# Identification of bovine lymphocyte subpopulations by a combined bacterial adherence and fluorescent antibody technique



by

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# LITERATURE REVIEW

Lymphocytes are immunologically important cells and over 50% of the circulating white blood cells of cattle are lymphocytes.

Prior to the early 1960s, lymphocytes were cells recognized as a distinct subpopulation of cells based on morphological characterization. The cellular morphology provided little information about possible functional roles of lymphocytes. Advances in cellular immunology indicated that there were three main lymphocyte populations. These included B-lymphocytes, T-lymphocytes and null cells. Figure 1 presents a schematic diagram of lymphocyte differentiation. The general derivation and characterization of lymphocyte populations has been described previously.[1,2,3]

The B-lymphocytes are derived from the bone marrow in mammals and are processed in the bursa of Fabricius in birds. The B-cells make up a relatively small percentage of the circulating lymphocyte population; however, they are the major type of lymphocyte found in the follicles of lymph nodes and the spleen.

The functional role of B-cells is to participate in humoral immune responses via production of antibodies (Ab). B-cells synthesize immunoglobulins (Ig) and incorporate them into their plasma membranes. B-cells react with antigens (Ag) via their surface Ig (SIg).

Upon Ag stimulation, some mature B-cells will multiply and differentiate into Ig-secreting plasma cells. The plasma cells synthesize and secrete Ig of one class of heavy chain and one type of

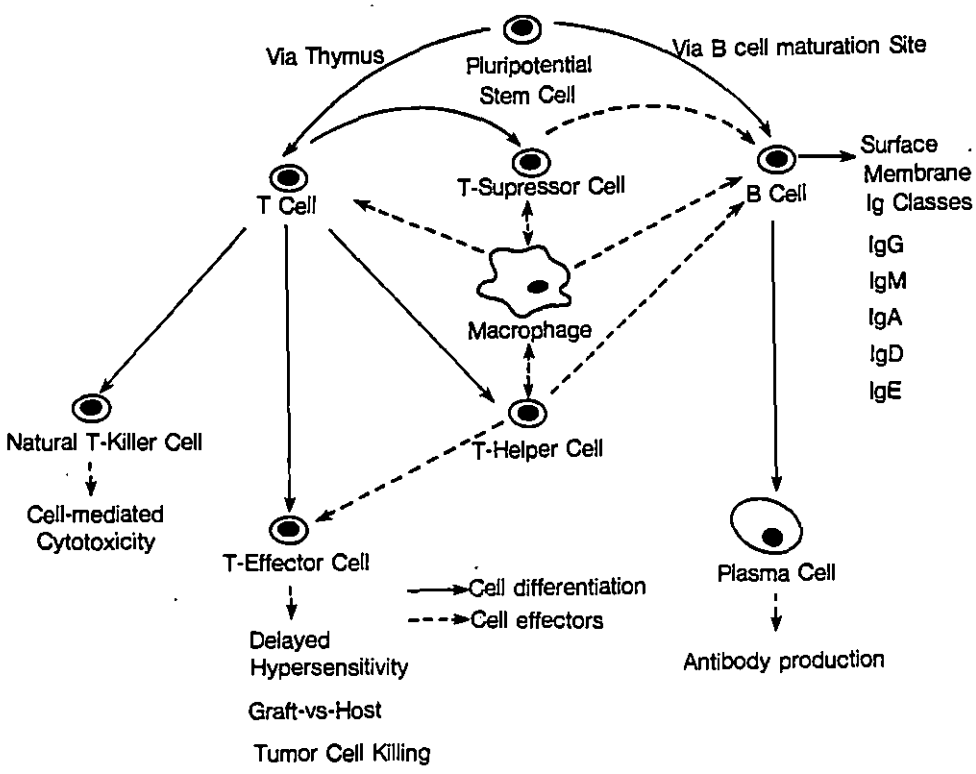


Figure 1. Diagramatic representation of general lymphocyte differentiation and function. Modified from Jabs et al.[3]

light chain. The secreted Ig is produced by the polyribosomes which synthesize heavy and light chains that are released into an intracellular "pool" where the four chain moiety is assembled.

Differentiation of B-cells following Ag stimulation occurs in several steps. First, the membrane-bound Ig forms a "cap" enabled by membrane fluidity. This is followed by receptor regeneration and proliferation of B-cells with the same Ag specificity as the parental cell. These cells are referred to as a "clone". The differentiation to plasma cells may require T-cell interaction depending on the thymic dependence of the Ag. The induced plasma cells secrete Ig of the same specificity as the parental B-cell.

In addition to SIg, B-cells also have other surface molecules including: receptors for the Fc portion of Ig, receptors for the third component ($C_3$) of the complement cascade and certain histocompatibility molecules.

It should be noted that B-cells specific for an Ag are found in an organism prior to exposure to that Ag. Upon exposure, the Ag is bound to B-lymphocytes with a higher affinity for that Ag.

T-lymphocytes are derived from the thymus. These cells comprise the majority of the circulating lymphocyte population. The functional role of T-cells is to participate in cell-mediated immune responses. T-cells can be further characterized into functionally important subgroups. These include: helper, suppressor, effector, memory and killer T-lymphocytes.

T-helper cells aid in regulation of B-cell activities by enhancing the Ab production of these cells. This enhancement is produced by the action of lymphokines which are soluble factors of variable properties secreted by activated T-cells. T-suppressor cells regulate B-cell function by depressing Ab production by B-cells. This is also due to the effects of lymphokines. T-helper cells have receptors for the Fc portion of IgM whereas T-suppressor cells bear receptors for the Fc portion of IgG. T-effector cells participate in a number of cell-mediated responses including delayed hypersensitivity, chemotaxis, blastogenesis, transplantation immunity and tumor cell killing reactions. Natural T-killer cells mediate direct cytotoxic effects upon tumor cells which express specific surface receptors.

T-cells work in combination with macrophages. Macrophages process the Ag by engulfing it and altering it chemically thereby increasing its immunogenicity. After processing of Ag has occurred, T-cells interact with the macrophage-bound Ag leading to T-cell activation. Macrophages also produce soluble T-cell reactive factors called interleukins.

T-cells react with Ag through antigen specific factors. These factors generally show three properties. First, they bind Ag specifically. Second, they have determinants coded for by genes of the major histocompatibility complex. Finally, they mediate regulatory functions i.e. suppressor or helper activities.

The null lymphocyte population is composed of cells which do not express either SIg or receptors for heterologous erythrocytes; thus, they can be referred to as non-B, non-T cells. Recent evidence indicates that the number of null cells present within peripheral blood may be due to lack of sensitivity of the methods used to detect B and T-cells.

The lymphocyte characterization described above is primarily a result of work involving human and murine lymphocytes. Much of this work has been verified with bovine lymphocytes. The presence of bovine $Ig^+$ B-cells has been documented by many researchers.[4,5,6,7,8,9,10,11] In addition, many of the T-cell functional subgroups have been described. Both helper ($FcM^+$) and suppressor ($FcG^+$) cells have been described.[5,10,12,13] Approximately 9-27% of the peripheral blood T-lymphocytes of normal cattle are suppressor T-cells. Although there is some variability in values reported, there are approximately 25% helper T-cells present in the circulating T-cell population of normal animals.[10] Bovine effector T-cells were identified using separation of peripheral blood lymphocytes (PBL) with plant agglutinins and monitoring the effects on cytotoxicity.[14] Both cytotoxic and natural killer T-cells have been described in the bovine system.[15]

Lymphocyte populations are identified and enumerated by surface marker analysis of the membrane receptors described above. Most of the techniques detect either receptors for heterologous erythrocytes (T-cells) or SIg (B-cells).

In 1969, Bach et al.[16] described formation of sheep red blood cell (SRBC) rosettes around a majority of normal human lymphocytes. This spontaneous erythrocyte rosette (ER) formation was later confirmed by other researchers.[17,18,19] The numbers of ER forming lymphocytes indicated that this reaction was not a result of immune response against SRBC. Studies by Jondal et al.[20] indicated that the rosette forming lymphocytes were T-lymphocytes. This work was later confirmed by several researchers.[21,22,23,24] Subsequent studies have shown that T-cells from guinea-pigs,[25] cats,[26,27] dogs,[28] and pigs[29] have the ability to form spontaneous ER with heterologous erythrocytes of various species.

Rosette forming cells were shown to be T-cells by several approaches. First, ER forming cells were shown to lack the characteristic SIg associated with B-cells.[20,21,22] Bentwick et al.[30] indicated that approximately 5% of human ER forming lymphocytes additionally possessed SIg. Second, using lymphocytes isolated from the thymus, nearly 100% of the lymphocytes formed ER.[19,20,23] Because the thymus contains less than 0.1% B-cells as described by Greaves et al.[31], ER formation must be primarily a T-cell property. Similar results have also been described in non-human primates[32] and pigs.[29] Finally, Coombs et al.[18] showed that only viable lymphocytes were capable of ER formation. Using complement and heterologous anti-T-cell sera to kill human T-cells, it was demonstrated that this treatment lowered the numbers of ER forming cells detectable in peripheral blood.[33] This reaction was also described in pigs[29] and

cats.[27]

Previous studies have indicated that lymphocytes from a given species form rosettes only with species-heterologous erythrocytes.[25,34]

In the human system, considerable variations in the numbers of ER forming cells among PBL populations have been described. These range from 5% to 50-80%.[20,35] Variations in these results have been attributed to differences in technical procedures used by various researchers. Some of these procedural differences included: presence of a protein source in the cell suspension medium, time and temperature of incubation and the ratio of red cells to lymphocytes.

The presence of serum in the rosetting medium results in increased numbers and stability of rosette forming cells detected. In the human system, either 10-25% human AB serum[23,30] or 25% fetal calf serum (FCS)[34] gave the best results. Recent studies by Pattengale and Reichelderfer[36] indicated enhancement and increased stability of ER with human T-cells in the presence of high molecular weight substances such as serum albumin, dextran and Ficoll.

Treatment of both lymphocytes and erythrocytes with either papain or neuraminidase has been reported to enhance human ER formation.[37,38] These claims were refuted by Bentwick et al.[30] on the basis that the enhanced ER formation by neuraminidase-treated lymphocytes can be accounted for by B-cell rosette formation. In 1974, Kaplan and Clark demonstrated that treatment of erythrocytes with 2-aminoethylisothiouronium bromide (AET) resulted in increased ER formation with human

lymphocytes.[39]

The effects of time and temperature of incubation on ER formation by human lymphocytes have been investigated. Spontaneous ER dissociate at 37°C, but remain stable at 0-4°C.[19] Overnight incubation at 0-4°C has been reported to give the highest number of rosettes.[20,22,35] In addition, a two-step temperature treatment was necessary for optimal rosette formation.[19,20] This treatment consisted of a 10-15 minute preincubation of the erythrocyte-lymphocyte mixture at 37°C prior to centrifugation and a second overnight incubation at 0-4°C.

The ER test has been found to be a reliable marker for T lymphocytes in several species as described above. Development of a suitable bovine ER test has followed a similar derivation.

In 1976, Grewal et al.[40] first described an ER technique for the identification of bovine T-cells. Using 100% FCS, neuraminidase treatment of SRBC and lymphocytes and a two step temperature treatment, 38% of PBL and 52% of fetal thymocytes were found to form ER. These researchers noted that changes in lots of FCS resulted in variable numbers of ER forming cells detected. Verification that ER forming bovine lymphocytes were T-cells was accomplished as described for other species. Higgins and Stack[41] reported that neuraminidase treatment of SRBC enhanced ER formation by bovine thymocytes, but not PBL. Other researchers used neuraminidase-treated SRBC and 10% FCS in the rosetting medium and reported enhanced ER formation with bovine PBL.[6,42] Approximately 49% of PBL and 92% of fetal thymocytes were

found to form ER. These workers also noted increased variability of results with changes in FCS lots, SRBC donors and incubation temperatures.

Procedures using large molecular weight compounds to enhance ER formation by bovine lymphocytes have also been described. Wardley[43] added 6% dextran to tissue culture medium containing 10% FCS. This solution was used to suspend SRBC and PBL. He obtained 72% ER formation by PBL and 96% by fetal thymocytes. It should be noted that when neuraminidase treated SRBC were used in this system, only 52% of PBL were found to form spontaneous ER. Binns reported that approximately 37% of PBL were found to form spontaneous rosettes with SRBC when the rosetting medium contained 6% dextran and 10% FCS.[44] The variability of these results may be due to differences in the source or molecular weights of the dextran used by each researcher. Kuchroo and Jennings have reported enhanced ER formation between bovine PBL and autologous erythrocytes in the presence of 6% dextran.[45] This procedure yielded spontaneous rosetting with 10% of PBL and 30% of fetal thymocytes. Outteridge and Duffy reported 83% of PBL formed ER with SRBC when the erythrocytes were first suspended in saline containing 17% Ficoll 400 and 84 iu/ml of preservative-free heparin.[5] To date, no other researchers have reported using Ficoll for enhancement of bovine ER formation.

Paul et al.[46] demonstrated that SRBC treated with 0.1M AET have markedly enhanced capacity to form spontaneous ER with bovine PBL. Using this technique, approximately 63% of PBL were found to form

rosettes. This study also verified the usefulness of 10% FCS for enhancing rosette formation by bovine PBL. Using Paul's technique, Schore et al.[7] found only 39% ER forming cells in the PBL of lactating cows. No explanation for this discrepancy was given. The exact mechanism of action of AET is currently unknown.

As described, it should be apparent that ER formation by bovine PBL is not a reliable marker for the identification and enumeration of bovine T lymphocytes. As in other species, procedural differences can lead to drastically different results. The validity of the various treatments used to enhance the reaction is questionable since it is not possible to distinguish whether further populations of T-cells are actually being detected or if the increased numbers of rosette forming cells detected are artifacts of the technique used. Higgins et al.[41] suggested that ER forming cells are actually a subpopulation of bovine T lymphocytes.

Several other methods for detection of bovine T-cells have been proposed recently. Splitter et al.[12] have described a method which uses peanut agglutinin (PNA) conjugated with fluorescein isothiocyanate (FITC) to stain bovine T-cells for immunofluorescence. Yang has previously described a goat anti-bovine thymocyte immunoglobulin which could be used to stain bovine T-cells by an indirect fluorescent antibody (FA) technique with rabbit anti-goat-FITC antiglobulin.[9] Approximately 70% of bovine PBL could be identified as being T-cells by this procedure. This method provided highly specific and reproducible results.

As described above, the presence of SIg incorporated into the plasma membranes of B-cells has been well-documented. The majority of techniques used to identify and enumerate B-cells involve the use of these SIg as markers on these cells.

In 1961, Moller reported that a population of mouse lymphocytes gave membrane fluorescence upon reaction with FITC labeled anti-Ig serum.[47] Later research indicated that SIg molecules acted as Ag specific receptors on B lymphocytes.[48,49] Some researchers have postulated that there may be some SIg present on both B and T-cells, but the density and exposure of SIg on T lymphocytes was very low.[50] The direct and indirect FA tests have been the most commonly used methods of detecting B-cell populations. The direct FA test involves the use of antiglobulin sera conjugated with FITC to label SIg bearing cells. The indirect FA test involves the use of antiglobulin directed against the SIg of the B-cells and of FITC conjugated antiglobulin specific for the first Ab. In both procedures, the B-cells detected are surrounded by a ring of membrane fluorescence. The direct FA test has been used successfully to detect B-cells in rabbits,[51] chickens,[52] pigs[53] and rats.[54] Many researchers have used these procedures to study bovine B-cell populations.[4,5,8,9,10,11] These studies have indicated that there are approximately 11-27% B-cells among the PBL of cattle. These variable results are most likely due to differences in the techniques used by each group of researchers. Nairn has shown that the indirect FA test is more reliable than the direct test due to increased sensitivity.[55] One disadvantage of these techniques is that

it is possible for the Fc portion of the antiglobulin molecule to become passively attached to Fc receptors on the surface of T lymphocytes, thus giving false positive results.[56] This difficulty can be avoided by using only the $F(ab')_2$ portion of the antiglobulin molecule in the FA procedure.

Another method used to detect SIg bearing cells involves the coupling of antiglobulin molecules to the surface of solid support markers such as erythrocytes or polyacrylamide beads. These tests operate on the same basic principles as the FA tests described above. The antiglobulin molecules react with the SIg leading to rosetting of the supporting particles around the lymphocyte. Many researchers have coupled antiglobulins to erythrocytes for the identification of bovine B-cells. These erythrocyte-Ab (EA) rosettes are also referred to as direct antiglobulin rosetting reactions (DARR) and mixed antiglobulin rosetting reactions (MARR). The direct procedures involve the use of a single antiglobulin mediator, while the mixed test uses a first and second antiglobulin system similar to the indirect FA procedure. Using these techniques, various researchers have found approximately 28% of the PBL of cattle to be B-cells.[6,7,8,13]

A slight modification of these techniques has been described by Chao and Yokoyama[57] in which antiglobulin coated polyacrylamide beads were used to identify populations of human peripheral blood B lymphocytes.

In general, the techniques used to identify bovine peripheral blood B-cells are very reliable and relatively simple to perform.

However, the use of the FA procedures described is more desirable then other methods for several reasons. First, the use of FITC labeled antiglobulins provides a more sensitive probe for the detection of SIg due to the lack of possible steric hindrance associated with EA and bead procedures. Second, the reagents utilized in FA procedures are relatively stable when compared with erythrocytes used in rosetting assays. Finally, when the SIg bearing cells are labeled by fluorescent methods, it is possible to enumerate and separate these cells using a fluorescence activated cell sorter (FACS). This equipment allows the results to be determined in an objective manner.

In 1977, Teodorescu et al.[58] identified five human lymphocyte subpopulations by their differential binding of various strains of bacteria. In this initial study, B-cells and four T-cell subpopulations could be identified. B-cells were identified by a strain of Brucella melitensis or by anti-light chain antibody coated on Escherichia coli. T-cell subpopulations were identified using a combination of several genera of bacteria including: Arizona, Escherichia, Bacillus, Staphylococcus, Sarcina, Salmonella and Corynebacterium. Further work[59] indicated that there were actually two subpopulations of B-cells present in human PBL (Figure 2).

Using differential binding of lymphocytes to monolayers of bacteria and removal of non-adherent cells, Kleinman and Teodorescu[60] and Teodorescu et al.[59] separated PBL subpopulations. These subpopulations were then assayed for mitogen response and cytotoxic properties to determine their functional capabilities. The $T_3$ and $T_4$

subpopulations (their designation) gave greatly reduced responses to stimulation by ConA, PHA, Slo and PWM when compared with responses of $T_1$ cells, $T_2$ cells or whole PBL preparations.[60] Using assays for detecting specific cytotoxicity and natural killer activity, the $T_3$ and $T_4$ cells killed allogeneic target cells without stimulation by those cells. Further, when compared to $T_1$, $T_2$ cells and unseparated PBL, the $T_3$ and $T_4$ cells had a significantly higher spontaneous killer activity. $T_1$ cells and $T_2$ cells had similar values to those of unseparated PBL. When $T_3$ and $T_4$ cells were separated, the $T_4$ cells had significantly higher spontaneous killer activity then any other subpopulation. Thus, the $T_4$ cells appeared to be primarily responsible for spontaneous killer activity.[59] In a separate study, it was shown that the $T_2$ subpopulaton of PBL contained the effector cells capable of suppressing natural killer activity.[61]

In concurrent work, Mayer et al.[62] and Chen et al.[63] used the bacterial rosette technique to identify six murine lymphocyte subpopulations and functionally characterize them. This work resulted in identification of three B-cell ($B_1$, $B_2$ and $B_3$) and three T-cell ($T_1$, $T_2$ and $T_3$) subpopulations. Using a Jerne plaque assay in combination with separation of lymphocytes on bacterial monolayers, the Ig-secreting cells were found to be within the $B_1$ subpopulation. Chen et al.[64] determined the relationship between the Ly-1-2 and -3 alloantigens and the three murine T-cell subpopulations. They found the $T_1$ subpopulation contained most of the Ly-$1^+2^+3^+$ cells (helper and cytotoxic T-cell precursors) and the $T_2$ subpopulation contained some

Ly-$1^+2^-3^-$ (helper T-cells) and some Ly-$1^-2^+3^+$ (cytotoxic and suppressor T-cells) cells. The $T_3$ subpopulation contained the remainder of the Ly-$1^+2^+3^+$, Ly-$1^+2^-3^-$ and Ly-$1^-2^+3^+$ cells. In addition, a majority of the cytotoxic T-cells was present in the $T_3$ subpopulation.

Teodorescu et al.[65] and Nelson et al.[66] have shown that the bacterial rosette procedure can be used to identify and enumerate human leukemic lymphocytes. This work indicated that there was a marked increase in the numbers of B-cells detected by B-cell labeling bacteria among the PBL of chronic lymphocytic leukemia patients. In addition, by comparing the relative binding affinities of symptomatic versus non-symptomatic chronic lymphocytic leukemia patients, there was decreased bacterial binding of lymphocytes among patients in symptomatic stages. Relatively high levels of bacterial rosetting were found in the PBL of asymptomatic patients.

At this time, it is believed that the bacteria interact with lymphocytes through a carbohydrate on their surface and a lectin on the lymphocyte surface.[59] Based on these results, it was suggested that with the progression of leukemia, lymphocytes with less lectin recognition potential are selected and escape any control mechanisms of proliferation.[66]

Following normal donors over a one year period, Bratescu and Teodorescu[67] found a twofold increase in the number of human B-cells present among PBL in the winter months as compared to the number of B-cells present in summer months. Total leukocyte counts and numbers of

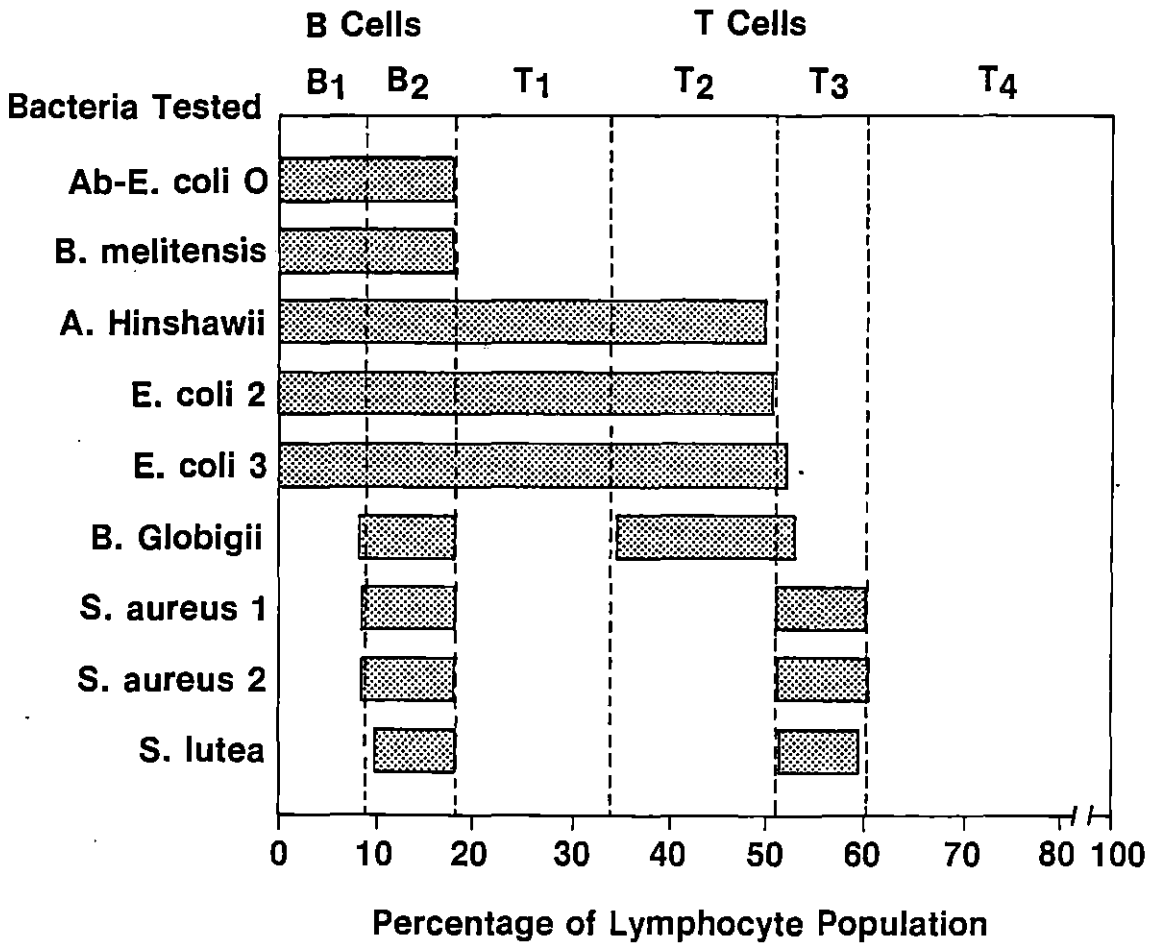


Figure 2. Human lymphocyte subpopulation "map" developed by Teodorescu et al.[59]

lymphocytes remained constant. In addition to the B-cell increase, variations in the numbers of $T_1$ cells and $T_2$ cells were identified. It was speculated that hormonal factors were responsible for these changes.

# SECTION I. IDENTIFICATION OF BOVINE LYMPHOCYTE SUBPOPULATIONS BY A COMBINED BACTERIAL ADHERENCE AND FLUORESCENT ANTIBODY TECHNIQUE

## SUMMARY

It is known that certain strains of bacteria bind selectively to subpopulations of human peripheral blood lymphocytes. We have developed a technique which used the specificity of bacterial binding concurrently with fluorescent antibody staining methods to identify five B-cell and five T-cell subpopulations of bovine lymphocytes. In addition, greater than 95% of the peripheral blood lymphocytes could be positively identified as being either T-cells or B-cells. Using ethidium bromide-stained bacteria and lymphocytes in combination with fluorescent antibody staining to detect surface immunoglobulins or T-cell antigens, the method provided a simple yet highly specific technique for the enumeration of both B and T-cells within a single preparation of peripheral blood lymphocytes. The use of bacterial rosetting with fluorescent antibody staining was found to be easier and more reliable then the methods currently used to identify bovine B and T-lymphocyte subpopulations. Using five Brucella abortus strains of known pathogenicity, we found no apparent relationship between the pathogenicity of a bacterium for a host and its ability to form rosettes with that host's peripheral blood lymphocytes.

# INTRODUCTION

Lymphocytes are immunologically important cells and more than half of the circulating white blood cells of cattle are lymphocytes.

Standard tests used to identify T and B-lymphocyte populations include the erythrocyte rosette (ER) test (T-cells) and the fluorescent antibody (FA) test to detect $Ig^+$ cells (B-cells). The indirect FA test used to detect surface immunoglobulins provides specific and reproducible results. The bovine ER test provides conflicting results. Studies of ER forming cells suggest that variations in procedural techniques such as neuraminidase treatment of the erythrocytes or changes in the incubation temperature can account for significant differences in the percentages of ER forming cells detectable among populations of peripheral blood lymphocytes (PBL) of cattle.[1,2,3]

Spontaneous rosetting of human lymphocytes with a variety of gram negative bacteria was observed by Teodorescu et al.[4] Two subpopulations of B-cells and four subpopulations of T-cells could be distinguished by these investigators.

The present investigation consisted of a combination of bacterial rosetting (BR) of bovine PBL, specific FA staining of B and T-cells, and counterstaining of bacterial and cellular nucleic acids with ethidium bromide to optimally identify bovine lymphocyte subpopulations. In addition, five strains of Brucella abortus of

known pathogenicity were evaluated to determine if there was any relationship between the pathogenicity of a bacterium and its BR capabilities.

# MATERIALS AND METHODS

## Bacterial preparation

Strains of bacteria found to rosette human lymphocytes were obtained from Dr. Aurel Bratescu (Department of Microbiology, University of Illinois, at the Medical Center, Chicago, IL 60612). These included Escherichia coli (Ec #2, Ec #3) and Brucella melitensis (Bm). A strain of Corynebacterium pyogenes (Cp #2) was isolated from a case of bovine mastitis at the College of Veterinary Medicine at Iowa State University. Five strains of Brucella abortus (45/20, 19, 458, 2308 smooth and 2308 rough) were obtained from Dr. Billy Deyoe (National Animal Disease Center, USDA, Ames, Iowa 50010). The bacteria were grown in Roux flasks containing trypticase soy agar and fixed with 10% formaldehyde in phosphate-buffered saline (PBS) and washed as described by Bratescu et al.[5] Bacteria were suspended in PBS containing 0.02% $NaN_3$ and standardized to $1X10^9$ cells/ml spectrophotometrically.

## Peripheral blood lymphocyte preparation

Ten apparently healthy dairy or beef breed cattle were used in this experimentation. Venous blood (15 ml) was collected and placed in tubes containing 2.0 ml of acid citrate dextrose solution (ACD). The blood was centrifuged at 1000 X g for twenty minutes and the buffy coat cells were removed. These cells were washed once with Medium-199 containing 10% fetal calf serum and 0.02% $NaN_3$ (M-199-FCS). The buffy

coat cells were standardized to $1X10^7$ cells/ml in M-199-FCS.

Ethidium bromide staining of bacteria

0.1 ml of standardized bacteria was mixed with an equal volume of 10ug/ml ethidium bromide. The solution was allowed to incubate for approximately two hours in the dark at room temperature.

Indirect fluorescent antibody staining of lymphocytes

0.2 ml of standardized PBL were mixed with 0.1 ml of either a 1:40 dilution of rabbit anti-bovine globulin serum when labeling B-cells or a 1:30 dilution of goat anti-bovine thymocyte serum when labeling T-cells. The anti-bovine globulin serum was produced by repeated injections of rabbits with rabbit red blood cells coated with normal bovine agglutinins. The goat anti-thymocyte serum was provided by Dr. Thomas Yang (Department of Pathobiology, University of Connecticut, Storrs, Connecticut, 06268). This serum had previously been shown to react specifically with bovine T-lymphocytes.[6] The PBL mixture was incubated at $4^oC$ for 30 min. and then washed twice with M-199-FCS by centrifugation at 256 X g. Following washing, 0.05 ml of either fluorescein (FITC) conjugated goat anti-rabbit IgG (heavy and light chain specific, Zymed Laboratories, Burlingame, California) (B-cells) or FITC rabbit anti-goat IgG (heavy and light chain specific, Zymed Laboratories, Burlingame, California) (T-cells) was added to the PBL. Following incubation at $4^oC$ for one hr., the cells were washed once with a solution containing 1.0 ml each of M-199-FCS and PBS containing 0.01 M $NaN_3$.

Bacterial rosette formation

The method of binding stained bacteria to PBL was essentially that described for binding of unlabeled bacteria to lymphocytes in blood smears by Bratescu et al.[5] Briefly, 0.1 ml of stained bacteria was added with 0.2 ml of M-199-FCS to 2 X $10^6$ labeled PBL. The mixture was then centrifuged at 900 X g at 4°C for 6 minutes. The supernatant was removed and 0.1 ml of mounting fluid was added to the cells. The mounting fluid consisted of 10.0 ml of PBS containing 100 mg of p-phenylenediamine in 90.0 ml of glycerol.[7] In addition, 0.1 ml of ethidium bromide was added to the cell suspension. Following incubation for twenty minutes at 4°C, one drop was applied to a cold slide and covered with a cold coverslip. Rosettes were counted using a fluorescence microscope (Leitz Ortholux II, Ernst Leitz Ltd., Midland, Ontario, Canada). At least 200 cells were counted and a cell was considered rosette positive if there were at least three bacteria surrounding it.

# RESULTS

Using the indirect FA tests for the detection of B and T-cells, we found a mean of 27.8 $\pm$ 4.2% B-cells and 70.3 $\pm$ 2.2% T-cells in the peripheral blood of cattle tested. These figures agree closely with those reported by Yang using indirect fluorescent T-cell staining.[6] Using T-cell staining simultaneously with the B-cell staining procedure, we found less than 5% null cells in PBL of cattle. In control preparations omitting the first antibody in the indirect procedure, we found there was less than 1% non-specific staining.

Satisfactory fluorescent-staining of bacteria was achieved using ethidium bromide. It has several unique properties which make it amenable for use in this procedure. First, it fluoresces bright orange when exposed to U.V. light. This provides a strong contrast to the green color of fluorescein. Second, it only fluoresces once it has intercalated the cellular nucleic acids, thus reducing non-specific fluorescence unless there are free nucleic acids in the preparation from lysed lymphocytes. Finally, the lymphocyte nucleic acids are also stained by the ethidium bromide which allows all of the lymphocytes to be seen under dark field. The high visibility of lymphocytes and bacteria allow for a high accuracy of counts.

Four types of lymphocytes were distinguished by the combined anti-globulin FA-BR technique (Fig. 1). The first type was

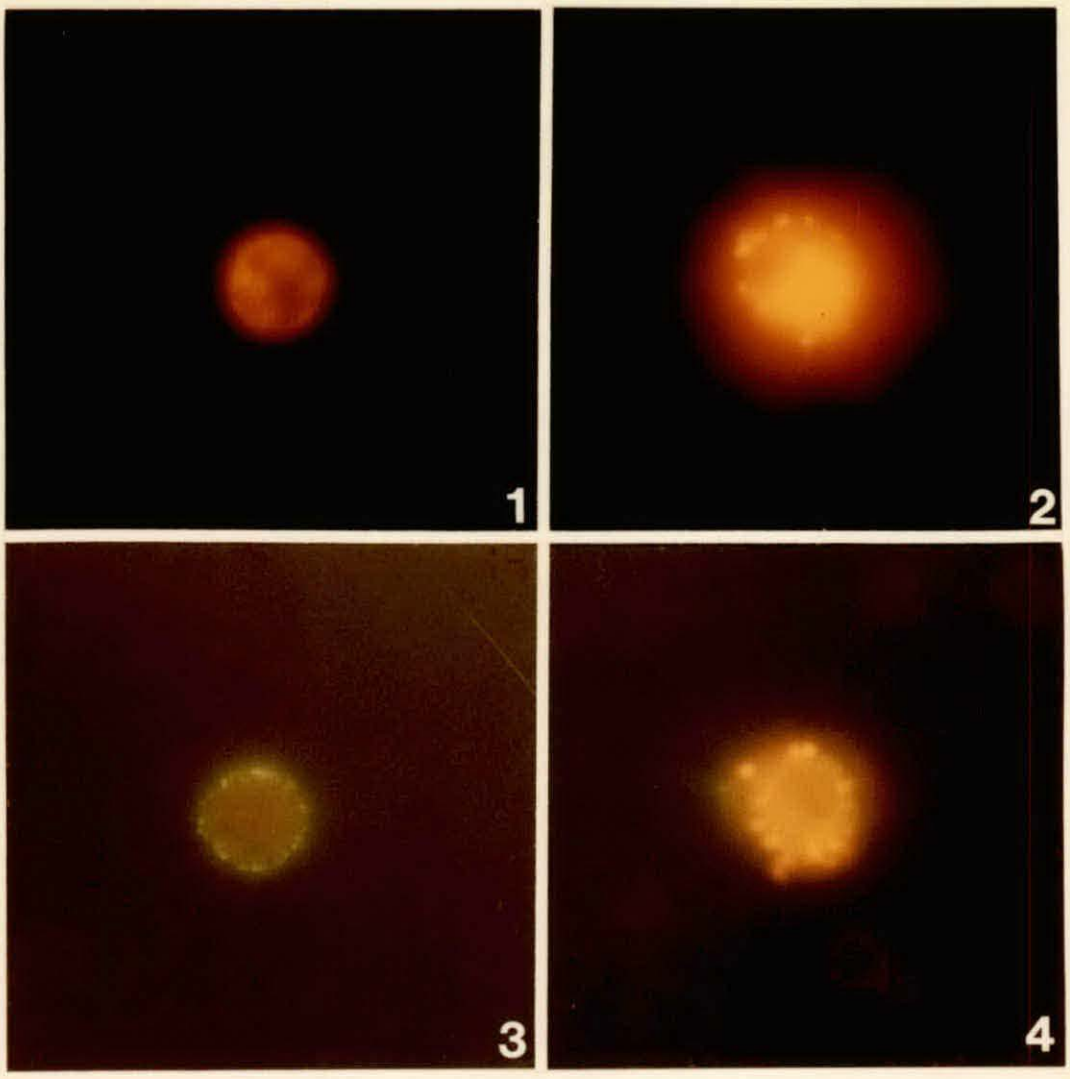


Figure 1. Binding of the bacterium Ec #2 to bovine peripheral bloodlymphocytes. 1) Non-rosetting T-cell 2) Rosetting T-cell 3)Non-rosetting B-cell 4) Rosetting B-cell. The cells were photographed at a 330 X magnification

non-rosetting T-cells with orange stained nuclei. The second type was rosetting T-cells which appeared as cells with orange nuclei surrounded by orange bacteria. The third type was non-rosetting B-cells which appeared as cells surrounded by a green ring due to membrane fluorescence. Finally, the rosetting B-cells were seen as cells surrounded by green membrane fluorescence and orange bacteria. Occasionally, the nuclei of the B-cells were also stained orange by the ethidium bromide.

Table 1 shows the percentages of T and B-lymphocytes rosetted by the various bacteria. Using the combined FA-BR test with the anti-thymocyte serum, we were able to cross check our anti-globulin FA-BR results. Close agreement was achieved when comparing the two methods (Table 2).

After determining whether a strain of bacteria labeled B or T-cells and the percentage of each, we performed double bacterial labeling experiments to determine if the bacteria were binding to the same or different lymphocyte subpopulations. In these experiments, the lymphocytes were mixed with a combination of two different bacteria and the percentage of lymphocytes labeled was recorded (Tables 3 and 4).

The results obtained with the various bacterial combinations were analyzed as described by Mayer et al.[8] Briefly, the analysis considers two bacteria: a which binds to X% of the lymphocyte population and b which binds to Y%. When a and b are both used in the same test, the percentage of the lymphocyte population with bacteria

TABLE 2. Percentages of Bovine PBL Identified By Combined FA-BR Test Using Goat Anti-Thymocyte Serum to Label T-cells (% Mean ± Standard Error)[a]

| Bacteria | % of Tot. T-cells (FITC Labelled) | % of Tot. B-cells (non T-cells) | %PBL Bovine | %PBL Human[b] |
|---|---|---|---|---|
| C. pyogenes #2 | 23.4 ± 1.1 | 8.2 ± 1.2 | 18.3 ± .27 | ND |
| E. coli #2[c] | 53.3 ± .94 | 35.3 ± 2.2 | 48.1 ± 2.0 | 56.0 ± 0.9 |
| E. coli #3[c] | 68.2 ± .60 | 53.5 ± 1.1 | 63.4 ± 1.9 | 61.0 ± 1.2 |
| B. melitensis[c] | 7.7 ± .92 | 40.4 ± 1.7 | 17.0 ± 1.4 | 16.0 ± 0.8 |
| none (FA only) | 70.3 ± 2.2[d] | | | |

[a]Means and S.E. calculated on a pool of four runs on ten animals.

[b]Data as reported by Teodorescu et al.[9] using the direct bacterial rosette method.

[c]Culture obtained from A. Bratescu, Department of Microbiology, University of Illinois, at the Medical Center, Chicago, IL 60612.

[d]Data obtained using FA staining and ethidium bromide to identify percentages of B and T-cells in PBL.

TABLE 2. Pecentages of Bovine PBL Identified By Combined FA-BR Test Using Goat Anti-Thymocyte Serum to Label T-cells (% Mean ± Standard Error)[a]

| Bacteria | % of Tot. T-cells (FITC Labelled) | % of Tot. B-cells (non T-cells) | %PBL Bovine | %PBL Human[b] |
|---|---|---|---|---|
| C. pyogenes #2 | 23.4 ± 1.1 | 8.2 ± 1.2 | 18.3 ± .27 | ND |
| E. coli #2[c] | 53.3 ± .94 | 35.3 ± 2.2 | 48.1 ± 2.0 | 56.0 ± 0.9 |
| E. coli #3[c] | 68.2 ± .60 | 53.5 ± 1.1 | 63.4 ± 1.9 | 61.0 ± 1.2 |
| B. melitensis[c] | 7.7 ± .92 | 40.4 ± 1.7 | 17.0 ± 1.4 | 16.0 ± 0.8 |
| none (FA only) | 70.3 ± 2.2[d] | | | |

[a]Means and S.E. calculated on a pool of four runs on ten animals.

[b]Data as reported by Teodorescu et al.[9] using the direct bacterial rosette method.

[c]Culture obtained from A. Bratescu, Department of Microbiology, University of Illinois, at the Medical Center, Chicago, IL 60612.

[d]Data obtained using FA staining and ethidium bromide to identify percentages of B and T-cells in PBL.

Table 3. Mean Percentages of B-cells Identified by Combinations of Two Bacteria Using Double Bacterial Labeling Experiments[a]

| Bacteria | None | Ec #2[b] | Ec #3[b] | Cp #2[b] | Bm[b] |
|---|---|---|---|---|---|
| Ec #2 | 34.4 | - | 67.9 | 45.5 | 70.3 |
| Ec #3 | 51.8 | 67.9 | - | 51.5 | 48.5 |
| Cp #2 | 8.2 | 45.5 | 51.5 | - | 38.2 |
| Bm | 37.3 | 70.3 | 48.5 | 38.2 | - |

[a] Means calculated on a pool of three runs on four animals.

[b] (Ec #2) *E. coli* #2, (Ec #3) *E. coli* #3, (Cp #2) *C. pyogenes* #2 and (Bm) *B. melitensis*.

TABLE 4. Mean Percentages of T-cells Identified by Combinations of Two Bacteria Using Double Bacterial Labeling Experiments[a]

| Bacteria | None | Ec #2[b] | Ec #3[b] | Cp #2[b] | Bm[b] |
|---|---|---|---|---|---|
| Ec #2 | 52.7 | - | 81.9 | 55.2 | 53.4 |
| Ec #3 | 67.7 | 81.9 | - | 70.8 | 76.9 |
| Cp #2 | 23.5 | 55.2 | 70.8 | - | 31.5 |
| Bm | 6.5 | 53.4 | 76.9 | 31.5 | - |

[a] Means calculated on a pool of three runs on four animals.

[b] (Ec #2) E. coli #2, (Ec #3) E. coli #3, (Cp #2) C. pyogenes #2 and (Bm) B. melitensis.

attached may be:

1) equal to X+Y in which case a and b identify different subsets of cells, i.e. Ec #2-Cp #2 when labeling B-cells (Table 3);
2) equal to X (where $X \gg Y$) in which case b is a subset of a, i.e. Ec #2-Cp #2 when labeling T-cells (Table 4);
3) equal to Y (where $Y \gg X$) in which case a is a subset of b, i.e. Cp #2-Ec #3 when labeling B-cells (Table 3);
4) greater than X (where $X > Y$) in which case only some of the cells identified by b must also be identified by a, i.e., there are at least three subsets of cells, one identified only by a, one by a and b, and one only by b, i.e. Ec #3-Ec #2 when labeling T-cells (Table 4);
5) greater than Y (where $Y > X$) in which case only some of the cells identified by a must also be identified by b, i.e. Ec #2-Ec #3 when labeling B-cells (Table 3);

Other combinations of results are possible, but were not encountered in our work.

This analysis was used to develop "maps" of lymphocyte subpopulations (Fig. 2 and 3). Using four strains of bacteria, five $Ig^+$ B-cell subpopulations ($B_1$, $B_2$, $B_3$, $B_4$ and $B_5$) and five $Ig^-$, presumably T-cell, subpopulations ($T_1$, $T_2$, $T_3$, $T_4$ and $T_5$) were identified. $B_1$ cells were labeled by Ec #2. $B_2$ cells were labeled by Ec #2 and Ec #3. $B_3$ cells were identified by Bm, Cp #2 and Ec #3. $B_4$ cells were labeled by Bm and Ec #3. $B_5$ cells were not identified by any of the bacteria tested, but represent a significant percentage of

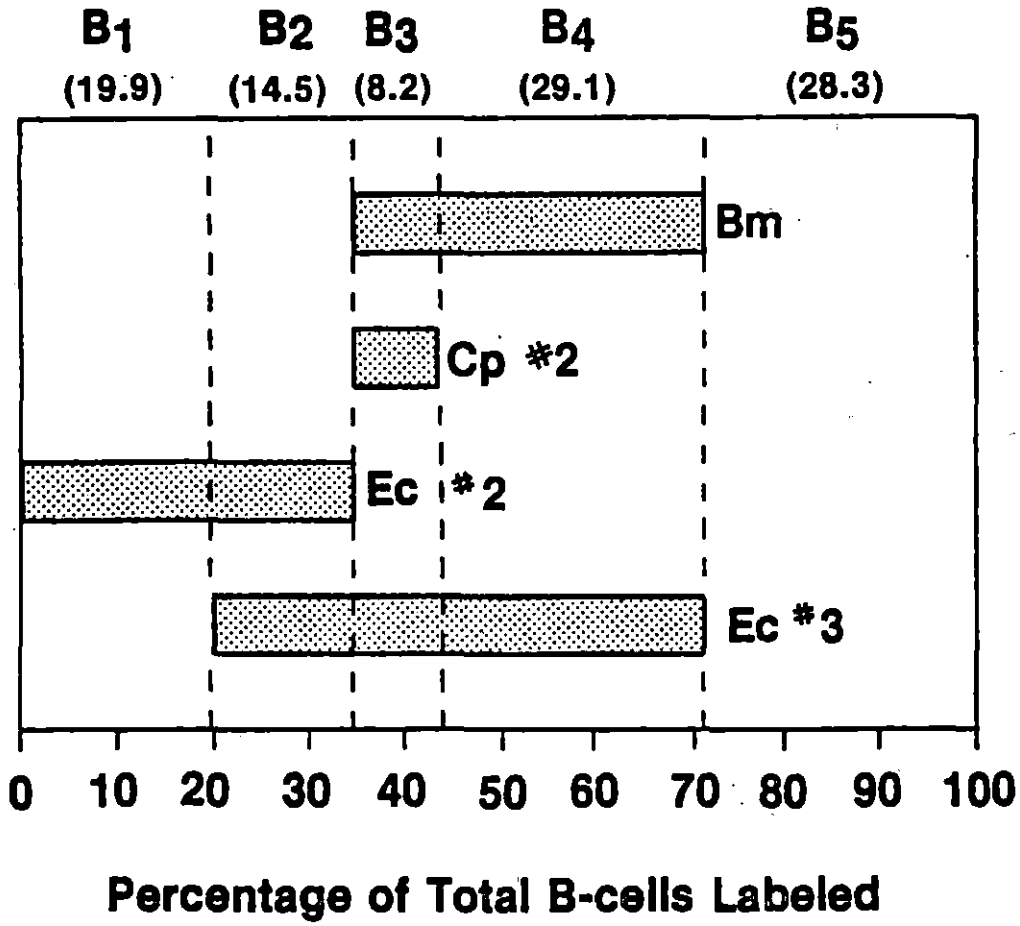


Figure 2. Percentile distribution of bovine B-lymphocyte subpopulations as identified by the bacteria tested: (Ec #2) E. coli #2, (Ec #3) E. coli #3, (Cp #2) C. pyogenes #2 and (Bm) B. melitensis.

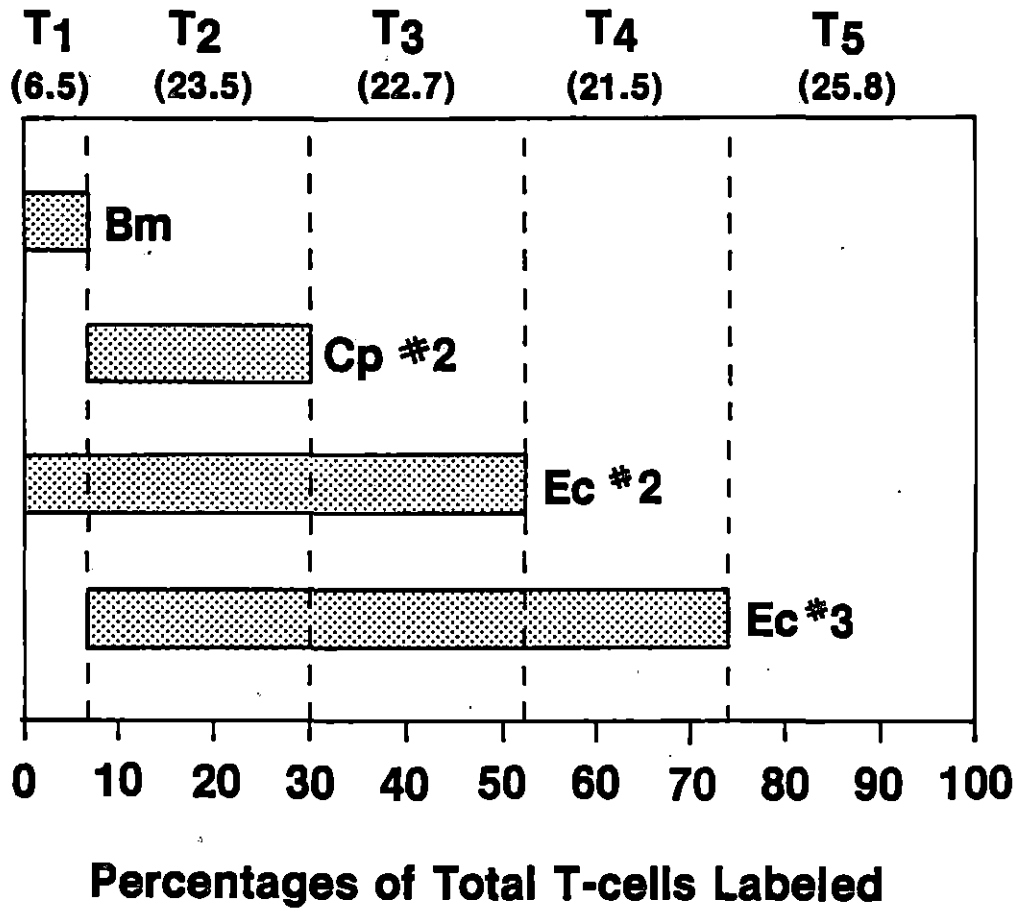


Figure 3. Percentile distribution of bovine T-lymphocyte subpopulations as identified by the bacteria tested: (Ec #2) E. coli #2, (Ec #3) E. coli #3, (Cp #2) C. pyogenes #2 and (Bm) B. melitensis.

the total B-cell population (28.3%). $T_1$ cells were labeled by Ec #2, and Bm. $T_2$ cells bound Ec #3, Ec #2 and Cp #2. $T_3$ cells bound Ec #2 and Ec #3. $T_4$ cells were labeled by Ec #3. $T_5$ cells were not labeled by any of the bacteria tested, but represent 25.8% of the total T-cell population.

Of the five strains of B. abortus tested, none was found to rosette greater than five percent of the PBL of cattle tested.

# DISCUSSION

Studies by Teodorescu et al.[4] indicated that the differential binding of bacteria to human lymphocytes can be used to identify human lymphocyte subpopulations. We investigated the possibility of combining the direct BR test with immunofluorescent staining of B and T-lymphocytes and ethidium bromide stained bacteria in order to develop a reliable method of identification for bovine lymphocyte subpopulations.

Our data show that certain bacteria bind specifically to subpopulations of bovine lymphocytes. The combination of the bacterial rosette technique with the fluorescent methods described did not influence the binding properties of the bacteria tested.

The results obtained for percentages of bovine PBL labeled by these bacteria agree closely with those reported by Teodorescu et al. for human PBL (Tables 1 and 2).

The bacteria used in these tests were stored and used over a period of one year during which their binding properties remained constant. Thus, the bacteria can be considered stable shelf reagents. This is not the case with conventional ER techniques which require that fresh reagents be prepared for each assay.

The combined FA-BR test provided a highly sensitive method of determining specific bacterial adherence to T-cells or B-cells. It also allows visual detection and enumeration of each lymphocyte

subpopulation based on the known patterns of specific bacterial adherence. The test eliminated the need to separate T and B-cells for accurate enumeration.

Previous work by Bratescu et al.[10] suggested that there may be a direct relationship between the pathogenicity of a bacterium for a host and its ability to form rosettes with that host's lymphocytes. Our results indicate that this is not the case for B. abortus.

It should be noted that some of the bacteria used in this study are natural saprophytes or pathogens of cattle. The possibility exists that the presence or absence of lymphocyte receptors in bovine cells may help define the role of lymphocytes in various cattle diseases. The method may also be used to aid in the identification of functional populations of lymphocytes in immunologic processes as has been described in man and mice.[4,11]

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# SECTION II. IDENTIFICATION OF CHANGES IN BOVINE LYMPHOCYTE SUBPOPULATIONS IN *Brucella* INFECTED CATTLE BY A COMBINED BACTERIAL ADHERENCE AND FLUORESCENT ANTIBODY TECHNIQUE

# SUMMARY

Serological tests are widely used to study the immune responses of cattle to brucellosis. In addition, assays to detect lymphocyte responses to specific Brucella abortus antigens have been developed. Changes in bovine lymphocyte populations associated with Brucella infections are relatively unknown. Using a technique which combines specific bacterial binding with fluorescent antibody staining techniques to identify subpopulations of bovine lymphocytes, we were able to monitor changes within these subpopulations associated with B. abortus infection. An increase of 11% in the total number of B-cells in peripheral blood as well as a 11% increase within one subpopulation ($B_2$) was detected using this procedure. The 2308 strain of B. abortus used to experimentally infect cows was used to examine the relationship between the ability of a bacterium to elicit a lymphocyte subpopulation shift and its ability to detect that lymphocyte response. This work indicated no apparent relationship.

# INTRODUCTION

Brucellosis in cattle continues to be a major disease associated with agricultural economic losses and of public health risk in this country.

Much of the current and past work in brucellosis control has involved the development and application of serological surveillance techniques. Some of the most commonly used methods include the standard tube agglutination test (SAT), complement fixation test (CFT)[1] and Rivanol plate agglutination test (RIV)[2].

In addition to the serologic techniques currently used, many investigators have studied the response of bovine lymphocytes to specific Brucella associated antigens.[3,4,5] However, there are several problems associated with using the lymphocyte blastogenesis test (LBT) to measure cell-mediated immune responses. Some of the difficulties include variations in techniques employed by different researchers such as incubation time and temperature, tissue culture media and concentration of antigen. As reported by Schultz,[6] variations in antigen nature, cell source and interpretation of results will affect the results obtained. Schultz[6] further reports that Brucella antigen LBT results vary markedly from day to day. It was also found that even when using a variety of Brucella antigens the LBT test is not a reliable method of detecting Brucella infection in cattle. Finally, cell-mediated immunity alone has not been shown conclusively to

provide protective immunity in any cattle disease.[6]

Not much is known concerning the changes in lymphocyte populations associated with brucellosis in cattle. Patsula and Kondaurov[7] reported a general trend for decreased numbers of T lymphocytes and increased numbers of B lymphocytes in blood of experimentally infected calves. Approximately four weeks post exposure, there was a marked increase in the number of circulating B-cells and concurrent appearance of agglutinins and complement-fixing antibodies in the blood serum.

Spontaneous rosetting of human lymphocytes with a variety of gram negative bacteria was observed by Teodorescu et al.[8] Two subpopulations of B-cells and four subpopulations of T-cells could be distinguished by these investigators.

Studies by Teodorescu et al.[9] and Nelson et al.[10] have shown that bacterial adherence techniques can be used to monitor changes in human lymphocyte subpopulations in patients with chronic lymphocytic leukemia. Increases in the number of B-cells and changes in bacterial binding affinities corresponding to symptomatic stages of the disease were detected by these investigators.

The present investigation involved monitoring relative changes in lymphocyte subpopulations associated with Brucella infected cattle using a combined bacterial rosetting (BR)-fluorescent antibody (FA) technique described previously by Canning et al.[11]

# MATERIALS AND METHODS

## Bacterial preparation

Strains of bacteria found to rosette human lymphocytes were obtained from Dr. Aurel Bratescu (Department of Microbiology, University of Illinois, at the Medical Center, Chicago, IL 60612). These included Escherichia coli (Ec #2, Ec #3) and Brucella melitensis (Bm). A strain of Corynebacterium pyogenes (Cp #2) was isolated from a case of bovine mastitis at the College of Veterinary Medicine at Iowa State University. The bacteria were grown in Roux flasks containing trypticase soy agar and fixed with 10% formaldehyde in phosphate-buffered saline (PBS) and washed as described by Bratescu et al.[12] Bacteria were suspended in PBS containing 0.02% $NaN_3$ and standardized to $1X10^9$ cells/ml spectrophotometrically.

## Selection of animals

Two apparently healthy Holstein-Friesian cows which had calved within thirty days were used as controls. Four Brucella infected cows used in other experiments were made available by Dr. Billy Deyoe (National Animal Disease Center, USDA, Ames, Iowa 50010). These animals were experimentally infected with strain 2308 of B. abortus and selected on the basis of high serologic titers using SAT, RIV and mercaptoethanol tests. In addition, these infected animals had aborted prior to use in this experiment. B. abortus was isolated from the uterus of all cows at the time of abortion (Table 1).

Table 1. Status of B. abortus Infected Cattle Used in the Experimentation

| Animal Number | Days Post Infection | Days Post Abortion | Serologic Titers SAT[a] | ME[a] | RIV[a] |
|---|---|---|---|---|---|
| 200 | 154 | 84 | I 800 | + 400 | + 400 |
| 221 | 157 | 54 | + 800 | + 1600 | I 100 |
| 222 | 155 | 80 | + 400 | I 400 | I 400 |
| 225 | 161 | 100 | + 1600 | + 1600 | + 400 |

[a] (SAT) standard tube agglutination test, (ME) mercaptoethanol agglutination test and (RIV) Rivanol plate agglutination test.

Peripheral blood lymphocyte preparation

Venous blood (15 ml) was collected and placed in tubes containing 2.0 ml of acid citrate dextrose solution (ACD). The blood was centrifuged at 1000 X g for twenty minutes and the buffy coat cells were removed. These cells were then washed once with Medium-199 containing 10% fetal calf serum and 0.02% $NaN_3$ (M-199-FCS). The buffy coat cells were then standardized to $1X10^7$ cells/ml in M-199-FCS.

Ethidium bromide staining of bacteria

Standardized bacteria (0.1 ml) was mixed with an equal volume of 10ug/ml ethidium bromide. The solution was allowed to incubate for approximately two hours in the dark at room temperature.

Indirect fluorescent antibody staining of lymphocytes

Standardized PBL (0.2 ml) was mixed with 0.1 ml of either a 1:40 dilution of rabbit anti-bovine globulin serum when labeling B-cells or a 1:30 dilution of goat anti-bovine thymocyte serum when labeling T-cells. The anti-bovine globulin serum was produced by repeated injections of rabbits with rabbit red blood cells coated with normal bovine agglutinins. The goat anti-thymocyte serum was provided by Dr. Thomas Yang (Department of Pathobiology, University of Connecticut, Storrs, Connecticut 06268). This serum had previously been shown to react specifically with bovine T-lymphocytes.[13] The PBL mixture was incubated at $4^{o}C$ for 30 min. and then washed twice with M-199-FCS by centrifugation at 256 X g. Following washing, 0.05 ml of either fluorescein (FITC) conjugated goat anti-rabbit IgG (heavy and light chain specific, Zymed Laboratories, Burlingame, California) (B-cells)

or FITC rabbit anti-goat IgG (heavy and light chain specific, Zymed Laboratories, Burlingame, California) (T-cells) was added to the PBL. Following incubation at 4°C for one hr., the cells were washed once with a solution containing 1.0 ml each of M-199-FCS and PBS containing 0.01 M $NaN_3$.

Bacterial rosette formation

The method of binding stained bacteria to PBL was essentially that described for binding of unlabeled bacteria to lymphocytes in blood smears by Bratescu et al.[12] Briefly, 0.1 ml of stained bacteria was added with 0.2 ml of M-199-FCS to 2 X $10^6$ labeled PBL. The mixture was then centrifuged at 900 X g at 4°C for 6 minutes. The supernatant was removed and 0.1 ml of mounting fluid was added to the cells. The mounting fluid consisted of 10.0 ml of PBS containing 100 mg of p-phenylenediamine in 90.0 ml of glycerol.[14] In addition, 0.1 ml of ethidium bromide was added to the cell suspension. Following incubation for twenty minutes at 4°C, one drop was applied to a cold slide and covered with a cold coverslip. Rosettes were counted using a fluorescence microscope (Leitz Ortholux II, Ernst Leitz Ltd., Midland, Ontario, Canada). At least 200 cells were counted and a cell was considered rosette positive if there were at least three bacteria surrounding it.

# RESULTS

Using the indirect FA tests for the detection of B and T-cells, we found a mean of 33.0 $\pm$ 0.7% B-cells and 66.0 $\pm$ 1.4% T-cells in the peripheral blood of control cows. Experimentally infected animals had values of 44.5 $\pm$ 1.7% B-cells and 55.3$\pm$ 1.5% T-cells. Thus, there was an approximate 11% increase in the total number of B-cells present. As previously reported,[11] using T-cell staining simultaneously with the B-cell staining procedure, we found less than 5% null cells in the PBL of cattle tested. In control preparations omitting the first antibody in the indirect procedure, we found less than 1% non-specific staining.

Satisfactory fluorescent-staining of bacteria and lymphocytes was achieved using ethidium bromide. The high visibility of lymphocytes and bacteria allowed for a high accuracy of counts.

Four types of lymphocytes were distinguished by the combined FA-BR technique. The first type was non-rosetting T-cells with orange stained nuclei. The second type was rosetting T-cells which appeared as cells with orange nuclei surrounded by orange bacteria. The third type was non-rosetting B-cells which appeared as cells surrounded by a green ring due to membrane fluorescence. Finally, the rosetting B-cells were seen as cells surrounded by green membrane fluorescence and orange bacteria. Occasionally, the nuclei of the B-cells were also stained orange by the ethidium bromide.

Table 2 shows the percentages of T and B-lymphocytes from control and infected animals labeled by the various bacteria. The numbers of rosetting T-cells for each bacterial strain tested did not vary appreciably between control and infected animals. However, there was an increase in the number of B-cells labeled by Ec #2 (15%) and Ec #3 (10.9%) when lymphocytes from infected cows and control cows were compared.

After determining the percentage of B and T-cells labeled by each strain of bacteria, we performed double bacterial labeling experiments to determine if the bacteria were binding to the same or different lymphocyte subpopulations as shown previously.[11] In these experiments, the lymphocytes were mixed with a combination of two different bacteria and the percentage of lymphocytes labeled was recorded (Tables 3 and 4).

The results obtained with the various bacterial combinations were analyzed as described by Mayer et al.[15] Briefly, the analysis considers two bacteria: a which binds to X% of the lymphocyte population and b which binds to Y%. When a and b are both used in the same test, the percentage of the lymphocyte population with bacteria attached may be:

1) equal to X+Y in which case a and b identify different subsets of cells, i.e. Ec #2-Cp #2 when labeling B-cells (Table 3);
2) equal to X (where $X > Y$) in which case b is a subset of a, i.e. Ec #2-Cp #2 when labeling T-cells (Table 4);
3) equal to Y (where $Y > X$) in which case a is a subset of b, i.e. Cp

#2-Ec #3 when labeling B-cells (Table 3);

4) greater than X (where X > Y) in which case only some of the cells identified by b must also be identified by a, i.e., there are at least three subsets of cells, one identified only by a, one by a and b, and one only by b, i.e. Ec #3-Ec #2 when labeling T-cells (Table 4);

5) greater than Y (where Y > X) in which case only some of the cells identified by a must also be identified by b, i.e. Ec #2-Ec #3 when labeling B-cells (Table 3);

Other combinations of results are possible, but were not encountered in our work.

This analysis was used to develop "maps" of lymphocyte subpopulations. Figures 1 and 2 show the relative sizes and positions of the PBL subpopulations of control cows. Using four strains of bacteria, five $Ig^+$ B-cell subpopulations ($B_1$, $B_2$,$B_3$, $B_4$ and $B_5$) and five $Ig^-$, presumably T-cell, subpopulations ($T_1$, $T_2$, $T_3$, $T_4$ and $T_5$) were identified. $B_1$ cells were labeled by Ec #2. $B_2$ cells were labeled by Ec #2 and Ec #3. $B_3$ cells were identified by Bm, Cp #2 and Ec #3. $B_4$ cells were labeled by Bm and Ec #3. $B_5$ cells were not identified by any of the bacteria tested, but represent a significant percentage of the total B-cell population (32.0%). $T_1$ cells were labeled by Ec #2, and Bm. $T_2$ cells bound Ec #3, Ec #2 and Cp #2. $T_3$ cells bound Ec #2 and Ec #3. $T_4$ cells were labeled by Ec #3. $T_5$ cells were not labeled by any of the bacteria tested, but represent 28.9% of the total T-cell population. When these "maps" were compared

Table 2. Percentages of PBL and lymphocyte subpopulations from Brucella infected and normal animals labeled by the combined FA-BR technique (mean ± S.E.)[a]

| Bacteria | | Control Animals | Infected Animals |
|---|---|---|---|
| Bm[b]- | %T cells | 7.4 ± 0.3 | 7.8 ± 0.2 |
| | %B cells | 33.2 ± 1.4 | 32.4 ± 0.9 |
| | %PBL | 19.3 ± 0.7 | 20.1 ± 0.4 |
| Cp #2[b]- | %T cells | 17.4 ± 1.0 | 17.4 ± 0.5 |
| | %B cells | 10.7 ± 1.1 | 9.7 ± 0.5 |
| | %PBL | 15.5 ± 0.8 | 14.1 ± 0.3 |
| Ec #2[b]- | %T cells | 53.3 ± 1.1 | 49.5 ± 0.5 |
| | %B cells | 34.8 ± 0.3 | 49.8 ± 1.4 |
| | %PBL | 46.0 ± 0.4 | 49.6 ± 0.1 |
| Ec #3[b]- | %T cells | 63.7 ± 0.6 | 64.5 ± 1.2 |
| | %B cells | 48.0 ± 0.9 | 58.9 ± 1.4 |
| | %PBL | 58.5 ± 0.4 | 60.9 ± 0.7 |

[a] Means and S.E. calculated on a pool of three runs on two control animals and three runs on four infected animals.

[b] (Ec #2) E. coli #2, (Ec #3) E. coli #3, (Cp #2) C. pyogenes #2 and (Bm) B. melitensis.

Table 3. Mean Percentages of T-cells From *Brucella* Infected Cattle Labeled by Combinations of Two Bacteria Using Double Bacterial Labeling Experiments[a]

| | Bm[b] | Cp #2[b] | Ec #2[b] | Ec #3[b] |
|---|---|---|---|---|
| Bm | 8.2 | 24.2 | 48.8 | 67.5 |
| Cp #2 | 24.2 | 17.1 | 48.6 | 66.8 |
| Ec #2 | 48.8 | 48.6 | 48.3 | 65.6 |
| Ec #3 | 67.5 | 66.8 | 65.6 | 61.9 |

[a] Means calculated on a pool of three runs on four animals.

[b] (Ec #2) *E. coli* #2, (Ec #3) *E. coli* #3, (Cp #2) *C. pyogenes* #2 and (Bm) *B. melitensis*.

Table 4. Mean Percentages of B-cells From *Brucella* Infected Cattle Labeled by Combinations of Two Bacteria Using Double Bacterial Labeling Experiments[a]

| | Bm[b] | Cp #2[b] | Ec #2[b] | Ec #3[b] |
|---|---|---|---|---|
| Bm | 33.9 | 32.1 | 82.4 | 62.2 |
| Cp #2 | 32.1 | 10.1 | 60.0 | 59.4 |
| Ec #2 | 82.4 | 60.0 | 51.7 | 66.7 |
| Ec #3 | 62.2 | 59.4 | 66.7 | 59.8 |

[a] Means calculated on a pool of three runs on four animals.

[b] (Ec #2) *E. coli* #2, (Ec #3) *E. coli* #3, (Cp #2) *C. pyogenes* #2 and (Bm) *B. melitensis*.

with those from previous work,[11] there was close agreement. When the subpopulation "maps" for control animals were compared with those for Brucella infected animals (Figs. 3 and 4) the relative size and position of T-cell supopulations remained constant. However, comparison of the B-cell subpopulations showed an increase in both the $B_2$ subpopulation (approximately 11.7%) and the total number of B-cells labeled by the 4 strains of bacteria tested (approximately 15.2%).

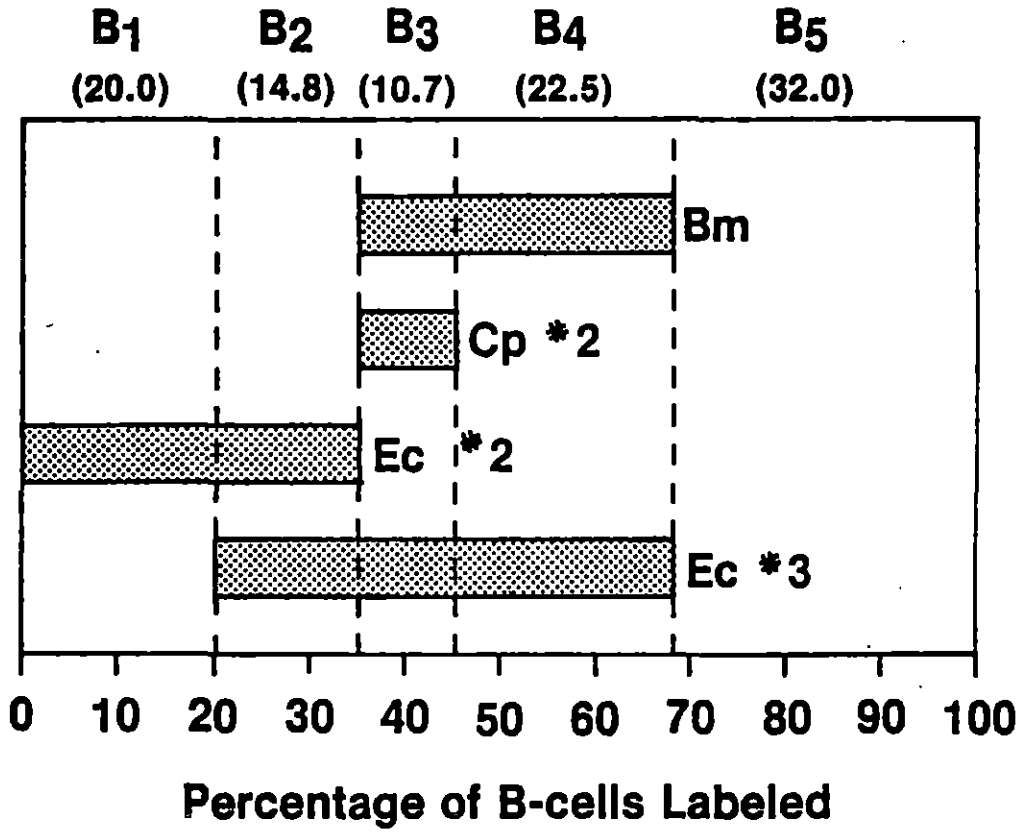


Figure 1. Percentile distribution of normal bovine B-lymphocyte subpopulations as identified by the bacteria tested: (Ec #2) E. coli #2, (Ec #3) E. coli #3, (Cp #2) C. pyogenes #2 and (Bm) B. melitensis

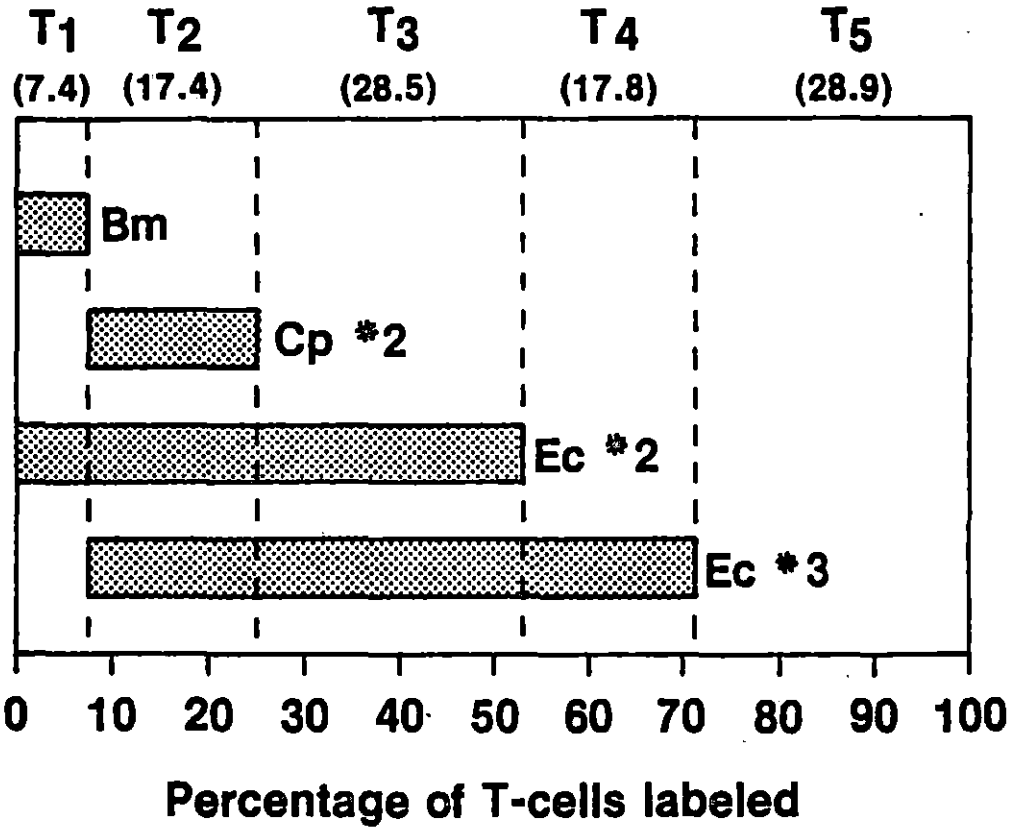


Figure 2. Percentile distribution of normal bovine T-lymphocyte subpopulations as identified by the bacteria tested: (Ec #2) E. coli #2, (Ec #3) E. coli #3, (Cp #2) C. pyogenes #2 and (Bm) B. melitensis

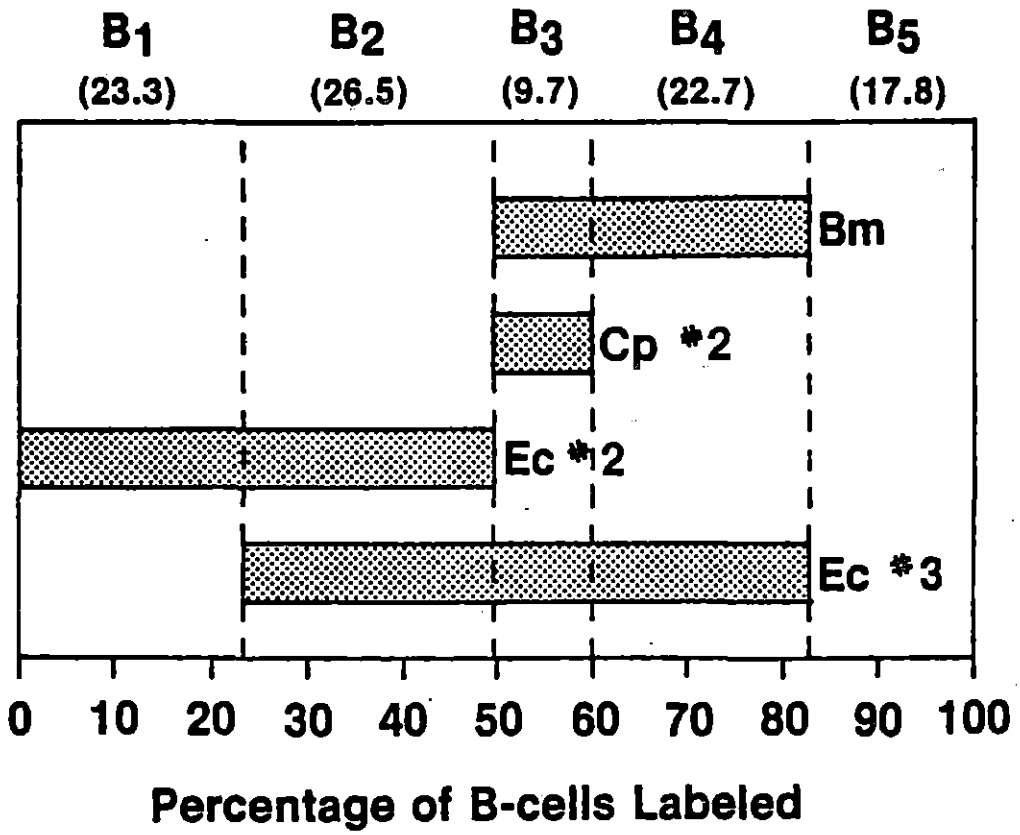


Figure 3. Percentile distribution of Brucella infected bovine B-lymphocyte subpopulations as identified by the bacteria tested: (Ec #2) E. coli #2, (Ec #3) E. coli #3, (Cp #2) C. pyogenes #2 and (Bm) B. melitensis

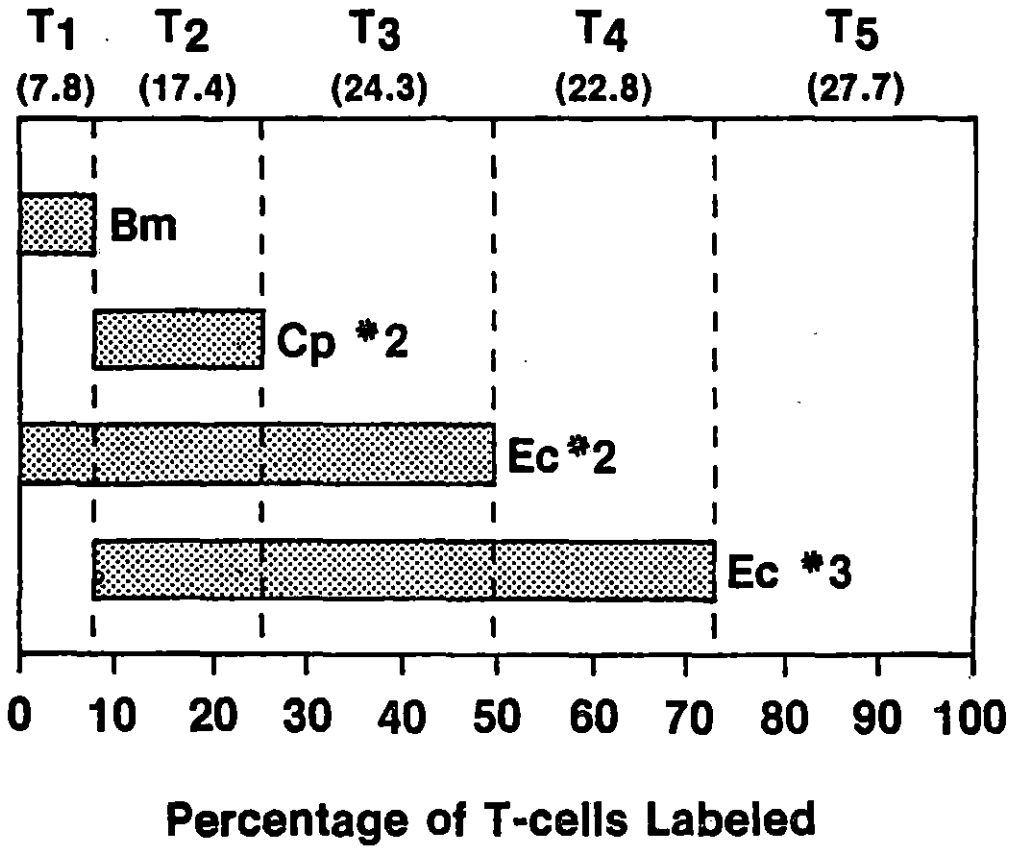


Figure 4. Percentile distribution of Brucella infected bovine T-lymphocyte subpopulations as identified by the bacteria tested: (Ec #2) E. coli #2, (Ec #3) E. coli #3, (Cp #2) C. pyogenes #2 and (Bm) B. melitensis

# DISCUSSION

Previous work[11] has shown that the differential binding of certain bacteria in combination with immunofluorescent staining methods provides a highly sensitive method of detecting bovine lymphocyte subpopulations.

In the current study, various strains of bacteria were evaluated for their binding to lymphocytes from B. abortus infected cattle and from normal cows. Marked increases (approximately 11%) in the number of B-cells detected by FA staining were noted when comparing PBL of infected animals and normal animals. In addition, an eleven percent increase in the $B_2$ subpopulation of lymphocytes was also detected by Ec #2 and Ec #3. No apparent changes in the number or relative size of the T-cell subpopulations was noted when comparing PBL of normal and infected animals.

Performing the combined FA-BR procedure with the 2308 smooth strain of B. abortus used to infect the cows, we found that it rosetted wih less than five percent of the PBL of both normal and infected cows. Thus, there is no apparent relationship between the ability of a bacterium to produce a lymphocyte subpopulation shift and its ability to detect lymphocytes stimulated by it.

It is possible that the increased number of B-cells detected may be a result of normal clonal expansion as a result of exposure to an antigen. This seems likely due to the recrudescent nature of

B. abortus infections. If indeed this is the case, this procedure may be of diagnostic value for early detection of cattle in active stages of Brucella infection. This capability would be highly desirable since clonal expansion theoretically occurs prior to any humoral response such as those being detected by current diagnostic serological techniques. However, more work would be needed to determine if the detected growth in the $B_2$ subpopulation is specific to B. abortus infection. In addition, a study comparing the size and number of subpopulations associated with vaccine treated and nonvaccinated animals may aid in differentiation between vaccinated and actively infected animals.

A long term study following animals throughout the course of the infection is needed. It is possible that such a study may detect shifts in several of the lymphocyte subpopulations, with each one being associated with a particular stage of the disease. This study would require a more sophisticated plan for controling possible variables whch may influence the number and size of the subpopulations detected. Teodorescu[67] has shown previously that among humans there is an approximate twofold increase in the number of B-cells present in the peripheral blood. It was proposed that this change was due to hormonal effects. Thus, it seems that housing the cattle in a controled environment would be needed to control variations within the subpopulations due to changes in temperature. Other variables such as stage in pregnancy of infection, challenge dose, route of infection and age of the animals would also have to be controled to prevent

nonspecific changes among the subpopulations due to differences in these variables. Monitoring the animals throughout the pregnancy and infection periods would allow evaluation of the relationship between shifts in subpopulations due to pregnancy and those associated with B. abortus infection.

Finally, analysis of the bacterial lymphocyte subpopulations to determine the nature of the receptors involved in the BR reaction as well as evaluating their functional roles in immunological processes could add greatly to our knowledge of the role of lymphocytes in host protection against brucellosis and other cattle diseases.

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# GENERAL SUMMARY

It is known that certain strains of bacteria bind selectively to subpopulations of human peripheral blood lymphocytes. The major objective of this experimentation was to develop a suitable bacterial adherence technique for the identification of subpopulations of bovine peripheral blood lymphocytes. In addition, the secondary objective was to apply this technique to observe changes in lymphocyte subpopulations associated with the pathogenesis of bovine brucellosis.

As a result of this experimentation, we have developed a technique which used the specificity of bacterial binding concurrently with fluorescent antibody staining methods to identify five B-cell and five T-cell subpopulations of bovine lymphocytes. This method provided a simple yet highly specific technique for the enumeration of both B and T-cells within a single preparation of peripheral blood lymphocytes. In addition, the use of the combined FA-BR procedure was found to be easier and more reliable than methods previously used to identify bovine B and T lymphocyte subpopulations.

Using five B. abortus strains of known pathogenicity, we found no apparent relationship between the pathogenicity of a bacterium for a host and its ability to form rosettes with that host's peripheral blood lymphocytes. The use of the combined FA-BR test to monitor changes in bovine lymphocyte subpopulations associated with B. abortus infection resulted in the detection of an eleven percent increase in

the total number of B-cells present in the peripheral blood. In addition, an eleven percent increase within one subpopulation ($B_2$) was detected using this procedure. It is not known if these changes are specific to Brucella infection. Further work involving a variety of Ags and highly controlled housing conditions would be needed to confirm this. Functional studies of the lymphocyte subpopulations is needed to determine which type of lymphocytes are required for competent immune response to bovine brucellosis and other diseases.

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