1974

Isolation of Rabbit Lymphocytes and Serological Study of Their Alloantigens

Michael I. Luster
Loyola University Chicago

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by

Michael I. Luster

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University of Chicago, in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

May

1974

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INTRODUCTION

The rabbit is an ideal animal to study the problems of transplantation for a number of reasons: (1) Skin grafting experiments in the rabbit (18) have revealed that the rabbit, like man, is unique in that the major histocompatibility locus is not a blood group locus. In all other experimental animals so far tested, only the rabbit and the dog have this feature in common with man (30); (2) The rabbit is large enough so that sufficient serum and cells may be obtained for studies of individual animals, whereas much of the data obtained from mouse experiments has required the use of pooled cells and/or serum; (3) The rabbit is suitable for surgery (16) and; (4) The genetic control of rabbit serum proteins has been extensively studied (43). Hence the relationship, if any, of the rabbit lymphocyte allotypes to the allotypes of rabbit IgG-immunoglobulin, IgA-immunoglobulin, IgM-immunoglobulin, 2M-macroglobulin, low density lipoproteins and transferrin molecules can readily be determined.

The characterization and chemical isolation of the lymphocyte antigens, especially the HL-A antigens, have become an active field of investigation in the last ten years (44). With the actual delineation of a number of lymphocyte isoantigens, and with the concurrent clinical

preoccupation with organ transplantation, interest has centered on the problem of preventing allograft rejection. From the standpoint of making organ transplantation practical, much of the research is directed toward obtaining purified antigens which might be applicable for the induction of tolerance. Studies on the immunochemical characterization of the lymphocyte antigens are also important from the standpoint of providing information regarding structural concepts of the genetic region controlling the synthesis of the lymphocyte antigens.

In this thesis a method is described for the isolation and purification of circulating rabbit lymphocytes utilizing a combination and modification of a number of known techniques. The techniques include: removal of the vast majority of erythrocytes by an appropriate sedimentation agent (88); passage of the white cell-rich supernatant through a glass bead column in order to remove glass adhering cells such as platelets and granulocytes (64,78); and the removal of remaining erythrocytes by a mild red blood cell (rbc) lysing agent (1). This technique resulted in the isolation of up to 52% of the circulating lymphocytes from whole blood with little contamination with red blood cells or other white cell types.

Studies were next performed to determine whether

the isolation procedure selected for a particular lymphocyte cell type. Vast evidence has accumulated indicating the existence of two lymphocyte types in peripheral blood, the bone marrow-derived or B-cell and the thymus-derived or T-cell (23). It appears that both cell types are required in the immune response (56). Raff has detected surface immunoglobulins on the B-cell population of circulating lymphocytes in mice by means of indirect immunofluorescence but not on T-cells (67). That the isolation technique did not specifically select for B or T-derived lymphocytes was indicated by utilization of the fluorescent labeled anti-IgG immunoglobulin technique as described by Raff (65).

Isoantisera were then prepared by deliberate immunization with purified allogeneic lymphocytes. The isoantibodies were identified by a modified Terasaki cytotoxicity test (86). Since lymphocytotoxic antisera are often multispecific, two methods were utilized to determine the degree of specificity of the most potent isoantisera produced. These methods included serological typing against a large population of animals and cross-adsorption studies. These studies indicated that the cytotoxic antisera in question were monospecific or nearly monospecific.

After the question of specificity of the antisera was resolved, the phenotypic profiles of the defined lymphocyte antigens were determined in 38 rabbits. The results indicated the existence of at least one major lymphocyte alloantigen locus which controlled at least three antigenic determinants.

The relative concentration of one of these alloantigens (Ly-1) was determined in various organ systems. The highest concentration of antigen was found in the spleen followed in descending order by the lymph nodes, lung, skin, kidney, heart and liver. Erythrocytes and fat cells contained little, if any, antigen. In addition, serological typing studies suggested a possible relationship in the genetic control of antigenic determinants on immunoglobulin molecules and lymphocyte alloantigens. However, the anti-lymphocyte antisera failed to form any precipitin arc when tested against sera from rabbits that possessed the respective lymphocyte alloantigens in the Ouchterlony test. In addition, anti-immunoglobulin allotype antisera when incubated with leukocytes failed to destroy any cells as demonstrated by the cytotoxicity test.

The purposes of this study are to immunologically define the rabbit lymphocyte alloantigen system and to determine whether it could be used as a model system for the study of lymphocyte alloantigens in man. A method was

developed to isolate lymphocytes from the blood of rabbits. These lymphocytes were used to produce a number of cytotoxic isoantisera. Subsequently, the isoantisera were utilized to obtain the relative distribution of the lymphocyte alloantigen(s) in a rabbit population as well as their relative concentration in various organs. Finally, the relationship of these lymphocyte alloantigens to immunoglobulin allotypes was investigated.

LITERATURE REVIEW

I Methods for Isolation of Lymphocytes

Numerous methods have been developed for the isolation of human lymphocytes from peripheral blood. The vast majority of these separation techniques rely on the physical characteristics of the cells such as adherence properties (31), size (63), density sedimentation rate (78), phagocytic activity (48), or the electrophoretic mobility (95).

The physical properties of the cells usually determine the experimental design of choice. In addition, one must consider the ultimate use to which the purified cell population is to be put. In this study, purified lymphocytes were required for isoimmunization between various rabbit strains. The techniques used to provide lymphocytes to be used for this purpose should meet the following criteria: 1) a sufficient number of lymphocytes must be obtained to insure a representative population; 2) little, if any, contamination by other cell types should occur, and; 3) maximum viability of lymphocytes should be retained.

A combination of techniques were utilized to take advantage of differences both in sedimentation properties and adhesive properties of rabbit blood cells. Rbc and

granulocytes removed by density sedimentation with an appropriate gelatin and by subsequent passage of the cell preparations through glass bead columns. Rbc, which have a specific gravity of 1.092 sediment more rapidly than leukocytes which have a specific gravity of 1.065 (73). After incubation of heparinized blood with an appropriate sedimentation fluid, erythrocytes are found to sediment to the lower one-third of the test tube while approximately 75% of the leukocytes are found in the top two-thirds of the tube (64). More than 95% of the lymphocytes can be shown to be viable in all of the examined systems as observed by exclusion of trypan blue dye (62).

In contrast to sedimentation, adherence depends on the metabolic activity of the cell, and in addition requires the presence of calcium and magnesium. This adherence effect has been exploited by running blood cells through glass bead, glass wool or nylon columns, which retain granulocytes and platelets but allow lymphocytes to pass through (31). Following passage through glass bead columns, approximately 70-80% of the lymphocytes originally placed in the columns can be recovered, while 80% of the recovered lymphocytes remain viable (78).

II Methods for the Detection of Lymphocyte Isoantibodies

Once lymphocytes are prepared and used for isoimmun-

izations a number of techniques are currently available for detection of the anti-lymphocyte antibodies. The three most commonly utilized techniques include platelet or leukocyte complement fixation (80), leukoagglutination (14), and cytotoxicity testing (86).

Complement fixation tests have been primarily applied to studies in which antisera against platelets are tested and only rarely employed when the antisera against lymphocytes are tested. Complement fixation is the least sensitive albeit one of the most quantitative techniques for measuring histocompatibility antibodies. The test can detect high-titered antibodies which, under these conditions are apt to behave as if they are monospecific.

Although the reproducibility of positive leukoagglutination tests is 95%, the reproducibility of negative tests is only 66% (14). The major difficulty encountered with the leukoagglutination test is nonspecific clumping. This occurs as a result of a number of factors such as the increased adhesiveness of the cells due to gelatin which is used in the isolation procedure, adherence of the leukocytes to microscopic debris and variation of leukocyte stability in blood samples drawn at various times. Certain physiologic states such as mild excercise or overeating may lead to reduction in leukocyte reactivity and to false

negatives, whereas excessive exercise leads to false positives (93).

Cytotoxicity methods for detection of lymphocyte antibodies were first applied by Terasaki and McClelland (86). In this test, lymphocytes are obtained from heparinized or defibrinated blood. The cells are incubated in an excess of immune sera. The cell-antisera mixtures are centrifuged in a tube or allowed to settle in the well under oil. The cells attach to the glass bottom of the well and this allows the cells to be brought from a volume to a plane surface so that the test can be read. In the cytotoxicity test the percentage of damaged cells increases as the cell concentration is decreased in the presence of a constant amount of antibody and complement (12). For this reason all tests, including negative controls, should be run in triplicate. The tests are usually read by adding a dye such as eosin-Y or trypan blue (87). Viable cells remain unstained while nonviable cells take up the dye which is presumably an indication that they have been damaged by antibody-complement reaction.

Although Terasaki's microcytotoxicity test is currently the most accurate, reproducibility is still no greater than 90 or 95%. This is primarily due to the time factor involved in the reading of the test. Since

a number of factors cause lysis of cells when viewed under a microscope, insufficient time exists to perform complete cell counts; instead, only partial cell counts (20-30 cells) or subjective readings are made.

III The Major Histocompatibility Antigens in Various Species

Substantial evidence has accumulated in recent years that the alloantigens found on circulating lymphocytes are also histocompatibility antigens (77). Histocompatibility antigens are responsible for the immunological response against transplanted grafts. This rejection phenomenon is due to the antigenic variation of these histocompatibility antigens within individuals of a particular species which results in an immunological response.

A comprehensive review of the major histcompatibility antigens in different species would be much too lengthy to discuss in this thesis. Instead, a brief review limiting the discussion to pertinent aspects of lymphocyte antigens will be presented. The primary goal in this discussion is to indicate the basic similarities and differences in both the control and expression of lymphocyte antigens in the various species which have been studied as a model system for man. Secondly, the significance of elucidating the histocompatibility system of the rabbit will be discussed with respect to the use of the rabbit system as a model system

to study man.

A The Human Lymphocyte Antigen (HL-A) System

In man, the majority of established lymphocyte alloantigens are known to form a single complex, designated as HL-A. The HL-A specificities segregate as units in family studies. The HL-A system, like the animal systems to be described later reveals extensive polymorphism, multiple allelism, cross-reacting antibodies and possible gene interactions.

The HL-A system was first demonstrated by Payne who detected the presence of leukocyte isoantibodies in sera from pregnant women (59). These isoantibodies identified an allelic pair of antigens which were designated LA-1 and LA-2. Dausset demonstrated the existence of an antigen on leukocytes which was subsequently designated Mac (20). During the first half of the 1960's, numerous antigens belonging to the same system were described (51). Among the most ambitious investigators were van Rood who identified over fourteen antigens and Dausset who described ten leukocyte antigens, characterized by a very high frequency of positive and negative association with one another (21,22,69).

In order to alleviate the dilemma which occurred when identical antigens were given more than one designation,

a group of the leading workers met and compared the reactions of their antisera against a defined cell panel (13). It was at this international workshop that a uniform notation and the term HL-A was adopted.

Data obtained as a result of this workshop and from later studies which utilized operationally monospecific antisera (13) revealed that the major leukocyte antigens were determined by two closely linked loci at a single chromosomal region. The two genetic regions are referred to as the LA and 4 subloci or more commonly the 1st and 2nd series. Preliminary evidence has indicated the existence of a third subloci (85).

The 1st and 2nd series have subsequently been found to be responsible for a set of mutually exclusive alleles. The evidence to support this is as follows: first, each individual tested contains only four of the major antigenic determinants, two from each parent; secondly, the genetic information for two of these antigenic determinants segregate independently from the corresponding haplotype. There are 11 and 16 alleles in the 1st and 2nd series respectively (51). It has been estimated that 2% of the genes in the 1st series and 6% of the genes in the 2nd series are as yet unidentified (22). Taking these genes into account, it is possible to generate 127 different HL-A haplotypes in the general population which, in various combinations,

could be expressed as some 8,000 different genotypes (44).

Since the synthesis of human histocompatibility antigens is under the control of a multiple allelic system, it is essential that monospecific reagents be used to determine the complexities of the system. It is, however, very difficult to obtain such monospecific antiserum. Much of the antisera is obtained from multiply transfused individuals and these antisera are usually very polyspecific. Somewhat more specific antisera can be obtained from multiparous women, in which case it is assumed that these women were immunized by the leukocytes of the fetus which express the HL-A haplotype of the father. It is possible to produce lymphocytotoxic antisera of narrow specificity in man and various animals by selective immunization and/or skin grafts followed by selective adsorption (89). Monospecificity for the antiserum can then be established either by showing that the antisera can be completely adsorbed by all cells in a test panel in cross adsorption studies or by showing that the sera react in a similar way with a known cell panel (93). The ideal situation is, of course, to utilize both methods.

In general, it is very difficult to prove that two antisera are completely identical and it is often necessary to compare the results obtained by typing with individual reagents. To demonstrate that the two antisera are, in fact,

similar, the two antisera are usually first typed against the same panel of cells and coefficients of correlation are determined (60). It should be apparent, therefore, that present methods to describe the HL-A system are often inadequate since they are based on studies which have utilized antisera which may be neither monospecific nor identical in reactivity.

Studies of the relative distribution of alloantigens on human organs have been hampered due to a limited supply of operationally monospecific antisera. However, through the combined studies of a number of investigators it has been surmized that the HL-A antigens may be present on all tissues except erythrocytes. Operationally monospecific antisera has been used in adsorption studies to demonstrate the presence of HL-A antigens on platelets, placenta, kidney and spleen (70). Berah has shown that the HL-A2 antigen is present on the spleen in highest concentration followed in order by lung, liver, intestine, kidney and heart. No HL-A2 antigen could be demonstrated in the brain (7).

B Experimental Animals Which Have Been Used as a Model System for the Study of Lymphocyte Antigens in Man

1) B System of Chickens

The major histocompatibility system in chickens is

referred to as the B system and was originally found on chicken rbc (52). Through utilization of the lymphoagglutination technique with anti-B antisera, the antigen was also found to be present on circulating lymphocytes (74). The B system is a highly polymorphic immunogenetic system composed of at least 12 alleles controlled by one genetic locus (52). The system is further complicated by the fact that each allele produces a number of serological specificities, presently referred to as antigens. That the B system is highly polymorphic is evidenced by the fact that no two distinct chicken breeds have been found which possess the same antigen. Moreover, in non-inbred populations, no two identical individuals have been found (38). Alloantisera obtained by cross-immunization with leukocytes or lymphocytes have been found to be extremely polyspecific. Adsorption of the antisera with randomly chosen cells decreased the activity of the antisera but did not totally remove all antibody activity (38).

2) H-1 of Rats

Cross-adsorption and typing studies with anti-allotype antisera have revealed the existence of a major histocompatibility system in rats controlled by a single locus. This system has been designated H-1 by Stark (83), and Ag-B by Palm (58). The H-1 locus has been found to

consist of 9 alleles which produce a total of 21 different antigenic specificities. The number of antigenic specificities produced by each individual allele ranges between 4 and 10. Like the majority of histocompatibility systems from other species, the antigens were detected on erythrocytes. The histocompatibility antigens were later demonstrated to be found on lymphocytes by utilization of the lymphocyte cytotoxicity test (83).

3) Histocompatibility in the Dog

To demonstrate the occurrence of lymphocyte alloantigens in dogs, an approach similar to that used in humans has been utilized. Cytotoxic antisera were produced by injection of allogeneic lymphocytes and two alloantigens were subsequently identified (17). The distribution of these two antigens in various dog populations indicates the existence of one major lymphocyte antigen system. Compatibility for the two antigens correlated with skin graft survival. However, as in humans, the blood group locus in dogs is not part of the histocompatibility locus (30).

4) H-2 of Mice

The most widely studied histcompatibility system is the H-2 complex in mice. The H-2 system was originally discovered by Gorer (34) while working with four blood

group antigens in inbred strains of mice. One of these erythrocyte antigens was found to be on numerous tissues and was partially responsible for transplanted tumor rejection (35). These widely distributed antigens were later designated H-2 antigens and were found to be controlled by a gene complex located on the 9th linkage group (36). This gene complex appears to consist of two loci designated H-2D and H-2K (81).

In inbred strains of mice it has been shown that there are at least 21 distinct chromosomes. The H-2K region has at least 10 alleles while the H-2D region has at least 11 unique alleles. In wild mice 30 chromosomes, alleles of which are not yet assigned to either the D locus or K locus, have been determined (47). These 30 chromosomes found in wild mice are distinct from the 21 chromosomes found in inbred strains resulting in a total of at least 51 distinct chromosomes.

When tested with monospecific antisera against H-2 antigens of inbred strains, the wild mice were shown to possess antigens which can be divided into two classes, private and public (45). Private H-2 antigens occur with very low frequency in the local populations of wild mice. The public antigens, in contrast, occur with high frequency both in wild mice and inbred strains. Preliminary

evidence indicates that typing for the private H-2 antigens will allow for the detection of at least an additional 20 chromosomes. Thus, studies of wild mice indicates a complexity of the H-2 system which far exceeds the complexity of other serological systems.

It has been approximated that there are 500 genes separating the H-2K locus from the H-2D locus (47). Four of these genes have already been determined and include the Ss-Slp, Ir-IgA, Ir-IgG and the Ir-1 genes. The Ss gene is responsible for the concentration of a particular -globulin in the serum. The Slp gene, which is functional only in male mice, controls the levels in sera of a closely related serum protein (79). The Ss gene is important in the study of the immunogenetics of the H-2 system because it can be used as a marker to divide the K or left hand region from the D or right hand region.

In the mouse, as well as in other species, a large number of immune response (Ir) genes exist which play an important role in the activation of immune responses to various antigens (64). Most of these Ir genes appear to be linked to histocompatibility genes. The first of the immune response genes discovered was the PPL gene in guinea pigs, which is required for the immunological response to poly-L-lysine (49). A large number of Ir genes

have since been found in mice linked to the H-2 complex (6).

A locus, designated the Ir-IgA locus, controls the immune response to allotypes on IgA-immunoglobulins while a closely linked but separate locus, designated the Ir-IgG locus, controls the immune response to allotypes present on IgG-immunoglobulin molecules in mice (50). The Ir-IgA and Ir-IgG genes are associated only with the $H\text{-}2^b$ and $H\text{-}2^a$ alleles, respectively. Reisfield has suggested that the genes controlling the synthesis of H-2 and IgA allotypic determinants are linked (68). A third gene, designated Ir-1, has been shown to control the immunological response to various synthetic polypeptides (6). The Ir-IgA, Ir-IgG, and Ir-1 genes are all located between the H-2K and H-2D loci (6).

Histocompatibility antigens are important because they play a primary role in the immunological response to organ transplantation. This immunological response is due to a combination of their allotypy and ubiquitous distribution in the body of an individual. It is, therefore, important in regards to transplantation studies to determine the relative distribution of these antigens on various organs in representative individuals. Various qualitative bioassay procedures have shown that the H-2 antigens are present on most, if not all, of the tissues and organs

of the mouse including erythrocytes (55). Subsequent studies have revealed large differneces in the amounts of H-2 antigens in various tissues and organs (3,39).

These investigations, however, were not always conducted using operationally monospecific antiserum against known alleles of both the H-2^d and H-2^k loci, nor were the same methods of testing utilized. For example, Pizarro tested various mouse organs for the presence of H-2 antigens by agglutination and was only able to place them into three groups (61). The first group contained the largest quantity of antigen and included spleen and liver. The second group contained an intermediate quantity of histocompatibility antigen and included kidney and skin. The third group, consisting of testes, muscle and brain, contained little, if any, antigen. In a more recent study in which operationally monospecific antiserum were utilized, Heberman has confirmed that all of the H-2 antigens tested in various organs showed a similar pattern of distribution (39). The H-2 antigens were found in the highest concentration in spleen followed by liver, lymph nodes, lung, kidney, and skeletal muscle in descending order. No detectable amount of any H-2 antigen was found in the brain or fat.

5) Rhesus Monkeys

Balner and Dersjant (2) have utilized anti-allotype antiserum produced by random immunization to identify five alloantigens in Rhesus monkeys. Mating studies have indicated that these 5 alloantigens are alleles at a single locus. Skin grafting experiments have indicated that these alloantigens are responsible for tissue rejection and they have thus been termed histocompatibility antigens.

Bogden (9,10,11) has produced a large spectrum of "leukocyte typing" reagents in the Rhesus monkey by isoimmunization with allogeneic leukocytes. Several of these reagents appeared to be monospecific as shown by extensive cross-adsorption studies; however, the vast majority of these reagents were polyspecific. Although 12 unique antisera were identified, a number of animals in the population tested did not react with any of the leukocyte antisera. Bogden and Gray have shown the presence of the antigens determined by the anti-leukocyte antisera on lymphocyte membranes and erythrocytes of the monkey.

Like Balner, Rogentine (72) recently identified five alloantigens in Rhesus monkeys. However, Rogentine's results indicate that the 5 alloantigens behave as a part of a single genetic system that can be subdivided into two closely linked segregated series.

6) Other Mammalian Species

In addition to the animals previously discussed, limited studies have been undertaken of the histocompatibility systems in the guinea pig, hamster, cattle and swine. The major histocompatibility system in these animals has been shown to be characterized by extensive genetic polymorphism (40). Moreover, the major histocompatibility antigens in each of these species have been shown to be part of the major blood group system (5,90). The elucidation of histocompatibility systems in both guinea pigs and hamsters have been hampered due to the inability to evoke a strong immunological response when attempting to produce cytotoxic antisera against the lymphocyte alloantigens (40).

7) Histocompatibility in the Rabbit

Only recently has the interest initiated by Medawar (54) concerning the immunogenetics of transplantation in the rabbit been renewed. Demant (24) has used random skin allografts on rabbits to produce a number of alloimmune sera as shown by cytotoxic tests on a panel of rabbit cells. Sera which reacted similarly were grouped and seven antigens were thus determined. The distribution pattern in the panel of cells tested indicated the existence of a major histocompatibility locus. In addition, Demant (25) found longer

survival time of maternal versus paternal skin grafts in newborn rabbits. Compatibility for the defined specificities correlated with prolonged survival of maternal skin grafts, while accelerated rejection was observed in incompatable donors.

Black (8) has prepared a panel of isoantisera by injecting allogeneic rabbit spleen cells in adjuvant into rabbits. Most of the antisera were of narrow specificity and appeared to demonstrate antigens at one genetic locus. There were at least three alleles which could occupy this locus. Skin graft survival appeared to be correlated with compatibility for the described specificities.

A partially inbred colony of rabbits was used by Tissot and Cohen (88) to produce a large number of cytotoxic antisera. Multiple skin allografts were performed between allogeneic rabbits. The antisera produced detected antigens controlled by a single genetic locus. This locus, which controls a complex series of antigens, has been designated the RL-A locus and is probably the same locus described by Black (8).

Although the RL-A locus appears to be the major histocompatibility locus in the rabbit, data presented by Tissot and Cohen (88) indicates that other minor loci are also involved in skin graft rejection. Since their

attempts to produce monospecific antisera by specific adsorption were not successful and since a large number of identical antisera exist, they suggested that these antisera may be already monospecific. No attempts were made to quantitate the number of alleles controlled by the RL-A locus since the rabbits used in Tissot's colony were inbred and did not represent a true random population.

IV Concluding Remarks

It is apparent then, in all of the histocompatibility systems studied, the number of histocompatibility loci and the corresponding number of alleles which can be detected are determined and limited by the experimental approach and general definitions utilized by the particular investigator(s). For example, not only do the methods for producing and defining cytotoxic antisera vary but there are also numerous methods used to determine positive and negative reactors once the antiserum has been produced. For as yet undetermined reasons, the methods used to test isoantisera against positive and negative reacting cells (e.g., lymphoagglutination and cytotoxicity tests) seldom result in identical patterns. In addition, terms such as alleles, antigens and specificities have never been well defined. Even with these obstacles, however, several

generalities can be observed. First, in all animals studied to date, there appears to exist more than one histocompatibility system; however, only one of these evokes a strong immunological response as a result of isoimmunization. This particular system has been designated the "major histocompatibility system" (MHS). Secondly, in all MHS so far studied, the system was found to be highly complex and polymorphic. The final point that deserves mentioning is the fact that, in most species, the major histocompatibility locus is found to be a blood group locus. Only in the rabbit, dog and human is the primary blood group system distinct from the MHS.

MATERIALS AND METHODS

Rabbits:

Rabbits used in these studies were obtained from several sources. Fifteen of the rabbits were obtained from Dr. Sheldon Dray at the University of Illinois in Chicago. These rabbits were progeny from crosses of closed colonies of Flemish giants and New Zealand Whites (FG/NZW), originally obtained from the National Institute of Health. Five ACEP and 4 C Race rabbits obtained from Bar Harbor, Maine, were also utilized. In addition, 22 rabbits were purchased from breeders in the Chicago area. These were all New Zealand Whites (NZW). All of the rabbits used were female.

Barbital Buffer:

The barbital buffer (pH 7.3) utilized in the isolation procedure was a general physiological buffer of the following composition: 8.5 g NaCl; 0.028 g $CaCl_2$; 0.185 g sodium barbital; 0.575 g diethyl barbituate and 0.668 g $MgCl_2$ in 1 liter of distilled water.

Tris-NH_4Cl (TAC) Buffer:

The TAC buffer was made by mixing 9 parts NH_4Cl (8.3 g/l) with 1 part Tris (hydroxymethyl) aminomethane

(Tris) (20.6 g/l). The Tris was adjusted to pH 7.2-7.4 with 1N HCl prior to the addition of the NH_4Cl.

Trypan Blue:

Trypan blue (Allied Chemical) was utilized as an indicator dye in the cytotoxicity test. Four ml of a 0.4% aqueous solution of trypan blue was mixed before use with 1 ml of a fivefold concentrated Hank's Balanced Salt Solution (Grand Island Biological Company).

Columns:

The columns used in the purification of lymphocytes were 10 ml disposable serological glass pipets (Kimble Products) that measured 30 cm in length by 1 cm in diameter. A column of this size was found to be satisfactory for the application and retention of up to 5 ml of cell suspension.

The columns were packed about 75% full with siliconized glass beads (Type 100-5005, 3M Co.). A thin layer of commercially siliconized glass wool (Analabs) was used for the bottom layer to prevent loss of glass beads. A piece of rubber tubing with a clamp was fitted tightly over the bottom of the pipet and used as a means of controlling flow rate.

Preparation of Glassware:

Glass beads were washed with concentrated nitric

acid followed by exhaustive tap and distilled water rinses. All glassware including columns and glass beads were siliconized with siliclad (Clay-Adams Inc.) (64). Siliconizing prevented non-specific adherence of the cells to the glass.

Collection of Blood:

When attempting to produce purified lymphocytes, a total of 30 ml of blood was collected from the marginal ear vein of a rabbit into three 15 ml test tubes, each containing 150 units of heparin in 0.15 ml physiological saline (Sigma Chemical Co.). The tubes were then mixed for 10 min. to disperse the anticoagulant.

Cell Counts:

Prior to use of purification methods, the total cell counts were determined. All cell counts were performed on a Coulter Counter "Model S" (Coulter Electronics Inc.). In addition, smears of the cell preparations obtained following each step in the purification procedure were stained with Wright's stain and checked for cell types present under oil immersion.

Methods for Rbc Sedimentation:

I Pig Skin Gelatin:

A modification of the method described by Tissot

and Cohen (88) was utilized. In this technique, 30 ml of heparinized blood was warmed to 37 C and mixed with 12 ml of 3% pig skin gelatin in physiological saline. The tubes were then placed in a 37 C waterbath at a 45 degree angle. After 20 to 30 min. of settling, the white cell-rich supernatant was harvested with a Pasteur pipet, pooled and centrifuged at 2000 RPM for 10 min. (Sorvall, GLC-1) and the supernatant discarded.

II Plasmagel:

The blood was mixed with one-third volume of plasmagel and allowed to stand at room temperature for 30 to 45 min. as described by Amos (1). The plasmagel solution (Labatoire Roger Bellon, Neuilly, France) was composed of 3.0 g of modified gelatin fluid, 0.7 g NaCl and 0.2 g $CaCl_2$ in 100 ml of distilled water. Following sedimentation of the erythrocytes, the supernatant and buffy coat were removed and centrifuged to obtain a cell button as in Method I.

III Dextran:

Dextrans (Pharmacia) possessing molecular weights of 150,000, 250,000, and 500,000 were tested as sedimentation agents (64). The technique employed was similar to that described for pig skin gelatin. Sedimentation of cells was allowed to continue until the erythrocytes had settled

to the lower one-third of the test tube and the leukocytes were then harvested by a Pasteur pipet.

Each of the sedimentation agents (pig skin gelatin, plasmagel and dextran) were tested at a concentration of 1.5, 3.0, and 5.0%. Three percent pig skin gelatin was eventually chosen for use in the isolation procedure.

The Columns:

The leukocyte-rich button obtained following the sedimentation procedure was resuspended in 0.5 ml of barbital buffer and passed through a 'dry' glass bead column. The suspension was forced into the column by means of an air pump and the column was sealed at the bottom end with a clamped rubber tube. The column was incubated at 37 C for 30 min. and the cells were eluted with 5 ml of barbital buffer warmed to 37 C. The barbital buffer was forced through the column at a flow rate of 1 ml per 30 seconds. The eluant was collected and centrifuged for 5 min. at 1,000 RPM, (GLC-1, Sorvall) to obtain a cell button.

Methods for the Removal of Contaminating Rbc:

I Distilled Water

In this method (94), the lymphocyte-rich cell button obtained following column passage was resuspended in 2 ml of isotonic saline at 4 C. In order to lyse the contaminating

erythrocytes, 5 ml of cold distilled water was added rapidly. This hypotonic suspension was agitated for 30 sec. and isotonicity was restored by the addition of 1.5 ml of cold, 4 C, fivefold concentrated Hank's Balanced Salt Solution. The cells were centrifuged at a low speed (1,000 RPM, GLC-1, Sorvall) to remove cell ghosts and the cells were resuspended in 1 ml of barbital buffer.

II TAC Buffer:

As previously described by Amos et al. (1), 6 ml of TAC buffer, previously warmed to 37 C, was added to the cell button obtained following column passage and the tube was shaken vigorously in a 37 C water bath for 4 minutes. The lymphocyte suspension was centrifuged at low speed at room temperature to remove erythrocyte stroma (1,000 RPM, GLC-1, Sorvall). The procedure was repeated using 4 ml of TAC buffer or until no rbc remained as observed microscopically. After the rbc were removed, the lymphocytes were resuspended in 1 ml of barbital buffer.

III 0.1 N HCl

The cell button was resuspended in 3 ml of barbital buffer. Seven ml of 0.1 N HCl was added to the suspension and the tube was shaken gently for 30 seconds. Then 1.1 ml of a ten-fold concentrated Hank's Balanced Salt

Solution was added drop wise while the tube was shaken constantly. The solution was centrifuged at a low speed (1,000 RPM, GLC-1, Sorvall) to remove stroma and the lymphocyte button was resuspended in 1 ml of barbital buffer.

Immunofluorescent Staining of Lymphocyte Population:

Fluorescein labeled goat anti-rabbit antiserum was obtained from Dr. C. F. Lange at Loyola University Medical Center, Maywood, Illinois. The antiserum was tested for specificity by immunoelectophoresis (IEP) with normal rabbit serum as antigen and goat anti-rabbit whole antiserum as the control. IEP plates consisted of 1.5% Noble agar in 0.05 M barbital buffer at pH 8.6. The running buffer was 0.05 M barbital buffer pH 8.6. Immunoelectrophoresis revealed a strong band in the IgG region and a weak band in the IgM region.

Cell fractions were prepared from 40 ml of heparinized blood prior to and following passage through a glass column (as described under the section entitled "Columns" in Materials and Methods). Aliquots of each fraction were smeared on two glass slides and both of the slides were stained by the Wright's method. The remainder of the aliquot from the fractions were treated with TAC buffer in order to lyse the contaminating erythrocytes and washed twice in oxoid barbital buffer. To the washed

cell suspensions, each of which contained approximately 3.0×10^4 lymphocytes in 0.025 ml, was added 0.025 ml of fluorescein labeled goat anti-rabbit IgG/IgM antiserum. The cell mixture was incubated for 30 min. at 37 C, washed three times with 1 ml of barbital buffer and resuspended in 0.05 ml of the same buffer. A drop of the cell suspension was placed on a glass slide, covered with a cover glass, and then the cover glass was sealed to the slide with paraffin (65). The slides were examined immediately with an A. O. Spencer Fluorescent microscope equipped with an Osram HBO 200 high-pressure mercury lamp and a dark-field condenser for immersion oil.

Immunization with Allogenic Lymphocytes:

Although the immunization schedule varied slightly, all animals required multiple injections of lymphocytes before an antibody response was detected as determined by the cytotoxicity test. Each injection consisted of approximately 4.0×10^7 lymphocytes. In the primary injection, the lymphocytes were incorporated into Freund's complete adjuvant and injected intradermally into the back of the necks of the recipients. A second or booster injection was given two weeks after the initial injection. For the second injection, the lymphocytes were again incorporated into Freund's complete adjuvant and injected intramuscularly

into each thigh. Additional booster injections, which consisted of lymphocytes incorporated into barbital buffer, were given intradermally into the ear. Each animal was tested for antibody production two weeks following each of the booster injections by the cytotoxicity test and then reimmunized when required. Generally at least 3 to 8 additional booster injections were required before antibody production could be detected. In the text, antisera are designated by the recipient rabbit number.

Sera:

Blood was obtained from normal and immunized rabbits by ear venipuncture. After allowing the blood to stand for 24 hours at 5 C, the retracted clot was removed, the remainder of the blood was centrifuged and the serum was separated. Complement was inactivated by heating the sera at 56 C for 30 minutes. Serum was then stored at -20 C until use.

Preparation of Lymphocytes for Cytotoxicity Testing:

Since it has been shown that granulocytes do not interfere with lymphocyte cytotoxicity testing (93) passage of cell preparations through columns were not performed in the preparation of leukocytes for cytotoxicity testing. In this procedure, 10 ml of blood were collected into a 25 ml Erlenmeyer flask which contained 10-12 glass

beads of approximately 1 cm diameter. The flask was gently shaken and the clear clot which resulted was removed. The defibrinated blood was allowed to incubate with 5 ml of 3% pig skin gelatin (Eastman Organic Chem.) in saline at a 45 degree angle in a 37 C waterbath. After 15-30 min. of settling, the lymphocyte-rich supernatant was harvested. The cell-rich supernatant was centrifuged to obtain a cell button and the supernatant discarded. Five ml of Tris-NH_4Cl maintained at 37 C was added to the button and the tube was mixed with a Vortex mixer and shaken vigorously for 4 min. in a 37 C waterbath. The suspension was lightly centrifuged to remove erythrocyte ghosts from the supernatant and the cells were resuspended in 1.0 ml barbital buffer. The Tris-NH_4Cl treatment can be repeated two or three times depending upon the extent of rbc contamination in the suspension. An almost pure leukocyte suspension can thus be obtained. The suspension was adjusted to contain approximately 10,000 cells per drop with barbital buffer.

Cytotoxicity Testing:

Cytotoxicity tests, including controls, were performed in triplicate or quadruplicate (87). To each well in the Microtest Tissue Culture Plate (3034: Falcon Plastics, Oxford, California) a drop of mineral oil was

first added to prevent evaporation. To the appropriate wells were then added either 1 or 2 l of antisera followed by 1 l of leukocyte suspension (250 cells). The antisera or cells were dispensed with a 50 l Hamilton syringe. The plate was left standing at room temperature for 5 min. after which 5 l of complement were added. The source of complement was fresh rabbit donor serum. The plate was gently shaken and then reincubated for 40 min. in a 37 C waterbath. Following incubation, one drop of barbital buffer was added to each well and left to stand for 10 min. The buffer was removed with a Pasteur pipet. This barbital buffer wash was used to remove accumulated debris as well as mineral oil from the well. One drop of trypan blue dye was then added to each well and left to stand for 10 min. The dye was flicked off with a rapid hand motion. The wells were refilled with barbital buffer and were read under a Nikon inverted phase microscope at 1000X power.

Lymphocyte Typing Studies:

The cytotoxicity test, as previously described, was utilized for serological typing of the rabbit population. Cytotoxicity tests for each animal were performed in triplicate or quadruplicate. Controls, which consisted of wells lacking in either complement or antiserum, were also

performed in triplicate or quadruplicate. Since the time factor involved in reading the cytotoxicity test is limited, total cell counts were not performed. An overall impression of the percent cell death in each well was obtained by subjective readings or by randomly counting stained and unstained cells. Cytotoxic values were then determined by averaging percent cell death over the negative control in all tests. This procedure provides a method for obtaining a measure of the relative degree of the cytotoxic reaction and the values are not intended to represent an absolute "quantitative" value. Wells which contained approximately 0-13% dead cells above that of the negative control, as determined by uptake of trypan blue dye, were considered negative (-), while 13-15% stained cells above the negative control were considered borderline (+-). Cytotoxicity tests in which there were approximately 15% or more stained cells above the negaitve control were considered positive (+).

Statistical Analysis:

The number of similar and dissimilar reactions in every possible pair of functional antisera were counted by comparing the incidence of positive and negative reactions from the lymphocyte typing studies. Fisher's 2x2 test was used to calculate the degree of association between the

antiserum pairs (60). The 2x2 tables are represented in the following:

		1st Antiserum	
		+	-
2nd Antiserum	+	a	b
	-	c	d

a, b, c, and d represent the number of observations of the four possible types of reactions. Coefficients of correlation were determined as follows:

(1) If b=c=0, the antisera are identical

(2) If b=0, serum 2 is contained within serum 1, and vice versa if c=0

(3) If ad-bc > 0, then the antisera are positively associated, while if ad-bc < 0, then the antisera are negatively associated

The Chi-square (with the Yate's correction) was used to determine the significance of the coefficients of correlation values and was calculated as follows:

$$x^2 = \frac{(\text{Observed} - \text{Expected}) - 0.5^2}{\text{Expected}}$$

Adsorption Studies:

For adsorption studies, the five most reactive antisera were diluted with saline to a point where a residual titer of 1:4 remained. Dilution has been found

essential for adsorption in leukocyte alloantigen systems (93). This was the last dilution that resulted in an easily readable positive cytotoxicity test when tested against cells of the respective immunizing donor.

Approximatley 0.4 ml of 1:4 diluted antisera was incubated with 5.8×10^7 leukocytes obtained from the original donor animal. The cell antiserum mixtures were incubated at 37 C for 1 hour followed by incubation at 4 C for 2 hours. The cell suspensions were centrifuged at 4 C for 10 min. at 1085 g. The serum was removed and tested by the cytotoxicity test for remaining antibody activity against the respective homologous cells. In all cases, the adsorption procedure had to be repeated before all antibody activity was eliminated. Controls consisting of cells which did not react with the particular antiserum as demonstrated by the cytotoxicity test were also included.

Cross-Adsorption Studies:

Since only strongly reactive antisera can be tested for relative specificity, for the reasons that will be described later, anti-2 antiserum was selected for further study. The specificity of the reactivity of anti-2 antiserum was determined by means of adsorption of the antiserum with selected allogeneic leukocytes. The donors of these allogeneic leukocytes, referred to as the test

panel, were selected on the basis that their cells revealed a high cytotoxicity index when tested against anti-2 antiserum. In addition, negative controls, which consisted of cells that did not react with the anti-2 antiserum, were utilized. These adsorption studies were performed in a manner identical to that described for the homologous adsorption studies.

Complement Fixation Tests on Rabbit Erythrocytes:

Donor rabbits whose lymphocytes induced a potent cytotoxic antisera when injected into a recipient animal were tested for the presence of the homologous lymphocyte antigen(s) on their erythrocytes. Ten ml of heparinized blood collected from each of these original donor animals were centrifuged at 1,000 RPM (GLC-1, Sorvall) for 5 minutes. The remaining rabbit erythrocytes were washed 3-4 times in isotonic saline. The top layer of red blood cells was removed after each centrifugation. A 2% solution of erythrocytes in saline was prepared for use in the test (93).

To 10 x 1 cm glass test tubes were added 0.5 ml of the 2% erythrocyte suspension, 2 ml of a 1:4 dilution of the appropriate lymphocytotoxic antisera or sheep hemolysin (Difco) and 0.3 ml of 1:30 diluted guinea pig complement (Kalstead Laboratories). Controls which

consisted of tubes lacking either complement or antisera were run with each test. The mixtures were incubated a total of 40 min. at 37 C, and were gently agitated at 10 min. intervals. The tubes were then centrifuged at 1085 g at 4 C and the degree of hemolysis determined.

Detection of a Lymphocyte Alloantigen in Organ Homogenates:

Various organs were obtained from two animals whose lymphocytes had previously demonstrated a strong positive cytotoxic reaction when tested against anti-2 antiserum. After the animals were sacrificed by intravenous injection of 7 ml of Nebutol, the following organs were removed and placed on ice: heart, kidney, liver, spleen, lung, lymph nodes, and sections of skin and fat. The organs were immediately taken to the laboratory, placed in vials and frozen by immersion in an acetone-dry ice mixture. The samples were stored at -45 C.

Prior to homogenation, the organs were thawed, freed of extraneous material, rinsed in saline and weighed. Homogenates were prepared by grinding 1-5 g of organ with a volume of saline equivalent to 5 times the organ weight. The organs were homogenized in a commercial blendor (Waring) at high speed until a homogenous suspension was obtained (generally 0.5-1.5 min.). For preparation of skin homogenates, the hair was first removed and the skin sections were placed

on top of dry ice. When the skin tissue was completely frozen (brittle) it was cut into thin sections and homogenized at high speed.

The homogenates were centrifuged at 3020 g for 10 min.. The supernatnant was removed and the cell pellet was resuspended in sterile saline. The cell pellet was recentrifuged and the saline wash was repeated until the supernatant became clear. Wet weights were then determined for each organ. The entire homogenation procedure was performed in the cold room at 4 C.

Operationally monospecific anti-2 antiserum, as determined by serological studies was diluted 1:4 with saline, and was utilized throughout these experiments. Four-tenths ml of the diluted antiserum was adsorbed with various concentrations of crude antigen suspension ranging between 4 and 500 mg. Incubation of the mixture was carried out at 37 C for 1 hour with periodic agitation of the tubes. The tubes were next incubated at 4 C in the cold room for two hours, followed by centrifugation at 1,085 g for 5 min. at 4 C. The serum supernatants were collected and tested for remaining antibody activity against a positive leukocyte cell donor by the cytotoxicity test.

Immunoglobulin Typing:

The Ouchterlony technique (32) was utilized for the determination of the immunoglobulin allotypes in rabbit sera. Double diffusion experiments were performed in 1.5% (W/V) Noble agar in 0.2 M borate, 0.15 M NaCl, pH 8.1 buffer. Monospecific anti-allotype antisera, previously produced in our laboratory, were used for typing (92). All rabbits in the population were tested for the presence of the a1, a2, and a3 allotypic specificities of the heavy chain and the b4, b5, b6, b9, c7 and c21 allotypic specificities of rabbit IgG light chains. Rabbit sera possessing known allotypic determinants were used for reference controls. Coalescence of the precipitin band of an unknown serum with a known reference serum was the criterion for assigning specific allotypes to unknown animals.

In addition, the Ouchterlony technique was used to compare anti-lymphocyte antisera and anti-IgG allotypic antisera. In these studies, serum obtained from an animal positive for both the lymphocyte and immunoglobulin allotypes was reacted against the respective anti-allotype antisera in agar gel plates.

To determine whether the observed cytotoxic reactions might be due to antibodies directed against IgG allotypes which are found on some lymphocytes, experiments

were also performed in which anti-IgG allotypic antisera was substituted for lymphocytotoxic antisera in the cytotoxicity test.

RESULTS

I Lymphocyte Isolation

Rbc Sedimentation:

The 3% pig skin gelatin and the dextrans with molecular weights ranging from 1.5×10^5 to 5.0×10^5 were found to be satisfactory sedimentation agents for erythrocytes since these agents sediemtned approximately 97% of the rbc. In contrast, the erythrocytes did not sediment to any appreciable extent in the plasmagel, and the yield of leukocytes in the supernatant when the plasmagel was utilized was less than 2%. As shown in Table 1, 3% pig skin gelatin was the most efficient sedimentation agent since up to 76% of the leukocytes could be recovered in the supernatnant, while a maximum of 18% of the leukocytes were recovered after sedimentation with dextran.

Passage of Leukocyte-Rich Preparations Through the Columns:

Following sedimentation of the erythrocytes with 3% pig skin gelatin, the leukocyte-rich fractions were resuspended in 3-4 ml of barbital buffer and were passed through a column packed with glass beads. The lymphocytes and contaminating erythrocytes were eluted from the column first with 4 ml of eluting barbital buffer (Fig. 1). In contrast, granulocytes adhered strongly to the beads in

Table 1. Leukocyte yields at various concentrations of sedimentation agents[1]

Sedimentation Fluid	Leukocyte Yield[2] Percent Sedimentation Fluid		
	1.5%	3.0%	5.0%
Dextran MW 150,000	4.0×10^6	1.4×10^7	8.0×10^6
Dextran MW 250,000	4.0×10^6	9.0×10^6	4.0×10^6
Dextran MW 500,000	ND[3]	1.0×10^7	1.2×10^7
Pig Skin Gelatin	3.9×10^7	6.0×10^7	2.4×10^7
Plasmagel	1.0×10^6	1.0×10^6	1.0×10^6

[1] Values are given as the number of leukocytes recovered in the supernatant from 10 ml of heparinized blood (average of 7.8×10^7 leukocytes) after incubation with sedimentation fluid.

[2] Each value represents an average from three determinations.

[3] None detectable.

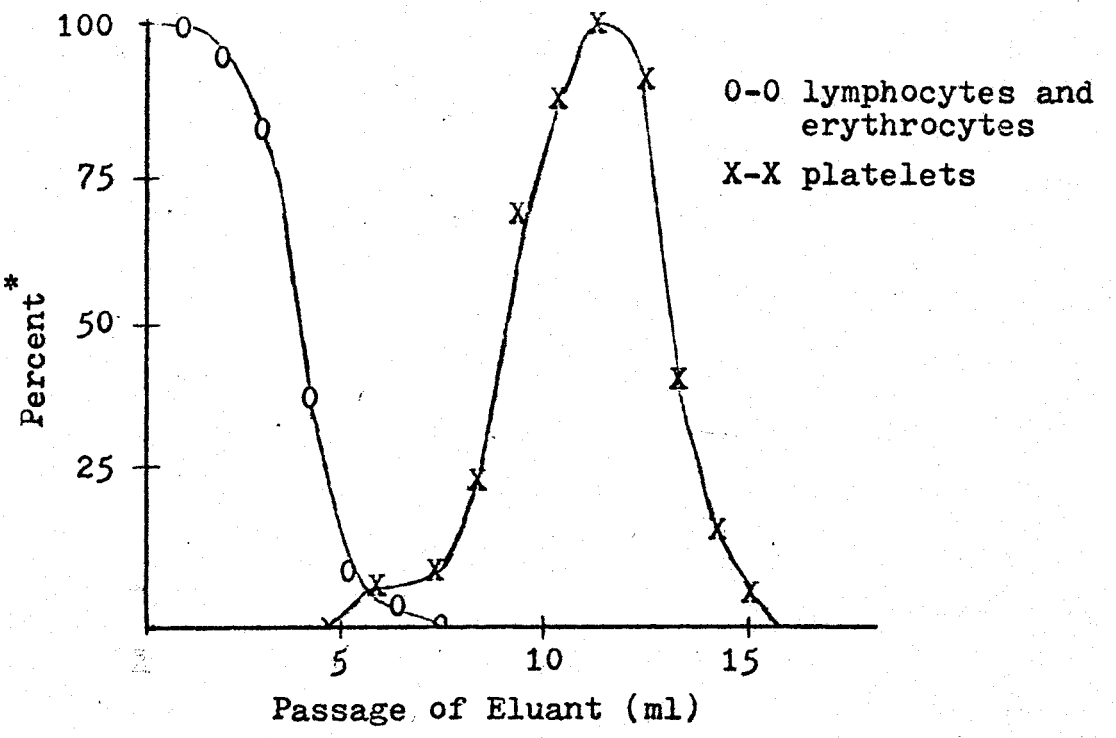

Figure 1. Elution of cells from glass bead columns (30cm x 1cm ID). Leukocyte-rich preparations were obtained from 30 ml of rabbit peripheral blood followed by sedimentation with pig skin gelatin. Cell types were determined on prepared slides, stained with Wright's stain and examined under oil immersion. Cell counts were determined by the "Model S" coulter counter.

* Values are represented as the percent of a given cell type at the peak output of that particular cell type.

the column. Platelets did not adhere firmly to the glass beads but were retarded sufficiently during passage through the columns to allow complete separation from the lymphocytes. The vast majority of platelets were eluted with the passage of approximately 6-15 ml of eluant (Fig. 1).

After the lymphocytes and platelets were eluted, the columns could be completely freed of residual lymphocytes and contaminating erythrocytes by continued washing with barbital buffer; however, the granulocytes continued to adhere. The first 5 ml of eluant which contained the most lymphocyte-rich fractions were collected and centrifuged to obtain a cell button and the supernatant was discarded. Only these purified fractions were used for isoimmunizations.

Comparison of Methods for Lysis of Contaminating Rbc:

The lymphocyte-rich cell buttons obtained following passage through glass bead columns, were resuspended in various agents in an attempt to remove the remaining erythrocytes. The distilled water technique was unsatisfactory for use since it lysed not only the erythrocytes but also caused lysis of the lymphocytes. Although HCl lysed the contaminating erythrocytes with only minimal loss of lymphocytes, the 0.1 N HCl was also unsatisfactory since it caused gross observable denaturation and discoloration of the lymphocytes. The TAC buffer, as described

previously, was selected as the appropriate agent since it lysed essentially all of the contaminating erythrocytes and did not cause any observable damage to, or loss of, the lymphocytes.

Examination of Lymphocyte Preparations for Purity:

Smears were prepared of the purified lymphocyte preparations, stained with Wright's stain and then examined for purity. In a field of 100 lymphocytes, generally fewer than 2 granulocytes and 1 erythrocyte were observed (Fig. 2). The Model S Coulter Counter and Wright's stain were used to determine the yield of purified lymphocytes. The yield was found, on the average, to be approximately 48% (Table 2). The percentage of cells recovered ranged from 42% to 52%. A yield of 48% is equivalent to approximately 1.6×10^7 lymphocytes in 10 ml of whole blood.

Determination of Lymphocyte Cell Type:

Cell preparations obtained prior to and following passage through glass bead columns were reacted with fluorescein labeled goat anti-rabbit antiserum that possessed antibodies against both IgG and IgM. Three cell types could be detected due to variations in fluorescent staining. These cell types included: 1) nonstained cells probably consisting of granulocytes and thymocytes; 2) partially stained cells probably composed of bone marrow-derived

Figure 2A. Photograph of whole blood preparation from rabbit K343-6 stained by the Wright's method. The clear ("halo") cells represent erythrocytes while stained cells represent various white blood cell types. Magnification: 400X.

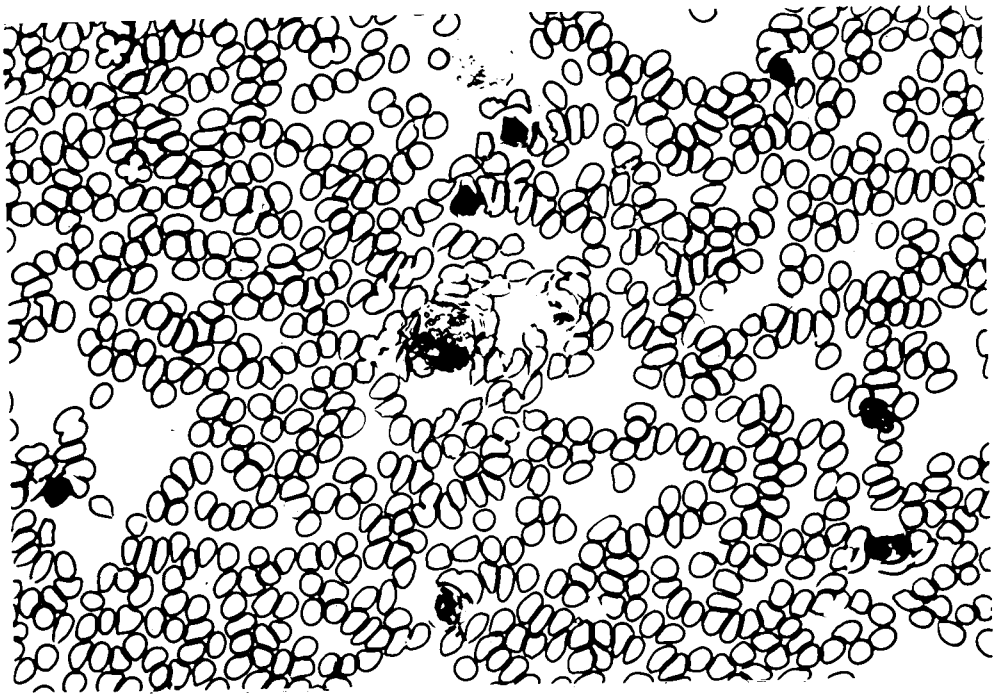

Figure 2B. Photograph of purified lymphocyte preparation from rabbit K343-6 stained by the Wright's method (20-fold concentration). Only lymphocytes are observed in this cell preparation. Magnification: 400X.

Table 2. Percentage of lymphocytes recovered from 10 ml of blood[1]

Animal Number	Number of Lymphocytes in 10 ml Sample	Number of Lymphocytes Recovered	Percent Recovery
A	3.1×10^7	1.3×10^7	42.7
13	3.8×10^7	2.0×10^7	51.9
G203-4	2.5×10^7	1.1×10^7	44.5
J120	2.1×10^7	1.1×10^7	52.4

[1] Each value represents an average from three determinations. Values were determined by cell counts under the microscope and use of the coulter counter.

lymphocytes with IgM-immunoglobulin cell surface receptors; and 3) fully stained cells consisting of bone marrow-derived lymphocytes with IgG-immunoglobulin cell surface receptors (Fig. 3 and 4).

Table 3 indicates the percentage of fluorescent stained cells in addition to the percentage of lymphocytes and granulocytes in cell preparations tested prior to and following passage through glass bead columns. From these results it is possible to calculate the ratio of B to T cells prior to and following passage through the glass bead columns. For example, in rabbit K343-6 the total number of lymphocytes prior to column passage was 42% of the total leukocyte population as revealed by the Wright's smear. The percentage of B cells as indicated by percentage of fluorescent stained cells was approximately 18%. Subtraction of these two values gave the total percent of T cells in the white cell preparation (24%). Following passage through the column, the total number of lymphocytes in the preparation was found to be 97%, of which 42% were B cells. The remainder, 55%, constitutes the percent of T cells in the leukocyte population. In summary, then, the ratio of B to T cells prior to column passage was 18 to 24 or 1 to 1.32 and following passage was 42 to 55 or 1 to 1.31, thus indicating that the liklihood for selection

Figure 3. Photograph of cell preparations prepared prior to passage through glass bead columns (rabbit K343-6). The cell preparation was reacted with fluorescein labeled goat anti-rabbit antiserum containing antibodies directed against both rabbit IgG and IgM immunoglobulins. Unstained cells probably represent thymus-derived lymphocytes and granulocytes while partially or fully stained cells probably represent bone marrow-derived lymphocytes. Magnification: 400X.

Figure 4: Photograph of cell preparations prepared after passage through glass bead columns (rabbit K343-6). The cell preparation was reacted with fluorescein labeled goat anti-rabbit antiserum containing antibodies directed against both rabbit IgG and IgM immunoglobulins. Unstained cells probably represent thymus-derived lymphocytes while partially or fully stained cells probably represent bone marrow-derived lymphocytes. Magnification: 400X.

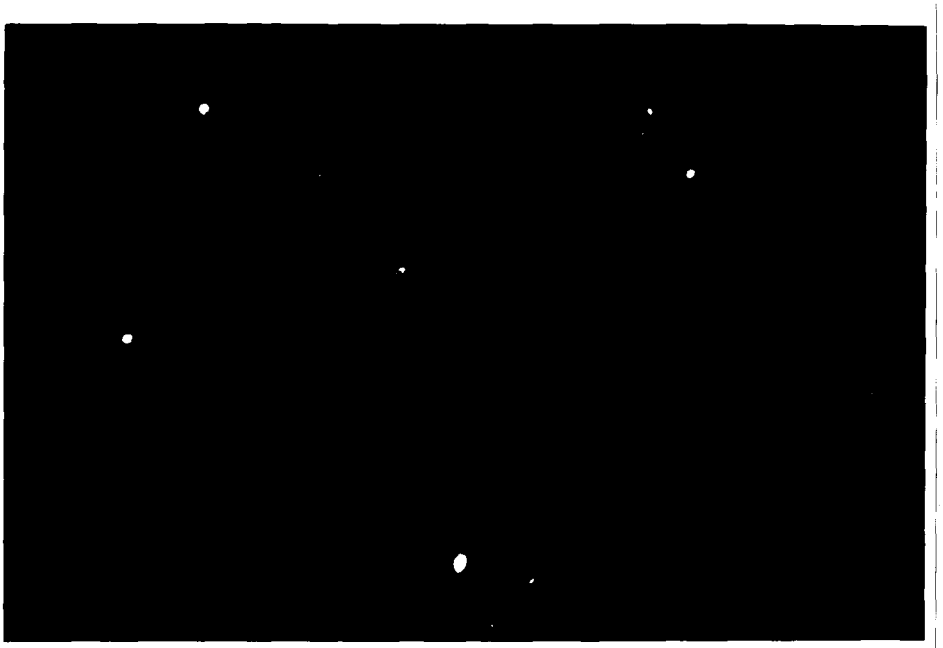

Table 3. Determination of the percentage of B and T lymphocytes prior to and following passage through a glass bead column[1]

Rabbit Number	Cell Preparation	Fluorescent Staining Reaction[2] (Per cent)			Wright's Stain Reaction (Per cent)	
		Negative	Partially Stained	Fully Stained	Lymphocytes	Granulocytes
1R	Pre-column	78	18	4	47	53
	Post-column	49	44	7	99	1
K343-6	Pre-column	82	15	3	42	58
	Post-column	57	36	6	97	3

[1] Cells were reacted with fluorescein labled goat anti-rabbit antiserum directed against both IgG and IgM

[2] Approximately 3×10^4 lymphocytes (erythrocytes were not included in the count) were reacted with fluorescein labeled goat anti-rabbit antiserum containing antibodies directed against both rabbit IgG and IgM immunoglobulins. Unstained cells probably represent thymus-derived lymphocytes while partially or fully stained cells probably represnt bone marrow-derived lymphocytes.

of B or T cells by glass bead columns is remote. The results from animal 1R were similar. In this animal, the ratio of B to T cells was 22 to 25 or 1 to 1.14 prior to column passage as compared to 51 to 48 or 1 to 0.94 following column passage.

II Immunological Studies

Isoimmunization with Allogeneic Lymphocytes:

Table 4 indicates the donor-recipient pairs used for isoimmunization with purified allogeneic lymphocytes. Although all animals required multiple injections before an antibody response was detected by the cytotoxicity tests, the number of booster injections required for an antibody response varied from one animal to another. For example, both animal 2 and animal 12 required six injections before an antibody response could be detected against donor lymphocytes; however, animal 5 required ten injections. Seven of the thirteen recipients (54%) injected with allogeneic lymphocytes produced at least a weakly detectable response as demonstrated by the lymphocyte cytotoxicity tests. These animals included: 2, 4, 5, 7, 12, 18 and 90791. However, two of these cytotoxic antisera, anti-7 and anti-18, were so weak that it made serological typing against cell panels difficult to interpret.

In addition, Table 4 indicates the particular

Table 4. Production of lymphocytotoxic antisera by isoimmunization of rabbits from various strains

Immunized Rabbit Number	Strain[1]	Donor Rabbit Number	Strain	Number of[2] Challenge Injections	Antibody[3] Production
2	NZW	3	NZW/FG	6	Positive[4]
4	NZW	15420	C Race	10	Positive
5	NZW/FG	91113	ACEP	10	Positive
7	NZW	0	NZW/FG	11	Weak
8	NZW	1	NZW	8	None
12	NZW/FG	6	NZW	6	Positive
13	NZW/FG	A	NZW	11	None
17	NZW	18	NZW	9	None
18	NZW	17	NZW	11	Weak
15420	C Race	4	NZW	10	None
90791	ACEP	G194-5	NZW/FG	10	Positive
F170-2	NZW/FG	T30	NZW/FG	10	None
2G18-2	NZW/FG	15570	C Race	10	None

[1] NZW- represents New Zealand White; NZW/FG- progeny of crosses between New Zealand Whites and Flemish Giants; ACEP- from Bar Harbor, Maine; C Race- from NIH

[2] Each injection consisted of purified lymphocytes isolated from 30 ml of periphereal blood (approx. 4.0×10^7).

[3] Antibody production was determined by the cytotoxicity test as follows: 0-13% lysed cells above the negative control was considered negative; 13-15% was borderline; while 15-100% was considered positive.

[4] In the text antisera are designated by recipient number; e.g., antiserum obtained from this animal was designated anti-2.

rabbit strains used for isoimmunization. Rabbits 17 and 18 were unique since these were the only pair to evoke a response, although weak, by isoimmunization with lymphocytes of the same strain. All other cytotoxic antisera were produced by isoimmunization of lymphocytes between different strains. This included the use of a NZW/FG, C Race, ACEP, NZW and a NZW/FG as recipient and a NZW, NZW, NZW/FG, NZW/FG and an ACEP as the respective immunizing donor rabbit.

Serological Typing:

The pattern of serologic response obtained by preliminary typing with seven cytotoxic antisera against leukocytes from 38 rabbits is shown in Table 5. The lymphocytotoxic reactions against cells from the immunizing donor animal have been encircled for identification. The reaction pattern of the lymphocytotoxic antisera against heterologous cells and especially against cells from the immunizing donor can be used as an indication of the relative potency of these antisera. Animals 1,4 5,12,13 and 90791 were not typed since they died prior to this particular study.

The potency of the antisera was determined by the relative percent of dead cells above that of the negative control: 0-13% (-), 13-15% (-+) and 15-100% (+). The cyto-

Table 5. Preliminary typing of a random population of rabbits with cytotoxic antisera produced by isoimmunization of purified lymphocytes[1]

Rabbit Number	Strain	Lymphocytotoxic Antisera						
		2	4	5	7	12	18	90791
0	NZW/FG	+	-	+	⊕	-	+-	+
2	NZW	-	-	-	-	+	+-	-
3	NZW/FG	⊕						
6	NZW	-		-		⊕	-	
7	NZW	-	-	-	-	-	-	-
8	NZW	-	-	-	-	-	+-	-
17	NZW	-	+	-	+	+	⊕	+
18	NZW	+	-	+-	+-	-	-	+-
20	NZW	-	-	-	-	-	-	-
13528	C Race	+	-	+-	+	+-	-	+
13622	C Race	-	-	-	-	-	-	-
15420	C Race	+	⊕	+	-	-		
22093	ACEP	-	-	-	-	-	-	-
22833	ACEP	+	-	+	-	-	-	+
91113	ACEP	-		⊕	+-		-	+
92072	ACEP	-	-	-	+-	-	-	-
A	NZW	-	-	-	-	-	+	+
B	NZW	-	-	-	-	+	+	-
BPP	NZW	-	+	-	+-	+	-	-
C	NZW	-	-	-	-	-	-	-
DSBNS	NZW	-	-	-	+	+	+	-
DSCLL	NZW	-	-	-	-	-	+-	-
DSCTL	NZW	-	-	-	-	+	+-	-
G194-5	NZW/FG	+	-	+	-	+	+	⊕
G203-4	NZW/FG	+	-	-	-	-	+-	-
2G18-2	NZW/FG	+	-	+	-	-	+	+
H322-3	NZW/FG	+	-	+	-	-	-	+
H325	NZW/FG	-	-	-	-	-	-	-
J120	NZW/FG	+	-	-	+-	-	-	+
J222-2	NZW/FG	+	-	+-	-	-	-	+
K343-6	NZW/FG	+	-	+	-	-	+-	+
MN	NZW	-	+	-	-	+	-	-
1R	NZW	-	-	-	-	-	-	+
2R	NZW	-	-	-	-	-	-	-
T30	NZW/FG	+	-	-	-	-	-	+
5X	NZW	+	-	+	-	-	-	+
12X	NZW	-	-	-	-	-	-	-
14X	NZW	-	-	-	-	-	-	-

[1] Results are given as percent dead cells (observed by uptake of trypan blue dye) over that of the negative control (no antiserum). 0-13% (-); 13-15% (+-); and 15-100% (+). All tests were run in quadruplicate.

[2] Circled results indicate lymphocytotoxic reaction against cells from the immunizing donor animals.

toxic antisera, anti-7 and anti-18 antisera, were not studied further because they reacted with too few animals to give meaningful correlations or consistent results. Anti-2 antiserum, in contrast, appeared to be a potent antiserum and was thus utilized in further studies. Other cytotoxic antisera which appeared to be of sufficient potency to perform serological studies included: anti-4, anti-5, anti-12 and anti-90791 antisera.

Certain of the cytotoxic antisera appeared to have similar reaction patterns against the cell panel: e.g.,anti-2, anti-5, and anti-90791 antisera reacted with the same group of rabbits (0,3,18,13528,15420,22833, 91113, G194-5,G203-4,2G18-2,H322-3,J120,J222-2,K343-6, T30 and 5X).Anti-4 and anti-12 antisera reacted with a second group, (6,17, B, BPP,DSBNS,DSCTL, and MN). In addition, a large number of animals do not react with either group of antisera (2,7,8,20,13622,22073,92072,A, C,DSC11, H325,1R,2R,12X and 14X).

The relationship of these cytotoxic antisera were analyzed by Fisher's 2x2 comparison (60). In this statistical evaluation, the number of similar and dissimilar reactions in every possible pair of the strong lymphocytotoxic antisera were counted (Table 6). It was observed that there existed at least one reaction pair for each of the four

Table 6. 2x2 comparison of lymphocytotoxic antisera

Antiserum 5 / Antiserum4

	+	-
+	1	3
-	10	22

Antiseum 12 / Antiserum 4

	+	-
+	3	1
-	5	27

Antiserum 90791 / Antiserum4

	+	-
+	1	2
-	15	17

Antiserum 12 / Antiserum 5

	+	-
+	1	10
-	8	18

Antiserum 90791 / Antiserum 5

	+	-
+	11	1
-	5	19

Antiserum 90791 / Antiserum 12

	+	-
+	2	6
-	13	14

Antiserum 4 / Antiserum 2

	+	-
+	1	14
-	3	18

Antiserum 5 / Antiserum 2

	+	-
+	11	4
-	1	22

Antiserum 12 / Antiserum 2

	+	-
+	1	14
-	8	14

Antiserum 90791 / Antiserum 2

	+	-
+	13	1
-	4	18

possible events in each antiserum analyzed. This suggested that no two of the lymphocytotoxic antisera were identical. The number of double positive antisera reactions ranged from 1 to 13 while the number of double negative reactions ranged from 14 to 27.

Once it was determined that no two cytotoxic antisera were identical, Fisher's 2x2 test was used to calculate the association between the antiserum pairs (Table 7). The results suggest that many antisera detect similar antigens. It was found that the reactions of anti-2, anti-5 and anti-90791 were positively correlated with one another indicating one group. The anti-4 and anti-12 antisera were positively correlated with one another indicating a second group. These 2x2 values, revealing positive correlation, ranged from +76 to +238. In addition, a negative correlation was consistently obtained between the reaction of the antisera in one group and those in the other group. These correlation values ranged from -8 to -98.

Chi-square values for each correlation test are shown in Table 8. A chi-square value of 3.84 (P=0.05) or greater was considered significant. Thus, the antisera were shown to detect alloantigens that fell into two distinct categories. Anti-2, anti-5 and anti-90791 antisera (Group I

Table 7. The coefficient of correlation, r^1, of 5 cytotoxic antisera

Antisera	Coefficient of Correlation			
	Animals Tested			
	90791	12	5	4
2	+ 230	- 98	+ 239	- 24
4	- 13	+ 76	- 8	-
5	+ 204	- 61	-	
12	- 50	-		

[1] r represnts coefficient of correlation derived from the equation r=ad-bc.

Table 8. The chi-square[1] values for the coefficient of correlation of 5 cytotoxic antisera

Antisera	Chi-Square Values			
	Animals Tested			
	90791	12	5	4
2	17.62	2.65	17.01	0.02
4	0.013	5.03	0.12	-
5	13.65	0.84	-	
12	0.54	-		

[1] A chi-square value of 3.84 (p=0.05) or greater was considered significant.

antisera) detected one alloantigen (Ly-1) while anti-4 and anti-12 antisera (Group II antisera) detected a second lymphocyte alloantigen (Ly-2). The chi-square values obtained when the antisera from Groups I and II were compared did not appear to be significant.

Homologous Adsorption Analysis:

Table 9 summarizes the results of the adsorption studies with five of the cytotoxic antisera: anti-2, anti-4, anti-5, anti-12 and anti-90791. Following two adsorptions of the antisera with 5.8×10^7 cells from the corresponding immunizing donor animals, the antisera were tested by the cytotoxicity test against immunizing donor lymphocytes. The percent cell death was determined as the number of dead cells in each well (observed by uptake of trypan blue dye) compared to total number of cells. Negative controls represented wells which contained cells and complement but no antibody. A value of 0-13% was considered negative while 13-15% was considered borderline. Values of 15% or greater were considered positive. Prior to adsorption, the cytotoxic indexes for the positive controls ranged between 23% and 30%. Following adsorption, the values decreased to betweeen 3 and 7%. These results indicated that adsorption with homologous cells eliminated the cytotoxic reaction of all five antisera.

Table 9. Cytotoxic activity remaining after two adsorptions of lymphocytotoxic antisera with 5.8×10^4 leukocytes from the corresponding original immunizing donor[1]

Antiserum Group	Cytotoxic Antiserum Number	Adsorbing Homologous Donor Cells	Positive Control	Adsorbed Antiserum
			Percent	
I	2	3	25	4
I	5	91113	23	4
I	90791	G194-5	26	7
II	4	15420	30	3
II	12	6	27	6

[1] Results are given as percent dead cells (uptake of trypan blue dye) above the negative control: 0-13%-negative; 13-15%-intermediate; 15-100%-positive.

Cross-Adsorption Analysis:

Although the serological patterns observed in the typing studies (Table 5) aided in revealing the nature of the cytotoxic antisera, additional studies were required to determine the number of specificities for a particular cytotoxic antiserum. In addition, since only potent antisera can be tested for relative specificity (for reasons that will be discussed later), only anti-2 antiserum was selected for further study. The specificity of the reactions of anti-2 antiserum was determined by means of adsorption with selected allogeneic leukocytes. The donor rabbits for these allogeneic leukocytes were selected from cells of animals that revealed a high cytotoxicity index (15% or greater) when tested against anti-2 antiserum (Table 5). The cells of these animals were retested by the cytotoxicity test against anti-2 antiserum to insure that their reactivity was sufficiently potent so that false negatives would not result (Table 10). The cytotoxic indexes of the lymphocyte cells chosen for use ranged between 15 and 20% above the negative control when tested against a 1:4 dilution of anti-2 antiserum. The cells obtained from the following rabbits were strongly reactive with anti-2 antiserum and, thus, were chosen to make up the cell panel: 0, 3 (immunizing donor), 13528, G194-5, G203-4, J120, J222-2, K343-6, and T30.

Table 10. Lymphocytotoxic reactions of anti-2 antiserum with the panel of cells chosen for use in the adsorption tests

Antiserum	Cytotoxic Reactions[1] Test Cells											
	0	18	13528	G194-5	G203-4	H322-3	J120	J222-2	K343-6	T30	20[2]	H325[2]
Anti-2 Antiserum Undiluted	18	16	19	19	18	15	20	17	20	18	3	0
Anti-2[3] Antiserum Diluted	20	14	17	16	18	15	21	17	20	16	1	0

[1] Results were determined by percent of dead cells (uptake of trypan blue dye) over that of the negative control (no antiserum): 0-13% represents a negative test; 13-15%-borderline; 15-100% represents a positive reaction. All tests were run in quadruplicate and results were averaged.

[2] Animals 20 and H325 were negative controls for the test.

[3] The anti-2 antiserum was diluted 1:4 with saline prior to adsorption tests.

Animals 18 and H322-3 were not included in the test panel although they reacted with anti-2 antiserum because of the weak cytotoxicity reaction their cells evoked when reacted against the antiserum. Several animals that had reacted with anti-2 antiserum in Table 10 had expired prior to the time adsorption tests were conducted. Cells from animals 20 and H325 were utilized as negative controls in the adsorption panel. In addition, the results in Table 10 indicate that dilution of the anti-2 antiserum with saline (1:4) did not significantly decrease the cytotoxic reactions. Prior to dilution, the cytotoxic values of the positive cells from the test panel ranged from 15% to 20% above the negative control. Following dilution of the anti-2 antiserum, the cytotoxic values ranged from 14% to 21%.

The results obtained by the methodical adsorption of antiserum anti-2 with the positive-reacting leukocytes from individual donors of a test panel are illustrated in Table 11. The positive control consisted of diluted (1:4) unadsorbed antiserum. The results are given as percent of cell death above negative control. The negative controls consisted of cells and complement but no antibody. A cytotoxicity index of 0-13% was considered negative (-) while 13-15% was considered borderline (+-). A cytotoxicity value of 15-100% was considered positive (+).

Table 11. Cytotoxic activity remaining after the adsorption of anti-2 antiserum with cells from the cross-adsorption panel[1]

Adsorbing Cells	Cells Used in Cytotoxicity Test	Cytotoxic Values Prior to Adsorption	Cytotoxic Values After Adsorption
0	0	+-	-
0	3	+	-
0	13528	+-	-
0	G194-5	+	-
0	G203-4	+	-
0	J120	+	-
0	J222-2	+	-
0	K343-6	+	-
0	T30	+	-
0	20	-	-
0	H325	-	-
3	0	+	-
3	3	+	-
3	13528	+	-
3	G194-5	+	-
3	G203-4	+	-
3	J120	+	-
3	J222-2	+	-
3	K343-6	+	-
3	T30	+	-
3	20	-	-
3	H325	-	-
13528	0	+	-
13528	3	+	-
13528	13528	+	-
13528	G194-5	+	-
13528	G203-4	+	-
13528	J120	+	-
13528	J222-2	+-	-
13528	K343-6	+	-
13528	T30	+	-
13528	20	-	-
13528	H325	-	-

Table 11. (Continued)

Adsorbing Cells	Cells Used in Cytotoxicity Test	Cytotoxic Values Prior to Adsorption	Cytotoxic Values After Adsorption
G194-5	0	+	-
G194-5	3	+	-
G194-5	13528	+	-
G194-5	G194-5	+	-
G194-5	G203-4	+	-
G194-5	J120	+-	-
G194-5	J222-2	+	-
G194-5	K343-6	+	-
G194-5	T30	+	-
G194-5	20	-	-
G194-5	H325	-	-
G203-4	0	+	-
G203-4	3	+	-
G203-4	13528	+	-
G203-4	G194-5	+	-
G203-4	G203-4	+	-
G203-4	J120	+	-
G203-4	J222-2	+	-
G203-4	K343-6	+-	-
G203-4	T30	+	-
G203-4	20	-	-
G203-4	H325	-	-
J120	0	+	-
J120	3	+	-
J120	13528	+-	-
J120	G194-5	+	-
J120	G203-4	+	-
J120	J120	+	-
J120	J222-2	+	-
J120	K343-6	+	-
J120	T30	+	-
J120	20	-	-
J120	H325	-	-

Table 11. (Continued)

Adsorbing Cells	Cells Used in Cytotoxicity Test	Cytotoxic Values Prior to Adsorption	Cytotoxic Values After Adsorption
J222-2	0	+	-
J222-2	3	+	-
J222-2	13528	+	-
J222-2	G194-5	+	-
J222-2	G203-4	+	-
J222-2	J120	+	-
J222-2	J222-2	+	-
J222-2	K343-6	+	-
J222-2	T30	+	-
J222-2	20	-	-
J222-2	H325	-	-
K343-6	0	+	-
K343-6	3	+	-
K343-6	13528	+	-
K343-6	G194-5	+	-
K343-6	G203-4	+	-
K343-6	J120	+	-
K343-6	J222-2	+	-
K343-6	K343-6	+-	-
K343-6	T30	+	-
K343-6	20	-	-
K343-6	H325	-	-
T30	0	+	-
T30	3	+	-
T30	13528	+	-
T30	G194-5	+	-
T30	G203-4	+	-
T30	J120	+	-
T30	J222-2	+	-
T30	K343-6	+	-
T30	T30	+	-
T30	20	-	-
T30	H325	-	-

Table 11. (Continued)

Adsorbing Cells	Cells Used in Cytotoxicity Testing	Cytotoxic Values Prior to Adsorption	Cytotoxic Values After Adsorption
20	0	+	+
20	3	+	+
20	13528	+	+
20	G194-5	+	+-
20	G203-4	+	+
20	J120	+	+
20	J222-2	+-	+
20	K343-6	+	+
20	T30	+	+
20	20	-	-
20	H325	-	-
H325	0	+	+
H325	3	+	+
H325	13528	+	+
H325	G194-5	+	+
H325	G203-4	+	+
H325	J120	+	+
H325	J222-2	+	+
H325	K343-6	+	+
H325	T30	+	+
H325	20	-	-
H325	H325	-	-

[1] Results were determined by percent of dead cells (uptake of trypan blue dye) over that of the negative control (no antiserum): 0-13%(-); 13-15%(+-); 15-100% (+). All tests were run in either triplicate or quadruplicate and values were averaged.

The cytotoxic values for cells tested against the adsorbed antisera ranged between 0 and 12%, the mean average being 3.4%. In contrast, the cytotoxic values of the positive control, consisting of unadsorbed antisera tested against the identical cells, ranged between 14 and 28% with the mean being approximately 16.5%. Since anti-2 antiserum adsorbed with each of the individual cells did not give a positive reaction when tested against a primary panel of leukocyte donors, it has been assumed that anti-2 antiserum is operationally monospecific. In contrast, cytotoxic values of antisera adsorbed with cells from animals 20 and H325 and tested against cells from the positive cell panel ranged between 14 and 21%. This indicated that anti-2 antiserum did not lose any significant cytotoxic activity when adsorbed with cells that were negative reactors against the antiserum. It, therefore, appears that non-specific adsorption of antibodies does not occur.

Complement Fixation Tests on Donor's Erythrocytes:

Direct complement fixation tests were performed with lymphocytotoxic antisera to detect the presence of lymphocyte alloantigens on erythrocytes (Table 12). The five potent cytotoxic antisera, anti-2, anti-4, anti-5, anti-12 and anti-90791 were tested against erythrocytes obtained from the respective original immunizing donor.

Table 12. Complement fixation tests to determine the presence of rabbit lymphocyte alloantigens on rabbit erythrocytes

Lymphocytotoxic Antisera	Antiserum 1:10 (ml)	Barbital Buffer (ml)	G. Pig[1] Complement (ml)	RBC Obtained From Rabbit[2]	2% Rabbit Erythrocytes (ml)	Hemolysis
2	2	0.2	0.3	3	0.5	-
2	2	0.5	-	3	0.5	-
-	-	2.2	0.3	3	0.5	-
4	2	0.2	0.3	15420	0.5	-
4	2	0.5	-	15420	0.5	-
-	-	2.2	0.3	15420	0.5	-
5	2	0.2	0.3	91113	0.5	-
5	2	0.5	-	91113	0.5	-
-	-	2.2	0.3	91113	0.5	-
7	2	0.2	0.3	0	0.5	-
7	2	0.5	-	0	0.5	-
-	-	2.2	0.3	0	0.5	-
12	2	0.2	0.3	6	0.5	-
12	2	0.5	0.3	6	0.5	-
-	-	2.2	0.3	6	0.5	-
18	2	0.2	0.3	17	0.5	-

Table 12. (Continued)

Lymphocytotoxic Antisera	Antiserum 1:10 (ml)	Barbital Buffer (ml)	G. Pig[1] Complement (ml)	RBC Obtained From Rabbit	2% Rabbit Erythrocytes (ml)	Hemolysis
18	2	0.5	-	17	0.5	-
-	-	2.2	0.3	17	0.5	-
90791	2	0.2	0.3	G194-5	0.5	-
90791	2	0.5	-	G194-5	0.5	-
-	-	2.2	0.3	G194-5	0.5	-
Hemolysin	2	0.2	0.3	3	0.5	+
Hemolysin	2	0.5	-	3	0.5	-
-	-	2.2	0.3	3	0.5	-

[1] Guinea pig serum diluted 1:30

[2] Original immunizing lymphocyte donors

No hemolysis could be observed when cytotoxic antisera were reacted against immunizing donor erythrocytes. In contrast, the control which consisted of sheep hemolysin lysed essentially all of the red blood cells. These results indicated that no lymphocyte alloantigens exist on rabbit erythrocytes as detected by complement fixation.

Detection of a Lymphocyte Alloantigen in Organ Homogenates:

The relative distribution of the Ly-1 alloantigen(s) on various organ homogenates in two representative individuals were determined by adsorbing anti-2 antiserum with various quantities of organ suspensions (Table 13). The various organs and tissues were obtained from rabbit 3, the original immunizing donor, and rabbit 13528. The cells from both of these animals had previously been shown to give a strong cytotoxic reaction when tested against anti-2 antiserum in the cytotoxicity test. Results are given as percent dead cells (as determined by uptake of trypan blue dye) above negative control; 0-13% was considered negative (-); 13-15% borderline (+-); while 15-100% was considered positive (+). Negative controls consisted of complement and cells with no antibody. Although the relative distribution of the Ly-1 alloantigen(s) was observed independently in each animal, a more accurate determination was obtained by averaging the results of the two animals.

Table 13. Cytotoxic activity remaining after the adsorption of anti-2 antiserum with various concentrations of organ homogenates from two positive reacting rabbits

Rabbit Tested	Quanity of Adsorbing Homogenate (mg)	Dead Cells (%) Above Negative Control[1] Antiserum Adsorbed With Homogenates Of							
		Spleen	Lymph Node	Lung	Skin	Kidney	Liver	Heart	Fat
3[2]	500.0	ND[3]	ND	ND	ND	-	-	ND	ND
3	250.0	ND	ND	ND	-	-	-	-	+
3	125.0	-	-	-	-	+-	+-	-	+
3	62.5	-	+-	+-	-	-	+	+-	+
3	31.3	-	-	-	+-	+	+	+-	+
3	15.6	-	+	+	+	+	+	+	+
3	8.0	+-	+	+	+	+	+	+	+
3	4.0	+	+	+	+	+	+	+	+
13528	500.0	ND	ND	-	ND	-	-	-	ND
13528	250.0	ND	-	-	-	-	-	-	ND
13528	125.0	-	-	-	-	-	-	-	+
13528	62.5	-	-	-	-	-	+-	-	+
13528	31.3	-	-	-	+	+-	+	+	+
13528	15.6	-	+	+	+	+	+	+	+
13528	8.0	-	+	+	+	+	+	+	+
13528	4.0	+	+	+	+	+	+	+	+

[1] Results were determined by percent of dye uptake by cells over that of the negative control (no antiserum). 0-13% (-); 13-15% (+-); and 15-100% (+). All tests were run in quadruplicate.

[2] Rabbit number three was the original immunizing donor used in the production of anti-2 antiserum.

[3] Not Done

In this case, it appears that the highest concentration of antigen exist in the spleen (7.0) followed in descending order by the lymph nodes (5.0), lung (5.0), skin (4.5), kidney (4.0), heart (4.0) and finally liver (3.5). Fat contained little, if any, lymphocyte antigens. No substantial differences in alloantigen concentration between the lymph node and the lung, and between the kidney and the heart were observed.

In contrast, if total average weight of normal organs is considered, the order then becomes skin (463), liver (225), lung (90), kidney (53), heart (46), spleen (24), and lymph nodes (18) (Table 14). The total weight of fat was not determined due to difficulty in obtaining total body fat. The relative units of activity for each organ was determined directly by their cytotoxicity indexes (Table 13) as follows:

Minimum concentration (mg) of antigen capable of eliminating a positive cytotoxic reaction	Designated unit value
4.0	8
8.0	7
15.6	6
31.3	5
62.5	4
125.0	3
250.0	2
500.0	1

Table 14. Relative distribution of lymphocyte alloantigens in rabbit organs and tissues with consideration of total organ weight

Organ	Total[1] Organ Weight (gm)	Units of[2] Activity	Total[3] Activity
Skin	103.16	4.5	463.5
Liver	64.20	3.5	224.7
Lung	17.94	5.0	89.7
Kidney	13.21	4.0	52.8
Heart	11.55	4.0	46.2
Spleen	3.49	7.0	23.8
Lymph Nodes	3.60	5.0	18.0

[1] Weights were determined by weighing the organ before homogenation

[2] Units of activity for each organ were determined directly by their cytotoxicity tests as follows:

Min. conc. of antigen (mg) capable of eliminating the cytotoxic reaction	Designated Unit Values
4.0	8
8.0	7
15.6	6
31.3	5
62.5	4
125.0	3
250.0	2
500.0	1

[3] The total activity was determined by multiplying organ weight by the designated unit of activity

The total activity was subsequently determined by multiplying the total organ weight by the designated unit value.

Thus, for example, 31.3 mg of lymph node was the minimum concentration of antigen required to remove antibody activity as determined by the cytotoxicity test. This value represents a unit of activity of five. Total activity could then be determined by multiplying the organ weight by the unit of activity for the specific organ. In the case of the lymph node this value would be 5 (unit activity) x 3.6 (organ weight) or a net activity of 18.0.

Immunoglobulin Typing:

Preliminary studies to determine the relationship of the genetic control of immunoglobulin antigenic determinants and lymphocyte alloantigens are summarized in Table 15. Rabbits are classified as either reacting with Group I lymphocyte alloantisera (I), Group II lymphocyte alloantisera (II) or neither (0). In addition, the a, b and c loci immunoglobulin determinants are given for each animal.

Animals possessing the Ly-1 lymphocyte alloantigen(s) (reacting with antisera from Group I) were found to possess the following immunoglobulin allotypes; 1, 2, 3, 5, 7, and/or 21. Animals possessing the Ly-2 lymphocyte alloantigen(s) (reacting with antisera from Group II) were found to possess

Table 15. Relationship of immunoglobulin alloantigens to the lymphocyte alloantigens in the rabbit

Rabbit Number	Immunoglobulin Allotype	Lymphocyte Alloantigen Group	Rabbit Number	Immunoglobulin Allotype	Lymphocyte Alloanti sera Group
0	11,557	I[1]	C	11,447	0
2	11,447	0[2]	DSBNS	11,447	II
3	11,557	I	DSCLL	11,447	0
6	11,447	II[3]	DSCTL	11,447	II
7	11,447	0	G194-5	11,557	I
8	11,447	0	G203-4	13,557	I
17	11,447	II	2G18-2	22,447	I
18	11,447	I	H322-3	22,447	I
20	11,447	0	H325	22,447	0
13528	11,447	I	J120	33,447	I
13622	11,447	0	J222-2	11,557	I
15420	11,447	I	K343-6	23,557	I
22093	11,4421	0	MN	11,447	II
22833	22,4421	I	1R	11.447	0
91113	33,4421	I	2R	11,447	0
92072	33,4421	0	T30	11,447	I
A	11,447	0	5X	11,447	I
B	11,447	II	12X	11,447	0
BPP	11,447	II	14X	11,447	0

[1] Group I animals reacted with lymphocytotoxic antisera 2, 5 and 90791 (Ly-1 alloantigen(s))

[2] Group 0 animals did not react with either lymphocytotoxic antisera group

[3] Group II animals reacted with lymphocytotoxi antisera 4 and 12 (Ly-2 alloantigen(s))

the following immunoglobulin allotypes: 1, 4, and 7. Animals which did not possess either lymphocyte alloantigens Ly-1 or Ly-2 were found to possess the 1, 2, 3, 4, 7 and/or 21 immunoglobulin allotype determinants.

The Ouchterlony technique was also used to compare anti-lymphocyte antisera and anti-IgG allotypic antisera. In this study, serum obtained from an animal positive for both the lymphocyte and immunoglobulin allotypes was reacted against the respective anti-allotype antisera in agar gel plates to determine reaction behavior. Only preliminary studies were completed since all lymphocytotoxic antisera tested failed to form a precipitin band against sera in agar gel plates (results not shown).

The results of studies to demonstrate whether the observed cytotoxicity reactions might be due to antibodies directed against IgG allotypes which are found on some circulating lymphocytes are shown in Table 16. Cells from four animals (H322-3, 0, 92072, MN) were chosen because they possessed the entire spectrum of allotypic determinants as well as the lymphocyte alloantigen groups (See Table 15). In all cases, anti-immunoglobulin antisera failed to produce a cytotoxic reaction.

Table 16. Cytotoxic reactions of various allotypic antisera against purified lymphocyte cells[1]

Allotypic Antisera	Test Cells From Rabbits			
	H322-3	0	92072	MN
anti-a1	-	-	-	-
anti-a2	-	-	-	-
anti-a3	-	-	-	-
anti-b4	-	-	-	-
anti-b5	-	-	-	-
anti-b6	-	-	-	-
anti-b9	-	-	-	-
anti-c7	-	-	-	-
anti-c21	-	-	-	-
anti-2	-	+	-	-
anti-4	-	-	-	+
anti-5	-	+	-	-
anti-12	-	-	-	+
anti-90791	-	+	-	-

[1] Results are given as percent dead cells (observed by uptake of trypan blue dye) over that of the negative control (no antiserum). 0-13% (-); 13-15% (+-); and 15-100% (+). All tests were run in quadruplicate

DISCUSSION

In this thesis a method is described for the isolation and purification of circulating lymphocytes using a combination and modification of a number of techniques. These techniques include: 1) removal of the vast majority of erythrocytes by the use of pig skin gelatin as a sedimentation agent (88); 2) passage of the white cell-rich fraction through a glass bead column to remove glass adhering cells such as platelets and granulocytes (78,64) and; 3) the removal of remaining erythrocytes by TAC buffer, a mild rbc lysing agent (1). Utilization of these techniques resulted in the isolation of up to 52% of the circulating lymphocytes. Little contamination with red blood cells or other white cell types was observed.

An early difficulty encountered in the isolation of rabbit lymphocytes was due to the low sedimentation rate of rabbit erythrocytes. Human erythrocytes, for example, are not extremely difficult to isolate since they sediment at a rate approaching 4-6 mm/hr. In contrast, rabbit erythrocytes sediment at a much slower rate of 2-4 mm/hr (73). It was not until an appropriate sedimentation agent (pig skin gelatin) was obtained that the red cells could separate from the leukocytes with satisfactory efficiency to insure obtaining a representative population

of leukocytes.

An additional difficulty encountered during attempted isolations was due to the extreme fragility of rabbit lymphocytes. Fortunately, TAC buffer was found to be satisfactory since it completely lysed erythrocytes and had no visible effect on the lymphocytes. However, one disadvantage of TAC buffer is that it caused occasional clumping of the lymphocytes. This could usually be circumvented by insuring that the buffer and cell suspension were incubated at a constant temperature of 37 C.

Since glass bead columns were utilized in the isolation of lymphocytes in this study, the possibility that a particular cell type was selected by this procedure was considered. Vast evidence has already accumulated that two lymphocyte types, the bone marrow-derived or B-cell and the thymus-derived or T-cell, exist in peripheral blood (23). Karniely (42), while attempting to study the response to dinitrophenyl hapten attached to positively and negatively charged synthetic polypeptide carriers, indicated that it is possible to distinguish T and B cells on the basis of their capacity to react with glass bead columns. It was observed that thymocytes will adhere to slightly acid or basic surfaces due to their net electrical charge. In contrast, bone marrow-derived lymphocytes passed through

the columns unhindered. To test for this possibility, the lymphocyte cell type was determined prior to and following passage of the cell preparations through glass bead columns. Cell types were distinguished by their ability to react with fluorescent labeled antiserum containing antibodies directed against rabbit IgG and IgM.

Although there is some controversy with regards to methods for distinguishing bone marrow-derived and thymus-derived lymphocytes in rabbit peripheral blood (76), it appears from various studies (66) that at least three cell types can be distinguished by utilization of this technique: 1) non-stained cells which probably represent granulocytes and T-cells; 2) partially stained cells probably representing the B-cell population with IgM-immunoglobulin surface receptors and ; 3) fully stained cells which probably represent B-cells with IgG-immunoglobulin surface receptors. It was subsequently demonstrated that the lymphocyte isolation technique employed in this study did not select for any particular cell type since the ratios of B to T cells in these preparations prior to and following column passage were nearly identical. The reason that the glass beads did not select for a particular cell type may be due to the fact that all glassware, including glass beads used as the column filler were

siliconized. Siliconization would tend to neutralize the charge on the glass surface. In any case, even if these fluorescent studies do not distinguish B and T cell types in the rabbit, they do provide strong evidence that whatever the cell type, no one type of stained cell was selected for by the isolation technique. It should be noted, however, that it is still possible that isoantisera produced in our later studies may have been directed against an alloantigen unique for one of the two lymphocyte cell types.

Once a method for purification of circulating lymphocytes was found, the lymphocytes were utilized for isoimmunizations in an attempt to produce cytotoxic isoantisera. This is in contrast to previous work in which antisera were prepared by skin allografts (24,88) or by injection of spleen cells into allogeneic animals (8).

In previous transplantation studies, either partially inbred or highly inbred colonies of rabbits were utilized (8,25,88). The various lines of rabbits involved in this study represent a new approach to the study of histocompatibility genetics in the rabbit. The rabbit population chosen consisted of a number of distinct inbred strains of rabbits. Individual strains were partially inbred to provide a limitied amount of homogeneity, but

were distinct enough to provide for a fair amount of heterogeneity between different strains. Isoimmunizations were performed both between and within each of the various rabbit lines for several reasons. First, this method of isoimmunization selected for the maximum number of alleles in the various strains. Secondly, this method most closely simulates the situation that is found in the human population. The human population is, in a manner of speaking, a relatively large number of partially closed colonies with occasional crossbreeding (27).

Once antisera are prepared against purified lymphocytes, the lymphocytotoxic antisera can be tested against a large animal population in order to detect the number of positive and negative reactors. As a result much information can be gained with respect to the distribution of the antigen(s) in the population and to the questions of allelism and linkage in the genetic control of the lymphocyte antigen(s) (21). As a more practical application, lymphocyte typing can be used to help to determine the success of an organ transplant (37). In a more experimentally orientated approach, typing data can be used to indicate identity or non-identity between two antisera. For example, the degree of similiarity of two antisera has been determined by Cepellini (15), who statistically analyzed typing data. In contrast,

Black (8) has analyzed serological patterns of reactivity and then calculated coefficients of correlation to determine similarity of antisera. All of the above approaches were utilized in this study.

The leukocytes from 38 rabbits from a variety of strains were tested against seven cytotoxic antisera. All animals were classified as either positive or negative reactors. The results obtained by serological typing were used to calculate the degree of association between the five most potent cytotoxic antisera by Fisher's 2x2 test. The results suggested that many of the antisera detected similar antigens. One group of antisera, (anti-2, anti-5, and anti-90791), reacted with a similar group of animals and were shown to be positively correlated with one another. A second group of antisera (anti-4 and anti-12) reacted similiarly with another distinct group of rabbits and were also positively correlated with one another. The lymphocyte alloantigen(s) identified by these antisera have been temporarily designated Ly-1 and Ly-2 respectively. In addition, a large number of animals did not react with either group of antisera indicating that there are, as yet, additional unidentified antigens. Several other cytotoxic antisera (anti-7 and anti-18) which were also produced by isoimmunization were not studied further since they

reacted too weakly in the cytotoxicity test to give consistent results.

Since all histocompatibility systems so far studied have revealed extensive polymorphism, it becomes necessary to obtain monospecific or operationally monospecific reagents. A number of workers are currently attempting to produce lymphocytotoxic antisera of narrowed specificity by selective immunization (89) and compatibility typing (33) followed by selective adsorption. However, these procedures require a minimum prior knowledge of the histocompatibility system of the experimental animal. Monospecificity for an antisera produced by an animal in which knowledge of the genetic makeup is limited, such as the rabbit, can be established by cross-adsorption studies or by showing that the serum reacts in a particular manner with a known cell panel. The ideal test for monospecificity is, of course, to utilize both methods.

The first indication of the degree of specificity of the cytotoxic antisera resulted from the serological typing studies (Table 5). Each of the five cytotoxic antisera, which were subsequently classified into one of two groups (Group I consisting of antisera anti-2, anti-5 and anti-90791 and Group II consisting of antisera anti-4 and anti-12), appeared to detect a relatively

small number of antigenic specificities. This was revealed by the fact that cytotoxic antisera within each group reacted similarly with the cell panel. There are two possibilities to explain the production of similarly reacting antisera within these two groups: 1) although the antisera were produced independently, they may already be monospecific since they reacted similarly with cells from almost every animal of the population. This is supported by the fact that the probability of producing antisera that detect the same antigens in two random immunizations increases as the number of antigens that can be detected by any one antiserum decreases. 2) There may be only a minimal number of alleles or antigens segregating within the colony that can react with a particular antiserum, thereby effecting a false correlation, because two apparently independent antigens occur only as the product of the same alleles.

To distinguish between the two alternatives, the cytotoxic antisera produced against the two lymphocyte alloantigen groups were examined with respect to the particular strains of rabbits used for donor-recipient pairs in the immunizations(Table 4). If rabbits from different strains have many antigens that are not in common, then antisera produced by injection between different

strains should be multispecific. This would indicate the existence of a few alleles each presumably with many strong antigens. The antisera would be directed to each of the antigens present in the donor but absent in the host. However, if the antisera produced by isoimmunization between various strains are monospecific or nearly monospecific, then only a few strong antigens are presumed to be active in inducing antibody formation.

In these studies the individual cytotoxic antisera produced against lymphocyte antigens Ly-1 and Ly-2 were produced by recepient-donor pairs of diverse lines (Table 4). In addition, similar antisera were produced by isoimmunization of lymphocytes into recepients from different strains. For example, cytotoxic anti-2 antiserum in Group I was prepared by isoimmunization of lymphocytes from a NZW/FG into a NZW, while anti-5 antiserum, also in Group I, was prepared by isoimmunization of lymphocytes from an ACEP into a NZW/FG. Similarly, cytotoxic anti-4 antiserum of Group II was produced by isoimmunization of lymphocytes from a C Race into a NZW, while anti-12 antiserum, also from Group II, was produced by isoimmunization between a NZW and a NZW/FG. In fact, no cytotoxic antisera were produced when donor-recepient pairs were utilized from the same strain, excluding the rabbit pair which produced the

anti-18 antiserum which was a weak cytotoxic antiserum. These facts would tend to support the first hypothesis, that is, each cytotoxic antiserum is already monospecific or nearly monospecific.

Although the serological patterns as observed in the typing studies tended to indicate that the cytotoxic antisera were monospecific or nearly monospecific, additional studies were performed to further investigate the number of specificities for a particular antiserum. These additional experiments were in the form of cross-adsorption studies utilizing the most reactive antiserum, anti-2.

Cross-adsorption studies are complicated by the rare occurrence of cross-reacting antibodies. For example, in the HL-A system, cells possessing the Ba(+), HL-A2(-) determinants will be adsorbed by anti-HL-A2 antiserum (84). Thus, it is possible that two distinct antibodies might be present in a serum and both may be adsorbed by either of two distinct antigens. An additional problem which exists concerning adsorption analysis is that, in some cases, two weak antibodies act synergistically and result in a positive cytotoxic reaction (41). Adsorption of either one of the antibodies would result in loss of enough activity to consider the test negative. Thus, a dispecific antiserum may appear monospecific. For this

reason, as well as the problem of nonspecific adsorption, weak cytotoxic antisera cannot be properly analyzed with adsorption methods. Due to these difficulties in cross-adsorption studies, it was decided that only anti-2 antiserum appeared potent enough that cross-adsorption analysis would be reliable.

Anti-2 antiserum appears to be a monospecific antiserum, since adsorption with any of the cells from the positive panel resulted in loss of antiserum cytotoxicity. This conclusion is based upon the theory that the original immunizing donor must possess all the antigenic factors to which the recipient has been sensitized (4). Hence, any third party individual whose cells adsorb out all antibodies reactive with the original donor must also possess the full spectrum of factors reactive with the antiserum. The probability that an antiserum is monospecific, therefore, increases as the number of cells included in the test panel is increased.

It is never possible to completely substantiate monospecificity by cross-adsorption studies since a number of irregularities can be found in a lymphocyte alloantigen system. For example, in the HL-A system, various specificities are found which are often highly associated. When Lc-10 is present, Lc-11 is also present over 85

percent of the time. Likewise, HL-A1 and HL-A8 are often associated with one another. A limited number of adsorption tests may not chance upon an individual who is Lc-10(+), Lc-11(-) or HL-A1(+), HL-A8(-) (71). No evidence exists, at present, as to whether rabbit histocompatibility antigens are associated. As the anti-2 antiserum is adsorbed with more positive reacting cells and it is demonstrated that these cells completely remove cytotoxic activity from the antiserum, then the liklihood that the antiserum possesses antibody activity against associated antigens (ie, is multispecific) will decrease.

Analysis of data on serological typing (Tables 5-8) has revealed that at least one major locus exists controlling the synthesis of lymphocyte alloantigens in rabbits. The determination of the number of alleles controlled by the locus is beyond the scope of this thesis. However, a minimum of three major determinants has been found to be expressed on the lymphocyte. This has been determined by the fact that cytotoxic antisera have been produced against two of these alloantigen(s), designated Ly-1 and Ly-2. The additional alloantigen(s) are represented by the fact that a large segment of the population did not react with the antisera of either Groups I or II. This would indicate the existence of a minimum of at least

one as yet unidentified alloantigen. The results presented here cannot confirm whether the defined lymphocyte alloantigens are alleles but their mutual exclusiveness as indicated by Fisher's 2x2 analysis would indicate that allelism is probable. The question of allelism for these alloantigens can be determined by extensive mating studies.

If the cytotoxic antisera truely detected antigens controlled by a single locus, which preliminary evidence would indicate, then the locus is probably the same locus as the RL-A locus described by Tissot (88) and the RLC locus described by Black (8). Black's antisera were all produced by injecting spleen cells into unrelated noninbred recipients and Tissot produced cytotoxic antisera by skin grafts between inbred recepients. Our results appear similar to those of Black (8) who was also able to distinguish two distinct groups of cytotoxic antisera. In addition, a large number of animals did not type with either of Black's groups of antisera. Thus, this adds weight to the theory that all cytotoxic antisera detect antigens controlled by a single "strong" genetic locus in the rabbit.

Fisher's 2x2 test was next applied to determine the coefficient of correlation between the five cytotoxic antisera (Table 6). Although correlation values between

cytotoxic antisera within a population group were highly significant, nonsignificant correlation values were obtained when comparing antisera between Groups I and II. However, in determining the association of antisera in this present context, Fisher's 2x2 tests may be misleading. These values are misleading when negative reactions occur in both the compared cytotoxic antisera, since they tend to increase the positive associations and to decrease the significance of the negative associations (8). This accounts for some of the extremely high X^2 values in Table 8 for positively associated antisera and the lack of significance for the negatively correlated antisera. In these correlations reduced significance should be attached to the occurence of double negatives, especially where the number of alleles at any locus is unknown.

Analysis of the serological patterns in the typing experiments indicated that the lymphocytotoxic antisera were not directed against a relatively large number of antigens (Table 5-8). This is evidenced by the fact that only two distinct groups of antisera were obtained and these two groups of cytotoxic antisera appear to be of relatively high specificity. If the system was composed of multiple antigens one would expect a large number of cytotoxic antisera which would be of relatively low

specificity. In any case, additional studies would be required before this hypothesis is proven since there are obviously other antigenic determinants that have not yet been detected. This is evidenced by the large number of animals whose lymphocytes did not react with the Group I or Group II antisera. Since the number of lymphocyte alloantigens appears to be limited, it is assumed that the randomly produced reagents in these studies recognize primarily the strong antigens. However, if our antisera are later found to be polyspecific, the term antigen would then refer to a frequently occurring combination of antigenic factors. For this reason lymphocytotoxic antisera that appear monospecific are usually referred to as operationally monospecific (93). In any case, the highly selective quality of these reagents has permitted the determinations of a relative antigenic profile of a rabbit population so that it is possible to gain a general impression of both the number of factors involved in the expression of lymphocyte alloantigens and their relative distribution in the population.

Medawar (55) has theorized that histocompatibility antigens are distributed more or less uniformly throughout all tissues except erythrocytes. To our knowledge no systematic and comparative study has been reported regarding

the presence and distribution of histocompatibility antigens within various organ systems of the rabbit. It is important, with respect to transplantation studies to determine the existence of and relative quantity of these antigens on various organs in representative individuals.

No lymphocyte alloantigens could be detected on rabbit erythrocytes by the complement fixation test. This would further substantiate Cohen's proposal (18) that the blood group system and Major Histocompatibility System are distinct. Although it appeared that alloantigens were present in most if not all of the tissues, excluding erythrocytes, distinct differences were observed in the concentration of antigens in these various organs. The highest concentration of antigen was found to exist in the spleen followed in descending order by the lymph nodes, lung, skin, kidney, heart, and finally liver. Fat contained little if any lymphocyte alloantigen(s). No substantial differences in alloantigen concentration was observed between lymph node and lung and between kidney and the heart. In contrast, if total average weight of normal organs is considered, the order then becomes skin, liver, lung, kidney, heart, spleen and lymph nodes. The total body fat could not be obtained. It should be noted that the results obtained in this study are not accurate tissue

antigen estimations, i.e., quantitative measurements for a particular organ, but are an approximate and comparative study of the *in toto* content of antigen in a number of organs.

The results obtained in the rabbit are in fairly good agreement with those obtained in man (7). The human lymphocyte alloantigen, HL-A2, also appears to be distributed in an analogous manner in the various organs of the human: a large amount in the spleen, lung and lymph nodes, less in the skin and kidney, a little in heart and liver and practically none in fat. The only variation in antigen distribution of any consequence between man and the rabbit was the increased concentration in the liver that can be observed in the HL-A system.

The final section of this study entailed the study of the relationship between the control of lymphocyte alloantigen(s) and rabbit immunoglobulin determinants. This study was undertaken on the basis of evidence that such a relationship does exist in the genetic control of alloantigens in other species. For example, following the elucidation of the Ss-1p gene (46) and the Ir-1 region (53) in the mouse, each of which maps within the H-2 segment, a simplifying concept has been proposed for the genetic structure of H-2. It appears possible

to map each of the H-2 antigenic specificities in one or the other of two closely linked genes called the H-2K and H-2D. Between these two genes on the chromosome are the Ss-Slp loci which control the presence of a serum protein and the Ir region or immune response region. An analogous situation may exist in the rabbit and the discovery of an associated or linked gene(s) would greatly facilitate the elucidation of the genetic control of rabbit histocompatibility antigens. Since both transplantation antigens and immunoglobulin determinants may have similar evolutionary origins (19,26) a likely candidate for such a gene would be the loci controlling allotypic determinants.

The rabbit is an ideal animal for these studies since the genetic control of rabbit immunoglobulin allotypes has already been well established. In 1960, Oudin (57) reported the presence of six anti-allotype antisera in rabbits. Genetic analysis indicated that the six allotypes are controlled by two series of three allelic genes at two unlinked loci. The allelic genes of the a locus, designated $\underline{a}^1$, $\underline{a}^2$, and $\underline{a}^3$, control the allotypic specificities a1, a2 and a3 of rabbit IgG-immunoglobulin heavy chains. The allelic genes of the b locus, designated $\underline{b}^4$, $\underline{b}^5$ and $\underline{b}^6$ control the allotypic specificities b4, b5 and b6 of rabbit light chains. The b5 and b6 allotypes have determinants in common, but

the two allotypes are distinct (43). Later, Dubiski identified an allotype which was designated b9, controlled by the $\underline{b}^9$ gene, which was found to segregate as an allele at the b locus (29). Immunoglobulin light chains lacking the b locus specificity were found to have allotypic specificities c7 and/or c21 of the c locus (91). The c locus is not linked to the a or b loci, nor to the sex chromosome (32). Antisera against each of the above described immunoglobulin allotypes were used in this study.

The relationship between the immunoglobulin and lymphocyte alloantigens was first studied by comparison of the reactivity of the lymphocytotoxic antisera and anti-immunoglobulin antisera in immunodiffusion and cytotoxicity tests. In agar gel experiments, anti-immunoglobulin antisera formed a precipitin band while lymphocytotoxic antisera failed to form a precipitin band when tested against normal sera from animals possessing both determinants. This would tend to indicate that the two allotypic antisera are not detecting the same antigens. It should be kept in mind, however, that the anti-lymphocyte antibodies could be present in a quantity insufficient to produce a definite precipitin band.

In the cytotoxicity tests, only the lymphocytotoxic

antisera resulted in cell death when tested against cells from animals possessing both the immunoglobulin and lymphocyte alloantigen determinants. Anti-immunoglobulin antisera produced no cytotoxic reaction. Again, this would indicate that anti-immunoglobulin antisera and lymphocytotoxic antisera are detecting different antigens.

Some rabbit peripheral lymphocytes have, in fact, been shown to possess immunoglobulin allotypic determinants presumably on immunoglobulin molecules associated with the cell membrane (75). Moreover, these same lymphocytes could presumably react with anti-lymphocyte antisera in a cytotoxic reaction (66). From our studies, however, it appears that the two phenomena are not related.

The results obtained from serological typing of the rabbit population for both lymphocyte and immunoglobulin allotypes indicated the existence of a possible relationship between the b5 allotypic determinant on rabbit immunoglobulin light chains and the Ly-1 lymphocyte alloantigen(s). This was evidenced by the fact that all animals possessing the b5 determinant also possessed the Ly-1 lymphocyte alloantigen(s). However, the reverse reaction did not hold; all animals possessing the Ly-1 alloantigen(s) did not possess the b5 determinant. These results are only preliminary and in no way indicate an

absolute genetic association between the various immunoglobulin and lymphocyte allotypic determinants. However, if found to be true, the results could be explained by a previous genetic cross-over event involving the Ly-1 alloantigen(s) followed by segregation. This would indicate that only b5 animals at one time possessed the Ly-1 alloantigen(s) and at some period during the evolutionary process, rabbits possessing b-light chain determinants other than the b5 determinant captured the Ly-1 lymphocyte alloantigen(s).

Further studies could now be undertaken to determine the possibility that rabbit lymphocyte alloantigens are associated with a particular rabbit strain. For example, 10 of 16 animals that possessed the Ly-1 alloantigen(s) in the population are from the NZW/FG strain. In addition, two of the three original immunizing donors used in the production of antisera against the Ly-1 alloantigen(s) are of the NZW/FG strain. The third immunizing donor was an ACEP. In contrast, the seven rabbits in the population that possessed the Ly-2 alloantigen(s) were all from the NZW strain. The two original immunizing donors used to produce antisera against the Ly-2 alloantigen(s) were from the NZW and C race strains. In the large group of rabbits that did not possess either lymphocyte alloantigen, the vast

majority (11 of 15) were of the NZW race while two were ACEP and one each were from the C race and NZW/FG strains. It must be taken into consideration in these studies that the various strains are only partially inbred (less than 15 cross matings). Thus a relative amount of heterogeneity still remains in each population. This would explain the relative inconsistencies that can be noted when trying to associate an alloantigen group with a particular strain of rabbit. It would also explain the instances in which animals react with some antisera from both groups, in which case they would reflect a heterozygous animal. In any case, additional serological studies would be required to elucidate this point.

In summary, by understanding the genetic control of the lymphocyte alloantigen locus and its corresponding alloantigens in the rabbit, it should be possible to use this system as a model in understanding the histocompatibility loci and its corresponding alloantigens in man. The rabbit has now been shown to possess a lymphocyte alloantigen system that is apparently similar in complexity to that found in the human system but should be easier to study. In addition, the concentration and relative distribution of the lymphocyte alloantigens in various organs of rabbit and man appear similar. The rabbit is a laboratory animal which is easy

to work with and the genetic control of a number of other allotypic systems in rabbit has already been well documented. Furthermore, the rabbit is a large enough animal to be used for organ transplant studies. Thus, it seems reasonable that the rabbit could be used in the future as a model system for man.

SUMMARY

A method is described for the isolation and purification of rabbit lymphocytes. The isolation technique did not specifically select for bone marrow-derived or thymus-derived lymphocytes as indicated by the fluorescent labeled anti-IgG immunoglobulin technique.

Isoantisera prepared by deliberate immunization with allogeneic lymphocytes were used to determine the phenotypic profile of the defined lymphocyte alloantigen(s) in 38 rabbits. The existence of at least one major lymphocyte alloantigen locus which controlled at least three antigenic determinants is suggested.

Since lymphocytotoxic antisera are often multispecific, two methods were utilized to determine the degree of specificity of the most potent isoantisera produced. These methods included serological typing against a large population of animals and cross-adsorption studies. The cytotoxic antisera in question were found to be monospecific or nearly monospecific.

The relative distribution of one of the alloantigens (Ly-1) was determined in various organ homogenates. The highest concentration in mg wet weight of antigen was found in the spleen followed in descending order by the lymph nodes, lung, skin, kidney, heart and liver. Rbc and fat

homogenates contain little, if any, antigen. In addition, typing studies suggested a possible relationship in the genetic control of antigenic determinants on immunoglobulin molecules and lymphocyte alloantigens. However, attempts to demonstrate identity between anti-allotype antisera and anti-lymphocyte antisera by Ouchterlony tests and cytotoxicity tests were not successful.

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APPROVAL SHEET

The dissertation submitted by Michael I. Luster has been read and approved by the undersigned faculty members.

The final copies have been examined by members of the Dissertation Committee and the signatures which appear below verify the fact that any necessary changes have been incorporated and that the dissertation is now given final approval with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

John L. Vice — May 3, 1974

Dr. J. L. Vice, Advisor — Date

E W Bermes

Dr. E. Bermes

J Filkins

Dr. J. Filkins

Dr. T. Hashimoto

Charles F. Lange

Dr. C. F. Lange