CHAPTER I

INTRODUCTION

1.1 Statement of problems

World wide, acquired immunodeficiency syndrome (AIDS) now ranks as the fourth leading cause of death after heart disease, stroke, and acute lower respiratory infection. AIDS have produced a formidable worldwide pandemic by 42 million people that were infected with human immunodeficiency virus in the world and 95% of them are currently living in the developing world (1). There were about 3.5 million persons newly infected in sub-Saharan Africa (2). During 2002, AIDS caused the deaths of an estimated 3.1 million people globally. It is estimated that by the year 2005, about 100 million people will have been infected worldwide (3).

AIDS is an infectious disease caused by Human Immunodeficiency Virus (HIV). In HIV infected persons, HIV infects cells that possess CD4 receptor (4-6). This is reserved as the primary target cells, the helper T cell or CD4+ T lymphocyte cells, and it is reasoning of gradually the CD4+ T lymphocytes decreased. As CD4+ T lymphocytes play an important role to organize the body's immune responses, immunodeficiency is then occurred after HIV infection. When the number of CD4+ T lymphocytes decreases less than 500 cells/μl, patients may be infected with opportunistic pathogens. If it decreases less than 200 cells/μl, the opportunistic infection and malignancy will be occurred in higher rate (7-8). Progressive depletion of CD4+ T lymphocytes is associated with an increase likelihood of clinical complication (9-10).

The CD4+ T lymphocyte levels must be monitored every 3 to 6 months in all HIV infected persons (11). The CD4+ T lymphocyte counts are very important marker used to assess the degree of immune deterioration and speed of progression toward AIDS (12-13). This marker is also used to monitor the efficacy of antiretroviral therapy (14-15), and to determine the optimum timing for prophylaxis of opportunistic infections (16). When the absolute CD4 T lymphocyte count is less than 350 cells/μl, antiretroviral therapy should be started and if the number is less than 200 cells/μl, prophylaxis of opportunistic infection combine with antiretroviral therapy should be administrated (17). Consequently, the enumerations of CD4+ cells are also used as a tool to improve AIDS surveillance (18), and to monitor the efficacy of therapy (19). Moreover, the CD4+ T lymphocyte levels are a criterion for categorizing HIV related clinical condition by CDC's classification system in HIV infections and surveillance case definition for AIDS among adults and adolescents (20). Recently, the CD4+ T lymphocyte count has been used to assess prognosis for progression to AIDS or death, to formulate the differential diagnosis in a symptomatic patients, and to make therapeutic decisions regarding antiviral treatment and prophylaxis for opportunistic pathogens. In addition, it is used as indicators to assist a decision regarding in when should start or change antiretroviral therapy in order to assess treatment responses. Therefore, monitoring of CD4+ lymphocytes is a very important tool used for successful HIV/AIDS management and care (18, 20).

Currently, flow cytometry is as the standard method for determining CD4+ T lymphocytes (21-22). This technique requires flow cytometer and monoclonal antibody reagents that are very expensive, together with a considerable technical expertise or well-trained technical staffs are necessary in performing the assay and maintaining the quality services. Therefore, this method is inaccessible in resource poor countries. The cost of CD4 count per test is about 25 US dollar. The high costs for equipment involved in this

technique have been represented of the limitations for CD4+ cell counting in developing countries and made flow cytometry is not implementable in poor setting areas.

Alternative technologies non-flow cytometry have been reported (23-31). Some of them were based on microscopes (optical or fluorescene) such as the Cyto-Spheres (Coulter Corporation, USA) and the Dynabead (Dynal AS, Norway). Other alternative methods, based on enzyme immunoassays such as TRAxELISA (Innogenetics, Belgium), Zymmune (Zynaxis Inc., USA) and Capcellia (Bio-Rad, USA) were also developed for replacing the flow cytometric method.

By microscope-based techniques, e.g., Cyto-Spheres and Dynabead assay, the methods are simple and inexpensive compared to conventional flow cytometry. They require inexpensive equipment. However, counting under the microscope is times consuming and laborious. In Cyto-Sphere technique, the sample is stable for only 15 minutes after additional of lysis/stain reagent, which limits the batch size to ~2 samples. To differentiate monocytes from lymphocyte, smaller spheres were used to recognize the difference. Several groups of researchers have found a good correlation between CD4 cell count measured by this method compared to flow cytometry (r=0.91-0.93), whereas another group who analyzed only 10 samples, has reported poorer correlation (r=0.45) (23). Dynabead method, monocytes, although, are removed to avoid confusion with lymphocyte when counting. But, cell counting must be done within 1 hr after addition of lysis reagent this made the batch size to be limited to <6 samples. This method is easier to interpret and more closely correlating with flow cytometric method (n=54; r=0.96) than the Cyto-sphere (n=27, r=0.69) (24). However, both methods require a visual counting which was subjective and laborious, the limiting of 2-3 samples per bath were processed and only smaller number of cells were counted. Therefore, both techniques are suitable for smaller regional centers when less than 10 samples are requested per day (25, 26, 31).

Immunoenzymatic assays, such as Capcellia® CD4/CD8 immunoassay or TRAx™ CD4 test kit, require multi-steps of testing performances. It was taken almost 4 hours to complete the processes. As a ELISA based technique, washing steps are concerned and the batch running system requires promptly lysate, therefore, the prepared lysate should store frozen until all clinical specimens could be run in a batch. Since the principle of this assay is based on the detection of total CD4 protein in a whole blood lysate to predict CD4+ T lymphocytes per microliter. The results are not dependent on intact or viable cells as with other cytometric techniques. In addition, some diseases such as leukemia, lymphoma and rheumatoid arthritis which may possible have biological factors perturb the ratio of CD4 molecules per cell or the amount of soluble CD4 compared with those for the HIV-sero-positive and control specimens. Therefore, the immunoalkaline phosphatase assay is no longer available in the market, even it still promising as technology but it is considered to be obsolete (27, 28, 31).

Immunofluorescence assay, Zymmune assay that is a magnetic based fluorescent immunoassay. The format combines a mixture of magnetic and fluorescent microspheres. Magnetic particles are used for cell separation and fluorescent particles for detection system. This method is relatively with many washing cycles and uses the calibrator curves; the numbers of CD4 and CD8+ T lymphocytes were obtained without using flow cytometry and automated hematology analyzer. The Zymmune assay, however, is more cost effective than traditional flow cytometry (29-31).

The purpose of this study is to develop an alternative non-flow cytometry to enumerate the CD4+ T lymphocytes in whole blood. The developed method has to be simple, inexpensive, and affordable technique for resource limited-countries. The analysis system does not require flow cytometer, but use only a automated hematology analyzer, which is available in almost hospital laboratories. To enumeration of CD4+ lymphocytes,

CD4 mAb coated magnetic beads (MT4 bead reagent) and method will be developed. The developed method is involved in the incubation of whole blood with MT4 beads reagent, after incubation the CD4+ T lymphocytes will bind directly with MT4 beads. By using magnet concentrator, the bound cell-beads will be depleted, unbound cells will be examined by using automate cell counter. The absolute CD4+ T lymphocyte number is then obtained by subtraction of absolute lymphocyte number before and after CD4 cells depleted. Comparing with standard flow cytometric method will validate the developed method and reagent.

1.2 Literature review

1.2.1 AIDS (Acquired Immunodeficiency Syndrome)

Human Immunodeficiency Virus (HIV) is one of the most significant emerging infectious pathogens. It belongs to a class of viruses' known as *retroviruses* which causes most sever clinical manifestation, acquired immunodeficiency syndrome (AIDS), and have produced a formidable worldwide pandemic (1).

- 1981 AIDS was recognized.
- 1983 HIV was first isolated.
- 1984 HIV was generally accepted as the cause of AIDS.
- 1985 the HIV enzyme-linked immunosorbent assay (ELISA) antibody test became available for clinical use and screening of blood donations.
- 1987 azidothymidine (AZT; now known as zidovudine or ZDV) was made available as an investigational agent and approved for AIDS patients.
- 1989 early intervention treatment guidelines for HIV infected adults were established.
- 1992 the CDC guidelines concerning CD4+ T lymphocyte determinations were first published in the morbidity and mortality weekly report (MMWR) to provide laboratories with the most complete information about how to measure CD4+ T lymphocytes in blood from HIV infected persons by using flow cytometry.
- 1993-94 the guidelines were revised and published.
- 1997 the CDC developed the revised guidelines for laboratories performing lymphocyte immunophenotyping assays in HIV infected persons and this report updated previous recommendations and reflected current technology in a field that was rapidly changing.

❖ 2002 summary of finding for CD4 assays were discussed at workshop sponsored by the forum for collaborative HIV research, Washington DC.

HIV is most often transmitted through sexual contact by rapidly binding to cells that are present in the cervical, vaginal, penile, urethral and rectal mucosa. To expose to blood or blood products, and perinatal transmission during pregnancy or during the postpartum period are the risk. The transmission of HIV through male homosexual contact is increased when having rectal trauma. The presence of ulcerative genital lesions caused by either herpes simplex virus or syphilis has been shown to increase the risk of HIV transmission by heterosexual (32).

1.2.2 Immunology of HIV infection

1.2.2.1 Characterization

HIV belongs to a class of viruses called *retroviruses* and is a member of the subfamily of lentiviruses. The structure of a HIV is shown in Figure 1-1. The genetic information of a virus is RNA. This RNA is covered with a viral *protein coat;* together, the viral RNA and protein coat make up a *core particle*. The virus gene that specific for synthesizing this protein is called the *gag gene*. For HIV, there are three gag proteins produced, p17, p24, and p10. The core particle also contains several virus-specific enzymes. The gene that codes for these enzymes is a *pol gene* to produce enzyme reverse transcriptase, as well as some other enzymes used in viral replication. *Reverse transcriptase* functions to transcribe the viral genomic RNA into a DNA copy that ultimately intergrates into the host cell genome. The other viral specified by the *pol gene* are p*rotease* and *intergrase*. *Protease* is involved in maturation of viral proteins as the virus particle bud from the cell, and *intergrase* is responsible for integration of the viral

DNA into the cells chromosomal DNA. A viral envelope surrounds the core particle. The gene code for these proteins is the *env gene*. This protein is responsible for binding the virus to the cell receptor. For HIV, there are two env proteins, gp120, and gp41. (33-35).

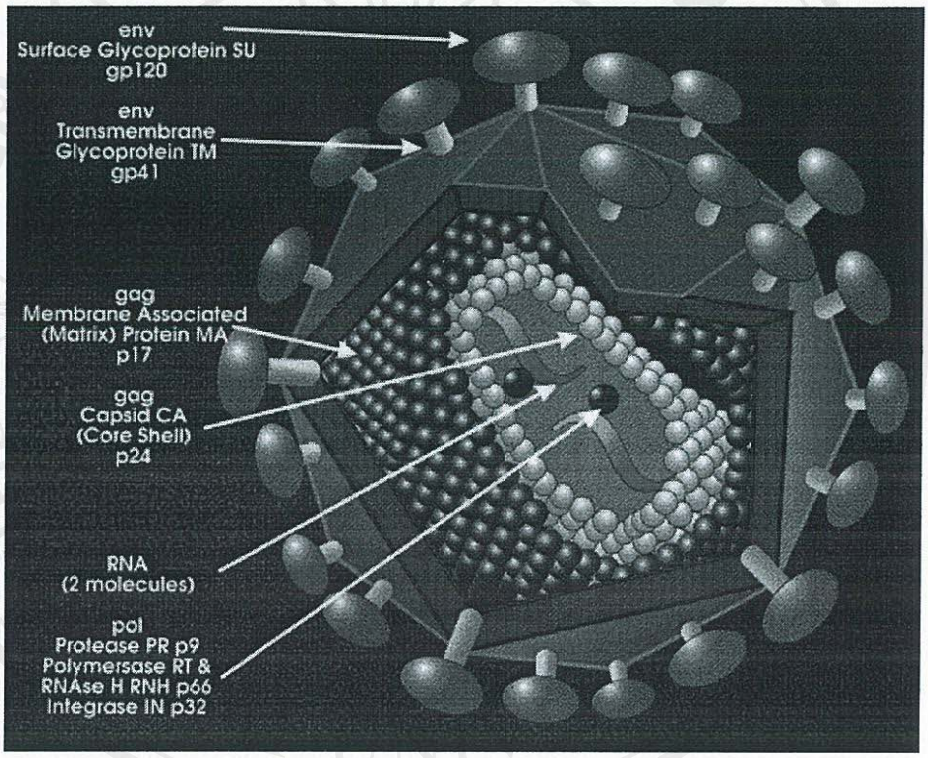


Figure 1-1: The structure of the HIV and its RNA genetic material (1)

1.2.2.2 Target infection cells

CD4 protein is a 55-kDa protein found majoring on the helper T lymphocytes. CD4 protein is a primary targeted for the initiated binding of the HIV's envelope glycoprotein, gp120, which is present on the surface of the virion (38). HIV also infects monocytes/macrophages since it expresses weak CD4 molecules on their surface. Viruses released from CD4+ T cells *in vivo* can preferentially infect monocytoid cells *in vitro*. Besides CD4 expressed cells, HIV can also infect B lymphocytes, megakaryocytes, natural killer (NK) cells, eosinophils, langerhans cells of skin, fibroblasts, laryngeal and bowel epithelial cells, dendritic cells, microglia, astrocytes and brain capillary and thymic endothelial cells. Many of these cells, however, do not have surface CD4 protein. HIV entry into CD4 negative cells may be mediated through the fusion between the fusion domain at the amino terminus of gp41 and the fusion receptor on the cell surface, such as galactosyl ceramide on brain and bowel cells. In addition, HIV entry may also be mediated through the Fc and complement receptors when antibody to HIV is present such as opsonization as is the case for monocytes and macrophages (39).

1.2.2.3 Replication

The life cycle of HIV is shown in Figure 1-2. The virus first binds to the surface of target cells by recognizing receptor using its envelope glycoprotein, gp120, which is present on the surface of the virion, to a cell-surface molecule, CD4, which is found predominantly on the helper-inducer subset of T lymphocytes and on certain other immune cells, including monocytes/macrophages and dendritic cell (36). During this process, the viral envelope is removed, leaving the core particle. Once this happens, a unique virus-specified enzyme called *reverse transcriptase* is activated. This enzyme reads the viral RNA and makes viral DNA. The host cell lacks such an enzyme. The viral DNA then moves to the nucleus of the cell, where it is incorporated using *integrase* into

the host cell's DNA in the chromosomes. Once this viral DNA is integrated into the chromosome, it resembles any other cell gene. As a result, the normal cell machinery reads the integrated viral DNA to make more copies of viral RNA. This viral RNA is then used for two purposes: firstly, some of the viral RNA moves to the cytoplasm and functions as viral mRNA to program the formation of viral proteins; secondly, the rest of the viral RNA becomes genetic material for new virus particles by moving to the cytoplasm and combining with viral proteins. These virus particles are formed at the cell surface and leave the cell by a process called budding. When virus particles initially bud from the cell, they are immature. This is because the viral proteins have not assumed their final form. The viral enzyme protease is responsible for conversion of immature virus particles into mature ones (37). The retrovirus life cycle has several important characteristics. First, most of them do not kill the cells they infect. Second, because viruses integrate their DNA into host chromosomes, they can establish a stable carrier state within the infected cell. As a result, once cells are infected with most retroviruses, they continually produce virus without dying. For some retroviruses, a latent state may also be established in which the retroviral DNA is integrated into the host chromosomes, but it does not program formation of new virus particles. However, sometimes years later, the latent viral DNA may become activated and virus will be produced which is probably important in AIDS (1).

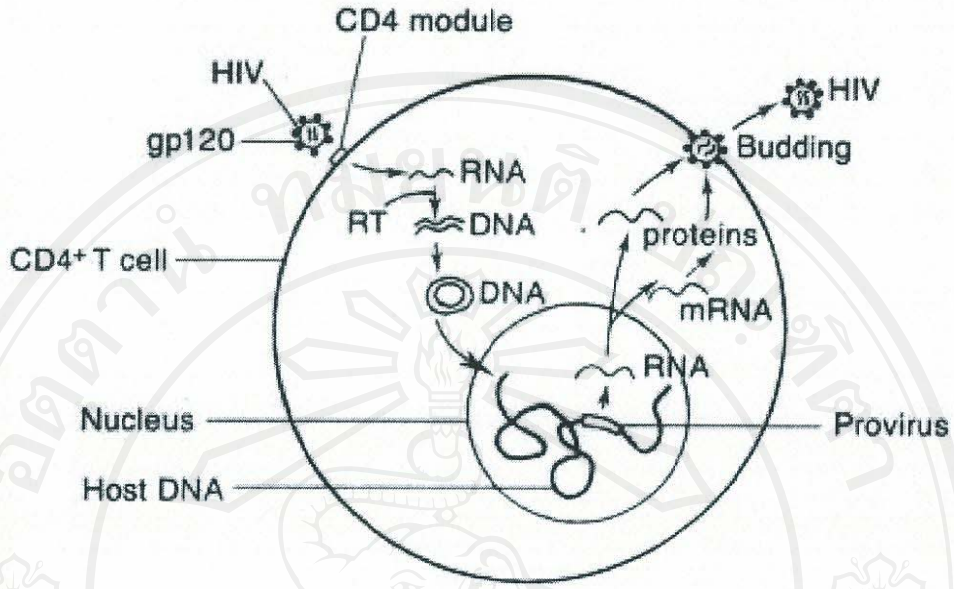


Figure 1-2: The life cycle of human immunodeficiency virus (1)

1.2.3 Host immune response and CD4+ T lymphocyte depletion caused by HIV infection

The immune system has an important role to play in defending body from pathogens and rejection of tumors. The system consists of a wide range of distinct cell types, each with important roles to play, and their ability to recognize foreign molecules (antigens). CD4+ T lymphocytes play a central role in both humoral and cell-mediated immunity. In humoral immunity, it provides the second signal necessary for B-lymphocytes that has bound antigen to divide and secrete antibodies. The B-cells will not complete maturation if its antigen specificity to T-helper cells is absent. CD4+ T lymphocytes also play an important role in cell-mediated immunity (1).

In AIDS patients, the main characteristic of immune disruption is the progressive destruction of the CD4+ lymphocyte population. Many causes of these cells destruction have been posited. A significant decrease of these immune cells resulted in susceptibility to opportunistic infections and malignancy. It is estimated that the time between sexual exposure to HIV and viral dissemination may be up to 72 hrs and approximately 10 billion viral particles are produced each day in untreated HIV-infected person. The initial event is the acute retroviral syndrome, which is accompanied by a precipitous decline in CD4+ T lymphocyte counts, and high concentration of HIV RNA in plasma, reflecting development of cytotoxic T-cell response. The CD4+ cell count was decrease as due to HIV-induced cell death (1). The slope of the CD4+ cell was decline depends on the viral load as shown in Figure 1-3. HIV RNA concentration in plasma shows an initial burst during acute infection and then decline to a set point because of sero-conversion and development of an immune response. With continued infection, viral replication is ongoing, leading to a loss of approximately 10% of CD4+ T lymphocytes per year in most

patients, HIV RNA levels gradually increase and late stage disease is characterized by a CD4+ T lymphocytes count <200 cell/μl and the development of opportunistic infection, selected tumors, wasting, and neurologic complications. The median time between primary HIV infection and the development of AIDS is approximately 11 years. In an untreated patient, the median survival after the cell has fallen to that number is 3.7 years (1, 4, 6).

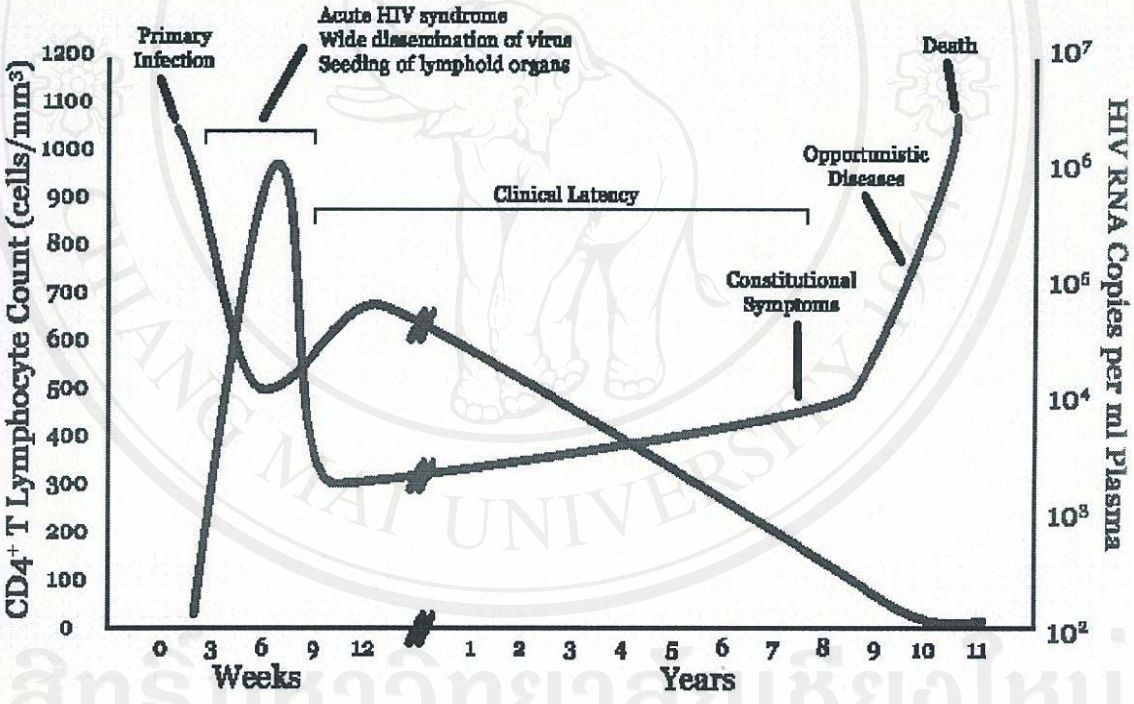


Figure 1-3: CD4+ T lymphocyte numbers over course of HIV infection (2)

1.2.4 Mechanism of CD4+ T cell depletion

1.2.4.1 Direct infection by HIV

Direct infection of CD4+ T lymphocytes i*n vitro* by HIV can result in cell death via a number of discrete mechanisms, including destruction of the cell membrane by large quantities of budding virions. HIV-mediated interruption of normal RNA processes and accumulates the high levels of heterodisperse RNAs, high levels of viral RNA, which dominate cellular protein synthesis capabilities, and accumulation of high levels of unintegrated viral DNA (40).

Syncytia formation plays a role in HIV-mediated cytopathocity of CD4+ cells in *vivo* and has been invoked to explain how uninfected CD4+ T cells may be killed indirectly by fusing with infected cells. Uninfected CD4+ lymphocytes are killed in *vitro* by fusion with an HIV-infected cell; fusion occurs when CD4 molecules on the surface of the uninfected cell bind to gp120 on the infected cell. Multinucleated syncytial cells are also common features of both SIV- and HIV-induced encephalitis. Anti-HIV gp120 antibody can recognize these bound gp120 molecules and cause the elimination of these cells by an antibody-dependent cell-mediated cytotoxicity (ADCC) pathway. Similarly, virus and/or viral proteins may adhere to peripheral blood or follicular dendritic cells and mark them for destruction by ADCC, CD8 lymphocytes, or other unidentified mechanism (41).

1.2.4.2 Indirect mechanism of CD4+ T cell destruction

An additional hypothesis to explain T-cell depletion in HIV infection involves the mechanism of programmed cell death or apoptosis in mature CD4+ cells. In HIV-infected individual, cross-linking of the CD4 receptor may occur as the result of the binding of either gp120 or gp120/anti-gp120 complexes to CD4 molecule, thus setting the stage for apoptosis following antigenic stimulation of the TCR. The results of several recent studies

support the role of apoptosis in CD4+ lymphocyte depletion (42). In addition, it has recently been reported that CD4+ cells from asymptomatic HIV-infected individuals undergo apoptosis (43).

1.2.5 The clinical spectrum of AIDS in relation to CD4+ T lymphocytes

1.2.5.1 Classification of HIV infection

HIV infection induces a chronic and progressive process with a broad spectrum of manifestations and complications from acute primary infection to life-threatening opportunistic infections and malignancies. Increasing levels of viral replication, emergence of a more virulent strain, and progressive destruction of the immune system with dysfunction and depletion of CD4+ lymphocytes and resultant life-threatening processes mark the course of the disease. Symptoms and complications after HIV infection are as outlined in Table 1-1

Table 1.1: World Health organization stages of HIV infection (2)

Stage and clinical features	Typical duration
Viral transmission	2-3 weeks
Acute HIV syndrome	2-3 weeks
Recovery and sero-conversion	1-3 weeks
Asymptomatic chronic HIV infection	Average 8 years
Symptomatic HIV infection/AIDS defining complication	Average 1.3 years

*** HIV infection can be classified into several stages as follows:**

A. Clinical stage I

- Symptomatic
- Persistent generalized lymphadenopathy

B. Clinical stage II

- Weight loss, <10% of body weight
- Minor mucocutaneous manifestation (seborrhoeic dermatitis, fungal nail infections, recurrent oral ulcerations, angular chilitis)
- Herpes zoster within the last five years
- Recurrent upper respiratory tract infection (i.e. bacterial sinusitis)

C. Clinical stage III

- Weight loss, >10% of bodies weight
- Unexplained chronic diarrhea, >1 month
- Unexplained prolonged fever, > 1 month
- Oral candidiasis
- Oral hairy leukoplakia
- Pulmonary tuberculosis within the past year
- Severe bacterial infections (i.e. pneumonia, pyomyositis)

D. Clinical stage IV

- HIV wasting syndrome, as defined by the CDC and Prevention
- *Pneumocystis carinii* pneumonia
- Toxoplasmosis of the brain
- Cryptosporidiosis with diarrhea >1 month
- Crytococcosis, extrapulmonary

- Cytomegalovirus disease of an organ other than liver, spleen or lymph nodes
- Herpes simplex virus infection, mucocutaneous> 1 month
- Progressive multifocal leukoencephalopathy
- Any disseminated endemic mycosis (i.e. histoplasmosis, coccidioidomycosis)
- Candiasis of the oesophagus, trachea, bronchi, or lungs
- Atypical mycobacteriosis, disseminated
- Non-typhoid Salmonella septicaemia
- Extra pulmonary tuberculosis
- Lymphoma
- Kaposi's sarcoma
- HIV encephalopathy, as defined by the Centers for Disease Control and Prevention

Table 1.2: Correlation between CD4+ cells count and HIV complication (2)

CD4 (cells/μl)	Infectious	Non-infectious
>500	Acute HIV syndrome, candidal vaginitis.	Persistent generalized lymphadenopathy Guillain-Barre syndrome, myopathy, and aseptic meningitis.
200-500	Pneumococcal and other bacterial pneumonia, pulmonary tuberculosis, herpes zoster, oropharyngeal candidiasis, cyptosporidiosis, self limited, Kaposis sarcoma, oral hairy lekoplakia.	Cervical intraepithelial neoplasis, cervical cancer, B-cell lymphoma, Anemia, mononeuronal multiplex, idiopathic thrombocytopenic purpura, mononeuronal multiplex, Hodgkin's lymphoma, lymphocytic interstitial pneumonitis.
<200	*Pneumocystis carinii* pneumonia, disseminated histoplasmosis and coccidiomycosis, military/ extrapulmonary TB,progressive multifocal leuko-encephalopathy.	Wasting, peripheral neuropathy, HIV-associated dementia, caridomyopathy, vacuolar myelopathy, progressive polyradiculopathy, non-Hodgkin's lymphoma.
<100	Disseminated cytomegalovirus, toxoplamosis, cryptococosis, chronic, microsporidiosis.	
<50	Disseminated cytomegalovirus, disseminated *Mycobacterium avium* complex .	Central nervous system lymphoma.

1.2.5.2 Prognosis

As HIV infection cause the reduction of CD4+ lymphocytes, absolute number of CD4+ T lymphocytes can be used to predict complications in HIV infected persons as summarized in Figure 1-4. *Mycobacterium avium* and cytomegalovirus (CMV) infections will occur in persons whose have absolute number of CD4+ T lymphocytes less than 60 cells/μl whereas *Pneumocystis carinii* pneumonia will occur in persons whose have absolute number of CD4+ T lymphocytes more than 100 cells/μl. The clinical complications were correlated with CD4+ cells count as shown in Table 1-2 (44)

1.2.5.3 Clinical evaluation and monitoring of HIV infection

At time of first contact to HIV, both clinical and laboratory assessments are very important. One approach is to stratify asymptomatic individuals based on complete blood count and CD4 studies. Patients with CD4+ T lymphocyte count greater than or equal to 500-cells/μl, physician does not give antiretroviral therapy. However, if CD4+ T lymphocyte count is in between 200 and 350 cells/μl and viral load greater than 20000 copies/ml, physician will start antiretroviral therapy. When patients have CD4+ T lymphocyte count less than 200 cells/μl or have symptomatic of AIDS, physician should give prophylaxis against *Pneumocystis carinii* pneumonia and antiretroviral therapy (46)

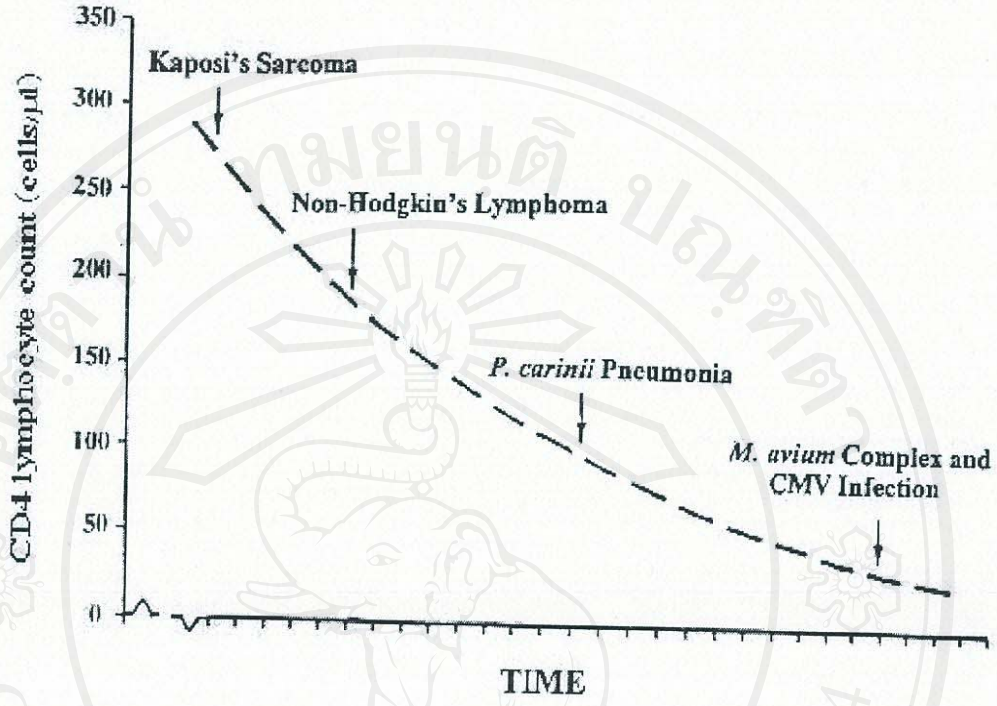


Figure 1-4: Correlation of complication with CD4+ T lymphocyte counts

(See Arch intern Med 1994; 155:1537)

1.2.6 Tests for enumeration of CD4+ T lymphocytes

1.2.6.1 The standard method: Flow cytometry

Flow cytometry technology has many significant advantages for biomedical science development. It is also as a standard method to enumerate CD4+ T lymphocytes (21-22). The stage in the process of measuring the percentage of CD4+ T lymphocytes in the whole blood sample is referred to as "immunophenotyping by flow cytometry". Immunophenotyping refers to the detection of antigenic determinants (which are unique to particular cell types) on the surface of white blood cells using antigen-specific monoclonal antibodies that have been labeled with a fluorescent dye or fluorochrome. Then, the fluorochrome-labeled cells are analyzed by using a flow cytometer. Its ability to analyze thousands of cells in a very short time and to identify a lymphocyte population within the complex mixture of blood elements which categorizes individual cells according to size, granularity, fluorochrome, and intensity of fluorescence. Size and granularity were detected by light scattering and characterize the types of white blood cells. Fluorochrome-labeled antibodies distinguish populations and subpopulations of white blood cells. However, this technology requires expensive instrument, extensive training in running the machine, troubleshooting and data analysis and flow cytometric immunophenotyping is a highly complex technology to perform in limited resources of laboratories in developing countries (25).

1.2.6.2. Alternative technologies for CD4+ T lymphocyte counts

A. Microscopic assay

Cytosphere assay (Coulter Corporation, USA) is a simple, manual method utilizes latex beads coated with CD4 antibody that bind to CD4+ T lymphocytes to form a cell sphere rosette, detected by light microscopy. Binding to smaller spheres coated with anti-CD14 identifies monocytes, which also express CD4 on their surface. The cytosphere assay proved to be easy to perform and interpret, highly reliable in identifying those individual cells, and correlative to flow cytometry in cell counts of HIV infected patients at various levels of immunocompromise. The only instruments needed to perform the assay are a reliable pipettor, a hemacytometer, and a light microscope, making this test extremely beneficial in limited resources. The cost of reagent per test is about 4-8 UD$. However, since monocytes were not depleted this made monocytes and lymphocytes can be difficult to differentiate, visual counting was subjective and manual counting was laborious with smaller number of cell counted. Even several groups have found a good correlation between CD4+ cell count measured by the cytosphere method and flow cytometry, but another group, who analyzed only 10 samples, has found poorer correlation with flow cytometry (r=0.45) (23).

Dynalbeads technique (Daynal AS, Norway) uses magnetic beads coated with anti-CD4 monoclonal antibodies to capture and isolate CD4+ T lymphocytes from whole blood. The sample should be processed with in 24 hrs. Monocytes are removed by using additional beads coated with anti-CD14, thus avoiding potential confusion when counting cells under the microscope as same as Cytosphere assay. CD4+ T lymphocytes are isolated by use of the magnet and lysed, and nuclei are counted after staining with Sternheimer-Malbin staining solution (containing crystal violet, safranin O, ammonium oxalate, and ethanol). This method showed good correlation with flow cytometry (r=0.9)

(24). As described above is indicated that Cyto-Spheres and Dynabead assays are simple and inexpensive compared to conventional flow cytometry. The limitations were described above for example counting under the microscope was both times consuming and laborious and only smaller number of cells were counted. Therefore, there are suitable for smaller regional centers when less than 10 samples are requested per day.

B. Immunoenzymatic assay

Capcelilia CD4/CD8 whole blood immunoassay (Bio-Rad, USA) is another an enzyme immunoassay (EIA) that uses mAb-coated paramagnetic microparticles to identify T cells, by using of anti-CD2 mAb, followed by anti-CD4 (or anti-CD8) mAb-peroxidase conjugate. The assay is in a 90-well format, and up to 43 samples can be assayed in a single run per kit (plus standards). A magnetic frame, plate reader manual washing minifold, and pipettes (including a multi channel pipette) are used for the assay. The Capcellia assay provides an absolute CD4+ cell count calculated from a 4-point standard curve. The measurement of CD4+ cells was well correlated with flow cytometry. However, the correlation with flow cytometry is variable for example in European laboratories (r=0.81), although its performance was lower in West Africa (r=0.61) (27).

TRAxELISA (Innogenetics, Belgium) is a sandwich enzyme immunoassay (EIA), which uses horseradish peroxidase-labelled Mabs directed against CD4 molecules in a micro-titer plate format. Pretreatment of blood results in lysis of cell membranes and release of CD4 molecules. A standard curve is prepared from the 6 concentration of standard CD4 protein. Control and specimen values are determined from the standard curve and reported as cells/μl. Results provide CD4 concentrations, which can be correlated with an absolute CD4+ lymphocyte count. Since the principle of this assay is

based on detection of total CD4 protein in a whole blood lysate to predict CD4+ T cells per micro liter. The results are not dependent on intact or viable cells as with other cytometric techniques. In addition, some diseases such as leukemia, lymphoma and rheumatoid arthritis which may possible have biological factors perturb the ratio of CD4 molecules per cell or the amount of soluble CD4 compared with those for the HIV-sero-positive and control specimens. It may not possible to use the TRAx CD4 assay to predict the absolute CD4+ cells count for all HIV-sero-positive patients. It is expanded needed to define with what frequency soluble CD4 may be present in HIV-sero-positive individuals and to determine whether this has any biological significance. Monocytes are not differentiated from T lymphocytes in this assay, and the test has performed relatively poorly compared with flow cytometry in several reported studies, particularly for patients with CD4 cell counts of less than 200 cells/μl. Therefore, the TRAx CD4 Test kit assay is no longer available in the market (28).

C. Immunofluorescence assay

Zymmune™ assay (Zynaxis Inc., USA) is a magnetic bead based fluorescent immunoassay that provides absolute CD4+ and CD8+ T lymphocyte counts without the use of flow cytometry and hematology. Whole blood is incubated with a mixture of the magnetic and fluorescent bead in the wells of a microplate. During incubation, the monoclonal antibody coated beads from rosettes around the appropriate target cells. The assay plate is then placed on a magnet which drawn down the magnetic beads and any rosette target cells, leaving unbound fluorescent beads and unlabeled cells in suspension. The Zymmune assay format combines a mixture of magnetic and fluorescent microspheres, each targeted to the same antigen. The magnetic particles constitute the separation system for the assay, while the fluorescent particles provide the detection

system. Discrimination between CD4+ T lymphocytes (or CD8 lymphocytes) and monocytes (or NK cells) is provided by antigen density difference. The antigen density being high on the target cells and low on the contaminating cell types. Shear forces generated during the incubation mediate the selection of high-antigen-expressing CD4+ or CD8+ T lymphocyte populations over low-antigen-expressing monocytes or NK cells. The cells and particles in suspension are removed by several wash cycles. Calibrator reagents are added to the designed wells on the plate. The washed rosettes are then re-suspended and the signal measured using a micro-plate fluorescence reader. The numbers of the CD4+ and CD8+ T lymphocytes are then calculated using the calibrator curves. The Zymmune^{TM} system is more cost effective than traditional flow cytometry (29-30).

1.2.7 Monoclonal antibody

1.2.7.1 Introduction of monoclonal antibody

Since the introduction of techniques for producing monoclonal antibody (mAb) by Kohler and Milstein (47), there has been steady expansion of their applications into numerous areas. Kohler and Milstein discovered the hybridoma technique in fusible between an antibody-producing spleen cell and an immortal myeloma cell in 1975. The fused, "hybridoma", cell is adaptable to grow in tissue culture support and capable to produce specific antibody. Once cloned of mAb will secrete only one type of antibody that reacts against only one antigenic determinant. This specific produced antibody is referred to a "monoclonal antibody"

Monoclonal antibodies solved the problems of specificity and reproducibility associated with traditional antisera (47). Monoclonal antibody is now widely used in several areas such as it uses to discover and identify cell surface markers (48), to develop detection or diagnosis assays (49), to purify protein of interest (50), to identify interest

gene and their product, to use for disease therapy (51, 52) and to apply in chemistry testing (53).

1.2.7.2 Principle of monoclonal antibody production

The principle of monoclonal production is to fuse the antibody-secreting cells to the suitable immortal partner cells such as myeloma. In normal cell, there are two ways for DNA (nucleotide) synthesis by the Salvage pathway and the De novo pathway. Myeloma cell lacks of HGPRT (hypoxamthine guanine phosphoribosyl transferase) enzyme, which is the most important for the post-fusion hybridoma selection process. Lacking of this enzyme made myeloma cell impossible to use Salvage pathway for purine biosynthesis. After, myelomas were fused with B cells, which have HGPRT enzyme with the help hybidoma cells (myeloma fused with B-cell) to survive. Adding selective media containing hypoxantine, aminopterin and thymidine (HAT medium), the folic acid antagonist aminopterin blocks the De novo pathway, therefore, the un-fused cells such as animal cells, myeloma cells, fused cells between B cell or between myeloma cells will die. While the splenic B cell counterpart provides the hybrid cell with the support of enzyme HGPRT, have ability to grow by alternatively used the salvage pathway. The hybridomas can survive in tissue culture condition and could secret specific antibody unlimited.

1.2.7.3 Production of monoclonal antibody

To generate a monoclonal antibodies specific for defined antigens, a mouse's immune response could be raised by immunizing the protein of interest. Checking the interest antibody levels before fusion done, the spleen is removed and the cells are harvested. The spleen cells were then mixed with myeloma cells. At step of adding PEG (polyethylene glycol) it will promote cells' membrane for their fusion. Fusion mixture contains a drug which kills myelomas which is $HGPRT^-$, the spleen cells which is $HGPRT^+$ are not sensitive to the drug but die naturally in few weeks. The survival cells

are "hybridoma" combination advantage nature of myeloma which is immortal cell and HGPRT gene from immune B cell. This hybridoma can grow continuously *in vitro* and secrete a single monoclonal antibody. The hybridomas are assayed by limiting techniques to screen for interest clones as shown in figure 1.4. The clones can be grown *in vitro* such as tissue culture technologies for producing antibody or in *vivo* such as ascitic fluid producing in mouse. The process of monoclonal antibody production is shown in Figure 1-5.

1.2.7.4 Purification of monoclonal antibody by affinity chromatography

The technique described here is for IgM purification by using a fraction collector, AKTAprime, with HiTrap IgM purification HP columns as shown in Figure 1-6. The columns are packed with a thiophilic adsorption medium (2-mercaptopyridine coupled to Sepharose High Performance). The interaction between the protein and the ligand has been suggested to result from the combined electron donating and accepting action of the ligand in a mixed mode hydrophilic-hydrophobic interaction. The simple steps of purification by AKTA prime are shown in Figure 1-7 and 1-8

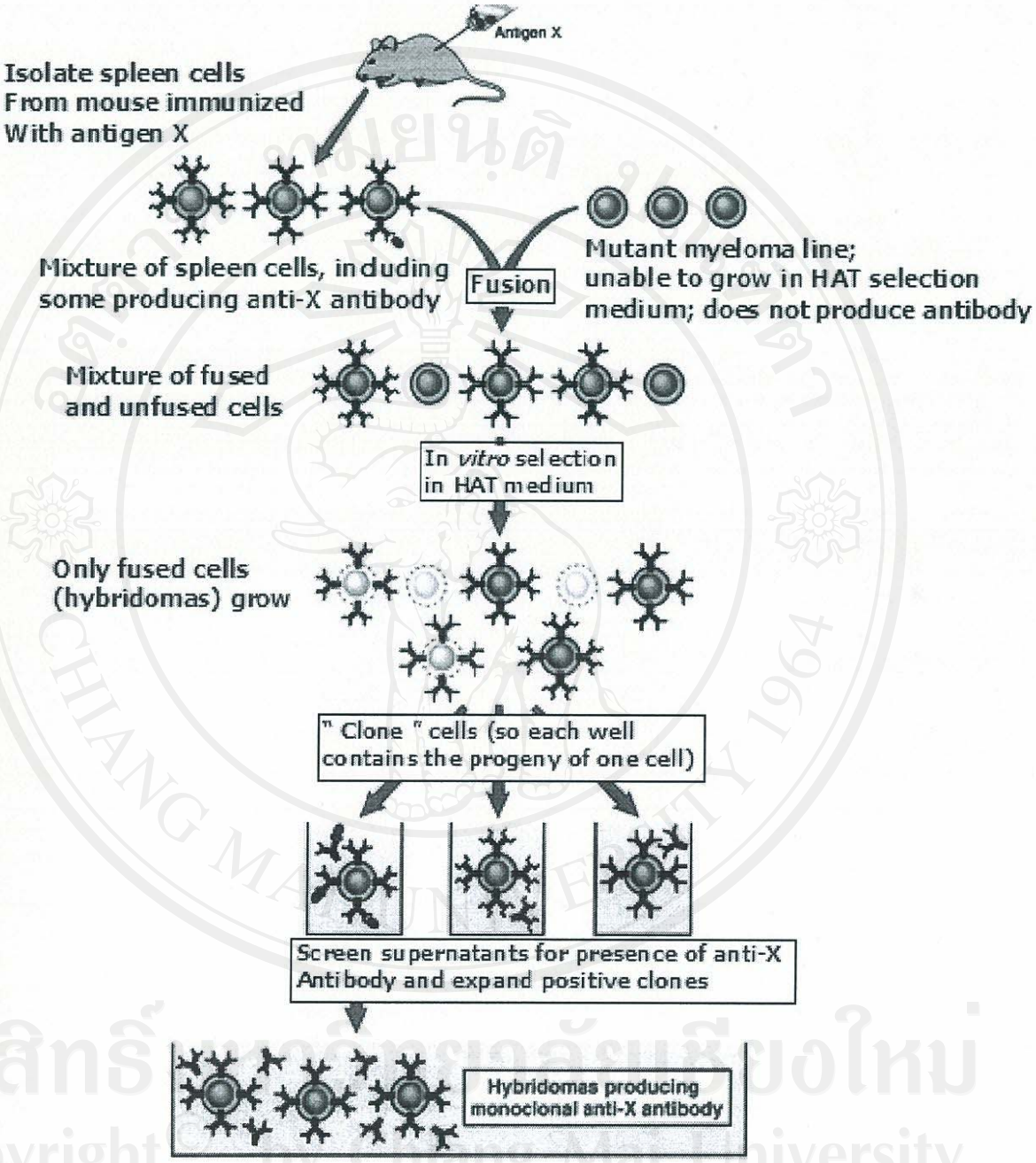


Figure 1-5: Monoclonal antibodies production (adapted from http://www.uccs.edu)

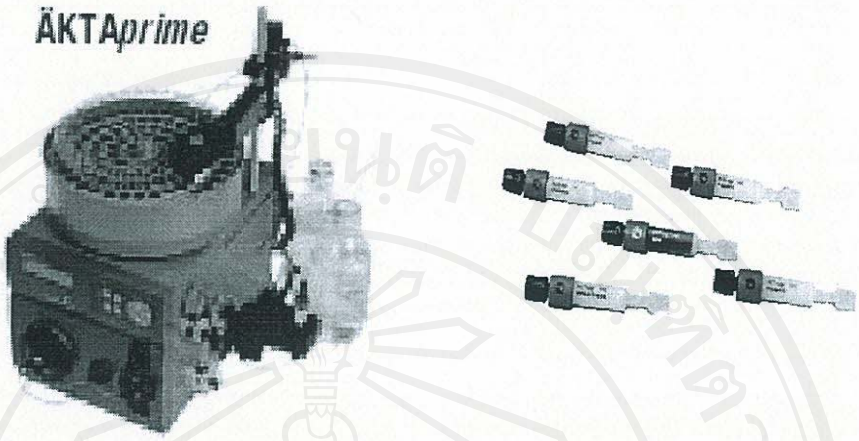


Figure 1-6: Automatic purification machine (AKTA prime) and HiTrap affinity columns (Adapted from Amersham Phamacia Biotech guideline)

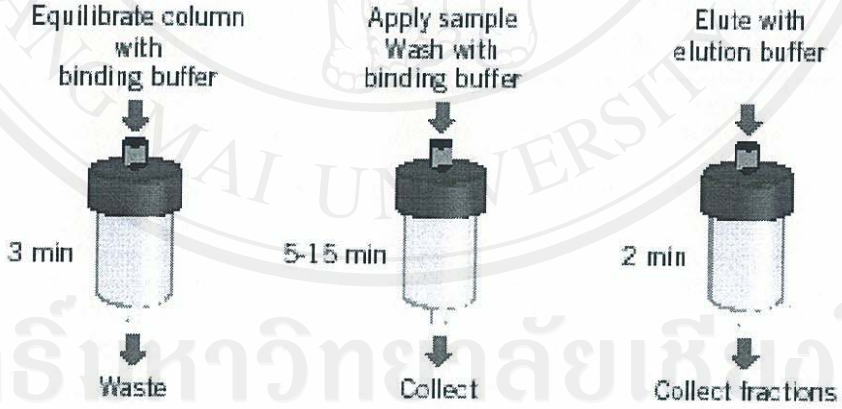
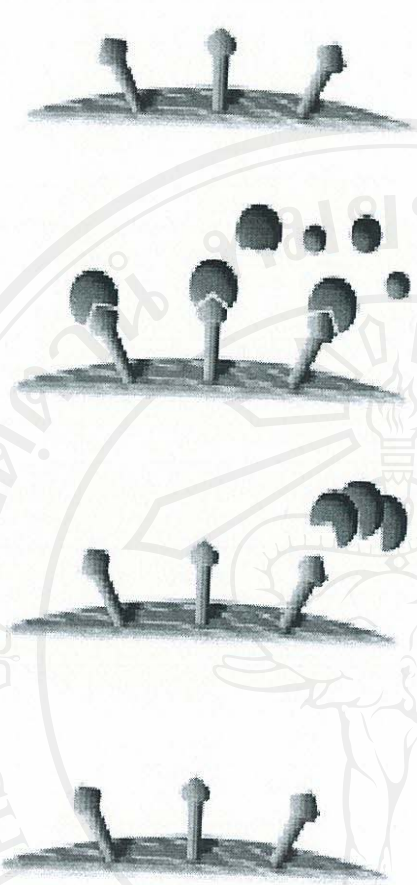


Figure 1-7: The simple procedure by affinity purification on pre-packed HiTap columns (Adapted from Amersham Phamacia Biotech guideline)



1. Affinity medium is equilibrated in binding buffer
2. Sample is applied under conditions that favor specific binding of the target molecules to a complementary binding substance (the ligand). Target substances binds specifically, but reversibly, to the ligand and unbound material washes through the column
3. Target protein is recovered by changing conditions to favor elution of the bound molecules. Elution is performed specifically, using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Target protein is collected in a purified, concentrated form
4. Affinity medium is re-equilibrated with binding buffer

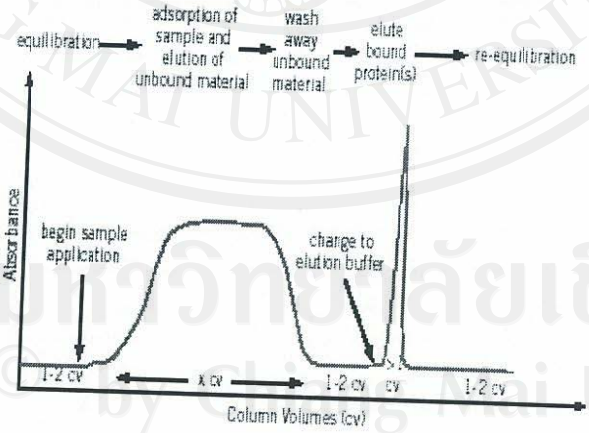


Figure 1-8: Purification steps by HiTrap IgM Purification HP column (Adapted from Amersham Phamacia Biotech guideline)

1.3 Objectives:

- To prepare and purify CD4 mAb from clone MT4.
- To develop reagent and method for enumeration of CD4+ lymphocytes in whole blood by non-flow cytometric method.
- To validate the developed technique and reagent by comparing CD4+ T lymphocytes enumeration to standard flow cytometric method.