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The Use of Antibody-Coated Latex Beads to Determine Single Positive and Double Positive Mouse Spleen Cells Expressing CD5 and/or CD19 Glycoproteins

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THE USE OF ANTIBODY-COATED LATEX BEADS TO DETERMINE SINGLE
POSITIVE AND DOUBLE POSITIVE MOUSE SPLEEN CELLS EXPRESSING CD5
AND/OR CD19 GLYCOPROTEINS

A thesis submitted in partial fulfillment of

the requirements for the degree of Master

of Science.

By

Abdulrazzag Abdulaziz Othman

B.S., Um Al Qura University, 2010

2015

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WRIGHT STATE UNIVERSITY
GRADUATE SCHOOL

April 15, 2015

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY ABDULRAZZAG ABDULAZIZ OTHMAN ENTITLED The Use of Antibody-Coated Latex Beads To Determine Single Positive and Double Positive Mouse Spleen Cells Expressing CD5 and/or CD19 Glycoproteins. BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Othman, Abdulrazzag. M.S. Department of Microbiology and Immunology, Wright State University, 2013. The Use of Antibody-Coated Latex Beads To Determine Single Positive and Double Positive Mouse Spleen Cells Expressing CD5 and/or CD19 Glycoproteins.

Flow cytometry is the standard method used to diagnose, stage, and monitor patients' response to the treatment given by counting the numbers of CD5, CD19 and CD5+ CD19+ B lymphocytes. In this study, a comparison was done between numbers of single CD5+, single CD19+ and dual CD5+ CD19+ mouse spleen B lymphocytes using flow cytometry and antibody-latex beads. The bead method involved antibody-coated latex bead and yielded results similar to those of flow cytometry. For cells exhibiting both markers (CD5+ CD19+), the bead method used antibody-coated beads of two different colors yielded similar results to those of flow cytometry results. These findings show that the antibody-coated beads are adequate to determine the numbers of single CD5+, CD19+positive cells and CD5+ CD 19+double positive B lymphocytes under circumstance where flow cytometry is not available.

HYPOTHESIS

The hypothesis of this study was that the bead method using antibody coated latex beads would give similar results to those of flow cytometry in identifying cells bearing two different CD markers. The null hypothesis was that the bead method would not give similar results to those of flow cytometry results in identifying cells bearing two different CD markers.

In this study, the CD markers selected were CD5 and CD19, used in the diagnosis and staging of chronic lymphocytic leukemia (CLL). To mimic the human situation, mouse spleen cells were used.

CD5 is a cluster of differentiation expressed on a subset of IgM secreting B cells called B-1 cells, and also on T cells (Antin et al., 1986). B-lymphocyte antigen CD19 also called CD19 (Cluster of Differentiation 19), is a protein which in humans is encoded by the CD19 gene and found on the surface of B-cells, a type of white blood cell (Tedder & Isaacs, 1989) Chronic lymphocytic leukemia is a disease resulting from the malignant transformation of an IgM secreting B-1 B cell.

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List of Abbreviations

CD = Cluster of differentiation

CLL = Chronic lymphocytic leukemia

CML= Carboxylate modified beads

IgM = Immunoglobulin M

IgD = Immunoglobulin D

MBA = Monoclonal B cell lymphocytosis

BCR = B- cell receptor

HTLV = Human T- lymphotropic virus

EBV = Epstein Barr virus

CBC = Complete blood count

CT = Computed tomography

MRI = Magnetic resonance imaging

FISH = Fluorescent in situ hybridization

BMT = Bone marrow transplantation

DMEM = Dulbecco's Modified Eagle's Medium

PBS = Phosphate- buffered saline

MES = 2-n-Morpholino-ethanesulfonic acid

BSA = Bovine serum albumin

FCS = Fetal calf serum

AIDS = Acquired immunodeficiency deficiency virus

HIV = Human immunodeficiency virus

PNH = Paroxysmal nocturnal haemoglobinuria

Acknowledgement

I would like to thank my advisor, Dr. Nancy Bigley for her guidance, effort and support in the success of this thesis. Thank you for your patience, guidance and endless support over the past two years. I would also like to thank my program director, Dr. Barbara Hull, and Dr. Cheryl Conley for their support, suggestions and being on my committee. I would like to thank all my lab mates and colleges for their supports. In addition, I would like to thank my family, especially Mam and Dad, for the love, believing in me and helping me every step of the way I work to achieve my goals. I would like to thank my wife, for her support, patience, and encouragement.

Dedication

I would like to dedicate my thesis project to my father (Abdulaziz Othman), my mother (Naima Bukhari), my siblings and my wife for their endless love, constant encouragement and support.

Introduction

Flow cytometry is a technique which allows fast and multi-parametric analysis of single cells and microparticles. Flow cytometry measures single cells through a laser detector beam. Fluorescent-labeled antibodies are used to detect specific cell-surface markers of interest that are glycoproteins called cluster of differentiation (CD) markers (Macey, 2007). Flow cytometry is prominent in the diagnosis chronic lymphocytic leukemia (CLL) by detecting malignant cells bearing CD5 and CD19 markers to determine whether the patients have the disease or not. After the patients are diagnosed with the disease, it is important to follow up the patients' condition and to monitor the effectiveness of treatment given by monitoring the numbers of CD5+ CD19+ (double-positive) cells.

Especially in the developing world countries, not every country or laboratory has the capability of having flow cytometry equipment. This may be due to the expensive price of the equipment, unreliable electric supply, maintenance cost, and/or the high price of reagents. The cost of flow cytometry ranges from $30,000 to $150,000 (Matthews, 2006). Flow cytometry needs a professional, highly skilled operator, and technical support. The beads method involves counting cells microscopically after attaching the

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antibody to coated latex beads. The use of two different sizes of colored beads permits the identification of two different cell surface molecules, making the beads method an alternative technique to flow cytometry. The main concept of the bead method is to coat inert latex spheres with CD5 or CD19 mouse monoclonal antibody and use these beads to identify and manually enumerate by light microscopy the numbers of single positive CD5+, CD19+, and double positive CD5+ CD19+ B lymphocytes in a fresh sample of mouse spleen cells suspension. Monoclonal antibodies are used to identify and enumerate T and B lymphocytes (Foon & Todd, 1986). The beads method can be used to monitor a patient's drug dosage and response. For example, some studies have evaluated fludarabine and cyclophosphamide treatment in untreated patients with CLL. The results show overall response rates of 76% of preventing the development of resistance to CLL when detected by flow cytometry using double positive CD5+ CD19+ cells (Yee & Brien, 2006).

Super Active Latex Beads

Super active latex beads are hydrophilic and have a very high density of functional groups for covalent coupling of proteins to the particles. The super active layer is a three-dimensional layer that increases the colloid stability of the particles and makes it easier for the protein to bind to the particles. This type of bead maintains the protein structure than the distorting proteins caused by absorption onto a rigid surface. Super active latex beads have five types: carboxylate modified beads (CML), chloromethyl beads, aldehyde/amidine beads, aldehyde/sulfate beads, and aliphatic amine. In this particular study, carboxylate modified beads were used which are produced by copolymerizing carboxylic acid containing polymers. The carboxylate modified beads offer an advantage of not introducing additional protein and

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are not broken down or degraded by phagosomes. The consequence is a latex polymer particle that has a highly charged and relatively hydrophilic surface with a pka of 5-9. These features create a perfect site for covalent bond formation and present less distortion of the antibody structure ("Super Active Latex Beads," n.d.).

Literature Review

Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is the most common leukemia among adults in the world and is 16-30% greater than other types of leukemia (Vural et al., 2014). CLL is characterized by a massive accumulation of CD5+ and CD19+ B lymphocytes (mature malignant B cells) in blood, bone marrow, and lymphoid organs. There are 15,000 new cases each year in the United States of diagnosed CLL in adults. CLL is more prevalent in men than women with a ratio of 2:1 (Ghia et al., 2007). In every 100,000 individuals, there are 2-6 cases each year and incidence increases with age. A recent study showed that CLL develops more frequently in people aged 65 years or older. An increase among younger people who are diagnosed before the age of 55 years account for one-third of the new cases (Ghia et al., 2007).

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Incidence and Epidemiology

CLL is the most common type of adult leukemia in Western societies and has highest incidence in Australia, Italy, Switzerland, the United States, and Ireland (Inamdar & Ramos, 2007). This disease is more common in whites than blacks (Redaelli et al., 2004). According to the American Cancer Society (ACS), approximately 4,600 cases of CLL were diagnosed in men and 3,500 in women of the total of 8,100 new cases in 2000 (Cherath, 2006). In fact, in the United States in 2010, approximately 14,990 people were diagnosed with CLL and the people died of the disease approximately 4,390 people (Parker & Strout, 2011).

Signs and Symptoms

Patients with CLL have a wide spectrum of symptoms and signs. Twenty five to fifty percent of patients with CLL are asymptomatic (Hallek et al., 2008). Symptoms can include: enlarged lymph nodes or spleen, fever, enlarged liver or spleen, continuous pain in the upper left part of the abdomen (which may be due to enlarged spleen), frequent infections, weight loss, loss of appetite, abnormal bruising (late-stage symptom), fatigue, or night sweats (Cheson et al., 1988).

Pathophysiology

In the majority of patients with CLL the B cells represent cells that are originally clonal B cells. These cells resemble the mature lymphocytes of the peripheral blood. When CLL lymphocytes are detected by CD19, CD5, and CD23 monoclonal antibodies, they express a B-cell surface antigen. CLL cells express low levels of surface immunoglobulin (Campo et al., 2011). These immunoglobulin are mainly

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immunoglobulin M (IgM) or IgM/IgD and IgD. CLL cells also express a low level of a single immunoglobulin light chain called kappa or lambda (Haeney, 2014). The monoclonal B cell lymphocytosis (MBL) in CLL patients has less than 5,000 monoclonal B cells in the peripheral blood. In elderly people MBL is observed in 5% of the patients (Gaidano et al., 2012).

Morphology

The leukemia cells in the blood smear are characterized by small, mature lymphocytes with narrowed cytoplasm edges, a heavy nucleus lacking recognizable nucleoli, and they do not show chromatin that is partially aggregated (Eichhorst et al., 2011). The blood smears of patients with CLL show exceptional fragility of the cell membrane that leads to the constant rupture of leukemic cells during the preparation of blood film. This characteristic of cells are called smudge cells or gumprecht nuclear shadows (Nowakowski et al., 2009).

Chromosomal Abnormalities In CLL

The most common chromosomal abnormalities in CLL contain 13q deletions, 11q and 7q deletions, trisomy 12 and 17p deletions. The 13q14 deletion is noticed in 50% of all CLL cases; only 18%–20% of cases do not show any chromosomal changes (Center et al., 2009).

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The B-Cell Receptor

The multimeric feature of B-cell receptor (BCR) that is formed by the accumulation of a non-covalently bound heterodimer Igα/Igβ (CD79A/ CD79B) with the surface of immunoglobulin (Ig) decreases in the B-cell receptor in CLL. The mechanism that would explain the poor expression of the B-cell receptor is still evasive. However, this low expression makes it hard for Ig to assemble and transport from endoplasmic reticulum to the cell surface due to the defect in folding and glycosylation property of the μ and CD79A chains. The high defect in CD79A results in poor expression of BCR in chronic lymphocytic leukemia. Most B-CLL cells express CD5 and IgM/IgD, which have a mantle zone-prevalent to that of naive cells. In a normal situation, these naïve cells express unmutated immunoglobulin genes (Dighiero & Hamblin, 2008). The low expression of the B-cell receptor is associated with reduced tyrosine kinase activity which causes a deficiency in tyrosine phosphorylation and intracellular calcium mobilization (Michel et al., 1993). Patients with previous history of pneumonia infection are more susceptible to develop chronic lymphocytic leukemia, as the infection with these encapsulated organisms may participate in the development of chronic lymphocytic leukemia (Landgren et al., 2007).

Cause

The cause of CLL is still unknown (Rozman & Montserrat, 1995). There is no link to radiation, drugs, or chemical exposure (Cronkite, 1987). However, CLL is shown to be linked to environmental factors and genetic abnormalities. For instance, a patient who has a close family member with CLL is up to seven times more likely to be diagnosed with CLL than other people. People with past exposure to human T-

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lymphotropic virus type I and II (HTLV-I and -II) and Epstein-Barr virus (EBV) infections are more likely to develop CLL than others (Cherath, 2006).

Cluster Differentiation (CD) Marker CD5 and CD19

CD5 is a cluster of differentiation expressed on a subset of IgM secreting B cells called B-1 cells, and also on T cells (Antin et al., 1986). The lack of the enzyme terminal deoxynucleotidyl transferase (TdT) in B-1 cells causing the limited diversity of their B-cell receptor. The B cell receptor signaling is negatively regulated by CD5 in the normal B-1 cells. The positive regulation of B cell receptor would results in the formation of cells resistant to undergo apoptosis. In normal B cells, CD5 functions as a negative regulator of BCR and antigen receptor activation in lymphocytes. The constant stimulation of BCR leads to the over-expression of CD5 in chronic lymphocytic leukemia, because CD5 is phosphorylated on tyrosine residue of BCR. The malignant B cells are produced in the bone marrow and proliferated in the lymph nodes causing defective apoptosis and massive accumulation initiated by CD5 molecules through an abnormal transformation of B cells. CD5 activates casein kinase 2 (CK2), The CD5-CK2 signaling pathway regulates T cell activation and Th differentiation (Pharm, 2009).

CD19 (Cluster of Differentiation 19), also called B-lymphocyte antigen CD19, is a transmembrane protein which in humans is encoded by the CD19 gene and found on the surface of B-cells, a type of white blood cell (Tedder & Isaacs, 1989). CD19 is part of the B cell co-receptor that functions to potentiate signaling through the B cell receptor. The formation of the receptor complex is induced by exogenous antigen and CD19. CD19 is also a B cell–specific antigen expressed on CLL cells. The expression of CD19

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stimulates homotypic aggregation in CLL. CD19 provokes phosphorylation of protein kinase B, cellular aggregation and production of autoantibodies, thus, stimulation plays an important role in the progression of CLL (Ho et al., 2009). Chronic lymphocytic leukemia is a disease resulting from the malignant transformation of an IgM secreting B-1 B cell.

Risk Factors

Several factors contribute to the development of CLL.

- Chemical exposure: some studies have shown a relationship between the exposure to an herbicide agent orange used particularly during Vietnam War and increasing risk of CLL (Brown et al., 1990). Some studies have linked farming exposure to some kinds of pesticides that might increase the risk of CLL (Brown et al., 1990; Miligi et al., 2003).

- Genetic Factors: a family history of a close family member being diagnosed with CLL would increase the chance more than twice (Capalbo et al., 2000).

- Gender: for an unknown reason CLL has been known more in males than females (Redaelli et al., 2004).

- Ethnicity: People in North America and Europe have higher risk of CLL than any other people in the world (Redaelli et al., 2004).

Diagnosis

Physical exam and history: the patient will be asked if he or she has any symptoms like fatigue, night sweats, abnormal bruising, or continuous pain in the upper left part of the abdomen. A doctor will also ask about a history of patient's past illnesses.

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Physical examination to check for any sign of enlarged lymph node in pelvic region, neck, and underarm. Also, careful examination for any sign of enlarged liver or spleen (Katz et al., 2010).

Complete blood count (CBC) with differential is a lab test that measures the different cells in blood. This test gives a clear picture about the numbers of red blood cells and platelets, numbers and types of white blood cells and level of hemoglobin, which is the protein that carries oxygen in red blood cells. High lymphocytes (lymphocytosis) that exceed more than 10,000 lymphocytes/mm³ of blood may indicate CLL is present. This patient will definitely have low red blood cells and platelet counts. When the blood smears are performed, the abnormal looking lymphocytes under the microscope will look like smudge cells (Cherath, 2006).

Bone marrow aspiration and biopsy is usually done before starting treatment and is a confirmatory test for the diagnosis of CLL. It is also helpful to tell in which stage is the patient and to determine if the treatment is effective. A bone marrow biopsy is usually done right after the aspiration. A small bone and marrow is removed. A patient with CLL has more than one fourth of his bone marrow composed of mature lymphocytes (Cherath, 2006).

Computed tomography scans (CT scans), and magnetic resonance imaging (MRI) might be used to look for an enlarged lymph node, spleen, and liver and whether leukemia has affected other organs of the body (Tran et al., 2005).

Immunoglobulin testing is done to see if patient has enough antibodies to fight infection. The beta-2-microglobulin may be tested. A high level of beta-2-microglobulin indicates a more advanced stage of CLL (Keating et al., 1995).

Immunophenotyping: a blood sample will be taken to see what immune cells are affected. In most cases B cells are affected by CLL and only very rare cases have T cells been affected (Cherath, 2006).

Cytogenetic analysis: bone marrow cells are grown in the lab to be tested microscopically. Cells from a normal person contain 23 pairs of chromosomes, but in some patients with CLL some chromosomes are missing or deleted. Most of the time, deletion occurs in some parts of chromosomes 13, 11, or 17 (Stilgenbauer et al., 2002). Knowing about chromosomal changes will be useful in determining which treatment is best (Shanafelt et al., 2006).

Flow cytometry is an important test that looks for a specific substance (marker) on the surface of cells that help to determine which type of cells are being affected. The sample of cells only binds to the antibody of interest if it is present on that sample. The bound cells are passed through a laser beam. The laser will emit light that will be measured and analyzed by a computer (Sklar, 2005). The coexpression of CD5 and B cell markers, e.g. CD19, is used to confirm the presence of CLL. In addition, peripheral blood lymphocytes are monitored in patients with lymphocytosis to detect malignancy if it is reactive. The absence of CD5 and CD19 coexpression omits B cell CLL, and indicates

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other B cell lymphoproliferative disorders, including follicular, or hairy-cell leukemia (Dillman, 2008).

Management & Treatment

Asymptotic patients or patients with an early stage are not treated unless needed. They are monitored closely by a doctor. They are only treated if they show active disease or rapid progression (Hallek et al., 2008). Treatment should be established in the early stage of CLL if one of the following circumstances apply: symptoms become worse, enlarged spleen, massive reduction in platelets or hemoglobin level, frequent infection, rapid increase in lymphocytes count, or progressive enlargement of liver or lymph node (Cherath, 2006).

Chemotherapy medications are used to treat people with CLL. A single drug or combination of drugs may be prescribed, depending on which stage the patients is. The most common know drugs are: cladribine, chlorambucil, cyclophosphamide, and fludarabine. Radiation therapy may be used to treat an enlarged lymph node and spleen. In severe cases, the spleen is removed surgically through a process called a splenectomy (Cherath, 2006).

Bone marrow transplantation (BMT) is the replacement of an affected marrow (patient) with another person's healthy (donor) bone marrow through a process called an allogeneic bone marrow transplant. The donor tissue is almost the same or very close to the patient's tissue. First, the patient's bone marrow is disposed by chemotherapy and radiation therapy. Then, the healthy bone marrow is transplanted to the patient through a

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needle in his vein. This represents the first type of bone marrow transplant that shows a positive result in younger people. However, the autologous bone marrow transplant uses the patient's own bone marrow after treated with anticancer drugs that kill all leukemia cells. This second type of bone marrow transplant shows a good result in older people. The bone marrow transplantation is not a good choice for treating patients with CLL as they are in a late stage and may not be in good health (Cherath, 2006).

Materials and Methods

Mice

Spleens of 4-month-old ICR female mice were harvested in the laboratory of Dr. Emily Dudley. Dulbecco's Modified Eagle's Medium (DMEM) (HyClone, Fisher Scientific, Pittsburgh, PA) was used to maintain the fresh harvested spleen samples and transferred to 063 B Medical Sciences laboratory. Using the frosted ends of two microscope slides, spleens were macerated and the harvested cells suspension centrifuged at 1500 rpm for 5 minutes. After centrifugation, cells were re-suspended in 5 ml 1x PBS and used in both beads and flow cytometry procedures.

The Bead Method

The beads method depends on using three types of reagents, which are labeling, blocking, and staining reagent in order to count whether cell expresses CD5+ or CD19+ or both CD5+ and CD19+ membrane markers. The labeling reagent is made up of 1.4 μm carboxylate modified super active latex beads (Life Technologies, Grand Island, NY) coated with either CD5 purified mouse monoclonal antibody (Biolegend, San Diego, CA), or CD19 purified mouse monoclonal antibody (eBiosciense, San Diego, CA). The CD5+ CD19+ (double-positive), labeling reagent consists of two colors of 1.0

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µm colored carboxylate modified super active latex beads. The blue carboxylate modified super active latex beads (Polysciences, Warrington, PA) are coated with CD19 purified mouse monoclonal antibody (eBiosciense, San Diego, CA). The red carboxylate modified super active latex beads (Polysciences, Warrington, PA) are coated with CD5 purified monoclonal antibody (Biolegend, San Diego, CA).

The blocking reagent consists of 0.5 µm in diameter carboxylate modified super active latex beads (Life Technologies, Grand Island, NY). The beads in the blocking reagent coated with CD14 purified mouse monoclonal antibody (Biolegend, San Diego, CA) to decrease all nonspecific protein binding that could interrupt with CD5 or CD19 coated beads. CD14 is lipopolysaccharides receptor on macrophage that could attack CD5+ or CD19+ and cause false positive result. The third reagent, which is the staining reagent, is made up of 2% acetic acid in distilled water and 0.025% crystal violet stain. The staining reagent lyses the RBCs and stain lymphocytes. The antibody-coated latex beads are used to bind to the surface of B lymphocytes expressing either CD5+ or CD19+ or both. Cell-latex beads rosette can be clearly seen by light microscopy (see Figure 2,3, and 5).

Coating Procedure

Antibodies are attached by passive adsorption to carboxylate modified super active latex beads. The materials needed for passive adsorption are:

- Carboxylate modified super active latex beads.
- Wash buffer (phosphate-buffered saline (PBS), 0.1 M, pH 7.2.
- 2- n-morpholino-ethanesulfonic acid (MES) buffer, 0.025 M, pH 6.

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- Storage buffer (phosphate-buffered saline (PBS), 0.1 M, pH 7.2, 0.1% glycine; 0.1% NaN3 sodium azide) ("Passive adsorption protocol," n.d.).

Preparation of 2- N-Morpholino-EthanesulfonicAcid (MES) Buffer

To make 10 ml of MES buffer, 0.025 M, pH 6. 48.8 mg of MES was weighed and added to 5 ml of double distilled water. At this point, the starting pH would be below 4.0. Using the pH meter the pH of the solution is adjusted to 6.0 with 1 N NaOH, transferred to a graduated cylinder or volumetric flask and diluted to a final volume of 10 ml with PBS. MES buffer was used to increase the protein density on the particle surface as the pH 6.0 is close to the isoelectric point of the antibodies used in this study.

Preparation of Storage Buffer

Storage buffer was made up by mixing 100 mg glycine, 100 mg sodium azide and brought to a volume of 10 ml with PBS. To fill any reactive sites on the microsphere that were not covered by the protein used, glycine may be used. Bovine serum albumin (BSA) could also be used to reduce non-specific binding ("Passive adsorption protocol," n.d.).

Determining Antibody and Latex Quantities

The following equation was used to calculate the optimal amount antibody needed for the beads.

Weight of the antibody $= \dfrac{\text{Weight the antibody for the total particle weight}}{\text{Diameter of the partical in } \mu m}$.

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100 mg of 1.4μm latex beads were coated with 2 mg of CD5 or CD19 antibody.

According to the equation, weight of the antibody $= \frac{2 \, mg}{1.4 \, \mu m} = 1.4$ mg of CD5 or CD19

antibody is needed to coat 100 mg of the beads. In a similar manner, 100 mg of 0.5μm

latex beads was coated with 1 mg of CD14 blocking antibody, according to the equation

$= \frac{1 \, mg}{0.5 \, \mu m} = 2$ mg of CD 14 antibody is needed to coat 100 mg of the beads ("Passive

adsorption protocol," n.d.).

Latex Preparation

2.5 ml (40mg/ml) latex microspheres were diluted with 10 ml MES buffer. The

mixture was centrifuged (~3,000g for ~20 min) to sediment the particles. The

supernatant formed was removed and the pellet dispersed in 10 ml MES buffer. The

mixture was centrifuged again and the supernatant formed removed from the particles.

The pellet was suspended in 10 ml MES buffer (10 mg/ml) for labeling beads with

antibodies specific be CD5+ or CD19+ or double positive CD5+ CD19+. For anti-CD

14 coated latex beads (blocking beads) the pellet was suspended in 5 ml MES buffer to

get 2% solids (i.e ~ 20 mg/ml) for blocking beads. The calculated amount of the

antibody was added to ensure the best coating of the particles with the least possibility

of aggregation. The latex/protein mixture was incubated overnight with gentle mixing at

room temperature

The next day, the protein-labeled latex beads were centrifuged to separate

particles from unbound protein. The pellet was suspended in 10 ml PBS and centrifuged

(~3,000g for ~20 min) to sediment the particles. The pellet was suspended again in 10

ml PBS and centrifuged (~3,000g for ~20 min) to sediment the particles twice more for

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a total of 3 washes. The final anti-CD14 latex beads were suspended in 5 ml Storage

Buffer to a final concentration of 2% solids and anti-CD5 or anti-CD19 latex beads in

10 ml storage buffer, giving final concentration of 1% solids. All reagents were stored at

4°C until used without freezing them ("Passive adsorption protocol," n.d.).

Measuring CD5 and CD19 Using Bead Method

For each sample two 12 x 75 mm tubes were labeled as L+B and S (figure 1 A).

100 µl of reagent S added into the test tube labeled S (figure 1 A). 100 µl of spleen cells

suspension placed at the bottom of the test tube labeled B+L (figure 1 B). Reagent B

was mixed and 10 µl of reagent B added to the spleen cells suspension in the L+B tube

(figure 1 C). The test tube was held vertically, mixed gently by hand for 2 minutes

immediately after adding reagent B (figure 1 C). Reagent L was mixed and 10 µl of

reagent L added to B+L test tube (figure 1 D). The test tube held vertical, mixed gently

by hand for 2 minutes immediately after adding reagent L. Any droplets placed around

the top of any test tube would result in non-lyse of red blood cells and erroneous CD5+

or CD9+ lymphocyte counts.

10 µl of the cell suspension latex sphere mixture was added from the B+L test

tube to the S test tube. To lyse the red blood cells in the C test tube, the test tube was

held vertically and mixed gently by hand for 10 to 15 seconds (figure 1 E). Both

chambers of the 0.1 mm deep hemacytometer (or one chamber of the 0.2 mm deep)

were loaded (figure 1 F). The hemacytometer was placed in a moist chamber (a towel

damped with PBS and covered with Petri dish cover) and the cells allowed to settle for 2

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to 3 minutes (the prepared sample is stable in a moisture chamber for 15 minutes) ("Manual CD4 count kit," n.d.).
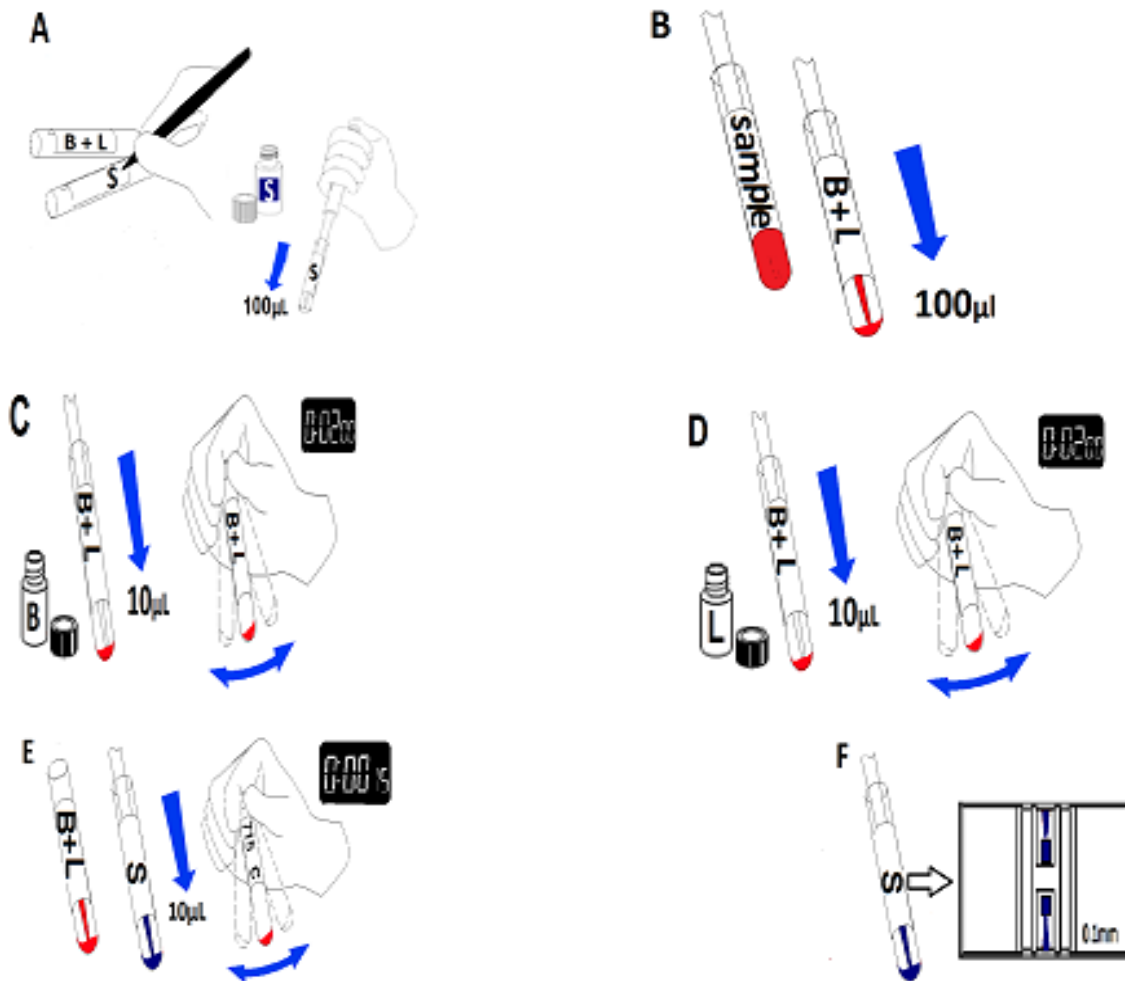

Figure 1. Bead Method Steps. (A-F). Figure illustrates the steps of bead method. The Symbol B + L represents blocking reagent + labeling reagent and the S symbol represents staining reagent. Figure adapted from ("Manual CD4 count kit," n.d.).

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Under the light microscope, the cells that had three or more large latex spheres attached were counted as CD5 + B lymphocytes (figure 2 A, B) or CD19 + B lymphocytes (figure 3A, B). Cells that had three or more bead attachments of both CD5 (red) and CD19 (blue) were counted as double positive CD5+ CD19+ B lymphocytes (see Figure 5).

Figure 2. Beads Method Result Shows Attachment of Three or More Beads.

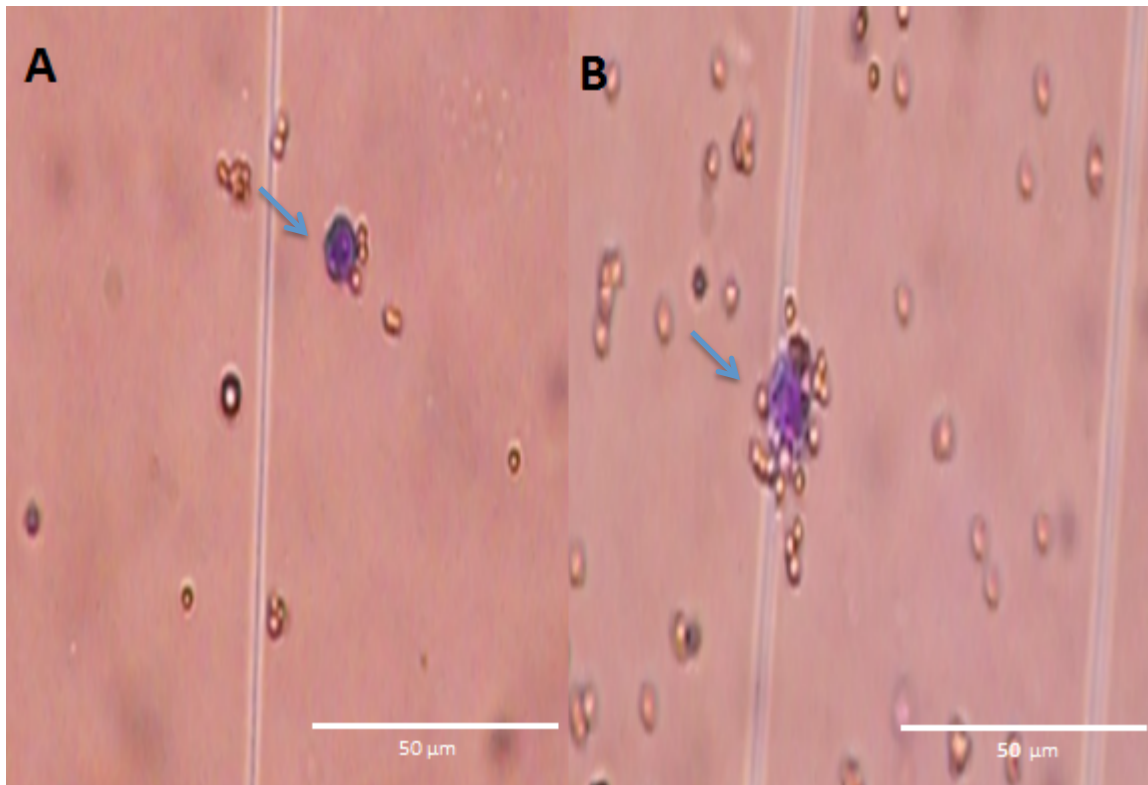Microscopic images demonstrate three beads and more attaching to CD5 B lymphocytes.
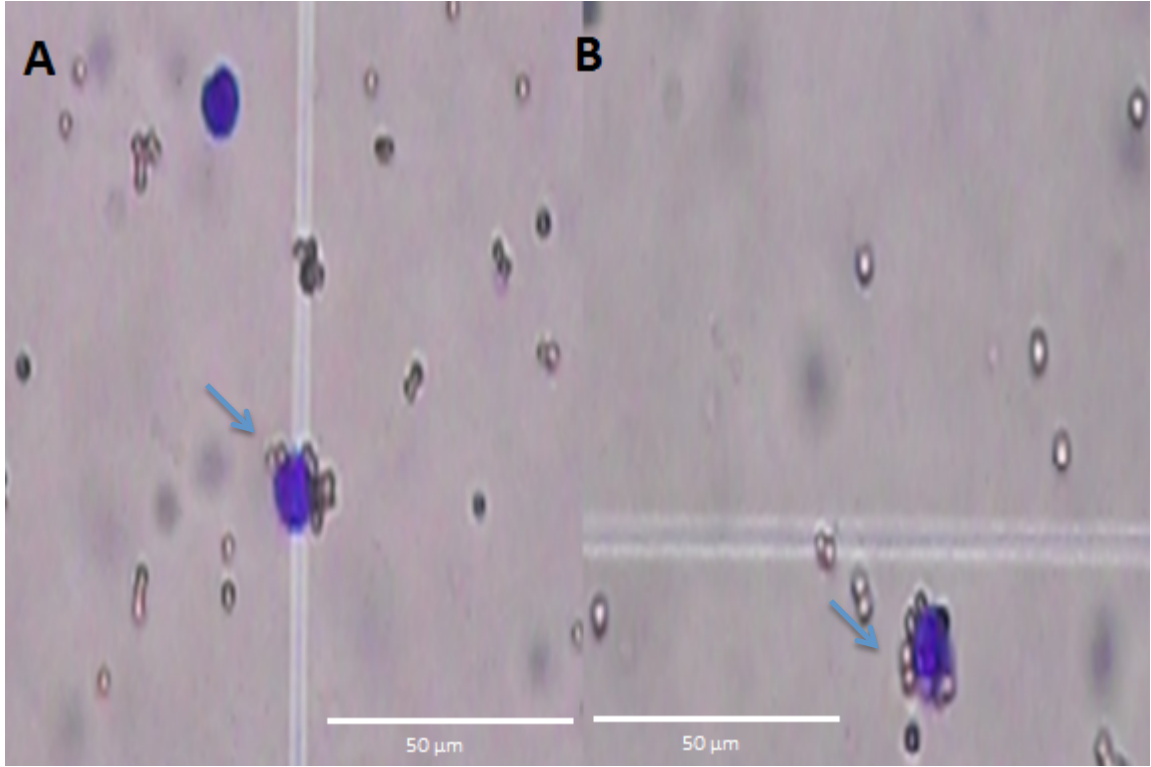
Figure 3. Beads Method Result Shows Attachment of Three or More Beads.

Microscopic images demonstrate three or more beads attaching to CD19 B lymphocytes.

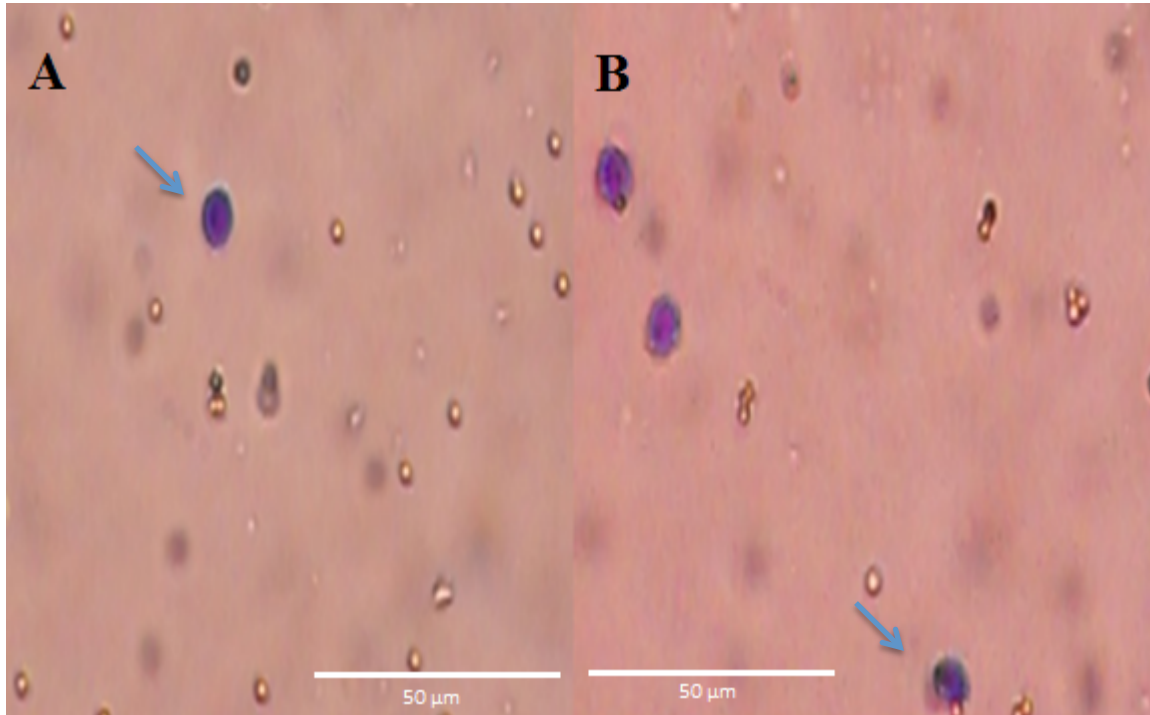**Figure 4**. **Beads Method Result Shows Attachment of One or No Beads.**

Microscopic images demonstrate no beads attached (A) and one bead attached
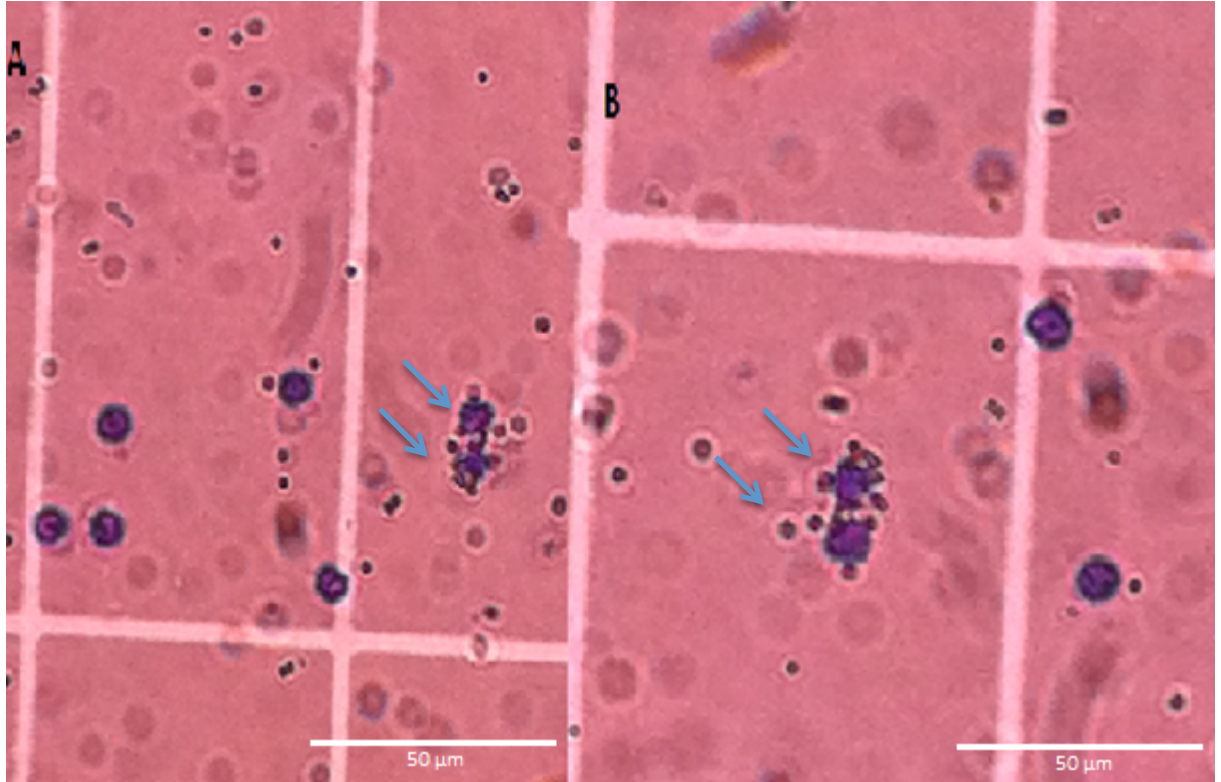
(B).

Figure 5. **Beads Method Result Shows Double Positive CD5+ CD19+ B Lymphocytes.** CD19 blue colored bead and CD5 in red colored bead. Microscopic images demonstrate cells being attached to beads conjugated CD19 (blue) and CD5 (red).

Absolute Count

Lymphocytes with no attached beads (figure 4 A), one bead attached (figure 4 B), or two

bead attachments were excluded from count.

The number of CD5+ or CD19+ lymphocytes or both CD5+ CD19+ lymphocytes/µl =

$$\frac{\text{CD5 + or CD19 + Lymphocyte count} \times \text{Chamber depth correction} \times \text{Sample dilution correction}}{\text{Surface area (mm}^2)}$$

Chamber depth correction $= \frac{1}{\text{Chamber depth}} = \frac{1}{0.1} = 10$ for 0.1 mm deep chamber

Correction for sample Dilution =

Primary dilution:

100 µl whole sample

+10 µl reagent L

+10 µl reagent B = 100/120

Secondary dilution:

10 µ of 100/120 primary dilution in 100 µl reagent C =

$$\frac{10 \text{ µl} \times \left(\frac{100}{120}\right)}{110 \text{ µl}} = \frac{1}{13.2}$$

Therefore the correction for sample dilution =

$$1 \div \frac{1}{13.2} = 13.2$$

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Surface area = 18 mm^2 for two sides of 0.1 mm deep chamber ("Manual CD4 count kit," n.d.)

For example if the CD5 count in both sides of 0.1 mm chamber was 20. The CD5 count could be calculated by applying the formula.

$$\frac{20 \times \text{Chamber depth correction (10)} \times \text{Sample dilution correction(13.2)}}{\text{Surface area (18 mm}^2)}$$

$20 \times 7.3 = 146$ CD5 + B lymphocytes/μl.

Flow Cytometry

Flow cytometry was used to count the number of CD5+ B lymphocytes and CD19+ B lymphocytes after marking them with fluorescent conjugated antibodies. FITC anti-mouse CD5 Antibody and FITC Rat IgG2a, κ Isotype Ctrl Antibody as control (Biolegend, San Diego, CA), were used to count CD5 B lymphocytes. PE/Cy5 anti-mouse CD19 Antibody and PE/Cy5 Rat IgG2a, κ Isotype Ctrl Antibody as control (Biolegend, San Diego, CA), were used to count CD19 B lymphocytes.

The spleen cell suspension was washed three times with 1% BSA (bovine serum albumin) and centrifuged at a speed of 1200 rpm for 5 minutes. After washing, cells were blocked with 3% BSA for 30 minutes incubation at room temperature. After incubation, cells were washed three times again with 1% BSA and centrifuged at a speed of 1200 rpm for 5 minutes. Cells were incubated with fluorochrome conjugated primary antibody specific for either CD5 or CD19 for 15 to 45 min 4°C in the dark 5 μg antibody / ml with 100 μl of 3% BSA for each sample. Then, cells were washed three

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times with 1% BSA and centrifuged at a speed of 1200 rpm for 5 minutes. After the last washing, cells were suspended in ice cold PBS, 10% FCS, 1% sodium azide at 4°C until analysis within 24 hours. Samples were analyzed using an Accuri flow cytometer. The results from flow cytometry were analyzed using the FCS Express program (figure 6-8).
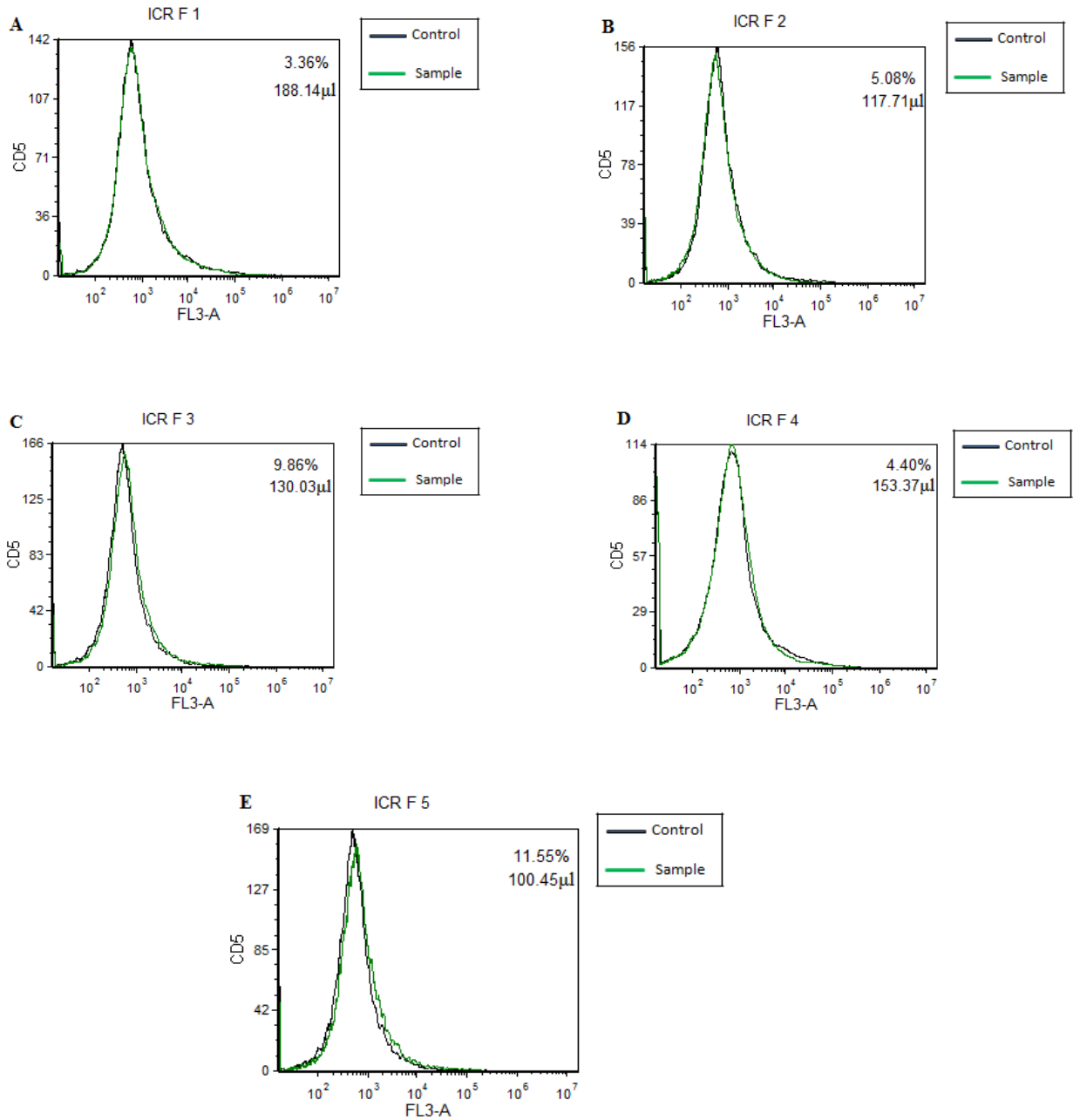
Figure 6. Flow Cytometry Analysis of CD5 Monoclonal Antibody (A-E).

The figure demonstrates flow cytometry analysis of ICR female mouse spleen

cells suspension using CD5 monoclonal antibody at 20,000 events. The number

of CD5+ cells per sample = 20,000/ volume at which 20,000 was detected.
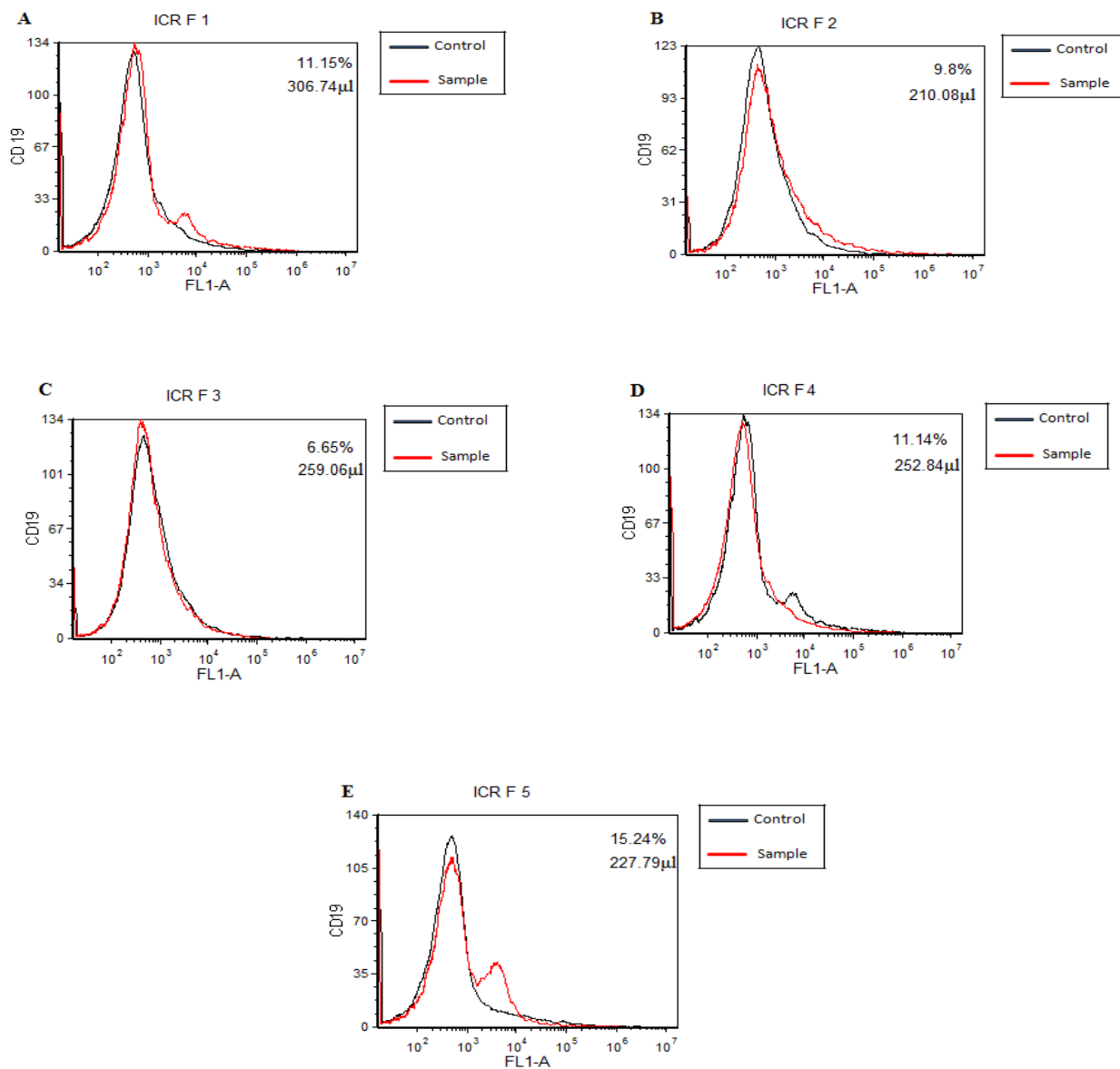
Figure 7. Flow Cytometry Analysis of CD19 Monoclonal Antibody (A-E).

The figure demonstrates flow cytometry analysis of ICR female mouse spleen

cells suspension using CD19 monoclonal antibody at 20,000 events. The

number of CD19+ cells per sample = 20,000/ volume at which 20,000 was

detected.

Figure 8. Flow Cytometry Analysis of CD5+ CD19+Double Positive antibodies (A-E). The figure demonstrates flow cytometry analysis of ICR female mouse spleen cells suspension using CD5+ CD19+ monoclonal antibodies at 20,000 events. The number of CD5+ CD19+ cells per sample = 20,000/ volume at which 20,000 was detected.
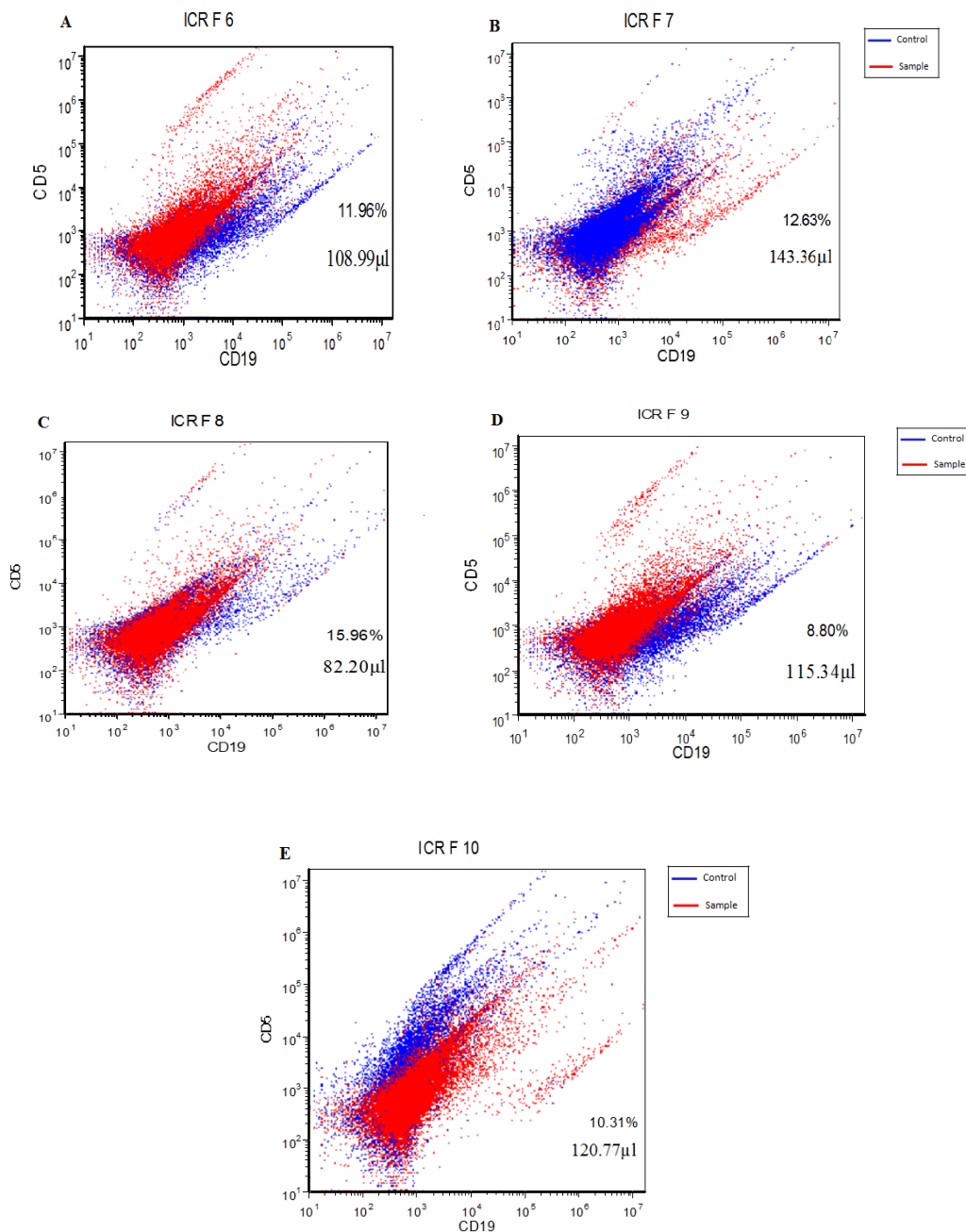
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Results

The numbers of CD5+ B lymphocytes, CD19+ B lymphocytes and CD5+ CD19+ B lymphocytes were measured using both flow cytometry and the bead method in cells from 4-month-old ICR female. The total number of lymphocytes in all spleens of ICR female mice was ~10 million. Although, the bead method results were slightly higher than flow cytometry in counting CD5+, CD19+ B lymphocytes and CD5+ CD19+ B lymphocytes, the difference was not significant. Table 1 shows the total numbers of CD5+ and CD19+ B lymphocytes individual samples from five ICR female mice by using flow cytometry and bead method. Significant differences (t-test) were not seen between the two methods (mean of the flow cytometry CD5+ = 146 ± 15, CD19+ = 251 ± 16 and mean of the bead method CD5+ = 145 ± 15, CD19+ = 257 ± 16; mean ± SEM).

Table 2 shows the total number of CD5+ cells and CD19+ cells per sample, with a ratio of approximately 1:1.7 CD5+: CD19+. The total number of lymphocytes was ~10 million in all samples. The percentage of the total numbers of single CD5+ cells and CD19+ B cells to the total number of lymphocytes is

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shown in Table 3. Again, no significance differences were observed between the two methods.

The numbers of dual labeled CD5+ CD19+ B lymphocytes from five ICR female mice are illustrated in Table 4. The results of bead method (118±9; mean ± SEM) are not significantly different (t-test) from those of flow cytometry (113±9; mean ± SEM).

Table 1.   CD5+ and CD19+ Splenic Cell Numbers Determined by Flow Cytometry and The Bead Method.

Mouse	Flow cytometry		Bead Method	
	CD5	CD19	CD5	CD19
ICR F 1	188/µl	306/µl	197/µl	314/µl
ICR F 2	117/µl	210/µl	124/µl	219/µl
ICR F 3	130/µl	259/µl	138/µl	263/µl
ICR F 4	153/µl	253/µl	160/µl	256/µl
ICR F 5	100/µl	227/µl	109/µl	234/µl

Mean of flow cytometry CD5+ = 146 ± 15, CD19+ = 251 ± 16, bead CD5+ = 145 ± 15, CD19+ = 257 ± 16; mean ± SEM).

Table 2. Number of Single CD5+ and CD19+B Lymphocytes Per Sample.

Mouse	Flow cytometry		Bead Method	
	CD5/Sample	CD19/Sample	CD5/Sample	CD19/Sample
ICR F 1	376000	612000	394000	628000
ICR F 2	234000	420000	248000	438000
ICR F 3	260000	518000	276000	526000
ICR F 4	306000	506000	320000	512000
ICR F 5	200000	454000	218000	468000

Mean of flow cytometry CD5+ = 146 ± 15, CD19+ = 251 ± 16, bead CD5+ =

145 ± 15, CD19+ = 257 ± 16; mean ± SEM).

Table 3. Percentage of Single CD5+ and CD19+ B Cells / Total Spleen.

Mouse	Flow cytometry		Beads Method	
	CD5/ Total lymphocytes	CD19/Total lymphocytes	CD5/ Total lymphocytes	CD19/ Total lymphocytes
ICR F 1	37	61	39	62
ICR F 2	23	42	24	43
ICR F 3	26	51	27	52
ICR F 4	30	50	32	51
ICR F 5	20	45	21	46

Mean of flow cytometry CD5+ = 146 ± 15, CD19+ = 251 ± 16, bead CD5+ = 145 ± 15, CD19+ = 257 ± 16; mean ± SEM).

Table 4. Number of Dual-labeled CD5+ CD19+B Lymphocytes Determined

by Flow Cytometry and The Bead Method.

Mouse	Flow cytometry	Bead Method
	CD5+ CD19+	CD5+ CD19+
ICR F 6	108/µl	116/µl
ICR F 7	143/µl	146/µl
ICR F 8	82/µl	87/µl
ICR F 9	115/µl	117/µl
ICR F 10	120/µl	124/µl

Mean of bead (118±9; mean ± SEM) and flow cytometry (113±9; mean ± SEM).

Discussion

The purpose of this study was to see whether the numbers of CD5+ and CD19+ B lymphocytes in mouse spleen suspensions were similar using flow cytometry and antibody-labeled latex beads for use in laboratories lacking flow cytometry. These antibody-labeled beads would enhance the laboratories ability for diagnosing, staging, and monitoring the treatment responses in CLL patients. This result confirms the findings of a previous study done by Allabidi (2014) in which the bead method could give similar results to those of flow cytometry results for counting CD4+ and CD8+ T lymphocytes. Since malignant B cells in chronic lymphocytic leukemia overexpress both CD5+ and CD19+, this method can be applied in monitoring disease status during therapy (Cabezudo et al., 1997).

The reagents needed to perform bead method are inexpensive and cost approximately $1200 for use with a regular laboratory microscope compared to the high cost of reagents and instrumentation for flow cytometry. The cost of flow cytometry equipment ranges from $30,000 to $150,000 (Matthews, 2006). In this study the bead method required a third of time needed for flow cytometry. The flow cytometry method requires more laboratory experience and expertise than the bead method. The bead

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method relies on the ability of monoclonal antibody-coated latex beads to bind to the surface of B lymphocytes that express either CD5 or CD19 or both. Upon the binding of CD5 or CD19 coated latex beads with the cell that has CD5 or CD19 cell surface antigen, a cell-latex beads rosette is formed which can be recognized by light microscopy.

There were no statistical differences observed using the bead and flow cytometry method for detecting either single labeled or dual-labeled cells in this study. The results of this study indicate that the bead method could be a substitute of flow cytometry in staging and monitoring treatment response in CLL patients. CD19 and CD5 are selective and discriminative markers between leukemic and normal B cells (Warriner, 2011). Significant difference have not been seen in the numbers of single positive CD5or CD19 cells in CLL and non-CLL patient samples (Cabezudo et al., 1999). Flow cytometric detection of dual-labeled CD5+CD19+ cells has been used as a screening tool to detect the early stage of CLL patient (Gupta et al., 2004). The results of bead and flow cytometry method were promising and sufficient to make us move forward to the next step of this research. The next step in our study is using human cells in staging and monitoring the treatment response in CLL patients.

Patient status is monitored after the organ transplant by analyzing the patient's peripheral blood lymphocytes for the number of circulating T cells using CD3 marker. This is done to detect early rejection and toxicity of bone marrow during immunosuppressive treatment. Also, it is important to monitor the effectiveness of anti-rejection therapy (Shanahan, 1997). Future studies can be done in postoperative monitoring after transplantation using the antibody-coated beads.

In future studies, it would be time and cost effective to use the bead method in diagnosing, staging, and monitoring the dosage of treatment given in HIV patients. Acquired immune deficiency syndrome (AIDS) is a disease that continues to constitute a major global threat caused by infection with human immunodeficiency virus (HIV) (Sepkowitz, 2001). About 35.3 million people had HIV all over the world in 2013. As of 2013, the numbers of new infections were 2.1 million. HIV disease is characterized by a massive decrease in circulating CD4+ T cells. In untreated HIV infection, the number of CD4 count decreases while the number of CD8 increases (Berberi & Noujeim, 2015).The ratio of CD4+:CD8+ T cells is used as a marker of disease progression . The usual ratio is 2:1, but in AIDS the ratio is reversed and CD4+ circulating T cells numbers are below 200 cells/µl (Zijenah et al., 2005). Similarly, a study by Allabidi (2014) found that the number of CD4+ T lymphocytes was more than the number of CD8+ T lymphocytes with a ratio of 2:1 CD4+: CD8+ using beads and flow cytometry method.

The two colors of antibody-coated beads could be used instead of flow cytometry in measuring the level of CD5+ CD19+ B cells in primary IgA nephropathy. The CD5+ CD19 + B cells are prominent producers of IgA and liked to contribute to autoimmune diseases. CD5+ CD19 + B cells play important role in the pathogenesis of IgA nephropathy. Patients with IgA nephropathy have elevated levels of dual positiveCD5+ CD19+ B cells. The change in CD5+ CD19 + level is used to monitor the treatment response in IgA nephropathy patients. As levels of dual-labeled cells decline with treatment (Yuling et al., 2008).

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Another disease where the bead method can be used is paroxysmal nocturnal haemoglobinuria (PNH) which is an acquired disease, that leads to intravascular hemolysis with infectious complications. Thus, because the white blood and red blood cells are dysfunctional this makes them more susceptible to lysis. This clonal stem cells disorder can be analyzed using the bead method to identify using CD55 and CD59, regulatory proteins in the complement system cascades leading to red cell lysis. (Richards et al., 2000). The CD55 molecule is the decay accelerating factor for complement and the CD59 glycoprotein also called MAC-inhibitory protein (MAC-IP), membrane inhibitor of reactive cell lysis (Shen et al., 2013).

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