Neutrophil Antigens and Antibodies in the Diagnosis of Immune Neutropenias*†

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ABSTRACT

Neutrophil specific antigens (NA) are expressed exclusively on human neutrophils and were identified using alloantibodies. Neutrophil specific antigens are polymorphic, and several of them (NA1, NA2, NB1, NB2, NC1, ND1, NE1, and 9A), are thought to define genes at different loci. Feto-maternal incompatibility of NA has resulted in alloimmune neonatal neutropenia. Also, NA are the target antigens for autoantibody production in infants and young children with autoimmune neutropenia of infancy and chronic idiopathic neutropenia in adults. Autoimmune neutropenia can occur secondary to several other diseases, including AIDS. Numerous assays are useful in detecting granulocyte antibodies in patients with neutropenia. Among these assays, granulocyte agglutination (GA) and granulocyte immunofluorescence (GIF) are available in some clinical laboratories. Both IgG and IgM agglutinins are detected by GA: in addition, IgG, IgM, and IgA are detected by GIF. Immune neutropenia (IN) occurs in all age groups. Originally thought to be rare, IN is being increasingly recognized in recent years. Further investigations should lead to a greater understanding of the role of NA in immune neutropenias and to identify as yet unknown NA specificities. With the availability of reproducible and sensitive assays to detect granulocyte antibodies and the increasing knowledge and understanding of various disease aspects of IN, proper diagnosis and appropriate clinical management are being applied.

Introduction

Neutrophil Antigens and Antibodies: Historical Perspectives and New Horizons

Human granulocyte antigens are heterogeneous with diverse properties. They...
may be shared with other blood and tissue cells (systemic) or develop exclusively on neutrophils (tissue specific). Clear-cut examples of tissue-specific alloantigens limited to a single cell lineage—the neutrophils, were described by Lalezari et al while investigating the causes of neonatal neutropenia, and neutrophil specific agglutinating antibodies were successfully documented in the maternal sera. These antibodies, being IgG, cross the placenta and react with fetal neutrophils causing neonatal neutropenia. Feto-maternal incompatibility in neutrophil antigen system (NA) is responsible for this disorder which is known as ‘alloimmune neonatal neutropenia’ (ANN). Alloimmune neonatal neutropenia is the neutrophil analog of hemolytic disease of the newborn. The paternally derived fetal leukocyte antigens actively cross the placenta during pregnancy and, when the mother lacks the antigens, cause alloimmunization. Forty percent of ANN is observed in the first born. Lalezari et al investigated several families with infants afflicted with ANN, characterized the antibodies involved, and identified the NA that caused the feto-maternal incompatibility. Neutrophil antigens are present in mature granulocytes and are fully expressed in cord neutrophils.

The first neutrophil antigen identified is designated as NA1, the letter N relating to neutrophils and the independent genetic loci being further described by alphabetic letters A, B, C, etc. Arabic numbers are used to define individual alleles. Other neutrophil antigens NA2, NB1, NB2, and NC1 were subsequently identified using sera of mothers of infants with ANN.

Several investigators have shown that NA are the targets for autoantibody production. Infants and young children less than two years of age have been shown to develop neutrophil specific autoantibodies. This disorder, known as autoimmune neutropenia of infancy (AINI), is analogous to autoimmune hemolytic anemia. Although the mechanism of the autoantibody production is not well understood, the clinical relevances of these antibodies and the antigens involved have been investigated. Neutrophil antigens NA1 and NA2 are frequently shown to be the target antigens in AINI. Antigens ND1 and NE1 have been identified less frequently in the sera of patients with autoimmune neutropenia.

The routine screening of the sera of multiparous women for leukocyte antibodies has led to the identification of two antigens, 9A and Mart. The antigen 9A appears to be granulocyte specific and related to or identical with NB2. Mart is present not only on granulocytes but also on monocytes and T-lymphocytes. These antigens are described in table I. Their gene frequencies are reported in many publications.

New Specificities

Neutrophil Specific Antigen ‘CNI’

It is clear that in the 28 years since the discovery of neutrophil antigens few new neutrophil specific antigens have been detected. Technical problems were a major difficulty; but it was speculated that there might be only a few polymorphic genes that code for such antigens. The majority of the NA were identified using maternal sera of ANN infants. Alloimmune neonatal neutropenia is considered to be a rare disease, although it is likely that mild cases go unnoticed. Since the examination of the blood of the neonate is not done routinely, the diagnosis of neutropenia is made usually because of infections in the child. Alloimmunization against neutrophils has been estimated to be in the order of 0.1 percent to three percent in multi-
TABLE I
Currently Recognized Neutrophil Antigens
the Method of Identification, Source of the Antisera
and Their Clinical Implications

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Source of Sera</th>
<th>Clinical Implications</th>
<th>Method of Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA1, NA2, NB1</td>
<td>Maternal, Infant</td>
<td>ANN, AINI, TR</td>
<td>GA, GIF</td>
</tr>
<tr>
<td>NB2</td>
<td>Maternal</td>
<td>ANN, TR</td>
<td>GA</td>
</tr>
<tr>
<td>NC1</td>
<td>Maternal</td>
<td>ANN</td>
<td>GA, GIF</td>
</tr>
<tr>
<td>ND1</td>
<td>Adult</td>
<td>AIN(A)</td>
<td>GA, GIF</td>
</tr>
<tr>
<td>NE1</td>
<td>Infant</td>
<td>AINI</td>
<td>GA, GIF</td>
</tr>
<tr>
<td>9A</td>
<td>Multiparous</td>
<td>-</td>
<td>GA</td>
</tr>
<tr>
<td>NGR-3</td>
<td>Multiparous, TR, IN</td>
<td>GC, GIF</td>
<td></td>
</tr>
<tr>
<td>CN1</td>
<td>Maternal</td>
<td>ANN</td>
<td>GA, GIF</td>
</tr>
</tbody>
</table>

ANN = Allergic neonatal neutropenia
AINI = Autoimmune neutropenia of infancy
AIN(A) = Autoimmune neutropenia in adults
TR = Transfusion reactions
IN = Immune neutropenia
GA = Granulocyte agglutination
GIF = Granulocyte immunofluorescence
GC = Granulocyte cytotoxicity
CN1 = Tentative designation of a new antigen identified in Charleston

In our investigations of newborns over a six month period, ANN was found in 16 percent of neonatal patients with infection or sepsis. This represented two in 1000 live births (0.2 percent) and 1.5 percent of all admissions to the neonatal special care unit. Incidentally, the specificity determination of one of the maternal sera led to the identification of a unique neutrophil antigen tentatively designated as CN1. This antigen is present in 31 percent of blacks and 1.5 percent in whites in data from our study. As pointed out by Engelfriet et al, the genes coding for NA all seem to have one allele with a very high and one allele with a very low frequency, suggesting rather recent mutation. Recently four additional and as yet unnamed specificities belonging to the N-series have been recognized in Lalezari’s laboratory. The CN1 would have gone unnoticed and unidentified if this infant with neutropenia had not been investigated by the present authors. More investigations of ANN cases will reveal other unidentified specificities and should lead to the expansion of NA polymorphism.

Methods Employed to Detect Neutrophil Antibodies

BENEFITS, PITFALLS and CLINICAL APPLICATIONS

Several methods are useful in detecting granulocyte antibodies. They were developed based on the type of antigen and antibody interactions, the various mechanisms of antibody mediated cell destruction, and the visible pattern by which the antigen-antibody interactions can be measured. They fall into three major categories. The first and second group of assays involve a primary recognition of antigen by antibody and their interactions result either in microscopically visible secondary reactions, as in the first group [granulocyte agglutination (GA), and granulocyte cytotoxicity (GC)], or in the alterations of the functional capacity of normal neutrophils, as in the second group (opsonization, and inhibition of hexose monophosphate shunt). The third group of assay systems are dependent only on primary recognition of antibody to the cell surface antigens. The binding of antibody is measured by a second probe (immunofluorescent enzyme-linked immunosorbent assays [ELISA] enzyme-linked or radio-labeled antihuman immunoglobulin assays).

Granulocyte Agglutination

Macro, micro, and capillary leukoagglutination techniques were initially employed to detect granulocyte antibodies. Among these three assays, the micro technique known as GA is simple, inexpensive, highly reproducible, and is used in many clinical laboratories.
Unlike red cell agglutination, GA is an active biphasic process involving primary interaction of antigen-antibody and cell movement which is time and temperature dependent. This necessitates the use of viable cells and longer incubation conditions. The availability of Ficoll-Hypaque density gradients to isolate viable cells and the use of disodium ethylene diamine tetra acetate (EDTA) to prevent non-specific aggregation of granulocytes, greatly facilitate the wider applicability of GA.

The IgG neutrophil agglutinins in ANN, IgG and IgM in AINI, and autoimmune neutropenia (AIN) in adults are detected by GA. Granulocyte agglutination is not only useful in detecting the allo- and auto-antibodies, but also extremely sensitive in identifying the antibody specificity directed against the N-series antigens that are involved in these disorder. Granulocyte agglutination is the only technique available to determine the neutrophil antigen phenotype of patients or normal donors. McCullough has shown that antibodies detected by GA correlate better with clinical findings than those detected by GC.21,22

**GRANULOCYTE IMMUNOFLOUORESCENCE**

Granulocyte antibodies that bind to cell surface antigens without resulting in visible reaction patterns are detectable by immunofluorescent techniques (GIF) with the help of a fluorescent labelled second antibody, usually produced in animals against human immunoglobulins. Granulocyte immunofluorescence has been modified for the use of micro quantities of the serum and is being employed in clinical laboratories.32 Despite the great success of GIF, it involves the manual reading of fluorescent positive cells under a fluorescent microscope, and, thus, is time consuming, tedious, and subjective. In recent years, the availability of flow cytometry has reduced the above drawbacks of GIF.33 Recently, a granulocyte flow cytometric micro method (GFCy) has been developed by us that utilizes five to 10 μl of the serum.29 Our newly developed GFCy is extremely simple and highly reproducible in detecting IgG granulocyte antibody. It should be useful in detecting IgM and IgA antibodies.*

**USE OF MULTIPLE METHODS**

Antibodies directed against N-series antigens are detected by GA and GIF, since the majority of the agglutinating...
antibodies have cell surface binding properties. Our own data and that of others have shown that in some cases of ANN, the maternal antibodies were detected only by GFCy,† GFCy is much more sensitive than GA, particularly when GA antibodies have a low titer. It remains to be seen whether or not antibodies detectable by GFCy are different from those detectable by GA. It has also been observed in several infants with AINI that autoantibodies detected by GFCy were stronger than those detected by GA. In some cases the autoantibodies were detected only by GFCy. It is important that both GA and GIF (GFCy) are employed simultaneously to detect granulocyte antibodies.

**Other Neutrophil Antibody Assays**

Other less commonly used assays are Staphylococcus protein A (SPA) assays, ELISA, solid phase radioimmunoassays (RIA), Fab-anti-Fab assays, Avidin-Biotin assay, and the functional assays such as opsonization and hexose monophosphate shunt inhibition. These assays require larger volumes of sera and cells and are technically more difficult to perform. The micro serological assays (GA, GIF, GFCy, and GC) have proved to be more useful, particularly for large scale population and family studies.

**Discussion**

Although immune neutropenia is being increasingly recognized in recent years, the role of different granulocyte antigen systems involved in the granulocytopenic disorders are poorly understood. N-series antigens belonging exclusively to neutrophils, G-series antigens common to all granulocytes, GM-series shared with monocytes and granulocytes, differentiation antigens, and those present in precursor cells are all known to induce antibodies and are implicated in patients with immune neutropenias. It is not known whether the antibodies detected by different assays have similar specificity, have the same mechanism of cell destruction, or have similar clinical relevance. No single technique has thus far been found to detect all clinically significant granulocyte antibodies. Investigations are needed to employ various assays simultaneously to detect granulocyte antibodies in granulocytopenic disorders. Expensive, cumbersome, or those assays that can not be used widely should be eliminated.

**Diagnosis, Natural History and Treatment**

In table II are given the details of the testing protocol to detect and identify the granulocyte antibody in various

**TABLE II**

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Test Sera</th>
<th>Test Cells (Granulocytes)</th>
<th>Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANN Infant</td>
<td>Infant</td>
<td>Father</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Mother</td>
<td>Infant*</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mother</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Donors†</td>
<td>Positive</td>
</tr>
<tr>
<td>Transitory</td>
<td>Infant</td>
<td>Mother</td>
<td>Positive</td>
</tr>
<tr>
<td>Neonatal Neutropenia</td>
<td>Mother</td>
<td>Infant*</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Father</td>
<td>Pos/Neg</td>
</tr>
<tr>
<td>AINI Infant</td>
<td>Infant</td>
<td>Father</td>
<td>Pos/Neg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mother</td>
<td>Pos/Neg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Infant*</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Donors†</td>
<td>Positive</td>
</tr>
<tr>
<td>AIN (Adults)</td>
<td>Autologous</td>
<td>Autologous*</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Donors†</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Sera (maternal or from patients with history of transfusions), should be tested for HLA antibodies and, if present, should be absorbed and then retested for granulocyte antibody.

* If available
† Selective
ANN = Alloimmune neonatal neutropenia
AIN = Auto-immune neutropenia of infancy
AIN = Auto-immune neutropenia

† Unpublished data.
immune neutropenias. The diagnosis of infants with ANN is made by the documentation of neutrophil specific antibodies in the mother and the infant that react with paternal neutrophils and not with maternal cells. Antibodies to human leucocyte antigens (HLA antibodies) that interfere with the assay should be tested, and, if present, should be absorbed and the maternal serum retested. The antibody may be identified in a cell panel. When the infant recovers from neutropenia, follow-up testing is useful to demonstrate the clearance of antibody. Infants with ANN are usually born with neutropenia, but neutropenia may not be seen until a few days after birth. The reason for this is unknown. Neutropenia persists from two weeks to three months, sometimes longer depending upon the strength of the maternal antibody. The neutropenia resolves with the simultaneous clearance of antibody. These infants may remain asymptomatic, but with severe and persisting neutropenia, they may become susceptible to infection, including sepsis.

Infections documented in infants with ANN are various forms of skin infections, omphalitis, otitis, fever, and respiratory and urinary infection. The bacteria involved are mostly staphylococci, Group A, B-hemolytic streptococcus, and *Echerichia coli*. Deaths have been observed in septic infants. Since steroids are ineffective, affected infants need appropriate antibiotics for serious infections. Neonatal neutrophil transfusion has been shown to be beneficial in some patients with sepsis. Cross-match testing should be done with infant serum to select appropriate donor neutrophils. Plasma exchange or the use of intravenous immune globulin may be alternative treatments, although they have not been tried as yet.

Neonatal neutropenia may also be caused by the transplacental passage of maternal neutrophil autoantibodies; this disorder is known as transitory neutropenia. This is documented by the presence of antibody in maternal and infant sera reacting with maternal and infant neutrophils and not with paternal cells. Neutropenia in the mother may be demonstrated by a complete blood count and differential.

Although the exact incidence is unknown, autoimmune neutropenia of infancy is the most common form of chronic neutropenia in infants. The clinical symptoms are described in detail in our earlier reports. Currently AINI is recognized by severe and persisting neutropenia between four to seven months of age. Autoimmune neutropenia of infancy is a self-limiting disorder, but it may become complicated by various forms of bacterial and fungal infections. It is diagnosed by the presence of neutrophil specific autoantibodies in the infant serum and their absence in the maternal serum. Since the autoantibodies are directed against alloantigens, the infant serum reacts with either one or both of the parent neutrophils when they share the same antigens. The antibody can be further identified by a cell panel. In general, these infants generally recover within one to four years, but AINI may continue beyond childhood. Epinephrine and steroid stimulation fail to increase the neutrophil count. The bone marrow may be normal with a significant decrease in mature neutrophils. Since these infants are susceptible to infections, antibiotics are necessary when indicated. In severe cases, IVIg has been successful. Steroids have been found to give a transient effect in some cases.

Autoimmune neutropenia may also occur in adults with chronic idiopathic neutropenia (CIN) or secondary to other autoimmune diseases, lymphatic malignancies, immune deficiency diseases including AIDS, and thyroid disorders...
The antibodies are generally detected by GIF assays. The antigens involved in these disorders are poorly understood. However, detection of granulocyte antibodies in the sera of these patients has proved helpful in understanding the cause of granulocytopenia and in instituting appropriate therapy for the clinical management of these patients. Various forms of secondary immune neutropenias have been recently reviewed by McCullough.22

Summary and Conclusions

Neutrophil specific alloantigens are polymorphic. Progress in uncovering the polymorphism and identifying many unknown antigens has been hampered since the first discovery by Lalezari et al 28 years ago. Technical problems in isolating viable granulocytes and the need for suitable, sensitive assays to achieve reproducible results greatly contributed to the discouragement faced by the initial investigators. With the availability of Ficoll-Hypaque density gradients to isolate pure and viable granulocytes and GA and GIF techniques to detect accurately the granulocyte antibodies, a renewed enthusiasm has been shown in recent years. Furthermore, the availability of flow cytometric assays replacing the manual GIF techniques should enhance the understanding of the granulocytopenic disorders. Currently, GA, GIF, and GFCy have been shown to have important clinical usefulness for the diagnosis of ANN, AINI, CIN, and secondary AIN. In addition, application of these assays will lead to a better understanding of the role of granulocyte antibodies in non-hemolytic transfusion and pulmonary reactions and may lead to their routine use in diagnosing these reactions. Although some serological and pathophysiological aspects of granulocytes are analogous to red cells, the antibodies detected by various techniques seem to suggest that granulocyte serology is much more complicated. Thus, there is a need for newer approaches to define various antigens, their biological and functional roles, and their clinical implications.5

The N-series antigens have important implications in ANN, AINI, and transfusion reactions. Identification of various polymorphic N-series antigens will lead to a greater understanding of their role in neonatal alloimmune and autoimmune neutropenias. These alloantigens have been shown to be useful as biological and genetic markers to evaluate the engraftment of donor bone marrow in ABO and HLA compatible bone marrow transplantation.34 Investigations are needed to support these initial observations.

Although the role of maternal antibodies in infants with ANN is known, the clinical significance of preformed antibodies in subsequent pregnancies has not been well investigated. In several instances, malformation, prematurity, and deaths have been shown to be asso-

| TABLE III |
| Granulocyte Antibodies in Primary and Secondary Immune Neutropenias |

<table>
<thead>
<tr>
<th>Immune Neutropenias (Antibody Mediated)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary</strong></td>
</tr>
<tr>
<td>I. Congenital (alloimmune)</td>
</tr>
<tr>
<td>(a) ANN</td>
</tr>
<tr>
<td>(b) Transitory neutropenias</td>
</tr>
<tr>
<td>II. Acquired (auto-immune)</td>
</tr>
<tr>
<td>(a) Idiopathic</td>
</tr>
<tr>
<td>(i) AIN</td>
</tr>
<tr>
<td>(ii) CIN or AIN</td>
</tr>
<tr>
<td><strong>Secondary</strong></td>
</tr>
<tr>
<td>III. Associated with systemic or other diseases</td>
</tr>
<tr>
<td>(1) Hematologic diseases</td>
</tr>
<tr>
<td>(2) Collagen vascular diseases</td>
</tr>
<tr>
<td>(3) Lymphoproliferative diseases</td>
</tr>
<tr>
<td>(4) Lymphatic malignancies</td>
</tr>
<tr>
<td>(5) Immunodeficiency diseases, AIDS</td>
</tr>
<tr>
<td>(6) Thyroid diseases</td>
</tr>
<tr>
<td>IV Drug induced</td>
</tr>
</tbody>
</table>

ANN = Alloimmune neonatal neutropenia
AIN = Auto-immune neutropenia of infancy
CIN = Chronic idiopathic neutropenia
AIN = Auto-immune neutropenia
AIDS = Acquired immune deficiency syndrome
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References


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