This is to certify that the

thesis entitled

FRACTIONATION OF BOVINE LYMPHOCYTES WITH AN IMMUNOABSORBENT COLUMN AND ANALYSIS OF CELL SURFACE CHARACTERISTICS

presented by

Daniel R. Pennell

has been accepted towards fulfillment of the requirements for

M.S. degree in Microbiology and Public Health

Norman B. McCullough
Major professor

Date 1/24/80

FRACTIONATION OF BOVINE LYMPHOCYTES WITH AN IMMUNOABSORBENT COLUMN AND ANALYSIS OF CELL SURFACE CHARACTERISTICS

By

Daniel R. Pennell

A THESIS

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

MASTER OF SCIENCE

Department of Microbiology and Public Health

1979

ABSTRACT

FRACTIONATION OF BOVINE LYMPHOCYTES WITH AN IMMUNOABSORBENT COLUMN AND ANALYSIS OF CELL SURFACE CHARACTERISTICS

By

Daniel R. Pennell

Bovine lymphocytes were fractionated into subpopulations enriched in surface immunoglobulin (sIg) positive and sIg negative cells and analyzed for the presence of immunoglobulin (Ig) and complement (C) receptors using EA and EAC rosette assays. Incubation of peripheral blood with zymosan allowed for the development of a leukocyte isolation procedure enabling differentiation between phagocytes and lymphocytes. Lymphocyte fractionations were performed using a column with matrix-bound anti-bovine IgG. Upon passage of lymphocytes through a column and elution with medium, cell isolates were obtained enriched in sIg negative lymphocytes. Elution with medium containing bovine gamma globulin allowed for the isolation of cells enriched in sIg positive lymphocytes. EA and EAC rosetting frequencies increased with increasing proportions of sIg positive lymphocytes suggesting that Ig and C receptors are largely B cell markers.

DEDICATION

To my family:

Parents, Patrick R. and Dolores R. Pennell

and sister, Tamera M. Schneider.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my research advisor, Dr. Norman B. McCullough, for his guidance and encouragement throughout this project. I would also like to thank Dr. Herbert W. Cox and Dr. Walter J. Esselman for their assistance during this study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Mammalian peripheral blood lymphocytes appear quite homogeneous morphologically under light microscopy. However these cells have been divided into subpopulations based on the presence of surface immunoglobulin (sIg) (74), and surface receptors for immunoglobulin (Ig) (25, 61), and complement (C) (6, 65). The manner in which these surface marker defined lymphocyte subpopulations interrelate has been established quite well for some species, e.g., human (29), but not for lymphocytes of other species. One approach in elucidating these interrelationships involves the isolation of lymphocyte fractions or subpopulations in relative purity with respect to one surface marker and subsequent analysis for the presence of the other markers (19).

Recently, assays for the detection of bovine lymphocyte sIg and receptors for Ig and C (43) have been developed. The study described herein was undertaken to develop a lymphocyte immunoabsorbent fractionation column so that bovine lymphocyte subpopulations with and without sIg could be obtained. Analysis of these cell isolates for the presence of receptors for Ig and C would then lend some insight into how these receptors are distributed amongst Ig-bearing and non-Ig-bearing lymphocytes.

LITERATURE REVIEW

Lymphocyte Heterogeneity

Lymphocytes were first divided into two separate and distinct groups based upon their development *in vivo*. They are the thymus-derived T lymphocytes and bursa- (avian) or bursa equivalent- (mammalian) derived B lymphocytes (29, 81). Both T and B cells are found in the circulating blood of birds and mammals. Both also populate specific regions of the lymph nodes and spleen of man and other mammalian species (29). B cells reside in the germinal centers of the lymph nodes and in areas around the splenic vein. T cells occupy the paracortical areas of the lymph nodes surrounding the germinal centers as well as the white pulp of the spleen and periarterial sheath around the splenic artery.

B lymphocytes are the precursors of memory and antibody secreting plasma cells (29, 81). Antibody production, the central ingredient of humoral immunity, is essential for normal protection against bacterial infection, especially encapsulated bacteria, as well as some immunity to infection of non-bacterial etiology (29). T lymphocytes are believed to mediate cell-mediated immune responses such as allograft rejection, graft-versus-

host reactions, and delayed type hypersensitivity (29, 81). Cellular immunity normally develops in response to viral, fungal, parasitic, and intracellular bacterial infection (29). T lymphocytes also play a major role in the early recognition and processing stages for some antigens (T dependent antigens) necessary for subsequent B cell activation and humoral response (29). In addition, T lymphocyte subpopulations (T helper and T suppressor cells) are thought to play regulatory roles in both cellular and humoral immune reactions (29, 61).

Other functional properties attributed to lymphocytes are thought to be mediated by lymphokines (23). Most of these lymphocyte produced and secreted soluble products are well characterized in vitro while their in vivo functions remain in question. Lymphokine production and secretion is generally attributed to T lymphocyte subpopulations although the well studied migration inhibition factor (MIF) has been found to be a product of both T and B lymphocytes (19).

B lymphocytes are identified in peripheral blood and other tissues as cells with readily detectable sIg (see Lymphocyte Surface Immunoglobulin section) and T lymphocytes by their ability to bind to heterologous erythrocytes (see Lymphocyte Receptors for Heterologous Erythrocytes section). This surface marker defining of

lymphocytes has led to identification of the so-called undefined lymphocytes (UL's) (25) or null cells (19) which lack either marker although these cells are probably either thymic or bursa equivalent in origin (19) or monocyte-macrophage related (25). Some caution is warranted in equating marker defined and ontogenetically defined T and B lymphocytes since some evidence has been collected suggesting that not all B cells have readily detectable sIg (19) and some sIg positive lymphocytes may bind to heterologous erythrocytes (26).

The subject of the lymphocyte surface has received considerable attention in recent years. This dynamic and complex membrane contains a number of cell markers in addition to sIg and receptors for heterologous erythrocytes, including receptors for Ig (see Lymphocyte Receptors for Immunoglobulin section) and C (see Lymphocyte Receptors for Complement section). For a discussion and references regarding the subject of other cell receptors, including those for mitogens and hormones, and antigens, including those of the histocompatibility complex, see Laudoulis et al. (52).

Isolation of Peripheral Blood Lymphocytes

By far the most commonly employed method for the isolation of blood lymphocytes entails centrifugation of

anticoagulated whole blood or buffy coat cells layered over a mixture of Ficoll and sodium metrizoate (Isopaque) (9, 10) or sodium diatrizoate (Hypaque) (32). Originally described by Boyum (9) in the late 1960s for human blood samples, the technique has since been adopted for use with lymphocytes from other animal species (10). Upon centrifugation at 800 to 1200 x g the white cell layer at the plasma-Ficoll-Isopaque (Hypaque) interface is removed. Although the presence of a small percentage of polymorphonuclear cells and erythrocytes is not infrequent, this cell layer consists principally of mononuclear cells (lymphocytes and monocytes) and platelets.

Removal of contaminating phagocytes has been accomplished by incubating whole blood or buffy coat cells with iron carbonyl prior to centrifugation over gradient (38). Phagocytic ingestion of the iron particles increases the density of the cells such that they will sediment to the bottom of the tube upon centrifugation. Removal of these iron laden leukocytes with a magnet is also possible. Alternatively, phagocytes may be labeled with latex (16) so that they may be differentiated from lymphocytes upon microscopic examination.

A useful modification of the Ficoll-Hypaque centrifugation procedure is outlined by English and Anderson (32). These authors have developed a discontinuous

gradient centrifugation technique using two Ficoll-Hypaque solutions of different concentrations of Hypaque. Solution 1 of specific gravity 1.077 gm/ml is layered over solution 2 with a specific gravity of 1.119 gm/ml. Diluted heparinized human blood was layered over the gradient. After centrifugation three distinct cell layers were observed; layer 1 (plasma-solution 1 interface) consisted of mononuclear cells and platelets, layer 2 (solution 1-solution 2 interface) of polymorphonuclear cells, and layer 3 of erythrocytes.

Lymphocyte Receptors for Heterologous Erythrocytes

Although Thy-1 and Ly antigens allow for rapid and specific immunological identification of mouse T lymphocytes (29), no such antigens have been identified for most other mammalian T cells. The observation that sheep erythrocytes (SRBC's) (11) as well as other heterologous RBC's (54, 82) attach to the surface of some human lymphocytes, i.e., form E rosettes, has led to the development of reliable methods of T cell detection and enumeration. Evidence that the E rosette assay detects T lymphocytes is based on a number of observations. It has been shown that lymphocytes with detectable sIg do not bind SRBC's to their surfaces (13, 48) with the exception of one study finding that 2-4% of peripheral blood lymphocytes with sIg bound neuraminidase-treated SRBC's (26). Mouse studies have shown that the

organ distribution of SRBC binding lymphocytes is inversely correlated to the presence of cells carrying Ig with the frequencies of rosette formation occurring at the expected values (77). Finally, the tissue distribution of E rosette forming lymphocytes were found to correspond to the thymus-dependent areas (80).

The E rosette assay is accomplished by incubating lymphocytes with erythrocytes and subsequent microscopic examination looking for lymphocytes with attached erythrocytes, i.e., rosettes. Procedures for detecting E rosetting lymphocytes vary considerably making it difficult to compare the results from one laboratory to another. A number of erythrocyte treatments have been introduced in order to minimize the variability and/or enhance the frequency of rosette formation including treatment with neuraminidase (26, 33, 54, 83), papain (33), and 2-aminoethylisothiouronium bromide (AET) (68, 69). Formation of rosettes in the presence of fetal calf serum (88) and dextran (8, 13) has also proved to enhance rosetting frequencies.

A modified rapid E rosette test has been described to identify "active" E rosette forming cells which are thought to represent a subpopulation of T lymphocytes (88, 89). The significance of this cell population has not been established. E rosette assays have been developed for a number of non-human lymphocytes (8, 33, 83) including lymphocytes of bovine origin (42, 68, 85).

Lymphocyte Surface Immunoglobulin

Although the concept of surface receptors for antigen had been proposed as early as 1900 by Ehrlich (31), it was not until 1961 when Moller found that anti-mouse Ig serum reacted with some mouse lymphocytes that the presence of surface-bound Ig was suggested (60). It is now generally accepted that lymphocytes with readily detectable sIg are B lymphocytes, a conclusion suggested by the distribution of sIg-bearing cells in lymphoid tissues (39). Receptors on T lymphocytes have been demonstrated (3, 59, 76) but the chemical nature of these receptors remains unresolved.

Routine procedures in both clinical and research laboratories for the detection and enumeration of B lymphocytes usually entails the use of fluorescenated antisera against polyvalent Ig of the species under investigation. Antisera labeled with peroxidase (39) can also demonstrate sIg. Enumeration of B cell subpopulations is possible with labeled antisera to heavy (class specific) or lambda and kappa light chains (1, 51, 70, 72, 73). A recent publication by Winchester et al. (87) recommends the use of labeled $F(ab')_2$ to avoid staining of lymphocytes bearing Fc receptors but lacking sIg. Some perform the staining procedure in the presence of sodium azide to prevent capping of sIg (51, 70).

Lymphocyte Receptors for Immunoglobulin

The subject of lymphocyte receptors for Ig became an extensive area of research interest in the late 1960s and continues to receive attention today. Receptors for IgG were the first to be demonstrated and characterized [see Dickler (25) for a recent review]. Since the observation of human lymphocyte receptors for IgM in 1975 (62) the attention has shifted considerably to this subject [see Moretta et al. (61) for recent review]. Recent investigations have demonstrated receptors for IgE on human B lymphocytes (40) and human and rat T lymphocytes (90) as well as IgA receptors on human T cells (56).

Early investigations into the nature of lymphocyte receptors for Ig demonstrated that binding is stable only when Ig is complexed to antigen (5) or in aggregated form (aggIg) (27). Early studies also showed the binding to be specific for the Fc fragment of IgG but not IgM (21, 36). Hence, lymphocytes with receptors for the Fc fragment of IgG (FcRL's for IgG) are most frequently detected with labeled aggIgG or erythrocytes sensitized with either heat inactivated anti-erythrocyte serum or purified IgG (EA or EAIgG).

The question as to whether the two assays detect the same receptor or two different receptors has been raised, since the EA rosette assay generally identifies

a lower percentage of cells as FcRL's (29). Depletion of rosetting cells from a population of lymphocytes incubated with EAIgG results in a subpopulation of lymphocytes with very few cells that can bind aggIgG (20) showing that the EAIgG assay detects a subset of those lymphocytes capable of binding aggIgG. This observation suggests that the receptors are the same and that the EA assay is simply less sensitive. Inhibition experiments also provide evidence that the same receptor binds both aggIg and antibody-antigen complexes (24).

Early studies with both mouse and human lymphocytes suggested that the Fc receptor for IgG was largey if not exclusively a B cell marker (5, 27). Later reports, however, show that at least some human T cells also carry this receptor (12, 21, 36).

FcRL's for IgG have been shown to mediate antibody-dependent cell-mediated cytotoxicity (ADCC). Incubation of lymphocytes with aggIgG inhibits this <u>in vitro</u> cytolytic phenomenon (24), whereby antibody coated target cells are lysed by non-sensitized lymphocytes. These effector cells are frequently referred to as killer (K) cells (29). ADCC is in fact another assay for lymphocytes with Fc receptors for IgG (29).

Receptors for IgM went undetected in all early studies due to the necessity for overnight incubation of

lymphocytes at 37°C in IgM free medium before EAIgM rosetting could occur (57, 62). One explanation that accounts for this incubation requirement is that IgM receptors are saturated with serum IgM in vivo and that during in vitro incubation new receptors are synthesized (34).

IgM receptors were first demonstrated on human T lymphocytes (57, 62) and later on B cells as well (34, 71). IgM receptors bind to the Fc portion of either monomeric (14, 75) or pentameric (14, 35) IgM. Moretta has found that human T FcRL's for IgG ($T._G$) and T FcRL's for IgM ($T._M$) to be separate and distinct cell populations (63). Moreover, T suppressor activity seems to reside within the $T._G$ population and T helper activity within the $T._M$ population suggesting a regulatory role for these receptors.

Considerable evidence has shown that sIg and Fc receptors for IgG and IgM are structurally independent of each other. As mentioned above no T cells carry both IgM and IgG receptors. In addition, IgM receptors on T lymphocytes are trypsin sensitive whereas receptors for IgG on T lymphocytes are trypsin resistant (58). On B lymphocytes, binding of EAIgM requires the presence of divalent cations and is not inhibited by preincubation of cells with aggIgG whereas binding of EAIgG does not require the presence of divalent cations and is inhibited by preincubation of cells

with aggIgG (14, 71). Furthermore, capping of sIg with anti-Ig serum does not effect the distribution of Fc receptors for IgG (24) or IgM (71).

Receptors for Ig of one class or another are found on mast cells (22), macrophages (22), monocytes (45, 53), neutrophils (53), and basophils (46). An EA rosette assay has been used to study bovine lymphocytes (43).

Lymphocyte Receptors for Complement

In 1965 Uhr observed that antibody-antigen-complement complexes bind to some lymphocytes (84). Since that observation a considerable amount of research effort has been directed toward the so-called complement receptor lymphocyte (CRL). [See Nussenzweig (65) and Bianco and Nussenzweig (6) for recent reviews.]

Detection of CRL's is usually accomplished by incubating lymphocytes with erythrocytes sensitized with antibody and a non-lytic complement source. The complement source may be non-lytic by nature, e.g., mouse serum, or diluted to a sublysing titer. Antibody sensitization of erythrocytes with IgM to avoid antibody binding to Fc receptors is suggested for human lymphocyte studies (2). Alternatively, erythrocytes are sensitized with anti-erythrocyte serum and the data is interpreted with the understanding that FcRL's may also be detected. Erythrocytes that bind poorly to T lymphocytes are preferred to

decrease the possibility of E rosette formation with EAC's (2).

The C receptor is generally considered a B cell marker although it may be that not all B lymphocytes are CRL's. Many report that there are fewer CRL's than lymphocytes with sIg in peripheral blood (29, 67, 77), although the EAC rosette assay may not be sensitive enough to detect all CRL's. CRL's correspond well with B cell ontogeny in that lymphocytes with C receptors are rare in the thymus (7, 53, 67) and show the characteristic distribution in thymus-independent tissue regions (28, 80).

Human lymphocyte EAC studies have shown that CRL's can bind both C3b and C3d (30). Unlike Fc receptors for IgG, C receptors are destroyed by trypsin (66). Capping of sIg has no effect on the distribution of C receptors (66). C receptors have been demonstrated on red blood cells (64), macrophages (53), monocytes (53) and granulocytes (30, 53). An EAC rosette assay has been used to study bovine lymphocytes (43, 50).

Isolation of Lymphocyte Subpopulations

Obtaining lymphocyte subpopulations in relative purity has contributed considerably to the understanding of lymphocyte marker interrelationships. There are a number of approaches used in obtaining such cell isolates.

Patients of immunodeficient status have been used as lymphocyte sources for this purpose. Thus, patients with X-linked Agammaglobulinemia have no CRL's (37).

A number of in vitro manipulations have been developed for isolating lymphocyte subpopulations. These procedures can be divided into two types.

1. There are methods which deplete or exclude a defined set of lymphocytes from the original lymphocyte population. The remaining cells are generally defined in the negative context, e.g., Fc receptor negative lymphocytes.
2. Lymphocyte fractionation results in two or more subpopulations, defining one from another according to some lymphocyte characteristic.

Selection of a lymphocyte separation technique depends to some degree upon the nature of the investigation. Factors to consider include: the time, cost and assessability of supplies necessary to perform the separation; the purity, viability, and cell recovery that can be expected; and the number of cells which can be processed.

Julius et al. (49) have described a now rather popular method for T lymphocyte isolation involving the incubation of lymphocytes in nylon wool. Subsequent elution of non-adhering cells results in a cell isolate with only 5% cells with sIg. Recovery of T lymphocytes ranged from

50-90%. The basis for retention of Ig-bearing lymphocytes with nylon wool is not known and selective loss of T cell subpopulations is possible.

Rosette depletion has been accomplished with E (18, 44, 78), EA (20, 67), and EAC (7, 18, 67) rosette forming cells. After lymphocytes are allowed to form rosettes, they are centrifuged over a gradient, e.g., Ficoll-Hypaque, allowing non-rosetting lymphocytes to be recovered. The success of this technique depends to a major extent upon the stability of the rosettes formed. When rosettes are stable, however, purity is generally very good and large numbers of cells can be processed. Saxon et al. (78) have recently described an E rosette depletion technique allowing for the isolation of the rosetting lymphocytes as well as the non-rosetting lymphocytes by lysing the RBC's of the rosetting cells after centrifugation.

Gutierrez et al. (41) have described a discontinuous density gradient centrifugation procedure which allows for very good recovery of highly purified sIg negative lymphocytes, however the B cell subpopulations are of relatively poor purity (72.8% with detectable sIg). This one step method is very simple and rapid and allows for large numbers of cells to be processed.

Methods offering the greatest success for obtaining highly pure lymphocyte subpopulations involve the separation of cells with an immunoabsorbent column. Such columns involve the binding of antigen specific antibody to column matrix. Upon application of cells to the column, antigen positive cells are retained while antigen negative cells can be eluted.

Wigzell et al. (86) first introduced such a column for the ısolation of sIg negative mouse lymphocytes by utilizing what they refer to as the "double layer principle." Plastic beads are first coated with mouse immunoglobulin. The antigen coated beads are then labeled with excess anti-Ig so that free antigen binding sites on the antibody molecules are available to interact with Ig-bearing lymphocytes. Although some non-specific retention of cells was noted, only 0.8% of those lymphocytes passing through the column were sIg positive. No attempt was made to recover bound cells.

Campbell and Grey (15) introduced a similar column also for mouse lymphocytes. This column, however, was made by binding anti-gamma globulin to plastic beads directly. Upon serial passage of lymphocytes through four columns only 0.2% were sIg positive although cell recovery was poor. Again, there was no attempt to recover bound cells.

Schlossman and Hudson introduced the first column allowing for the recovery of retained cells (79). This time, however, anti-serum was made to $F(ab')_2$ and the antibody fraction of the serum purified. The purified anti-$F(ab')_2$ was bound to dextran gel beads (Sephadex G-200). Retained mouse lymphocytes were eluted by digesting the beads with dextranase. Non-specific retention of cells was reduced considerably presumably due to the use of purified antibody. Dextranase eluted cells were difficult to analyze since they were covered with anti-$F(ab')_2$ but unbound lymphocytes had only 3-6% cells with sIg. A similar column for guinea pig lymphocytes allowed for the demonstration of transfer of delayed type hypersensitivity (DTH) from immune to non-immune animals with sIg negative lymphocytes (47). Attempts to determine whether the dextranase eluted cells could also transfer DTH failed due to an inflammatory reaction presumably caused by undigested dextran residue.

With only minor modification a similar immuno-absorbent column was developed for human lymphocytes (17). Again, purified anti-$F(ab')_2$ was bound to Sephadex G-200. Elution of retained cells, however, was accomplished by competitive inhibition with human gamma globulin. Data from ten lymphocyte fractionations resulted in unretained cells with an average of less than 2% of the cells positive

for sIg. Gamma globulin eluted cells averaged 95.2% cells positive for sIg. Viabilities were greater than 95% for all populations.

A lymphocyte immunoabsorbent fractionation column is somewhat time consuming to construct and operate but requires no specialized equipment and allows for the processing of a wide range of cell numbers.

MATERIALS AND METHODS

Animals

Adult New Zealand female rabbits (Spartan Research Animals, Inc., Haslett, Michigan) were immunized for the production of anti-bovine IgG.

Adult ICR female mice (Harlan Industries, Inc., Cumberland, Indiana) were used as a source of complement for EAC rosette studies.

Holstein heifers, used as a source of blood for leukocyte studies, were housed at the Large Animal Clinic at Michigan State University. All bovine data appearing in this report were collected using leukocytes from animal #223629, age 2, in good health according to clinic records.

Immunization

Rabbits were immunized by injecting 2 mg of bovine IgG (Miles Laboratories, Inc., Elkhart, Indiana) intramuscularly in Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan) and one week later the same dosage was injected subcutaneously in sterile saline. Rabbits were bled 8 weeks after the last injection. Sufficient humoral response to the immunization was determined when a visible precipitin band was observed using Ouchterlony gel diffusion.

Collection and Treatment of Sera

Rabbit blood, obtained from the marginal ear vein, was allowed to clot for 2 to 3 hr and spun at 1000 x g for 30 min at 4°C. The anti-bovine IgG serum was harvested, pooled and stored at -20°C.

Mouse blood was obtained from the subclavian artery, allowed to clot for 30 min in an ice bath and centrifuged at 3000 x g for 15 min at 4°C. The serum was harvested, pooled and absorbed against an equal volume of washed SRBC's for 45 min at 0°C. Absorbed serum was harvested by centrifugation at 3000 x g for 15 min at 4°C and stored in 0.2 ml aliquots at -70°C.

Rabbit anti-SRBC serum, used in all EA and EAC rosette studies, was obtained from Dr. E. Sanders, Michigan State University. The antiserum was heat inactivated at 56°C for 30 min and stored in 0.5 ml aliquots at -20°C.

Isolation of Bovine Leukocytes

Heifers were bled from the caudal vein using 20 ml vacutainer tubes (Becton-Dickinson, Rutherford, New Jersey) containing sodium heparin. Blood leukocytes were isolated using the Ficoll-Hypaque centrifugation method described by Boyum (10) with slight modification. Warm blood, diluted 1:2 with EDTA-phosphate buffered saline (EPS) was layered over 3 ml Ficoll-Hypaque and spun at 1100 x g for 30 min at room temperature. The white cell layer at the

Ficoll-Hypaque-supernatant interface was removed with a Pasteur pipette and washed three times with Dulbecco's phosphate saline (PBS) (Grand Island Biological Co., Grand Island, New York) recovering cells by centrifugation at 500 x g.

An alternate leukocyte isolation procedure enabling rapid differentiation between phagocytic and non-phagocytic cells was established. Twenty-five milligrams of washed (PBS) zymosan A (Sigma Chemical Co., St. Louis, Missouri) in 2 ml Hank's balanced salt solution (HBSS) (Grand Island Biological Co.) were added to 10 ml whole blood and incubated for 30 min with end-over-end mixing at 37°C. The zymosan-blood mixture was diluted 1:2 with EPS and leukocytes isolated as described above. Cells with intracellular zymosan were scored as phagocytes.

A comparison study involving the two leukocyte isolation procedures was conducted in order to establish zymosan treatment as an adequate method for the identification of phagocytic cells. Differentials were performed on leukocytes in whole blood with Wright's stain and Ficoll-Hypaque isolated white cells with Turk's stain from zymosan treated and untreated blood. Each luekocyte isolation procedure was performed in triplicate and cell differentials were determined by counting 200 cells microscopically. The zymosan-Ficoll-Hypaque isolation

procedure was adopted for use in subsequent bovine leukocyte fractionation studies.

Enrichment of Rabbit Anti-Bovine IgG Antibody

Antibody from anti-bovine IgG serum was enriched with an insoluble immunoabsorbent of bovine gamma globulin (Miles Laboratories) covalently bound to cyanogen bromide activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, New Jersey). The procedure used is a modification of that outlined by Chess and Schlossman (18). Thirty milligrams of CNBr activated sepharose 4B, swollen in 10^{-3} M HCl, were mixed with 200 mg bovine gamma globulin, dissolved in borate buffered saline (pH 8.3) (coupling buffer) and gently mixed for 2 hr at room temperature. The resulting protein-bead complex was washed with coupling buffer to remove unbound protein and resuspended in 0.5 M Tris-HCl (blocking reagent) for 2 hr at room temperature to block remaining active protein binding sites. Excess blocking reagent was washed out with three rounds of 0.1 M acetate buffer followed by coupling buffer before a final wash with several volumes of PBS. Antiserum (100 ml) was thawed to room temperature and slowly passed through 10 ml of immunoabsorbent packed in a 1 x 20 cm glass column. Unbound serum components were eluted with PBS, and bound antibody with 0.1 M glycine-HCl (pH 2.5) collected in 2.0 M phosphate buffer

(pH 8.0). The latter eluate was repeatedly lyophilized and dialyzed against coupling buffer to achieve a final concentration of 10 mg protein per ml and stored at -70°C. Protein determinations were performed as described by Lowry (55).

Analysis of Enriched Rabbit Anti-Bovine IgG

Rabbit anti-bovine IgG serum, enriched antibody, and normal human control serum were examined by electrophoresis to assess the effectiveness of the purification procedure. Fractionation of samples was performed on 60 x 75 mm Titan III cellulose acetate plates (Helena Laboratories, Beaumont, Texas) containing eight strips per plate. Plates were soaked in Electra HR buffer (Helena Laboratories) of pH 8.6 and ionic strength 0.05 and 5.5 µl of sample applied to each strip. A constant current of 6 ma was applied to each plate (0.78 ma per strip) in Electra HR buffer for 15 min. Samples were stained with Ponceau S (Gelman Instrument Co., Ann Arbor, Michigan), dried, and read with a Gelman ACD-18 automatic computing densitometer (Gelman Instrument Co.) at a wavelength of 500 nm. All samples were run in duplicate.

Precipitin activity of enriched antibody, diluted to the original serum volume, was compared with the activity of undiluted immune rabbit serum and a normal rabbit serum control using Ouchterlony gel diffusion.

Preparation of Cell Fractionation Immunoabsorbent Columns

The procedure for preparation of cell fractionation columns outlined by Chess and Schlossman (18) will be described briefly. Swelled Sephadex G-200 (60 ml) (Pharmacia Fine Chemicals) was activated with 100 mg of cyanogen bromide (Eastman Kodak Company, Rochester, New York), maintaining a constant pH of 10.2 with 1 N NaOH, and a constant temperature of 20°C with ice, with gentle mixing for 1 hr. The activated Sephadex, washed in coupling buffer was mixed with 20 mg of enriched anti-IgG antibody and incubated for 4 hr at room temperature. The Sephadex G-200-antibody conjugate was transferred to a sintered glass funnel and washed with cold coupling buffer. The resulting immunoabsorbent was stored in PBS containing 0.1% sodium azide (Eastman Kodak Company) at 4°C. Disposable 10 ml syringes (Becton-Dickinson) fitted with 35 mm pore polyethylene discs (Bell Art Products, Benawalk, New Jersey) were packed with 9 ml of immuno-absorbent and washed with medium 199 containing 5% fetal calf serum and 2.5 mM EDTA (cell medium) before use in cell fractionation.

Cell Preparation and Fractionation

The procedure used is a modification of the methods described by Chess and Schlossman (18). Briefly, bovine

leukocytes isolated by the zymosan-Ficoll-Hypaque procedure were washed three times with cell medium and concentrated to 1 x 10^7 cells per ml in cell medium before application to fractionation columns. Lymphocyte suspension (5-10 ml) was applied to columns, run at a flow rate of 0.3 to 0.5 ml per min. Unbound cells were eluted with 45-90 ml of cell medium and retained cells with 45 ml of cell medium containing 10 mg bovine gamma globulin per ml. During the elution of bound cells the column material was gently mixed with a Pasteur pipette. The recovered cell populations were then washed with cell medium before analysis. A retained portion of unfractionated cells was also analyzed. All cell populations were analyzed at the same time.

Rosetting Assays

EA and EAC rosette procedures were performed according to the methods of Higgins and Stack (43) with slight modification. Washed SRBC's from whole blood in Alsever's solution (Colorado Serum Company, Laboratories, Denver, Colorado) were suspended to 5% in complement fixation buffer (CFB) and incubated with an equal volume of anti-SRBC serum for 30 min at 37°C. The antibody sensitized erythrocytes (EA's) were washed three times with CFB and resuspended to 5% in CFB with 1% gelatin (CFBG). A portion of the EA suspension was incubated with an equal volume of mouse serum for 30 min at 37°C for complement sensitization.

The resulting antibody, complement sensitized erythrocytes (EAC's) and the retained portion of the EA's were washed three times in CFBG and resuspended to 1 x 10^8 cells per ml. A 0.15 ml portion of leukocyte suspension at 2 x 10^6 cells per ml was mixed with an equal volume of sensitized erythrocytes and incubated for 15 min at 37°C. The cell mixture was then spun at 200 x g for 5 min and reincubated for 16 to 18 hr in an ice bath. One drop of 1:5 diluted crystal violet was added to each tube and the cells gently resuspended. Lymphocytes, examined under phase microscopy, with three or more erythrocytes attached were scored as rosettes. At least 200 cells were counted from each tube. Each test was run in triplicate.

Optimal antibody and complement titers were determined by testing several dilutions of each and finding those dilutions resulting in the highest frequency of rosetting cells. E, EC, and EA controls were included in all titration studies. E controls were included with all leukocyte fractionation studies.

Detection of Surface Immunoglobulin

Surface immunoglobulin was detected with FITC conjugated IgG fraction of rabbit anti-bovine gamma globulin (Cappel Laboratories, Inc., Cochranville, Pennsylvania). A 0.1 ml portion of leukocyte suspension in PBS with 0.1% sodium azide at a concentration of 2 x 10^6

cells per ml was incubated with an equal volume of fluorescein-antibody conjugate for 30 min at 37°C and washed three times with PBS with 0.1% sodium azide. Fluorescent activity was studied with a Zeiss fluoroscope equipped with a dark field condenser and an Osram HBO 200 mercury lamp using exicter filter BG5 and barrier filters 47 and -65. For each test 200 cells were counted and scored.

Enumeration of Phagocytic Cells in Fractionation Studies

Enumeration of phagocytes in fractionation studies was determined by scoring 200 cells for intracellular zymosan using leukocytes in the E controls.

Viability Assay

Viabilities were determined with trypan blue (Grand Island Biological Company). A 0.1 ml portion of leukocyte suspension at a concentration of 2×10^6 cells per ml in HBSS were incubated with 0.05 ml of 1:5 diluted trypan blue for 15 min at 37°C. Two hundred cells were scored in each test. Viabilities of greater than 90% were achieved in all studies unless otherwise specified.

Photography

The photomicrograph appearing in this report was taken with Tri-X-pan Kodak film at an exposure time of 1/8 sec using a Zeiss microscope.

Statistics

All tests run in triplicate are expressed in terms of mean percent ± standard deviation ($\bar{x}$ ± S.D.). The student's t-test was used to evaluate results when indicated.

Preparation of Reagents

- Ficoll-Hypaque density gradient

  72.8 gm Ficoll 400 (Pharmacia Fine Chemicals)

  480 ml Hypaque (sodium diatrizoate) 25% aqueous solution (Winthrop Laboratories, New York, New York)

  720 ml Distilled water.

  The specific gravity was determined with a hydrometer (Arthur Thomas Company, Philadelphia, Pennsylvania) and always found to be between 1.076 and 1.079. The solution was filter sterilized with a 0.2 μm Nalgene filter unit (Sybron Corporation, Rochester, New York) and stored at 4°C.

- EDTA-phosphate buffered saline (EPS)

  3.58 gm EDTA-trisodium salt (Sigma Chemical Company)

  9.00 gm NaCl

  1.08 gm KH_2PO_4

  1.00 ℓ Distilled water.

  The solution was filter sterilized (0.2 μm) and stored at 4°C. The pH was adjusted to 7.2 to 7.4 before use.

- Complement fixation buffer (with 0.1% gelatin) (CFB and CFBG)

83.00 gm	NaCl
10.19 gm	Sodium 5,5-diethyl barbituric acid
34.60 ml	1N HCl
5.00 ml	Stock solution containing 1 M $MgCl_2$ and 0.3 M $CaCl_2$.

The above reagents were dissolved in distilled water and adjusted to 2 ℓ. The solution was filter sterilized (0.2 μm) and stored at 4°C. CFB, made by diluting a portion of the above stock CFB 1:5 with distilled water, was stored for no longer than 24 hr at 4°C. The pH was determined to be 7.3 to 7.4 before use. CFBG was made by dissolving 0.25 gm gelatin (General Foods Corporation, White Plains, New York) in 200 ml distilled water. The gelatin solution was mixed with 50 ml of stock CFB. CFBG was stored for no longer than 24 hr at 4°C. The pH was determined to be 7.3 to 7.4 before use.

- Cell medium

1 pkg	Medium 199 powder with Hank's salts and L-glutamine (Grand Island Biological Company)
50 ml	Fetal calf serum (Microbiological Associates Inc., Bethesda, Maryland)
0.73 gm	EDTA-trisodium salt
10 ml	200 mM L-glutamine (100x) (Microbiological Associates)

10 ml Penicillin (10,000 units per ml), streptomycin (10,000 µg per ml) (Grand Island Biological Company)

Cell medium reagents were diluted to 1 ℓ with distilled water, aliquoted into 100 ml portions, and stored at -20°C. Medium was thawed to room temperature and the pH adjusted to 7.2 to 7.4 before use.

- Turk's stain

5 ml Acetic acid

5 ml 1% Crystal violet

490 ml Distilled water.

Reagents were mixed and stored at room temperature. Leukocytes at a concentration of 2 x 10^6 cells per ml were mixed with an equal volume of stain. A wet mount was made and examined microscopically.

RESULTS

Detection of Phagocytic Leukocytes with Zymosan

Wright's stain differential examination of zymosan treated and untreated whole blood stained prior to Ficoll-Hypaque centrifugation resulted in cell types of comparable frequencies (Table 1). Basophils were seen in lowest frequency; 0.2% for both zymosan treated and untreated blood. Only minor variations in frequencies were seen with the remaining cell types. All phagocytic leukocytes, with the exception of basophils, injested zymosan to some degree, i.e., 90.0% of the monocytes, 79.0% of the neutrophils, and 86% of the eosinophils were seen with intracellular zymosan. Extracellular zymosan was seen throughout the blood smear.

Turk's stain examination of leukocytes, recovered from Ficoll-Hypaque centrifugation, of the same blood samples yielded 15.3% phagocytes when blood was preincubated with zymosan as compared to 11.3% for untreated blood (Table 2). Of the phagocytes counted, 92.8% were seen with intracellular zymosan. Extracellular zymosan was only rarely seen. Leukocytes were greater than 90% viable for all three untreated blood samples.

Table 1. Wright's stain examination of bovine leukocytes in zymosen treated and untreated whole blood

	Untreated blood	Zymosan treated blood		
	Percent leukocytes	Percent leukocytes	Percent leukocytes with intracellular zymosan	Percent phagocytes with intracellular zymosan[a]
Lymphocyte	38.3 ± 4.8	34.7 ± 1.2	0.0	--
Monocyte	3.7 ± 0.6	3.0 ± 0.5	2.7 ± 1.0	90.0
Neutrophil	34.0 ± 5.2[b]	41.5 ± 1.8[b]	32.8 ± 4.8	79.0
Eosinophil	23.8 ± 1.2	20.7 ± 1.8	17.8 ± 3.1	86.0
Basophil	0.2 ± 0.3	0.2 ± 0.3	0.0	0.0

[a]Values = ($\bar{x}$% leukocytes with intracellular zymosan of each cell type ÷ $\bar{x}$% leukocytes of the same cell type) X 100.

[b]Not statistically significant at $P < 0.05$.

Table 2. Turk's stain examination of bovine leukocytes isolated from Ficoll-Hypaque centrifugation of zymosan treated and untreated whole blood

	Untreated blood	Zymosan treated blood
Percent phagocytes	11.3 ± 2.3	15.3 ± 1.6
Percent leukocytes with intracellular zymosan	--	14.2 ± 1.5
Percent phagocytes with intracellular zymosan[a]	--	92.8
Percent leukocytes viable	94.0 ± 2.0	NT[b]

[a]Value = ($\bar{x}$% leukocytes with intracellular zymosan ÷ $\bar{x}$% phagocytes) X 100.

[b]NT = Not tested.

Antibody and Complement Titration for Rosette Assays

An antibody titration study was performed so that the optimal anti-SRBC dilution, i.e., that dilution resulting in the greatest frequency of rosette forming cells, could be ascertained. The results are shown in Table 3. Several dilutions between 1:50 and 1:1600 of antiserum were used to sensitize sheep erythrocytes for EA and EAC rosette studies. All EAC tests and an EC control were performed with complement (mouse serum) diluted 1:20. Dilutions of 1:50 and 1:100 resulted in agglutination of erythrocytes after the 30 min incubation making the assay difficult or impossible to read. The lowest dilution without visible microscopic erythrocyte agglutination (1:200) resulted in the highest mean frequencies of EA and EAC rosette formation (31.0% and 41.5%, respectively) and was chosen as the optimal antibody titer for all subsequent EA and EAC assays. Rosette formation decreased with increasing dilution of antiserum for both EA and EAC assays. EAC rosette frequencies were consistently higher than EA frequencies of the same antibody titer. An EC control resulted in more cells forming rosettes than did unsensitized erythrocytes but fewer cells forming rosettes than all EA and EAC tests performed.

A similar study to assess the optimal complement titer for EAC rosette studies was conducted. The results

Table 3. Rosette formation by bovine peripheral blood lymphocytes and sheep erythrocytes sensitized with variable dilutions of rabbit anti-sheep erythrocyte serum

Rabbit antiserum dilution	Percent rosettes with diluted mouse serum and no serum	
	1:20	No serum
1:50	Agg[a]	Agg
1:100	Agg	Agg
1:200	41.5 ± 3.1	31.0 ± 5.6
1:400	32.5 ± 3.5	27.5 ± 2.0
1:800	11.5 ± 1.3	6.0 ± 1.0
1:1600	9.0 ± 1.0	3.5 ± 2.1
No antiserum	3.0 ± 0.5	0.3 ± 0.3

[a]Agg = Agglutination.

are shown in Table 4. All EAC assays and an EA control were performed with anti-SRBC serum diluted 1:200. Incubation of antibody sensitized erythrocytes with undiluted mouse serum resulted in packed erythrocytes upon even gentle washing. The packed erythrocytes required prolonged mixing before the cells could be resuspended making undiluted mouse serum an impractical source of complement. The first mouse serum dilution (1:5) minimized this packing phenomenon considerably and resulted in the highest mean frequency of EAC rosettes of all dilutions studied. EAC rosette formation decreased with increasing complement dilution. EAC frequencies were consistently higher than EC controls of the same complement titer. An EA control resulted in fewer cells forming rosettes than all EAC tests and more cells forming rosettes than that seen with the E control. Unsensitized erythrocytes resulted in the lowest mean frequency of rosette formation with the exception of erythrocytes sensitized with mouse serum diluted 1:20. Mouse serum diluted 1:5 was chosen as the most appropriate complement titer for the EAC rosette assay and was used in all subsequent EAC rosette studies.

Analysis of Enriched Rabbit Anti-Bovine IgG

Electrophoretic analysis of immune rabbit serum and enriched anti-bovine IgG antibody (Figure 1) resulted in a demonstrated reduction in non-gamma globulin protein

Table 4. Rosette formation by bovine peripheral blood lymphocytes and sheep erythrocytes sensitized with variable dilutions of mouse serum

Mouse serum dilution	Percent rosettes with diluted rabbit antiserum and no antiserum	
	1:200	No antiserum
Undiluted	EP[a]	NT[b]
1:5	42.8 ± 6.5	2.3 ± 0.3
1:10	42.0 ± 4.3	1.3 ± 0.6
1:20	37.8 ± 1.5	0.8 ± 0.6
1:40	32.8 ± 2.3	1.2 ± 0.3
1:80	31.7 ± 4.5	1.3 ± 0.8
No serum	30.2 ± 3.6	1.0 ± 0.5

[a]EP = Erythrocyte packing.

[b]NT = Not tested.

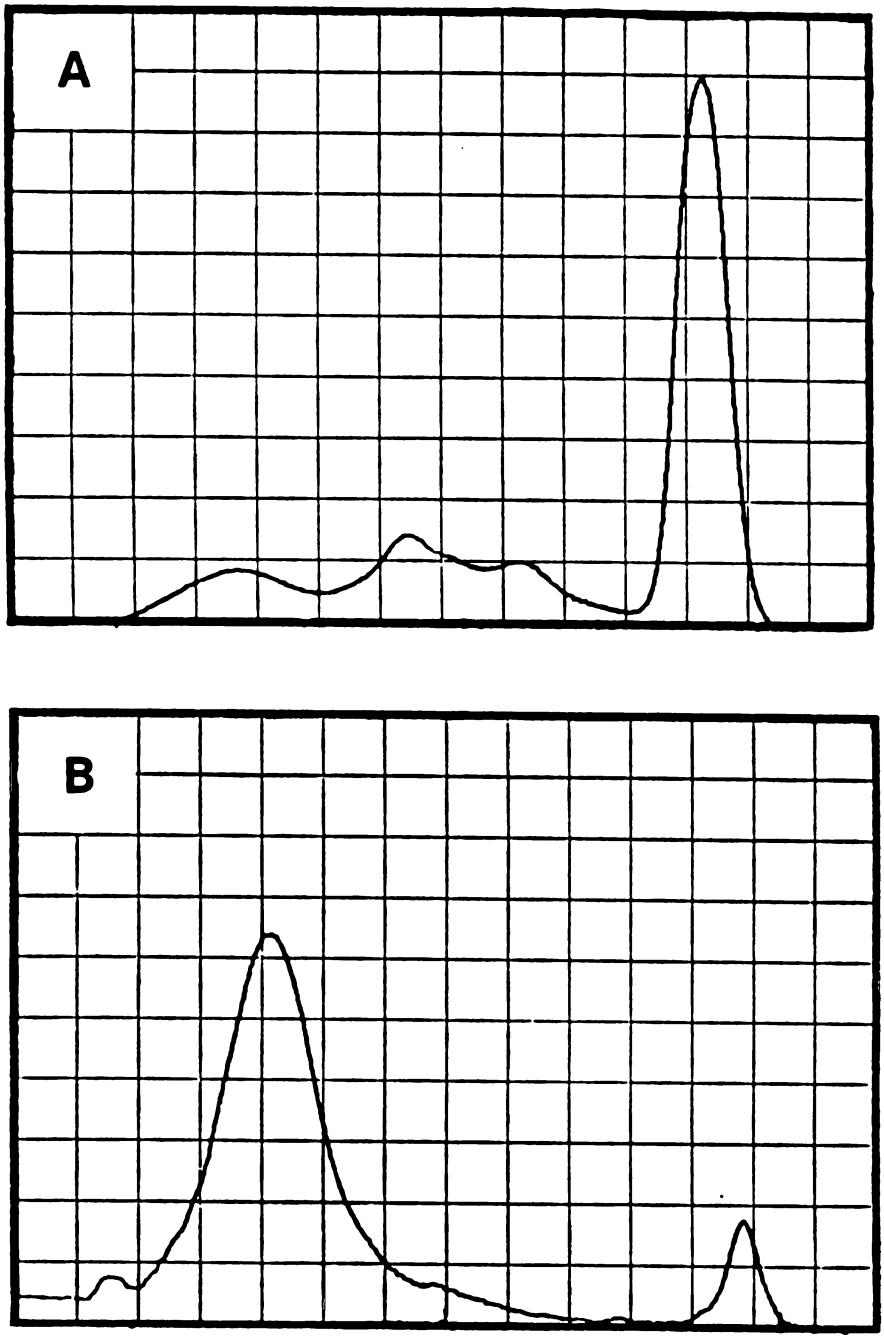

Figure 1. Electrophoretic patterns of rabbit anti-bovine IgG serum (A) showing a high albumin peak (far right) and a relatively low gamma globulin peak (far left) and enriched anti-IgG (B) showing considerable reduction in non-gamma globulin protein.

as a result of the enrichment technique. Gamma globulin protein accounted for 13.22% of the total protein of immune serum and 82.79% of the purified product according to densitometer computations. The highest contaminating protein of the enriched product was albumin (8.18%). Ouchterlony gel diffusion of immune serum and enriched antibody against IgG showed a retention of precipitin activity following antibody enrichment.

Fractionation of Bovine Leukocytes

Bovine leukocyte fractionation studies were performed with leukocytes isolated from zymosan treated peripheral whole blood. The number of cells recovered from cell medium and cell medium containing bovine gamma globulin elution reflects counts of both leukocytes with intracellular zymosan, henceforth referred to as phagocytes, and leukocytes without intracellular zymosan, henceforth referred to as lymphocytes. Viabilities, surface immunoglobulin, and rosetting assays are reported as percentages determined from lymphocyte counts.

The results of the first fractionation experiment are shown in Table 5. The column was adjusted to a flow rate 0.5 ml per min and 9.04×10^7 leukocytes were applied. The entire procedure was performed at room temperature. Of the leukocytes applied to the column 58.6 were recovered. Elution with cell medium resulted in a decrease in the

Table 5. Fractionation of 9.04×10^7 bovine leukocytes at 25°C and flow rate of 0.5 ml per min

	Unfractionated leukocytes	Leukocytes eluted with: Cell medium (45 ml)	Leukocytes eluted with: Cell medium with bovine gamma globulin (45 ml)
NR[a] x 10^6	--	49.6	3.4
% V[b]	75.5	89.0	76.3
% Z+[c]	9.0	0.5	11.5
% sIg[d]	23.0	7.0	44.0
% EAC[e]	14.2 ± 3.5	11.2 ± 6.8	28.3 ± 3.3
% EA[f]	8.3 ± 2.8	8.2 ± 0.6	13.7 ± 2.5
% E[g]	0.3 ± 0.6	0.3 ± 0.6	0.7 ± 0.8

[a]NR = Number of leukocytes recovered.

[b]% V = Percent lymphocytes viable.

[c]% Z+ = Percent leukocytes with intracellular zymosan.

[d]% sIg = Percent lymphocytes with surface immunoglobulin.

[e]% EAC = Percent lymphocytes forming EAC rosettes.

[f]% EA = Percent lymphocytes forming EA rosettes.

[g]% E = Percent lymphocytes forming E rosettes.

number of lymphocytes with surface immunoglobulin to 7.0% as compared to 23.0% seen in unfractionated cells. Elution with cell medium containing gamma globulin resulted in a cell population with 44.0% of the lymphocytes containing surface immunoglobulin. Phagocytes were markedly reduced from precolumn cells (9.0%) to 0.5% in cell medium eluted cells and enhanced to 11.5% of the gamma globulin eluted cells. Viabilities ranged from 75.5% (unfractionated cells) to 89.0% (cell medium eluted cells). EAC and EA rosette formation increased with increasing percentages of lymphocytes with surface immunoglobulin.

The results of the second leukocyte fractionation experiment are shown in Table 6. Several changes were made in the operation of this column, including: a reduction in the number of cells applied from 9.04 to 6.27 x 10^7 leukocytes; operation of the column at 37°C instead of 25°C; a decrease in flow rate from 0.5 to 0.3 ml per min. In addition, the cell medium elution was divided into 30 ml portions. Cells recovered from the first 30 ml portion, as well as unfractionated and gamma globulin eluted cells, were analyzed as in the previous study. Due to the low recovery of cells from the second and third 30 ml portions of cell medium, only viability and surface immunoglobulin determinations were made. Because of the prolonged duration of operation of this column, recovered and retained cells

Table 6. Fractionation of 6.27×10^7 bovine leukocytes at 37°C and flow rate of 0.3 ml per min

		Leukocytes eluted with			
		Cell medium			Cell medium with bovine gamma globulin
	Unfractionated leukocytes	First 30 ml	Second 30 ml	Third 30 ml	45 ml
NR[a] x 10^6	--	37.4	0.4	0.4	2.7
% V[b]	87.5	91.0	88.0	86.5	80.0
% Z+[c]	0.0	0.0	NT[h]	NT	3.0
% sIg[d]	26.0	7.0	6.0	24.0	80.0
% EAC[e]	29.2 ± 1.8	14.1 ± 1.8	NT	NT	45.0 ± 7.5
% EA[f]	9.3 ± 3.1	5.0 ± 1.5	NT	NT	21.2 ± 11.0
% E[g]	1.3 ± 1.5	3.2 ± 1.5	NT	NT	2.1 ± 0.8

[a]NR = Number of leukocytes recovered.

[b]% V = Percent lymphocytes viable.

[c]% Z+ = Percent leukocytes with intracellular zymosan.

[d]% sIg = Percent lymphocytes with surface immunoglobulin.

[e]% EAC = Percent lymphocytes forming EAC rosettes.

[f]% EA = Percent lymphocytes forming EA rosettes.

[g]% E = Percent lymphocytes forming E rosettes.

[h]NT = Not tested.

were periodically washed and resuspended in fresh cell medium while waiting for the remaining cells to elute from the column. All cells were kept at 37°C until analyzed.

Although this column took considerably longer to run cell viabilities improved (greater than 80% for all fractions). Cell recovery improved slightly (67.2%). Nearly all of the recovered leukocytes from cell medium were recovered from the first 30 ml. Phagocytes were observed only in the gamma globulin eluate fraction (3.0%). The first 30 ml of cell medium elution yielded a population of cells with 7.0% of the lymphocytes containing surface immunoglobulin as compared to 26.0% seen in unfractionated lymphocytes. Eighty percent of the lymphocytes eluted with gamma globulin contained surface immunoglobulin. EAC and EA rosette formation increased with increasing percentages of lymphocytes with surface immunoglobulin.

DISCUSSION

The isolation of bovine lymphocytes by gradient centrifugation of peripheral blood results in contamination with phagocytes as has been seen with the isolation of human lymphocytes using the same method (10). The contaminants are largely monocytes and on the average account for approximately 20% of the cells isolated when bovine leukocytes are isolated from normal animals (68). Monocytes are sometimes difficult to distinguish from lymphocytes using cell morphology alone. An investigation of lymphocytes should then include some means of phagocyte removal or make differentiation of these cells more apparent by some means of phagocyte detection. This is generally accomplished in human studies by removal with iron carbonyl (38) or detection with latex (16).

Several attempts to remove bovine phagocytes with iron carbonyl failed despite previous reports of success with this technique (43, 85). The discrepancy in results may be due to the source of iron particles used in this study. Microscopic examination of leukocytes incubated with iron carbonyl prior to gradient centrifugation showed no phagocytes with intracellular iron although most of the particles were quite large as compared to the average size

of the leukocytes. Smaller particles may allow for phagocyte removal with iron.

Attempts to detect bovine phagocytes with latex gave ambiguous results although Paul et al. (68) have since reported success with this technique. The problem encountered in this laboratory with respect to this technique was the frequent inability to distinguish between surface adherence and actual ingestion of latex.

The problem of phagocyte removal or detection was overcome by treating freshly drawn blood with zymosan. Examination of blood immediately following zymosan treatment showed that 90% of the monocytes ingested the particles (Table 1). Slightly lower percentages were seen with neutrophil and eosinophil populations although the principle contaminating phagocytes after centrifugation are mononuclear cells. Phagocytes accounted for 15.3% of the cells isolated upon centrifugation (Table 2). Of the phagocytes encountered in this cell isolate, 92.8% were seen with intracellular zymosan. Thus only 1.1% of the leukocytes counted were phagocytic without intracellular zymosan. Zymosan tagging of phagocytes proved successful for this study in that rosetting assays and fluorescent antibody test for sIg could be performed allowing for data collection with respect to lymphocytes only (Figure 2).

The results of the antibody (Table 3) and complement (Table 4) titration studies showed that anti-erythrocyte

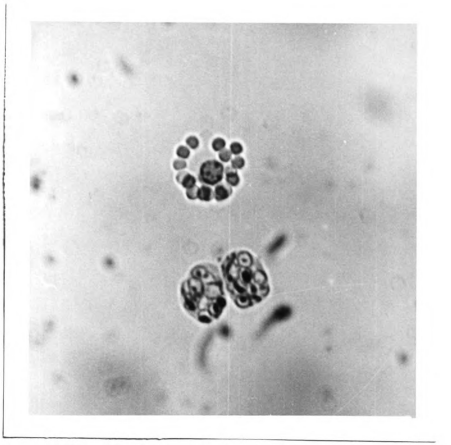

Figure 2. Photomicrograph of two phagocytes with intracellular zymosan and a rosetting lymphocyte at 400X.

serum diluted 1:200 and mouse serum diluted 1:20 resulted in the greatest frequency of EAC rosette formation. Both EA and EAC rosetting frequencies increased with decreasing dilution of antiserum. Similarly, EAC rosetting frequencies increased with decreasing dilutions of mouse serum. These results differ somewhat from those of Higgins and Stack (43) where EAC rosetting frequencies reached a peak value and then declined substantially with decreasing dilutions of both antibody and complement.

From a theoretical standpoint one would expect to see increasing rosetting cells with decreasing antibody and complement dilutions since the erythrocyte surface would accumulate an increasing amount of antibody and complement. With more antibody and complement on the erythrocyte surface the EAC assay should become more sensitive in detecting Ig and C lymphocyte receptors. It would be the hope, with any titration such as this, that a plateau would be reached. If such a plateau is observed, dilutions well into the plateau can be used and thereby assure that the assay is operating at its maximum capacity. No such plateau was demonstrated with respect to the titrations performed here. This would suggest that both the EA and EAC assays, as used in this study, are not sensitive enough to detect all lymphocytes with Ig or C receptors.

EC controls performed during the complement titration study resulted in low rosetting frequencies for all

mouse serum dilutions (range 0.8 to 2.3%). Little difference is seen from that obtained from the E control (1.0%). Since EC's are not sensitized with antibody, activation of complement is unlikely as is adherence of complement components to the erythrocyte surface. It is logical to assume then that EC rosetting is due to the erythrocyte receptors on lymphocytes.

Sheep erythrocytes prove to be an appropriate choice for performing EA and EAC assays since E rosetting is minimal. Thus, most of the EA rosettes should be due to the presence of lymphocyte Ig receptors. Some rosettes formed with EAC's may be due to the presence of lymphocyte Ig receptors as well as C receptors. This is supported by the observation that EAC rosetting frequencies are always higher than EA rosetting frequencies.

Results from the first fractionation experiment (Table 5) show that passage of bovine lymphocytes through an immunoabsorbent column allows for the isolation of two lymphocyte subpopulations: one enriched in Ig-bearing cells and the other enriched in non-Ig-bearing cells. Lymphocytes isolated from cell medium elution resulted in a lymphocyte subpopulation with only 7.0% of the lymphocytes positive for sIg. Lymphocytes isolated due to elution with cell medium containing bovine gamma globulin resulted in an enrichment of sIg positive lymphocytes to 44.0%. This

experiment also shows that both EA and EAC rosetting frequencies increase with an increase in lymphocytes with sIg. This observation is in agreement with that reported by others where both EA (43) and EAC (43, 50) rosetting frequencies decreased when these assays were performed on bovine lymphocytes passed through nylon wool.

In hopes that a more effective separation of sIg positive and sIg negative lymphocytes could be accomplished, a second fractionation study was undertaken involving several changes. Contamination of Ig-bearing lymphocytes in the cell medium eluate of the first column may have been due to the presence of too many sIg positive cells for the column to retain. Thus the number of cells applied to the second column was reduced to almost two-thirds of that applied to the first column. Furthermore, it was believed that by reducing the flow rate from 0.5 to 0.3 ml per min more time would be allowed for column antibody to react with sIg and possibly reduce sIg positive cells in the cell medium eluate. In running the second column at 37°C instead of room temperature it was hoped that cell viability would improve. By increasing the cell medium elution volume from 45 to 90 ml it was hoped that sIg negative cells would be more thoroughly eliminated from the column so that sIg positive cells could be obtained in greater purity. By dividing the cell medium elution into three-30 ml portions

and analyzing those cells eluted with each portion, it was hoped that some insight might be gained relating to the effectiveness of increasing the cell medium elution.

The net effect of these changes resulted in the isolation of sIg positive lymphocytes in higher purity upon elution with cell medium containing gamma globulin (80% positive for sIg). Cells eluted with the first 30 ml of cell medium resulted in a cell isolate similar to that obtained in the first fractionation study. Elution with cell medium beyond the first 30 ml further reduced the number of sIg negative cells in the column although loss of sIg positive cells increased with the last 30 ml as compared to the second 30 ml. Cell viability improved slightly. Again both EA and EAC rosetting frequencies increased with increasing enrichment of Ig-bearing lymphocytes suggesting that Ig and C receptors, as detected by these assays, are largely cell markers found on sIg positive cells.

Although some caution is warranted in equating ontogenetically defined lymphocytes with marker defined lymphocytes, there is general agreement that B cells, in contradistinction to T cells, possess readily detectable sIg. Separation of Ig-bearing (B) and non-Ig-bearing (T) lymphocytes of bovine origin with an immunoabsorbent column yielded results inferior to those obtained by Chess et al.

(17) for human lymphocytes. A more thorough and controlled investigation into the dynamics of such a column may be necessary before comparable results are obtained.

BIBLIOGRAPHY

1. Aisenberg, A. C., and K. J. Bloch. 1972. Immunoglobulins on the surface of neoplastic lymphocytes. N. Engl. J. Med. 287:272-276.

2. Aiuti, F., J.-C. Cerottini, A. Coombs, M. Cooper, H. B. Dickler, S. Froland, H. H. Fundenberg, M. F. Greaves, H. M. Greary, H. G. Kunkel, J. Natvig, J.-L. Preud'homme, E. Rabellino, R. E. Ritts, D. S. Rowe, M. Seligmann, F. P. Siegel, J. Stjernsward, W. D. Terry and J. Wybran. 1975. Identification, enumeration and isolation of B and T lymphocytes from human peripheral blood. Clin. Immunol. Immunopath. 3:584-597.

3. Ashman, R. F., and M. C. Raff. 1973. Direct demonstration of theta-positive antigen binding cells, with antigen-induced movement of thymus-dependent cell receptors. J. Exp. Med. 137:69-84.

4. Bankhurst, A. D., and N. L. Warner. 1971. Surface immunoglobulins on mouse lymphoid cells. J. Immunol. 107:368-373.

5. Basten, A., J. F. A. P. Miller, J. Sprent, and J. Pye. 1972. A receptor for antibody on B lymphocytes. I. Method of detection and functional significance. J. Exp. Med. 135:610-626.

6. Bianco, C., and V. Nussenzweig. 1977. Complement receptors. Contemp. Topics Mol. Immunol. 6:145-176.

7. Bianco, C., R. Patrick, and V. Nussenzweig. 1970. A population of lymphocytes bearing a membrane receptor for antigen-antibody-complement complexes. J. Exp. Med. 132:702-720.

8. Binns, R. M. 1978. Sheep erythrocyte rosettes in pigs, sheep, cattle, and goats demonstrated in the presence of dextran. J. Immunol. Meth. 2:197-210.

9. Boyum, A. 1968. Separation of leukocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. 21:Suppl. 97.

10. Boyum, A. 1976. Isolation of lymphocytes, granulocytes, and macrophages. Scand. J. Immunol. 5:9-15.

11. Brian, P., J. Gordon, and W. A. Willetts. 1970. Rosette formation by peripheral lymphocytes. Clin. Exp. Immunol. 6:681-688.

12. Brian, P., and R. H. Marston. 1973. Rosette formation by human T and B lymphocytes. Eur. J. Immunol. 3:6-9.

13. Brown, C. S., H. Halpern, and H. H. Wortis. 1975. Enhanced rosetting of sheep erythrocytes by peripheral blood T cells in the presence of dextran. Clin. Exp. Immunol. 20:505-512.

14. Burns, G. F., J. C. Cawley, and C. R. Barker. 1979. Characterization of the receptor for IgM on human B lymphocytes. Immunology 36:569-577.

15. Campbell, P. A., and H. M. Grey. 1972. Removal of immunoglobulin-bearing lymphocytes by anti-immunoglobulin-coated columns. Cellular Immunology 5:171-179.

16. Cavins, J. A., I. Djerassi, A. J. Roy, and E. Klein. 1965. Preservation of viable human granulocytes at low temperatures in dimethyl sulfoxide. Cryobiology 2:129-133.

17. Chess, L., R. P. MacDermott, and S. F. Schlossman. 1974. Immunologic functions of isolated human lymphocyte subpopulations. I. Quantitative isolation of human T and B cells and response to mitogens. J. Immunol. 113:1113-1121.

18. Chess, L., and S. F. Schlossman. 1976. Methods of separation of unique human lymphocyte subpopulations, pp. 77-80. In N. R. Rose and H. Friedman (ed.), Manual of clinical immunology, vol. 8.

19. Chess, L., and S. F. Schlossman. 1977. Human lymphocyte subpopulations. Adv. Immunol. 25:213-241.

20. Clements, P. J., and J. Levy. 1978. Receptors for IgG (Fc receptors) on human lymphocytes; re-evaluation by multiple techniques. Clin. Exp. Immunol. 34:281-287.

21. Cline, M. J., J. Sprent, N. L. Warner, and A. W. Harris. 1972. Receptors for immunoglobulin on lymphocytes and cells of a cultured plasma cell tumor. J. Immunol. 108:1126-1128.

22. Cline, M. J., and N. L. Warner. 1972. Immunoglobulin receptors on a mouse mast cell tumor. J. Immunol. 108:339-345.

23. David, J. P. 1973. Lymphocyte mediators and cellular hypersensitivity. N. Engl. J. Med. 288:143-149.

24. Dickler, H. B. 1974. Studies of the human lymphocyte receptor for heat-aggregated or antigen-complexed immunoglobulin. J. Exp. Med. 140:508-522.

25. Dickler, H. B. 1976. Lymphocyte receptors for immunoglobulin. Adv. Immunol. 24:167-214.

26. Dickler, H. B., N. F. Adkinson, and W. D. Terry. 1974. Evidence for individual human peripheral blood lymphocytes bearing B and T cell markers. Nature 247:213-215.

27. Dickler, H. B., and H. G. Kunkel. 1972. Interaction of aggregated γ-globulin with B lymphocytes. J. Exp. Med. 136:191-196.

28. Dukor, P., C. Bianco, and V. Nussenzweig. 1972. Bone marrow origin of complement-receptor lymphocytes. Eur. J. Immunol. 1:491-494.

29. Dwyer, J. M. 1976. Identifying and enumerating human T and B lymphocytes. Prog. Allergy 21:178-260.

30. Eden, A., G. W. Miller, and V. Nussenzweig. 1973. Human lymphocytes bear membrane receptors for C3b and C3d. J. Clin. Invest. 52:3239-3242.

31. Ehrlich, P. 1900. On immunity with special reference to cell life. Proc. R. Soc. Lond. 66:424-448.

32. English, D., and B. R. Anderson. 1974. Single-step separation of red blood cells, granulocytes, and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque. J. Immunol. Meth. 5:249-252.

33. Escajadillo, C., and R. M. Binns. 1975. Rosette formation of pig T lymphocytes with sheep erythrocytes. Int. Archs. Allergy Appl. Immunol. 49:325-331.

34. Ferrarini, M., T. Hoffman, S. M. Fu, R. Winchester, and E. Kunkel. 1977. Receptors for IgM on certain human B lymphocytes. J. Immunol. 119:1525-1529.

35. Ferrarini, M., L. Moretta, M. C. Mingari, and B. Pernis. 1976. Human T cell receptors for IgM; specificity for the pentameric Fc fragment. Eur. J. Immunol. 6:520-521.

36. Froland, S. S., F. Wisloff, and T. E. Michaelsen. 1974. Human lymphocytes with receptors for IgG. Int. Arch. Allergy Appl. Immunol. 47:124-138.

37. Geha, R. S., F. S. Rosen, and E. Merler. 1973. Identification and characterization of sub-populations of lymphocytes in peripheral blood after fractionation of discontinuous gradients of albumin: The cellular defect in x-linked aggammaglobulinemia. J. Clin. Invest. 52: 1726-1734.

38. Goldrosen, M. H., P. J. Gannon, M. Lutz, and E. D. Holyke. 1977. Isolation of human peripheral blood lymphocytes: Modification of a density discontinuous density gradient of Ficoll-Hypaque. J. Immunol. Meth. 14:15-17.

39. Gonatas, K., J. C. Antoine, A. Stieber, and S. Arrameas. 1972. Surface immunoglobulins of thymus and lymph node cells demonstrated by the peroxydase coupling technique. Lab. Invest. 26:253-261.

40. Gonzalaz-Molina, A., and H. L. Spiegelberg. 1977. A subpopulation of normal human B lymphocytes that bind IgE. J. Clin. Invest. 59:616-624.

41. Gutierrez, C., R. R. Barnabe, J. Vega, and M. Keisler. 1979. Purification of human T and B cells by a discontinuous density gradient of percoll. J. Immunol. Meth. 29:57-63.

42. Higgins, D. A., and M. J. Stack. 1977. Bovine lymphocytes recognition of cells forming spontaneous (E) rosettes. Clin. Exp. Immunol. 27:348-356.

43. Higgins, D. A., and M. J. Stack. 1978. Some parameters of the erythrocyte-antibody-complement (EAC) rosette system for recognition of bovine B-cells. J. Immunol. Meth. 20:211-224.

44. Hokland, P., M. Hokland, and I Heron. 1976. An improved technique for obtaining E rosettes with human lymphocytes and its use for B cell purification. J. Immunol. Meth. 13:175-182.

45. Huber, H., and H. H. Fudenberg. 1966. Receptor sites of human monocytes for IgG. Int. Arch. Allergy Appl. Immunol. 34:18-31.

46. Ishizaka, T., C. S. Soto, and K. Ishizaka. 1973. Mechanisms of passive sensitization. III. Number of IgE molecules and their receptor sites on human basophil granulocytes. J. Immunol. 111:500-511.

47. Jaffer, A. M., G. Jones, E. J. Kasdon, and S. F. Schlossman. 1973. Local transfer of delayed hypersensitivity by T lymphocytes. J. Immunol. 111:1268-1269.

48. Jondal, M., G. Holm, and H. Wigzell. 1972. Surface markers on T and B lymphocytes. I. A large population of lymphocytes forming nonimmune rosettes in sheep red cells. J. Exp. Med. 136:207-215.

49. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol. 3:645-649.

50. Kately, J. R., and S. J. Bazzell. 1978. Immunologic studies in Holstein-Friesian cattle: An immunocompetence profile. Am. J. Vet. Res. 39:1683-1687.

51. Kincade, P., A. Lawton, and M. Cooper. 1971. Restriction of surface immunoglobulin determinants to lymphocytes of the plasma cell line. J. Immunol. 106:1421-1423.

52. Laudoulis, C. T., T. J. Gill III, S. H. Chen, and D. M. Misra. 1975. The structure and metabolism of lymphocyte membranes. Prog. Allergy 18:205-288.

53. Lay, W. H., and V. Nussenzweig. 1968. Receptors for complement on leukocytes. J. Exp. Med. 128:991-1007.

54. Lohrmann, M. P., and L. Novikovs. 1974. Rosette formation between human T lymphocytes and unsensitized Rhesus monkey erythrocytes. Clin. Immunol. Immunopath. 3:99-111.

55. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

56. Lum, L. G., A. V. Muchmore, D. Karen, J. Decker, I. Koski, W. Strober, and R. M. Blaese. 1979. A receptor for IgA on human T lymphocytes. J. Immunol. 122:65-69.

57. McConnell, I., and C. M. Hurd. 1976. Lymphocyte receptors. II. Receptors for rabbit IgM on human T lymphocytes. Immunology 30:835-839.

58. Mingari, M. C., L. Moretta, A. Moretta, M. Ferrarini, and J. L.-Preud-Homme. 1978. Fc receptors for IgG and IgM immunoglobulins on human T lymphocytes: Mode of re-expression after proteolysis or interaction with immune complexes. J. Immunol. 121: 767-770.

59. Moller, E., W. W. Bullock, and O. Makela. 1973. Affinity of T and B lymphocyte receptors for hapten determinants. Eur. J. Immunol. 3:172-179.

60. Moller, G. 1961. Demonstration of mouse iso-antigens at the cellular level by the fluorescent antibody technique. J. Exp. Med. 114:415-434.

61. Moretta, L., M. Ferrarini, and M. D. Cooper. 1978. Characterization of human T-cell subpopulations as defined by specific receptors for immunoglobulins. Contemp. Topics Immunobiol. 8:19-67.

62. Moretta, L., M. Ferrarini, M. L. Durante, and M. C. Mingari. 1975. Expression of a receptor for IgM by human T cells *in vitro*. Eur. J. Immunol. 5:565-569.

63. Moretta, L., S. R. Webb, C. E. Grossi, P. M. Lydyard, and M. D. Cooper. 1977. Functional analysis of two human T-cell subpopulations: Help and supression of B cell responses by T cells bearing receptors for IgM ($T._M$) or IgG ($T._G$). J. Exp. Med. 146:184-200.

64. Nelson, R. A. 1953. The immune adherence phenomenon: An immunologically specific reaction between microorganisms and erythrocytes. Science 118:733-737.

65. Nussenzweig, V. 1974. Receptors for immune complexes on lymphocytes. Adv. Immunol. 19:217-294.

66. Parish, C. R., and J. A. Hayward. 1974. The lymphocyte surface. I. Relationship between Fc receptors, C3 receptors, and surface immunoglobulin. Proc. R. Soc. Lond. Ser. B 187:47-63.

67. Parish, C. R., and J. A. Hayward. 1974. The lymphocyte surface. II. Separation of Fc receptor, C3 receptor, and surface immunoglobulin-rearing lymphocytes. Proc. R. Soc. Lond. Ser. B 187: 65-81.

68. Paul, P. S., D. R. Senogles, C. C. Muscoplat, and D. W. Johnson. 1979. Enumeration of T cells, B cells and monocytes in peripheral blood of normal and lymphocytic cattle. Clin. Exp. Immunol. 36:306-316.

69. Pellegrino, M. A., S. Ferrone, M. P. Dierich, and R. A. Riesfeld. 1975. Enhancement of sheep red blood cell, human lymphocyte rosette formation by the sulfhydryl compound 2-amino ethylisothiouronium bromide. Clin. Immunol. Immunopath. 3:324-333.

70. Pernis, B., L. Forni, and L. Amente. 1970. Immunoglobulin spots on the surface of rabbit lymphocytes. J. Exp. Med. 132:1001-1018.

71. Pichler, W. J., and W. Knapp. 1978. Receptors for IgM on human B lymphocytes. Scand. J. Immunol. 7:105-109.

72. Piessens, W. F., P. H. Schur, W. C. Maloney, and W. H. Chruchill. 1973. Lymphocyte surface immunoglobulins: Distribution and frequency in lymphproliferative diseases. N. Engl. J. Med. 288:176-180.

73. Popamichail, M., J. C. Brown, and E. J. Holborow. 1971. Immunoglobulin on the surface of human lymphocytes. Lancet 2:850-852.

74. Preud'Homme, J.-L., and M. Seligmann. 1974. Surface immunoglobulins on human lymphoid cells. Prog. Clin. Immunol. 2:121-174.

75. Preud'Homme, J.-L., L. M. Gonnot, A. Tsapis, J. C. Brovet, and C. Mihaesco. 1977. Human T receptors for IgM: Reactivity with monomeric 8s subunits. J. Immunol. 119:2206-2208.

76. Roelants, G. E. 1972. Quantitation of antigen specific T and B lymphocytes in mouse spleens. Nature (New Biol) 236:252-255.

77. Ross, G. D., E. M. Rabellino, M. J. Polley, and H. W. Grey. 1973. Combined studies of complement receptor and surface immunoglobulin-bearing cells and sheep erythrocyte rosette-forming cells in normal and leukemic human lymphocytes. J. Clin. Invest. 52:377-385.

78. Sakon, A., J. Feldhaus, and R. A. Robins. 1976. Single step separation of human T and B cells using AET treated SRBC rosettes. J. Immunol. Meth. 12:285-288.

79. Schlossman, S. F., and C. Hudson. 1973. Specific purification of lymphocyte populations on a digestible immunoabsorbent. J. Immunol. 110: 313-315.

80. Silveira, N. P. A., N. F. Mendes, and M. E. A. Tolnai. 1972. Tissue localization of two populations of human lymphocytes distinguished by membrane receptors. J. Immunol. 108:1456-1460.

81. Sprent, J. 1977. Recirculating lymphocytes, pp. 43-112. In J. J. Marchalonis (ed.), The Lymphocyte. Structure and Function. Part I.

82. Stathopoulos, G., and E. V. Elliott. 1974. Formation of mouse or sheep red blood cell rosettes by lymphocytes from normal and leukaemic individuals. Lancet 1:600-601.

83. Tarr, M. J., R. G. Olsen, G. S. Krakowka, G. L. Cockerell, and A. A. Gabel. 1977. Erythrocyte rosette formation of equine peripheral blood lymphocytes. Am. J. Vet. Res. 38:1775-1779.

84. Uhr, J. W. 1965. Passive sensitization of lymphocytes and macrophages by antigen-antibody complexes. Proc. Nat. Acad. Sci. USA 54:1599-1606.

85. Wardley, R. 1977. An improved E rosetting technique for cattle. Br. Vet. J. 133:432-434.

86. Wigzell, H., K. G. Sundquist, and T. O. Yoshida. 1972. Separation of cells according to surface antigens by the use of antibody-coated columns. Fractionation of cells carrying immunoglobulins and blood group antigen. Scand. J. Immunol. 1:75-87.

87. Winchester, R. J., and S. M. Fu. 1976. Lymphocyte surface membrane immunoglobulin. Scand. J. Immunol. 5:77-82.

88. Woody, J. N., and K. W. Sell. 1975. Characteristics of the 'active rosette test.' Technical considerations of the test and comments. J. Immunol. Meth. 8:331-338.

89. Wybran, J., and H. H. Fudenberg. 1973. Thymus-derived rosette-forming cells in various human disease states: Cancer, lymphoma, bacterial and viral infections and other diseases. J. Clin. Invest. 52:1026-1032.

90. Yodoi, J., and K. Ishizaka. 1979. Lymphocytes bearing Fc receptors for IgE. I. Presence of human and rat T lymphocytes with Fc_{ε} receptors. J. Immunol. 122:2577-2583.