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Comparison Between Flow Cytometry and Bead Method in Counting Cd4 and Cd8 T Lymphocytes in Mouse Spleen Cells Suspension

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COMPARISON BETWEEN FLOW CYTOMETRY AND BEAD METHOD IN COUNTING CD4 AND CD8 T LYMPHOCYTES IN MOUSE SPLEEN CELLS SUSPENSION

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY <u>Abdulrahman Abdalla Allabidi</u> ENTITLED <u>Comparison Between Flow cytometry And Bead Method In Counting CD4 And CD8 T Lymphocytes In Mouse Spleen Cells Suspension.</u> BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF <u>Master of Science.</u>

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ABSTRACT

Abdulrahman Allabidi. M.S. Department of Microbiology and Immunology, Wright State University, 2013. Comparison Between Flow cytometry And Bead Method In Counting CD4 And CD8 T Lymphocytes In Mouse Spleen Cells Suspension.

The flow cytometry is gold standard method to count CD4, CD8 human T

lymphocytes, and calculate CD8 to CD4 ratio in HIV patients peripheral blood to

stage them and monitor their response to treatment. In this study, a comparison was

made by counting CD4and CD8 mouse spleen T lymphocytes by flow cytometry, and a

method involving antibody coated latex beads. The bead method yielded results

comparable to those obtained by flow cytometry. These results indicate that antibody

coated beads are suitable to determine CD4 and CD8 + T lymphocytes counts in situation

where flow cytometry is not readily available.

HYPOTHESIS

The hypothesis of this study was that the bead method using antibody coated latex beads would give results comparable to those of flow cytometry.

The null hypothesis was that the bead method would not give results comparable to these of flow cytometry.

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LIST OF ABBREVIATIONS

HIV = Human Immunodeficiency Virus.

gp = Glycoprotein.

LTR = Long terminal repeat.

cDNA = Complementary DNA.

PrEP = Pre-exposure prophylaxis.

PEP = Post-exposure prophylaxis.

CD = Cluster of Differentiation.

NNRTI = Non-nucleoside reverse transcriptase inhibitor.

NRTIs = Nucleoside analogue reverse transcriptase inhibitors.

ACKNOWLEDGEMENT

Introduction

The human immunodeficiency virus (HIV) is a member of the genus Lentivirus, which is part of the family Retroviridae. Lentiviruses have many shared morphologies and biological properties, and are responsible for illnesses of a long incubation period (Levy, 1993). Lentiviruses are single-stranded, positive-sense, enveloped RNA viruses. Lentiviruses use reverse transcriptase enzyme to replicate their genome. HIV is structurally different from other retroviruses in that it is roughly spherical with a diameter of about 120 nm which is around 60 times smaller than a red blood cell (McGovern et al., 2002). HIV contains two copies of positive single-stranded RNA that codes for the virus's nine genes. Enclosing the virus genome a conical shaped capsid which is composed of 2,000 copies of the viral protein p24 (Kuiken et al., 2008). The single-stranded RNA is strongly attached to nucleocapsid proteins, p7, and enzymes needed for the synthesis of the virion. These enzymes are reverse transcriptase, proteases, ribonuclease and integrase. The virion particle integrity is maintained by a matrix composed of the viral protein p 17 surrounding the capsid. Two layers of phospholipids form the viral envelop. These two layers are taken from the membrane of a human cell when a newly formed virus particle buds from the cell. Rooted in the viral envelope proteins from the host cell as well as 70 copies of a complex HIV protein that emerge through the surface of the virus particle (Kuiken et al., 2008). This Env protein consists of a cap made of three glycoprotein (gp 120) molecules, and a stalk consisting of three gp 41 molecules that anchor the structure in to the viral envelope (Chan et al., 1997). Attachment to and fusion with target cells is

the function of this glycoprotein complex (Chan et al., 1997).Both of these surface

proteins have been considered as targets of future treatments or vaccines against HIV

with more concentration on gp 120 (Wyman, 1998). HIV 1 and HIV 2 are the two strains

of HIV. HIV-1 is the virus that was initially discovered and termed both

lymphadenopathy-associated virus LAV and human T-lymphotrophic virus- III HTLV-

III. HIV1 is more virulent, and infectious than HIV 2 (Gilbert et al., 2003), making

HIV 1 the main cause of the HIV infections around the world. HIV 2 is confined to West

Africa because of its relatively poor capacity for transmission (Reeves et al., 2002).

Literature Review And Preliminary Data.

HIV Genome

The HIV genome consists of seven structural landmarks and nine genes. The

seven structural landmarks are LTR, TAR, RRE, PE, SLIP, CRS, and INS. The nine

genes are gag, pol, env, tat, rev, nef, vif, vpr, vpu. In some cases a tenth tev gene a fusion

of tat env and rev can be found. The nine or ten genes encode for 19 proteins. Function

of some HIV gene and proteins are not well known, however the function of some others

is well known. For example, the structural proteins for new virus particles need the

genetic information in the genes gag, pol, and env (Kuiken et al., 2008). The six

remaining genes, tat, rev, nef, vif, vpr, and vpu (or vpx in the case of HIV-2), regulate the

proteins that control HIV's ability to infect cells, produce new copies of virus, or cause

disease (Kuiken et al., 2008). HIV uses the Vpr protein (p14) to arrest cell division at

G2/M. CD4 (the main HIV receptor), as well as the MHC class I and class II molecules are down-regulated by Nef protein (p27) (Garcia et al., 1991; Stumptner-Cuvelette et al., 2001). The release of new virus particles from infected cells is controlled by Vpu protein (p16). The RNA sequence known as the long terminal repeat (LTR) is located at the ends of each strand of HIV RNA, and contains certain regions that control the production of new viruses and can be activated by viral or host cell proteins (Kuiken et al., 2008).

Tropism

CD4+ T cells, macrophages, and microglial cells are the main targets for HIV-1. This viral tropism makes HIV target the backbone of human immune system. The virion envelope glycoprotein (gp120) attaches to the CD4 molecule and chemokine coreceptors on CD 4 cells. This interaction will allow HIV-1 to start the cell entry process. Gp 41, another glycoprotein on the viron envelope binds to the chemokine receptors and promotes viral and cellular membrane fusion and delivery of viral contents to the host cell (Chan et al., 1997). Non-syncitia-inducing strains of HIV1 (R5 viruses) use the β-chemokine receptor CCR5 for entry to CD4+ T cells and macrophages. Syncitia-inducing strain of HIV1 (X4 viruses) uses the α-chemokine receptor, CXCR4, for entry in to macrophages and CD4+ T cells (Berger et al., 1998). Macrophages are the first cells infected by HIV and the source of HIV production when CD4+ cells severely decrease in number. Microglial cells act as a reservoir of HIV in the central nervous system.

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Macrophages fuse into multinucleated giant cells that produce large number of virus in tonsils and adenoids of HIV-infected patients (Rappaport, 2014). People with CCR5-Δ32 mutation are resistant to infection with R5 virus, because this mutation will prevent the R5 viruses from attaching to CCR5 co receptor, decreasing its ability to infect target cells (Tanga & Kaslowa, 2003). Seminal fluid carries both X4 and R5 HIV variants, making sexual intercourse one of the main routes of HIV transmission. After infection, the virions will target immune cells CD4 and macrophages and disseminate throughout the body. By a selection process the R5 virus becomes the predominant strain to be transmitted by this pathway (Zhu et al., 1996). R5 HIV is selectively carried by spermatozoas as they possess both CCR3 and CCR5 but not CXCR4 on their surface. Furthermore, the genital epithelial cells also sequester the X4 virus (Muciaccia et al., 2005). At the late stage of HIV infection, there is usually a co-receptor switch In subtype B HIV-1 infected patients. This switch will lead to appearance of T-tropic variants that can infect a variety of T cells through CXCR4. These variants have a high replication rate and are more virulent, causing a tremendous decrease in CD4 cell count , immune system failure, and fulminant opportunistic infections (Clevestig et al., 2005). Another way in which HIV infection progresses to AIDS is adaptation of the virus to use CXCR4 instead of CCR5 (Moore, 1997).

HIV Replication Cycle

Replication of HIV starts with cell entry and ends with release of virions from the infected cells. Adsorption of glycoproteins on HIV surface to receptors on the target cells is the starting point of the entry process. The adsorption process starts with the interaction of the trimeric envelope complex gp160 spike (gp 120 and gp 41) with CD4 and a chemokine receptor (CCR5 or CXCR4) on the cell surface (figure 1). The next step in the entry process is fusion of the viral envelope with the cell membrane and the release of the HIV capsid content into the cell. When gp120 is bound to CD4 protein, structural changes in the viral envelope complex lead to exposure of the chemokine binding domains of gp120, allowing them to interact with the target chemokine receptor. Gp120 and CD4 interaction will lead to stabilization of the attachment between the virus and the target cell. This reinforcement of the attachment will allows the N-terminal fusion peptide of gp41 to penetrate the cell membrane. Collapse of the extracellular portion of gp41 into a hairpin occurs when the repeated sequences in gp41, HR1, and HR2 interact. This loop structure brings HIV and the target cell close together, allowing fusion of the viral envelope with the target cell membrane and entry of the viral capsid (Chan, & Kim 1998).

Fusion of HIV with the target cell will deliver the HIV RNA and various enzymes, including reverse transcriptase, protease, integrase, and ribonuclease, into the

target cell (figure 1). The viral single-strand RNA genome will be transcribed into

double-strand DNA by reverse transcriptase. The transcription occurs during the

microtubule-facilitated transport to the nucleus. The viral double-strand DNA
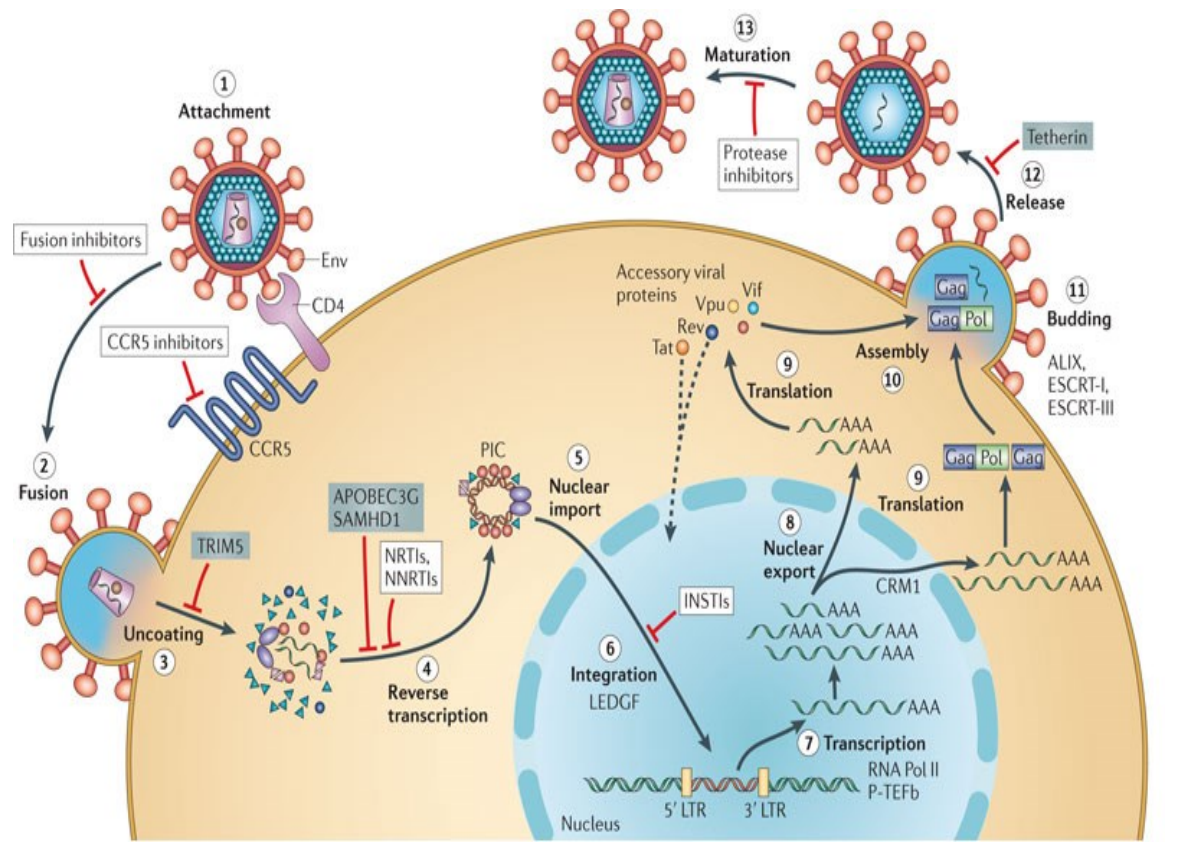
Figure 1. HIV Replication Cycle And The Site Of Antiretroviral Drug Action. Figure

adapted from (Engelman, & Cherepanov, 2012).

now is ready to be integrated into a target cell chromosome (Chan, & Kim 1998). The process of reverse transcription lacks proof reading leading to a high number of mutations. These mutations account for the development of drug resistance and help the virus to evade the immune system.

The ribonuclease activity of reverse transcriptase allows it to degrade the viral RNA during the synthesis of complementary DNA (cDNA). Furthermore, reverse transcriptase has a DNA-dependent DNA polymerase activity that produces a sense DNA from the antisense cDNA. The sense DNA and its complement form a double-stranded viral DNA. The viral double-stranded DNA is then transported into the target cell nucleus. The integration of the viral DNA into the target cell's genome is executed by the viral integrase enzyme (Zheng et al., 2005). After integration, the integrated viral DNA may then lie dormant. The dormant viral DNA is the main cause of the latent HIV infection stage. Certain cellular transcription factors especially NF-κB need to be present to actively produce the virus. Intracellular levels of NF-κB increase when the T-cells are in active status (Zheng et al., 2005). This supports the concept that T- cells that most likely undergoing HIV replication are those currently involved in fighting infection (Hiscott et al., 2001).

Viral replication starts when the integrated DNA is transcribed into mRNA. The mRNA is then spliced into smaller pieces and exported from the nucleus into the cytoplasm. Regulatory proteins Tat (which induces new virus production) and Rev are produced by translation of the mRNA in the cytoplasm. Accumulation of the newly formed Rev protein in the nucleus makes Rev protein binds to viral mRNAs and helps unspliced RNAs to leave the nucleus (Zheng et al., 2005). The full-length mRNA is the coding sequence for the structural proteins Gag and Env. Moreover, the full-length RNA is the HIV genome which will binds to the Gag protein to produce new virus particles after packaging. HIV1 is more dominant then HIV2 possibly because of the difference in the packaging process. HIV-2 will specifically bind to the mRNA that was used to create the Gag protein itself. On the other hand, HIV-1 will bind to any suitable RNA. This manner of HIV interaction with RNA indicates that HIV-1 is more mutable than HIV-2 and for this reason HIV-1 is the most common HIV strain worldwide (Pollard, & Malim 1998).

In the final stage of the of HIV replication, assembly of new HIV-1 virions starts at the plasma membrane of the infected cell. Gp160 will be processed by endoplasmic reticulum and Golgi complex. Furin will cleave the gp 160 in to HIV envelope glycoproteins gp41 and gp120 at Golgi complex. Gp41 and gp120 will be transported to the plasma membrane of the infected cell where gp41 anchors gp120 to the membrane. The inner surface of the infected cell plasma membrane is the attachment site for Gag

(p55) and Gag-Pol (p160) along with the HIV genomic RNA. Subsequent to these attachments the virion begins to bud from the infected cell. The mature virus will be formed after cleavage of the gag polyproteins into capsid, nucleocapsid proteins, and the actual matrix. Viral protease is the main mediator of this cleavage process , also the target for antiretroviral drugs of the protease inhibitor class (Figure 1). Only mature viruses are able to infect another cell (Hallenberger et al., 2001). High genetic variability is one crucial characteristic of HIV. This resulted from its fast replication rate, with approximate formation of 1010 virions every day, its high h mutation rate of approximately 3 x 10^{-5} per nucleotide base per cycle of replication , and its reverse transcriptase recombination ability. This high genetic variability can lead to the production of large number of HIV variants in a single infected person during one day. Increase of this variability occurs when a single cell is infected by two or more different strains of HIV in the same time (Robertson et al., 1995).

Based on the differences in the envelope region, HIV-1 can be characterized into three groups M, N, and O. Group M can be subdivided into eight subtypes, and is the most common world wild (Jean K et al., 1998). Subtype B is the dominant form in Europe and North America. Subtypes A and D are found mainly in Africa, while subtype C is found mainly in Asia and Africa. Subtype B is the focus of most laboratories. Few laboratories focus on the other subtypes (Osmanov et al., 2002).

Cluster Designation Marker CD4 And CD8

CD4 (cluster of designation 4) is a glycoprotein found on the surface of immune cells, especially T helper cells. CD4+ T helper cells are white blood cells that are an essential part of the human immune system. They are called helper cells because one of their main roles is to organize the action of other cells in the immune system. The role that CD4+ T helper cells play make them the main core of the immune system and depletion of these cell leads to severe failure in the immune system like in HIV and DiGeorge syndrome. CD4+ T helper cells use T cell receptor to communicate with MHC class II molecules on the surface of the antigen-presenting cell. CD4 is a co-receptor that assists the T cell receptor in the process of communication. The intracellular domain of CD4 amplifies the signal generated by the T cell receptor by recruiting tyrosine kinase Lck enzyme. This enzyme will active other enzymes and molecular components of the signaling cascade inside activated T cells.

CD8 (cluster of designation 8) is a glycoprotein found on the surface of immune cells especially cytotoxic T cells. Recognizing and eliminating cancer cells, and virus infected cells are the two main functions of CD8 + T cells. By recognizing any changes in the MCH Class I molecules, the extracellular domain of CD8 receptor will attach to MCH Class I molecules and if any change is recognized by this receptor the cytotoxic T cell will get activated and eliminate the target cell.

Statistics And Epidemiology

Of the estimated 35.3 million people with HIV/AIDS worldwide 9.7 million

people receive high active antiretroviral therapy (HAART). The number of infected

Females is 16.8 million of the infected people and 3.4 million are less than 15 years old

(UNAIDS, 2012). The number of new infections at 2012 is 2.3 million. Compared with

3.1 million new infections recorded at 2001. The number of death from AIDS in 2012 is

about 1.6 million which has been reduced if compared to 2.2 million in 2005 (UNAIDS,

2011). In 2010, about 68% (22.9 million) of all HIV cases and 66% of all deaths (1.2

million) were recorded in Sub-Saharan Africa. Making Sub-Saharan Africa the region

most affected with this global epidemic. AIDS causes 10% of all deaths in children in

Sub-Saharan Africa. The largest population of people with HIV 5.9 million is in South

Africa. Life expectancy in countries with high HIV rate has fallen from 65 to 35 years

like in Botswana (UNAIDS, 2011).

Transmissibility Of HIV

The three main routes of HIV transmission are sexual contact, exposure to

infected body fluids or tissues, and vertical transmission from mother to child during

pregnancy, delivery, or breastfeeding. Sexual contact is considered to be the most

frequent mode of HIV transmission where heterosexual contact represents the majority of

all transmissions worldwide. In the United States male homosexual population accounts

for 64% of all new cases, making homosexual contact the most common cause of HIV transmission in USA (Rom & Markowitz, 2007). Anal intercourse has a high risk of transmission (1.4-1.7 % per act) compared with vaginal intercourse 0.38% per act (Boily et al., 2009). The risk of transmission from oral sex is relatively low 0–0.04% per act (Pattman et al., 2010). The risk of female-to-male transmission in settings involving prostitution in low income countries has been estimated as 2.4% per act and male-to-female transmission as 0.05% per act (Boily et al., 2009).

Sexually transmitted disease with genital ulcers increases the risk of HIV transmission to approximately five-fold. Smaller increase in the risk of HIV transmission is associated with other sexually transmitted disease, and by the parenteral route which is between 0.63 and 2.4% per act (Rom & Markowitz, 2007). 0.3 % per act is the risk of contracting HIV from a needle stick from an HIV-infected person. Furthermore, mucous membrane exposure to infected blood has the risk of 0.09% or 1 in 1000 per act (Baggaley et al., 2006). 8% of all new cases of HIV in the United States are from IV drug abusers in 2010 (CDC, 2012). The risk of contracting HIV from infected blood is about 93%. Because of HIV screening for the blood donor and donated blood, the risk of HIV transmission from blood transfusion is extremely low in developed countries(Baggaley et al., 2006). On the other hand, only half of transfusions may be appropriately screened in low income countries. Because of this, up to 15% of HIV infections in these areas happen due to transfusion of infected blood and blood products (UNAIDS, 2011).

According to the CDC, tattoos, and piercings carry theoretical risk of transmitting HIV, yet no confirmed cases have been documented (CDC, 2012). Mosquitoes and other blood sucking insects cannot transmit HIV because it cannot survive the digestive systems of these insects (Crans, 2010).

Pregnancy, delivery, and breastfeeding are the three possible routes of HIV transmission from mother to child. Without treatment of the infected mother, the risk of HIV transmission before or during delivery is about 20% and with breastfeeding the risk will increase to 35% (Coustsoudis et al., 2010). 90% of HIV cases in children are caused by vertical transmission. The high risk of vertical transmission can be reduced to about 1 % with appropriate management and treatment. For infected mother taking antiretroviral during pregnancy and delivery, doing elective caesarean delivery, avoiding breastfeeding, and administering antiretroviral drugs to the newborn are considered adequate to reduce the risk of vertical transmission (Coustsoudis et al., 2010). Exposure to feces, nasal secretions, saliva, sputum, sweat, tears, urine, or vomit carry no risk of transmitting HIV unless these materials are contaminated with blood (Kripke, 2007).

HIV Signs And symptoms.

Acute infection, latency, and AIDS are the three stages of HIV infection. Acute infection is the first stage after incubation period. The duration of acute infection or primary HIV infection is several weeks. Acute HIV infection or acute seroconversion

syndrome symptoms are similar to the influenza or infectious mononucleosis symptoms. These symptoms include sore throat, fever, lymphadenopathy, headache, malaise, rash, and myalgia. Infected individuals can be asymptomatic (Kahn & Walker, 1998). Nonspecific symptoms of acute HIV make it very difficult to diagnose the acute stage. Yet some points in the patients' history like unprotected sex and multiple sexual partners may provide a strong clue. Recognizing HIV infection during acute stage is very important because the patient is highly infectious during this period (Darr et al., 2001).

According to the virus activity and host immunity, the latency stage takes from seven to ten years (Colledge et al., 2010) During this phase the host immunity will reduce the number of viral particles in the circulation, and the host will be symptom free. However, HIV is still active within lymph nodes leading to persisting swelling of the involved lymph nodes because of large amounts of virus will become trapped in the follicular dendritic cells (Burton al,. 2002).

AIDS

AIDS is the final stage of HIV infection. It is defined by low CD4+ T cell counts (less than 200 per microliter), various opportunistic infections, cancers and other conditions. Opportunistic infections symptoms are the main symptoms of AIDS. Most opportunistic infections are caused by bacteria, viruses, fungi and parasites that are normally eliminated by cellular immunity, especially CD4 + T lymphocytes. Beside

opportunistic infections, patients with AIDS have a high risk of developing certain kinds of cancers like Kaposi's sarcoma, cancers of the immune system   like lymphoma, and cervical cancer (Holmes et al., 2003).

AIDS Pathophysiology

A massive increase in virus numbers in the peripheral blood occurs after a period of rapid viral replication. This increase happens immediately after virus entrance to the human body. During primary infection, the level of HIV may reach several million virus copies per milliliter of blood (Piatak et al., 1993). In the same time, CD4 counts will decrease and CD8 + T cells will be activated due to acute viremia. After seroconversion the activated CD8 + T cells will attack and kill HIV-infected cells with help of antibodies. Killing of HIV infected cells by CD8 + T cells shows the importance of CD8+ T cell in controlling virus levels and restoring the CD4+ T cell counts. Many patients with a good CD8+ T cell response demonstrate slower disease progression and a better prognosis (Pantaleo et al., 1997). Depletion of CD4 T cells is the main cause of AIDS. There is a difference between the mechanism of CD4 T cell depletion in the acute stage and chronic stage. In the acute phase, HIV causes CD4 cells depletion by cell lysis and killing of infected T helper cells by cytotoxic T cells (Hel et al., 2006). On the other hand, the generalized immune activation and gradual loss of the ability of the immune system to generate new T cells are the two main reasons for CD4 T lymphocytes depletion during the chronic phase (Zuckerman et al., 2007).

Prevention

Near 80% risk reduction of HIV transmission can be achieved if condom s are used regularly. The risk of HIV infection is less than 1% per year if a couple ,of which one is infected, used condoms consistently (Crosby & Bounse, 2012).Tenofovir vaginal gel application immediately before intercourse shows 40% infection risk reduction among African women (Celum & Baeten, 2012). On the other hand, regular use of the spermicide nonoxynol-9 is associated with increased risk of transmission due to its tendency to cause vaginal and rectal irritation (Baptista & Ramalho-Santos, 2009). By circumcision only, the risk of HIV transmission can reduced in heterosexual men by between 38% and 66% over 24 months (Siegfried et al., 2009). Based on this study and others, recommendations were made by the World Health Organization and The Joint United Nations Programme on HIV and AIDS to consider male circumcision as a method of preventing female-to-male HIV transmission in 2007. Decrease in high risk behaviors was noticed after implementation of comprehensive sexual education at school (Ljubojević & Lipozenčić, 2010). Counseling and free HIV test do not change high risk behaviors in those who test negative, however it does increase condom use in those who test positive (Fonner et al., 2012).

Pre-exposure prophylaxis (PrEP) can be defined as administration of antiretroviral drugs in high risk people where studies have shown it to be beneficial. Starting

antiretroviral treatment in patients with HIV whose CD4 count $\geq$ 350cells/µL protects

96% of their partners from HIV (Anglemyer et al., 2011) Daily use of Tenofovir, with

or without Emtricitabine decreased risk of HIV transmission in heterosexual male, young

heterosexuals in Africa, and heterosexual couples where one is HIV positive (Celum &

Baeten, 2012). Following recommended precautions within the health care facilities is

effective in decreasing the risk of HIV transmission ("Recommendations for prevention

of HIV transmission in health-care settings," 1987). Needle-exchange programs and

opioid substitution therapy are two ways to decrease risk of HIV infection among

intravenous drug abusers (MacArthur et al., 2012).

Post-exposure prophylaxis (PEP) is a course of antiretrovirals administered within

48 to 72 hours after exposure to HIV-positive blood or genital secretions. Following a

needle-stick injury, administration of zidovudine reduces the risk of a HIV infection

five-fold. In contrast, the use of tenofovir, emtricitabine, and raltegravir as PEP will

reduce the risk more than five-fold (Kuhar et al., 2013). In case of sexual assault, PEP

treatment is recommended for four weeks to reduce the risk of HIV transmission (Young

et al., 2007). Measures recommended to reduce the risk of HIV vertical transmission

from mothers to children include the use of a combination of antiviral drugs during

pregnancy and after birth, bottle feeding instead of breastfeeding, and cesarean section.

These measures can reduce rates of transmission by 92–99 % (Coutsoudis et al., 2010).

Super Active Latex Beads.

Super active latex beads are hydrophilic and contain a very high density of functional groups for covalent coupling of proteins to the particles. The super active layer is a three-dimensional layer which increases the colloid stability of the particles and provides a 'soft landing' for the proteins. There is less distortion of the protein structure than if it were physically adsorbed to a rigid surface. There are 5 types of the super active latex beads Carboxylate Modified beads (CML) , Chloromethyl beads, Aldehyde/Sulfate beads, Aldehyde/Amidine beads, and Aliphatic Amine. In this study, we used carboxylate modified beads. CML beads are produced by copolymerizing carboxylic acid containing polymers. The result is a latex polymer particle with a highly charged, relatively hydrophilic surface , and pka of 5-9. These characteristics will help the CML to be used in plasma, and in aqueous environment with pH range from 5-9 with less tendency of aggregation (Super Active Latex Beads," n.d.) .

Bead Method

The main objective of the bead method is to coat inert latex spheres with CD4 or CD8 mouse monoclonal antibody, and use these beads to identify and manually enumerate by light microscopy the absolute count of CD4+ or CD8+ lymphocytes in a

fresh sample of mouse spleen cells. The lymphocyte population of mouse spleen is composed of different cell types, including CD4 T lymphocytes and CD8 T lymphocytes. These cells types are morphologically indistinguishable by light microscopy but can be differentiated by characteristic antigenic differences in their cell membranes. The main purpose of developing monoclonal antibodies is to identify and enumerate T and B lymphocytes (Foon KA and Todd RF 1986). In comparison to the relatively nonspecific polyclonal antibodies produced against these cellular populations, monoclonal antibodies can more specifically identify T cell and B-cell surface antigens. The main idea of the beads method is to coat inert latex spheres with CD4 or CD8 mouse monoclonal antibody, and use these beads to identify and manually enumerate by light microscopy the absolute count of CD4+ or CD8+ lymphocytes in fresh sample, of mouse spleen cells suspensions.

Diagnosis

Because AIDS has nonspecific symptoms, many HIV-positive people are unaware that they are infected with the virus. In 2001, HIV testing was performed on less than 1% of the sexually active urban population in Africa. Similarly, in urban women health care clinic only 0.5% of pregnant women were counselled, tested or provided with their test results (Kumaranayake & Watts, 2001). The screening test for HIV is enzyme-linked immunosorbent assay (ELISA). The screening test detects antibodies to HIV. Patients with nonreactive results from ELISA are considered HIV-negative. A patient

with a reactive ELISA result is retested in duplicate . If the result of either duplicate test is reactive, the patient is reported as repeatedly reactive and undergoes confirmatory testing. Western blot is the confirmatory test for HIV. Patients who are repeatedly reactive by ELISA and positive Western blot are considered HIV-positive indicating HIV infection. Patients with repeatedly reactive ELISA and an indeterminate Western blot result are considered to show either an incomplete antibody response to HIV or nonspecific reactions in an uninfected person. In case of indeterminate test result Western blot test should be repeated after one month (CDC, 2006). According to the CDC HIV can be classified in to three stages according to CD4 count and clinical symptoms.

Stage 1: CD4 count $\geq$ 500 cells/μl and no AIDS defining conditions.

Stage 2: CD4 count 200 to 500 cells/μl and no AIDS defining conditions.

Stage 3: CD4 count $\leq$ 200 cells/μl or AIDS defining conditions(CDC, 2008).

The ratio of CD4 to CD8 gives an idea about the impact of HIV on the Immune system. The normal ratio of CD4/CD8 is 2 to 1. In AIDS patients the CD4/CD8 ratio is reversed because of the decrease in the CD4 counts. However, with anti-retroviral treatment, this ratio will return to normal 2 to 1. The main use of CD4/CD8 ratio is to monitor the response of HIV patients to antiretroviral treatment. CD4/CD8 ratio is also used in the diagnosis of HIV infection in infants under the age of 18 months. The CD4/CD8 ratio test is 98.3% specific and $\geq$ 98.7% sensitive for identifying infant HIV-1 infection (all subtype C) (Zijenah et al., 2006).

Another test used to monitor HIV infection is viral load in patients' serum. Viral load can be calculated by estimating the virus RNA in the plasma in the form of RNA copies per milliliter of plasma. The relationship between the viral load and CD4 + T lymphocytes is inverse relationship. Knowing the absolute CD 4 + T lymphocytes will indicate the viral load level if it is high or low. Viral load is considered a very important prognostic marker of disease progression and provide valuable information about patients response for the therapeutic strategy. On the other hand , CD4 + T lymphocytes counts provide important clues about when to initiate treatment with antiretroviral therapy ,and the time to start opportunistic infections prophylaxis (Saag et al., 1996). For these reasons we chose CD4 + and CD8 + T lymphocytes as a markers to stage and monitor HIV patients in our study. For sure using CD4 + T lymphocytes and Viral load will give a more holistic view about the patients conditions , even though our study where directed toward a regions with limited resources and laboratory ability.

Management

Currently, there is no cure or effective HIV vaccine. The main treatment for HIV consists of a mixture of antiretroviral drugs HAART. The medication regime does not eliminate HIV, yet it slows the progression of the disease to its final stages (May & Ingle et al., 2011). HAART is a combination of at least three medications belonging to two

different classes of antiretroviral agents. The initial regimen consists of a non-nucleoside

reverse transcriptase inhibitor (NNRTI) plus two nucleoside analogue reverse

transcriptase inhibitors (NRTIs) (Colledge et al., 2010). The most common used NRTIs

include zidovudine (AZT) or lamivudine and tenofovir (TDF). If the above regimen is not

effective in controlling the disease, combinations of agents which include protease

inhibitors (PI) are used (Colledge et al., 2010). WHO recommends starting antiretrovirals

in all adolescents, adults and pregnant women with a CD4 count less than 500/µl.

However, treatment can be started if patients have symptoms regardless of CD4 count.

Beginning treatment when CD4 counts are less than 500/µl reduces the risk of death

(Siegfried et al., 2010).

Once treatment is begun it should be continued without breaking. The main goal

of treatment is to maintain plasma HIV-RNA count below 5000 copies/mL. If treatment

is effective, levels fall below 5000 copies/ ml after four weeks. Once 5000 copies/mL

viral load is reached, viral load should be measured every three to four months. More

than 5000 copies/ml viral load is considered inadequate control of HIV activity (Saag, M

S, 1996). During the first year of treatment, HAART is effective in more than 95% of

patients (Vogel et al., 2010). HAART helps in decreasing the risk of HIV transmission to

sexual partners and from mother to child, progression to AIDS, and a decreased risk of

death due to HIV (Sterne et al., 2009). The risk of acquiring tuberculosis is reduced by

70% with treatment. The most common side effects of protease inhibitors are

lipodystrophy syndrome, dyslipidemia, and diabetes mellitus (Fantry, 2003). Nucleoside

and non-nucleoside reverse transcriptase inhibitors can cause different side effects like

fatigue, myalgia, proximal weakness, dilated cardiomyopathy, and peripheral lipoatrophy

(Carr & Cooper, 2000). Efavirenz should be avoided in the first trimester of pregnancy

because of reported cases of fetal neural tube defects (Vogel et al., 2010).All this

spectrum of sides effects make it necessary to give antiretroviral drugs for the patients

who need them. To decide if a patient needs to be treated or not, physician must put the

patients in the correct HIV stage. Staging HIV patients will make them avoid unnecessary

exposure to the possible side effects of the antiretroviral therapy, and provide good

distribution of the antiretroviral therapy in resources limited regions.

In a situation where there is no access to flow cytometry it is impossible to stage

HIV patients. Flow cytometry requires a reliable power supply, the cost of a flow

cytometry ranges from $30,000 to $150,000, and the reagents needed for determining the

lymphocyte surface markers by this method are very costly. In addition, use of flow

cytometry requires technical and operational expertise. Taking into consideration all

these factors, it is clear that flow cytometry is not always available in developing world

countries. These facts make the bead method the best alternative for the flow cytometry

in the developing countries. The bead method will help in staging HIV patients, and

initiating HAART. Furthermore, monitoring patients and determining if the patients are

responding to the treatment is another important use of the bead method.

Chemoprophylaxis against opportunistic infections is started at different levels of CD4 counts. For example, when CD4 count is less than 200 cell /μl chemoprophylaxis against Pneumocystis Jirovecii is started. This is other area where the bead method can be used to determine when to initiate chemoprophylaxis against opportunistic infections. Recently, WHO and the Global Fund to Fight AIDS have launched global campaigns against AIDS in Africa and other developed countries. One of the campaign targets is to distribute HAART to millions of patients. Without a reliable readily accessible method to count CD4 T cells, these major efforts will not be able to provide medications where they are most needed.

MATERIALS AND METHODS

Animals And Spleen Harvesting

Spleens from 4 month old ICR female mice were harvested in the laboratory of Dr. Emily Dudly. Harvested spleens were kept in Dulbecco's Modified Eagle's Medium (DMEM) (HyClone, Fisher Scientific, Pittsburgh,Pa). Spleens were macerated between the frosted ends of two microscope slides. The resulting cells suspension was centrifuged for 5 minutes at 1500 rpm. The cells were re-suspended in 5 ml 1x PBS and the spleen cells suspension was ready to be analyzed by the Bead method, and flow cytometry.

Principles Of The Bead Method

Three reagents are required to count CD4 or CD8 using beads method. These reagents are labeling reagent, blocking reagent, staining reagent (Figure 2) .The Labeling reagent consists of 1.4 μm carboxylate modified super-active latex Beads (Life Technologies, Grand Island, NY) coated with CD4 purified mouse monoclonal antibody (Biolagend , San Diego, CA). The bead method depends on the ability of monoclonal antibody-coated latex beads to bind to the surface of T lymphocytes expressing CD4 or CD8. When the CD4 or CD8 coated latex spheres come in contact with a cell that has the CD4 or CD 8 cell surface antigen, the two bind, forming a cell-latex beads rosette that is readily recognized by light microscopy. The blocking Reagent contains 0.5 μm in diameter carboxylate modified super active latex beads (Life Technologies, Grand Island, NY). The beads in this reagent are coated with CD 14 purified mouse monoclonal antibody (Biolagend, San Diego, CA) to minimize the reactivity of monocytes against CD4 coated latex spheres and reduce nonspecific binding with CD4 coated beads. The staining reagent consists of 2% acetic acid in distilled water and 0.025% crystal violet stain. The function of staining reagent is to lyse red blood cells, and stain lymphocytes.

-0.5 µm Latex beads coated with anti CD 14 antibodies

-1.4 µm Latex beads coated with anti CD 4 or 8 antibodies

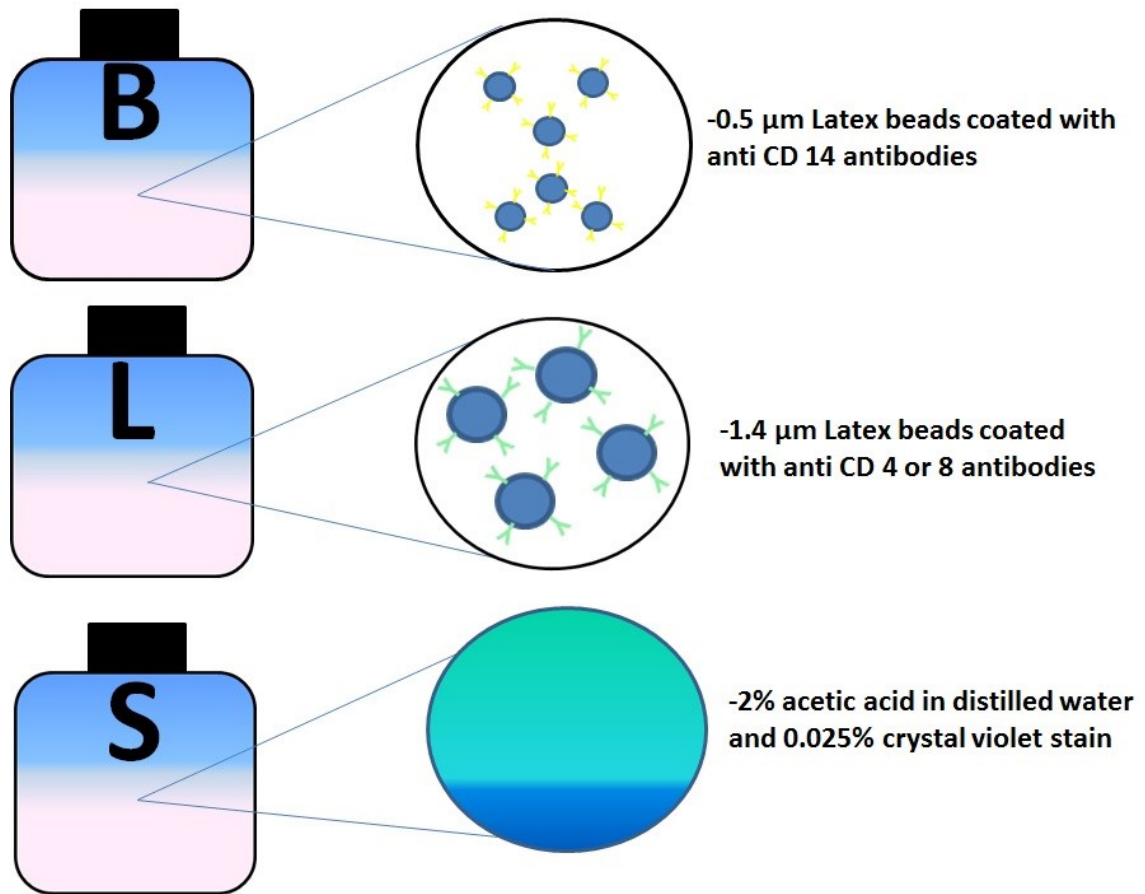-2% acetic acid in distilled water and 0.025% crystal violet stain

Figure 2. Bead Method Reagents. **B** blocking reagent, **L** labeling reagent, and **S** staining reagent.

Reagents Preparation

For the blocking and labeling reagent passive adsorption method was used to coat the beads. The passive adsorption method is a simple method for the attachment of antibodies by physical adsorption to carboxylate modified super active latex beads. The materials needed for this method are carboxylate modified super active latex beads, 2- n-morpholino-ethanesulfonic acid (MES) (Fisher Scientific, Pittsburgh, PA) buffer, 0.025 M, pH 6, wash buffer phosphate-buffered saline (PBS), 0.1 M, pH 7.2 , and storage Buffer phosphate-buffered saline (PBS), 0.1 M, pH 7.2,0.1% glycine; 0.1% NaN3 sodium azide . We chose MES buffer because its pH value is close to the isoelectric point of the antibodies used in this study, maximizing the protein density on the particle surface. Buffer systems contain multivalent anions (phosphate or borate) are not used in coating the latex microspheres because these ion species will compromise the colloidal stability of the latex beads.

MES Buffer Preparation

To prepare 10 ml of MES buffer with 0.025 M, and pH 6 we weighted 48,8 mg of MES and added it to 5 ml of Double distilled water. Since the starting pH would be below 4.0, a pH electrode was used to titrate to pH 6.0 with 1 N NaOH. Once the solution was adjusted to the correct pH, it was transferred to a graduated cylinder or volumetric flask and diluted to a final volume of 10 mL.

Storage Buffer Preparation

Glycine was used in the storage buffer to fill any reactive sites on the microsphere surface which had not been covered by the protein, and to reduce non-specific binding. Bovine serum albumin (BSA) might be used for the same purpose. The NaN3 was present as a biocide. If the latex is kept sterile, NaN3, which is not compatible with cell or tissue culture, can be omitted. Storage buffer was prepared by mixing 100 mg glycine, 100 mg sodium azide, and brought to a volume of 10 ml with PBS.

Determining Antibody And Latex Quantities

By using the following equation the optimal amount antibody needed for the beads coating was calculated.

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

In this study 100 mg of 1.4 μm latex beads were coated with 2 mg of CD4 antibody. According to the equation, weight of the antibody $= \frac{2 \text{ mg}}{1.4 \text{ μm}} = 1.4$ mg of CD4 antibody is needed to coat 100 mg of the beads. The same amount of CD8 antibodies was used to cote beads with CD8 antibody. Similarly, we coated 100 mg of 0.5 μm latex beads with 1 mg of CD14 blocking antibody , and according to the equation $= \frac{1 \text{ mg}}{0.5 \text{ μm}} = 2$ mg of CD 14 antibody is needed to coat 100 mg of the beads.

Latex Beads Coating

To coat latex beads with CD4, CD8, or CD14 we pipetted 2.5 ml (40 mg/ml) latex microspheres and diluted with 10 ml MES buffer. Then we centrifuged the mixture to sediment the particles with 3,000g for 20 minutes. We removed the supernatant and re-suspend the pellet in 10 ml MES buffer. We centrifuged it again and removed the supernatant from the particles. These washing steps were done to prepare the beads for coating with CD4, CD8, or CD 14 monoclonal antibodies. After that, we re-suspend pellet in 5 ml MES buffer to make.The latex suspension approximately 2 % solids (20 mg/ml). We added 5 ml MES buffer to prepare CD4 or CD8 coated beads (labeling beads). For CD 14 coated beads (blocking beads), the pellet was re-suspended in 10 ml MES Buffer to get 1% solids percentage (10 mg/ml). We added the calculated amount of the antibody. The calculated amount of the antibody would ensure the best coating of the particles with the least possibility of aggregation. Latex/protein mixture was incubated with gentle mixing at room temperature overnight.

In the next day, we centrifuged the latex/protein mixture to separate the protein-labeled latex particles from unbound protein. In the next step, we Re-suspend the pellet in 10 ml PBS. We washed the protein-labeled beads three times by centrifugation and suspension in 10 ml PBS. In the last step , we re-suspended the final latex of CD14 in 10 ml Storage Buffer giving a final percentage of 1% solids, and CD4 or CD8 in 5 ml

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storage buffer giving final percentage of 2% solids. We stored the reagents at 4°C, and we did not freeze them.

Measuring CD4 And CD8 Using Beads Method

To count the CD4 or CD8 T cells by using bead method we labeled two 12 x 75 mm tube as L+B and S (figure 3 A). We Put 100 µl of reagent S in to the test tube labeled S (figure 3 A). We placed 100 µl of spleen cells suspension at the bottom of the test tube labeled   B+L (figure 3 B). We Mixed reagent B before Adding 10 µl of reagent B to the Spleen cells suspension in the L+B Tube (figure 3 C). Then we Held the test tube vertically, and mixed gently by hand for 2 minutes immediately after adding reagent B (figure 3 C). In the same way, we Mixed reagent L, and added 10 µl of reagent L to B+L test tube (figure 3 D). We Held the test tube vertically, and mixed gently by hand for two minutes immediately after adding reagent L. after two minutes, we added 10 µl of the cell suspension latex spheres mixture from the B+L test tube to the S test tube (figure 3D). Droplets around the top of any test tube will result in non-lysis of red blood cells and erroneous CD4+ or CD8+ lymphocyte counts. To lyse the red blood cells in the C test tube, we held the test tube vertically, and mixed gently for 10 to 15 seconds (figure 3 E). In the next step, we loaded both chambers of the 0.1 mm deep (or one chamber of the 0.2 mm deep) Hemacytometer with the sample from step 9 (figure 3 F). We placed the Hemacytometer in a moisture chamber and allow the cells to settle for 2 to 3 minutes. Under the light microscope, we counted the cells that had three or more large latex spheres attached to them as CD4 + T lymphocytes (figure 4 A,B) or CD8 + T

lymphocytes (figure 4 C, D). We used the following equation to calculate the absolute count of CD4 or CD8 + T lymphocyte.

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Absolute CD4 or CD8 + T lymphocytes/µL =

CD4 or CD8 +T lymphocyte count x Chamber depth correction x Sample dilution correction
Surface area (mm2)

where:

Surface Area = 18 mm^2 for two sides of 0.1 mm deep chamber

Chamber Depth Correction = $\frac{1}{chember\ depth}$ = $\frac{1}{0.1}$ = 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 = 100/20
Secondary dilution:
10 µL of 100/120 primary dilution
in 100 µL Reagent S =

$$\frac{10\mu l\ x\ (\frac{100}{120})}{110\ \mu l} = \frac{1}{13.2}$$

Therefore the Correction for Sample Dilution =
$1\div \frac{1}{13.2} = 13.2$

For example if the CD4 count in 0.1 mm chamber was 60. So the CD4 count in the sample can be calculated by applying the formula.

60 x Chamber depth correction (10) *x Sample dilution correction* (13.2)
Surface area (18 mm2)

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60x 7.3 = 438 CD 4 + T lymphocytes /μl.

From the equation a conversation matrix was created to know the concentration of CD 4 or CD8 + T lymphocyte in the sample (Table 1). Lymphocytes with no attached beads (figure 5 A,C) , one (figure 5 B,C) , or two bead attachments will not be counted .
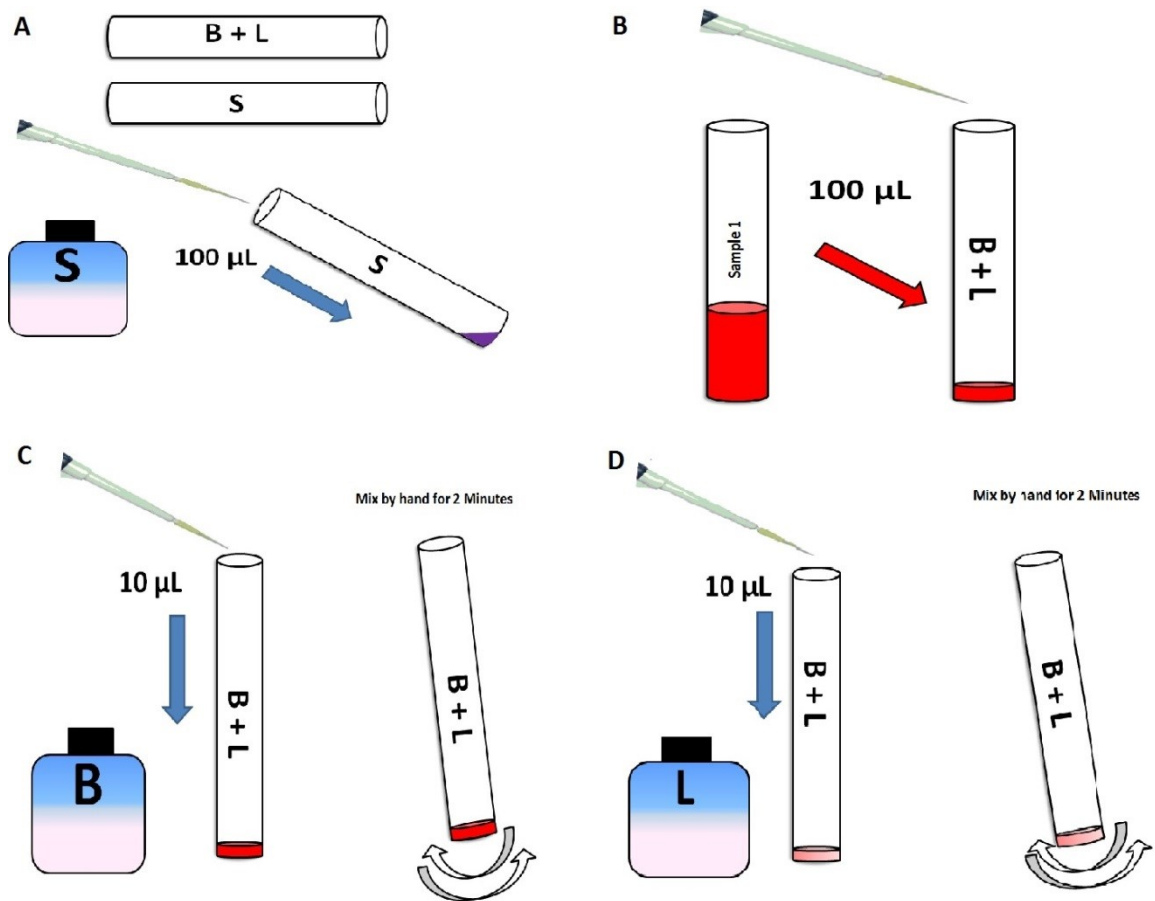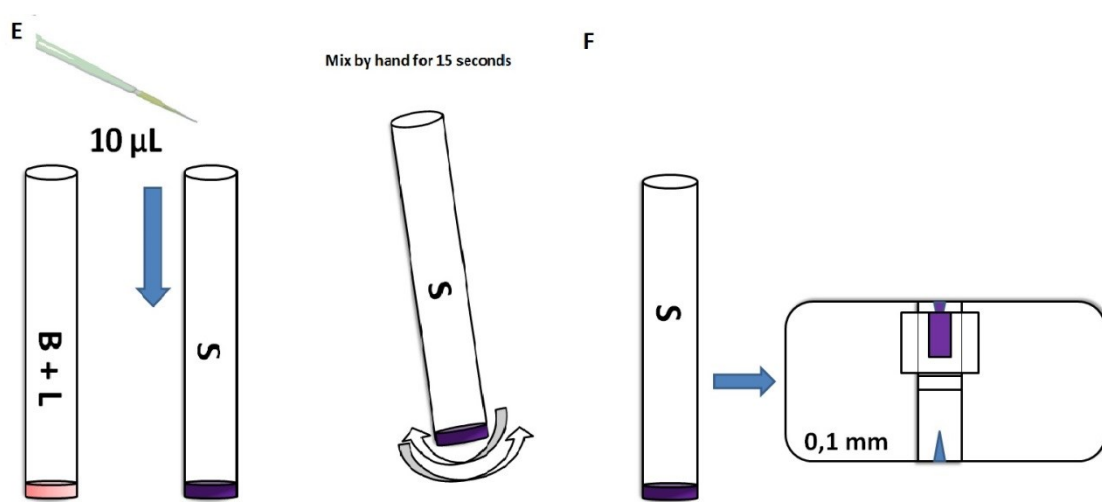
Figure 3. Bead Method Protocol. (A-F). Figure shows different steps of the bead method.
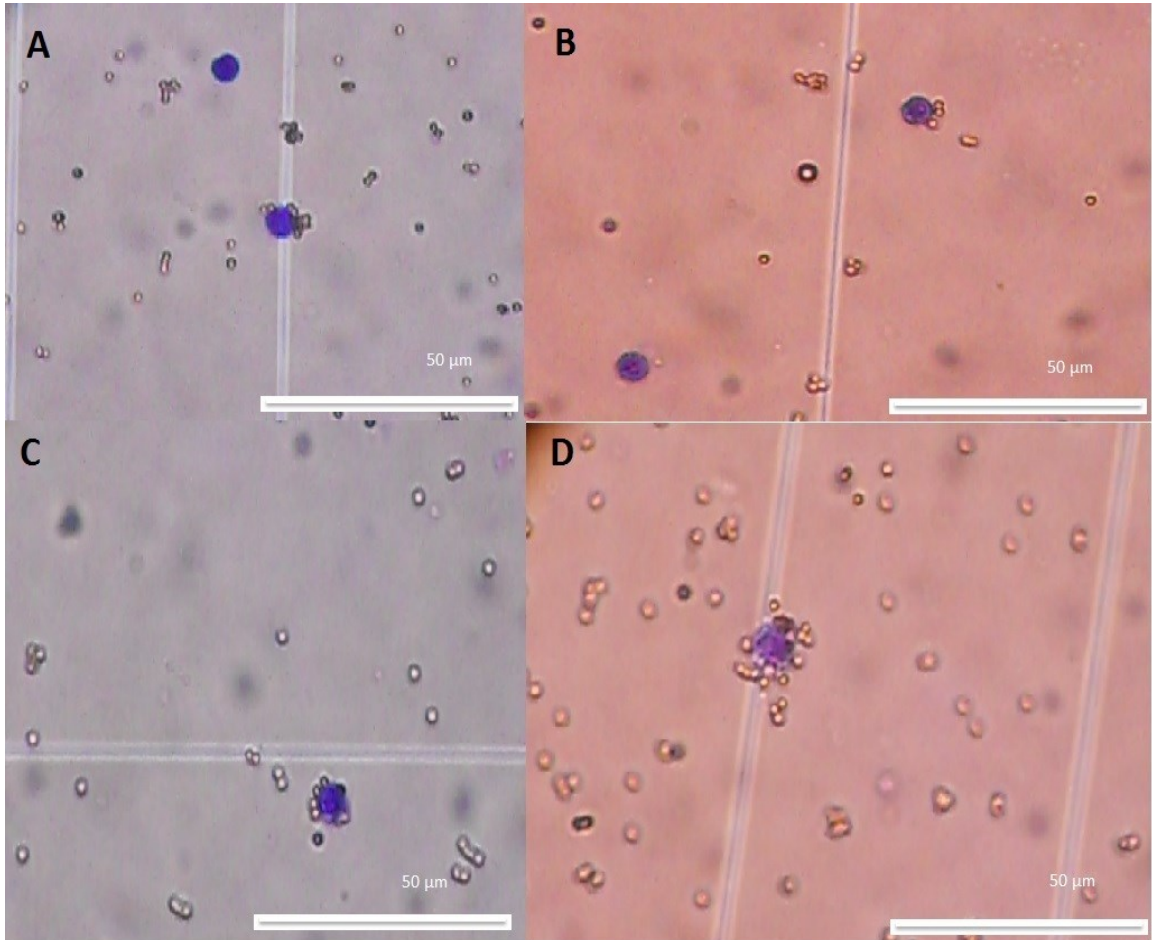
Figure 4. Bead Method Results Three Or More Beads Attachment. Microscopic images at 40X magnification demonstrate three beads and more attaching to CD4 and CD8 T lymphocytes (A,and B CD 4. C, and D CD8).

Figure 5. Bead Method Results One Or No Beads Attachment. Microscopic images at
40X magnification demonstrate no beads attached (A,C) and one bead attached (B, and
C).

CD8+ and CD4+ COUNT CONVERSION MATRIX

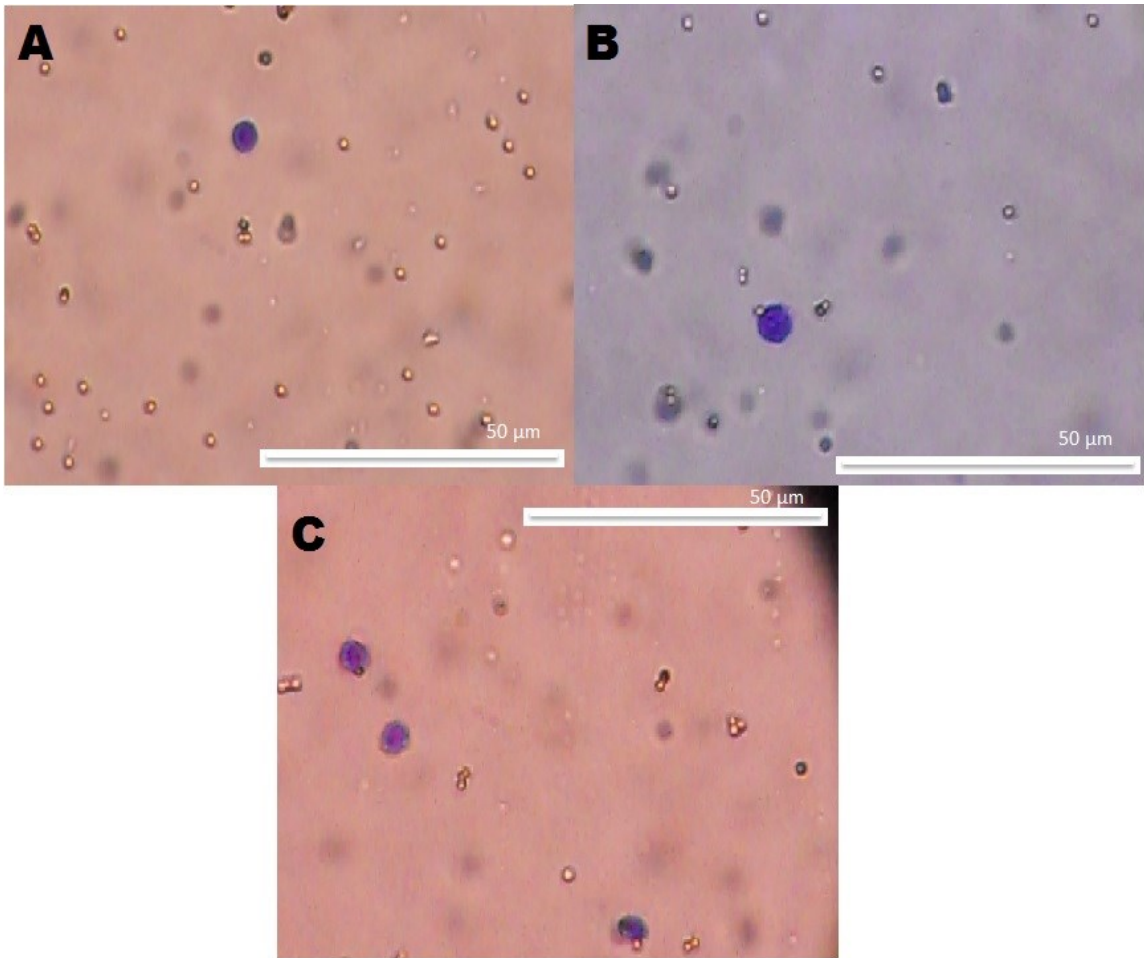| # Cells	CD8 or CD4+/µl	# Cells	CD8 or CD4+/µl	# Cells	CD8 or CD4+/µl	# Cells	CD8 or CD4+/µl	# Cells	CD8 or CD4+/µl
< 5	< 30								
5	37	60	438	115	840	170	1241	225	1643
6	44	61	445	116	847	171	1248	226	1650
7	51	62	453	117	854	172	1256	227	1657
8	58	63	460	118	861	173	1263	228	1664
9	66	64	467	119	869	174	1270	229	1672
10	73	65	475	120	876	175	1278	230	1679
11	80	66	482	121	883	176	1285	231	1686
12	88	67	489	122	891	177	1292	232	1694
13	95	68	496	123	898	178	1299	233	1701
14	102	69	504	124	905	179	1307	234	1708
15	110	70	511	125	913	180	1314	235	1716
16	117	71	518	126	920	181	1321	236	1723
17	124	72	526	127	927	182	1329	237	1730
18	131	73	533	128	934	183	1336	238	1737
19	139	74	540	129	942	184	1343	239	1745
20	146	75	548	130	949	185	1351	240	1752
21	153	76	555	131	956	186	1358	241	1959
22	161	77	562	132	964	187	1365	242	1767
23	168	78	569	133	971	188	1372	243	1774
24	175	79	577	134	978	189	1380	244	1781
25	183	80	584	135	986	190	1387	245	1789
26	190	81	591	136	993	191	1394	246	1796
27	197	82	599	137	1000	192	1402	247	1803
28	204	83	606	138	1007	193	1409	248	1810
29	212	84	613	139	1015	194	1416	249	1818
30	219	85	621	140	1022	195	1424	250	1825
31	226	86	628	141	1029	196	1431	251	1832
32	234	87	635	142	1037	197	1438	252	1840
33	241	88	642	143	1044	198	1445	253	1847
34	248	89	650	144	1051	199	1453	254	1854
35	256	90	657	145	1059	200	1460	255	1862
36	263	91	664	146	1066	201	1467	256	1869
37	270	92	672	147	1073	202	1473	257	1876
38	277	93	679	148	1080	203	1482	258	1883
39	285	94	686	149	1088	204	1489	259	1891
40	292	95	694	150	1095	205	1497	260	1898
41	299	96	701	151	1102	206	1504	261	1905
42	307	97	708	152	1110	207	1511	262	1913
43	314	98	715	153	1117	208	1518	263	1920
44	321	99	723	154	1124	209	1526	264	1927
45	329	100	730	155	1132	210	1533	265	1935
46	336	101	737	156	1139	211	1540	266	1942
47	343	102	745	157	1146	212	1548	267	1949
48	350	103	752	158	1153	213	1555	268	1956
49	358	104	759	159	1161	214	1562	269	1964
50	365	105	767	160	1168	215	1570	270	1971
51	372	106	774	161	1175	216	1577	271	1978
52	380	107	781	162	1183	217	1584	272	1986
53	387	108	788	163	1190	218	1591	273	1993
54	394	109	796	164	1197	219	1599	274	2000
55	402	110	803	165	1205	220	1606		
56	409	111	810	166	1212	221	1613		
57	416	112	818	167	1219	222	1621		
58	423	113	825	168	1226	223	1628		
59	431	114	832	169	1234	224	1635		

Table 1. CD4 And CD8 T lymphocytes Count Conversation Matrix.

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Flow Cytometry CD4 And CD8 Counting

Flow cytometry uses laser and fluidic principles for cell sorting, counting, and biomarker detection. By using fluidic principles, the flow cytometry aligns the cells so they pass in single file through the laser beam for analysis. In our study, flow cytometry was used to count the absolute number of CD4 T lymphocytes and CD8 T lymphocytes after marking them with fluorescent conjugated antibodies. Control antibody were used in every experiment. For CD4 T lymphocytes counts we used FITC anti-mouse CD4 Antibody (Biolegend San Diego, CA,) , and FITC Rat IgG2a, κ Isotype Ctrl Antibody as control (Biolegend San Diego, CA,). For CD8 T lymphocytes we used PE/Cy5 anti-mouse CD8a Antibody (Biolegend, San Diego, CA), and PE/Cy5 Rat IgG2a, κ Isotype Ctrl Antibody as control (Biolagend, San Diego, CA).

To count CD4 and CD8 + T lymphocytes we washed cell suspension 3 times with 1% (bovine serum albumin) BSA, and centrifugation speed of 1200 rpm for 5 minutes. After washing, we blocked the sample with 3% BSA and 30 minutes incubation. After the incubation, we washed the sample 3 times with 1% BSA, and centrifugation speed of 1200 rpm for 5 minutes. Conjugation with fluorochrome conjugated primary CD4 or CD8 antibody and there controls was the next step after washing. The amount of the antibody that was 5 μg antibody / ml with 100 μl of 3% BSA for each sample. We

Incubated the sample with fluorochrome conjugated antibody for 45 minutes at 4°C in
the dark. Then, we washed the sample with 1% BSA and centrifugation speed of 1200
rpm for 5 minutes 3 times. After the last step of washing, we fixed the sample by re-
suspension in cold PBS, 10% fetal calf serum , and 1% sodium azide at 4°C until analysis
within 24 hours. FCS Express program was used to analysis the results form flow
cytometry (figure 6-7).
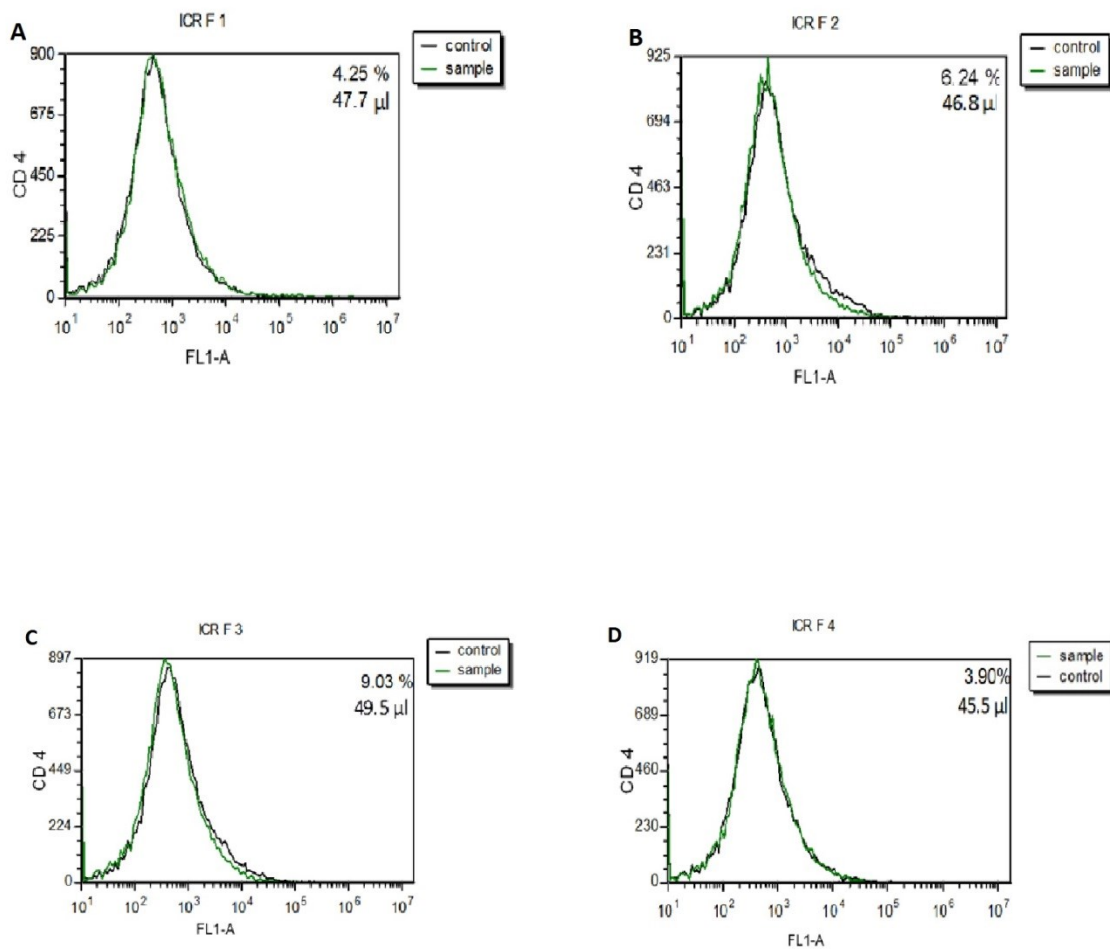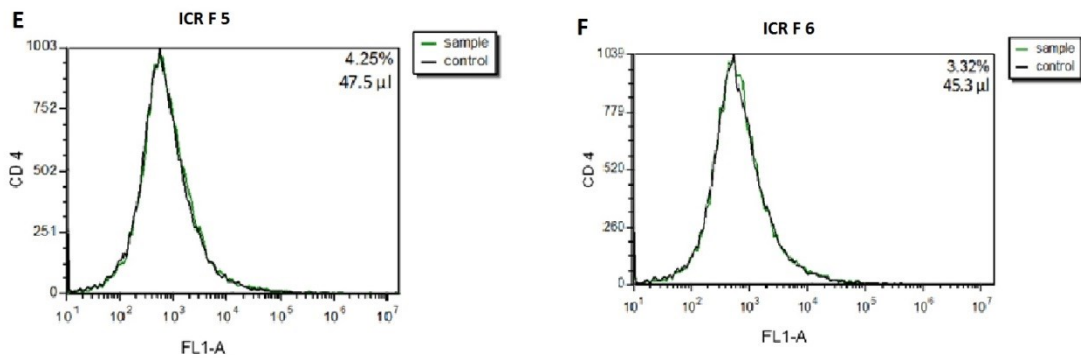
Figure 6. Flow Cytometry Results Of Sample Analysis Using CD4 Monoclonal Antibody(A-F). The figure shows flow cytometry analysis of ICR female mouse spleen cells suspenstion using CD4 monoclonal antibody. The machine was adjusted to stop after detecting 20,000 event. Knowing the volume at which 20,000 was detected we calculated how many CD4 per sample = 20,000/ volume at which 20,000 was detected.

A ICR F 1

sample
control

2.88%
100.5 µl

B ICR F 2

sample
control

2.54%
93 µl

C ICR F 3

sample
control

4.65 %
90.4 µl

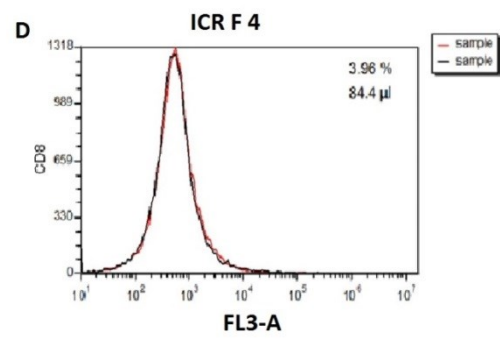**D** ICR F 4
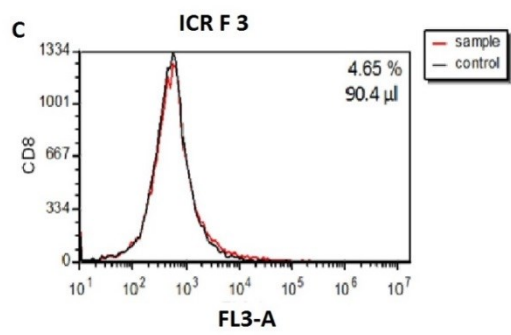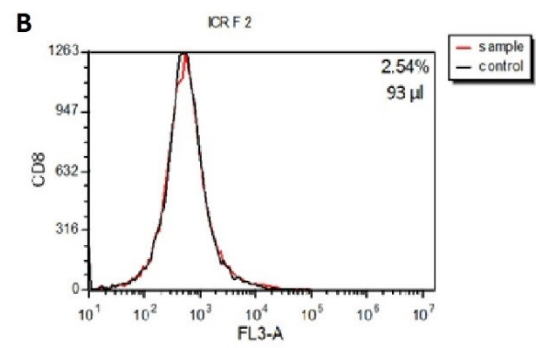
sample
sample

3.96 %
84.4 µl

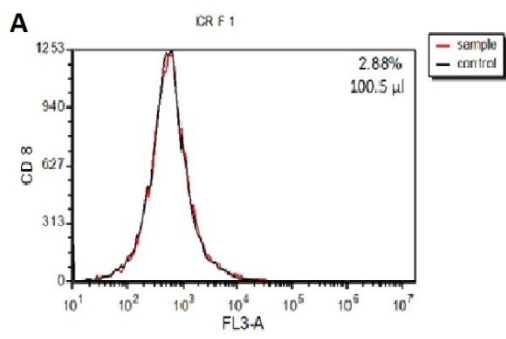**Figure 7. Flow Cytometry Results Of Sample Analysis Using CD8 Monoclonal Antibody(A-F).** The figure shows flow cytometry analysis of ICR female mouse spleen cells suspension using CD8 monoclonal antibody. The machine was adjusted to stop after detecting 20,000 event. Knowing the volume at which 20,000 was detected we calculated how many CD8 per sample = 20,000/ volume at which 20,000 was detected.
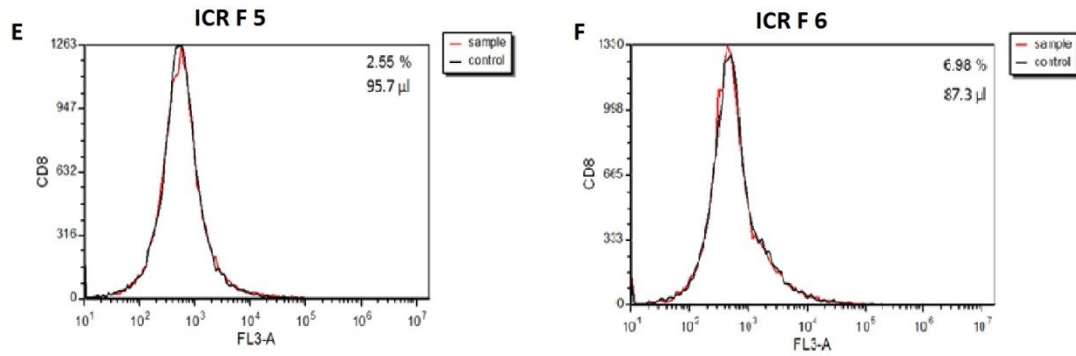
RESULTS

The absolute number of CD4+ T lymphocytes and CD8+ T lymphocytes was determined using both flow cytometry and the bead method in cells from six female ICR mouse four month of age. The total number of lymphocytes in ICR female mouse spleens was ~10 million in all six samples. The ratio of total CD4 T lymphocytes to the total lymphocytes counts was ~ 0.8 and ratio of total CD8 T lymphocytes counts to the total number lymphocytes counts was ~ 0.4 (Table 3-4). The results show that the number of CD4 + T lymphocytes is more than the number CD8 + T lymphocytes. The numbers of CD4 + T lymphocytes were around 400 Cells /μl, and the number of CD8 + T lymphocytes was around 200. The ratio of CD4 to CD8 was 2:1 (Table 2) (figure 8). The results were analyzed by using Sigma Plot. Paired t-test shows that the correlation between beads and flow cytometry is greater than would be expected by chance and this correlation is a statistically significant (P = <0.001). One Way Analysis of Variance demonstrate that the differences in the mean between beads method and flow cytometry is greater than would be expected by chance; there is a statistically significant difference (P = <0.001). Since the p value is less than 0.005 the null hypothesis can be rejected, and the bead method can give enough accurate estimation of the CD4 and CD8 T lymphocytes counts in ICR mouse spleen cells suspension. These results of this study provide the basis for using the bead method in situation where flow cytometry is not available.

Mouse	Flow cytometry		Bead Method	
	CD4	CD8	CD4	CD8
ICR F 1	419/µl	199/µl	423/µl	204/µl
ICR F 2	427/µl	215/µl	431/µl	219/µl
ICR F 3	404/µl	221/µl	409/µl	226/µl
ICR F 4	439/µl	237/µl	445/µl	241/µ
ICR F 5	421/µl	209/µl	423/µl	212/µl
ICR F 6	441/µl	229/µl	445/µl	226/µl

Table 2. Result Of CD4 And CD8 T Cells Counts Using Flow Cytometry And Bead Method. The table shows CD4 and CD8 T lymphocytes counts in 6 Female ICR mouse by using flow cytometry and Bead Method. The results of both methods are not identical for the same sample; however, the results from the beads method are not far from flow cytometry results. For all the samples Bead Method estamition was higher than the flow cytometry.(Mean of the flow cytometry CD4=425, CD8=218 , Mean of the Bead Method CD 4=429 , CD8 = 221)

Mouse	Flow cytometry		Bead Method	
	CD4/Sample	CD8/Sample	CD4/Sample	CD8/Sample
ICR F 1	838000	398000	846000	408000
ICR F 2	854000	430000	862000	438000
ICR F 3	808000	442000	818000	452000
ICR F 4	878000	474000	890000	482000
ICR F 5	842000	418000	846000	424000
ICR F 6	882000	458000	890000	452000

Table 3. Total CD4 And CD8 T Lymphocytes Counts. The table shows the total CD4 T cells and CD8 T cells counts per sample. This table was created to calculate the ratio of total CD4 T cells and CD8 T cells to the total number of lymphocytes.

Mouse	Flow cytometry		Beads Method	
	CD4/ Total lymphocytes	CD8/ Total lymphocytes	CD4/ Total lymphocytes	CD8/ Total lymphocytes
ICR F 1	0.83	0.4	0.84	0.4
ICR F 2	0.85	0.43	0.86	0.43
ICR F 3	0.80	0.44	0.81	0.45
ICR F 4	0.87	0.47	0.89	0.48
ICR F 5	0.84	0.41	0.84	0.42
ICR F 6	0.88	0.45	0.89	0.45

Table 4. CD4 And CD8 T Lymphocytes Ratio To Total Lymphocytes Count. The table demonstriates the ratio of ratio of total CD4 T cells and CD8 T cells to the total number of lymphocytes. This ratio shows no large difference between the two methods and supports the idea of using the beads method in staging, and monitoring HIV infected patients.
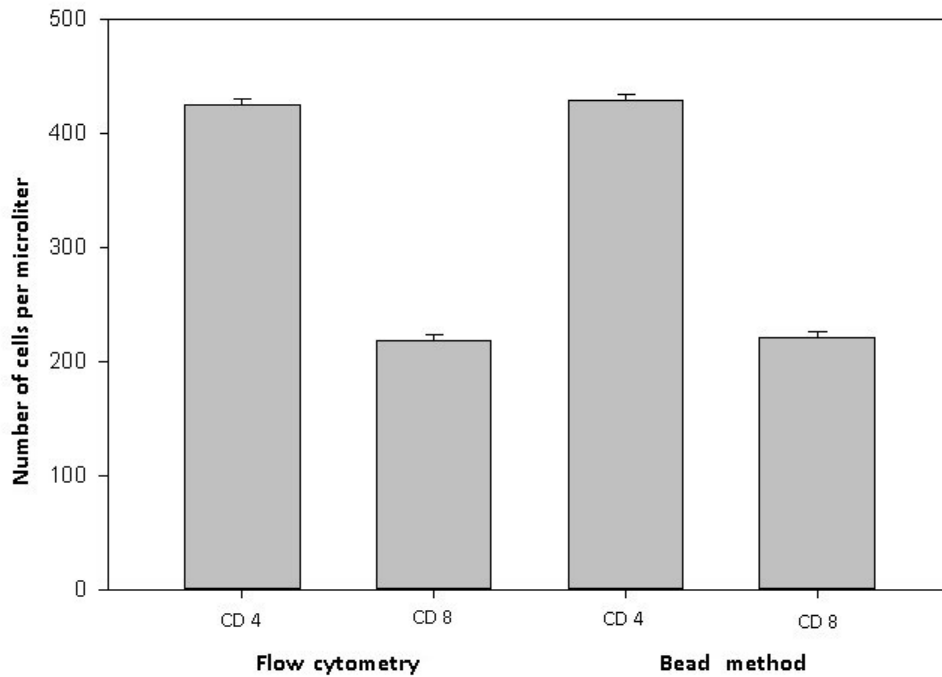
Figure 8. Bar Chart For Flow Cytometry And Bead Method Results. Bar chart shows the results of flow cytometry and bead method. The results of CD4 T lymphocytes counts are around 400 Cell/μl in both methods. In the same way, the results of CD8 T lymphocytes counts are around 200 cell/μl in both beads method and flow cytometry.

DISCUSSION

The main goal of this study was to compare the results of counting the absolute number of CD4 + T lymphocytes and CD8 + T lymphocytes in mouse spleen cell suspension using flow cytometry (gold standard method), with the bead method. This was done in anticipation of using the bead method in staging and monitoring treatment response in HIV infected patients. The bead method depends on the ability of monoclonal antibody-coated latex beads to bind to the surface of T lymphocytes expressing CD4 or CD8. When the CD4 or CD8 coated latex spheres come in contact with a cell that has the CD4 or CD8 cell surface antigen, the two bind forming a cell-latex beads rosette that is readily recognized by light microscopy.

The bead method was not time consuming in comparison to the flow cytometry method. The bead method took about two hours to do all 6 samples while the flow cytometry method took about 4 hours. The bead method did not require a lot of laboratory experience. In the other hand, the flow cytometry method required more laboratory training and experience than the bead method. The cost of the bead method reagents is about $1,000, in contrast the flow cytometry cost ranges from $30,000 to $150,000, and the reagents needed for determining lymphocytes surface markers by this method are very costly.

The bead method slightly over estimates CD 4 and CD8 + T lymphocytes. In clinical situation this slight over estimation can be acceptable because the clinical decision whether to treat patients or not depends on CD 4 and CD8 + T lymphocytes counts, and the clinical condition of the patients. Using the bead method with blood samples from HIV patients carries the risk of HIV infection. Yet, the risk of HIV infection in laboratory workers dealing with blood samples from HIV patients is approximately 0.3% (Kuhar et al., 2013).

In our study we did not use peripheral mice blood; instead we used mice spleen cell suspension. The relationship between splenic CD4 and CD8 + T lymphocytes and the peripheral blood CD4 and CD8 + T lymphocytes is different according the strain, sex, and age of the mice. In BALB/C mice the percentage of CD4 and CD8 + T lymphocytes in peripheral blood and in the spleen was the same in early age. However, in old age mice the percentage of CD4 and CD8 + T lymphocytes decline in the peripheral blood and stay the same in the spleen (Pinchuk & Filipov, 2008). The use of spleen cell suspension does not exactly simulate the clinical situation at which the bead method intended to be used, however the results continuity were very promising and satisfactory to make us advance to the next step in this research. The next step in our study is to compare staging of HIV infected patients using bead method, and flow cytometry.

In general, reducing the exposures to body fluids and blood is the most important strategy for preventing occupationally acquired HIV infection. To reduce the risk of contracting HIV infection while working with the bead method, we suggest adding a fixation step with PBS, fetal calf serum, and sodium azide. In addition, lab workers should deal carefully with needles and sharp objects, and use gloves when handling materials containing HIV. Gloves should be changed after any contact with a potentially contaminated surface or material. Hand washing is other way to reduce the risk of infection with HIV. During working with the bead method lab workers should avoid hand contact with mouth, eyes, ears, and nose. This requirement can be made easier by wearing goggles or a face shield. Decontamination of work surfaces after finishing working with the bead method can also help with reducing the risk of contracting HIV infection (Weiss et al., 2014).

Flow cytometry uses laser and fluidics principles for cell sorting, counting, and biomarker detection. By using fluidics principles, the flow cytometry aligns the cells so they pass in single file through the laser beam for analysis. In this study, flow cytometry was used to count the absolute number of CD4 T lymphocytes and CD8 T lymphocytes after marking them with fluorescent conjugated antibodies. The results from the flow cytometry and the beads method demonstrated that the beads method will give estimation of the absolute CD4 and CD8 + T lymphocytes accurate enough to substitute the flow cytometry in case of staging, and monitoring treatment response in HIV infected patients.

In comparison to polyclonal antibodies, monoclonal antibodies can specifically identify T cell and B-cell surface antigens. Moreover , monoclonal antibodies allow not only for lymphocyte measurements but in conjunction with other cell markers like TdT (Terminal deoxynucleotidyl transferase) they help in identification of distinct stages of T-cell and B-cell differentiation. TdT is an enzyme that plays important role in B and T lymphocytes Maturation. TdT randomly adds nucleotide to the DNA of Maturating T or B lymphocytes in order to help them to generate Immunoglobulin surface receptors capable of recognizing different pathogens. The addition of the nucleotides does not correspond to any DNA germline sequence (Pharm, 2009).

Leukemia is tumor of the white blood cells. Failure of cell maturation is the main cause of acute leukemia. The immature cells Proliferate and accumulate in the bone marrow. The high rate of leukemic cells proliferation will take up the space and nutrient at the expense of the normal haematopoietic cells. When the bone marrow runs out space the malignant cells will spill into the blood. Bone marrow failure clinical features are the clinical features of leukemia.   Since the bone marrow is responsible for production of red blood cells, maturation of white blood cells, and platelet synthesis, the three main clinical features of bone marrow failure are anemia, infection, and bleeding (Colledge, 2010). In case of acute lymphoblastic leukemia, the expression of TdT on the cells surface increases (Janossy et al., 1979). Future studies can be done about the concept of over

expression of TdT in acute lymphoblastic leukemia to assess if the bead method will help in diagnosis and staging of this kind of leukemia.

Another place where the bead method can be used is detection of CD5 and CD19 expressed on the same white blood cells which may help in diagnosis and staging of chronic lymphocytic leukemia, and primary IgA nephropathy. CD5 is a cluster designation found on the surface of IgM-secreting B cells (B-1 cells), and also on T cells. Due to lack of TdT enzyme B-1 cells have limited diversity of their B-cell receptor. The main function of CD5 is to mitigate activating signals from the B cell receptor. This mechanism B-1 cells activation will make them activated only by very strong stimuli like bacterial proteins (Pharm, 2009). The B-cell co-receptor is a complex of three proteins CD19, CD81, and CD21. The function of CD 19 is signaling chain of the receptor.CD21 recognizes the iC3b and C3d break down products of C3b fragments (one of the complement factors).CD81 function is not clearly understood, however in case of hepatitis C virus infection CD81 acts as receptor for the virus (Pharm, 2009).

Chronic lymphocytic leukemia is the most common type of leukemia. Thirty percent of leukemia patients having chronic lymphocytic leukemia. Sixtys to seventy years is the median age group for lymphoma with 2:1 male: female ratio. B lymphocytes in chronic lymphocytic leukemia fail to respond probably to antigens and transform in to plasma cells. The abnormal B lymphocytes will accumulate in the bone marrow leading

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to bone marrow failure (Colledge, 2010). Chronic lymphocytic leukemia is latent malignancy, and near 70% of the cases are diagnosed incidentally on a routine blood checkup. Clinical symptoms of chronic lymphocytic leukemia appear in the advanced stage of the disease. Anemia, recurrent infections, and weight loss are the main clinical features of chronic lymphocytic leukemia (Colledge, 2010). Many of the malignant B cells in chronic lymphocytic leukemia over express CD5, and CD19. These two CD markers can be used in monitoring disease status during therapy (Cabezudo et al., 1997). In this situation, the bead method can be used to assess the effectiveness of the therapeutic strategies instead of flow cytometry.

Other condition where the beads method can be used instead of flow cytometry is primary IgA nephropathy. Patients with primary IgA nephropathy will have polymeric IgA-dominant Ig deposition in the mesangium of kidney (Yuling et al., 2008). The main clinical features of primary IgA nephropathy are hematuria, headache, and fatigue. Most of the patients with IgA nephropathy will progress to renal failure without treatment. One of the pathological markers of IgA nephropathy is the present of CD5 CD19 + B Cells in the peripheral blood, and peritoneal fluid. The level of CD5 CD19 + B Cells decreases with treatment ,and This change in the level of CD5 CD19 + B Cells can be used to monitor the response of IgA nephropathy patients to the treatment (Yuling et al., 2008). Using the bead method to measure the level of CD5 CD19 + B cells is another clinical setting where the bead method can be used instead of flow cytometry.

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