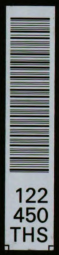

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NEUTROPHIL ANTIGENS AND ANTIBODIES:
A MICROAGGLUTINATION DETECTION TECHNIQUE
AND POPULATION STUDY

presented by

MARGARET A. PURDY

has been accepted towards fulfillment
of the requirements for

MASTER OF SCIENCE degree in CLINICAL LABORATORY SCIENCE

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Date June 6, 1984

NEUTROPHIL ANTIGENS AND ANTIBODIES:

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AND POPULATION STUDY

by

Margaret A. Purdy

A THESIS

submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Pathology

1984

ABSTRACT

NEUTROPHIL ANTIGENS AND ANTIBODIES:
A MICROAGGLUTINATION DETECTION TECHNIQUE
AND POPULATION STUDY

By

Margaret A. Purdy

This investigation centers on the use of an in-vitro microagglutination assay as a means to study the incidence of neutrophil antigens and their combinations in a random population. Neutrophil antigen typing was also evaluted for its possible use as an additional marker in cases of disputed paternity.

A study of the literature involving those antigens found on neutrophils was conducted. Also reviewed are the major methodologies used to examine neutrophil antigen-antibody systems.

Three selected populations were studied to determine the incidence of neutrophil antibodies. These populations consisted of patients with the previous diagnosis of: 1) systemic lupus erythematosus (SLE), 2) multiple sclerosis (MS), and 3) post-partum women. The clinical problems encountered with neutrophil antibodies in the management of 1) immune neutropenias, 2) granulocyte transfusions, 3) transfusion reactions, and 4) organ transplantation are presented.

ACKNOWLEDGEMENTS

My sincere appreciation and gratitude is expressed to J. R. Kateley, Ph.D., Director of Immunology, Edward W. Sparrow Hospital, for his assistance in the selection of a research project, his help in securing patient specimens and reagents, and for allowing me to use his department's space and equipment to complete my project.

W. E. Maldonado, M.D., Director of Laboratories, Edward W. Sparrow Hospital, for allowing me to use reagents and equipment.

M. Thomas, M.S., M.T. (ASCP); J. R. Kateley, Ph.D.; M. Boss, M.S., M.T. (ASCP) and S. Zablotney, Ph.D., M.T. (ASCP), my advisory committee.

F. K. Neumann, Administrator of Edward W. Sparrow Hospital, for the financial support received during the graduate program.

R. A. Purdy, M.A., for his patience, support and proof reading of all materials.

D. E. Emmons for her skillful typing of this paper. Without her assistance this project could not have been completed.

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INTRODUCTION

The first neutrophil antigens were discovered in 1960. Since that time a large number of antigens have been demonstrated on the surface of neutrophils. Some of these antigens are found exclusively on neutrophils (neutrophil specific antigens), while others are also present on erythyrocytes, lymphocytes and monocytes (systemic neutrophil antigens). These antigens are of significance; a) because of the rich genetic polymorphism they comprise, and b) because the antibodies that react with these neutrophil antigens have been implicated in immune neutropenia, transfusion reactions and reduced clinical effectiveness of granulocyte transfusions.

Because neutrophils play a key role in the immune function, antibodies directed against neutrophil specific antigens can interfere with normal neutrophil function and cause clinically significant problems. Antibodies against neutrophils are of importance for the pathogenesis of neonatal and acquired neutropenia and may be a limiting factor in the efficacy of transfusions. Neutrophil antigens, the target of such antibodies, are also important as additional markers for evaluation of allogenic bone marrow engraftment and are under investigation for their use in paternity testing.

REVIEW OF LITERATURE

The white blood cells or leukocytes are divided into three major morphologic classes; the monocyte, lymphocyte and granulocyte. Granulocytes are divided into three cell lines; the eosinophils, the basophils and the neutrophils (see Figure 1). The mature granulocyte of the neutrophil cell line is referred to as a polymorphonuclear cell or PMN.

The leukocyte antigens may be classified into two major classes: 1) systemic antigens - those shared between leukocytes and other tissues such as HLA, ABH and blood group antigens, and 2) specific antigens - those present only on a specific cell line, for example lymphocyte specific and neutrophil specific antigens. Much attention has been focused on the lymphocyte antigens since their discovery by Daussett (1958), while comparatively little investigation has been performed on the neutrophil antigens until recently. Lalezari (1960) discovered several antigens which are restricted to neutrophils. This sparked an interest in the role of the neutrophil as an immune effector cell.

Systemic Neutrophil Antigens

Systemic antigens are those antigens found on neutrophils and on other cells of varying lineage. These may represent antigens that are present on primitive cells and retained as the cell lines differentiate into specialized tissues and blood cells.

Figure 1. Granulocyte cell lines and maturation sequence

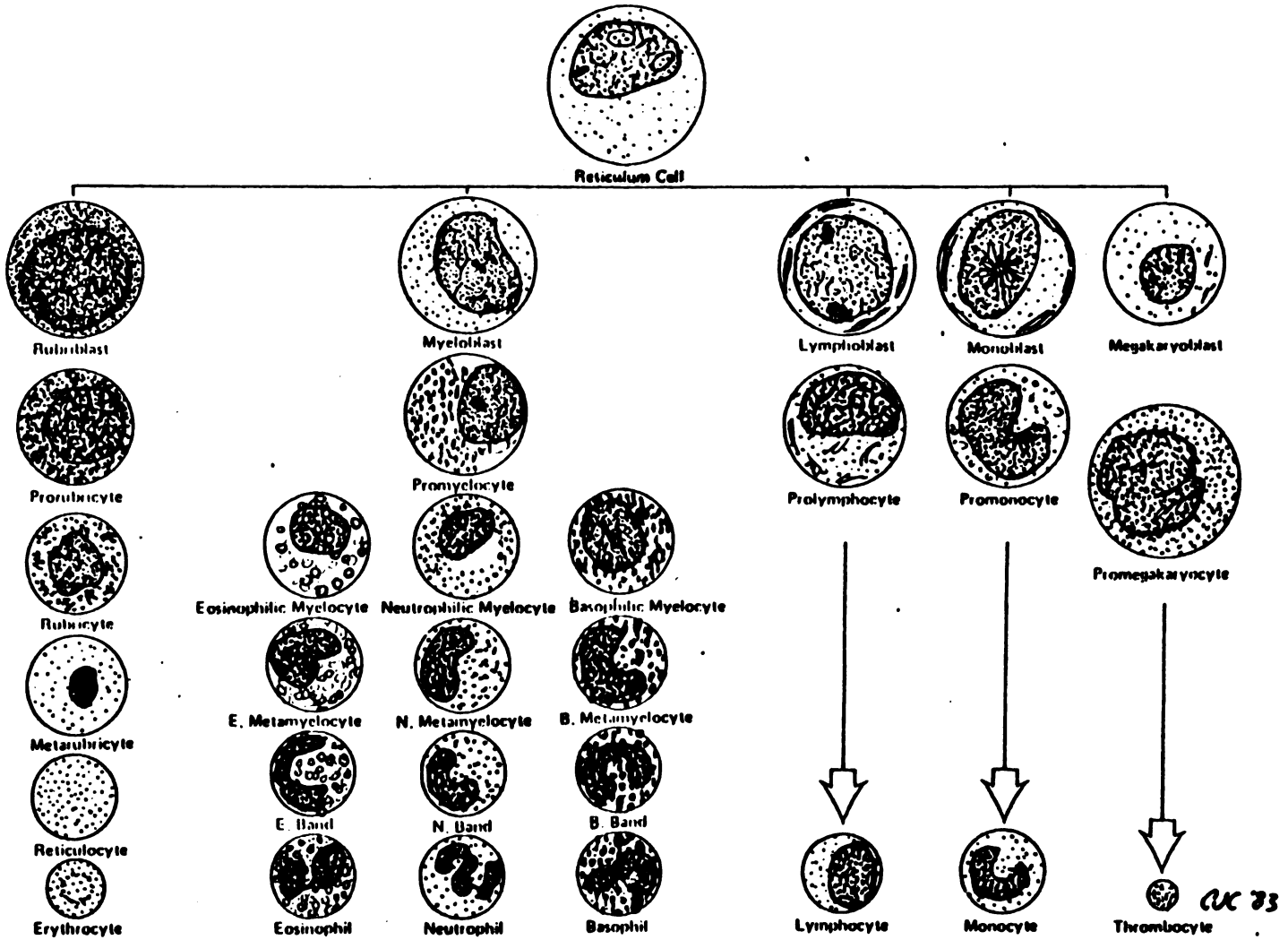

Reticulum Cell
Rubriblast
Prorubricyte
Rubricyte
Metarubricyte
Reticulocyte
Erythrocyte
Myeloblast
Promyelocyte
Eosinophilic Myelocyte
Neutrophilic Myelocyte
Basophilic Myelocyte
E. Metamyelocyte
N. Metamyelocyte
B. Metamyelocyte
E. Band
N. Band
B. Band
Eosinophil
Neutrophil
Basophil
Lymphoblast
Monoblast
Megakaryoblast
Prolymphocyte
Promonocyte
Promegakaryocyte
Lymphocyte
Monocyte
Thrombocyte

Figure 1

ABH Antigens

Antigens of the ABH system are found on almost all tissue and blood cells. An interesting characteristic of ABH antigens is that they may occur in a soluble form in almost all body fluids; these soluble antigens can absorb passively to cells. When ABH antigens are detected on a cell line efforts must be made to determine whether the antigens are integral membrane components or are only passively absorbed soluble antigens. ABH antigens have been demonstrated on neutrophils. These antigens can only be demonstrated by absorption and elution techniques (Marsh, 1975).

Erythrocyte Antigens

More antigens have been recognized on erythrocytes than on any other cell line. Red cell antigens predominate because of simple methodologies used to detect them, their early discovery and massive immunization that has occurred from red blood cell transfusions. Only some antigens that have been found on red blood cells can be detected on neutrophils. However, the red cell antigens that are present on neutrophils are all high incidence antigens; that is, antigens that are found on the vast majority of erythrocytes. The Gerbich antigen (GE), the Lewis antigens, Le^a and Le^b, and the Kidd system antigens JK^a and JK^b, have all been shown to occur on neutrophils (Marsh, 1975). In the MNSsU systems, only the U antigen has been identified (Marsh, 1974). The I and i antigens, which occur on all erythrocytes, have been demonstrated on neutrophils (Lalezari, 1967). As found in erythrocyte tests, the anti-I and anti-i antibodies that react with neutrophils are predominately cold reactive. Other cold reactive antibodies against

neutrophils that are not absorbed by adult or cord red blood cells probably represent a distinct specificity. The I and i antigen on neutrophils can be demonstrated by both agglutination and by cytotoxicity assays, in contrast to all the other red blood cell antigens which can only be demonstrated by absorption techniques. In the Kell system only the K^x antigen is demonstrated on neutrophils (Marsh, 1975). This antigen is of particular interest because it was shown to be absent in three children suffering from X-linked chronic granulomatous disease, a rare disorder characterized by severe recurrent infections caused by abnormal neutrophil functions (Marsh, 1975).

HLA Antigens

The HLA system is now recognized as the most complex polymorphic system in man. At least sixty different HLA antigens have been identified. HLA antigens are controlled by alleles at four separate but closely linked loci, named HLA-A, B, C and DR. Antigens produced by genes at the A, B, C and DR loci are detected by lymphocytotoxicity tests (serologically defined); while the D locus-produced antigens are detected by their ability to stimulate lymphocyte proliferation in the mixed lymphocyte culture reaction (cell defined). Granulocytes carry both HLA-A and HLA-B antigenic determinants, but it has not been conclusively established whether they carry the HLA-C or HLA-D determinants (Thompson, 1980). Table 1 depicts the antigens shared by neutrophils with other tissues.

Table 1. Classification of Antigens of Cells of Hematopoietic Origin Based on Concentration and Distribution Patterns. (Lalezari, 1980)

Antigen Class	Examples
Antigens present on both the mature and immature cells	
(a) Shared by all other tissue cells	A, B, H, group 5
(b) Restricted to cells of common lineages	GEM*, EM, G
Antigens mainly present on immature cells (concentration decreases with maturation)	HLA-A, B, C, D, DR
Antigens mainly present on mature cells (concentration increases with maturation)	Neutrophil, thrombocytes, erythrocyte-specific antigens

*GEM = granulocyte, endothelial cell, and monocyte.

Neutrophil Specific Antigens

A major category of antigens found on neutrophils are those not detectable on any other cell line. Neutrophil specific antibodies are very often revealed only after repeated absorption of antisera with plateletes and/or other cell types to remove HLA and other non-neutrophil specific antibodies. Lalezari (1960) discovered the first neutrophil specific antigens. Van Rood (1965) performed absorption studies and, using a 2x2 chi square contingency, isolated an additional number of neutrophil antibodies which he termed the 4^a and 4^b system. Payne et al. (1965) demonstrated the existence of two other leukocyte antigens which they termed the LA1 and LA2 systems. This multiplicity of antigens with individual systems of nomenclature led to increasing

confusion until a meeting of all the major investigators was held at a 1967 workshop in Turin, Italy. A standard nomenclature was agreed upon and has been used internationally since that time.

Neutrophil Antigen Nomenclature

The neutrophil specific antigens are divided into two categories, the N series and the G series.

The letter N indicates neutrophil specificity; N is followed by the letters of the alphabet, each of which indicates a genetic locus in chronological order of discovery. The letters are followed by Arabic numerals which identify alleles. Thus NA1 identifies the first allele of the antigens in the locus A, the first locus identified. The NA system appears to be a dialletic system composed of NA1 and NA2. There are at least five neutrophil loci recognized and designated NA through NE.

A new series of antigens controlled from a different locus was reported by Thompson (1980). Seven new distinct antigenic specificities were identified. Genetic analysis showed that five of these antigens are controlled from a new locus designated the human granulocyte antigen locus 3 (HGA-3). These five new antigens have been designated HGA-3a, b, c, d and e. An additional granulocyte antigen HGA-1 was distinct from the HGA-3 series because it segregated independently. Also HGA-1 was detected on monocytes and granulocytes while the HGA-3 antigens are granulocyte specific. All of the HG-A antigens segregate independently from the HLA, 5a-5b, and NA, NB loci, indicating that they are truly an independent system. The nomenclature of neutrophil antigens is summarized in Table 2.

Table 2. Neutrophil Antigen Nomenclature - 1984

Locus	Antigen
NA	NA1
	NA2
NB	NB1
NC	NC1
ND	ND1
NE	NE1
HGA-1	HGA-1
HGA-3	HGA-3A
	HGA-3B
	HGA-3C
	HGA-3D
	HGA-3E

Maturation of Neutrophils and Antigen Expression

Cells are known to undergo membrane modification during their differentiation and maturation and acquire new antigens in relation to their identity and specialized functions. These "organ-specific antigens" are genetically independent of the "systemic antigens" such as HLA and ABH which have wide tissue distribution. In the process of evolution, organ-specific antigens have been modified by mutation, giving rise to polymorphism and the development of organ-specific alloantigens. Such antigens have been described for the polymorphonuclear neutrophils (Lalezari, 1977). The classification of antigens of hematopoietic cells on the basis of concentration and distribution patterns should be helpful in the understanding of their potential in-vivo reactions. The antigens that are mainly present on mature cells

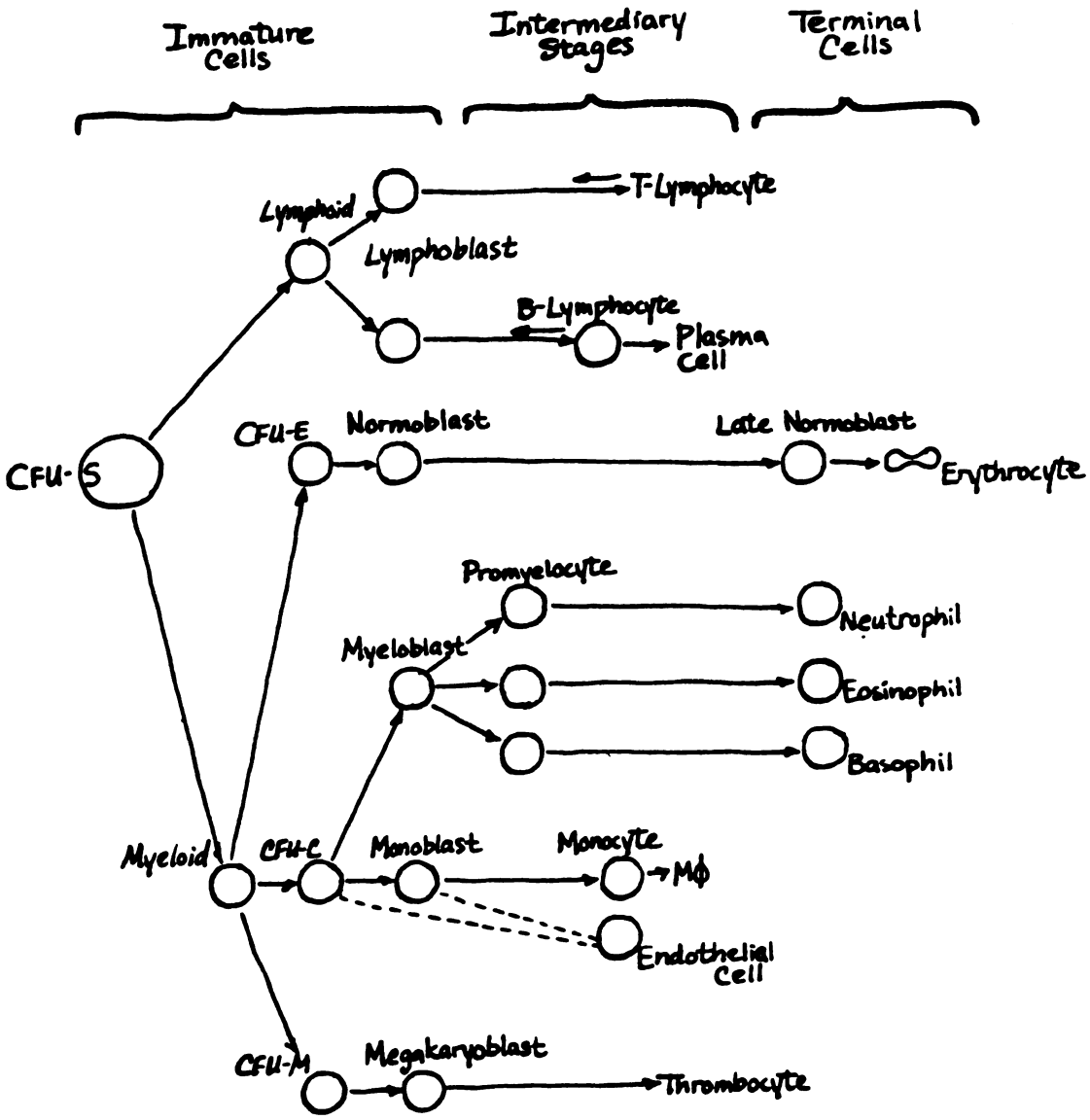
are absent from progenitor cells but develop during cell differentiation. In the case of neutrophil antigens, evidence exists that they are not expressed on the normal immature cells. Fitchen (1977) has shown that the treatment of bone marrow cells with anti-NA1 and anti-NA2 antibodies does not inhibit in-vitro growth of colony forming units - granulocytic cells (CFU-c cells). An increase in the density of neutrophil-specific antigens has been demonstrated by keeping blood neutrophils at room temperature. This treatment, which may be considered as an in-vitro aging process, is revealed by a steady rise in the immunofluorescence produced by the antigen-antibody reaction as measured by microfluorometry (Lalezari, 1980). Figure 2 is a representation of the cells of hematopoietic orgin shown in the comparable stages of maturation. The scheme serves to point out antigens present on various cell lineages and expressed at various stages of maturation.

Serologic Characteristics of Neutrophil Antibodies

The serologic, immunochemical, and immunocytological properties of neutrophil specific antibodies produced by sensitization to a neutrophil specific antigen have been studied by Verheugh (1978). These studies indicate that neutrophil antibodies are IgG, IgM, IgA or a combination of these classes. IgD and IgE antibodies were not found by these researchers to react with neutrophils.

The IgG antibodies are mainly of the IgG_1 and/or IgG_3 subclasses. Neutrophil agglutination occurs with both IgG and IgM antibodies; IgG antibodies produce round agglutinates while IgM agglutinates have more bizarre shapes. Drew (1978) concurs with previous investigators that differences between IgG and IgM antibodies are also seen in the granulocyte cytotoxicity test. In general, granulocyte cytotoxicity antibodies were found to be IgM; IgG antibodies react weakly if at all.

Figure 2. Antigens present on various cell lines and expressed at various stages of maturation. CFU-s, CFU-e, CFU-m, CFU-c, and MΦ are colony-forming stem cells, erythroid, megakayocytic, granulocytic cells, and mononuclear phagocytes, respectively. (Lalezari, 1980)



Antigens	ABH	HLA-A,B,C	HLA-D,DR	Maturity Specific
	+	+	+	−
	+	+	+	−
	+	+	+	−
	+	+	+	−
	+	+	+	+
	+	+	−	+
	+	±	−	+

CC '83

Figure 2

Methodologies Used To Investigate Neutrophil Antigens and Antibodies

The attempt to find a sensitive, yet specific, test has led to the development of numerous serological techniques to examine neutrophil antigen-antibody systems. The most widely used methods are agglutination, complement-dependent cytotoxicity, surface binding and functional assays. Various kinds of neutrophil antibodies appear to have different optimal requirements for reactivity. Therefore, the techniques cannot be used interchangeably nor are the test sensitivities parallel.

Neutrophil Separation

The ability to obtain a pure population of polymorphonuclear leukocytes is an important first step in the investigation of neutrophil antigen-antibody systems. Numerous investigators have relied upon density gradient separation of mononuclear and polymorphonuclear leukocytes from whole blood. English and Andersen (1974) used a discontinuous Ficoll-Hypaque gradient for the simultaneous separation of mononuclear leukocytes, neutrophils and erythrocytes. McCullough (1980) modified this procedure to obtain neutrophil suspensions that contain greater than 95 percent PMN's.

Agglutination Assays

The neutrophil agglutination reaction is not analogous to the familiar red cell agglutination in that simple mixing of the cells with antibodies and centrifugation of the mixture does not produce cell aggregation. In contrast, the reaction is slow and develops during the incubation of cell-antibody mixtures. Lalezari (1974) has demonstrated

by phase contrast microscopy and microcinematography, that the process involves participation of motile cells which appear to be activated by the antibody, and which move toward each other to form aggregates. Factors such as short exposure to heat, which do not destroy antigens but immobilize cells, prevent agglutination by anti-neutrophil antibodies. Thus, in contrast to the erythrocyte reaction, in neutrophil agglutination there is active cell participation. The method employed for cell preparation and the reaction conditions in which cell-antibody mixtures are incubated both have a major impact on the outcome of the neutrophil agglutination reaction (Lalezari, 1974). The mechanism of granulocyte agglutination requires active cell participation and the presence of disodium ethylenediametetraacetate (EDTA). The mechanism of this dependence on EDTA is not clear; it may be related to the protection that EDTA affords against cell death and disintegration. Leukocytes of blood collected in EDTA appear to retain their morphologic integrity and motility longer than with other anticoagulants (Lalezari, 1974).

Granulocyte agglutination is temperature and time dependent, and is biphasic, including a sensitization phase and an agglutination phase in which cell viability is crucial (Lalezari, 1977). Microagglutination tests, as currently performed, utilize pure suspensions of neutrophils, require micro (1-5 ul) amounts of serum and cells, and are usually done in microtest plates under oil.

Cytotoxic Assays

First utilized with lymphocytes, cytotoxicity methods have been modified and applied to the detection of granulocyte cytotoxic antibodies (Thompson, 1980). In these procedures test granulocytes are incubated

with antisera and rabbit complement. Antigen-antibody reactions capable of fixing and activating complement result in cell membrane damage. The damaged nonviable cells allow intracellular penetration of a vital dye, such as eosin or trypan blue, and can be distinguished from viable cells microscopically. Various modifications of the granulocyte cytotoxic assay have been used by investigators attempting to identify a granulocyte antigen system solely by cytotoxins. McCullough (1981) modified the assay of Drew (1977) to study the clinical significance of the cytotoxic assay in patients who received radiolabeled granulocyte transfusions.

A method applying fluorochromasia to granulocyte testing has been described by Thompson, et al. (1980). They have reported a series of granulocyte, monocyte and endothelial antigens detectable with a double fluorochromasia study. This assay has been used to study granulocyte antigens associated with drug-induced neutropenia.

Non-complement dependent cytotoxic antibodies can be investigated with the use of antibody dependent cell-mediated cytotoxicity (ADCC) assay of Louge, et al. (1978). In this assay an effector cell (lymphocyte) is cytotoxic to an antibody-coated target cell (granulocyte), by recognition of the F_c region on the cell-bound antibody through an F_c receptor on the effector lymphocyte.

Surface Binding Assays

More than two decades ago Engelfriet and van Loghem (1961) reported a class of leukocyte antibodies which could not be detected by agglutination or cytotoxicity techniques. Attempts have since been made to detect these antibodies on the surface of sensitized neutrophils

using secondary labeled antibodies. Verheught (1978) has reported the detection of neutrophil allo- and auto-antibodies with the use of an immunofluorescent test. Neutrophils sensitized in-vivo and in-vitro with antibody are detected with a fluorescent conjugated anti-immunoglobulin. The developed assay detected previously identified neutrophil specific antibodies NA1, NA2, NB1, NC1 and two heretofore unidentified antibodies ND1 and NE1.

Staphylococcal protein A (SPA), derived from Staphylococcus aureus, binds specifically to the F_c portion of IgG and has been used to detect neutrophil antibodies by Harmon, et al. (1980). This assay provides the means for direct and indirect antibody binding, and quantitation of antibody. But SPA binds primarily to IgG subclasses IgG_1, IgG_2, and IgG_4, fails to bind with IgG_3 and binds only to a limited degree with IgM.

The enzyme linked immunospecific assay (ELISA) technique is currently being investigated by Clay (1981) to detect surface bound neutrophil antibodies. A suitable conjugate and substrate must be found, so that the numerous enzymes contained by neutrophils will not interfere with the conjugate-substrate interaction.

Functional Assays

The biologic function of neutrophil antibodies was analyzed by Boxer and Stossel (1974) using either rabbit alveolar macrophages or human neutrophils to detect and ingest neutrophils coated with opsonizing antibody. The quantitative analysis of this immune phagocytic reaction involves spectrophotometric measurement of nitroblue tetrazolium reduction and scintillation spectrophotometric measurement of $^{14}CO_2$.

Neutrophil antibody inhibition of myeloid maturation, inhibition of the hexose monophosphate shunt activity, inhibition of neutrophil erythrophagocytosis, and the cell elastimetry technique are other functional assay systems that have been developed. However, the technical, serologic and cellular requirements of functional assays tend to diminish their practicality in routine or large-scale screening analysis.

See Table 3 for a summary of methodologies used to investigate neutrophil antigen-antibody systems.

Table 3. Methodologies Used To Investigate The Neutrophil Antigen-Antibody Systems.

Assays	Technique	Researcher
Agglutination	-pure suspension of neutrophils -biphasic agglutination phase -active cell participation	Lalezari (1974) Thompson (1980)
Cytotoxicity	-target granulocytes with antisera -complement dependent damaged cell membranes	Drew (1977) McCullough (1981)
	-double fluorochromasia assay	Thompson (1980)
	-ADCC assay non-complement	Logue (1978)
Surface Binding Assay	-sensitized neutrophil conjugated with a fluorescent anti-immunoglobulin	Verheught (1978)
	-SPA assay binds to F_c portion	Harmon (1980)
	-ELISA techniques	Clay (1981)
Functional Assay	-ingestion of neutrophils coated with opsonizing antibody	Boxer (1974)

CLINICAL CONSIDERATIONS

Renal Transplantation and Neutrophil Antigens

Neutrophil antigen typing has been studied for its clinical applications in transplantation of organs and bone marrow engraftment. Studies by Blaschke (1977) of renal transplantation suggest that the neutrophil antigens are not expressed on the kidney cells and found no correlation between granulocyte antigen compatibility and renal allograft fate. It appears that the granulocyte antigens do not participate in renal transplant rejection.

Bone Marrow Engraftment

A new clinical area for neutrophil antigen typing is its usefulness in bone marrow engraftment. Schacter (1980) has described bone marrow transplantation in which eight patients with HLA identical sibling dnnors had neutrophil antigen markers differing between donor and recipient. Conversion to donor neutrophil type occurred in four bone marrow engraftments and signaled successful engraftment long before peripheral blood counts began to elevate. Neutrophil antigens are thus seen as an additional marker for predicting the success of allogenic bone marrow engraftment especially in instances of an identical HLA match.

Bone marrow transplantation has been successful and has been accomplished even in the presence of a neutrophil specific antibody. Warkentin (1980) reported a case of successful bone marrow engraftment without delayed neutrophil recovery in the presence of neutrophil

specific antibody NA1. The sibling bone marrow donor was NA1 positive. This study suggests that neutrophil specific antigens are not functionally present on the pluripotential stem cell and correlate with Lalezari's (1980) restriction of these antigens to mature cells.

Neutrophil Antigens and Cases of Disputed Paternity

Neutrophil antigens exhibit normal somatic Mendelian inheritance patterns as shown by Lalezari (1960). In cases of disputed paternity, the neutrophil antigen is therefore being studied as a possible additional marker to exclude a falsely accused putative father. The combined prior probability of exclusion of a falsely accused putative father based on the NA, NB and HGA systems estimated by the method of Boyde is 0.178. This compares well with the power of the ABO and Rh systems as studied by Verheught (1978). The use of neutrophil antigen typing as a tool in paternity testing has only recently begun to be investigated.

Neutrophil Antibodies

Because neutrophils play a key role in the immune functions, antibodies directed against neutrophil specific antigens have been found to interfere with normal neutrophil function and have also been found to be clinically significant.

Transfusion Reactions

For many years leukocyte antibodies have been suspected of playing a role in the pathogenesis of transfusion reactions. Van Loghem (1956) reported a patient who tolerated leukocyte poor blood transfusions better than whole blood transfusion. Britingham and Chaplin (1957) have

shown that blood containing leukoagglutinating antibody, when transfused to a normal donor, can cause a relatively severe transfusion reaction. A high fever results from the pyrogens released following the neutrophil antigen-antibody reactions. Post transfusion pulmonary problems such as dyspnea, coughs and pulmonary infiltrates are also associated with neutrophil antibodies.

These types of reactions may be caused by infusing leukocytes into recipients with antibodies, or infusing plasma containing leukoagglutinins into a normal recipient (Mollison, 1979). Greenwalt (1962) showed the key role of granulocytes in mediating these reactions by demonstrating that 75 of 82 patients who had a history of febrile transfusion reactions failed to have these reactions if the granulocytes were removed, even when lymphocytes were transfused. Granulocyte antibodies are a much more likely cause of transfusion reactions than HLA antibodies, which must be very potent to cause any reactions (Lalezari, 1980).

Patients with granulocyte antibodies may be successfully transfused by using red blood cell products in which a majority of leukocytes have been removed (Greenwalt, 1962), for example, by using buffy coat poor red blood cells or deglycerolized red blood cells.

Granulocyte Transfusion

The role of leukocyte serology in granulocyte transfusion has been difficult to establish. Early studies demonstrated that in patients who had leukoagglutinating or lymphocytotoxic antibodies, incompatible granulocytes had a decreased intravascular recovery and failed to localize at sites of infection. Ungerleider (1979) correlated post-transfusion neutrophil increment counts and the occurrence of non-

hemolytic transfusion reactions. These are often difficult to determine, due to the clinical status of the patient. In addition, variations in infusion techniques and the technical difficulties associated with obtaining accurate counts, when counting low levels of neutrophils, reduce the efficacy of increment counts as an effective transfusion indicator. Thus, without a method to define a clinically successful transfusion, it is impossible to study meaningfully the association between the serologic assays and the in-vivo fate of transfused granulocytes. McCullough (1981) has studied the effect of granulocyte agglutinating, granulocyte cytotoxic and lymphocytotoxic antibodies on the intravascular recovery, half-life and the extravascular localization of Indium-111 labeled granulocytes in fifty patients. From these studies, it appeares that granulocyte agglutinating antibodies interfere with the intravascular kinetics and localization of granulocytes at infected sites while granulocyte cytotoxic and lymphocytotoxic antibodies do not cause this same interference.

Immune Neutropenias

The immune neutropenias are the most highly studied clinical problem areas. This group of disorders is characterized by the in-vivo destruction of neutrophils by antibodies. The clinical characteristic of these disorders is a marked increase in susceptibility to serious infections that tend to be refractory to antibiotic therapy.

Isoimmune Neonotal Neutropenia (INN)

Neutrophil antibodies were first identified while studying isoimmune neonatal neutropenia (INN) due to fetal-maternal incompatability. This disease is analagous to hemolytic disease of the

newborn in origin. A mother produces an alloantibody that is directed against an antigen on the infant's neutrophils. If the antibody is of the IgG class, it can cross the placenta and cause the destruction of fetal neutrophils in-vivo. Unlike hemolytic disease of the newborn, there is usually no adverse effect on the infant in-utero. After birth, however, the maternal antibody present in the fetal circulation causes a selective and transient absence of segmented neutrophil leukocytes in the blood and bone marrow of the newborn. The other blood elements are not affected by this antibody. After birth, the infant may be susceptible to various overwhelming infections. The total leukocyte count on these infants may be near normal. The lack of neutrophils in a differential blood count or an absolute neutrophil count may be the only reliable clue to define the disorder.

The disease appears to be relatively common, but not often recognized. Lalezari (1977) has observed 28 afflicted children in 21 families, and many additional cases have been reported by other investigators, including two cases resulting in infant fatalities. The disease is self-limiting, lasting only as long as maternal antibody is present in the infant.

Primary Autoimmune Neutropenia

When a patient produces an autoantibody that is reactive with an antigen located on the neutrophils, primary autoimmune neutropenia results.

A typical case described by Lalezari (1975) is one in which the patient had a history of fevers, respiratory infections, otitis and skin rashes. Both anti-NA1 and anti-NA2 have been found in the sera of patients with similar histories. Steroid treatments have been helpful

in some affected patients. Mothers who themselves suffer from autoimmune neutropenia will often passively transfer this autoantibody to their unborn children who as newborn infants will then suffer from a transient immune neonatal neutropenia.

In adults autoimmune neutropenia has been recognized for a number of years. Butler (1958) has shown that when plasma containing autoantibodies from these patients is transfused to a normal recipient, a temporary neutropenia is induced. Sometimes a particular neutrophil specific antibody can be identified, as in the case due to anti-NA1 reported by Lalezari (1977). These patients often respond favorably to steroid treatments.

Secondary Autoimmune Neutropenia

Patients with a previously diagnosed autoimmune disease such as autoimmune chronic active hepatitis or rheumatoid arthritis have also been found to have high titers of neutrophil specific antibodies. It is believed that the original autoimmune disease stimulates the production of an anti-neutrophil antibody to cause an autoimmune neutropenia secondary to the primary disease. These patients often respond favorably to steroid treatments.

OBJECTIVES

The objectives of these studies were:

1. To produce a reliable testing mechanism for the detection of neutrophil antigens and antibodies by attempting various modifications of currently employed microagglutination techniques.

2. To determine the incidence of detectable neutrophil antigens in a population of pre-partum women and random laboratory volunteers using the above modified technique.

3. To evaluate the use of neutrophil antigens as a method to exclude falsely accused putative fathers in paternity testing.

4. To determine the incidence of neutrophil antibodies in three populations. The populations include: post-partum women, patients diagnosed as having systemic lupus erythematosus and multiple sclerosis patients who have received transfer factor, a dialysable leukocyte extract. These populations were selected for the possible greater incidence of the presence of neutrophil antibodies.

METHODS

Source of Specimens

Neutrophil Antigen Specimens

The neutrophil antigen studies require a minimum of 4 ml of anticoagulated blood. Blood samples were routinely collected on pre-partum women for a partial blood count (PBC) upon admission to the hospital immediately prior to giving birth. After the PBC was performed the remaining blood was used in the neutrophil antigen screening program. Random volunteers from the laboratory population were also solicited to provide additional blood samples for analysis. Blood samples collected on all patients for paternity testing were also analyzed for neutrophil antigens.

The blood specimens on all donors were obtained using a sterile venipuncture technique. The blood was collected in 7 ml sterile vacutainer tubes (Scientific Products, Minneapolis, MN) containing one of three anticoagulants: heparin, acid-citrate-dextrose (ACD) or potassium ethylenediametetraacetic acid (EDTA).

Neutrophil Antibody Specimens

The neutrophil antibody studies require a serum sample. The three populations studied were chosen for the possibility of a higher incidence of neutrophil antibodies than the random population. Post-partum women had a serum sample collected for blood-bank studies. The remaining

serum was made available for neutrophil antibody testing. Patients with SLE were tested for antinuclear antibody (ANA). Sera remaining after the ANA testing was complete were used to test for neutrophil antibodies. Patients diagnosed as having the disease multiple sclerosis had a blood sample collected after they had participated in a program where transfer factor (a dialysable leukocyte extract) was injected.

All serum samples were obtained by collecting 10 ml of blood from each donor using a sterile venipuncture technique. The blood was placed in a sterile red-top vacutainer tube and allowed to clot at room temperature for 20 minutes. These tubes were than centrifuged in a Sorvall refrigerated centrifuge (Sorvall Company, New York, N.Y.) at 3,000 rpm, 10C, for 15 minutes. The serum was removed and frozen in a 12 x 75 mm plastic tube at -20C until initiation of testing.

Serum Preparation

Sera to be used in the assay were allowed to thaw at room temperature then clarified by centrifugation in a Fisher Model 59 centrifuge (Fisher Scientific Company, Silver Spring, MD) at 4,000 G for three minutes.

Lyophilized sera, provided by the National Institute of Health (NIH, Bethesda, MD) were reconstituted with 1.0 ml of sterile distilled water and centrifuged as above.

Microagglutination Tray Preparation

Each serum or antiserum was assigned a number and listed accurately on the master sheet for the tray position it would occupy.

Using a repeating pipette (MLA Scientific Products, Minneapolis, MN) or automatic oiler, 15 ul of high viscosity mineral oil (Fisher

Scientific Company, Itasca, IL) was placed in the appropriate wells of a microtest tissue culture plate (Falcon Plastics, Oxnard, CA).

Using a Hamilton syringe (Hamilton Company Inc., Reno, NV) with repeating dispenser, 3 ul of each test serum or antiserum was placed under the oil in the center of each well.

These microagglutination trays were prepared in 100 tray lots and stored at -80C until needed.

Granulocyte Isolation

Blood was collected from donors in 7 ml vacutainer tubes (containing EDTA anticoagulant) with a minimum of stasis. Each anticoagulated blood sample was mixed and added to 2.0 ml of a 1% methyl cellulose solution in a 16 x 100 mm plastic culture tube. The tube was slanted at a 45° angle and held at room temperature for 20 minutes.

While the red blood cells were sedimenting in the methyl cellulose solution the Ficoll-Hypaque gradient tubes were prepared. See Appendix. One (1.0) ml of Ficoll-Hypaque solution specific gravity 1.119 (Solution II) was added to a 12 x 75 mm plastic tube. A 1.0 ml volume of a second Ficoll-Hypaque solution specific gravity 1.077 (Solution I) was carefully layered onto Solution II, being sure not to disturb the interface.

With a pasteur pipette the white cell rich supernate from the methyl cellulose tube was carefully layered onto the Ficoll-Hypaque gradient. The gradient tube was centrifuged at 1,200 rpm for 12 minutes at room temperature in a horizontal rotator. Figure 3 is a diagramatic representation of the Ficoll-Hypaque leukocyte-rich plasma gradient after centrifugation.

Figure 3. **Isolation of mononuclear leukocytes (layer 1), polymorphonuclear leukocytes (layer 2) and erythrocytes (layer 3) by centrifugation of leukocyte-rich plasma on a Ficoll-Hypaque double-density gradient at 1,200g for 12 minutes.**

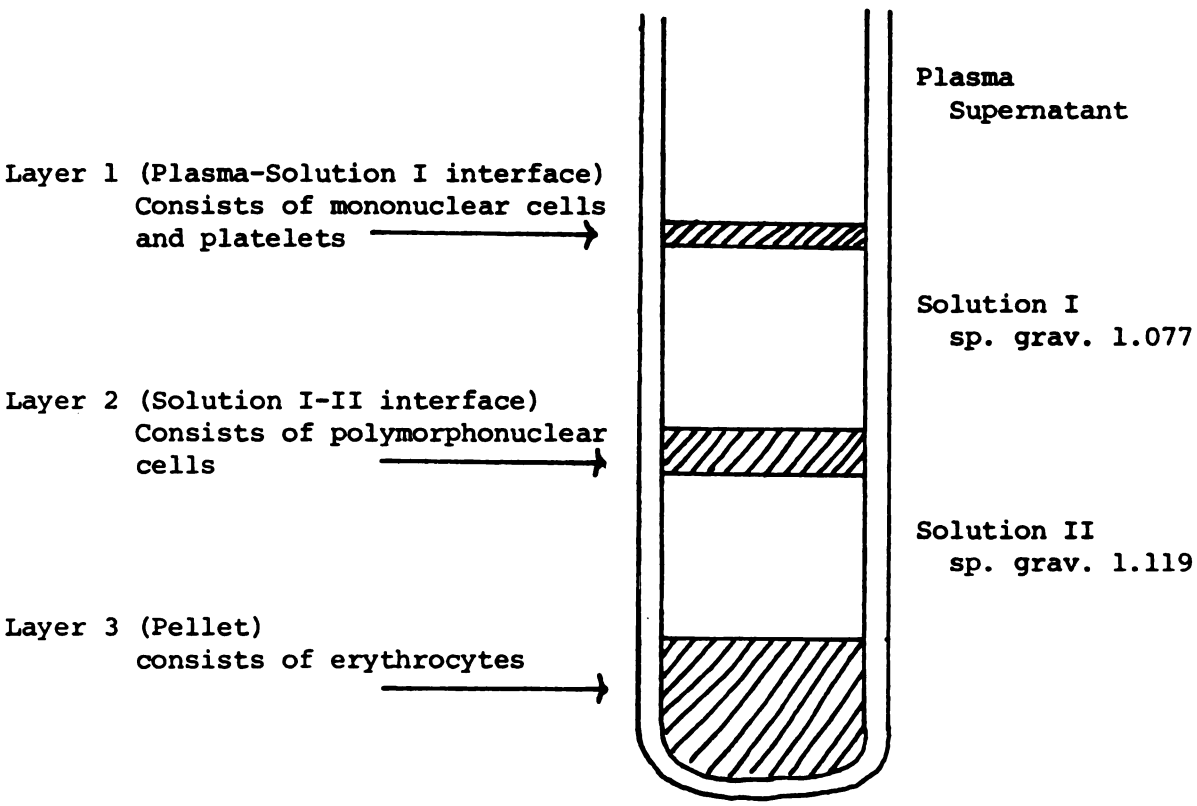

Plasma
Supernatant
Layer 1 (Plasma-Solution I interface)
Consists of mononuclear cells
and platelets
Solution I
sp. grav. 1.077
Layer 2 (Solution I-II interface)
Consists of polymorphonuclear
cells
Solution II
sp. grav. 1.119
Layer 3 (Pellet)
consists of erythrocytes

Figure 3

Using a pasteur pipette the plasma supernate with mononuclear cells and platelets was removed and discarded. Using a clean pasteur pipette, layer II consisting of greater than 95% polymorphonuclear cells, was removed and transferred to a 12 x 75 mm test tube.

These isolated granulocytes were washed with 1.0 ml of Hank's buffered saline solution without calcium and magnesium (Hank's Incomplete) then certrifuged at 1,000 rpm for eight minutes. The cell pellet was gently resuspended by flicking the tube. A second wash was performed with 1.0 ml of phosphate buffered saline (PBS) and the centrifugation at 1,000 rpm for eight minutes was repeated. See the Appendix for the formulation of the PBS solution. After the second centrifugation, the cell pellet was resuspended in 0.5 ml of granulocyte resuspension solution (GRS), a mixture of EDTA, bovine serum albumin, and phosphate buffered saline (see Appendix).

The PMN's were counted using a counting chamber or a Coulter ZBI particle analyzer (Coulter Corporation, Hialeah, FL) and adjusted to 5×10^6 cells/ml in the GRS medium. The cells were morphologically shown to be 100% blood neutrophils by placing a drop of cell suspension on a mocroscope slide. The dried cell suspension was stained with a Wright-Giemsa staining technique and a differential count performed.

K562 Cell Line

A continuous cell line, K562, was also used to screen patient sera for group specific granulocyte antigens. The K562 cells were grown in Roswell Park Memorial Institute 1640 (RPMI 1640) medium supplemented with 10% fetal calf serum (FCS). Cells for agglutination studies were harvested 48 hours after splitting the cultures. The cells were washed by centrifugation at 2,000 rpm to form a pellet and resuspended in RPMI 1640 medium to a concentration of 2.5×10^6 cells/ml.

Microagglutination Test Procedure

The microagglutination trays were removed from the freezer and allowed to thaw at room temperature for a minimum of 15 minutes. When the sera were completely thawed 2 ul of a granulocyte cell suspension or K562 cells were added using a Hamilton microtiter syringe. The microagglutination trays with the added granulocytes were then incubated in a 37C dry air incubator for 8 hours. After incubation individual reaction wells were evaluated for neutrophil agglutination using an inverted phase-contrast microscope. The agglutination reaction was graded based on the proportion of cells participating. No visible agglutination was scored as a negative reaction. Agglutination of 25% of the cells was scored as a 1+ reaction, 50% agglutination a 2+ reaction, 75% agglutination a 3+ reaction and 100% agglutination a 4+ reaction. Any agglutination of 25% or greater was considered a positive reaction. Figures 4 and 5 depict the microagglutination reaction as seen with the inverted phase-contrast microscope.

Figure 4. Positive microagglutination reaction of granulocytes and antiserum incubated at 37C for 8 hours. This is a 4+ positive reaction as viewed with an inverted phase-contrast microscope. Large clumps of PMN's are formed along the edge of the reaction well in the microagglutination tray. (x200)

Figure 5. Negative microagglutination reaction of granulocytes and antiserum incubated at 37C for 8 hours. The granulocytes display a negative reaction as evidenced by the smooth, uniform appearance of the cells in the reaction well. (x200)

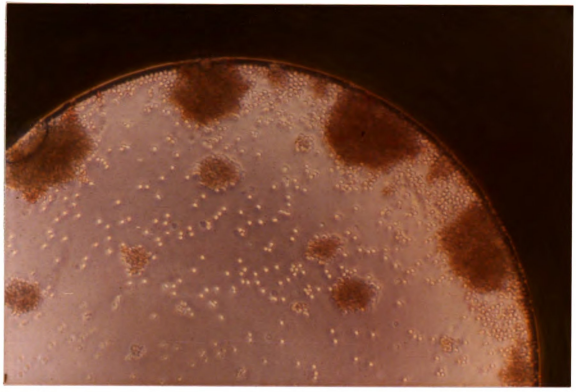


Figure 4

Figure 5

RESULTS

In an effort to produce a reliable testing mechanism for the detection of neutrophil antigens and antibodies several variables of the microagglutination technique as reported by Lalezari (1980) were evaluated.

Method Modification Studies

The first modification was to evaluate the need for the 20 minute methyl cellulose sedimentation step. Varying amounts of anticoagulated blood were layered directly onto varying volumes of gradient solution and centrifuged as shown in Table 4. Eight different patients were tested at each concentration for a total of 72 attempts to use anticoagulated blood layered directly onto the double gradient. These studies showed

Table 4. Combination of anticoagulated blood layered directly onto various volumes of double gradient solution.

Gradient Volume	Blood Volume	Final Gradient Volume
2.0 ml	1.0 ml	3.0 ml
2.0 ml	2.0 ml	4.0 ml
2.0 ml	3.0 ml	5.0 ml
3.0 ml	1.0 ml	4.0 ml
3.0 ml	2.0 ml	5.0 ml
3.0 ml	3.0 ml	6.0 ml
4.0 ml	1.0 ml	5.0 ml
4.0 ml	2.0 ml	6.0 ml
4.0 ml	3.0 ml	7.9 ml

that numerous erythrocytes were present in the granulocyte suspension which obscured the agglutination reaction.

The anticoagulant used in blood collection was evaluated next. Blood was collected in three different anticoagulants: sodium heparin, ACD and potassium EDTA. Blood from the same individual was collected in each anticoagulant. Four subjects were collected in this manner. The isolated granulocytes were tested as described in the Methods Section. All anticoagulants studied demonstrated equal ease of granulocyte separation and all showed comparable agglutination reactions. Blood anticoagulated with EDTA was used preferentially in testing only because of the greater availability of these samples. See Table 5 for a list of the agglutination reactions observed.

Table 5. Agglutination reactions of granulocytes isolated from blood anticoagulated with sodium heparin, ACD and EDTA.

		Antiserum NA_1	Antiserum NA_2	Antiserum NB_1	Antiserum NA_u
Subject 1	heparin	++	–	–	++
	ACD	++	–	–	++
	EDTA	++	–	–	++
Subject 2	heparin	++++	+	++	++
	ACD	++++	+	++	++
	ADTA	++++	+	++	++
Subject 3	heparin	+	++	–	+
	ACD	+	++	–	+
	ADTA	+	++	–	+
Subject 4	heparin	–	–	–	+++
	ACD	–	–	–	+++
	EDTA	–	–	–	+++

In an attempt to determine if granulocyte antigen identification could be performed on blood collected and stored for varying lengths of time at varying temperatures, blood was stored for 24 hours at room temperature and the isolated granulocyte suspensions were stored at room temperature for 24 and 48 hours and 5 days. Granulocytes were tested immediately after isolation and then stored at room temperature in GRS for 24 hours, 48 hours or 5 days and then retested. Viability studies using the vital dye methylene blue indicated the granulocytes were 90 percent viable even after 5 days; however all agglutination reactions that were noted on immediate testing were negative on re-testing. See Table 6.

Table 6. Agglutination reactions of granulocytes tested immediately after isolation (fresh) and after storage for 24 hours, 48 hours and 5 days at room temperature.

	Fresh	24 hours	48 hours	5 days
Subject 1	++++	–	–	–
Subject 2	+++	–	–	–
Subject 3	++++	–	–	–
Subject 4	++	–	–	–
Subject 5	+	–	–	–

Immediate testing of patient samples is not always possible. To determine if blood storage of granulocytes would eliminate the need for immediate testing, 100 blood samples were collected in EDTA anticoagulant and stored at room temperature for 24 hours. The granulocyte isolation

and testing were performed as described above. Uniformly negative reactions were observed. These studies emphasize that blood must be tested soon after collection to preserve the agglutination reaction of the granulocytes.

An attempt to enhance visualization of the agglutination reactions was investigated. Eosin dye was added to the microagglutination tray immediately prior to microscopic evaluation of the granulocyte agglutination. The eosin imparted an overall bright red appearance to all the cells but also to the background serum as well. Eosin dye was not helpful in visualization of the agglutination reaction.

Incubation time was also studied as a variable in the agglutination reaction. The granulocyte agglutination reactions of 15 subjects were graded after a 2 hour, 4 hour, 8 hour and 24 hour incubation at 37C. See Table 7.

The incubation of the microagglutination tray for 24 hours resulted in loose aggregates of cells and "false positive" reactions. The 2 hour and 4 hour incubations saw the reactions strengthen, while the most satisfactory and reproducible results were observed after an eight hour incubation at 37C.

Table 8 summarizes the utility of variables studied to achieve a reproducible assay.

With a satisfactory microagglutination procedure in place, the second phase was begun. In this series of experiments, the neutrophil antigen frequency in a random population was assessed.

Table 7. Agglutination reactions of granulocytes evaluated after a 2 hour, 4 hour, 8 hour and 24 hour incubation of the microagglutination tray at 37C.

Incubation Time in Hours		Antisera					Antigen Type
		NA_1	NA_2	NB_1	NA_u	Neg. Control	
Subject a	2 hr	—	—	—	—	—	NA_2
	4 hr	—	++	—	—	—	
	8 hr	—	+++	—	—	—	
	24 hr	±	+++	±	±	+	
Subject b	2 hr	—	—	—	+	—	NA_u
	4 hr	—	—	—	++	—	
	8 hr	—	—	—	++	—	
	24 hr	+	+	+	++	++	
Subject c	2 hr	—	++	—	—	—	NA_1/NA_2
	4 hr	+	+++	—	—	—	
	8 hr	++	++++	—	—	—	
	24 hr	++	++++	±	±	+	
Subject d	2 hr	+	—	—	—	—	NA_1/NA_u
	4 hr	++	—	—	+	—	
	8 hr	+++	—	—	++	—	
	24 hr	+++	±	±	++	++	
Subject e	2 hr	+	+	—	+	—	$NA_1/NA_2/NA_u$
	4 hr	+	++	—	++	—	
	8 hr	++	+++	—	+++	—	
	24 hr	++	+++	±	+++	—	
Subject f	2 hr	+	+	+	—	—	$NA_1/NA_2/NB_1$
	4 hr	+	++	++	—	—	
	8 hr	++	++	+++	—	—	
	24 hr	+++	+++	++++	+	+	
Subject g	2 hr	—	+	+	++	—	$NA_2/NB_1/NA_u$
	4 hr	—	++	++	++	—	
	8 hr	—	++	+++	++	—	
	24 hr	+	++	+++	++	+	
Subject h	2 hr	—	+	—	++	—	NA_2/NA_u
	4 hr	—	++	—	+++	—	
	8 hr	—	+++	—	++++	—	
	24 hr	±	+++	±	++++	—	
Subject i	2 hr	+	—	+	—	—	$NA_1/NA_2/NB_1/NA_u$
	4 hr	++	++	++	++	—	
	8 hr	++++	+++	++++	++	—	
	24 hr	++++	++++	++++	++++	±	

Table 7. (continued)

Incubation Time in Hours		Antisera					Antigen Type
		NA_1	NA_2	NB_1	NA_u	Neg. Control	
Subject j	2 hr	–	–	–	–	–	NA_2/NA_u
	4 hr	–	+	–	+	–	
	8 hr	–	+++	–	++	–	
	24 hr	±	++++	±	+++	+	
Subject k	2 hr	–	++	–	–	–	NA_2
	4 hr	–	++	–	–	–	
	8 hr	–	++++	–	–	–	
	24 hr	+	++++	+	+	+	
Subject l	2 hr	–	++	–	+	–	NA_2/NA_u
	4 hr	–	++	–	++	–	
	8 hr	–	–	+++	–	+++	
	24 hr	±	+++	±	+++	+	
Subject m	2 hr	–	–	–	–	–	No Antigens
	4 hr	–	–	–	–	–	
	8 hr	–	–	–	–	–	
	24 hr	±	–	±	–	+	
Subject n	2 hr	–	+	–	++	–	NA_2/NA_u
	4 hr	–	++	–	++	–	
	8 hr	–	++++	–	++++	–	
	24 hr	±	++++	±	++++	+	
Subject o	2 hr	–	++	–	–	–	NA_2
	4 hr	–	++	–	–	–	
	8 hr	–	+++	–	–	–	
	24 hr	–	++++	±	+	+	

Table 8. Summary of Method Modification Studies

Modification Attempted	Result
Blood layered directly onto double gradient	Not Acceptable
EDTA anticoagulant	Acceptable
ACD anticoagulant	Acceptable
Heparin anticoagulant	Acceptable
Granulocyte storage for 24 hours at room temperature	Not Acceptable
Granulocyte storage for 48 hours at room temperature	Not Acceptable
Granulocyte storage for 5 days at room temperature	Not Acceptable
Blood storage of granulocytes for 24 hours at room temperature	Not Acceptable
Eosin enhanced reactions	Not Acceptable
2 hour microagglutination tray incubation at 37C	Not Acceptable
4 hour microagglutination tray incubation at 37C	Not Acceptable
8 hour microagglutination tray incubation at 37C	Acceptable
24 hour microagglutination tray incubation at 37C	Not Acceptable

Incidence of Neutrophil Antigens

The population studied included 68 pre-partum women and 33 volunteer blood donors.

The frequency of four (4) neutrophil antigens was determined using NIH reagents.

Table 9 depicts the results of this antigen testing and demonstrates that some subjects had more than one neutrophil antigen type while others had no detectable antigens.

Table 9. Neutrophil Antigen Population Incidence

Antigen	Incidence in Lansing, Michigan Population	Incidence in Minnesota Population (Clay, 1980)
NA_1	49.5%	34.0%
NA_2	69.3%	66.0%
NA_u	60.3%	72.0%
NB_1	13.9%	83.0%
No detectable antigens	5.9%	not reported

The population incidence of the neutrophil antigens NA1 and NA2 in this study compares favorably with the neutrophil antigen incidence found in a Minnesota population studied by Clay (1980). However, a markedly decreased number of people in this study were positive for the NB1 antigen. This may reflect the differences in the typing sera used or the antigen NB_1 may react under different conditions than the NA series. Further investigation in this area is needed.

Many subjects had more than one antigen on their neutrophils and the incidence of the combination of neutrophil antigens in this population is presented in Table 10.

Table 10. Incidence of Neutrophil Antigen Combinations

Antigen Combinations	Lansing, Michigan, Population Incidence
NA_1 (only)	4.9%
NA_2 (only)	12.8%
NA_u (only)	12.8%
NA_1/NA_2	13.8%
NA_1/NA_u	3.9%
NA_2/NA_u	11.8%
NB_1/NA_u	0.9%
$NA_1/NA_2/NA_u$	19.8%
$NA_1/NA_2/NB_1$	1.9%
$NA_1/NA_u/NB_1$	1.9%
$NA_2/NA_u/NB_1$	5.9%
$NA_1/NA_2/NA_u/NB_1$	2.9%
No detectable antigens	5.9%

There is no similar listing of the neutrophil antigen combinations found in other studies to compare with these findings. Insufficient data exists on the neutrophil antigen types of patients to determine if those with a specific neutrophil antigen type have a greater risk of developing anti-neutrophil antibodies.

The subjects who were positive for all four neutrophil antigens also donated their granulocytes to serve as a broad spectrum screen

cell in the detection of neutrophil antibodies. It is interesting to note that antigen NB1 is found in 14% of this population but no donor was typed as solely NB1. This antigen was always found in combination with one of the NA antigens.

Paternity Exclusion of Neutrophil Antigens

Phase three of this study was to examine the use of neutrophil antigens as a method to exclude falsely accused putative fathers in paternity testing. Four cases of disputed parentage were studied. The putative father, mother and child were evaluated for neutrophil antigens. None of the putative fathers in this small series was excluded by HLA testing, and this was confirmed by the neutrophil antigen studies. Table 11 depicts the neutrophil antigens of the child, the mother and the putative father. In each case, the man was not excluded from possible paternity by either HLA or neutrophil antigen testing.

Table 11. Neutrophil Exclusions in Paternity Testing

CASE	PUTATIVE FATHER	MOTHER	CHILD	NEUTROPHIL EXCLUSIONS	HLA EXCLUSIONS
P 007-83	NA_2/NA_u	NA_1/NA_u	NA_2/NA_u	Not Excluded	Not Excluded
P 019-83	NA_2/NA_u	NA_2/NA_u	NA_2	Not Excluded	Not Excluded
P 061-83	NA_1/NA_u	None Detectable	NA_u	Not Excluded	Not Excluded
P 144-83	NA_2/NA_u	NA_1	NA_2	Not Excluded	Not Excluded

Neutrophil Antibody Incidence in Selected Populations

The final phase of this study was a survey of the incidence of neutrophil antibodies present in three selected populations. The three

populations selected to be studied were 1) post-partum women, 2) patients diagnosed as having systemic lupus erythematosus (SLE) and a high titer positive antinuclear antibody (ANA) test, 3) patients diagnosed as having multiple sclerosis and who have also received the leukocyte product Transfer Factor (TF).

Donors previously typed for neutrophil antigens supplied a source of granulocytes (now of a known neutrophil antigen type) which were isolated and used as target cells. The cell line K562 was also used as a source of target cells. Microagglutination trays were prepared with sera from the three populations and the target cells were added. If neutrophil antibodies were present in the sera of these three selected populations, aggregation should occur when target cells demonstrating the antigen against which that antibody was directed were added to the sera.

One SLE patient of the 28 patients tested reacted with the K562 cell line to demonstrate the neutrophil antibody reaction.

The sera from 360 post-partum women tested were all negative as were the sera from the 21 MS patients tested. Table 12 summarizes the findings of this study.

Table 12. Neutrophil Antibody Incidence in Three Populations

Population Type	Number of Patients Tested	Neutrophil Antibody Incidence
Post-partum women	360	None Detected
Multiple sclerosis patients	21	None Detected
Systemic lupus erythematosus patients	28	3.6%

Previous reports in the literature demonstrate as high as 52.6% of SLE patients with neutrophil antibodies (Drew, 1978) and 23.6% of multiparous females have been shown to demonstrate a neutrophil antibody (Thompson, 1977). The studies presented here have obtained a much lower incidence of neutrophil antibodies. There is speculation that some neutrophil antibodies may be complement dependent (Gazit, 1983) and/or cold reacting (McCullough, 1981). The detection and screening system used in this study does not detect either of these types of antibodies. A variety of detection techniques may be necessary in future testing to detect all varieties of neutrophil antibodies.

DISCUSSION OF RESULTS

The experiments reported above suggest that the analysis of patients for their neutrophil antigen genotype may be an important clinical consideration. The prevalence of neutrophil antigens and antigen combinations found in this study (see Table 9 and 10) compare favorably with the antigen incidence reported by Clay (1983). The literature does not contain previous reports of the frequency of neutrophil antigen combinations. However the presence of patients with auto-antibodies against multiple neutrophil antigens and the normal somatic Mendelian inheritance pattern of these antigens argues for the frequent presence of multiple neutrophil antigens.

There are several methodologies available to determine neutrophil antigens but the microagglutination method of Lalezari (1974) with the reported modifications gives reproducible results using a minimum amount of blood. One problem inherent in any procedure which necessitates leukocyte separation is that of obtaining sufficient quantities of blood to perform the testing from pediatric patients and those with very low leukocyte counts.

In the method modifications studied in this project the most critical step appears to be allowing the methyl cellulose-whole blood mixture to settle for exactly twenty minutes. Less settling time results in an increased number of RBC's in the white cell rich layer, obscuring the agglutination reaction in the later phases.

Sedimentation time of greater than twenty minutes results in the granulocytes settling out along with the red blood cells to give a very low total yield and often leaves an insufficient number of neutrophils to perform the assay.

A second critical step is the use of a freshly collected sample. The neutrophils begin to lose the ability to aggregate if more than four hours is allowed to elapse between sample collection and cell separation. The agglutination phase of neutrophil testing requires viable cells which are fully capable of locomotion, and this response appears to be related to chemotaxis (Lalezari, 1977). This lack of agglutination can lead to false negative results if the test is not performed on a fresh sample.

The third critical step is the centrifugation speed and centrifugation time used in processing the double gradient phase. A centrifuge speed of more than 1,200 rpm and/or a centrifugation time of longer than twelve minutes results in the neutrophils being forced through the double gradient and onto the red cell layer. This causes an insufficient yield to perform the testing. A centrifuge speed below 1,200 rpm or a centrifugation time of less than twelve minutes does not give a clear separation of lymphocytes and neutrophils resulting in a neutrophil layer heavily contaminated with lymphocytes and therefore unsuitable for testing.

The use of neutrophil antigens as a tool to exclude a falsely accused putative father has good potential and needs further investigation. The normal somatic Mendelian inheritance pattern of neutrophil antigens, as well as the rich genetic polymorphism they comprise, would make neutrophil phenotyping a useful procedure in exclusionary screening studies. A current drawback for using neutrophil typing in paternity testing is the

lack of commercially available antisera. Only four of the thirteen known neutrophil antisera are available from the NIH. A limited amount of serum is given to qualified investigators, but the amount provided is not sufficient for a large scale screening program. While the availability of reagent currently limits the application of neutrophil antigen testing, neutrophil phenotyping and genotyping should be more prevalent when commercial sources of sera are available.

Patient testing for neutrophil specific antibodies seems to have the most clinical usefulness currently. A determination of the presence of neutrophil antibodies may resolve a symptomatic dilemma or alter the course of drug treatment in some instances. Drew and Terasaki (1978) had previously reported a total of 52.6% of systemic lupus erythematosus patients had granulocyte autoantibodies compared with approximately 10% in normal males or females. Gazit and Gil (1983) found approximately 10% of the pregnancy sera they tested reacted positively with the K562 cell line indicating the presence of granulocyte alloantibodies.

In this study, two different target cells were used to detect the presence of neutrophil antibodies in sera from patients with SLE. Donor neutrophils, typed with antisera from the National Institute of Health (Bethesda, MD), were used, as were K562 cells. In 1975, Lozzio and Lozzio reported the establishment of the K562 line derived from a pleural fluid of a patient with chronic myeloid leukemia in blast crisis. It was later determined this cell line did not exhibit either HLA or beta$_2$ microglobulin determinants (Dres et al., 1977; Ziegler et al., 1981). Very recently, it has been shown by radioactive binding assay that these cells do exhibit HLA antigens on their surface, though in a density too low to be detected by antibodies and complement (Ziegler et al., 1981).

It has also been shown that K562 cells exhibit the Philadelphia chromosome. This cell line appears to be an erythroleukemia cell, with characteristics analagous to those of the murine Friend erythroleukemia cells (Gazit, 1983). The K562 cell membrane glycoprotein determinants are similar to those of normal human erythrocytes. In particular, these cells synthesize glycophorin A, have spectrin and express the "i" surface antigen (Fitchen et al., 1981; Koeffler and Golde, 1981). When appropriately induced, these cells synthesize hemoglobin. However, the cell line retains its myelogenous surface markers since it possesses group specific granulocyte antigens (Drew et al., 1977a). It was found that some K562 reactive antibodies can be characterized as cold-reacting, and antigens specific for these antibodies are present on mature granulocytes. The K562 cell line was suggested as an efficient target in screening for the presence of granulocyte alloantibodies (Gazit and Gil, 1983).

Use of K562 cells in screening for granulocyte antibodies is very convenient since they can be easily maintained in culture and can thus provide an unlimited number of target cells. Also, their lack of reactivity with HLA-ABC and DR antibodies makes them an ideal target for identification of granulocyte specific alloantibodies. The shortcoming is, however, that this cell line exhibits probably only a limited number of granulocyte specific alleles, two of the thirteen known determinants (Lalezari and Radel, 1974; Drew et al., 1977a).

In this series of experiments only 3% of the SLE patients tested demonstrated a granulocyte alloantibody, while none of the 360 serum samples from post-partum women or the 21 serum samples from the multiple sclerosis patients exhibited any reaction with either of the target granulocyte methods used.

One theory for the lack of reactivity with the cell line is that these antibodies may be complement dependent. The previous studies by others used a granulocytotoxicity technique, which evaluated cell lysis as the endpoint. The greatest number of reactions were also found at 4C while all of the above reaction were carried out at room temperature (23C).

Although the K562 cell line is convenient, its lack of numerous granulocyte antigens necessarily limits its effectiveness as a target cell. The use of a granulocyte donor panel would appear to be the method of choice to offer the widest array of granulocyte antigens as target cells. It also appears that the current methodology may require the use of more than one detection technique in an attempt to detect all the neutrophil specific antibodies present.

CONCLUSION

Neutrophil antigens and antibodies are becoming a clinical concern. With a reproducible and technically feasible assay available to test for neutrophil antigens, this trend will continue. A common incidence of neutrophil antigens in the population along with an increasing realization of the importance of neutrophil antibodies as the causative agent in disease processes, highlights the urgency of future work in this area.

This study has produced a reliable testing mechanism for the detection of neutrophil antigens. The method gives consistent results if neutrophils are tested soon after collection.

The neutrophil antigen incidence found in the study population is similar to the incidence reported by others with the exception of a decreased incidence of the NB1 antigen.

The difficulty in obtaining a fresh blood sample in sufficient volume from all three parties involved in the cases of disputed paternity makes it impossible to draw definite conclusions from the small number of cases tested. It appears that neutrophil antigens may be helpful in cases of disputed paternity but further studies are needed.

The precentage of patients with neutrophil antibodies was much lower in this study than previous reports in the literature. The method used here to detect neutrophil antibodies appears limited to warm-reacting, non-complement dependent antibodies and may not detect all of

the possible neutrophil antibodies some of which are postulated to be cold-reacting and complement dependent. Several detection systems appear to be necessary to investigate neutrophil antibodies.

Neutrophil antibodies have been shown to play an important role in bone marrow transplantation, leukocyte transfusions, blood transfusion reactions, and autoimmune neutropenia.

Identification of neutrophil antigens may be a new step forward in the ability to exclude a falsely accused putative father and provide additional factors to consider in bone marrow transplantation.

The realization of this importance has spurred interest in neutrophil serology and its potential role as another histocompatibility parameter that can be useful in donor selection and graft survival.

The testing to detect neutrophil antigens and antibodies is an exciting and emerging field. As the number of commercial typing sera to detect neutrophil antigens increases and screening procedures for neutrophil antibodies improve; typing for neutrophil antigens and neutrophil antibody detection may become as commonplace as erythrocyte and lymphocyte typing and antibody detection.

APPENDIX

APPENDIX

Reagents

33.9% Hypaque (sodium diatrizoate) - Winthrop Laboratories, NY

50 ml 75% Hypaque
60.5 ml sterile water

50% Hypaque

250 gm Hypaque
500 ml sterile water

9% Ficoll (400,000 M.W.) - Pharmacia, Piscataway, NJ

9 gms Ficoll
100 ml sterile water
dissolve at 4C for 24 hours

Solution I (upper gradient fluid)

10 parts 33.9% Hypaque
24 parts 9% Ficoll
specific gravity must be 1.077
filter using a 0.20 u filter (Millipore Corp., Bedford, MA)
store at 4C

Solution II (lower gradient fluid)

10 parts 50% Hypaque
24.5 parts 9% Ficoll
specific gravity must be 1.119
filter using a 0.20 u filter
store at 4C

Adjusting specific gravity - if solution is too heavy, add 9% Ficoll. If the solution is too light, add the appropriate Hypaque reagent.

1% Methyl Cellulose

1.0 gm methyl cellulose
100 ml 0.9% sterile saline
dissolve at 4C for 24 hours and store at 4C

Phosphate Buffered Saline (PBS)

Na Cl	8.0 grams
K Cl	0.2 grams
Na_2 HPO4	1.15 grams
K H_2 PO4	0.2 grams

Place in a volumetric flask and add sterile water until a final volume of 1,000 cc is reached. Adjust the pH of PBS to 7.2. Filter with a .20 u filter and store at 4C.

Bovine Serum Albumin-EDTA Solution (BSA-EDTA)

22% BSA	3.00 ml
10% EDTA	0.88 ml
PBS	18.12 ml

10% EDTA = 10 gms Na_2 EDTA dissolved in 100 ml sterile water. May need to warm EDTA in 37C water bath to get EDTA into solution. Store at 4C. Adjust pH of BSA-EDTA to 7.2 with 1.0N NaOH. Store at 4C.

Granulocyte Resuspension Solution (GRS)

PBS	10.00 ml
22% BSA	0.25 ml
10% EDTA	0.50 ml

Adjust pH of GRS to 7.2 with 1.0N NaOH. Store at 4C.

The following may be obtained from GIBCO - Grand Island Biological Corporation, Grand Island, NY.

RPMI 1640 with 25 mM Hepes Buffer (with glutamine - without antibiotics)

Hanks' Balanced Salt Solution - HBSS

Hanks' Incomplete - HBSS without calcium, magnesium, sodium bicarbonate

Fetal Calf Serum (FCS) - Reheis #50-204

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VITA

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