IMMUNOLOGICAL ASPECTS OF SHORTENED NEUTROPHIL HALF-DISAPPEARANCE TIMES AFTER ISOLOGOUS NEUTROPHIL TRANSFUSIONS

by

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ABSTRACT

Organ and tissue transplants have decreased the morbidity associated with such medical problems as uremia and heart disease. The success of organ transplants has paralleled advances in transplantation immunology. Medical problems associated with bone marrow destruction or replacement with its resultant neutropenia could be temporarily alleviated by the use of leukocyte transfusions. Though leukocyte transfusions have been done, the efficacy has not been evaluated by direct measurement of physiological function of the transfused cells.

To determine the potential efficacy of leukocyte transfusion in 12 normal subjects, neutrophil kinetic studies and immunological tests to detect leukocyte antibodies were employed. The physiological behavior of the neutrophils was followed by labeling them with radioactive diisoprophylfluorophosphate ($DF^{32}P$) *in vitro*, and then transfusing them. Interval sampling of these labeled cells allowed their half-disappearance time ($T_{\frac{1}{2}}$) to be computed. This was first done with autologous neutrophils to obtain the normal $T_{\frac{1}{2}}$ of the subject's own neutrophils. Labeled isologous neutrophils were then transfused into that subject and the $T_{\frac{1}{2}}$ of the donor cells in the recipient's circulation was measured. Post transfusion sera were collected from each recipient after 1, 6 and 13 days, and tested for leukoagglutinins.

Erythrocyte and lymphocyte cytotoxic antibodies were tested for as well, but none were found in these sera.

Four of the 12 recipients had accelerated isologous neutrophil disappearance rates, while 3 of the 12 demonstrated leukoagglutinin formation. Two of the 4 subjects with shortened neutrophil survival times formed leukoagglutinins, and 2/3 of those with leukoagglutinins had shortened survival times. A 2x2 Chi-square evaluation of these data indicated no significant association between the neutrophil survival time and leukocyte antibody formation ($p = 0.15$).

INTRODUCTION

The value of whole blood transfusion has been known for many years. It is a life-saving procedure after hemorrhage and in disease states causing anemia. Blood transfusion, however, only became a relatively safe procedure after the discovery of the main red cell isoantigens by Landsteiner in 1901 (Weiner, 1962). Even after the discovery of the main blood groups and cross matching procedures, blood transfusion has undergone continuous change. Today, the expectation of finding new red cell antigens is just as high as ever (Zmijewski, 1968).

Today the emphasis in research is focused on organ and tissue transplantation; leukocyte transfusion is a tissue transplant. Leukocyte transfusion, for therapy, could benefit neutropenic patients (Graw, 1971). Neutropenia, or leukopenia in general, is most commonly associated with acute luekemias or other marrow replacement disease, marrow depression, and cytotoxic marrow damage. Even with the stimulus of an infection, these patients fail to produce neutrophilic leukocytes. The use of leukocyte-poor blood, although of no help to a neutrophenic, will help cut down tremendously the formation of leukocyte isoantibodies. However, if these patients are going to be typed for histocompatibility, they should have blood drawn before transfusion (histocompatibility is a matching of donor and recipient tissue antigens). Zmijewski (1967) found that as little

as 1-8% of isologous leukocytes in a blood sample taken from a recently transfused patient could invalidate typing tests.

To be of therapeutic value, the transfused neutrophils must function normally (Haab, 1961). Past leukokinetic studies have indicated that many times isologous neutrophils (those obtained from another person) do not function in a normal fashion. These studies have shown that in as many as one-third of normal subjects isologous neutrophils will have an abnormally short disappearance time. Even though a subject has a normal neutrophil survival value following isologous transfusion, are the isologous leukocytes going to function in a physiologically helpful manner? Kauder, et al. (1965), found that even though the transfused granulocytes marginate normally and leave the circulation, they fail to enter inflammatory exudates as readily as homologous granulocytes.

The purpose of this study is to investigate whether a shortened neutrophil disappearance time is associated with an antibody against the leukocytes, or is independent of an antigen-antibody reaction.

REVIEW OF LITERATURE

The criterion of success of early leukocyte transfusions was clinical improvement in the transfused subject, without regard to white cell antigens or leukokinetics. Yankee, et al. (1964) reported improved clinical condition in infected neutropenic patients transfused with granulocytes from normal donors. Cures of septicemia were reported in acute leukemics after transfusion with leukocytes collected from patients with chronic myelogenous leukemia (Freireich, 1964; also Galbraith, 1965). Both of these groups worked out leukocyte dosage schedules so that "cures" could be obtained. In a controlled study with dogs, marrow failure was produced with radiation, and a septicemia established by injecting Escherichia coli. After the dogs became very ill, they were transfused with leukocytes collected from normal dogs. The leukocytes were collected using a NCI-IBM Blood Cell Separator. Disappearance of the septicemia determined the success of the transfusion (Epstein, 1969).

Methods have been devised in the last decade which make it possible to follow the behavior of neutrophils after transfusion, thus allowing a better assessment of transfusion success. Neutrophil kinetics can be studied by labeling the cells with radioactive diisoprophylfluorophosphate ($DF^{32}P$), <u>in vitro</u>. $DF^{32}P$ is a potent irreversible esterase inhibitor which becomes firmly attached to the neutrophils. The other cells and formed elements of the blood are labeled little or not

at all (Athens, 1959). After transfusion, the labeled netrophils leave the circulation in a random (exponential) fashion, with a mean half disappearance time ($T_{\frac{1}{2}}$) of 6.7 hours in normal subjects (Cartwright, 1964).

Mauer, et al. (1960) chose ten normal pairs of subjects for an isologous transfusion leukokinetic study. None had received blood transfusions previously. Autologous transfusion, with $DF^{32}P$ labeled granulocytes, showed that each subject had a normal blood neutrophil $T_{\frac{1}{2}}$. After isologous transfusion of labeled neutrophils, however, two of the ten had a $T_{\frac{1}{2}}$ of 2.5 and 1.2 hours. A patient who had been transfused many times previously had a $T_{\frac{1}{2}}$ of 0.8 hours. Similar results were obtained later by this same group, when twleve subjects received isologous transfusions. After transfusion the $T_{\frac{1}{2}}$ of the isologous granulocytes was abnormally short in five of the twelve, compared with their autologous studies. It was suggested that antigenic differences, between donor and recipient, were responsible for the rapid disappearance of the granulocytes from the circulation in these five subjects (Kauder, 1965).

It has been confirmed that the erythrocyte antigens systems (ABO, Rh, MNSs, Kell, Jk, Duffy), platelet antigens (Ko) and serum alloantigens (Gm, Gc, Lp, Ag) have no significant effect on histocompatibility. As a consequence, most attention has been given to the white cell antigens (Dausset, 1970). The first leukocyte group (Mac) was described by Dausset in 1958. The other antigens of the HL-A (Human Leukocyte-locus A) system have been discovered through continued study. HL-A antigens are found on the leukocytes, platelets,

and the solid tissues of the body. They are not found on erythrocytes or in serum.

Agglutinating antibodies against the neutrophilic white cells were first brought to light in a transient state known as neonatal neutropenia. Because the neutropenia disappeared in 2-4 weeks, it was thought that maternal antibodies could be responsible; as in erythroblastosis fetalis. By incubating the newborn's leukocytes with its mother's serum, it was found that agglutination of the leukocytes took place (Lalezari, 1965; Van Rood, 1961; Payne, 1964). Since then leukoagglutinins have been demonstrated in the sera of multiparous females where fetal leukocytes appeared to be the only antigenic stimulus. The fact that passive transfer of leukoagglutinins across the placental barrier occurs in man, places pathogenic significance on these antibodies. The antibodies in the mother are directed against some of the antigens of the father. They are also directed against some of the antigens inherited from the father and present in other siblings. They can be detected in an infant's serum during the period of neutropenia. Bone marrow examination of the neutropenic infant reveals a predominance of immature myeloid elements with a complete absence of mature neutrophils (Lalezari, 1966b). To date three distinct neutrophil specific antigens have been found. They have been designated NA1, NB1, and VAZ (Lalezari, 1970a), and are so called "high incidence" antigens. Antisers to these antigens will agglutinate leukocytes of the general population with an incidence of 56%, 97% and 89.7% respectively. These antigens are not found on erythrocytes, solid tissues, or any leukocyte except the neutrophil.

The development of leukocyte iso-antibodies also takes place in multritransfused patients (Killman, 1956; Payne, 1958). Ceppellini (1964), in an effort to produce strong monospecific antisers, repeatedly transfused white cells from a single donor to the same recipient. He divided recipients into two groups according to different transfusion schedules. The first group received 14 to 16 whole blood transfusions of 80 ml a week. Resting periods were inserted every four weeks sufficient to spread the transfusions over a full year. Twenty-seven percent of these recipients developed strong leukoagglutinins. The second group received transfusions of 250 ml of whole blood every 45 to 60 days. Twelve percent of these recipients developed strong leukoagglutinins within one year. The first schedule was considered a more efficient method of developing agglutinins, requiring 400 ml of blood to develop the first detectable leukoagglutinin. The second schedule required 750 ml of blood before an agglutinin was detectable.

The evidence that white cells are a source of transplantation antigens, is supported by their ability to sensitize a recipient against a skin graft (Friedman, 1961). Leukocyte transfusion is in fact a tissue graft, and as such could be expected to reject rapidly in a sensitized individual. The correlation of graft rejection and antibody formation should be simple, based on one antigen to one antibody relationship, but it is not. There are several technical difficulties which together make the correlation a hard task, including: non-availability of strong antisers directed against one antigen, no cell panels convenient at the time needed and test results that can not be duplicated (Ceppellini, 1964; Zmijewski, 1965).

Leukocyte antibodies can be detected by several different techniques, however, the leukoagglutination and lymphocytotixic methods have been used more extensively and have been more intensively studied (Zmijewski, 1966).

In response to this problem, an attempt was made to correlate the agglutinating and cytotoxic antibodies (Bodmer, et al. 1966). Leukocyte antisers were tested against a white cell panel using both leukogglutination and lymphocyte cytotoxic techniques. They found good agreement between the two assays, but with consistently more positive results using the agglutination test (X^2 = 85.3 with 1 df). When differences existed, the antisers usually had very strong agglutinating properties. Cells sufficient in quantity to remove the agglutinating activity also removed all cytotoxic activity. The differences between the two tests could be attributed to the sera, which were selected on the basis of their agglutinating activity. Ceppellini (1965b), however, said that no correlation should be drawn between the EDTA-type leukoagglutinins and any of the antibodies detected by cytotoxicity. Zmijewski (1966) is in general agreement with this opinion, as he concluded that many sera could have both agglutinating and cytotoxic activity which is directed against different antigens. Use of the cytotoxic methods as a test of choice has become more prevalent since organ transplantation problems have been realized. Walford (1964) suggested that cytotoxicity is a better method to detect lymphocyte antigens than leukoagglutination. Some papers have stressed that both agglutination and cytotoxic methods

must be used when testing for histocompatibility, at least until a more definite association can be drawn between the antigens detected by the two tests (Zmijewski, 1966).

Lymphocyte cytotoxicity is complicated by some of the same problems as leukoagglutination. In a study to better determine the relationship between the HL-A antigens, Dausset, et al. (1970), confirmed their earlier findings (Dausset, 1968) that the HL-A region of the human chromosome includes at least two subloci, with at least six antigens determined at the first sublocus and at least eight antigens at the second. Population studies have been complicated by sera which react with cells of other specificities. Such cross reactions have been observed between HL-A2 and Da15, HL-A5 and Da6, HL-A9 and Da7. A cytotoxicity-negative, absorption-positive phenomenon was observed when HL-A5 and HL-A7 antisers did not kill, but were absorbed by Da6 and Da9 cells respectively. Cytotoxicity-negative, absorption-positive reactions were first noted by Ceppellini (1965a), and are analogous to agglutination-negative, absorption-positive reactions in leukoagglutination tests. Cross absorption procedures are used when checking for monospecific antisera (Zmijewski, 1968). During a cross absorption study to select monospecific leukoagglutinins, it was observed that some cell samples selectively absorbed antibody, but were not agglutinated by it. This phenomenon has been termed agglutination-negative, absorption-positive, and is a potential source of error (Van Rood, 1961).

Agglutination Tests

Zmijewski (1968) indicated that even the best leukocyte agglutination tests are fickle techniques, complicated by results that seem to go against the facts of serology. The antibodies detected by the leukoagglutination tests react with antigens common to leukocytes, platelets, and some solid tissues.

A test should be sensitive enough to detect low concentrations of antibody, and yet not give a false positive result. Sensitivity seems to be correlated with how the white cell suspension is prepared and how long the cells are incubated with the antisers. The blood samples for most leukoagglutination methods are anticoagluated with 5% Na_2EDTA, although 10% Na_2EDTA and difibrination have also been used (Zmijewski, 1968). Leukocytes prepared from blood anticoagulated with EDTA are preferred for testing since these leukocytes show better reproducibility (80%) than the test leukocytes from defibrinated blood (50-60%). Reproducibility is determined by testing sera against the same donors' leukocytes on different days (Van Rood, 1963). Complete removal of platelets is said to reduce the number of false positives (Lalezari, 1964; Zmijewski, 1968). Van Rood (1963), however, said that platelets do not cause any complications. He based his reasoning on the fact that platelets have many of the same antigens as white cells, not taking into account the fact that the stickiness of platelets will cause a white cell suspension to appear as though it were agglutinated.

Incubation times vary tremendously with the agglutination

method used, being anywhere from 15 minutes (Payne, 1965) to 18 hours (Lalezari, 1964). Incubation time is limited by the ability of the leukocytes to retain their immunological and morphological characteristics, and this seems to be determined by the agent used to sediment the red cells. Lalezari (1961, 1962, 1964) found the use of Polybrene as a red cell sedimentor permits prolonged incubation of the white cells, whereas those preparations using dextran, gelatine, and polyvinylpyrrolidone (PVP), do not give good results when incubated any more than three hours. With long incubation times to detect trace concentrations of antibody, and the addition of excess EDTA to reaction mixtures to inhibit false positives, one must consider the possibility of false negatives occurring. More recently some of the leukoggluti-nation methods have been modified to allow micro techniques to be used (Payne, 1967; Lalezari, 1970b).

The physiologic activity of the cell donor can also affect agglutination results. Leukocyte reactivity is increased after strenuous exercise, overeating, and excess alcohol consumption. It is reduced following mild exercise, cold, or heat. For consistent results in testing, blood should be drawn from a resting, fasting subject (Zmijewski, 1967).

Cytotoxic Tests

Lymphocytotoxic methods can be used in conjunction with the agglutination tests to obtain a better estimate of the number or type of antibodies present in a serum. The cytotoxic tests have shown about the same amount of diversity prevalent in leukoagglutination tests. Cytotoxic serum is obtained from multiparous women,

multitransfused patients, and immunized subjects (Walford, 1964). Specific immunization is obtained by sensitization of a subject with leukocyte injections or skin allografts (Dausset, 1970). Cytotoxic antibodies detect antigens common to leukocytes, platelets (Lalezari, 1970) and the solid tissues. Anticoagulation of blood for cell samples has been accomplished by either defibrination (Walford, 1964), or heparinization (Terasaki, 1964; Engelfreit, 1966). Early tests obtained white cells free of erythrocytes by differential centrifugation, osmotic shock (Walford, 1964) or gelatine precipitation (Walford, 1964). Later tests have used dextran (Zmijewski, 1966; Engelfreit, 1966), PVP, PVP with specific red cell antiserum, or specific red cell antiserum alone (Mittal, 1968). Engelfreit (1966) used a suspension of mixed leukocytes for his cytotoxicity test, whereas all other tests used a suspension of lymphocytes only. Granulocytic cells can be removed by absorption on a cover glass (Terasaki, 1964), nylon fleece (Walford, 1964), or on small glass beads (Mittal, 1968). After incubation of the cells (30 minutes--all tests) with antisers at room temperature, all of the different test methods agree that exposure of the cells to complement is necessary. Cytotoxicity is determined by exclusion of trypan blue or eosin dye by living cells.

MATERIALS AND METHODS

I. Experimental Protocol

Subjects were chosen from inmates of the Utah State Prison. They were healthy men who had not received prior blood transfusion and had no history of venereal disease or hepatitis. Subjects were selected as pairs on the basis of red cell antigens and cross match compatibility (Ross, 1960). Prior to transfusion, all subjects' sera were tested for pre-existing leukoagglutinins against their respective partner. Recipients' sera were tested again 1, 6 and 13 days post transfusion for leukoagglutinins. Sera demonstrating leukoagglutinins against the specific blood donor were then tested against a panel of 11 different leukocytes. The leukocytes for this panel were obtained from normal volunteer donors.

A leukokinetic study using the *in vitro* $DF^{32}P$ labeling technique previously reported (Mauer, 1960) was done on each subject, giving the half-disappearance time of his own neutrophils from his blood (autologous $T_{\frac{1}{2}}$). Two days later a unit of the donor's blood was labeled again with $DF^{32}P$ and transfused into the recipient. The resulting half disappearance time of donor's cells from the recipient's blood (isologous $T_{\frac{1}{2}}$) was compared to the autologous $T_{\frac{1}{2}}$. When the isologous $T_{\frac{1}{2}}$ was more than 40% shorter than the autologous $T_{\frac{1}{2}}$ it was considered significantly shortened. The transfusion volume was greater than 400 ml of whole blood, thus more than 3.8×10^9 leukocytes were

given in each case.

The sera of all recipients showing an abnormally short isologous $T_{\frac{1}{2}}$, as well as those developing a leukogglutinin, were tested for lymphocyte cytotoxic antibodies. All recipients' post transfusion sera were tested against a commercial red cell panel.

II. Immunological Studies

A. Leukoagglutination Test (Lalezari, 1964).

1. Antibody Sources.

The known positive serum used in this study was obtained from National Institutes of Health (Vasques, #2-64-9-12-26-01, NIAID, Transplantation and Immunology Branch, Bethesda, Md.)

Fasting blood was collected and anticoagulated with 100th volume of 10% Na_2EDTA. Cell-poor plasma was collected after centrifugation at 1,350 G for 30 minutes. Plasma specimens were stored in 1 ml aliquots at -20° C. Plasmas have been stored for over four years with no appreciable effect on the activity of various antibodies (Lalezari, 1964). Heat inactivation of plasmas before testing is not necessary, but is serum is used complement must be inactivated by heating the sample to 56° C for 30 minutes.

2. Leukocyte Preparation.

Leukocytes are prepared at room temperature using siliconized glassware. Fasting blood is collected with a plastic syringe (usually 20 ml) and anticoagluated with 100th volume of 10% Na_2EDTA. 10 ml samples are transferred to 125X15 mm test tubes. Platelets are removed by differential centrifugation as follows: Tubes are centrifuged at 350 G for 15 minutes. Leukocytes layer on top of the red cells and

platelets remain suspended. Deceleration of the centrifuge must be slow to avoid resuspension of loosely sedimented leukocytes. The platelet rich supernate is removed completely with a pasteur pipette and placed in another test tube. The supernate is then centrifuged at 1,350 G for 30 minutes. The resulting platelet poor plasma is returned to the tube containing the red cells and leukocytes. 0.2 ml of 1% Polybrene (Abbott Laboratories, North Chicago, Ill.) in saline is added and the tube contents are mixed. The tube is tilted at about 45^{o} and left at room temperature for 30 minutes, allowing the red cells to sediment. In a swinging head centrifuge the tubes are centrifuged at 15 G for 5 minutes, which leaves the leukocytes suspended.

Contaminating red cells usually form a line on the side of the tube. These cells are sedimented by additional 5-minute centrifugations at 15 G after rotating the tube 180^{o} around its long axis. This is repeated until no red cell line forms (decelerate slowly). The leukocyte rich plasma is removed to another tube using a pasteur pipette, and the cell concentration is adjusted to about $4000/mm^{3}$ by dilution with autologous plasma. To each 9 volume of cell suspension 1 volume of 10% Na_2EDTA is added. Cells thus prepared are kept at room temperature and should be used within 7 hours from the time blood was collected.

3. Test Procedure.

The leukocyte agglutination test is performed with serial double fold dilutions of test plasmas, from full strength plasma to 1:16 dilution. 0.1 ml volumes are used, with saline as diluent in 75X12

mm nonsiliconized test tubes. 0.05 ml cell suspension is added to each dilution. Controls are plasmas obtained from the respective leukocyte donors and their own cells, and one tube containing cells and physiologic saline. The tubes are sealed with Parafilm and placed in a 37° C water bath. Results of the test are determined after 5 and 18 hours incubation.

4. Interpretation of Tests.

The tubes that are negative appear to have a clear supernate with the nonagglutinated cells forming a homogenous film on the bottom of the tube. When agglutination occurs, the agglutinated cells appear as floating white particles. Gross examination is confirmed by 100X microscopy in dim light. The reactions are graded microscopically as follows: 4+; all of the cells participate in one or a few large clumps, no free cells. 3+; aggregates are smaller and about 25% of the cells are free. 2+; smaller aggregations just visible with the naked eye, 50% of the cells are free. 1+; agglutination is not grossly visible, 75% of the cells are free. ± no aggregates but groups of 3 or 4 cells are fairly common. Negative tubes have no agglutinated cells or only occasional pairing. The type of cells involved in the agglutination reaction can be classified by spreading the suspension on a slide and staining after it has dried.

5. Comments.

Qualitative studies of leukocytes prepared with Polybrene reveal intact viability, phagocytosis, and cell mobility. In this technique lymphocytes are partially removed. Polymorphonuclear cell recovery

is over 50%. Prolonged incubation of the cell suspension with ABO incompatible plasmas containing no leukocyte agglutinins, even in the presence of EDTA, results in occasional mixed erythrocyte-leukocyte agglutination. This reaction can be distinguished microscopically from true leukoagglutination on the basis of red cells appearing in the aggregate, which does not occur with true leukoagglutination. With prolonged storage, the leukocyte reactivity diminishes, but no significant changes occur within the first 5-7 hours after a blood sample is drawn.

Leukoagglutinins are not detectable in nontransfused donors or nulliparous females. It may be assumed that a positive reaction indicates an acquired antibody (Lalezari, 1964).

B. Lymphocyte Cytotoxicity Test (modified from Terasaki, 1964).

1. Antibody Sources.

Fasting blood is drawn into clean glassware and allowed to clot. Cell free serum is obtained and is either froxen in bulk or placed in disposable polystyrene micro-droplet-testing trays (Falcon Plastics, Los Angeles, Calif.). 0.001 ml of serum is placed in the tray well, and covered with 1 drop of mineral oil. Each tray can accommodate 60 antiserum samples, and are frozen at -60° C until used (Mittal, 1968).

2. Lymphocyte Preparation.

10 ml of blood are collected and anticoagulated with 0.5 ml heparin (Heparin sodium, preservative free, River Labos, Northridge, Calif.). The blood is mixed well and placed in a large test tube with 2.5 ml of 4% polyvinylpyrrolidone (PVP, K-60, avg mol wt 160,000,

Matheson Coleman and Bell, East Rutherford, N.J.) in saline, to sediment the red cells. After the red cells have sedimented sufficiently, the supernate is drawn off with a pasteur pipette and placed into a 20 ml disposable plastic syringe packed with nylon fibers (Leuko-Pak, Fenwal Laboratories, Morton Grove, Illinois). This is allowed to sit for 20 minutes at room temperature, which allows the platelets and granulocytes to adhere to the nylon fibers. With the syringe plunger the plasma, with the lymphocytes suspended, is expressed into 15 ml centrifuge tubes. The fleece is washed twice with 5 ml Hanks' solution (Hyland Labs, Los Angeles, Calif.) and both washes are pressed into the centrifuge tubes with the plunger. These suspensions of lymphocytes are centrifugated at about 1000 G for 10 minutes. The buffy coats are aspirated with a pasteur pipette and placed in a microcentrifuge tube. The tubes are centrifuged for 10 seconds at 11,000 RPM in a micro-centrifuge (Beckman Spinco model 152), the supernate is removed and the buffy coat is washed loose by squirting Hanks' solution from a pasteur pipette. The cell suspension is placed in a 5 ml beaker and to it 0.0025 ml specific red cell antiserum is added. Red cell agglutination is accomplished by horizontal shaking for 2 minutes, after which the lymphocyte suspension is placed in a microcentrifuge tube and spun for 2 seconds at slow RPM (about 1/3 speed). The supernate is removed to another tube and the low speed spins are repeated until no contaminating red cells are seen. The supernate is removed and spun again at 11,000 RPM for 10 seconds to remove residual red cell antiserum. The lymphocytes are resuspended

in 0.4 ml Hanks' solution and the cell concentration is adjusted to 1000,000/mm^3. Lymphocytes thus prepared can be used within 24 hours without undue loss of activity, and are stored at 2-8° C until used.

3. Test Procedure.

All of the components of the test system are placed in the wells with microsyringes (Hamilton Syringe Co., Fullerton, Calif.) operated by a pushbutton repeating dispensor (Hamilton PB-100).

0.001 ml of lymphocyte suspension is placed in each well of antiserum and incubated for 30 minutes at room temperature. 0.005 ml C' is added to each well and incubated for 1 hour. The cells are stained in 1-2 minutes after adding 0.005 ml 5% aqueous eosin. The cells are fixed by adding 0.005 ml 40% formaldehyde at a pH of 7.0. Mineral oil is gently poured over the entire surface of the testing tray and the cover is replaced.

4. Interpretation of Tests.

Reactions are read at 100X with an inverted phase contrast microscope. Living cells are round and refractile, while dead cells appear dark and somewhat larger. The reactions are scored numerically. 1; negative, all cells are alive, refractile and with distinct cell membranes. 2; negative but with 10% dead cells. 4; questionable, with 50% dead cells. 6; positive, with 75% dead cells; 25% cells still viable. 8; 100% dead cells.

5. Comments.

Repeated freezing and thawing of lymphocytotoxic sera results in some decrease in antibody activity. Mittal, et al. (1968), found a 7.6% increase in negative reactions after alternately freezing and

thawing sera 7 times. Prozone effects are evident in about 10% of sera tested, and may be due to anticomplementary factors in the undiluted serum. For this reason the large volume of C' is added to the incubation mixture.

Cell concentration is critical, 1000 lymphocytes being the optimal number per well. After eosin is added to the well, some time must elapse (1-2 minutes) before adding the formalin. False negative reactions have been observed when eosin and formalin are added simultaneously. After fixation, tests can be read within 24 hours (Mittal, 1968).

C. Red Cell Antibody Test.

1. Antibody Sources.

Serum should be obtained from clotted blood and stored at 2-8° C until used. Serum can be frozen if testing is to be delayed for long periods. Serum does not require heat inactivation.

2. Red Cell Preparation.

Red cell panels for antibody testing are best obtained commercially. For best detection of antibodies an 8 and 16 cell panel is desirable (Panocell and Panocell-16, Pfizer Diagnostics, New Yor, N.Y.). No additional processing of the red cell suspensions is needed with commerical products.

3. Test Procedure.

A detailed test procedure is included with the red cell panel when purchased. In summary, the following methods are included: For the immediate spin test, 2 drops of serum and 1 drop red cell suspension are placed in each test tube and centrifuged at 1500 RPM for 1 minute,

after which the tubes are read. 2 drops of 30% bovine albumin are then added and the tubes are incubated at 37° C for 30 minutes. The tubes are read again after centrifugation as before. An indirect Coombs test is then performed on the red cell after washing them three times in saline.

4. Test Interpretation.

Agglutination during any of the steps of the procedure indicates the presence of an antibody in the serum. The type of antibody present can be found by referring to a master list of antibodies included with the kit, and comparing the agglutination pattern with it.

5. Comments.

It has been generally accepted that the red cell antigenic system is in no way associated with the white cell antigens, however, this is still open to question and study (Dausset, 1970; Ceppellini, 1964). A transfusion reaction caused by red cell incompatibility can be detected with these panels.

EXPERIMENTAL RESULTS

I. Pretransfusion Leukoagglutination Studies

No leukoagglutinins were found in any donors' or recipients' plasmas before transfusion (Table 1). Each subject's plasma was tested against his partner's leukocytes. Controls were set up with the donor or recipient's own plasma and leukocytes and a known positive antiserum (see methods). Tests with this known antiserum produced agglutination of leukocytes in 58% of the donors (Table 1). The agglutination pattern was mixed (neutrophils and lymphocytes) up to a dilution of 1:4 and at higher dilutions consisted of aggregates of only neutrophilic granulocytes.

II. Neutrophil $T_{\frac{1}{2}}$ Studies

Of the 12 isologous neutrophil disappearance rates, 4 were more than 40% shorter than their autologous $T_{\frac{1}{2}}$ value. The variation that can be expected in the blood neutrophil $T_{\frac{1}{2}}$ value is $\pm$ 25% (Cartwright, 1964), therefore, when the isologous $T_{\frac{1}{2}}$ was more than 40% shorter than the recipient's autologous $T_{\frac{1}{2}}$ it was considered significantly shortened. Except for recipient 9, all donors and recipients had normal autologous neutrophil kinetics (Table 2, Autologous $T_{\frac{1}{2}}$), therefore, subsequent abnormal results were not because of a pre-existing abnormality.

The biphasic curve in pair number 7 is interpreted as a B type curve (Athens, 1965) in which case there is a short initial component

followed by an exponential with a normal (slower) $T_{\frac{1}{2}}$.

It can be seen that there was no positive correlation between the volume of blood infused and the appearance of an agglutinin (Table 2).

Recipient 9's abnormally short isologous $T_{\frac{1}{2}}$ is not nearly as short when compared to his unexplainably short autologous $T_{\frac{1}{2}}$.

III. Post-transfusion Leukoagglutination Studies

Three recipients developed leukoagglutinins after isologous transfusion (Table 3). An agglutinin was found in the serum of recipient 11 on day 6 and in the sera of recipients 9 and 12 on day 13. A fourth subject's serum exhibited equivocal activity at 24 hours (number 2). In no case was a positive result seen that early.

Of the reactive sera, number 9 exhibited the strongest activity, a 2+ reaction at 13 days post-transfusion. This agglutinin caused 25% of the leukocytes of the leukocyte panel to agglutinate. Only the full strength serum showed any activity against the cell panel, while specific donor cells gave a + reaction at 1:2 dilution (Table 4). The other two positive sera, numbers 11 and 12, agglutinated only the specific donor leukocytes. Only the full strength sera of recipients 11 and 12 showed agglutinating activity. All agglutination reactions were of the mixed type, involving neutrophils and lymphocytes, which is consistent with the observations of Zmijewski (1968), who observed that lymphocytes will agglutinate in the presence of a minor population of agglutinating neutrophilic cells. Lymphocytes in pure suspension are not agglutinated by leukoagglutinins (Zmijewski, 1968).

IV. Lymphocyte Cytotoxicity Studies

No lymphocyte cytotoxic antibodies were found in the post-transfusion sera of the recipients forming leukoagglutinins. These sera (numbers 2, 9, 11, 12) were tested against their respective cell donors and a panel of lymphocytes. Lymphocytes for the panel were obtained from normal blood donors. Though 0.001 ml of serum is normally used, this test was set up using 0.001 and 0.002 ml of serum for each specific lymphocyte suspension tested. This was done to increase the chance that any low titered antibodies present in the serum would be present in sufficient quantity to react with the lymphocytes in the well.

V. Red Cell Antibody Studies

No red cell antibodies were found when post-transfusion sera were tested against 8 and 16 cell commercial screening panels (Panocell and Panocell-16, Pfizer Diagnostics, New York). All twelve recipients' post-transfusion sera were tested. Red cell agglutination was checked for microscopically after the immediate spin, warm incubation, high protein and indirect Coombs' test portions of the procedure.

Recipient 2 had a detectable but very weak incompatibility when he was cross matched with his partner post-transfusion. No incompatibility was detectable before transfusion.

TABLE 1

Pretransfusion Leukoagglutination Tests

Transfusion pair	Donor serum and cells	Recipient serum and cells	Donor cells recipient serum	Donor serum recipient cells	Positive control sera with donor cells 1:2	1:4	1:8	1:16*
1	-	-	-	-	2+	+	+	-
2	-	-	-	-	+	2+	-	-
3	-	-	-	-	-	±	-	-
4	-	-	-	-	-	-	-	-
5	-	-	-	-	+	+	±	-
6	-	-	-	-	-	-	-	-
7	-	-	-	-	+	-	-	-
8	-	-	-	-	-	-	-	-
9	-	-	-	-	2+	3+	+	±
10	-	-	-	-	-	-	±	-
11	-	-	-	-	2+	2+	+	-
12	-	-	-	-	+	±	-	-

*Dilutions of serum Vasques

TABLE 2

Neutrophil $T_{\frac{1}{2}}$ Values Following Autologous and Isologous Transfusion

Transfusion pair	Autologous Donor $T_{\frac{1}{2}}$ (hrs.)	Recipient $T_{\frac{1}{2}}$ (hrs.)	Recipient isologous transfusion $T_{\frac{1}{2}}$ (hrs.)	Significantly shortened $T_{\frac{1}{2}}$	Recipient isologous transfusion volume (ml)
1	7.3	9.0	5.0	Yes	481.0
2	5.5	5.7	6.6	No	578.5
3	7.0	4.7	4.6	No	500.0
4	5.8	6.3	4.8	No	623.5
5	7.0	7.7	5.2	No	619.5
6	8.0	5.0	5.0	No	568.7
7	9.0	5.0	$\frac{1}{2}$ & 6*	No	507.0
8	5.7	6.5	6.3	No	534.5
9	6.6	2.3	1.5	No	562.0
10	11.6	9.3	4.3	Yes	409.0
11	8.6	7.5	4.2	Yes	515.0
12	7.75	10.2	1.0	Yes	530.5

*Biphasic curve, see Discussion for explanation.

TABLE 3

Appearance of Serum Leukoagglutinins Following Isologous Transfusion

Recipient	24 hr. Post-* transfusion	6 Day Post-* transfusion	13 Day Post-* transfusion
1	-	-	0
2	±	0	0
3	-	0	0
4	-	-	0
5	-	-	0
6	-	0	0
7	-	0	0
8	-	-	0
9	-	0	2/
10	-	-	0
11	-	/	0
12	-	±	/

* Donor cells vs donor plasma, recipient cells vs recipient plasma and recipient cells vs donor plasma controls were always positive.

Reaction key: 0 = not tested

TABLE 4

Leukoagglutination Results of Reactive Sera* against Two of the Donor Leukocytes and a Random Leukocyte Panel**

Random leukocyte or donor	Recipient serum number 9	11	12
Donor 9	2+	-	-
Donor 12	+	-	+
A	±	±	±
B	-	-	-
C	+	-	-
D	+	-	±
E	-	-	-
F	-	-	-
G	-	-	-
H	±	-	-
I	-	-	-
J	±	±	-
K	-	-	-

*See Table 3 **Specific donor leukocytes for serum 11 were not available.

DISCUSSION

I. Experimental Results

No leukoagglutinins were found in the pretransfusion serum of any subject used in this study. These results support those of Lalezari (1964) who noted that pregnancy or precious blood transfusion were necessary prerequisites for agglutinin formation. Only one subject (donor 9) out of the twelve pairs used admitted to previous transfusion. The fact that his serum contained no agglutinin is not surprising when compared with the experiments done by Ceppellini (1964), who over a period of one year transfused as much as 1200 ml of whole blood in multiple small doses. Many of his recipients failed to develop agglutinins against the donor.

The strong known antiserum (see results, Table 1) produced agglutination of 58% of a donor cell panel. The reaction pattern of mixed agglutination at dilutions less than 1:8 and neutrophils only at higher dilutions, supports previous findings with neutrophil specific antiserum (Lalezari, 1970). There was an apparent prozone effect caused by the concentrated antiserum (Table 1, 1:2 dilution of antiserum with donors 2 and 9).

Previous studies have indicated that 7 of 22 isologous neutrophil $T_{\frac{1}{2}}$s were abnormally short (Mauer, 1960; Kauder, 1965), which correlates well with the 4 of 12 of this study (Table 2).

A 'B' type curve (Table 2, recipient 7) is thought to be caused

by an influx of cells from the bone marrow to the circulation which will dilute the $DF^{32}P$ labeled cells, or a reutilization of the label from cells that are in some way disrupted. A comparison of columns 2, 3 and 4 of Table 2 suggests that donor neutrophils transfused to an isologous recipient have a $T_{\frac{1}{2}}$ similar to the recipients' autologous $T_{\frac{1}{2}}$, when the recipients' isologous $T_{\frac{1}{2}}$ is not changed. The transfused leukocytes behave as though they were the recipients' own leukocytes.

Recipient 9 (Table 2) had an abnormally short autologous $T_{\frac{1}{2}}$. He is a confirmed drug addict and was taking drugs i.v. just previous to the study. The negative medical history that qualified him as a subject should be suspect, as should his apparent normal physiology.

The leukoagglutination test of Lalezari is highly reproducible. The results listed in Table 4 were obtained 4-6 months after those in Table 3, and show that reactions of positive sera with specific donor leukocytes are not changed. The ± reaction of recipient 2 (Table 3) was also ± at this later testing, while having a normal autologous control. Weak reactions in control tubes were not seen. If agglutination does occur in control tubes it can be eliminated by having a low leukocyte concentration ($4000/mm^3$) and by adding extra EDTA to the cell suspension. The weak (±) reactions seen with recipient 2, 12 (Table 3), and with several members of the leukocyte panel (Table 4) are considered not to be positive. This is because ± reactions tend to be non-reproducible and inconsistent as well as nonspecific (Lalezari, 1964). Note, however, that recipient 12

showed ± reactivity of day 6 and developed a positive result on day 13. The higher titered agglutinin seen in recipient 9 may be the result of the isologous transfusion or an anamnestic response to previous sensitization. His serum agglutinated 25% of the leukocytes of the cell panel and the specific donor leukocytes, but not his own leukocytes.

In three subjects leukogglutinins were formed post-transfusion and on testing no lymphocyte cytotoxic antibodies were detectable in these same sera. It has been reported by other workers that leukoagglutination reactions can take place using a specific serum without cytotoxic antibodies being demonstrable (Walford, 1969). These leukoagglutinins probably detect antigens different from those detected by the cytotoxic technique, as suggested by Zmijewski (1966).

The weak red cell incompatibility found in recipient 2 post-transfusion seems to have no affect on the leukocytes. Post-transfusion leukocyte agglutination studies were not positive and his isologous $T_{\frac{1}{2}}$ was normal.

II. Appearance of a Leukoagglutinin and the Possible Correlation with Shortened Isologous $T_{\frac{1}{2}}$

There is an 85% chance that antigenic differences between donor and recipient are responsible for shortened isologous $T_{\frac{1}{2}}$ values (Table 5, $p = 0.15$). Two-thirds of the recipients developing a post-transfusion agglutinin had a shortened $T_{\frac{1}{2}}$, while 2/4 of the recipients with a shortened $T_{\frac{1}{2}}$ value developed leukoagglutinins (Table 5). There is an inherent sensitivity difference between the leukokinetic test and the leukoagglutination test, in that the leukokinetic test

is done *in vivo* while the agglutination test is done *in vitro*. It is well established that the body is a much more sensitive detector of physiologic and biochemical differences, as evidenced by graft rejection, transfusion reactions, etc. Therefore, it could be that the *in vivo* leukokinetic test detected a difference between donor and recipient that was not significant enough to cause a detectable agglutinin to be formed. In his attempt to produce leukoagglutinins Ceppellini brought out several significant facts. Even though a recipient develops no detectable leukoagglutinins after transfusion he is still sensitized, as evidenced by a second set rejection of a tissue transplant from this same donor. "In other words, not all histocompatibility factors manifest themselves as leukoagglutinins after multiple i.v. transfusions, and graft (tissue) rejection is a much more sensitive test for detecting isoantigenic differences than the appearance of leukoagglutinins" (Ceppellini, 1964).

Blood is a tissue and when transfused to a recipient could be thought of as an erythrocyte and leukocyte graft. The $T_{\frac{1}{2}}$ could be a measure of neutrophil 'graft' rejection.

There are several suggestions that would make a future study more definitive. A stable population should be chosen for the study that could be available for up to 2 years. A larger more statistically significant population should be used (expensive) with the pairs being used two, three, or even four times for transfusion on a suitable schedule. In fact in this study an attempt was made to follow up the short $T_{\frac{1}{2}}$ in recipient 10. Pair 10 was taken through an autologous study again, and were to be used in another isologous

study, in an attempt to stimulate agglutinin production. However, they took a leave of absence before this could be accomplished (unauthorized leave).

Such future follow up studies would allow the possible formation of leukocyte antibodies to be detected, as well as additional effects of the leukokinetic study.

TABLE 5

Correlation* of the Development of a Leukoagglutinin and a Shortened ** $T_{\frac{1}{2}}$

Recipient	$T_{\frac{1}{2}}$ Significantly* Shortened	Leukoagglutinin Demonstrated
1	Yes	No
9	No	Yes
10	Yes	No
11	Yes	Yes
12	Yes	Yes

*By 2 X 2 X^2, p = 0.15.

**Isologous $T_{\frac{1}{2}}$ more than 40% shorter than autologous $T_{\frac{1}{2}}$.

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