Granulocyte serology: current concepts and clinical signifcance

M.E. Clay, R.M. Schuller, and G.J. Bachowski

Applying serologic procedures to the detection of RBC and lymphocyte antigens has facilitated the identification of granulocyte antigens with established clinical significance, which are now classified in the human neutrophil antigen system. Granulocyte alloantibodies and autoantibodies have been implicated in a variety of clinical conditions including alloimmune neutropenia, autoimmune neutropenia, febrile and severe pulmonary transfusion reactions, drug-induced neutropenia, refractoriness to granulocyte transfusions, and immune neutropenia after hematopoietic stem cell transplantation. Although the intrinsically fragile nature of granulocytes contributes to the inherent challenges of granulocyte serology, several advances in laboratory procedures have improved detection of granulocyte antibodies. This review will provide a current perspective about the importance and use of granulocyte serology for detection of granulocyte antibodies that have significant medical effects.
***Immunohematology* 2010;26:11–21.**

Key Words: granulocyte, neutrophil, antigens, antibodies, HNA, alloimmune neonatal neutropenia, autoimmune neutropenia, transfusion-related acute lung injury, TRALI

It has now been half a century since Lalezari[1] described the first case of neonatal neutropenia attributable to transplacental transfer of granulocyte-specific antibodies, thus initiating the field of granulocyte serology. This event fostered several decades of work directed at the identification of granulocyte-specific antigens (and antibodies), methods for their detection, and an appreciation for their clinical significance. In many ways, granulocyte serology developed along the laboratory footprints of RBC and human lymphocyte antigen (HLA) serology with one major exception—granulocytes could not be preserved for testing purposes. The requirement for fresh cells was and continues to be a major impediment for wide-scale implementation of granulocyte immunobiology studies. Although the number of laboratories worldwide that specialize in this technology is limited, the clinical significance of granulocyte antibodies has not diminished and is now supporting the development of new methodologies that have the potential to enhance the utility of testing and growth of this field. Therefore, as we move ahead, it is important to (1) focus on the clinical events that support the need for granulocyte serology, (2) understand the benefits and limitations of the current serologic assays, and (3) evaluate the application of new technologies for the detection of granulocyte antigens and antibodies.

Clinical Significance

Alloimmune Neonatal Neutropenia

Neutropenia observed in the neonate is primarily the result of neutrophil-specific alloantibodies transplacentally transferred to the fetus from the maternal circulation. The presence of these human neutrophil antigen (HNA) antibodies most likely is the result of prenatal maternal sensitization by fetal neutrophils crossing the placental barrier, although the antibodies may have also arisen in association with autoimmune diseases such as systemic lupus erythematosus or rheumatoid arthritis.[2,3] Maternal alloimmunization can occur anytime after the first trimester of pregnancy. Once present in the fetal circulation, maternal HNA antibodies will bind to the mature fetal cells expressing the corresponding neutrophil alloantigens, as the neutrophil precursor cells are spared.[4] Neonates affected by alloimmune neonatal neutropenia (ANN) are almost always neutropenic at birth, although cases have been reported in which the neutropenia has been delayed by 1 to 3 days.[4] ANN is often asymptomatic and goes undiscovered unless the deficit of the neutrophils is profound enough (absolute neutrophil count < 1.5×10^9/L) that the newborn becomes susceptible and succumbs to infection caused by bacterial or fungal pathogens. These children commonly present with fever, malaise, lethargy, skin infections such as cellulitis and omphalitis, mucosal and respiratory infections (stomatitis, otitis media, and pneumonia), urinary infections, and very rarely septicemia. These infections are usually mild, but fatalities have been reported in 5 percent of cases.[5] As there are numerous reasons for neonatal neutropenia (i.e., congenital, antibody-mediated destruction, or infection that decreases hemopoietic cell production), detection of these maternal neutrophil antibodies is very important for determining the mechanism causing neutropenia. This allows the physician to focus on the appropriate treatment. The presence of maternal antibodies can persist up to 6 months after birth although antibodies and clinical effects usually dissipate more quickly. The incidence of ANN has been estimated to be 0.1 to 0.2 percent.[6,7] Currently, neutrophil antibodies with the following specificities have been implicated in cases of ANN: HNA-1a,[8–10] HNA-1b,[11] HNA-1c,[12] FcγRIIIb (CD16),[13,14] HNA-2a,[15] HNA-3a,[16] and HNA-4a.[17]

Autoimmune Neutropenia

Autoimmune neutropenia (AIN) occurs in both infants and adults. It is the most common form of chronic neutropenia in infants. Severe neutropenia is usually recognized 4 to 7 months after birth.[4] In the vast majority of AIN cases infants exhibit relatively mild clinical issues such as stomatitis, otitis, and respiratory infections. The presence of clinical symptoms lessens as the child becomes older. Neutropenia in infants is self-limited with complete resolution commonly observed within 7 to 24 months.[18] However, this condition necessitates aggressive hygienic care of infants to prevent infection and vigorous therapy that may include severe antibiotic therapy along with treatment with recombinant G-CSF and IVIG when infection occurs. Cell destruction can occur in the peripheral circulation in addition to interference with myelopoiesis in the bone marrow. Complete blood counts demonstrate absolute neutropenia, although random fluctuations in the neutrophil count can range from zero to near normal.

AIN is also observed in adults. Monocytosis with or without lymphopenia may also be present.[19] As in infants, the bone marrow commonly demonstrates an absence of mature cells with an increase in myeloid precursors. This apparent imbalance of precursors to mature segmented neutrophils is distinct in cause from the maturation arrest that occurs in congenital defects of neutrophils or hematologic malignancy. Unlike RBC autoantibodies, antibodies in AIN are often reported to have specificity that can include HNA-1a, HNA-1b, and HNA-2a.[18–20]

Drug-Induced Neutropenia

Although neutrophil antibodies are believed to be involved in drug-induced neutropenia, neither the precise mechanisms nor the particular antigens on the cell surface have yet been clarified. Some cases of drug-induced granulocytopenias probably result from direct marrow toxicity, although immune-mediated processes also occur. Quinine, quinidine, ibuprofen, and psychotropic medications such as clozapine are recognized as potential causes of neutropenia.[21–23] Drug-dependent antibodies have been found to react with both granulocytes and granulocyte precursors. The FcγRIIIb and CD177 neutrophil glycoproteins have been reported to form neoantigens with drugs or their metabolites, which are recognized by drug-dependent neutrophil antibodies.

Transfusion Reactions

Antibodies to neutrophil and HLA antigens can result in a variety of transfusion reactions. The spectrum of severity can range from mild febrile reactions to death in the case of severe transfusion-related acute lung injury (TRALI). Recently TRALI has been shown to be the most common cause of transfusion-related fatalities in the United States.[24]

Febrile nonhemolytic transfusion reactions are common and can occur when blood products containing WBCs are transfused into patients who have leukocyte antibodies.[25] The occurrence of febrile reactions has been mitigated with the use of leukocyte-reduced RBCs and platelet products.

As early as 1951 blood transfusions were implicated in noncardiogenic lung edema.[26] The term TRALI was conceived in 1985 when Popovsky and Moore[27] investigated a series of 36 patients with well-defined transfusion-related acute lung injury. They detected leukocyte (HLA and HNA) antibodies in 89 percent of the implicated donors. Seventy-two percent of these patients were treated with mechanical ventilation, and 6 percent died. The leading theories for the cause of TRALI involve the priming or activation of neutrophils with antigen-antibody interactions, inducing an acute inflammatory response.[28] Antibodies to HLA class I and class II as well as HNAs have all been implicated in the development of TRALI.[28]

Even though HLA class I alloantibodies are the most frequently encountered WBC antibodies in implicated blood donors, they may act as much weaker triggers of acute lung injury when compared with HLA class II and HNA antibodies. In a study designed to investigate leukocyte antibody specificities in severe TRALI reactions, Reil et al.[29] reported that even though 73 percent of leukocyte antibodies identified in their donor population were HLA class I, HLA class II and HNA antibodies were associated with 81 percent of the antibody-mediated TRALI cases.[29] Specifically, in their 36 reported cases, 17 were elicited by blood products containing HLA class II antibodies, and HNA antibodies were implicated in 10 cases. Of the 10 fatal outcomes, 6 were linked to HNA-3a antibodies in the transfused blood products, and HLA class II antibodies were associated with 3 fatalities.

Although leukocyte agglutinating (aggregating) antibodies have been frequently associated with TRALI,[30–34] the ability of HNA-3a antibodies to also prime and activate neutrophils in the pulmonary vasculature is thought to be central to severe and fatal TRALI.[35] Studies have now shown that in addition to HNA-3a antibodies, HNA-2a and HNA-4a antibodies also have the ability to prime neutrophils in vitro.[36–38]

Although the pathophysiology of TRALI appears complex and remains under investigation,[39] substantial clinical and scientific information suggests that HLA (especially class II) and HNA antibodies do play a significant role in this disorder.[28] Efforts are now being focused on detecting HLA and HNA antibodies in donors both to retrospectively investigate the cause of acute lung injury in blood recipients and to serve as a valuable strategy in preventing TRALI by identifying donors who may be responsible for the transfusion reaction.

Granulocyte Antigens

Granulocyte-specific antigens are those with a tissue distribution restricted to granulocytes (neutrophils, eosinophils, and basophils), whereas neutrophil-specific antigens are only present on neutrophils. Because of the difficulty in characterizing antigens on basophils and eosinophils, many

antigens described as neutrophil-specific have not been tested to determine whether they are present only on neutrophils or on all granulocytes. Although granulocyte (neutrophil) antigen systems were initially identified through studies of ANN and AIN, both terms, granulocyte and neutrophil, have been used to describe the alloantigens.

Nomenclature

Lalezari and colleagues[8] identified the first granulocyte antigen during their investigation of a neonate with transient ANN and subsequently proposed the first nomenclature system for these antigens. Because their studies indicated that the antigen was neutrophil-specific, they designated it the N system and the antigens were named in chronologic order of discovery. The antigens were labeled alphabetically and the alleles were described numerically. The system eventually classified seven antigens: NA1,[8] NA2,[40] NB1,[41] NB2,[42,43] NC1,[44] ND1,[45] and NE1,[46] which became the foundation for granulocyte serology.

During this same period and subsequently, several different granulocyte antigens were identified. Some were specific for granulocytes (or neutrophils): HGA-3, GA, GB, GC, Gr1, Gr2[47–49]; some were shared with other cells or tissues: HGA-1, 5a, 5b, 9, MART[47,50–52]; and some had unclassified distribution: CN1, KEN, LAN, LEA, SL.[53–56] All of these granulocyte antigens were detected with sera from patients with autoantibodies or alloantibodies directed against granulocytes, using traditional blood group serologic techniques such as agglutination, immunofluorescence, and cytotoxicity. Unfortunately, most of these antigens have not been further classified owing to the lack of sufficient biologic material for study.

Although this led to a consolidation of individuals working in this field, advances were made and efforts were directed at immunobiochemical, genetic, and structural or functional studies for a few of the well-classified granulocyte antigens. With the advent of this information came the need for a standardized granulocyte antigen nomenclature system based on the molecules carrying the antigens and the genes encoding each allele. In 1999, the Granulocyte Antigen Working Party of the International Society of Blood Transfusion (ISBT) established a new nomenclature system for the well-characterized granulocyte antigens, which was based on the glycoprotein location of the antigens.[57] In this system the granulocyte antigens are called human neutrophil alloantigens to indicate they are expressed on neutrophils; however, this does not imply that they are neutrophil-specific. Each antigen group is assigned a number, and polymorphisms within the group are designated alphabetically in sequential order of detection. The HNA system currently includes seven antigens, which are restricted to five antigen groups. The key features of the HNA system are summarized in Table 1. An extensive amount of information is now known about these antigens, and the reader is referred to Bux's recent and comprehensive review about the antigens that constitute this system.[58]

HNA-1 is the best-characterized group, and it contains three antigens: HNA-1a, HNA-1b, and HNA-1c. The HNA-1 antigens are expressed only on neutrophils, and antibodies to these antigens are frequently implicated in cases of alloimmune or autoimmune neutropenia.[18,58] The antigens are epitopes that occur on the neutrophil low-affinity FcγRIIIb receptor (CD16). This molecule binds the Fc region of IgG antibodies complexed to other antigens or immunoglobulins. The gene frequencies of HNA-1a, HNA-1b, and HNA-1c vary among different ethnic populations.[58] Furthermore, individuals lacking FcγRIIIb have been identified. These individuals do not express the HNA-1 antigens on their neutrophils, thus presenting as an HNA-1 null phenotype.[54,62]

The HNA-2 group has one characterized antigen—HNA-2a—which is located on a 58- to 64-kDa glycoprotein that is expressed only on neutrophils.[63] Although HNA-2a is a high-frequency antigen phenotype (i.e., greater than 90% for most populations), it is unique in that it is expressed on subpopulations of neutrophils among antigen-positive people. The expression of HNA-2a is greater on neutrophils from women than men, and two or three subpopulations of expression are often detected: one population that lacks HNA-2a expression and one or two populations that express the antigen but with different intensities.[64] The functional

Table 1. Human neutrophil antigens†

Antigen groups	Antigens	Former name	Antigen location/ glycoprotein CD number	Coding gene alleles‡	Clinical significance
HNA-1	HNA-1a HNA-1b HNA-1c	NA1 NA2 SH	FcγRIIIb/CD16b FcγRIIIb/CD16b FcγRIIIb/CD16b	*FCGR3B*01* *FCGR3B*02* *FCGR3B*03*	ANN, AIN, TRALI,§
HNA-2	HNA-2a	NB1	58–64 kDa/CD177	Unknown	ANN, AIN, TRALI, febrile transfusion reactions, drug-dependent neutropenia
HNA-3	HNA-3a HNA-3b	5b 5a	CTL2/Unknown CTL2/Unknown		ANN, TRALI , febrile transfusion reactions
HNA-4	HNA-4a	MART	CR3/CD11b (α M subunit)	*ITGAM*01* (230G)	ANN
HNA-5	HNA-5a	OND	LFA-1/CD11a (α L subunit)	*ITGAL*01* (2372G)	Unknown

†Information from references 20, 58, 59, 60.
‡Alleles of the coding genes are named according to the ISGN Guidelines for Human Gene Nomenclature.[61]
§Except HNA-1c.
AIN = autoimmune neutropenia; ANN = alloimmune neonatal neutropenia; CD = clusters of differentiation; CR3 = complement receptor 3; CTL2 = choline transporter-like protein 2; FcγRIIIb = Fc gamma receptor IIIb; HNA = human neutrophil antigen; LFA-1 = leukocyte function antigen-1; TRALI = transfusion-related acute lung injury.

role of HNA-2a is not known, and antibodies to this antigen have been associated with AIN,[20] ANN,[65] febrile transfusion reactions,[66] TRALI,[67] and drug-dependent neutropenia.[68]

HNA-3a is expressed on granulocytes, lymphocytes, platelets, endothelial cells, kidney, spleen, and placental cells.[50] It is a high-frequency antigen located on choline transporter-like protein 2 (CTL2), and its function is not known.[59,60] Alloantibodies to HNA-3a have been associated with occasional cases of febrile transfusion reactions[69] and one case of ANN.[70] Although antibodies to HNA-3a are rare, their major clinical significance has been their association with serious and fatal TRALI events.[32–34]

The HNA-4 and HNA-5 antigens are located on subunits of the β2 integrin family (CD11a and CD11b molecules). Integrins are a large family of adhesive receptors that are essential for cell-cell interactions and cell trafficking. HNA-4a is an epitope on the α M (CD11b) chain of CD11b/CD18 (the CR3 molecule), and HNA-5a is an epitope on the α L chain (CD11a) of CD11a/CD18 (the leukocyte function antigen-1 [LFA-1] molecule).[71] HNA-4a is expressed on granulocytes, monocytes, and natural killer cells, whereas HNA-5a is expressed on all leukocytes.[58] Alloantibodies to HNA-4a can cause ANN,[72] and the CD11b/CD18 complex has been reported to be the target of autoantibodies.[18,73] To date, HNA-5a antibodies have not been clinically associated with neutropenia.

A general consensus exists that granulocytes do not express ABO antigens[74] or HLA class II antigens.[75] Although the density of HLA class I antigens on granulocytes is fairly low, it can vary among individuals.[76] Therefore, these antigens must be taken into consideration when patient samples are tested for granulocyte antibodies. HLA class I antigens may be of particular interest during granulocyte transfusion for neutropenic patients as these antigens, in granulocyte concentrates, may be introduced in high amounts to recipients with preformed class I antibodies. Transfusion reactions including TRALI have been observed in this situation, and the antigen-antibody interaction has been proposed to limit effectiveness of the transfusion.[77–79]

Serologic Testing

The detection of HNA antibodies is labor intensive and technically challenging. Unlike HLA testing, commercial test kits are not readily available for the detection of granulocyte antibodies by solid-phase methodologies. Therefore, intact viable granulocytes are required for granulocyte antibody screening. Granulocytes are intrinsically designed to respond to physiologic priming signals, so they must be handled very carefully to prevent activation. Once activated it is impossible to interpret any serologic test results involving these cells, as false-positive test results abound. Granulocytes are also extremely labile and must be used within 24 hours of collection, necessitating ready access to panel cell donors with the needed antigen phenotypes. Although many have tried, attempts to develop short-term or long-term granulocyte preservation procedures have been unsuccessful, thus requiring that fresh suspensions of granulocytes be prepared daily for testing procedures. Finally, the presence of HLA antibodies in test sera makes identifying HNA antibodies difficult because granulocytes also express HLA class I antigens on their cell surface.

Preparation of Pure Granulocyte Suspensions

The isolation of pure granulocyte suspensions from peripheral blood was greatly facilitated and simplified with Bøyum's introduction in 1968 of the Ficoll-Isopaque gradient technique.[80] Double-density gradient centrifugation is now the most widely used approach for the isolation and purification of granulocytes for serologic testing. The procedure makes use of discontinuous Ficoll gradients, and numerous publications exist describing the methodology and the recovery, purity, functional integrity, and activity of the isolated granulocytes.[81–84]

Granulocyte Antibody Detection and Antigen Typing

Laboratory approaches for the detection of granulocyte antibodies require procedures that are accurate, reproducible, and practical. Although numerous granulocyte serologic assays have been described, not all meet these criteria. In addition to the assay used, the ability to detect antibodies in test specimens or antigens on test cells may also depend on target cell antigen density and the concentration and immunoglobulin type of the antibody in the test reagent. Therefore, the successful detection of granulocyte antibodies often requires that a combination of methods be used.

Although several methods for the detection of granulocyte-specific antibodies are used by the few laboratories that perform this testing, the ISBT Working Party on Granulocyte Immunobiology, a worldwide consortium of 16 laboratories that participate in the annual International Granulocyte Immunology Workshop (IGIW), have recommended that granulocyte antibodies should be investigated using a minimum of two methods: the granulocyte immunofluorescence test (GIFT) and the granulocyte agglutination test (GAT).[85] Multiple workshops have shown the sensitivity of GIFT to far exceed that of GAT; however, agglutinating antibodies such as HNA-1c, HNA-3a, and HNA-4a are much more readily detectable in GAT and in many cases it is the only reliable method for detecting these specificities. For example, the seventh IGIW included a test specimen that contained an HNA-3a antibody that could only be detected by GAT and the granulocyte chemiluminescence test (GCLT). Although GCLT has been useful in selective laboratory settings, it is not routinely used for clinical granulocyte antibody detection and is not discussed.

Antibody Detection Methods

Granulocyte Agglutination Test

Lalezari[86] developed the basis of this assay in the early 1960s. Since that time, micro techniques and the use of pure granulocyte suspensions with optimized concentrations of EDTA have vastly improved the performance of this test. Typically, a panel of three to five donors is selected to include all known neutrophil antigens currently defined by the ISBT consortium. Purified granulocytes are isolated from EDTA-anticoagulated peripheral whole blood using a double-density gradient. These granulocytes are then washed in PBS before use. The test is biphasic and is based on the intrinsic response of granulocytes to aggregate when stimulated by antibodies reacting to corresponding cell surface antigens. The resulting agglutination is the consequence of the granulocyte activation that occurs during the sensitization phase, which induces the cells to form pseudopods and slowly migrate toward one another during the aggregation phase, until membrane contact is established. Typically, the serum or plasma from the specimen being investigated is incubated with the granulocyte suspension for 4.5 to 6 hours at 30°C.[87] The reactions are evaluated using an inverted-phase microscope and graded from negative to 4+ on the basis of the percentage of cells that are agglutinated. Our laboratory has determined cutoff scores for both adult and pediatric patients (<6 years old) on the basis of the reaction grades established during microscopic evaluation. Both IgG and IgM antibodies are detected by this method.

Granulocyte Immunofluorescence Test

Developed by Verheugt et al.[88] in the late 1970s, this fluorescent "antiglobulin" technique is used for the detection of granulocyte alloantibodies and autoantibodies that are circulating and cell bound. As with the GAT, an antibody screening panel of three to five donors is selected to include all currently defined HNA antigens. A purified granulocyte suspension is prepared and treated with 1% paraformaldehyde for a short time to prevent nonspecific immunoglobulin binding to the neutrophil Fc receptors and to stabilize the cell membrane. Serum or plasma from the patient is then incubated with an optimized concentration of granulocytes for 30 minutes at 37°C. After a wash step that removes unbound antibodies, the granulocytes are then incubated with $F(ab')_2$ fragments of a fluorescent conjugated anti-human antibody for approximately 30 minutes at room temperature in a dark environment. The assay's performance is optimized with the use of a fluorescent secondary probe that can detect both IgM and IgG antibodies (to ensure the detection of both primary and secondary immune responses) and the use of $F(ab')_2$ Ig fragments to prevent the probe from binding to the high concentrations of Fc receptors on granulocyte surface membranes. The cells undergo another wash cycle, are resuspended, and then are analyzed. Detection of the immunofluorescence reactions can be accomplished by using either a fluorescence-detecting microscope or a flow cytometer. Evaluation by a flow cytometer has in most cases replaced the fluorescence microscope as more cells can be analyzed in a shorter time, resulting in improved assay sensitivity and reproducibility, and it does not require the expertise needed to evaluate the characteristic staining pattern seen with specific granulocyte antibodies by microscopic analysis.

Monoclonal Antibody Immobilization of Granulocyte Antigens

The monoclonal antibody immobilization of granulocyte antigens assay (MAIGA) is based on the platelet version (MAIPA) developed by Kiefel et al. in 1987.[89] MAIGA relies on the capture of neutrophil-specific antigen-antibody complexes by a murine monoclonal antibody onto a solid-phase surface.[90] The benefits of this test are twofold: first, this is currently the most sensitive assay for the detection of granulocyte antibodies, and second, the assay is designed to detect only HNA antibodies even when HLA antibodies are present in the test specimen. The disadvantage of this procedure is that it is very complex and requires highly skilled staff to perform the detailed techniques.

Granulocytes are incubated with the test serum or plasma for 30 minutes at 37°C. This granulocyte suspension is then washed to remove any unbound immunoglobulins and incubated with a murine monoclonal antibody to a specific neutrophil glycoprotein for an additional 30 minutes. After another wash step, the granulocyte membranes are disrupted in a mild detergent and centrifuged. The resulting lysate is then transferred to polystyrene microwells coated with anti-mouse immunoglobulins for incubation. The trimolecular neutrophil antigen–patient HNA antibody–murine monoclonal antibody complex present in the lysate is captured on the solid phase, whereas any HLA antibody-antigen complexes (if present) are removed by a subsequent wash step. Remaining complexes are then detected by the addition of anti-human IgG conjugated to horseradish peroxidase followed by a substrate (OPD dissolved in 30% H_2O_2), and the reaction is analyzed with a spectrophotometer.

MAIGA can be used to detect antibodies specific to HNA-1a, HNA-1b, and HNA-1c antigens located on FcγRIIIb (CD16); HNA-2a present on gp 58- to 64-kDa (CD177); HNA-4a on the complement component receptor C3bi (CD11b); and HNA-5a on the LFA-1 receptor (CD11a). HNA-4a and HNA-5a antibodies can also be detected by a common CD18 monoclonal antibody.

Antigen Detection and Typing Methods

The same methodology that is used to detect antibodies in serum and plasma can be used to characterize the antigen phenotype of an individual's granulocytes. Just as the short lifespan of granulocytes is a limitation to obtaining panel cells for antibody testing, it also restricts the ability to do antigen typing because granulocyte specimens must be tested

within 24 hours of collection to obtain reliable results. A sufficient volume of whole blood must be collected to obtain an adequate number of granulocytes for phenotyping. This can be a dilemma in pediatric patients or patients who are neutropenic. Because ample volume, freshness, and availability of granulocytes is critical, this also acts as a practical barrier to doing donor-recipient crossmatch testing during investigations of suspected granulocyte-antibody–mediated transfusion reactions such as TRALI. The highly characterized antisera used to phenotype granulocytes should be free of HLA class I antibodies as their presence can result in a false-positive HNA typing. At a minimum, the titer of the HLA antibody should be weaker than the HNA antibody as it can then be readily diluted to a nonreactive level. Another constraint to phenotyping granulocytes is the limited availability of qualified antisera for the currently defined HNA antigens.

Monoclonal antibodies specific to several HNA antigens are commercially available and have been used to type granulocytes by GIFT using the flow cytometer. An advantage to phenotyping by this method is that it can be done with whole blood instead of with isolated granulocytes.

Because phenotyping granulocytes cannot often be performed or can be unreliable if samples are not handled in a careful and timely manner, genotyping by PCR, which has less stringent sample age and handling restraints, can serve either as an alternative to or as confirmation of serologic results.

Granulocyte Genotyping

The discovery of the PCR in the 1980s facilitated the development of molecular methods that have now been applied to delineate the genetics and allelic variations of the HNAs. Initially, the characterization of the genes encoding the HNA-1 antigen system (HNA-1a, HNA-1b, and HNA-1c) allowed for the development of PCR assays using sequence-specific primers (SSP) to differentiate the alleles. To complicate matters there is a high degree of homology between the *FCGR3A* gene that encodes the FcγRIIIa granulocyte receptor and the *FCGR3B* gene where the three different HNA-1 polymorphisms reside. HNA-1a, HNA-1b, and HNA-1c are encoded by *FCGR3B*1, FCGR3B*2*, and *FCGR3B*3* genes, respectively. *FCGR3B*1* differs from *FCGR3B*2* by five nucleotide bases, and a single nucleotide polymorphism (SNP) differentiates *FCGR3B*2* from *FCGR3B*3* (Table 2). *FCGR3A* differs from *FCGR3B* at five different nucleotide locations (Table 2).

Methods are also available to genotype the HNA-4a and HNA-5a alleles.[71] Both of these polymorphisms are the result of SNPs. Even though the molecular sequence of HNA-2a has been identified, genotyping methods to detect the gene that encodes this alloantigen are not available. The HNA-2a negative phenotype is attributable to CD177 mRNA splicing defects, and no mutations have been detected in the CD177 introns or exons in these individuals.[93] Because the gene encoding HNA-3a has only recently been identified, there has not been a genotyping procedure for this antigen, but the clinical importance of this antigen is supporting efforts to get a molecular method developed.

Genomic DNA (gDNA) can be isolated and purified from anticoagulated blood using any number of published methods. A unique set of sense and antisense oligonucleotide primers that border the DNA fragment to be amplified are added to a master mix of reagents that includes deoxynucleoside triphosphates (dNTPs), *Taq* polymerase, a buffer solution containing Mg^{2+}, and the individual's gDNA. The master mix is subjected to a series of 30 to 40 temperature changes that define the amplification cycles. Each cycle consists of three temperature steps that are critical in the amplification of the DNA target strands. The first step in the cycle typically involves heating the master mix to a temperature of 95°C. This denaturation step results in double-stranded DNA separating into single strands by disrupting the hydrogen bonds between each nucleotide base pair. The annealing step follows in which the reaction temperature is lowered to 50°C to 65°C. This allows the primers to hybridize to the opposing strands of the target DNA. The final extension step heats the master mix from 71°C to 72°C, which results in DNA synthesis in the presence of *Taq* polymerase and dNTPs complementary to the oligonucleotide primers. A final extension step is added after all cycles have been completed to ensure that any remaining single-stranded DNA is fully elongated. The amplified double-stranded DNA (amplicon) is separated by size using agarose gel electrophoresis, stained with ethidium bromide, and visualized by fluorescence using ultraviolet light. The sizes of the amplicons are determined by comparison with a DNA ladder, which contains DNA fragments of known sizes that were run alongside the PCR products during the agarose gel electrophoresis step.

Future Directions

Solid-Phase Technology

The complement-dependent lymphocyte cytotoxicity assay was the first platform, developed nearly 50 years ago, for identifying HLA antibodies and remains the standard for the development of new methodologies in this field.

Table 2. Nucleotide differences for the three HNA-1 alleles (1a, 1b, and 1c) on FcγRIIIb and among the FcγRIII genes†

HNA	Gene	Polymorphic nucleotides										
		141	**147**	**227**	**266**	**277**	**349**	**473**	**505**	**559**	**641**	**733**
1a	*FCGR3B*1*	G	C	**A**	C	G	**G**	A	C	G	C	T
1b	*FCGR3B*2*	**C**	**T**	G	C	**A**	A	A	C	G	C	T
1c	*FCGR3B*3*	**C**	**T**	G	**A**	**A**	A	A	C	G	C	T
	FCGR3A	G	C	G	C	G	A	**G**	**T**	**T**	**T**	**C**

†Information from references 91 and 92.
NOTE: Boldface indicates substituted nucleotides.
FcγRIIIb = Fc gamma receptor IIIb; HNA = human neutrophil antigen.

Multiple new technologies are now being used by HLA laboratories, and in the past few years solid-phase assays have been developed in which purified HLA molecules are bound to a well in a microtiter plate or to a bead. HLA antibody is then detected either using an enzyme-linked immunosorbent assay (ELISA) technique or by analyzing the beads with a flow cytometer, which uses a new technology developed by Luminex (Austin, TX).[94] A critical advantage of this technology is its ability to measure multiple analytes simultaneously in a single reaction system. The commercially available test kits use purified HLA antigens bound to microbeads, which can be individually differentiated by varying ratios of internal fluorescent dye prepared during the manufacturing process. The test involves incubation of test sera with the panel of microbeads, and the bound HLA antibody is detected by a secondary R-phycoerythrin (PE) conjugated IgG (or IgM)–specific antibody.

Analysis is done with the use of a Luminex flow analyzer in which the beads pass through two lasers, similarly to classic flow cytometry. One laser identifies the specific bead being analyzed, thereby identifying the unique HLA antigen. The other laser detects the presence of bound Ig on the surface of the microbead. The color signals are then detected and processed into data for each reaction. A significant fluorescence shift from the negative control range indicates the presence of specific antibody in the test sample.

The widespread use of Luminex technology by HLA laboratories has fostered commercial activity directed at using this technology for the detection of HNA antibodies that have been related to febrile and pulmonary transfusion reactions. One Lambda, Inc. (Canoga Park, CA), has developed recombinant cell lines for HNA-1a, -1b, -1c, -2a, and -4a, and these cell lines have allowed the production of purified proteins that have been individually immobilized on Luminex microbeads for use in their LABScreen Multiflow bead assay. Stroncek and colleagues[95] evaluated the specificity and sensitivity of this new procedure using 22 sera that had known HNA antibodies detected with the GA assay and 140 samples from nontransfused men. All HNA antibody specificities (except 4a) recognized by GAT were detected in the solid-phase flow bead system (8 HNA-1a, 6 HNA-1b, 1 HNA-1c, and 8 HNA-2a positive sera), and HNA-1c reactivity was detected in four specimens that were negative by GAT. In addition, all of the 140 negative control samples had negative reactions when tested by LABSreen Multi, and the system was able to detect both HNA and HLA antibodies (both class I and class II) in some individual specimens.[95] Although preliminary, these results suggest that this solid-phase technology may emerge as a major achievement in the development of a new sensitive and specific platform for the detection of granulocyte antibodies in an automated manner with limited complexity and high cost efficiency.

Cell Lines Expressing HNA Antigens

Another approach to eliminate the need for freshly isolated granulocytes for granulocyte antibody screening has been the establishment of cell lines that express HNA antigens. In 1999 Bux's group transfected Chinese hamster ovary (CHO) cells with HNA-1a, HNA-1b, and HNA-1c cDNA and established stable cell lines expressing these antigens.[96] Although the cell lines were stable for 1 month at 4°C, they demonstrated high background fluorescence in the GIF assay and could only be used for the MAIGA, which limited their overall utility. More recently, Yasui et al.[97,98] transduced HNA genes into the human erythroleukemia cell line K562 and established a panel of K562 cell lines that stably expressed HNA-1a, -1b, -1c, -2a, -4a, -4b, -5a, and -5b for at least 1 year while being maintained in culture. They were not able to establish a cell line expressing HNA-3a because of the previous lack of biochemical and genetic information for this antigen. The group's report demonstrated the utility of using the cultured cells in the GIF assay to detect HNA antibodies in serum samples from patients with HNA antibody–related disorders, but experience with this new cell line technology is very limited and it remains to be seen whether the cell lines can be maintained or preserved in a manner that would support the use of these cells for routine (nonresearch) granulocyte serology applications.

ISBT Working Party on Granulocyte Immunobiology

In 1989, Professor Alan Waters organized the UK Platelet and Granulocyte Serology Working Group, and 11 international laboratories participated in the First International Workshop on Granulocyte Serology. The objectives of this first workshop were as follows: (1) to initiate an exchange of sera among laboratories to evaluate the techniques being used for the detection of granulocyte antibodies, (2) to foster working relationships among laboratories involved in this field, and (3) to support the development of the science and technology for the detection of granulocyte antibodies.[99]

The Working Party was initially affiliated with the British Blood Transfusion Society but is now affiliated with the ISBT. The goals of the Second Workshop, in 1996, were the (1) establishment of typed granulocyte panels by well-defined typing sera, (2) proficiency testing of granulocyte antibody detection, (3) investigation of uncharacterized sera, (4) proficiency testing of HNA genotyping, and (5) exchange of information to establish standards in granulocyte serology.[100] Fifteen international laboratories participated in the Fourth Workshop, in 2001, with the main objective being the establishment of a formal quality assurance (QA) scheme for granulocyte serology and molecular typing methods.[85]

To date, the Working Party has conducted nine Granulocyte Serology Workshops and comprises 16 international member laboratories, which are listed in Table 3. Also, the American Red Cross Mid-America Blood Services Neutrophil & Platelet Immunology Laboratory has been a member of this consortium since its inception in 1989. The workshops continue to focus on the QA and standardization of the test systems, and the Working Party recently published its

serologic screening recommendations for the investigation and prevention of leukocyte antibody-mediated TRALI.[101]

Conclusions

Granulocyte antibody and antigen testing has played and continues to play a critical role for diagnosing and investigating relatively rare but potentially lethal clinical conditions such as TRALI and immune neutropenias. Although mitigation strategies are already being implemented nationwide, TRALI still appears to be a leading and preventable cause of transfusion fatalities. As advancing technology allows granulocyte antibody testing to be done on a less manual and larger scale with more standardized and reproducible results, it now has the potential to become even more valuable as a tool to increase blood safety by actually preventing transfusion reactions without compromising an adequate supply of blood.

Table 3. Members of the ISBT Working Party on Granulocyte Immunobiology

- Platelet and Leucocyte Immunology Laboratory, EFS Ile de France, Hopital Henri Mondor, Creteil, France
- Leucocyte and Platelet Immunology Laboratory, Blood Service West of the German Red Cross, Hagen, Germany
- The BloodCenter of Wisconsin, Platelet and Neutrophil Immunology Laboratory, Milwaukee, WI, USA
- Institute of Transfusion Medicine, University of Schleswig-Holstein, Kiel, Germany
- Australian Red Cross Blood Service-Queensland, Brisbane, Australia
- Platelet and Granulocyte Immunology Laboratory, National Blood Se vice, Bristol, UK
- Blood Transfusion Center of Slovenia, Ljubljana, Slovenia
- Centre de Transfusio I Banc de Teixitis, Hospital Vall d'Hebron, P. Vall d'Hebron, Barcelona, Spain
- Sanguin Diagnostics, Immunohaematology Diagnostic Department, Amsterdam, The Netherlands
- Institute for Clinical Immunology and Transfusion Medicine, Justus Liebig University, Giessen, Germany
- American Red Cross Mid-America Blood Services, Neutrophil & Platelet Immunology Laboratory, St. Paul, MN, USA
- Department of Transfusion Medicine, Graduate School of Medical Sc ences, University of Tokyo, Tokyo, Japan
- Department of Immunohaematology and Transfusion Medicine, Institute of Haematology and Blood Transfusion, Warsaw, Poland
- Aberdeen and North East Scotland Blood Centre, Scottish National Blood Transfusion Service, Aberdeen, UK
- Platelet and Leucocyte Immunology Laboratory, Institute of Biology, Nantes, France
- Department of Transfusion Medicine, Karolinska University Hospital, Stockholm, Sweden

Acknowledgments

The authors would like to thank Penny Milne and Bobbie Gibson for their helpful assistance with the preparation of the manuscript.

References

1. Lalezari P, Nussbaum M, Gelman, et al. Neonatal neutropenia due to maternal isoimmunization. Blood 1960;15:236–43.
2. Bux J, Jung KD, Kauth T, et al. Serological and clinical aspects of granulocyte antibodies leading to alloimmune neonatal neutropenia. Transfus Med 1992;2:143–9.
3. Rodwell RL, Gray PH, Taylor KM, et al. Granulocyte colony stimulating factor treatment for alloimmune neonatal neutropenia. Arch Dis Child Fetal Neonatal Ed 1996;75:F57–8.
4. Rodney GE, Lalezari P. HLA and neutrophil antigen and antibody systems. In: Hoffman R, Benz E, Shattil SJ, eds. Hematology basic principles and practice. 3rd ed. New York: Churchill Livingstone, 2000:2220–41.
5. Lalezari P. Alloimmune neonatal neutropenia. In: Engelfriet CP,Van Logham JJ, von dem Borne AEGKr, eds. Immunohaematology. Amsterdam: Elsevier Science Publishers, 1984:179–86.
6. Clay M, Kline W, McCullough J. Serological examination of postpartum sera for the presence of neutrophil-specific antibodies (abstract). Transfusion 1981;21:616.
7. Levine DH, Madyastha P, Wade R, Levkoff AH. Neonatal isoimmune neutropenia (abstract). Pediatr Res 1981;15:296A.
8. Lalezari P, Bernard GE. An isologous antigen-antibody reaction with human neutrophils, related to neonatal neutropenia. J Clin Invest 1966;45:1741–50.
9. Verheugt FWA, van Noord-Bokhorst JC, von dem Borne AEGKr, et al. A family with alloimmune neonatal neutropenia: group specific pathogenicity of maternal antibodies. Vox Sang 1979;36:1–8.
10. van der Weerdt CM, Lalezari P. Another example of isoimmune neonatal neutropenia due to anti-NA1. Vox Sang 1972;22:438–46.
11. Boxer LA, Yokoyama M, Lalezari P. Isoimmune neonatal neutropenia. J Pediatr 1972;80:783–7.
12. Bux J, Ernst-Ludwig Stein, Bierling P, et al. Characterization of a new alloantigen (SH) on the human neutrophil Fc gamma receptor IIIb. Blood 1997;89:1027–34.
13. Schuller RM, Stroncek DF, Alpan O, et al. Different responses observed between G-CSF and GM-CSF therapy in a case of neonatal alloimmune neutropenia (abstract). Transfusion 2008;48(Suppl):53A.
14. Bachowski GJ, Mair DC, Schuller RM, et al. Alloimmune neonatal neutropenia in monozygous twins caused by antibody to FcγRIIIb in a mother lacking CD16 antigen (abstract). Transfusion 2007;47(Suppl):21A.
15. Stroncek DF, Sharpiro RS, Filipovich AH, et al. Prolonged neutropenia resulting from antibodies to neutrophil-specific antigen NB1 following marrow transplantation. Transfusion 1993;33:158–63.

16. de Haas M, Muniz-Diaz E, Alonso LG, et al. Neutrophil antigen 5b is carried by a protein, migrating from 70 to 95 kDa, and may be involved in neonatal alloimmune neutropenia. Transfusion 2000;40:222–7.
17. Fung L, Pitcher LA, Willett JE, et al. Alloimmune neonatal neutropenia linked to anti-HNA-4a. Transfus Med 2003;13:49–52.
18. Bux J, Behrens G, Jaeger G, et al. Diagnosis and clinical course of autoimmune neutropenia in infancy: analysis of 240 cases. Blood 1998;91:181–6.
19. Bux J, Kissel K, Nowak K, et al. Autoimmune neutropenia: clinical and laboratory studies in 143 patients. Ann Hematol 1991;63:249–52.
20. Stroncek D. Granulocyte antigens and antibody detection. Vox Sang 2004;87(Suppl 1):91–4.
21. Sperner-Unterweger B, Czeipek I, Gaggl S, Geissler D, Spiel G, Fleischhacker WW. Treatment of severe clozapine-induced neutropenia with granulocyte colony stimulating factor (G-CSF). Remission despite continuous treatment with clozapine. Br J Psychiatry 1998;172:82–4.
22. Stroncek DF. Drug-induced immune neutropenia. Transfus Med Rev 1993;7:268–74.
23. Mamus SW, Burton JD, Groat JD, et al. Ibuprofen-associated pure white cell aplasia. N Engl J Med 1986;314:624–5.
24. Holness L, Knippen MA, Simmons L, et al. Fatalities caused by TRALI. Transfus Med Rev 2004;18:184–8.
25. Brittingham TE. Immunologic studies on leukocytes. Vox Sang 1952;2:242–8.
26. Barnard, RD. Indiscriminate transfusion: a critique of case reports illustrating hypersensitivity reactions. N Y State J Med 1951;51:2399–402.
27. Popovsky MA, Moore SB. Diagnostic and pathogenic considerations in transfusion-related acute lung injury. Transfusion 1985;25:573–7.
28. Bux J, Sachs UJ. The pathogenesis of transfusion-related acute lung injury (TRALI). Br J Haematol 2007;136:788–99.
29. Reil A, Keller-Stanislawski B, Gunay S, et al. Specificities of leukocyte alloantibodies in transfusion-related acute lung injury and results of leukocyte antibody screening of blood donors. Vox Sang 2008;95:313–17.
30. Ward HN. Pulmonary infiltrates associated with leukoagglutinin transfusion reactions. Ann Intern Med 1970;73:689–94.
31. Thompson JS, Severson CD, Parmely MJ, et al. Pulmonary 'hypersensitivity' reactions induced by transfusion of non-HLA lekoagglutinins. N Engl J Med 1971;284:1120–5.
32. Nordhagen R, Conradi M, Drotorp SM. Pulmonary reaction associated with transfusion of plasma containing anti-5b. Vox Sang 1986;51:102–7.
33. Kopko PM, Marshall CS, MacKenzie MR, et al. Transfusion-related acute lung injury. Report of a clinical look-back investigation. JAMA 2002;287:1968–71.
34. Davoren A, Curtis BR, Shulman IA, et al. TRALI due to granulocyte-agglutinating human neutrophil antigen-3a (5b) alloantibodies in donor plasma: a report of 2 fatalities. Transfusion 2003;43:641–5.
35. Silliman CC, Curtis BR, Kopko PM, et al. Donor antibodies to HNA-3a implicated in TRALI reactions prime neutrophils and cause PMN-mediated damage to human pulmonary microvascular endothelial cells in a two-event in vitro model. Blood 2007;109:1752–5.
36. Sachs UJ, Chavakis T, Fung L, et al. Human alloantibody anti-MART interferes with Mac-1-dependent leukocyte adhesion. Blood 2004;104:727–34.
37. Kopko PM, Curtis BR, Kelher M, et al. Merging the pathogenesis of transfusion-related acute lung injury: the priming activity of the 5b (HNA-3) antibody (abstract). Transfusion 2004;44(Suppl 1):22A.
38. Sachs UJ, Hattar K, Weissman N, et al. Antibody-induced neutrophil activation as a trigger for transfusion-related acute lung injury in an ex vivo rat lung model. Blood 2006;107:1217–9.
39. Fung YL, Silliman CS. The role of neutrophils in the pathogenesis of transfusion-related acute lung injury. Transfusion Med Rev 2009;23:266–83.
40. Lalezari P, Radel E. Neutrophil-specific antigens: immunology and clinical significance. Semin Hematol 1974;11:281–90.
41. Lalezari P, Murphy GB, Allen FH. NB1, a new neutrophil antigen involved in the pathogenesis of neonatal neutropenia. J Clin Invest 1971;50:1108–15.
42. Huang ST, Lin J, McGowan El, et al. NB2, a new allele of NB1 antigen involved in febrile transfusion reaction (abstract). Transfusion 1982;22:426.
43. Lalezari P, Petrosova M, Jiang AF. NB2, an allele of NB1 neutrophil specific antigen: relationship to 9a (abstract). Transfusion 1982;22:433.
44. Lalezari P, Thelenfeld B, Weinstein WJ. The third neutrophil antigen. In: Terasaki PI, ed. Histocompatibility testing. Baltimore: Williams & Wilkins, 1970:319–22.
45. Verheugt FWA, von dem Borne AEGKr, van Noord-Bokhorst JC, et al. ND1, a new neutrophil granulocyte antigen. Vox Sang 1978;35:13–17.
46. Claas FHJ, Langerak J, Sabbe LJM, et al. NE1, a new neutrophil specific antigen. Tissue Antigens 1979;13:129–34.
47. Thompson JS, Overlin VL, Herbick JM, et al. New granulocyte antigens demonstrated by microgranulocytotoxicity assay. J Clin Invest 1980;65:1431–9.
48. Hasegawa T, Bergh OJ, Mickay MR, et al. Preliminary human granulocyte specificities. Transplant Pro 1975;7(Suppl 1):75–80.

49. Caplan SN, Berkman EM, Babior BM. Cytotoxins against a granulocyte antigen system: detection by a new method employing cytochalasin-B-treated cells. Vox Sang 1977;33:206–11.
50. Van Leeuwen A, Eerrise JG, van Room JJ. A new leukocyte group with two alleles: leukocyte group five. Vox Sang 1964;9:431–7.
51. Van Rood JJ, van Leeuwen A, Schippers AMJ, et al. Immunogenetics of the group four, five and nine systems. In: Curton ES, Mattiuz PI, Tosi RM, eds. Histocompatibility testing. Baltimore: Williams & Wilkins, 1967:203–19.
52. Kline WE, Press C, Clay M, et al. Three sera defining a new granulocyte-monocyte–T lymphocyte antigen. Vox Sang 1986;50:181–6.
53. Madyastha PR, Glassman A, Levine D, et al. Identification of a new neutrophil antigen (CN1) more prevalent among American blacks (abstract). Transfusion 1983;23:426.
54. Schnell M, Halligan G, Herman J. A new granulocyte antibody directed at a high frequency antigen causing neonatal alloimmune neutropenia (abstract). Transfusion 1989;29(Suppl):46S.
55. Rodwell RL, Tudehope DI, O'Regan P, et al. Alloimmune neonatal neutropenia in Australian aboriginals: an unrecognized disorder? Transfusion Med 1991;1:63–7.
56. Stroncek DF, Ramsey G, Herr GP, et al. Identification of a new white cell antigen. Transfusion 1994;34:706–11.
57. Bux J, Bierling P, von dem Borne AE, et al. ISBT Granulocyte Antigen Working Party. Nomenclature of granulocyte alloantigens. (letter).Vox Sang 1999;77:251.
58. Bux J. Human neutrophil alloantigens. Vox Sang 2008;94:277–85.
59. Greinacher A, Wesche J, Hammer E, et al. Characterization of the human neutrophil alloantigen-3a. Nature Med 2010;16:45–8.
60. Curtis BR, Cox NJ, Sullivan MJ, et al. The neutrophil alloantigen HNA-3a (5b) is located on choline transporter-like protein 2 (CTL2) and appears to be encoded by an R>Q 154 amino acid substitution. Blood 2010;115:2073-6.
61. Shows TB, McAlpine PJ, Boucheix C, et al. Guidelines for human gene nomenclature. An international system for human gene nomenclature (ISGN, 1987). Cytogenet Cell Genet 1987;46:11–28.
62. Stroncek DF, Skubitz KM, Plachta LB, et al. Alloimmune neonatal neutropenia due to an antibody to the neutrophil Fc-gamma receptor III with maternal deficiency of CD16 antigen. Blood 1991;77:1572–80.
63. Stroncek DF, Skubitz K, McCullough JJ. Biochemical nature of the neutrophil-specific antigen NB1. Blood 1990;75:744–55.
64. Matsuo K, Lin A, Procter JL, et al. Variations in the expression of granulocyte antigen NB1. Transfusion 2000;40:654–62.
65. Bux J, Jung KD, Kauth T, et al. Serological and clinical aspects of granulocyte antibodies leading to alloimmune neonatal neutropenia. Transfusion Med 1992;2:143–9.
66. Bux J. Granulocyte antibody mediated neutropenias and transfusion reactions. Infusions Ther Transfusion Med 1999;26:152–7.
67. Bux J, Becker F, Seeger W, et al. Transfusion-related acute lung injury due to HLR-A2-specific antibodies in recipient and NB1-specific antibodies in donor blood. Br J Haematol 1996;93:707–13.
68. Stroncek DF, Shankar RH, Herr GP. Quinine-dependent antibodies to neutrophils react with a 60 kD glycoprotein on which neutrophil-specific antigen NB1 is located and an 85 kD glycosylphosphatidylinositol–linked N-glycosylated plasma membrane protein. Blood 1993;81:2758–66.
69. Lalezari P, Bernard GE. Identification of a specific leukocyte antigen: another presumed example of 5b. Transfusion 1965;5:132–42.
70. de Haas M, Muniz-Diaz E, Alonso LG, et al. Neutrophil 5b is carried by a protein, migrating from 70 to 95 kDa, and may be involved in neonatal alloimmune neutropenia. Transfusion 2000;40:222–7.
71. Simsek S, van der Schoot CE, Daams M, et al. Molecular characterization of antigenic polymorphisms (OND[a] and MART[a]) of the $ß_2$ family recognized by human leukocyte alloantisera. Blood 1996;88:1350–8.
72. Fung L, Pitcher LA, Willett JE, et al. Alloimmune neonatal neutropenia linked to anti-HNA-4a. Transfusion Med 2003;13:49–52.
73. Hartman KR, Wright DG. Identification of autoantibodies specific for the neutrophil adhesion glycoproteins CD11b/CD18 in patients with autoimmune neutropenia. Blood 1991;78:1096–104.
74. Kelton JG, Bebenek G. Granulocytes do not have surface ABO antigens. Transfusion 1985;25:567–9.
75. Dunstan RA, Simpson MB, Sanfilippo FP. Absence of specific HLA-DR antigens on human platelets and neutrophils (abstract). Blood 1984;64:85a.
76. Thorsby E. HLA antigens on human granulocytes studied with cytotoxic iso-antisera obtained by skin grafting. Scan J Haematol 1969;6:119–27.
77. Sachs UJ, Bux J. TRALI after transfusion of cross-match-positive granulocytes. Transfusion 2003;43:1683–6.
78. Stroncek DF, Leonard K, Eiber G, et al. Alloimmunization after granulocyte transfusions. Transfusion 1996;36:1009–15.
79. McCullough J, Clay M, Hurd D, et al. Effect of leukocyte antibodies and HLA matching on the intravascular recovery, survival, and tissue localization of 111-indium granulocytes. Blood 1986;67:522–8.

80. Bøyum A. Isolation of mononuclear cells and granulocytes from blood. Isolation of mononuclear cells by one centrifugation and of granulocytes by combining centrifugation and sedimentation at 1g. Scan J Clin Lab Invest 1968;21(Suppl 97):77–89.
81. English D, Anderson BR. Single-strep separation of red blood cells, granulocytes, and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque. J Immunol Methods 1975;5:249–52.
82. Ferrante A, Thong YH. A rapid one-step procedure for purification of mononuclear and polymorphonuclear leukocytes from human blood using a modification of the Hypaque-Ficoll technique. J Immunol Methods 1978;24:389–93.
83. Ferrante A, Thong HY. Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leukocytes from human blood by the Hypaque-Ficoll method. J Immunol Methods 1980;36:109–17.
84. Madyastha P, Madyastha KR, Wade T, et al. An improved method for rapid layering of Ficoll-Hypaque double density gradients suitable for granulocyte separation. J Immunol Methods 1982;42:281–6.
85. Lucas G, Rogers S, de Haas M, et al. Report on the Fourth International Granulocyte Immunology Workshop: progress toward quality assessment. Transfusion 2002;42:462–8.
86. Lalezari P, Pryce S. Detection of neutrophil and platelet antibodies in immunologically induced neutropenia and thrombocytopenia. In: Rose NR, Friedman H, eds. Manual of clinical immunology. Washington, DC: American Society of Microbiology 1980;744–9.
87. McCullough J, Clay ME, Press C, et al. Granulocyte serology: a clinical and laboratory guide. Chicago: ASCP Press, 1988:12–14, 168–71.
88. Verheugt FWA, Von dern Borne AEGKr, Decary S, et al. The detection of granulocyte alloantibodies with an indirect immunofluorescence test. Br J Haematol 1977;36:533–44.
89. Kiefel V, Santoso S, Weisheit M, et al. Monoclonal antibody-specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet reactive antibodies. Blood 1987;70:1722–6.
90. Minchinton R, Noonan K, Johnson TJ. Examining technical aspects of the monoclonal antibody immobilization of granulocyte antigen assay. Vox Sang 1997;73:87–92.
91. Stroncek D. Neutrophil alloantigens. Transfusion Med Rev 2002;16:67–75.
92. Wang E, Marincola FM, Stroncek D. Human leukocyte antigens and human neutrophil antigens systems. In: Hoffman R, Benz E, Shattil Sj, et al., eds. Hematology: basic principel and practices 4th ed. Philadelphia: Elsevier, 2005:2401–22.
93. Kissel K, Scheffler S, Kerowgan M, et al. Molecular basis of NB1 (HNA-2a, CD177) deficiency. Blood 2002;99:4231–3.
94. Vigrali DA. Multiplexed particle-based flow cytometric assays. J Immunol Methods 2000;243:243–55.
95. Stroncek DF, Adams S, Lee JH, et al. Neutrophil antibody testing. ASHI Q 2008;32:68–72.
96. Bux J, Kissel K, Hofmann C, et al. The use of allele-specific recombinant Fcγ receptor IIIb antigens for the detection of granulocyte antibodies. Blood 1999;93:357–62.
97. Yasui K, Miyazaki T, Matsuyama N, et al. Establishment of cell lines stably expressing HNA-1a, 1b, and -2a antigen with low background reactivity in flow cytometric analysis. Transfusion 2007;47:478–86.
98. Yasui K, Hirayama F, Matsuyama N, et al. New cell lines express HNA-1c, -4a, -4b, -5a, or -5b for identification of HNA antibodies. Transfusion 2008;48:1037–9.
99. Lucas GF, Carrington PA. Results of the First International Granulocyte Serology Workshop. Vox Sang 1990;59:251–6.
100. Bux J, Chapman J. Report on the Second International Granulocyte Serology Workshop. Transfusion 1997;37:977–83.
101. ISBT Working Party on Granulocyte Immunobiology. Recommendations of the ISBT Working Party on Granulocyte Immunobiology for leukocyte antibody screening in the investigation and prevention of antibody-mediated transfusion-related acute lung injury. Vox Sang 2009;96:266–7.

Mary E. Clay, MS, MT(ASCP), (corresponding author) Director, Transfusion Medicine, Research and Development Program, University of Minnesota Medical School, Department of Laboratory Medicine and Pathology, MMC 198, D-251 Mayo Building, 420 Delaware Street, SE, Minneapolis, MN 55455, and Consultant, Neutrophil & Platelet Immunology Laboratory, American Red Cross, Mid-America Blood Services Region, 100 South Roberts Street, St. Paul, MN 55107, Randy M. Schuller, BS, MT(ASCP), Supervisor, and Gary J. Bachowski, MD, PhD, Assistant Medical Director, Neutrophil and Immunobiology Laboratory, American Red Cross, Mid-America Blood Services Region, St. Paul, MN.

For information concerning the National Reference Laboratory for Blood Group Serology, including the American Rare Donor Program, please contact Sandra Nance, by phone at (215) 451-4362, by fax at (215) 451-2538, or by e-mail at snance@usa.redcross.org