been developed. The characterization of the genes encoding the HNA-1 antigens has allowed for the development of genotyping assays for these antigens.^{43–45} HNA-1a, HNA-1b, and HNA-1c are encoded by *FCGR3B*1*, *FCGR3B*2*, and *FCGR3B*3*, respectively. *FCGR3B*1* and *FCGR3B*2* differ at 5 nucleotides, one of which is silent. One nucleotide differs between *FCGR3B*2* and *FCGR3B*3*.²⁰ While distinguishing single nucleotide polymorphisms is usually simple, genotyping for *FCGR3B* alleles is complicated by the high degree of homology between *FCGR3B* and the gene that encodes FcγRIIIa, *FCGR3A*. Among the 5 nucleotides that differ between *FCGR3B*1* and *FCGR3B*2*, *FCGR3A* is the same as *FCGR3B*1* at 3 nucleotides and the same as *FCGR3B*2* at 2 nucleotides. As a result, most laboratories use PCR and sequence-specific primers to distinguish *FCGR3B* alleles. A unique set of primers is used to amplify each of the 3 alleles.

Methods to genotype neutrophil antigens HNA-4a and HNA-5a have been described.²⁸ Both of these polymorphisms are due to a single nucleotide polymorphism. HNA-4a is due to a single nucleotide substitution in the α_M subunit of the β_2 integrin, G302A that predicts an Arg61His amino acid polymorphism. HNA-5a is due to a single nucleotide substitution in α_L subunit of the β_2 integrin, G2466C, that predicts an Arg766Thr amino acid polymorphism. A variety of methods can be used to type these alleles. An PCR-SSP method is being used to type HNA-4a.⁴⁶ HNA-5a can be typed using a PCR-SSP method⁴⁷ or PCR restriction fragment length polymorphism method.⁴⁸

While the molecular basis of HNA-2a has been described, HNA-2a genotyping methods are not available. The HNA-2a-negative phenotype is due to *CD177* mRNA splicing defects.⁴⁹ The *CD177* mRNA from people with HNA-2a-negative neutrophils contains additional sequences of varying length that are homologous to *CD177* intron sequences. However, no mutations have been detected in the *CD177* introns or exons from people with HNA-2a negative neutrophils. It may be possible to distinguish the HNA-2a-positive from-negative phenotypes by analyzing neutrophil *CD177* mRNA for accessory sequences, but working with mRNA is much more difficult than working with DNA and no laboratory is currently analyzing granulocyte mRNA to assess HNA-2a antigen expression. Since the gene encoding HNA-3a has not been identified, no genotyping assays is available for this antigen.

LEUKOCYTE ANTIBODY DETECTION

HLA and neutrophil-specific antibodies were first characterized in the 1960's and 1970's and several methods were established for antibody screening and antigen typing. While the methods used for HLA antibody testing have changed markedly, the methods used for neutrophil antibody testing have remained largely unchanged.

Detecting HLA antibodies

While the CDC assay was the standard method for detecting HLA antibodies for many years, this assay has several shortcomings. Panels of lymphocytes from many donors must be prepared on a regular basis. This involves phenotyping the donors, collecting their blood, isolating their lymphocytes and preparing typing trays with lymphocytes from one donor in each well. The CDC assay involved adding sera to be tested to each well of the typing tray and incubating the cells and sera with complement. Cytotoxic HLA antibodies were measured by dye exclusion using microscopy. This assay was labor intensive and required a highly skilled staff.

The cellular based CDC typing assay has largely been replaced with solid phase assays. Solid phase assays make use of isolated HLA antigens fixed to well or microbeads. Antibody reactions to the antigen-coated surfaces are typically detected with an antihuman antibody conjugated to an enzyme detection system (ELISA) or a fluorochrome and the reactions are measured with a spectrophotometer or flow cytometer.

For the first solid phase assays HLA antigens were isolated from cell extracts using monoclonal antibodies attached to affinity columns.⁵⁰ The number of antigens that can be isolated is limited by the availability of monoclonal antibodies. To overcome this limitation, methods were developed to produce recombinant HLA antigens in *Escherichia coli*.⁵¹ These recombinant techniques allow for the production of individual antigens. However, proteins produced in *E. coli* are not glycosylated. Since the lack of glycosylation could affect some epitopes, methods have been developed to produce recombinant HLA molecules in human cell expression systems which produce mature glycosylated HLA molecules that are indistinguishable from native HLA antigens.⁵² Both HLA class I and class II antigens can be produced using human cell expression systems and used in solid phase antibody detection assays.

The initial solid phase assays used antigens fixed to wells and enzyme detection systems. Later, assays were developed that involved the coupling of HLA antigens to microbeads that could easily pass through a flow cytometer and antibody detection kits were developed that used flow cytometry to assess the binding of antibodies to the microbeads coated with individual HLA molecules. A further improvement in HLA antibody testing was the use of 100 color-coded polystyrene microbeads that are similar to those used for HLA SSOP typing, but have been adapted for detecting HLA antibodies.^{52,53} The microbeads are coated with HLA antigen rather than oligonucleotide probes. After serum or plasma is incubated with the antigen coated microbeads, fluorochrome labeled antihuman IgG is added and flow cytometry can be used to determine the color-code of the reactive beads and hence the specific antigen or antigens to which the antibody is reactive.⁵²

Flow cytometry based assays using pools of single antigen coated microbeads containing a variety of HLA antigens can be used to assess "panel reactive antibodies" or PRA. The percent PRA is determined by calculating the percentage of microbeads that are reactive to antibodies in serum samples.⁵⁴ Flow cytometry based microbead assays can also distinguish antibodies specific to class I and class II antigens, even when a sample contains a mix of antibodies to class I and class II antigens (Figure 3).⁵³ These antigen specific color-coded microbeads can be used with modified flow cytometers for high throughput testing of HLA antibodies.

Neutrophil Antibodies

While commercially available reagents and kits have greatly improved the detection of HLA antibodies, screening for neutrophil antibodies remains technically challenging. Antibodies to some neutrophil antigens can be detected using solid phase assays, however, the clinically important antibodies HNA-1 antigens have been difficult to detect in solid phase assays. In addition, since the gene encoding HNA-3a has not been identified, recombinant technology cannot be used to produce to HNA-3a antigen. As a result, most laboratories are using intact neutrophils for antibody screening assays. Unfortunately, neutrophils have a short life span so fresh neutrophils must be prepared daily for testing. Neutrophils are prepared from fresh whole blood using density gradient separation. Patient sera are tested in one of several assays against panels of neutrophils prepared from donors with known phenotypes to distinguish antibodies with different specificities. The presence of HLA antibodies can make the detection of neutrophil antibodies difficult. HLA-specific antibodies can be separated from neutrophil-specific antibodies by absorbing serum with platelets.⁵⁵ Alternatively, monoclonal antibody capture assays can be used to test for antibodies specific to neutrophil membrane glycoproteins. Mammalian cell expression systems have been used to express HNA-1a, HNA-1b and HNA-2a and these can be used to assess antibodies in flow cytometry, but these cells are not commercially available.

The assays used to detect neutrophil antibodies are granulocyte agglutination, granulocyte immunofluorescence or flow cytometry and monoclonal antibody immobilization of granulocyte antigens. In the granulocyte agglutination assay antibodies cause neutrophils to actively agglutinate.⁵⁵ Cells and serum are incubated for 4 to 6 hours at 30° C. When antibodies are present, neutrophils clump. The granulocyte agglutination assay is very reliable but less sensitive than other assays. It can detect antibodies to HNA-1, -2, -3, -4, and -5 antigens and it is the assay that can best identify antibodies specific for HNA-3a.

In the granulocyte immunofluorescence assay or flow cytometry assay, antigen-antibody reactions are detected using fluorescence-conjugated secondary antibodies and a fluorescent microscope.⁵⁵ Prior to incubation with sera, neutrophils are treated with 1% paraformaldehyde for 5 minutes at 20 to 24° C to help prevent nonspecific binding of antibodies to neutrophil Fc receptors and to stabilize the cell membranes. Binding of antibody to the neutrophils is detected with a fluorochrome conjugated secondary antibody. When analyzed by fluorescent microscopy the binding of antibodies in the test serum results in a uniform staining of the outside of the neutrophils. Strong reactions are readily distinguished, but considerable training is required to distinguish weak reactions from background staining. Testing for neutrophil antibodies with flow cytometry is the same as the granulocyte immunofluorescence assay except that neutrophils are evaluated with a flow cytometer rather than a fluorescent microscope.

The monoclonal antibody immobilization of granulocyte antigens (MAIGA) assay allows the detection of antibodies to specific neutrophil membrane glycoproteins.^{56–58} In this assay, neutrophils are incubated with test sera, washed, incubated with a murine monoclonal antibody to a specific neutrophil glycoprotein. The neutrophils are then dissolved in a mild detergent. The soluble glycoprotein-monoclonal antibody complex is “captured” in a well with an antibody specific to mouse IgG fixed to the well bottom. An antibody specific to human IgG conjugated to alkaline phosphatase is added followed by a substrate, and the reaction is detected with a spectrophotometer.

The MAIGA assay can be used to detect antibodies specific to HNA-1a, HNA-1b, and HNA-1c antibodies on FcγRIIIb (CD16), HNA-2a on NB1 gp (CD177), HNA-4a on complement component C3bi receptor (CR3 or CD11b), and HNA-5a on leukocyte function antigen-1 (LFA-1 or CD11a). The use of neutrophils from panels of donors with known HNA-1

phenotypes allows the identification of antibodies specific to HNA-1a, HNA-1b and HNA-1c. In addition, antibodies are sometimes detected that are directed to FcγRIIIb but are not specific to HNA-1a, HNA-1b, or HNA-1c. The MAIGA assay permits the recognition of antibodies to specific neutrophil glycoproteins even when antibodies to HLA antigens are present.

EVALUATION OF PATIENTS AND DONORS INVOLVED IN TRALI

When a patient experiences TRALI or a severe pulmonary transfusion reaction, a possible role for leukocyte antibodies in the transfusion reaction should be investigated. Even if the patient doesn't experience a reaction severe enough to meet the criteria for TRALI, a leukocyte antibody investigation may be indicated especially if the patient becomes neutropenic.⁵⁹ The purpose of testing for leukocyte antibodies is not to make or exclude a diagnosis of TRALI, but to identify donors whose blood components are at high risk for causing transfusion reactions. A determination that the transfusion was responsible for the reaction should be made before the donors are tested for leukocyte antibodies. Donors need only be tested if the transfusion is felt to be responsible for the transfusion reaction.

The first step in the investigation is to test all implicated blood donors for leukocyte antibodies (Figure 4). The evaluation should not be restricted only to women or multiparous women. Testing should not be limited to HLA antibodies. All male and female donors should be tested for antibodies to both neutrophil and HLA antibodies. HLA antibody testing should include both HLA class I and class II antibodies. Recipients who were transfused with neutrophil antibodies should be typed for neutrophils antigens and those transfused with HLA antibodies should be typed for HLA antigens.

Generally, it is not necessary to test the transfusion recipient for leukocyte antibodies because the leukocyte antibodies in the recipient have not been proven to cause TRALI. Granulocyte transfusion recipients are the exception. Recipients of granulocyte transfusion components who experience pulmonary transfusion reactions or TRALI should also be tested for both neutrophil and HLA antibodies.

Strategy for Antibody Detection

The implicated donor should be tested for both HLA and neutrophil antibodies (Figure 5). Most laboratories are using solid phase assays to test for HLA antibodies. These assays are available to rapidly and economically screen for the presence or absence of antibodies to HLA class I and class II antibodies. While these screening assays indicate that antibodies are present or absent, they provide no information concerning the specificity of the antibodies. Other solid phase assays are available that can determine the specificity of the HLA antibodies. Since the screening assays are less expensive than those which identify specific antibodies, testing all implicated donors in a two-step process maybe worthwhile. All implicated donors are tested in the screening assay, but only implicated donors found to have a reactive screening assay are tested in a second assay capable of identifying specific antibodies.

Most laboratories screen for neutrophil antibodies by testing serum against panels of granulocytes in granulocyte agglutination, granulocyte immunofluorescence or flow cytometry assays. Since HLA antibodies can react with neutrophils in granulocyte agglutination, immunofluorescence and flow cytometry assays, if HLA antibodies are present in a reactive sample, it must be tested in the MAIGA or a similar assay to determine if both HLA and neutrophil-specific antibodies are present. Since the MAIGA assay sometimes identifies antibodies that cannot be detected in other assays, some laboratories test all serum samples in MAIGA assays. An alternative to use of the MAIGA assay to determine if an antibody is specific to neutrophil or HLA antigens is to absorb that reactive sample with platelets and

repeat the testing. Since platelets express HLA Class I antigens, but not neutrophil specific antigens, platelet absorption selectively removes HLA Class I antigens.

Deferring Donors with Leukocyte Antibodies

It is widely accepted that both HLA and neutrophil-specific antibodies are responsible for febrile transfusion reactions that occur following the transfusion of leukocyte-containing blood components. However, the role of leukocyte antibodies in TRALI is complex. There have been many case reports that link the transfusion of neutrophil specific and HLA antibodies to acute lung injury, but often the transfusion of leukocyte antibodies does not result in a transfusion reaction.

In addition to case reports, animal models and clinical studies support a role of neutrophil antibodies in causing TRALI. Animal models have also shown that the transfusion of neutrophil antibody HNA-2a can cause acute lung injury.^{60,61} A prospective study that compared the outcome of the transfusion of FFP from multiparous women with control FFP suggests that neutrophil antibodies cause TRALI.⁶² The probability that a recipient of a plasma-containing blood component with a neutrophil antibody will experience a transfusion reaction appears to be high. “Look back” studies evaluating recipients of blood components with neutrophil antibodies implicated in TRALI or pulmonary transfusion reactions have shown that up to 36% of recipients of blood components with neutrophil specific antibodies experience transfusion reactions.^{59,63}

An animal model supports a role for HLA class I antibodies in acute lung injury.⁶⁴ The transfusion of MHC I antibodies to BALB/c mice resulted in acute lung injury. Lung injury required the presence of neutrophils and the expression of Fcγ Receptors by the mice. The MHC I antibodies bound diffusely to the lung and kidney microvascular. These findings suggested that the lung injury was due to adherence and activation of neutrophils to the Fc portion of MHC I antibodies bound to pulmonary endothelial cells.

While case reports and animal models suggest that antibodies to HLA class I and class II antigens cause TRALI, look back studies suggest that HLA antibodies infrequently cause TRALI. Look-back studies suggest that HLA antibodies are an infrequent cause of pulmonary transfusion reactions or TRALI. Studies by Toy et al have found that only one of 103 patients who transfused a blood component with broadly reactive HLA class I and class II antigens experienced TRALI.⁶⁵ Another look-back study by Cooling found pulmonary transfusion reactions in only 3 of 20 recipients of TRALI- implicated units containing broadly reactive HLA antibodies.⁶⁶

Since the incidence of neutrophil specific antibodies in healthy blood donors is low and the probability that the transfusion reaction of a blood component with neutrophil specific antibodies is high, at most centers donors with neutrophil-specific antibodies are deferred from further donations. Since HLA antibodies can often be found in healthy donors and since the probability that an HLA antibody causes a transfusion reaction is low, the decision to exclude donors with HLA antibodies is more difficult. If the reaction is severe or only one donor is implicated in a reaction, then it seems prudent to defer the donor with HLA antibodies from further donations. However, if a pulmonary transfusion reaction is mild or several donors are implicated in the reaction, it is reasonable to allow the donor with HLA antibodies to continue to donate. Demonstrating that the transfusion recipient expressed a HLA antigen corresponding to the antibody in the blood component or that the donor has been implicated in other transfusion reactions provides further support for a decision to defer the donor. The exclusion of all implicated donors with HLA antibodies probably unnecessarily prevents many people from donating blood.

Screening Donors for Leukocyte Antibodies

Some centers are only transfusing plasma products prepared from blood collected from male donors. While this approach will likely reduce the incidence of TRALI, it is not a practical method for reducing the risk TRALI from platelet transfusions. Elimination of all female platelet donors would create a shortage of platelets. A better approach would be to test platelet donors for leukocyte antibodies and not allow those with antibodies to donate platelets by apheresis or their whole blood donation to be used to produce platelets. The advent of high throughput multiplex assays to screen for HLA antibodies using color-coded beads and a modified flow cytometer makes it possible to screen platelet donors for HLA antibodies. Unfortunately, no such system is available for neutrophil antibody testing and testing large numbers of platelet donors for neutrophil antibodies with cellular assays would not be practical.

CONCLUSIONS

When evaluating a patient experiencing TRALI and the implicated donors, HLA antigen typing and antibody testing technology allows for readily available rapid typing of HLA antigens and screening for HLA antibodies. However, a lack of reagents and availability of commercial kits continues to limit neutrophil antigen typing and antibody testing to reference and research laboratories. Donors with neutrophil specific antibodies should be deferred from blood donations, but the decision to defer donors with HLA antibodies requires the consideration of clinical and donor factors.

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Figure 1.

Color-coded beads are being used with a modified flow cytometer for HLA antigen genotyping and antibody detection. Microbeads (panel A) are color-coded with a blend of fluorescent intensities of two dyes (panel B). One hundred different color-coded microbeads can be produced (panel C) which can be identified with a modified two laser flow cytometry. The gates of the modified flow cytometer can analyze up to 100 different color-coded microbeads in a single sample (panel D).

Figure 2.

Microbeads can be used for HLA sequence specific oligonucleotide probe (SSOP) genotyping. Genomic DNA from for a specific HLA locus is amplified by PCR and labeled with biotin (panel A). The amplified DNA is hybridized to a mixture of color-coded beads each of which is labeled with an oligonucleotide probe (panel B). Microbeads with reactive probes are identified with streptavidin-PE (SAPE) that binds to the biotinylated DNA (panel C).

Figure 3.

Detection of HLA antibody with microbeads. HLA antigens are bound to color-coded microbeads. Binding of alloantibodies in serum samples to specific HLA antigens can be detected with fluorescent labeled anti-human IgG.

Figure 4.

An approach to evaluating a transfusion recipient experiencing a severe pulmonary transfusion reaction and the implicated donor(s).

Figure 5.

Evaluation of neutrophil-specific and HLA antibodies in donors implicated in pulmonary transfusion reactions.