A RADIOIMMUNE MICROFILTER PLATE ASSAY FOR THE DETECTION OF ANTI-GRANULOCYTE ANTIBODIES

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ABSTRACT

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An assay for IgG anti-granulocyte antibodies has been developed which utilizes small plasma volumes and low numbers of granulocytes in a microfilter plate. Granulocytes are isolated by a single density ficoll-hypaque gradient procedure with RBC removal by lysis and washing. Anti-granulocyte IgG coating the cells are detected by the addition of ^{125}I labeled anti-human IgG. This technique is capable of detecting antibodies which are reactive in granulocyte agglutination and immuno-fluorescence tests as demonstrated with known anti-NA-1, anti-NB-1, and anti-Mart sera. Positive results were obtained with plasma from two patients with autoimmune granulocytopenia, and three multiply transfused patient with febrile transfusion reactions believed to be secondary to allogeneic granulocytic antibodies. Plasmas with anti-A or anti-B titers of greater than 1:256 are positive against corresponding granulocytes. Preliminary absorption and elution studies show that anti-A eluted from RBCs is

reactive against A granulocytes, suggesting the presence of A antigens on granulocytes.

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LIST OF ABBREVIATIONS

WBC	White Blood Cell
AGA	Anti-Granulocyte Antibody
Ab	Antibody
Ag	Antigen
INN	Isoimmune Neonatal Neutropenia
AIN	Autoimmune Neutropenia
CBNI	Chronic Benign Neutropenia of Infants
CAIN	Chronic Autoimmune Neutropenia
AINN	Autoimmune Neonatal Neutropenia
INP	Idiopathic Neutropenia
CIN	Chronic Idiopathic Neutropenia
SN	Secondary Neutropenia
SLE	Systemic Lupus Erythematosis
RA	Rheumatoid Arthritis
FS	Felty's Syndrome
AIDS	Acquired Immune Deficiency Syndrome
IDIN	Idiosyncratic Drug-Induced Neutropenia
WB	Whole Blood
ACD	Acid Citrate Dextrose
CPD	Citrate Phosphate Dextrose
EDTA	Ethylenediaminetetraacetic Acid
PBS	Phosphate Buffered Saline

EPS	PBS with EDTA
PFA	Paraformaldehyde
GAT	Granulocyte Agglutination Test
AHG	Anti-Human Globulin
GIFT	Granulocyte Immune-Fluorescence Test
GCT	Granulocyte Cytotoxicity Test
ACT	AHG Consumption Test
SPA	Staphylococcal Protein-A
HLA	Histocompatibility Complex
BSA	Bovine Serum Albumin
2nd Ab	Secondary Antibody
RI	Refractive Index
MFPGA	Microfilter Plate Anti-Granulocyte Antibody Assay

INTRODUCTION

Antibodies which react with granulocytes can occur in a variety of disorders and may result in neutropenia with a subsequently increased risk of infection. Numerous methods are available to detect these anti-granulocyte antibodies but no single assay is capable of detecting every specificity of antibody. The primary purpose of this research was to develop a modification of an established microfilter plate IgG anti-platelet antibody assay for use with granulocytes. To this end, studies of optimum granulocyte isolation and storage protocols, plasma and granulocyte concentrations, incubation times, and wash solution composition were carried out. Granulocyte typings with sera known to contain antibodies for selected granulocyte specific antigens were performed for comparison to results obtained by established anti-granulocyte antibody assays. Preliminary investigations into the ABH reactivity of granulocytes were also carried out.

LITERATURE REVIEW

Normal Granulocyte Kinetics

Granulocytes account for approximately 35% of the white blood cells (WBCs) in the peripheral blood of normal human infants. The percentage of WBCs which are granulocytes rises during the first 12 years of life until the normal adult value of 60% is reached.(2) The distribution of mature granulocytes in the adult is normally 95% neutrophils, 4% eosinophils, and 1% basophils with slightly higher percentages of the latter two types in infants.(2)

All three types of mature granulocytes seen in the peripheral blood arise from precursor cells in the bone marrow.(112) Briefly, the sequence of maturation for granulocytes is as follows: stem cell, myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, and segmented cell.(112) As cells proceed through these maturation stages they become progressively smaller, the cytoplasmic to nuclear volume ratio increases, the nuclear chromatin condenses, and the cytoplasm becomes less basophilic.(11)

Two general classes of granules are seen in mature granulocytes, azurophilic (or primary) and specific (or secondary) granules. Azurophilic granules are first

detected in the promyelocyte and account for only 10-20% of the granules in the mature cell.(112) Specific granules are first observed in the myelocyte which is also the last cell of the series which is capable of cell division.(56) It is the composition of the specific granules which determines the classification of the mature granulocyte as a neutrophil, eosinophil, or basophil.(112)

Studies of neutrophils in the bone marrow and peripheral blood have established five kinetic pools which are summarized in Figure 1. The time frame for maturation from myelocyte to segmented neutrophil has been estimated at 6-14 days.(86) The half-life of neutrophils in the peripheral blood is approximately 6-7 hours with a subsequent life span of 4-5 days in the tissues after migration.(80,86) Once in the tissues, neutrophils may be shed from mucosal surfaces, be sequestered by the reticuloendothelial system, or die in the tissue.(112)

The release of the mature cells from the bone marrow to the peripheral blood occurs in an orderly fashion with the most mature cells in the storage pool being released first.(112) Neutrophils in the peripheral blood are equally divided between the circulating and marginal pools, the latter of which represents those sequestered in the capillaries.(8) Migration of neutrophils into the tissues appears to be unidirectional and occurs in a random manner without selection by age or maturity of the cells.(56,112) Extravascular neutrophils have a propensity to accumulate in

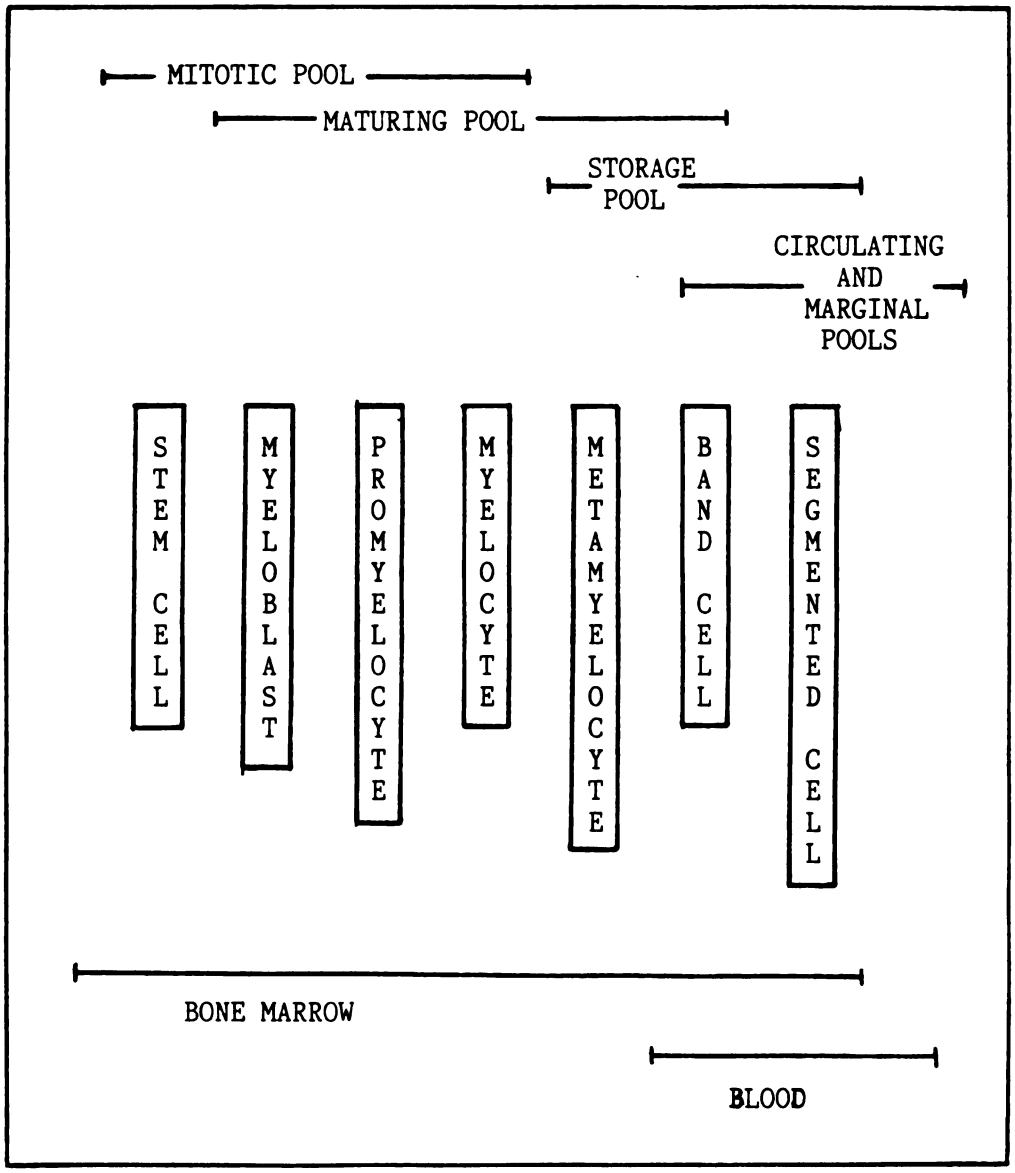

FIGURE 1: Neutrophil kinetic pools of the bone marrow and blood.

the liver, lungs, and spleen.(56) Under normal conditions 60% of the total number of neutrophils are in the bone marrow, 0.7% in the peripheral blood, and 40% in the tissues.(81)

The normal rate of neutrophil production in the bone marrow is estimated at between 0.85 and 1.6 x 10^9 cells/kg/day.(2) Numerous studies indicate the presence of various hormonal substances which are capable of stimulating granulopoiesis, aptly termed granulopoietins.(112) There is also evidence for the existence of so called "release factors", including certain glucocorticoids, which promote the movement of granulocytes from the storage pool to the circulating and/or marginal pools.(112)

The main function of neutrophils is to prevent pyrogenic infections.(112) Attracted to sites of infection by a number of chemotactic factors, neutrophils proceed to phagocytize and digest the offending microbe.(26) The ability of a neutrophil to locomote in response to chemotactic factors and to become adhesive at the site of infection is dependent on the presence of magnesium.(62) The presence of surface receptors on neutrophils for the Fc region of IgG antibodies and complement fragments C3b and C5a aide in the ingestion of opsonized microbes.(16,77)

Ingestion of a microbe is followed by degranulation of the neutrophil; the process by which phagosomes fuse with granules within the cell.(112) The granules of neutrophils

contain lysosomal enzymes which utilize both oxygen-dependent and independent mechanisms to kill and digest microbes.(26,35) It is the leakage of these same enzymes from active neutrophils that promote many of the symptoms of host tissue inflammation at the site of an infection.(112) The bacteriocidal activity of neutrophils varies as a function of maturation with metamyelocytes having only 28% of the killing activity observed for mature segmented nuclei neutrophils.(35) The proposed maturation sequence for the functional differentiation of the neutrophil is as follows: Fc receptors, immune phagocytosis, complement receptors, oxygen-independent microbial killing, oxygen-dependent microbial killing, and finally chemotaxis.(35)

Neutrophil function can be assayed by a variety of techniques such as observation of ameboid projections to asses motility, quantitation of latex particle phagocytosis, and the measurement of oxygen consumption or glucose oxidation during phagocytosis.(9,20)

Granulocytopenia

Neutropenia, which is often used interchangeably with the term granulocytopenia, is a condition associated with below normal numbers of neutrophils (granulocytes). Agranulocytosis implies the existence of a severe granulocytopenia with severely decreased or complete absence of granulocyte precursors in the marrow.(112) The major clinical symptoms noted in cases of neutropenia are

pyrogenic infections, the most common types being cutaneous cellulitis, abscesses, furunculosis, pneumonia, and septicemia.(23) There is a rough correlation between the degree of neutropenia and the risk of bacterial infection.(46,112) Neutropenic patients may have milder symptoms of local infection, such as heat and swelling, than non-neutropenic patients due to the smaller number of neutrophils involved in the inflammatory process.(90)

C. Finch has proposed the following classification of granulopenias based on 5 general kinetic types.(112)

Type I: Reduced Granulocytopoiesis (decreased total and effective granulocytopoiesis)

Type II: Increased Ineffective Granulocytopoiesis (increased intramedulary destruction)

Type III: Reduced Granulocyte Survival (increased destruction or utilization)

Type IV: Combination (Types I and II or II and III)

Type V: Pseudogranulocytopenia (decreased release from the bone marrow or increased shift from circulating to marginal pool)

Although kinetic classification of a particular case of granulocytopenia may be useful for diagnosis and/or treatment, it provides little information concerning the etiology of the disease. On the basis of etiology,

granulocytopenias can be divided into three broad groups: hereditary or constitutional, non-drug-induced acquired, and drug-induced acquired.(112) The non-drug-induced acquired granulocytopenias include those which are directly immune-related and those secondary to other immunological disorders

Immune-Related Neutropenias

Immunological mechanisms are implicated in a variety of acquired neutropenias. P. Lalezari has outlined four general categories of neutropenias which may be mediated by anti-granulocyte-antibodies (AGAs): fetal-maternal incompatibility, autoimmune neutropenia, neutropenia associated with other immunological disorders, and drug-induced neutropenia.(49) Unfortunately, some antibody-mediated neutropenias show little if any difference in clinical characteristics from those where no antibody (Ab) is detected.(23) This may be at least partially due to technical difficulties in the detection of AGAs and has resulted in a lack of standard nomenclature for the different types of neutropenia.

Antibody-mediated neutropenias that have been characterized are not restricted to a single kinetic classification.(112) Abs which coat neutrophils may directly result in opsonization or may cause deposition of complement, which in turn may opsonize or directly damage the cell.(38) In either of these cases the end result would be a reduced neutrophil life span. The primary target of an

AGA, mature cells in the peripheral blood or precursors in the bone marrow, is dependent on both the titer of the AGA and the specific antigen (Ag) which it recognizes; these factors will influence the severity of the neutropenia.(38,51) It is hypothesized that the neutropenia will become increasingly severe as the AGA is directed towards earlier precursor cells. On the other hand, an AGA specific for mature granulocytes may be of sufficient quantity and activity to overwhelm any compensatory response by the bone marrow.(38) In vitro AGAs are capable of altering neutrophil function. One study demonstrates that at low concentrations IgG AGAs can stimulate ingestion of particles by neutrophils whereas high concentrations inhibit this activity.(9)

Treatment for patients with immune-related neutropenia varies depending on the clinical manifestations of the disease. The use of steroid therapy, specifically prednisone, is successful in many patients, particularly those with chronic infections which do not respond to antibiotics.(87) The activity of steroids may be to decrease Ab production, increase bone marrow granulocyte production, promote early release of neutrophils such that more Ab is consumed, and/or decrease the rate of reticulo-endothelial system destruction of granulocytes.(51)

Fetal-Maternal Incompatibilities

In 1960 Lalezari first documented a case of neutropenia in an infant due to detectable fetal-maternal granulocyte incompatibility.(53) Extensive studies of 24 different families in the ensuing years have brought to light a number of general features of this disease.(49,103,107) Analogous in some ways to erythroblastosis fetalis (hemolytic disease of the newborn), it is named Isoimmune Neonatal Neutropenia (INN).(53)

The hematological characterization of INN includes moderate to severe neutropenia, variable cellularity of the bone marrow, and frequently compensatory monocytosis leading to normal total WBC counts.(48,53,54,102) The latter finding is probably of positive prognostic value.(49) The most common clinical findings are skin infections and pneumonia, with sepsis occurring in the most severe cases.(53,54,107) The typical duration of INN is from 2 to 17 weeks.(54) Occurrence of INN in the first born is not unusual and was reported in 40% of the families studied.(107) In 22 out of 24 cases of INN investigated, the Ab detected was IgG and reactive in vitro against granulocytes from the father and afflicted children but not the mother, thus confirming the isoimmune nature of the disease.(49,102,105).

Autoimmune Neutropenias

The basic criterion for the diagnosis of autoimmune neutropenia (AIN) are a chronic, non-cyclic, non-hereditary neutropenia with a well proliferating bone marrow, a lack of hepato-splenomegaly, and an auto-reactive AGA demonstrable by direct or indirect methods.(87) The latter point eliminates a number of "idiopathic" neutropenia cases but this may only represent inadequate testing for AGAs. This definition also serves to exclude neutropenias which are secondary to other disease states, drug-induced, or of a hereditary nature such as Kostman Syndrome.(87,101)

It is within the category of autoimmune neutropenia where the nomenclature of disease states suffers the most from the lack of standardization. Disorders which belong in this classification include Chronic Benign Neutropenia of Infants (CBNI), Chronic Autoimmune Neutropenia (CAIN), Primary and Secondary Autoimmune Neonatal Neutropenia (1st and 2nd AINN), Chronic Idiopathic Immunoneutropenia (CIIN), Autoimunne Neutropenia (AIN), and some cases of Idiopathic Neutropenia (INP) and Chronic Idiopathic Neutropenia (CIN).(13,23,49,72,101,105,106) These are essentially the same disorder and will be referred to as Autoimmune Neutropenia (AIN) for the sake of clarity.

IgA, IgG, and IgM AGAs have been reported in AIN.(72,105,106) In general, there is an inverse relationship between the strength of the AGA and the absolute neutrophil count as well as between the absolute

neutrophil count and the incidence of infection.(13,72,87) Although one of the criterion for AIN is a well proliferating bone marrow, it is not uncommon to observe a lack of mature granulocytes in the marrow.(13,51,72,99) Many patients with AIN have a compensatory monocytosis and an increased rate of monocyte migration to sites of infection.(23,87)

Autoimmune Neutropenia is a highly variable disorder with spontaneous resolution in less than a year or life-long affliction (31 years in one case).(46,84) The severity of AIN can range from benign to fatal.(49,87) Treatment of these patients, other than the use of antibiotics during infections, remains somewhat controversial, particularly the value of splenectomy.(41,59)

Autoimmune Neutropenia has been reported in patients as young as six months.(72) Although development of autoimmunity in children this young is rare, 6 cases have been reported in children under the age of one year in which no AGA against the child's granulocytes be detected in the maternal serum.(51,72,87) These cases represent true primary AIN in infants. The severity and duration of AIN in those under two years of age is generally reported as being less than that seen in adults.(72,84,87) It is postulated that the initial trigger of Ab production in these children are antigens present on maternal granulocytes.(51) This would imply that fetal granulocyte antigens are not identical to those found in adults.

One exception to this pattern demonstrated an Ab which was also reactive against mother's granulocytes, although the clinical disease in the the infant was similar to cases of INN. Further testing revealed the presence of an IgG Ab coating the neutrophils of the cord blood, confirming passage of the Ab from the mother to the child. The neutropenia of the infant in this situation is designated as Secondary Autoimmune Neutropenia (2nd AIN) to reflect the autoimmune nature of the maternal Ab and its passive transfer to the infant as opposed to Ab production by the infant as is seen in primary AIN.(49)

Neutropenias Associated With Other Immunological Disorders

Neutropenia is associated in various frequencies with numerous immunological disorders, particularly those involving autoimmunity. While the mechanisms behind many of these secondary neutropenias (SN) remains elusive, some do show evidence of Ab-mediation.(49,101) Unlike the general patterns of INN and AIN, the quantity and activity of an AGA in SN does not appear to necessarily correlate with the degree of neutropenia seen.(14,17,93) The most extensively studied SNs that may be Ab-mediated are those associated with autoimmune disorders, specifically Systemic Lupus Erythematosis (SLE), Rheumatoid Arthritis (RA), and Felty's Syndrome (FS).(49,101,112) Anti-granulocyte antibodies and neutropenia have also been noted in selected cases of infectious mononucleosis, acquired immune deficiency

syndrome (AIDS), AIDS-related complex, and Evan's Syndrome.(49,82,100)

Systemic Lupus Erythematosis

Approximately one half of the diagnosed cases of SLE have SN but only 5% are severe enough to increase the risk of infection.(24,59,93) Auto-AGAs can be demonstrated in at least 53% of SLE patients, but these patients are not necessarily the ones which exhibit SN.(31) It is suggested that this percentage may be significantly higher if the presence of Abs to granulocyte precursor cells are also sought.(59) The pathogenesis of this SN is still unclear and may be influenced by factors other than AGAs such as decreased production of colony stimulating activity due to a serum inhibitory factor.(17,101) Evidence for the pathological significance of AGAs in SN with SLE is suggested by the increase in granulocytes seen during steroid therapy.(59)

Rheumatoid Arthritis and Felty's Syndrome

Felty's Syndrome is a complication occurring in approximately 1% of RA patients and is characterized by splenomegaly and neutropenia.(17,112) At least 60% of FS patients have detectable AGAs but even in these cases immune destruction of granulocytes may only be one contributing factor to the neutropenia.(17,94) The role of splenomegaly in the pathogenesis of this disorder is demonstrated in some

cases by the resolution of the neutropenia after splenectomy.(59,60) There is an increased phagocytosis of neutrophils by splenic histiocytes in some patients but whether or not this phenomena is Ab-mediated has yet to be established.(6,94) The results of bone marrow examinations are highly variable indicating that the destruction of granulocyte precursors or suppression of granulocytopoiesis may be involved in the generation of the neutropenia.(6,17,60,94) Decreased granulocytopoiesis may be caused by Abs and/or serum inhibitory factors.(36,94,101) In many cases of FS the possibility of drug-induced neutropenia can not be ruled out.(59,60)

Drug-Induced Neutropenias

Examples of drug-induced neutropenia or agranulocytosis are well represented in the literature. There are two general classes of drug-induced neutropenia: predictable, for example with the use of chemotherapy agents which interfere with cell proliferation, and idiosyncratic.(112,113) Idiosyncratic drug-induced neutropenias (IDINs) are of particular significance because of their seemingly random occurrence and frequently high mortality rate.(113) A ten year study of adverse drug reactions in Sweden found IDIN to be the most common example of a fatal drug reaction.(113) It is estimated that almost 90% of unpredicted episodes of agranulocytosis can be attributed to the use of therapeutic drugs.(56) Over 100

different drugs are implicated in IDIN thus far including anti-microbials, phenothiazine derivatives, synthetic penicillins, and anti-thyroid drugs.(1,5,101,109,112,113)

Idiosyncratic drug-incuded neutropenias detected at an early stage may show a compensatory hypercellularity of the bone marrow.(112) The bone marrow tends to become progressively more hypocellular as drug administration continues with fewer and fewer mature granulocytes and may result in agranulocytosis.(113) In general, this agranulocytosis is an acute disorder which ends in recovery or death from infection within approximately two weeks of discontinuation of the offending drug.(113) Disease states with disturbances of immune function (such as SLE), increasing age, and certain genetic factors may all play a role in the predisposition of a patient to develop IDIN.(56,113)

Idiosyncratic drug-induced neutropenias can be divided into the following three operational classifications: differences in drug pharmacokinetics resulting in toxic levels of the drug or its metabolites in the bone marrow, abnormal sensitivity of precursor cells to normal drug concentrations, and adverse effects from immune response.(1,113) Although the actual mechanism causing the neutropenia has been identified in only a few cases, there are four different immunological mechanisms proposed which involve Ab-mediation.(109,113)

1. Drug Absorption: drug attached to granulocyte membrane acts as a hapten for Ab formation.
2. Innocent Bystander: Ab formed to drug and resulting complex non-specifically adheres to granulocyte membrane.
3. Protein Carrier: drug binds to carrier protein and complex then attaches to granulocyte membrane and acts as a hapten.
4. Spoiled Membrane: drug alters granulocyte membrane and Ab forms to new membrane configuration.

Any of these mechanisms can lead to increased destruction of mature granulocytes and/or suppression of granulocytopoiesis and a single drug may result in IDIN in different patients by way of different mechanisms.(113) Auto-AGAs are found in numerous IDINs, particularly when patient granulocytes are tested by direct methods.(4,5,19,32,57,78,79,98,108) Further studies indicate that in many of these cases the Ab detected is a drug-dependent Ab rather than a true AGA.(19,32,79,108)

Detection of Anti-Granulocyte Antibodies

Numerous types of assays are available for the detection of Abs specific for granulocytes but no single one is able to detect all AGAs.(49) Two of the major problems associated with the demonstration of AGAs are the ability to isolate a pure granulocyte population and the need to maintain granulocyte viability.(72) Variables that allow the detection of some AGAs by one method but not by another include the Ig class and subclass of the Ab, Ag density, Ab avidity and ability to fix complement, and the proximity of certain Ags to physiologically important receptors on granulocyte membranes.(17) Certain aspects of AGA testing are best summarized by McCullough.

> "It seems likely that leukocyte serology will resemble RBC serology in that different assays detect antibodies of different Ig class and clinical significance and that the clinical effect of an antibody may be related to its Ig class, optimum temperature of reactivity, and other factors."(72)

Collection, Isolation, and Storage of Granulocytes

Whole blood (WB) to be used for WBC or granulocyte isolation can be collected in a number of different anti-coagulants including heparin, acid-citrate-dextrose (ACD), citrate-phosphate-dextrose (CPD), and ethylenediaminetetraacetic acid (EDTA).(9,21,44,66,73,77,104) Whole blood collected in ACD or CPD and stored at 4C for 24 hours has granulocytes which appear morphologically normal but are somewhat functionally

deficient.(20,71) Granulocyte function in ACD or CPD WB is maintained better by storage at 20-24C.(71,73) These are the anticoagulants of choice when granulocytes are to be collected by filtration leukapheresis or continuous flow centrifuge leukapheresis.(70) For AGA testing procedures routine phlebotomy is a much more practical method for collection.(72) Collection in EDTA retains the morphology and functional integrity of granulocytes the longest.(54) Granulocytes can be isolated from EDTA WB up to 36 hours after collection, although yields are higher if isolation is done sooner, providing that the WB is kept at room temperature and in plastic tubes.(21)

The elimination of red blood cells (RBCs) from WBC or granulocyte preparations is of paramount importance in many AGA assays.(95) Early methods for the removal of RBCs included sedimentation and/or repeated centrifugations and were not overly successful.(42) The addition of rouleaux-forming agents such as dextran, polyvinylprolidine, or fibrinogen to sedimentation protocols was a common practice until studies showed that these agents contributed to granulocyte disintegration and non-specific aggregation.(9,49,54,66,69,72) The use of gelatin, methylcellulose, or polybrene to promote RBC sedimentation appear to circumvent these problems.(20,49,50,72) Agglutination of RBCs with anti-A, anti-B, or anti-H Abs and lectins removes the contaminating RBCs but may not be an acceptable practice for use in conjunction with certain

types of AGA assays.(40,73) Red blood cell contamination can also be removed by lysis and centrifugation methods.(44,47)

The isolation of granulocytes from other types of WBCs is not always done prior to AGA testing.(50,68) While the separation of a pure granulocyte population is not necessary for some assays, it is a critical point in others, particularly when attempting to establish the specificity of an Ab.(49) The most widely used methods for granulocyte isolation are by centrifugation thru a single or double density gradient of percoll, hypaque-dextran, ficoll-sodium diatrizoate, or ficoll-hypaque.(21,22,44,66,72,77,97,104) Washing and resuspension of granulocytes after density gradient isolation can be accomplished with phosphate buffered saline (PBS), PBS with EDTA added (EPS), Hank's balanced salt solution, or RPMI-1640.(21,44,73,77) The use of small amounts of EDTA and Na_3N in the final suspension diluent are suggested to minimize non-specific aggregation and bacterial growth during long incubations, respectively.(49,73) The optimum pH and temperature of reagents used in granulocyte isolation procedures and the final suspension media are disputed. Most authors advocate the use of a pH range of 7.0 to 7.7, noting a decreased motility at lower pH, while others observe a decrease in non-specific aggregation and occasionally enhancement of reactivity at a pH range of 6.5 to 6.2.(21,30,73,97) The

optimum temperature is generally considered to be 20-24C although some methods are successful at 4C.(20,37,73)

Granulocytes to be used in immunofluorescence tests are usually fixed with 1% paraformaldehyde (PFA).(44,103) Although this treatment may lengthen the storage time, it is primarily used to minimize the amount of non-specific absorbance of fluorescence by granulocytes.(104) Attempts to freeze granulocytes for long term storage have been unsuccessful with maximum recoveries of less than 30%.(76) The general consensus at this time is that granulocytes isolated for use in AGA assays should be used as soon as possible after collection.(49,50)

Agglutination Methods

The original agglutination tests for the detection of AGAs were performed in test tubes with a mixed population of WBCs and RBCs.(37,53) Although these protocols can detect the presence of Abs and agglutinates can be examined microscopically to determine the involvement of granulocytes, additional testing is needed to confirm the granulocyte specificity of an Ab.(37,50) Specificity of a tube agglutination assay is greatly enhanced by the use of a pure granulocyte preparation but the need for large amounts of sera and granulocytes make these assays impractical for screening or population testing.(9,49) Agglutination by some Abs can be detected in as little as 90 minutes, however, the optimum times for reading are after both 5 and

18 hours of incubation.(50,51,102) Granulocyte agglutination test (GAT) methods are able to detect both IgG and IgM AGAs.(105) The optimum temperature for incubations varies depending on the specificity of Ab being sought, 22C for some and 37C for others.(72,73) The presence of a chelating agent, such as EDTA, is often needed to demonstrate agglutination and its use is also generally recommended to minimize spontaneous aggregation of granulocytes, an inherent problem in GATs.(30)

To circumvent the problem of large volumes of sera and granulocytes needed in tube tests, micro-GATs have been developed.(49) One such method involves the use of microtiter plates with the examination of wells by inverted phase microscopy after 2 and 5 hours of incubation.(39,72,73) Papain treatment of granulocytes is successful in promoting the reactivity of some Abs detected in this manner.(21) A capillary tube technique is also described in which the distance of cellular flow after inversion is inversely related to the concentration of AGA present.(89)

The requirement for viable cells in GATs is critical because agglutination of granulocytes by Abs is an active process, unlike the passive process seen with RBCs.(30,54,105) The two phases involved in this process are the sensitization phase, during which the Ab attaches to and activates the granulocyte, and the agglutination phase, whereby activated cells form pseudopods which establish

membrane contact with other cells.(49) Due to stabilization of the cell membrane and higher negative charge of the cells, granulocytes fixed with PFA are unreactive in GAT techniques.(105) The inability of certain AGAs to cause agglutination may be related to their failure to activate the granulocytes after attachment.(49)

Fluorescence Methods

The use of fluorescent compounds conjugated to anti-human globulins (AHG) is a valuable tool for the visualization of AGAs.(42,45) Many, but not all, of the AGA specificities detected by GATs are also detectable by granulocyte immuno-fluorescence tests (GIFTs) although there is a lack of correlation with respect to Ab titer between the two.(105) In a comparison study of GAT and indirect GIFT (granulocytes incubated with sera prior to the addition of AHG) using sera from patients with febrile transfusion reactions, 78.5% were negative with both tests, 3.6% were positive with both tests, 2.6% were GAT positive and GIFT negative, and 15.2% were GAT negative and GIFT positive.(104) It is suggested that those which were positive only by GIFT may reflect AGAs that are unable to activate granulocytes or residual non-specific absorbance of fluorescence by the granulocytes.(10,49,104) The latter problem can be avoided by granulocyte fixation with PFA which may act by stripping non-specifically adhering IgG and/or altering the affinity of the granulocyte Fc

receptor.(104) Conversely, PFA fixation may alter specific granulocyte Ags, thus accounting for sera that are GAT positive and GIFT negative. A potential problem associated with the comparison of GIFT results to those obtained by other techniques is that GIFT methods may also detect Abs which have been internalized in addition to those on the cell surface.(93)

Direct GIFT (no incubation of sera with granulocytes) methods can be employed for the detection of auto-AGAs which may not be present in sufficient quantity in the serum to be demonstrated by indirect GIFT.(45,104,106) Unfortunately, particularly in cases of neutropenia secondary to other autoimmune disorders, a positive direct GIFT is not proof of Ab specificity for an identifiable granulocyte Ag.(10,60,106)

Cytotoxicity Tests

Granulocyte cytotoxicity tests (GCTs) for the detection of AGAs have been in use for many years and have undergone numerous modifications in an effort to obtain reproducible results.(44,78,105) Micro-GCTs currently in use were developed as a modification of micro-lymphocytotoxicity tests.(40) One of the inherent problems in this type of testing is the adherence of granulocytes to the microtiter plate, resulting in a high frequency of false positives.(40) Granulocytes were originally pretreated with iodoacetamide to minimize adherence but more recent studies advocate the

addition of alcohol to the cell suspension media for this purpose.(28,31,40) Treatment of granulocytes with cytochalasin-B, a microfilament fragmenting drug, can also be used to deter adherence.(15) Vital staining for quantitation of live and injured cells with eosin-Y, trypan blue, or methylene blue produce satisfactory results but much more dramatic differences between the two types of cells can be achieved by the use of a double-fluorescent vital stain.(7) Fresh granulocytes are significantly more reactive than those stored for 24 hours.(28) Particular attention must be given to the isolation of granulocytes for GCT procedures as lymphocyte contamination may result in false positives and the presence of RBCs tends to decrease granulocyte reactivity.(7) Papain treatment of granulocytes enhances the strength and percent reactivity and it is suggested that this effect may be due to a more complete exposure of certain Ags.(28,30,31) Incubation at 4C increases the sensitivity of the assay as opposed to 24 or 37C.(29,30) The clinical significance of AGAs detected only at 4C is questionable.(72)

Granulocyte cytotoxicity test techniques are designed to detect AGAs which are capable of binding complement which includes both IgG and IgM.(72) By current methods, GCTs are most useful for detecting cold reacting IgM auto-AGAs.(29) One study reports that 10% of healthy non-immunized donors have allo-reactive AGAs by GCT while another states the

frequency of positives as 15% and 11% in multi-transfused and multiparous populations, respectively.(15,31)

Comparison testing of GAT and GCT suggests that each method detects a different pattern of reactivity.(72) While some sera are both GCT and GAT positive, in many cases there is no relationship between GAT and GCT results.(24,39,96,104) One of the potential problems associated with the use of GCT as the sole testing method for AGA detection is pointed out by Hasegawa.

> "For crossmatch purposes, for example in multitransfused patients, granulocyte cytotoxicty tests alone would result in 24% false negative reactions in which granulocyte agglutinating antibodies had not been detected."(39)

Anti-Human Globulin Consumption Tests

Anti-human globulin consumption tests (ACTs) are based on the principle that Abs coating granulocytes will bind anti-human globulin (AHG) and thus decrease the titer of AHG.(43,99) The titer of AHG which remains unbound can be determined by adding Ab-sensitized RBCs and testing for their agglutination or measuring the amount of RBC hemolysis after the addition of complement.(25,48,60) The amount of agglutination or hemolysis is inversely related to the quantity of Ab coating the granulocyte.(24,60) Anti-human globulin consumption tests can be performed as a direct or indirect test, the former for the detection of auto-AGAs and the latter for auto- and/or iso-AGAs.(24)

With the exception of patients with SLE, the results of direct ACTs correlate well with the presence or absence of neutropenia, although not with the degree of neutropenia.(24) The ACT methods are suggested to be most useful in the study of neutropenia associated with FS or RA.(59,60) The problems associated with the use of ACTs include false positives due to "background consumption" of normal granulocytes and false negatives caused by the use of AHG reagents which do not react with all globulin Ags.(24,48)

Staphylococcal Protein-A Binding Methods

One of the more recently developed techniques for the detection of AGAs, Staphylococcal Protein-A (SPA) binding assays take advantage of the protein's ability to bind to the Fc region of some Abs.(111) More specifically, SPA binds best to IgG_1, IgG_2, and IgG_4 subclasses, the former of which tends to have the main anti-granulocyte activity.(69) One of the major advantages of SPA assays is that they detect bound IgG which may or may not cause agglutination or show complement fixation.(72) A potential disadvantage of this assay is the lack of sensitivity to IgG_3 Abs. The ease of labeling SPA with ^{125}I or fluorescein makes this technique useful both for the quantitation of AGAs and the visual monitoring of changes resulting from Ab binding.(38,69) Investigations with fluorescein-SPA show a

capping reaction of granulocytes exposed to AGAs which could be completely blocked by cytochalasin-B.(69)

Staphylococcal Protein-A binding assays are currently being used in conjunction with HL-60 cells, a human myeloid cell line of promyelocytic nature, and mature granulocytes to study the specificities of auto-AGAs for Ags that may be gained, lost, or conserved during granulocyte maturation.(22)

Functional Assays

A variety of assays for the detection of AGAs utilizing the normal functional capacities of granulocytes are available. Anti-granulocyte antibodies can be detected based on the Ab's ability to decrease granulocytes' bacteriocidal-phagocytic activity but this method is only useful for AGAs which are specific for Ags that are physiologically important for these granulocyte functions.(17,69) A pitfall of using direct functional assays is that functional injury can be inflicted upon the patient's granulocytes due to complement activation in the absence of AGAs.(17)

Patient granulocytes can be tested for the presence of surface IgG by incubation with normal donor granulocytes and assessment of the donor cells' phagocytic activity.(9,109) Donor granulocytes will show increased phagocytic activity in the presence of patient granulocytes coated with IgG and, in turn, an increase in glucose oxidation. The resulting

activity of the hexose monophosphate shunt in the donor granulocytes can be measured by the release of $^{14}CO_2$ from glucose-1-^{14}C or superoxide production.(5,47) The major problem associated with this and other types of functional assays is the requirement for large numbers of granulocytes.(9,69)

Granulocyte Antigens

There are two general classes of granulocyte Ags, those which are shared with other cell types and those which are unique to granulocytes.(49) Detection of an AGA by any of the various methods available generally gives little information as to the class of granulocyte Ag that the Ab is specific for. Studies to determine whether an AGA is specific for a shared or unique granulocyte Ag can be performed using standard absorption and elution techniques with pure populations of various cell types and repetition of the AGA testing with the absorbed sera and/or eluates.(5,29,45,66,102) Absorptions should be carried out at the optimum temperature for the particular Ab as determined by the reactivity pattern in AGA testing.(45,66,102) Heat, ether, and acid elution methods established for RBCs can readily be used for granulocytes.(5,110)

Shared Antigens

It is generally agreed that Ags of the histocompatibility complex (HLA) exist on the surface of granulocytes.(49) These Ags can be readily detected by GAT and GIFT methods but not by GCT even when strong monospecific antisera is used.(96,97,104) Granulocyte immune-fluorescence test results with HLA antisera are weaker than those observed in comparable tests with lymphocytes but this is somewhat expected in consideration of the lower density of HLA Ags on granulocytes.(9,104)

One of the longest ongoing debates in the literature concerning granulocyte serology is the question of whether or not these cells possess ABH Ags. Early investigations using mixed cell agglutination techniques found granulocytes to be involved in positive reactions when tested with anti-A and anti-B sera.(33,37,50) This reactivity was noted to occur even with cell preparations from non-secretors, suggesting that the ABH Ags are indeed an integral part of the granulocyte membrane.(33) Incubation of granulocytes from type O donors with plasma from type secretors failed to confer A reactivity, implying that the A Ag is not absorbed onto granulocytes as is the case with platelets.(3,43) With the advent of density gradient techniques for granulocyte isolation ABH Ags could no longer be demonstrated by GAT, nor by GCT or GIFT methods, although the anti-A and/or anti-B activity could be absorbed out of sera by the "cleaner" granulocyte preparations.(7,49,104) The most recent

testing, in which 97% pure granulocytes are used, fails to show any reactivity with anti-A or anti-B by micro-GAT, micro-GCT or a modified indirect ACT.(44) Substitution of RBCs for granulocytes in these protocols establishes the lower limits of sensitivity at 1-4 x 10^{10} A Ags and using this value it is calculated that granulocytes could have up to approximately 800 A Ags per cell and still go undetected.(44)

Testing for the presence of other RBC Ags on granulocytes has produced a number of conflicting reports. In the Rh system the D Ag can be detected on granulocytes but only by the use of mixed cell populations in fluorescence and AHG consumption tests.(25,42,55) Antibodies specific for Ge, U, Kx, Jk^a-Jk^b, I and i RBC Ags can be absorbed by granulocytes but only I and i are detectable by GAT methods.(49,64,65,66,67,68,85) The Kx Ag is somewhat unique in that it need not be present on the RBCs of a donor in order to be detected on the donor's granulocytes and vise versa.(67) The Bg Ag of RBCs, which is thought to be a weak variety of HLA Ag, can be detected by GAT and GCT methods.(85) Antibodies to the following RBC Ags can not be absorbed by granulocytes: D, C, M, N, S, s, Rh 29, LW, Fy^a, Fy^b, Fy^3, Lu^b, Lu^3, Lu^4, Lu^5, Xg^a, K, k, Kp^b, Js^a, Ku, K_{12}, K_{13}, K_{16}, and K_{18}.(9,64,66,67,68)

Antibodies to lymphocyte group 5 (5^a and 5^b) Ags react against granulocytes in GAT methods.(67,96) There is evidence to suggest another non-HLA Ag shared by

granulocytes and lymphocytes which can be detected by GCT assays but the exact identity of this Ag has yet to be established.(18) The Mart Ag is identified on granulocytes, monocytes, and T-lymphocytes.(45) This Ag is not associated with an HLA loci and has an Ag frequency of 0.991.(45) Antibodies to the common lymphoblastic leukemia Ag (CALLA) will react with granulocytes although studies of the Ag reveal that it is not identical to that which is found on lymphoid cells.(83)

Unique Antigens

Antigens which are unique to granulocytes are currently divided into three classes dependent upon the nature of the Abs used for detection: allogenic agglutinating, allogenic cytotoxic, or xenogeneic Abs.(49) Although many of the Ags defined thus far are common to all types of granulocytes there are some Ags which are only present on neutrophils, at least one which is only detected on basophils, and differences in Ag expression as a function of cell maturation stage.(38,61) The detection of Abs to Ags which are found only on granulocytes lends further support to the importance of other tissue specific Ags in transplantation and autoimmune disorders.(49,54)

The first allospecificities defined were for Ags detected by GAT methods.(49) The standard nomenclature system for these is based on the genetics of the Ag with the first letter "N" signifying neutrophil specificity, the

second letter for the locus, and the end number for the allele.(52,54) The NA locus is defined as a diallelic system including the NA-1 and NA-2 Ags and is independent of HLA, 9^a, ABO, MN, Rh, and Kidd loci.(51,52,53) The gene frequencies of these Ags are 0.337 for NA-1 and 0.663 for NA-2.(52,54) Anti-NA-1 or anti-NA-2 can be detected by GAT, GIFT, or ACT methods but usually not by GCT or SPA binding assays.(30,72,99,104) The density of NA-1 and NA-2 Ags does not vary appreciably in relation to granulocyte maturity.(105) The reactivity of NA-1 is not significantly altered by PFA fixation of granulocytes.(104) Antibodies to these Ags are capable of causing INN, AIN, and febrile transfusion reactions.(51,72,99,102,105,107)

A second locus, NB, is independent of NA, HLA, ABO, and 9^a loci.(52) One allele of this system is defined, NB-1, with a gene frequency of 0.83.(52,54,104) Anti-NB-1 is readily detectable by GAT and GIFT, gives variable results by CGT, and is implicated in INN and febrile transfusion reactions.(30,52,104,105) Although the nature of the Ag(s) involved in neutropenias secondary to other immunological disorders are rarely determined, one case of auto-anti-NB-1 in SLE is reported.(93,106)

Three other loci, NC, ND, and NE, each have one identified allele.(54,87,105) NC-1, previously called Vaz, has a gene frequency of 0.72 and is implicated in INN.(54,104,105) Anti-ND-1 is detected by both GAT and GIFT

and is reported in one case of AIN.(97,105,107) Anti-NE-1 is described in a child with AIN.(87)

In the 9 Ag system only the 9^a Ag is neutrophil specific, with a gene frequency of 0.345.(49,54) Anti-9^a is detectable only by GAT methods.(104)

Granulocyte Ags which are detected by allogenic cytotoxic Abs are not yet well defined.(49) There is evidence for a common Ag on all granulocytes, called G, detected by GCT and this may be the target of many auto-AGAs.(29,31) Two other Ags proposed from GCT results, Gr1 and Gr2, may be the products of allelic genes.(15) Gr1 and Gr2 are not detectable by GAT techniques and there is no correlation between these Ags and HLA, NA-1, NA-2, or NB-1 Ags.(15) Whether or not Gr1 and/or Gr2 Ags are present on other cell types is currently unknown. Other studies propose 5 granulocyte Ags identified by GCT, one of which is shared by monocytes and the other four which are found only on mature granulocytes.(28,97) The latter four may be allelic genes at the same locus, termed G-3.(97)

At least nine monoclonal Abs have been developed which react only with granulocytes: anti-AHN-1 to anti-AHN-7, 38D2, and 41D2.(27,91,92) Anti-AHN-1 can selectively inhibit a granulocyte's ability to phagocytize opsonized bacteria but does not appear to react with the Fc or C3b receptor.(92) None of the other 8 monoclonals are found to alter granulocyte function.(27,91) Anti-AHN-7 may recognize a myeloid differentiation Ag present on precursor cells and

mature granulocytes.(91) 38D2 and 41D2 recognize the same membrane glycoprotein which is present only on mature granulocytes.(27)

Significance of Granulocyte Specific Antibodies

Anti-granulocyte antibodies have been implicated in febrile transfusion reactions to various blood products for many years. It is suggested that the fever and pulmonary difficulties seen in these reactions are the result of the release of pyrogen from neutrophils and the accumulation of sensitized granulocytes in the pulmonary microvasculature, either of which can be Ab-mediated.(49) Although AGAs can not be demonstrated in many of these transfusion reactions, the lack of a febrile reaction to leuko-poor blood products in the same patients suggests the involvement of AGAs, particularly in the absence of anti-HLA Abs.(22,39,48,104) It is currently unknown whether or not all AGA specificities identified thus far are capable of inducing a febrile transfusion reaction.(49,54)

The pathological role of Abs specifically reactive against granulocytes is clearly demonstrated in INN and some cases of AIN.(45,54,102) The success of therapeutic granulocyte transfusions in these and other granulocytopenic patients may be at least partially dependent on the presence or absence of AGAs.(39,74,75)

MATERIALS AND METHODS

Specimen Collection

Granulocytes

Fresh whole blood was collected in 7 ml EDTA Vacutainers (Becton Dickinson, Rutherford, New Jersey) from healthy donors and granulocytopenic patients for the isolation of plasma, granulocytes, and RBCs. Informed consent was obtained from all participants in this study. Whole blood was separated into plasma, buffy coat, and RBC layers by centrifugation at 900 xg for 12 minutes.

Red Blood Cells

Red blood cells to be used in agglutination testing were washed 3 times with PBS, diluted to a 3% suspension with Alsever's solution, stored at 4C, and discarded when visual signs of hemolysis or contamination were noted. Red blood cells to be used in absorption procedures were collected fresh as needed, washed 3 times with PBS and used within 6 hours of collection.

Plasmas

Negative control plasmas (samples #13-21 and #30-41) were collected from healthy, non-immunized donors. Patients

(samples #1-12) used in this study were obtained from the Clinical Center at Michigan State University and St. Lawrence Hospital (Lansing, Michigan). Plasmas containing anti-NA-1, anti-NB-1, and anti-Mart were a gift from Dr. Jeffrey McCullough (St. Paul Regional Red Cross Blood Center, St. Paul, Minnesota). High titer anti-A and anti-B plasmas (samples #22-29) were a gift from Great Lakes Regional Red Cross (Lansing, Michigan). All plasmas were stored in aliquots at -80C prior to use and any residual plasma discarded after being thawed for the third time.

Granulocyte Isolation

Granulocyte isolation was performed by a single density ficoll-hypaque gradient method (12). All reagents were kept at room temperature during the procedure and plastic tubes were used. Buffy coats were prepared as described earlier from EDTA anticoagulated whole blood within 2 hours of collection, transferred to 15 ml Falcon tubes (Oxnard, California), and diluted to approximately 12 ml with EPS. Buffy coat-EPS suspensions were layered over 3 ml of 1.3570 R.I. ficoll-hypaque in a clean 15 ml Falcon tube and centrifuged at 700 xg for 12 minutes. All fluids and cells above the granulocyte-RBC pellet were removed and the pellet resuspended. The resuspended pellet was diluted to 6 ml with distilled H_2O, mixed gently by inversion for 15 seconds, immediately followed by the addition of 6 ml of 2xEPS, and again mixed by gentle inversion. The resulting

suspension was centrifuged at 300 xg for 8 minutes and the supernate discarded. Resuspension of the pellet and dilution with distilled H_2O followed by 2xEPS was repeated as needed, up to three additional times, to lyse contaminating RBCs.

Except where specifically noted, granulocyte preparations were diluted to the desired cell count with EPS, kept at room temperature, and used within 6 hours of the initial collection. All cell counts were performed on a Coulter Counter Model Fn (Coulter Electronics, Hialeah, Florida). Granulocyte purity of the final product was assessed by microscopic examination of smears stained with Wright's stain (58).

Granulocyte Storage

Fresh granulocytes were diluted to 2.0×10^5 cells/ml with PBS pH 7.0 containing 3% (w/v) bovine serum albumin (PBS-BSA), PBS-BSA diluted 1:1 with EPS (PBS-BSA-EPS), or EPS and allowed to stand at 24C or 4C for up to 24 hours before use. Granulocyte suspensions adjusted to 2.0×10^5 cells/ml with EPS were fixed with 5%, 1%, or 0.5% buffered formalin (pH 7.0) or 0.05% or 0.025% glutaraldehyde. Suspensions were then stored at 4C for 24 hours and tested simultaneously with freshly collected granulocytes.

Granulocyte Agglutination Test

The detection of anti-granulocyte antibodies by the Granulocyte Agglutination Test (GAT) was performed by a modification of the method of Lalezari and Bernard. (50) Granulocytes were isolated as previously described and adjusted to 8 x 10^4 cells/ml with EPS. Duplicate 12 x 75 plastic tubes (Sarstedt, Princeton, New Jersey) were set up for each test plasma and granulocyte donor combination to be tested: 100 ul plasma and 50 ul granulocyte suspension per tube. Negative controls were set up in duplicate for each granulocyte donor using 100 ul EPS in place of plasma. All tubes were sealed with Parafilm (American Can Co., Greenwich, Connecticut), mixed gently, and incubated in a 37C H_2O bath. Tubes were read for agglutination both macroscopically and microscopically: one tube from each set of duplicates after 5 hours of incubation, the remaining tube after 18 hours of incubation. Granulocyte agglutination grading was based on the following guidelines. (50)

4+ All cells agglutinated in large clumps; no free cells observed.

3+ Cells agglutinated in smaller clumps; less than 25% free cells.

2+ Cells agglutinated in tiny clumps still visible macroscopically; less than 50% free cells.

1+ Agglutination confirmed microscopically; less than 75% free cells.

+/- Occasional agglutinates observed microscopically; more than 75% free cells.

0 No agglutination or rare clumps of less than four cells observed microscopically; virtually all cells free.

Microfilter Plate Anti-Granulocyte Antibody Assay

The microfilter plate anti-granulocyte antibody assay (MFPGA) was developed as a modification of a microfilter plate assay currently in use for the detection of anti-platelet antibodies (88). Plasma samples were diluted with PBS-BSA-EPS. Except where specifically noted, granulocyte suspensions were adjusted to approximately 2 x 10^5 cells/ml with EPS.

Prior to use, 96 well microfilter plates with 0.2 um pore size filters (Millipore Corp., Bedford, Massachusetts) were pretreated with approximately 200 ul PBS-BSA-EPS diluent per well and allowed to stand at room temperature for 15 to 30 minutes. Plates were drained by a vacuum aspiration system and the back of the plate wiped dry.

To each set of wells the designated volume of diluted plasma was added, and if necessary additional PBS-BSA-EPS, to a total volume of 200 ul per well. Duplicate wells were

set up for each granulocyte donor to be tested at each plasma dilution plus a set of duplicate wells for each plasma dilution as plasma backgrounds. For each granulocyte donor used duplicate wells were set up with 200 ul PBS-BSA-EPS as a granulocyte background. To the appropriate duplicate wells, 40 to 50 ul of granulocytes were added: actual volume added was dependent on that needed to obtain approximately 800 granulocytes per well based on the cell count of the granulocyte suspension.

The plates were incubated for 60 minutes at room temperature with constant gentle rotation. At the end of the first incubation phase the plates were washed with approximately 200 ul PBS-BSA-EPS diluent per well 3 times, the back of the plate wiped dry, and 50 ul of diluted radiolabeled 2nd Ab added to each well. For each aliquot of 2nd Ab diluted three 12 x 75 plastic tubes were prepared with 50 ul of 2nd Ab in each and sealed with Parafilm to be used for total counts (TC) of 2nd Ab. Plates and tubes were then incubated for 18 to 22 hours at 4C.

After the second incubation phase plates were again washed with PBS-BSA-EPS 3 times and the backs dried. Standard size filter discs were punched from the bottom of each well and placed in individual plastic counting tubes. Previously prepared TC tubes were also placed in counting tubes and these were counted for 60 seconds.

Results from each set of duplicate wells were averaged. To calculate the corrected percent of 2nd Ab bound in each

plasma plus granulocyte well the background counts of both plasma only and granulocytes only wells were subtracted.

Secondary Antibody Preparation

The secondary antibody (2nd Ab) was radiolabeled by the method of Fraker and Speck. (34) To a test tube coated with 0.4 ug iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril) 50 ug of Anti-Human IgG (BRL, Bethesda, Maryland) was added. This was followed by the addition of 1.0 mCi $Na^{125}I$ (RPI, Mt. Prospect, Illinois). The reaction was conducted for 15 minutes on ice with continuous mixing. Gel filtration on G-25 Sephadex (Pharmacia, Piscataway, New Jersey) equilibrated with PBS pH 7.0 was used to remove free iodide. The protein peak was pooled and diluted to 5 ml with PBS-BSA. Aliquots of 125 ul were separated and diluted to 1 ml with PBS-BSA and stored at -20C. For use in the microfilter plate anti-granulocyte antibody assay (MFPGA) one aliquot was thawed per plate and diluted to 6 ml with PBS-BSA-EPS immediately prior to use.

ABO Procedures

ABO Typings

The ABO typing of donors was performed by standard RBC agglutination methods with typing reagents from Ortho Diagnositics (Raritan, New Jersey), including A subgroup typing.(110)

Anti-A and Anti-B Titers

IgG anti-A_1 and anti-B titers were performed by standard RBC agglutination methods using pooled RBCs from 3 different donors of each type.(110) Titers were incubated for 30 minutes at 37C and read at the antiglobulin phase using monoclonal anti-human-IgG (Ortho Diagnostics, Raritan, New Jersey). Anti-A_1 and anti-B antibody reactivity scores were determined by the method of Marsh (63).

Anti-A_1 Absorption and Elution

Absorption of anti-A_1 IgG antibodies were carried out for 60 minutes at 37C with a plasma:cell ratio of 1:2 using RBCs pooled from 3 different A_1 type donors. After washing the RBCs 4 times with PBS, antibodies were eluted off the cells by the addition of 1 ml PBS pH 3.0 per 2 ml packed RBCs and gentle agitation for 5 minutes. The pH of the resulting eluate was then adjusted to 7.0 and centrifuged for 10 minutes at 1000 xg to remove RBCs and stroma.

RESULTS

Granulocyte Isolation

The purity of granulocyte suspensions was assessed by differential counts of smears made and stained immediately after isolation. Smears were made from 18 different granulocyte suspensions. One hundred WBCs were counted from each smear. The results of the WBC differentials are summarized in Table 1. Seventeen of the smears were observed to be free of RBCs and only one RBC ghost was noted on the remaining slide. Platelets were noted on 4 of the smears with 2 platelets being the maximum number observed per smear.

Granulocyte-RBC pellets from type O donors, prior to RBC lysis and washing steps, were purposefully "contaminated" with 25 to 250 ul of packed A_1 RBCs (the latter quantity being significantly greater than the typical amount of RBC contamination noted in the pellets at this phase of the isolation). The resulting granulocyte preparations were then tested against high titer (> or = 1:512) anti-A_1 plasma. Addition of as little as 25 ul of A_1 RBCs resulted in weakly positive reactions. Larger amounts

TABLE 1: WBC differential percentages read from smears of isolated granulocyte suspensions. (N = 18)

WHITE BLOOD CELL TYPE	MEAN PERCENTAGE	S.D.
NEUTROPHILS	94.4	3.0
EOSINOPHILS	2.5	1.5
BASOPHILS	0.1	0.3
MONOCYTES	1.8	1.5
LYMPHOCYTES	1.2	1.4
TOTAL GRANULOCYTES	97.0	2.6

were tested against the same plasma.

Granulocyte Agglutination Test

A GAT was performed to detect the presence or absence of anti-granulocyte antibodies in the plasmas to be used in the initial set-up testing of the MFPGA assay procedure. The results are summarized in Table 2. All plasmas used in the GAT tested negative for atypical anti-RBC antibodies.

Initial Set Up of Anti-Granulocyte Antibody Testing

Plasma Concentration

To determine the minimum concentration of plasma needed to distinguish positive and negative reactions dilutions of plasmas #1 and #14, respectively, yielding effective concentrations from 0.1 to 2.0 ul, were tested against the same granulocyte donors used in the GAT. The values for plasma #1 concentrations below 0.5 ul appeared significantly higher than those for plasma #14 but the degree of difference between the two was somewhat erratic. Plasma concentrations of 0.5 ul and above showed at least a two-fold increase in 2nd Ab binding between #1 and #14 plasmas and greater linearity of plasma #1 results. The results of plasma concentration testing are summarized in Figure 2. All future testing was carried out using plasma concentrations of 1.0 and 2.0 ul.

TABLE 2: Granulocyte Agglutination Test results to establish positive and negative control plasmas for use in the Microfilter Plate Anti-Granulocyte Antibody Assay.

GRANULOCYTE DONOR	PLASMA DONOR	INCUBATION TIME 5 hours	18 hours
#14	EPS control	0	0
	#14	0	0
	#19	0	0
	#1	±	1+
#19	EPS control	0	0
	#14	0	0
	#19	0	0
	#1	0	1+

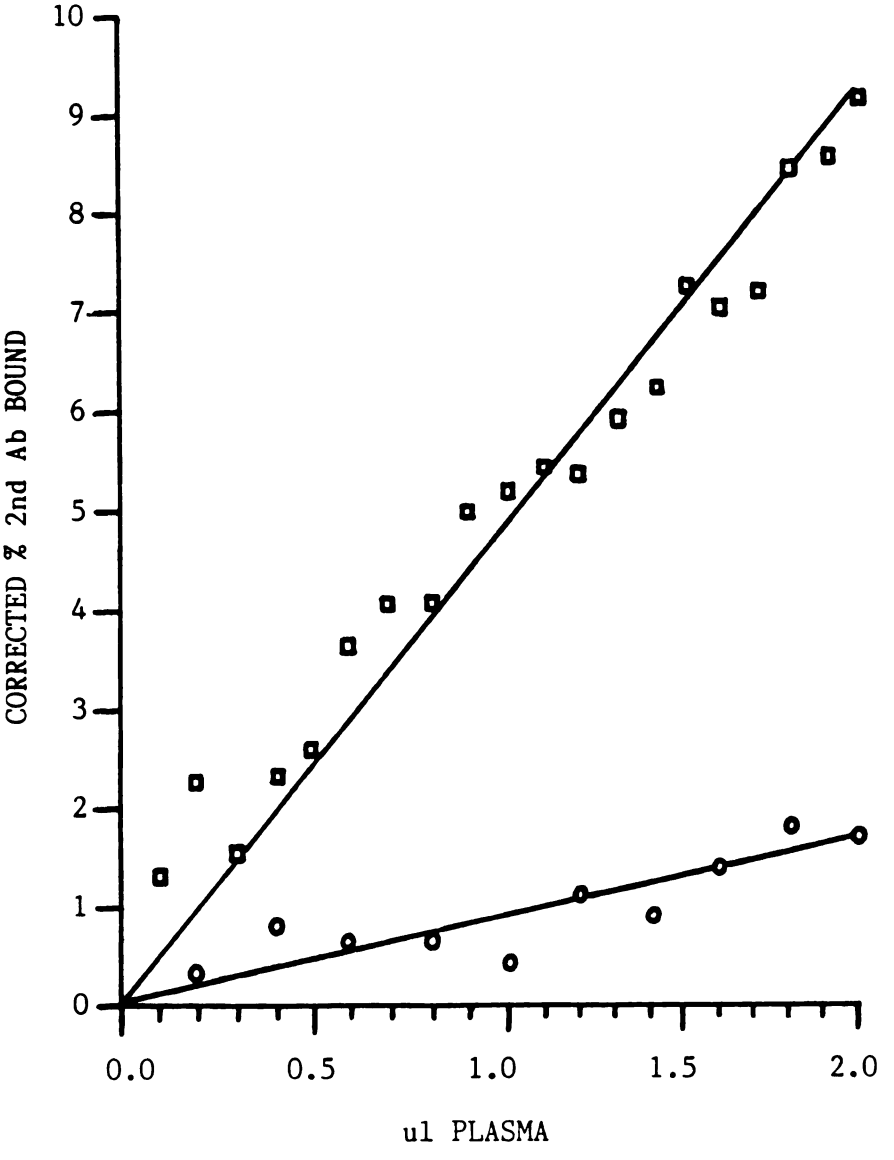

FIGURE 2: Results of increasing plasma concentration against allogenic granulocytes. (O = negative control plasma, □ = positive control plasma)

Granulocyte Concentration

Fifty ul of fresh granulocyte suspension diluted from 5.0 x 104 to 1.0 x 107 cells/ml were tested against plasmas #1 (positive control) and #14 (negative control) to determine the optimum concentration of granulocytes per well. Granulocyte concentrations above 400 cells/well gave consistently positive result against plasma #1. Use of concentrations above 1200 cells/well with either #1 or #14 plasma presented problems with drainage of the wells during washing and gave erratic results. All future testing was performed using 40 to 50 ul aliquots of granulocytes diluted from 1.6 to 2.0 x 105 cells/ml to give an effective concentration of approximately 800 cells/well.

Granulocyte Storage

Fresh granulocyte suspension prepared in either PBS-BSA or PBS-BSA-EPS and stored at 4 or 24C exhibited extensive clumping and were difficult to resuspend after as little as one hour. Fresh granulocytes suspended in EPS were more readily resuspended. The EPS granulocytes which were stored at 4C began clumping after 2 to 3 hours while those held at 24C showed no grossly visible aggregates until 4 to 5 hours of storage. Continuous gentle agitation of any fresh granulocyte suspension promoted rapid clumping.

Granulocytes which were fixed with buffered formalin or glutaraldehyde at any of the concentrations tested (0.025 to 5.0%) showed consistently lower results with positive plasma

samples as compared to fresh granulocytes. Microscopic examination of the fixed granulocytes revealed tightly rounded up cells without discernible granules.

Plasma Diluent and Wash Solution

Plasma diluent and wash solutions of PBS, PBS-BSA, EPS, and PBS-BSA-EPS were each tested. The use of PBS or PBS-BSA resulted in difficulties in draining the wells during washing. No drainage problems were noted with the use of EPS or PBS-BSA-EPS, but the former resulted in substantially less 2nd Ab binding than the latter. The optimum plasma diluent and wash solution was PBS-BSA-EPS.

Incubation

The optimum times for MFPGA assay primary and secondary incubations were tested. The results for the first incubation, tested at 30, 60, and 150 minutes, are shown in Figure 3. The maximum time for the second incubation was determined to be approximately 26 hours; longer incubations were observed to occasionally result in evaporation of the 2nd Ab. The minimum time for this incubation which yielded consistently positive results with 0.5 ul of plasma #1 was 14 hours. Subsequently, 60 minutes was chosen as the standard time for the first incubation and 18 to 22 hours for the second incubation in all future MFPGA assays.

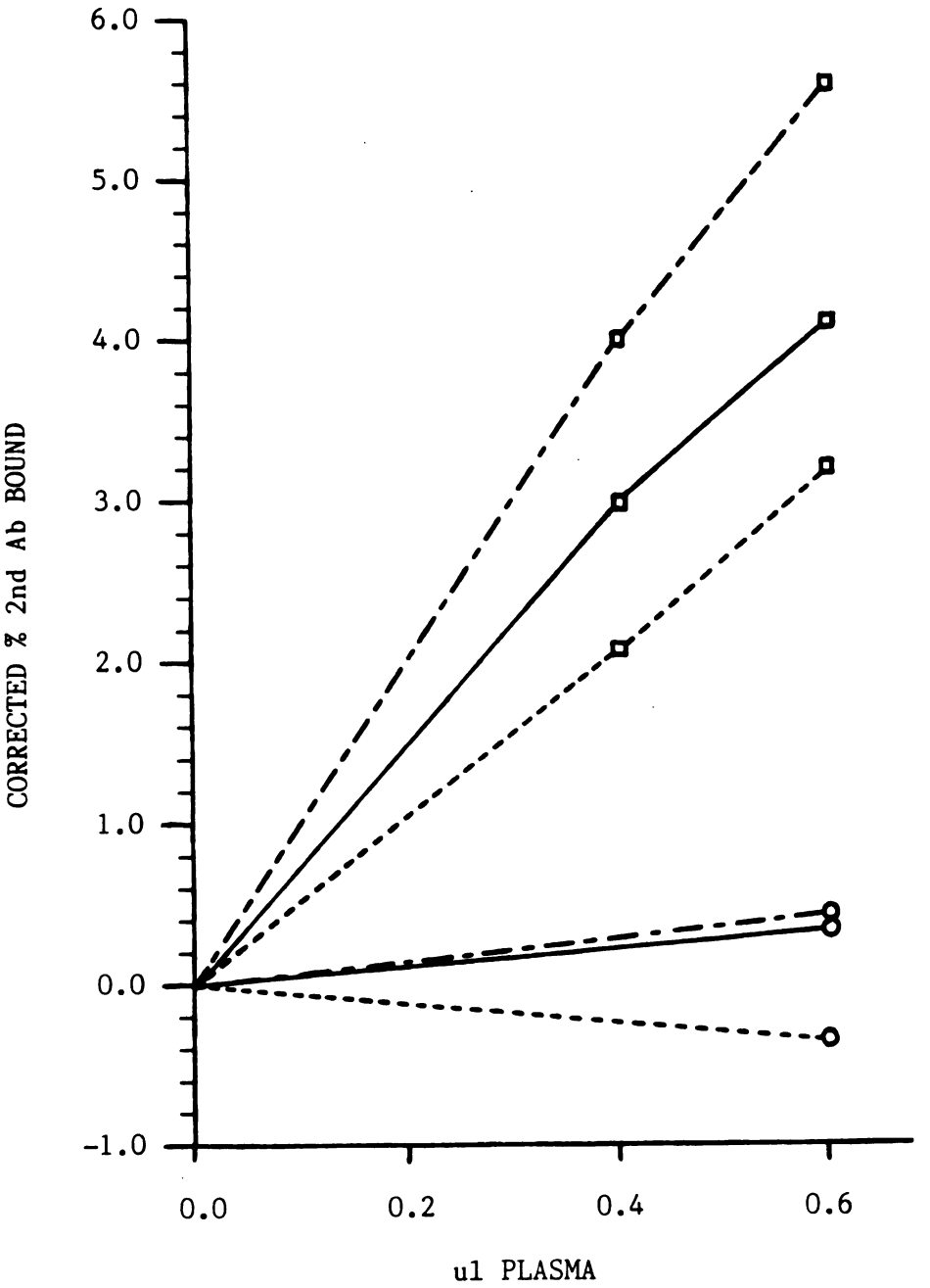

FIGURE 3: Results of increasing incubation time with allogenic plasma and granulocytes. (O = negative control plasma, □ = positive control plasma, ----- = 30 min. incubation, ——— = 60 min. incubation, -·——·- = 150 min. incubation)

Secondary Antibody Concentration

Using plasma #2 (strongly positive patient plasma) at high concentration (20 ul/well) 50 ul dilutions of 2nd Ab with effective concentrations ranging from 2ng to 45 ng per well were tested to determine the optimum concentration of 2nd Ab. The results are shown in Figure 4. The maximum ratio of 2nd Ab bound to 2nd Ab concentration was determined to be at 20 ng 2nd Ab per well and this concentration was used in all future testing.

Establishment of Normal Negative Ranges

Allo-Anti-Granulocyte Antibodies

To establish a normal negative range for the detection of IgG allo-anti-granulocyte antibodies in the MFPGA assay 20 healthy donors (samples #13-21 and #30-41) were tested at plasma volumes of 1.0 and 2.0 ul per well. All of these plasmas tested negative for atypical anti-RBC antibodies. Each plasma donor was tested against ABO compatible granulocyte donors. The results of these tests are summarized in Figures 5 and 6.

After establishing the upper limits of normal negatives for 1.0 and 2.0 ul of plasma at 2.0% and 3.5% 2nd Ab bound, respectively, future specimens tested were considered positive if above the upper limits at both 1.0 and 2.0 ul. Specimens which gave "positive" results at one plasma volume and "negative" results at the other plasma volume were repeated.

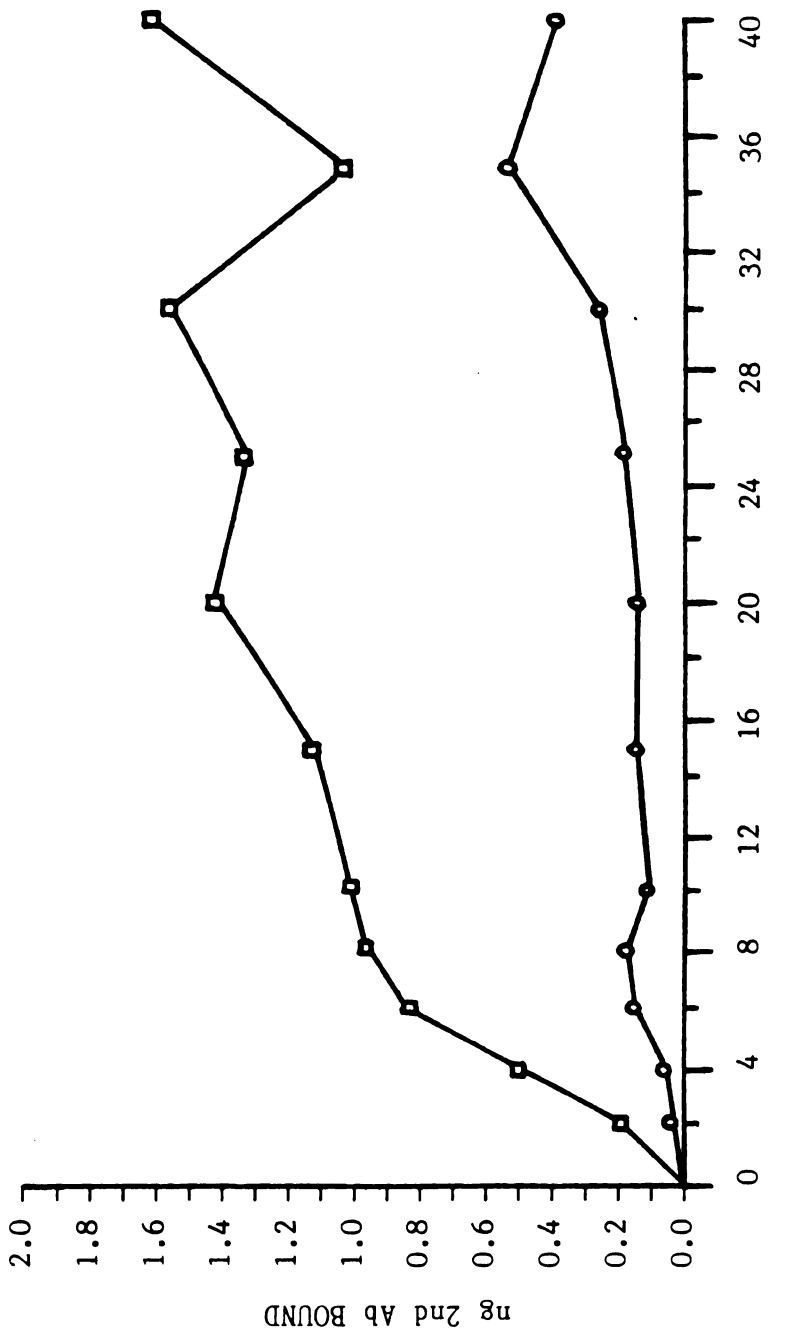

FIGURE 4: Results of incresing 2nd Ab concentration in the presence of excess plasma. (O = diluent, □ = positive control plasma)

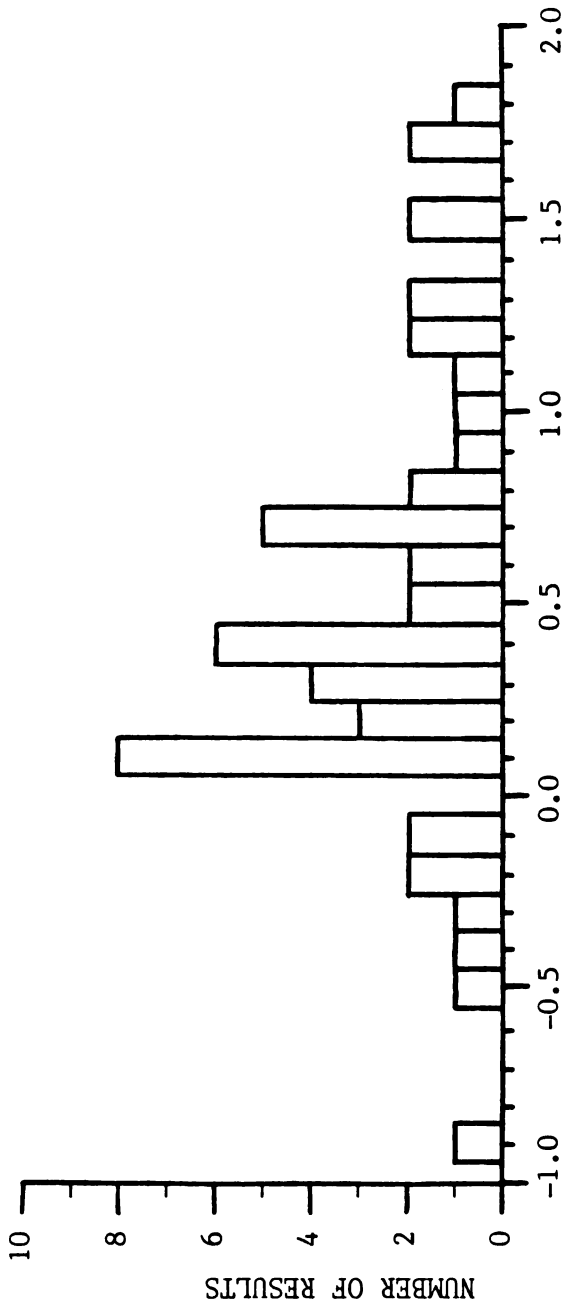

FIGURE 5: Distribution of results for negative plasma donors at 1.0 ul plasma volume for allo-anti-granulocyte IgG antibodies against ABO compatible granulocytes. ($N = 52$, $\bar{x} = 0.5\%$, s.d. = 0.6%)

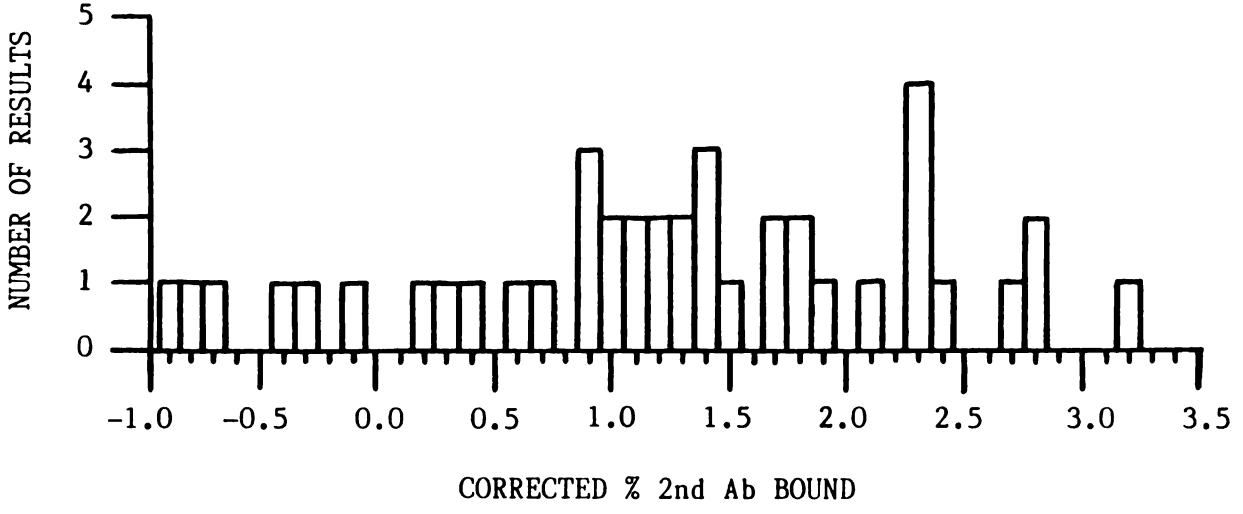

FIGURE 6: Distribution of results for negative plasma donors at 2.0 ul plasma volume for allo-anti-granulocyte IgG antibodies against ABO compatible granulocytes. (N = 41, $\bar{X}$ = 1.2%, s.d. = 1.0%)

Auto-Anti-Granulocyte Antibodies

Similar testing was carried out for the establishment of a normal negative range for IgG auto-anti-granulocyte antibodies. Fifteen healthy donors were tested and the upper limit values for percent 2nd Ab bound was set at 1.6% (x=0.6%, s.d.=0.5%, N=15) and 2.1% (x=0.9%, s.d.=0.6%, N=14) for 1.0 and 2.0 ul of plasma, respectively.

Patient Results

Twelve patients were tested for auto- and/or allo-IgG-AGAs. Due to the lack of an adequate method for granulocyte storage and the timing of specimen collection, auto-AGA testing could not performed on all of the patients. The results of AGA testing and the presumptive diagnosis for each patient are summarized in Table 3. Atypical anti-RBC Abs were detected in patients #2 and #9. No Ab specificity could readily be determined for known granulocyte Ags in those patients with positive results.

Antigen Typings

The results of granulocyte donor typings with anti-NA-1, anti-NB-1, and anti-Mart are shown in Table 4. All 3 antisera used were negative when tested for atypical anti-RBC antibodies. HLA antibody screening, performed in the laboratory of Dr. Jeffrey McCullough, showed anti-NA-1 and anti-NB-1 sera to be negative against all donor lymphocytes tested, 60 and 24 respectively. The anti-Mart sera was

TABLE 3: Patient results for allo- and/or auto-IgG AGAs and presumptive diagnosis.

PATIENT NUMBER	AUTO-AGA	ALLO-AGA	PRESUMPTIVE DIAGNOSIS
#1	NT*	POS (18)** NEG (1)	Immunethrombocytopenia, possible AIN
#2	NT	POS (20)	Febrile transfusion reactions, suspected allo-AGA
#3	NT	NEG (4)	Confirmed anti-$P1^{A1}$
#4	NT	NEG (3)	Possible AIN
#5	NEG	NEG (2)	Possible AIN
#6	NT	POS (5)	Possible AIN
#7	NT	NEG (4)	Possible AIN
#8	NT	POS (5)	Multitransfused, suspected allo-AGA
#9	NT	POS (2)	Multitransfused, suspected allo-AGA
#10	NEG	NEG (2)	Possible AIN
#11	NEG	NEG (2)	Possible AIN
#12	NEG	NEG (2)	Possible AIN

*NT = Not Tested
**() = Number of granulocyte donors tested

TABLE 4: Granulocyte donor antigen typings by Microfilter Plate Anti-Granulocyte Antibody Assay for NA-1, NB-1, and Mart: results obtained versus expected frequencies. (N = 11)

PERCENTAGE POSITVE	ANTIGEN		
	NA-1	NB-1	Mart
ACTUAL	54%	100%	100%
EXPECTED	56%	97%	99%

reactive against 21 out of 60 donor lymphocytes tested, but no HLA specificity could be determined. The ABO and Rh typing of the antisera donors was as follows: anti-NA-1/A Pos, anti-NB-1/A_2 Pos, anti-Mart/O Pos. Insufficient sera was available to perform anti-A and/or anti-B titers on these donors.

ABO

Anti-A_1 and Anti-B Titers

Plasmas from 16 individual donors (samples #13-28) and one pool of multiparous donors (sample #29) were titered for IgG anti-A_1 and/or anti-B and tested against various ABO type granulocyte donors in the MFPGA assay. The results are summarized in Table 5, showing positive results with anti-A and anti-B titers greater than 1:256. Each of the individual plasmas were tested and found to be negative for atypical anti-RBC antibodies.

Anti-A_1 Absorption and Elution

Two of the high titer (>or= 1:512) anti-A_1 plasma specimens, #24 and #26, were absorbed with A1 and O RBCs and eluates prepared from the RBCs. The results of absorbed plasma and eluate testing against granulocytes from type A1, A2, and O donors are shown in Table 6.

TABLE 5: Anti-A_1 and anti-B titers of plasma donors with results against various ABO type granulocyte donors. (NT = Not Tested, NA = Not Applicable)

PLASMA	PLASMA TYPE	ANTI-A_1 TITER	ANTI-B TITER	GRANULOCYTE DONOR TYPE (NUMBER OF DIFFERENT DONORS)			
				A_1	A_2	B	O
#13	B	1:32	NA	NEG (1)	NT	NEG (1)	NEG (1)
#14	A_1	NA	1:8	NEG (4)	NEG (2)	NEG (3)	NEG (7)
#15	O	1:64	1:32	NEG (1)	NT	NEG (1)	NEG (1)
#16	O	1:32	1:16	NEG (2)	NEG (1)	NEG (1)	NEG (1)
#17	O	1:128	1:128	NEG (2)	NEG (1)	NEG (1)	NEG (2)
#18	O	1:128	1:64	NEG (1)	NEG (1)	NEG (1)	NEG (1)
#19	O	1:128	1:128	NEG (1)	NEG (1)	NEG (1)	NEG (2)
#20	O	1:256	1:256	POS (1)	NT	NEG (1)	NEG (1)
#21	O	1:512	1:256	POS (3)	^{w}POS (1)	NEG (2)	NEG (3)
#25	O	1:512	1:128	POS (1)	NT	NEG (1)	NEG (1)
#26	O	1:512	1:512	POS (2)	NEG (1)	POS (1)	NEG (1)
#29	O	1:512	1:1024	POS (4)	^{w}POS (1) NEG (1)	POS (3)	NEG (5)
#22	O	1:512	1:1024	POS (1)	NT	NT	NEG (1)
#23	O	1:1024	1:1024	POS (1)	NT	NT	NEG (1)
#24	O	1:1024	1:512	POS (2)	^{w}POS (1)	POS (1)	NEG (1)
#27	O	1:512	NT	POS (1)	NEG (1)	NT	NEG (1)
#28	O	1:512	NT	POS (1)	NEG (1)	NT	NEG (1)

TABLE 6: Results of high titer anti-A_1 plasma absorptions and elutions against type A_1, A_2, and O granulocytes. (Results given as corrected % 2nd Ab bound for samples of 1.0 ul/2.0 ul)

HIGH TITER ANTI-A PLASMA	GRANULOCYTE DONOR TYPE		
	A_1	A_2	O
#24	17.9/28.6	2.3/6.4	0.2/3.0
absorbed with			
O RBCs	16.6/26.6	1.7/3.7	-0.4/0.3
A_1 RBCs	0.2/0.8	-0.6/1.0	0.0/1.2
eluate from			
O RBCs	0.1/1.3	0.4/0.8	0.1/0.9
A_1 RBCs	10.2/16.3	1.8/2.3	0.3/0.9
#26	9.7/16.7	1.5/4.0	1.2/1.1
absorbed with			
O RBCs	9.5/17.7	1.5/5.0	0.9/1.3
A_1 RBCs	0.1/0.2	0.1/0.2	1.3/1.1
eluate from			
O RBCs	0.8/1.1	0.1/0.9	0.4/1.1
A_1 RBCs	3.9/8.1	1.0/2.9	0.9/2.0

DISCUSSION

The advantages of the microfilter plate assay for the detection of AGAs include the requirements for only small volumes of plasma and low numbers of granulocytes. Testing for auto-AGAs can be accomplished with as few as 5 x 103 granulocytes. This is extremely beneficial when attempting to evaluate neutropenic patients. Allowing for positive and negative controls, a patient plasma can be tested for the presence of allo-AGAs against seven different granulocyte donors using only 50 ul of plasma. Similarly, nine granulocyte donors can be tested for a specific granulocyte Ag using less than 75 ul of typing sera. The latter point is particularly useful for performing population studies without depleting stocks of rare sera.

While the use of ^{125}I labeled anti-human IgG does not readily permit visualization of the morphological effects of AGAs on granulocytes it does allow for objective evaluation of results. Using the upper limit cut off values established for negative ranges, only two healthy donors (N = 59) had positive results for allo-IgG-AGAs. One of these positive healthy donors had been previously tranfused. Thus far no auto-IgG-AGAs have been detected in any healthy, non-immunized donors (N = 15).

The single density ficoll-hypaque gradient method used for granulocyte isolation resulted in greater than 90% pure granulocyte preparations as determined by microscopic examination. The lack of RBCs observed does not rule out the possibility of RBC membrane fragment contamination, particularly in consideration of the lysis and washing technique used for RBC removal. This point may be important when attempting to determine the granulocyte specific nature of a particular Ab or Ag but does not interfere with routine screening for AGAs. The possibility of significant platelet contamination in these granulocyte preparations is discounted by the observance of negative results when tested with high titer anti-PlA1 serum, known to react specifically and with a strong avidity for platelets.

The results of granulocyte donor typings with anti-NA-1 and anti-NB-1 sera demonstrate the ability of this assay to detect these Ab-Ag reactions which are specific for granulocytes. The observed frequencies of these Ags and the Mart Ag as determined by the microfilter plate assay correlate well with the expected frequencies determined by GAT and GIFT population studies.(45,52,54) Additional studies are needed to evaluate the ability of this assay to detect AGAs which are currently only demonstrated by GCT methods as sera containing these types of Abs have not been available for testing at this time.

Testing patient plasmas positive for allo-IgG-AGAs has failed to demonstrate the specificity of the Ab(s) present

in these samples. The reactivity of these sera against virtually every granulocyte donor tested suggests that these may contain agglutinating and/or cytotoxic Abs to high frequency Ags.

The microfilter plate AGA assay avoids many of the pitfalls of other methods currently available for the detection of AGAs. Although "micro" modifications of various methods are available, the quantity of cells and sera needed are still substantially larger than are required for this assay. There have been problems with reproducibility in other methods, particularly those involving agglutination techniques, which may simply be a consequence of the subjective nature of interpretation of results. While investigation into the ability of the microfilter plate assay to detect various specificities of Abs is not yet complete, preliminary results suggest that the Abs detected by this method may include both agglutinating and cytotoxic Abs. This is suggested by the results of Ag typing tests, all of which utilized agglutinating Abs, and the extensive reactivity of positive patient plasmas, possibly representing Abs to the G Ag as defined by GCT methods.(29) If this is indeed the case, this assay would offer a more comprehensive method of screening for AGAs than any other single method currently in use.

The use of overnight incubation with the microfilter plate method makes the turn around time comparable to that

for GAT methods.(50) Although GCT and GIFT techniques offer shorter turn around times, the microfilter plate assay allows for more objective interpretation of results than either of these methods.(7,15) Unlike with ACTs, the background binding of each granulocyte donor is not a problem which causes false positive results in this assay.(24)

Further investigation in the area of granulocyte storage is warranted, particularly concerning the use of PFA as a fixative for granulocytes to be use in this assay. One of the current drawbacks to the microfilter plate technique is the need for fresh granulocytes, which eliminates the possibility of storing selected Ag typed granulocytes for use in Ab specificity testing. Aside from the storage time benefits, fixation may also serve to increase the concentration of granulocytes which can be used per well by reducing the non-specific aggregation during incubations resulting in difficulties during the washing cycles. This in turn may increase the sensitivity of the assay for Abs which are directed to low density granulocyte Ags. Although PFA fixation has been shown not to alter the granulocyte Ags which can be detected by GIFT methods, the possible impact of PFA on Ag structure can not be ignored when investigating its use in other assay systems. Results of experimentation with formalin and glutaraldehyde fixation methods have thus far failed to allow for increased granulocyte storage times.

Recent investigation by Kelton failed to demonstrate the presence of A and B Ags on granulocytes by agglutination or AHG consumption methods.(44) The results obtained with the microfilter plate assay argue against those results. Reactivity in this assay with anti-A and anti-B sera was demonstrated, but only when sera with titers of 1:256 or greater were used. As shown by RBC absorption and elution studies with subsequent AGA testing against A_1, A_2, and O donor granulocytes, this was a specific anti-A reaction with A Ag rather than a non-specific reaction involving granulocyte Fc receptors. This suggests the presence of ABH Ags on granulocytes and the need for high titer sera to demonstrate this reactivity may be due to much lower quantities of the Ags on granulocytes as compared to RBCs. The possibility of RBC membrane fragment contamination of granulocyte preparations or the ability of granulocytes to adsorb A and B Ags accounting for the reactivity observed can not be ruled out at this time. Further investigations, including the use of alternate granulocyte isolation techniques, absorption and elution studies with granulocytes, and incubation of granulocytes with plasma from donors of known secretor status are needed to establish whether or not A and B Ags are produced by the granulocyte and are an integral part of the granulocyte membrane.

The ability to detect AGAs is clearly important in certain clinical situations. The demonstration and characterization of AGAs can be used to establish a

diagnosis of INN in neutropenic infants, evaluate tranfusion therapy in patients exhibiting severe febrile transfusion reactions, or document the involvement of auto-Abs in neutropenias secondary to other disorders. A future possibility, for which the microfilter plate method may be particularly well suited, is the use of granulocyte crossmatching techniques to insure immune compatibility with granulocyte transfusions.

APPENDICES

APPENDIX A

Granulocyte Isolation Procedure

Note: Keep all reagents and specimens at room temperature during the isolation procedure. Use plastic test tubes and pipets.

1. Centrifuge EDTA WB for 9 minutes at 900 xg.
2. Transfer plasma and buffy coats to individual clean tubes. Any plasma to be tested other than on the day of collection should be stored at -80C.
3. Dilute buffy coat to approximately 12 ml with EPS.
4. Carefully layer buffy coat-EPS suspension over 3 ml of 1.370 R.I. ficoll-hypaque in a clean test tube.
5. Centrifuge suspensions for 12 minutes at 700 xg. Carefully remove all cells and fluid above the granulocyte-RBC pellet at the bottom of the tube.
6. Resuspend the granulocyte-RBC pellet in 6 ml H_2O and mix gently for 15 seconds to lyse RBCs.
7. Immediately after mixing add 6 ml 2xEPS and mix gently by inversion.
8. Centrifuge the resulting suspension for 8 minutes at 300 xg and discard the supernate.

9. Repeat steps 6-8 as needed to lyse and remove residual RBCs. The final granulocyte pellet should have a faint greenish tinge.

10. Resuspend granulocytes and adjust the concentration to approximately 2 x 10^5 in EPS; keep at room temperature and use within 6 hours of initial collection.

APPENDIX B

Microfilter Plate Anti-Granulocyte Antibody Assay

1. Thaw aliquots of positive and negative control plasma, patient plasma, and typing sera as needed and dilute 1:100 with PBS-BSA-EPS.
2. Calculate the volume of each granulocyte suspension which will result in approximately 800 cells per well.
3. Pretreat microfilter plate(s) (96 well, 0.2 um pore size) with 200 ul PBS-BSA-EPS per well and let stand for 15-30 minutes.
4. Drain plate by vacuum aspiration system and wipe the back dry with several changes of absorbent tissue.
5. Add plasma and/or PBS-BSA-EPS diluent then granulocytes where appropriate to the following wells. Set up all wells in duplicate. A sample plate set up is shown in Figure 7.
    A. Diluent Backgrounds: 200 ul PBS-BSA-EPS per well.
    B. Plasma Backgrounds:
        a. 100 ul diluted plasma or typing sera plus 100 ul PBS-BSA-EPS.
        b. 200 ul diluted plasma or typing sera.
    C. Granulocyte Backgrounds:
        a. calculated volume of each granulocyte donor to give 800 cells/well.

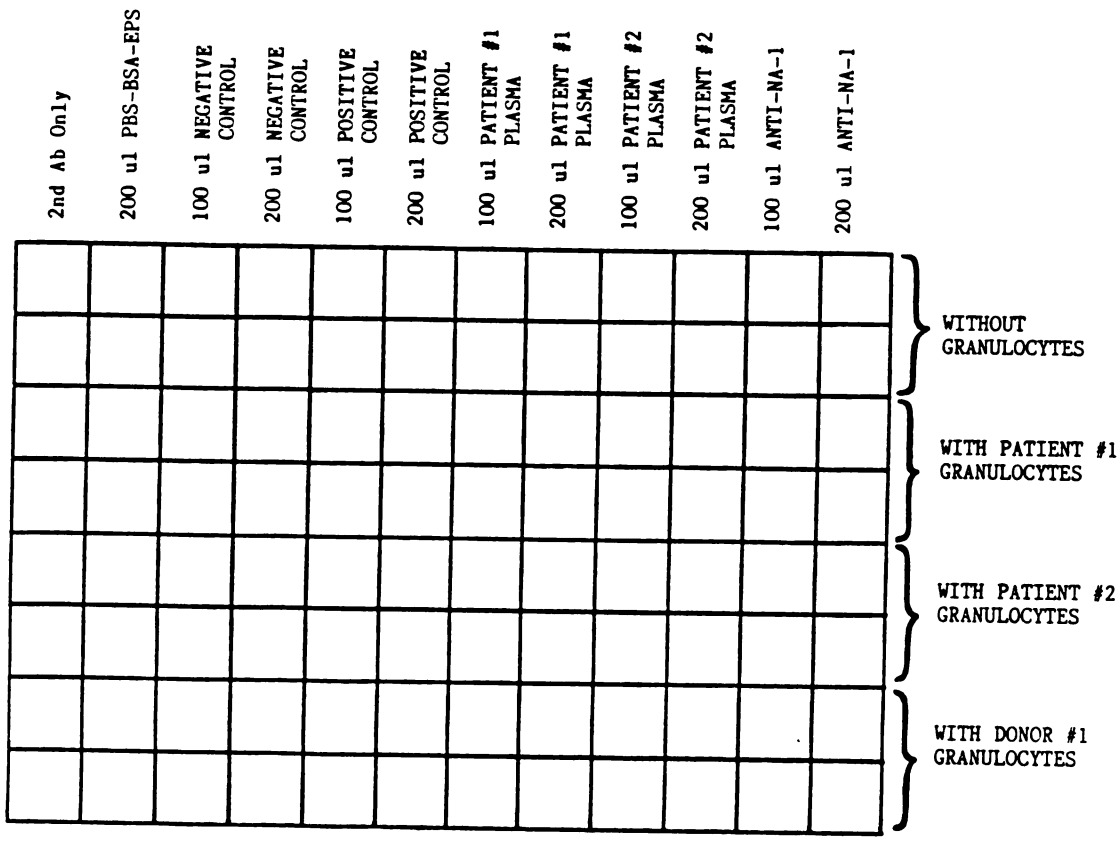

FIGURE 7: Sample microfilter plate plasma and granulocyte set up for anti-granulocyte antibody testing and granulocyte antigen typing.

b. calculated volume of each granulocyte donor plus 200 ul PBS-BSA-EPS.

D. Test Wells for Patients, Controls, and Typing Sera:

a. 100 ul of diluted plasma or sera, 100 ul PBS-BSA-EPS, and appropriate volume of granulocytes for each donor to be tested against.

b. 200 ul of diluted plasma or sera and appropriate volume of granulocytes for each donor to be tested against.

6. Cover plate and incubate at room temperature for 1 hour with continuous gentle rotation.

7. Thaw one aliquot of ^{125}I labeled secondary antibody for each plate set up and dilute with 5 ml PBS-BSA-EPS.

8. Wash the plate with 200 ul PBS-BSA-EPS per well and drain by vacuum aspiration. Repeat the wash cycle three times and dry the back of the plate after the final wash.

9. Add 50 ul of secondary antibody to each well and to each of three 12 x 75 plastic tubes (total count tubes).

10. Cover the plate and tubes and incubate at 4C for 18-22 hours.

11. Repeat wash cycle as described in step 8.

12. Punch out standard size filter discs from each well and place in individual tubes for counting.

13. Count total count tubes and filter disc tubes on gamma counter.

14. Average the results for each duplicate set of wells.

15. Calculate the % of secondary antibody bound for each of the wells set up in step 5D by subtracting the appropriate background values.

Interpretation of Corrected % 2nd Ab Bound

Negative: < or = 2.0% at 100 ul of diluted plasma and < or = 3.5% at 200 ul of diluted plasma.

Positive: > 2.0% at 100 ul diluted plasma and > 3.5% at 200 ul of diluted plasma.

Note: Results which appear negative at one plasma volume and positive at the other should be repeated if possible.

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VITAE

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