CHAPTER I

INTRODUCTION

1.1 Statement of problems

Infection with human immunodeficiency virus (HIV) induces a broad spectrum of manifestations from acute primary infection to progressive deterioration of the cell-mediated immune system, leading to secondary infection with a variety of opportunistic pathogens and the development of neoplasms, the hallmarks of acquired immunodeficiency syndrome (AIDS). HIV can infect several cell types, but most importantly the CD4 lymphocytes. The CD4 antigen on the host cell surface serves as the primary receptor for HIV. After entry, it may replicate then bud from infected cell to enter neighboring cells. The result of this is direct cell killing lead to CD4 lymphocyte depletion (1).

The depletion of CD4 lymphocytes is gradually and indicates severity or progression of disease. When the number of CD4 lymphocytes decrease less than 500 cells/μl, patients may infect with opportunistic pathogens. If it decreases less than 200 cells/μl, opportunistic infection and malignancy will occur with higher rate. Patients generally dead from these complication (2). Besides the number of CD4 lymphocytes indicates progression of AIDS, it is also useful to monitoring of therapy in HIV infected person. The U.S. Public Health Service (PHS) has recommended that CD4 lymphocyte levels must be monitored every 3 to 6 months in all HIV infected persons (3). If the absolute number of CD4 lymphocytes is less than 500 cells/μl, physician

should start antiretroviral therapy and when patients have the absolute number of CD4 lymphocytes less than 200 cells/µl, physician should give prophylaxis of opportunistic infection combine with antiretroviral therapy (4-5). Consequently, enumeration of CD4 lymphocytes is useful to classification of HIV infection, prediction of severity or progression of disease and guideline for monitoring of therapy in HIV infected persons.

At the present, the standard method for CD4 lymphocyte enumeration is immunophenotyping by flow cytometry. The result exhibits percentage of CD4 lymphocytes, which must be combined with white blood cell count and percentage of lymphocytes from differential white blood cell count (CBC) in order to calculate the absolute number of CD4 lymphocytes (6). This flow cytometric method is precise and reliable, however, the reagents used are imported and very expensive. Several types of reagents for enumeration of CD4 lymphocytes by flow cytometry, including two- and three-color reagents, have been developed. By two-color reagents, whole blood specimen is divided into four tubes, stained with two-color immunofluorescence using directly conjugated monoclonal antibody pairs and followed by lysis of erythrocytes, fixation of leukocytes and finally analysis by flow cytometer. By using these two-color reagents, a potential problem is that in some samples, particularly in blood samples obtained from AIDS patients on therapy, there may be contained lysis-resistant red blood cells. During flow cytometric analysis, these non-lysed red blood cell population is located in the lymphocyte light scatter gate. If significant numbers of contaminated red blood cells are present in the acquisition lymphocyte gate, the obtained CD4 lymphocyte number will be erroneously decreased. In contrast to the two-color reagent, a single tube whole blood

staining method is carried out when using three-color reagents. The three-color reagents used the third fluorochrome to discriminate red blood cells from white blood cells and therefore can overcome the contamination of red blood cell in lymphocyte gate.

In the present study, three-color reagents for CD4 lymphocyte enumeration by flow cytometry were developed. The three-color immunofluorescence monoclonal antibodies used in this study consisting of commercial anti-CD45 monoclonal antibody labeled with peridinin chlorophyll protein (PerCP), anti-CD4 monoclonal antibody labeled with fluorescein isothiocyanate (FITC) and anti-CD14 monoclonal antibody plus phycoerythrin (PE). Single tube of whole blood specimen was stained with the three-color immunofluorescence monoclonal antibodies. Then, red blood cells were lysed, and leukocytes were fixed and analyzed by a flow cytometer. In addition, the in-house red blood cells (RBC) lysing solution was also developed. For RBC lysing solution, several solutions such as 1% ammonium oxalate, 0.83% ammonium chloride, ammonium chloride tris buffer, hypotonic ammonium chloride and others were studied. Each solution were used as RBC lysing solution and compared with the commercial lysing solution for enumeration of CD4, CD8 and CD3 lymphocytes.

1.2 Literature review

1.2.1 HIV infection and AIDS

1.2.1.1 Historical milestones

AIDS was recognized in May 1981, when the first cases of *Pneumocystis carinii* pneumonia (PCP) were noted in previously healthy, young homosexual men from Los Angeles. Since that time, HIV infection has become a global health problem of enormous magnitude. In the fall of 1982, the Centers for Disease Control and Prevention (CDC) established a case definition of this newly recognized syndrome. Since then, there have been several revisions of the AIDS case definition. The 1987 revision which greatly increased the number of reportable AIDS cases, included HIV encephalopathy and wasting syndrome, and presumptive diagnoses made on the basis of laboratory evidence; it eliminated cases due to other causes of immunodeficiency. HIV was first isolated in May 1983 and generally accepted as the cause of AIDS in 1984. The HIV enzyme-linked immunosorbent assay (ELISA) antibody test became available for clinical use and screening of blood donations in March 1985. In March 1987, azidothymidine (AZT; now known as zidovudine or ZDV) was made available as an investigational agent and approved by the Food and Drug Administration 4 months later based on promising initial results in AIDS patients. Early intervention guidelines for HIV infected adults were established in 1989; these included ZDV for patients with CD4 cell counts of less than 500 cells/μl and prophylaxis against PCP for those with CD4 cell counts of less than 200 cells/μl (or with a prior episode for PCP regardless of CD4 count). Zidovudine was believed to improve survival in patients with AIDS (5). The CDC guidelines concerning CD4 lymphocyte determinations were first published in the morbidity and mortality weekly

report (MMWR) in 1992 to provide laboratorians with the most complete information about how to measure CD4 lymphocytes in blood from HIV infected persons by using flow cytometry. Then the guidelines were revised in 1993 and published in 1994. In January 1997, the CDC developed the revised guidelines for laboratories performing lymphocyte immunophenotyping assays in HIV infected persons again. This report updated previous recommendations and reflected current technology in a field that was rapidly changing (3).

1.2.1.2 HIV characteristic

HIV-1 and HIV-2, the causative of AIDS have about 50% homology at the nucleotide level, are closely related to several nonhuman primate immunodeficiency viruses and are some what distantly related to other lentiviruses and nonprimate mamalian immunodeficiency viruses. The HIV have the same general characteristic as other retroviruses such as they are enveloped viruses with a duplex RNA genome of approximately 10 Kb, replicating via a complementary DNA intermediate (cDNA) which becomes integrated into the host chromosome (as the HIV provirus). The complementary DNA is synthesized from an RNA genome via a virion-specified reverse transcriptase. However, in contrast to simple retroviruses, HIV-1 and HIV-2 have a much more complex genome, additional genes and gene products and a more complex replicative cycle (Table 1.1 and Figure 1.1). HIV like other enveloped viruses is susceptible to detergents, lipid solvents and heat but it is more resistant to these disinfection measures than many other enveloped viruses, especially when stabilized by host proteins (7).

Table1.1 Genes and proteins of human (and primate) immunodeficiency viruses (7).

Gene	Proteins	Function	Analogues found in:	
			Simple retroviruses	nonprimate lentiviruses
gag	Ca (p24)	Virion structure and assembly	X	X
	Ma (p17)	Virion structure and assembly	X	X
	NC (p9)	Stabilizes virion genomic RNA	X	X
	p7	Virion structure	X	X
pol	PR (p10)	Cleavage of Gag-Pol polyproteins	X	X
	RT (p51/66)	Reverse transcription	X	X
	RNAse-H	Degradation of RNA template during RT	X	X
	IN (p32)	Integration of virion cDNA	X	X
env	gp41	Transmembrane envelope glycoprotein; fusion protein	X	X
	gp120	External membrane glycoprotein; virion attachment (to CD4 and chemokine receptors)	X	X
tat	Tat	Augments transcription		X
rev	Rev	Blocks cytoplasmic transport of unspliced mRNA		X
nef	Nef	Complex; augments in vivo HIV replication		
vpu	Vpu	Complex; CD4 down-regulation		
vpr	Vpr	Complex; cell cycle arrest		
vif	Vif	Augments virion infectivity		
vpx[a]	Vpx	Complex; cell cycle arrest		

[a]*vpx* is a *vpr* analogue found only in HIV-2 and some SIV.

Key: Ca=capsid, Ma=matrix, NC=nucleocapsid, PR=protease

RT=reverse transcriptase, IN=integrase.

1.2.1.3 HIV life cycle

The CD4 antigen on the host cell surface serves as the primary receptor for HIV. It binds via the CD4-binding site on gp120. This results in displacement of gp120 and proteolytic cleavage of the third variable loop (V3), which allows interaction between a fusion domain on gp41 and a fusion receptor on the cell surface. Following membrane fusion, viral RNA enters into the cell and is reverse-transcribed into DNA by reverse transcriptase. The transcribed viral DNA provirus is transported into the nucleus and integrated into chromosomal DNA. The proviral DNA may remain latent in the cell for a variable period of time before starting to produce viral genomic RNA and viral mRNA. The viral mRNA is used to synthesize viral polypeptides. The viral genomic RNA is packaged into the viral capsid and buds through the cell membrane while incorporating the viral envelope glycoprotein present on the cell membrane. During the initial phase of HIV infection, approximately 10^9 virions are produced per day, of which 10^6 are mutant variants (8).
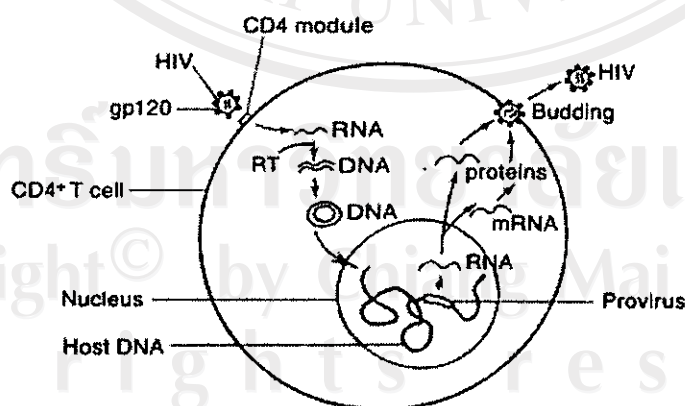

Figure 1.1 HIV life cycle (8).

Besides CD4 lymphocytes, HIV can also infect B lymphocytes, monocytes, macrophages, megakaryocytes, natural killer 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 do not have surface CD4. 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 (8).

1.2.1.4 CD4 lymphocyte depletion

After a person is infected with HIV, an enormous battle ensues between HIV and the immune system. As immune cells destroy virus particles, more virus particles are produced. As virus-infected cells are destroyed by T_c cells (CD8 lymphocytes), more immune cells replace those killed. Each day as HIV disease progresses, an estimated 1 billion virus particles are produced and destroyed as 2 billion immune cells are replaced. Although these early battles result in a draw, the virus eventually wins the war. As the years pass, it becomes more difficult to replace immune cells such as CD4 lymphocytes. In addition, because HIV can mutate rapidly, producing virus particles that progressively become more variable in structure, the immune system simply cannot keep up the fight. Without activated CD4 lymphocytes and macrophages, the immune system cannot see infectious microbes. Because CD4 lymphocytes are greatly diminished in number, B cells are not stimulated to form

plasma cells, which produce antibodies to combat infections. Similarly, cytokines are produced in amounts insufficient to activate macrophages and CD8 lymphocytes (9). Although it has often been assumed that depletion of CD4 lymphocytes and cells of the monocyte/macrophage lineage is a direct result of HIV or SIV infection, the extent to which the observed depletion is the result of the virus directly killing the cells is unclear. It is relatively easy to demonstrate a number of virus-related mechanisms of cell killing in vitro. However, the extent to which these mechanisms are responsible for the depletion of immune competent cells in vivo has not been defined and likely represents a combination of processes.

Direct killing of single cells: a number of mechanisms have been proposed for the killing in vitro of single HIV-infected cells. These include copious budding of virions from the cell surface with concomitant disruption of the integrity of the cell membrane, interference with cellular RNA processing, disruption of cellular protein synthesis due to high levels of viral RNA, and the accumulation of high levels of unintegrated viral DNA in the cells. The intracellular interaction between HIV gp120 and CD4 has also been implicated in cytopathicity. There are clear strain differences in the killing of cells by HIV-1; these are determined largely by gp120 sequences, providing strong support for the importance of *env* in this process. This role need not involve toxicity directly; rather, as with some simple retroviruses, certain strains could be less effective in inducing resistance to superinfection than others, allowing reinfection of a cell by the virus it produces and a consequent buildup of viral replication to toxic levels.

Syncytium formation: a potential explanation of the cause of the death of cells that are not directly infected with HIV is the formation of multinucleated giant cells or

syncytia. Some strains of HIV and SIV known as SI strains efficiently induce syncytia. 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. However, the relative contribution of syncytium formation to the depletion of CD4 lymphocytes in vivo remains unknown. Immune-mediated attack on infected cells and cells coated with viral proteins: both humoral and cell-mediated responses likely contribute to the partial suppression of virus and viral replication, acting both on the virus and on infected CD4 lymphocytes during the prolonged coursed of clinical latency. HIV-specific immune responses may also target uninfected immune competent cells that have viral proteins bound to their surfaces. Soluble gp120 can be shed from free virion and from HIV-infected cells and can bind to CD4 molecules on the surface of uninfected CD4 lymphocytes and monocyte/macrophages. 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. In addition, gp120-specific CD8 lymphocytes may target uninfected cells that have bound gp120. 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.

Apoptosis: it has been suggested that HIV infection causes apoptosis through an aberrant signaling mechanism. During HIV infection, cross-linking of the CD4 receptor could occur following binding of either gp120 or gp120/anti-gp120 complexes to CD4, thus providing the first of the two signals required for apoptosis.

The cell would then be primed for the second apoptotic signal such as activation of the T-cell receptor, which could be by either conventional antigens or superantigens. Several experiments have suggested a role for HIV-induced apoptosis in CD4 lymphocyte depletion. Complexes of gp120/anti-gp120 are present on the surface of CD4 lymphocytes in HIV-infected individuals. Although it has not been formally demonstrated that gp120-induced CD4 cross-linking results in signal transduction, extremely low concentrations of gp120 can prime cells for apoptosis. Apoptosis can be induced in CD4 lymphocytes from asymptomatic HIV-infection individuals if the cells are exposed in vitro to a superantigen or mitogen. High levels of anti-histone and anti-double-stranded DNA antibodies are seen in HIV-infected individuals. This observation could be explained by the release of nuclear contents during apoptosis. Both CD4 and CD8 lymphocytes from HIV-infected individuals can apparently undergo spontaneous apoptosis in vitro. However, most of the apoptosis in lymphoid tissue occurs in cells that are not infected with HIV and there is no correlation between apoptosis and the stage of HIV disease. Apoptosis in lymphoid tissue of HIV infected individuals may reflect the degree of activation of the lymphoid tissue (10). The recent study (11) provides a mechanistic view on how HIV-1 induces apoptotic death of infected primary human CD4 lymphocytes. The intrinsic mitochondrial pathway of apoptosis is the primary mechanism that induces CD4 lymphocytes to undergo apoptosis. Mitochondrial membrane permeabilization may be a consequence of the activation of the p53 pathway. Once phosphorylated, p53 induces up-regulation of Bax, which may translocate to the mitochondrial membrane and promote cytochrome *c* and apoptosis-inducing factor (AIF) release. This phenomenon may be especially relevant in primary acute infection when high levels

of virus are present and no potent mechanisms of viral control are yet fully operational. The primary infection stage has the highest proportion of CD4 lymphocytes infected during the course of HIV infection. This p53-mediated apoptosis may be responsible for the precipitous drop in CD4 lymphocytes seen in primary acute HIV-infected patients, with eventual stabilization of both CD4 and viral loads. These events set the stage for determining the propensity for progression to AIDS. The mechanism by which HIV mediates this process remains to be further clarified.

1.2.2 The clinical spectrum of AIDS in relation to CD4 lymphocytes
1.2.2.1 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. The course of the disease is marked by increasing levels of viral replication, emergence of a more virulent strain, and progressive destruction of the immune system with dysfunction and depletion of CD4 positive cells and resultant life-threatening processes. HIV disease can be more accurately staged by CD4 lymphocyte counts, HIV symptoms and complications as outlined in Table 1.2 (12).

Acute primary HIV infection: Primary infection with HIV is typically asymptomatic. However, 30-50% of patients may have symptoms. Acute primary HIV infection; known as acute HIV retroviral syndrome is characterized by fever, headache, malaise, myalgia, arthralgia, pharyngitis, nausea, anorexia, and a diffuse erythematous rash. Symmetrical lymphadenophthy is common. Hepatitis and

meningitis may be seen less frequently, as well as pneumonitis. Laboratory abnormalities are non-specific and suggestive of an acute viral illness. Leukopenia, lymphopenia, and thrombocytopenia are most common, and occasionally a leukocytosis is seen. Atypical lymphocytes may be note as patient's symptom resolve. An increase in CD8 lymphocyte counts with a resultant inversion in the CD4:CD8 ratio is frequently noted, and there may be a 50 to 100 cells/µl decline in the CD4 lymphocyte count as patients' symptom improve. Virological evaluation initially shows high levels of free infectious virions in the plasma. Plasma viremia is rapidly decreased with the development of a cellular immune response. HIV p24 antigenemia may be detected before HIV Gag and Env structural protein antibodies appear.

Asymptomatic HIV infection: Although diffuse lymphadenopathy and headache may be present, there are generally no chronic signs or symptoms attributable to HIV during the asymptomatic phase of the disease. There is, however, a wide variety of laboratory abnormalities, including anemia, neutopenia, thrombocytopenia, an increase transamenases. Hematological abnormalities are typically mild, although thrombocytopenia may be severe and require therapy. The CD4 lymphocyte count, the CD8 lymphocyte count and the serum p24 antigen level determenations are more direct measures of HIV disease and have prognostic value for disease progression and survival. The absolute number of circulating CD4 lymphocytes is predictive of the relative risk of various complication of HIV disease, including the broad spectrum of opportunistic infections. Over time, laboratory evidence of progressive immunodeficiency is note with a decline in the absolute number of circulating CD4 lymphocytes. Although variation in the CD4 lymphocyte

counts o ccur, a d ecline of 40-80 cells/µl per year in CD4 lymphocyte can be seen. This decline in the CD4 lymphocyte count varies from patient to patient; some patients may have a more rapid decline in the CD4 lymphocyte counts, whereas others may have more stable counts over longer period of time.

Early symptomatic HIV disease: Early clinical manifestations of HIV disease include constitutional symptoms such as headache, fatigue, malaise, myalgia, fever, night sweats, anorexia, diarrhea and weight loss. Symptoms may be present alone or in combination. Generalized lymphadenopathy involving extra inguinal sites may persist during both the asymptomatic and symptomatic phases of the disease. Persistent generalized lymphadenopathy may be present with or without other manifestations of HIV disease and is not predictive of disease progression. However, regression of lymphadenopathy may indicate an increase risk for disease progression, and enlargement of lymphnode may indicate non-Hodgkin's lymphoma or tuberculosis. Enlargement of the liver and spleen can also be seen and is typically nonspecific early in the disease. A wide variety of mucocutaneous conditions can occur and should alert the physician to the possible presence of HIV infection. Laboratory abnormalities include anemia, leukopenia, lymphopenia and thrombocytopenia may be note. Proteinuria, hypoalbuminemia, and elevation are serum blood urea nitrogen or creatinine levels may be indicative of HIV nephropathy. Mild elevations in serum transaminase are common and typically nonspecific. More modest elevations in serum transaminase should suggest the presence of an opportunistic infection, malignancy, or possible adverse reaction to a medication. Decreases in serum cholesterol and increase in serum globulin may also be seen. Progressive declines in CD4 lymphocyte count are commonly note. A decline in the

CD4 lymphocyte counts to fewer than 200 cells/µl presents an increased risk for disease progression. The risk of disease progression is approximately 20-30% over a 24-month period. Antiretroviral therapy will reduce this risk two to three folds.

Late symptomatic HIV disease: Late HIV disease is characterized by declining CD4 lymphocyte count (50-200 cells/µl), persistent or progressive constitutional symptoms, opportunistic infections, malignancies, wasting, and dementia. Common constitutional symptoms include fatigue, fever, anorexia, nausea, vomiting, diarrhea, and weight loss. Fatigue may be profound and signal the presence of an opportunistic infection. F ever should p rompt an e valuation for o pportunistic infection. Typically, fever is accompanied by symptoms that should direct the initial evaluation. Anorexia, nausea, and vomiting may suggest a gastric process secondary to *Candida*, *M. avium-complex* infection, cytomegalovirus infection, kaposi's sarcoma or lymphoma, or an adverse reaction to any number of medication. Vomiting may also reflect a central nervous system process. Diarrhea may be debilitating and result in progressive weight loss and wasting. Large volumes of watery diarrhea with abdominal pain and weight loss suggest an enteropathy. In addition to enteric pathogens, *Cryptosporidium*, *I. belli*, *Microsporidium*, and *M. avium-complex* infection should be suspected. Frequent small volumes of diarrhea with lower abdominal pain suggest colitis. The most frequent pathogen is cytomegalovirus.

Advanced HIV disease: Patients with advanced HIV disease have an increase likelihood of disseminated infection with *M. avium-complex* and cytomegalovirus, severe wasting, dementia, and death. Wasting syndrome is characterized by unexplained progressive weight loss with or without diarrhea and severe debilitation. Because several infections can lead to diarrhea and cachexia, a

careful evaluation for *Cryptosporidium*, cytomegalovirus, and *M. avium-complex* should be done. Patients with persistent fever of unknown etiology should be evaluated for *P. carrinii* pneumonia, mycobacterial infection, and disseminated cytomegalovirus infection. Evaluation should include chest radiography, blood cultures, bone marrow aspirate and biopsy, and liver biopsy if abnormalities in liver function are noted. AIDS-dementia complex is characterized by cognitive, behavioral, and motor dysfunction and is most common late in HIV disease after the development of HIV-related opportunistic infection or malignancies. Impaired concentration, forgetfulness, and slowed cognitive function are early symptoms of dementia. Changes in personality and behavior characterized by apathy and withdrawal may also be note. In rare cases, agitation, irritability, confusion, and psychosis may be seen. Motor symptoms, including gait disturbances, loss of coordination, and leg weakness, can also occur. With progressive complaints, confusion, psychosis, impaired rapid movements, hyperreflexia, release reflexes (snout, glabella, and grasp), weakness, ataxia, spasticity, bladder and bowel incontinence, and myoclonus can be found. Ataxia is a late feature and can be disabling in the face of progressive leg weakness. Cerebral atrophy and patchy or diffusely increased signal intensity in the white matter and less frequently the basal ganglia or thalamus are typical neuroimaging findings. Cerebral spinal fluid examination can show a mild elevation in protein and a mild pleocytosis with a predominance of mononuclear cells and can assist in the exclusion of meningitis or neurosyphilis.

Table 1.2 Stages of HIV disease (12).

Stage and clinical features	Typical duration	CD4 lymphocyte count Range (cells/μl)
Acute primary HIV infection	1-2 week	1000-500
Asymptomatic (No symptoms or signs other than lymphadenopathy)	>10 years	750-500
Early symptomatic (Non life-threatening infections, or chronic or intermittent symptoms)	0-5 years	500-100
Late symptomatic (increasingly severe symptoms, life-threatening infections, or cancers)	0-3 years	200-50
Advanced HIV disease (increasingly hazard of death, and serious opportunistic infections)	1-2 years	50-0

1.2.2.2 Prognosis

AIDS- free survival rate was studied in 813 HIV-seropositive persons (13). It was found that the persons whose absolute number of CD4 lymphocytes more than 500 cells/μl have higher survival rate than the persons whose absolute number of CD4 lymphocytes was lower. Survival rate of forty percent of the persons whose have absolute number of CD4 lymphocytes less than 163 cells/μl was one year while eighty percent of the persons whose have absolute number of CD4 lymphocytes more than 366 cells/μl was three years. Absolute number of CD4 lymphocytes can also use to predict complications in HIV infected person. *Mycobacterium avium* and

cytomegalovirus (CMV) infections will occur in persons whose have absolute number of CD4 lymphocytes less than 60 cells/µl whereas *Pneumocystis carinii* pneumonia will occur in persons whose have absolute number of CD4 lymphocytes more than 100 cells/µl as was shown in Figure 1.2 (14).
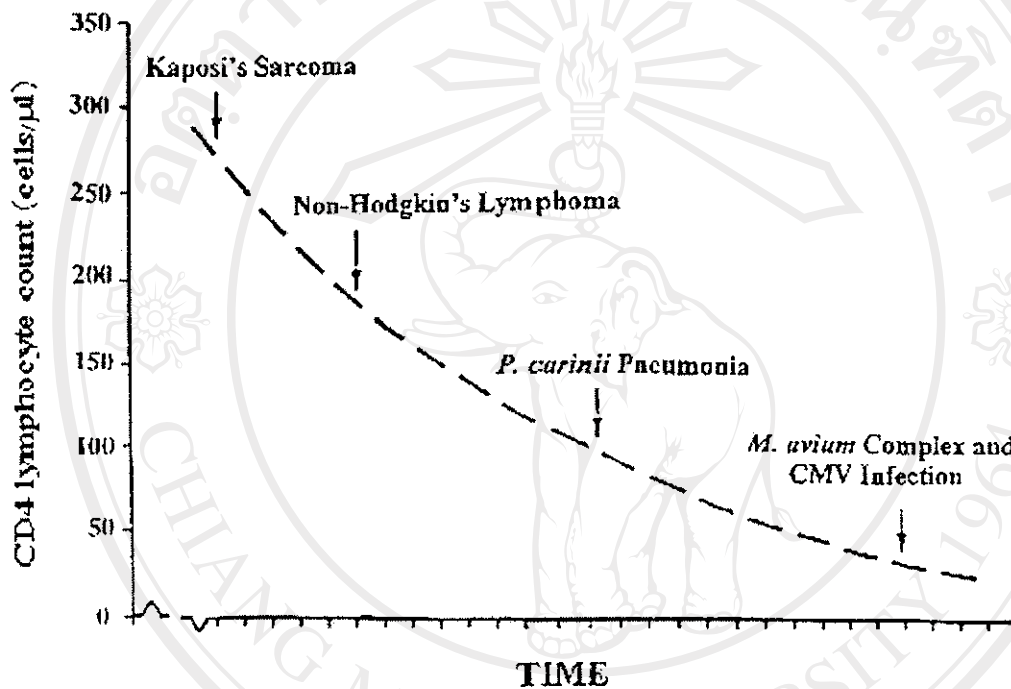
Figure 1.2 Complications and infections in HIV infected persons with absolute CD4 lymphocyte count less than 500 cells/µl (14).

1.2.2.3 Monitoring of therapy

At time of first contact, both clinical and laboratory assessments are important. One approach is to stratify asymptomatic individuals on the basis of complete blood count and CD4 studies. For those with normal results, an initial follow-up frequency of 3 to 6 months is adequate. In contrast, individuals with low,

intermediate, or rapidly declining CD4 values might be seen every 2 to 3 months, with a shorter follow-up period if symptoms are present. Once the decision is made to embark on medical therapy, follow-up recommendations intensify accordingly (4). Patients with CD4 lymphocyte count greater than or equal to 500 cells/μl, physician does not give antiretroviral therapy. If CD4 lymphocyte count between 200 and 500-cells/μl, physician should start antiretroviral therapy. When patients have CD4 lymphocyte count less than 200 cells/μl, physician should give prophylaxis against *Pneumocystis carinii* pneumonia and antiretroviral therapy (5).

1.2.3 The standard method for CD4 lymphocyte enumeration

Most laboratories measure absolute CD4 lymphocyte counts in whole blood by a multiplatform, three-stage process. The absolute number of CD4 lymphocytes is the product of three laboratory techniques: the white blood cell count, the percentage of lymphocytes from differential white blood cells, and the percentage of CD4 lymphocytes. The last stage in the process of measuring the percentage of CD4 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, 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. Consequently, flow cytometric immunophenotyping is a highly complex technology (3).

1.2.3.1 Monoclonal antibody panels

Monoclonal antibody panels must contain appropriate monoclonal antibody combinations to enumerate CD4 and CD8 lymphocytes and to ensure the quality of the results. CD4 lymphocytes must be identified as being positive for CD3 and CD4 as well as CD8 lymphocytes must be identified as being positive for both CD3 and CD8.

Two-color monoclonal antibody panels

The recommended two-color immunophenotyping antibody panel (Table 1.3) is delineated by CD nomenclature and fluorochrome. It provides data useful for defining the T-cell population and subpopulations, determining the recovery and purity of the lymphocytes in the gate, setting cursors for positivity, accounting for all lymphocytes in the sample, monitoring tube-to-tube variability and monitoring T-cell, B-cell, and natural killer (NK) cell levels. CD3 monoclonal antibody in every tube serves as a control for tube-to-tube variability and is also used to determine T-cell populations. If the CD3 value of a tube is greater than 3% of any of the others, that tube should be repeated.

Table 1.3 Recommended two-color monoclonal antibody panel (3).

Tube No.	FITC	PE	Cell populations identified
1	CD45	CD14	Lymphocytes, monocytes and granulocytes
2	Isotype	Isotype	-
3	CD3	CD4	CD4 lymphocytes
4	CD3	CD8	CD8 lymphocytes
5	CD3	CD19	T-cells, B-cells
6	CD3	CD16 and/or CD56	T-cells, NK-cells

Three-color monoclonal antibody panels

Three-color monoclonal antibody panels should fulfil the following basic requirements: enumerate CD4 and CD8 T-cells, validate the lymphocyte gate used, and provide some assessment of tube-to-tube variability. For determining T-cell subset percentages, the third color should be used to identify lymphocytes by following one of two procedures (Table 1.4).

A. Use CD45 as the third color to identify lymphocytes as those cells that are bright CD45 but have low side scattering properties (gate on CD45 and side scatter). In this case, the panel would consist of the following monoclonal antibodies: CD3/CD4/CD45; CD3/CD8/CD45; and CD3/CD19/CD45 (Table 1.4, Panel A).

B. Use lineage markers (T-cell, B-cell, and NK-cell) to identify lymphocytes. The panel would consist of the following monoclonal antibodies: CD3/CD19/CD16 and/or CD56; CD3/CD4/CD8; and isotype control (Table 1.4, Panel B).

A three-color monoclonal antibody panel must consist of at least two tubes, each with the same lineage marker. For the examples above, CD3 is the common lineage marker in each tube. Differences between replicate CD3 results should be less or equal to 2% (The variability of a CD3 result between two tubes is approximately half that of four tubes).

Four-color monoclonal antibody panels

Addition of CD45 to a single tube containing CD3, CD4, and CD8 allows the identification of lymphocytes based on CD45 and side scatter and the enumeration of CD4 and CD8 T-lymphocytes. A four-color monoclonal antibody panel must consist of at least two tubes, each with the same lineage marker. A second tube containing CD45, CD3, CD19, and CD16 and/or CD56 is recommended.

Table 1.4 Three-color monoclonal antibody panel (Adapted from Ref.3).

Panel	Tube No.	Monoclonal antibodies
A	1	CD3/CD4/CD45
	2	CD3/CD8/CD45
	3	CD3/CD19/CD45
B	1	CD3/CD19/CD16 and/or CD56
	2	CD3/CD4/CD8
	3	Isotype control

1.2.3.2 Standardization and quality control of immunophenotyping

<u>Negative and positive controls</u>

A. Negative (isotype) reagent control

1. Use this control to determine nonspecific binding of the mouse monoclonal antibody to the cells and to set markers for distinguishing fluorescence-negative and fluorescence-positive cell populations.

2. Use a monoclonal antibody with no specificity for human blood cells but of the same isotype as the test reagents. In many cases, the isotype control may not be optimal for controlling nonspecific fluorescence because of differences in F/P ratio, antibody concentration between the isotype control and the test reagents, and other characteristics of the immunoglobulin in the isotype control. Additionally, isotype control reagents from one manufacturer are not appropriate for use with test reagents from another manufacturer.

3. The isotype control is not needed for use with CD45 because CD45 is used to identify leukocyte populations based on fluorescence intensity.

4. For monoclonal antibody panels containing antibodies to CD3, CD4, and CD8 the isotype control may not be needed because labeling with these antibodies results in fluorescence patterns in which the unlabeled cells are clearly separated from the labeled

cells. In these instances, the negative cells in the histogram are the appropriate isotype control.

5. The isotype control must be used when a monoclonal antibody panel contains monoclonal antibodies that label populations that do not have a distinct negative population such as some CD16 or CD56 monoclonal antibodies.

B. Positive methodologic control

1. The methodologic control is used to determine whether procedures for preparing and processing the specimens are optimal. This control is prepared each time specimens from patients are prepared.

2. Use either a whole blood specimen from a control donor or commercial materials validated for this purpose. Ideally, this control will match the population of patients tested in the laboratory.

3. If the methodologic control falls outside established normal ranges, determine the reason. The purpose of the methodologic control is to detect problems in preparing and processing the specimens. Biologic factors that cause only the whole blood methodologic control to fall outside normal ranges do not invalidate the results from other specimens processed at the same time. Poor lysis or poor labeling in all specimens, including the methodologic control, invalidates results.

C. Positive control for testing reagents

    1. Use this control to test the labeling efficiency of new lots of reagents or when the labeling efficiency of the current lot is questioned. Prepare this control only when needed in parallel with lots of reagents of known acceptable performance.

    2. Use a whole blood specimen or other human lymphocyte preparation such as cryopreserved or commercially obtained lyophilized lymphocytes.

Flow cytometer quality control

A. Align optics daily. This ensures that the brightest and tightest peaks are produced in all parameters. Some clinical flow cytometers can be aligned by laboratory personnel whereas others can be aligned only by qualified service personnel.

    1. Align the flow cytometer by using stable calibration material (microbeads labeled with fluorochromes) that has measurable forward scatter, side scatter, and fluorescence peaks.

    2. Align the calibration particles optimally in the path of the laser beam and in relation to the collection lens so the brightest and tightest peaks are obtained.

    3. Align stream-in-air flow cytometers daily (at a minimum) and stream-in-cuvette flow cytometers (most clinical flow cytometers are this type) as recommended by the manufacturer.

B. Standardize daily. This ensures that the flow cytometer is performing optimally each day and that its performance is the same from day to day.

1. Select machine settings that are optimal for fluorochrome-labeled, whole blood specimens.

2. Use microbeads or other stable standardization material to place the scatter and fluorescence peaks in the same scatter and fluorescence channels each day. Adjust the flow cytometer as needed.

3. Maintain records of all daily standardizations. Monitor these to identify any changes in flow cytometer performance.

4. Retain machine standardization settings for the remaining quality control procedures (sensitivity and color compensation) and for reading the specimens.

C. Determine fluorescence resolution daily. The flow cytometer must differentiate between the dim peak and autofluorescence in each fluorescence channel.

1. Evaluate standardization/calibration material or cells that have low-level fluorescence that can be separated from autofluorescence (microbeads with low-level and negative fluorescence or CD56-labeled lymphocyte preparation).

2. Establish a minimal acceptable distance between peaks, monitor this difference, and correct any daily deviations.

D. Compensate for spectral overlap daily. This step corrects the spectral overlap of one fluorochrome into the fluorescence spectrum of another.

1. Use either microbead or cellular compensation material containing three populations for two-color immunofluorescence (no fluorescence, PE fluorescence only and FITC fluorescence only),

four populations for three-color immunofluorescence (the three above plus a population that is positive for only the third color), or five populations for four-color (the four above plus a population that is positive for only the fourth color).

2. Analyze this material and adjust the electronic compensation circuits on the flow cytometer to place the fluorescent populations in their respective fluorescence quadrants with no overlap into the double-positive quadrant. If three fluorochromes are used, compensation must be carried out in an appropriate sequence: FITC, PE, and the third color, respectively. For four-color monoclonal antibody panels, follow the flow cytometer manufacturer's instructions for four fluorochromes. Avoid over-compensation.

3. If standardization or calibration particles (microbeads) have been used to set compensation, confirm proper calibration by using lymphocytes labeled with FITC and PE labeled monoclonal antibodies (and a third-color or fourth-color labeled monoclonal antibody for three-color or four-color panels) that recognize separate cell populations but do not overlap. These populations should have the brightest expected signals. (If a dimmer than-expected signal is used to set compensation, suboptimal compensation for the brightest signal can result).

4. Reset compensation when photomultiplier tube voltages or optical filters are changed.

E. Repeat all four-instrument quality control procedures whenever instrument problems occur or if the instrument is serviced during the day.

F. Maintain instrument quality-control logs, and monitor them continually for changes in any of the parameters. In the logs, record instrument settings, peak channels, and coefficient of variation (CV) values for optical alignment, standardization, fluorescence resolution, and spectral compensation. Re-establish fluorescence levels for each quality-control procedure when lot numbers of beads are changed.

Sample analysis

A. For the two-color immunophenotyping panel using a light-scatter gate, analyze the sample tubes of each patient's specimen in the following order: 1) The tube containing CD45 and CD14 (gating reagent): read this tube first so that gates can be set around the lymphocyte cluster; 2) Isotype control: set cursors for differentiating positive and negative populations so that $\leq 2\%$ of the cells are positive; and 3) Remaining tubes in the panel.

    1. Count at least 2,500 gated lymphocytes in each sample. This number ensures with 95% confidence that the result is $\leq 2\%$ standard deviation (SD) of the "true" value (binomial sampling). This model assumes that variability determined from preparing and analyzing replicates is $\leq 2\%$ SD. Each laboratory must determine the level of variability by preparing and analyzing at least eight replicates of the last four tubes in the recommended panel. Measure

variability when first validating the methodology used and again when methodologic changes are made.

2. Examine light-scattering patterns on each sample tube. Determine whether lysis or sample preparation, which can affect light scattering, is the same in each sample tube of a patient's specimen. Deviation in a particular tube usually indicates sample preparation error, and the tube should be repeated such as a new aliquot of blood should be stained and lysed.

B. For three- or four-color monoclonal antibody panels using a CD45/side scatter gate, determine the lymphocyte population based on bright CD45 fluorescence and low side scattering properties. Draw a gate on this population and analyze the cell populations using this gate.

Data analysis

A. Light-scatter gate (for two-color panels).

1. Reading from the sample tube containing CD45 and CD14, draw lymphocyte gates using forward and side light-scattering patterns and fluorescence staining.

a. When using CD45 and CD14 and light-scattering patterns for drawing lymphocyte gates, define populations on the following basis:

- Lymphocytes stain brightly with CD45 and are negative for CD14.

- Monocytes and granulocytes have greater forward and side light-scattering properties than lymphocytes.

- Monocytes are positive for CD14 and have intermediate to high intensity for CD45.

- Granulocytes are dimly positive for CD14 and show less intense staining with CD45.

- Debris, red cells and platelets show lower forward scattering than lymphocytes and do not stain specifically with CD45 or CD14.

b. Using the above characteristics, draw a light-scattering gate around the lymphocyte population. Other methods for drawing a lymphocyte gate must accurately identify lymphocytes and account for non-lymphocyte contamination of the gate.

2. Verify the lymphocyte gate by determining the recovery of lymphocytes within the gate and the lymphocyte purity of the gate.

a. Definitions

- The lymphocyte recovery is the percentage of lymphocytes in the sample that are within the gate.

- The lymphocyte purity of the gate is the percentage of cells within the gate that are lymphocytes. The remainder may be monocytes, granulocytes, red cells, platelets, and debris.

b. Optimally, the lymphocyte recovery should be more than 95%.

    c. Optimally, the lymphocyte purity of the gate should be more than 90%.

    d. Optimal gates include as many lymphocytes and as few contaminants as possible.

    e. If the recommended recovery and purity of lymphocytes within the gate cannot be achieved, redraw the gate. If minimum levels still cannot be obtained, reprocess the specimen. If this fails, request another specimen.

B.   CD45 gating (for three- and four-color monoclonal panels).

    1. Identify lymphocytes as cells brightly labeled with CD45 and having low side scattering properties.

    2. Establish criteria for cluster identification based on a clear definition of lymphocytes that does not include basophils (less bright CD45, low side scatter) or monocytes (less bright CD45, moderate side scatter). Care must be taken to include all lymphocytes. B-cells may have slightly less CD45 fluorescence than the T-cells (the major cluster of lymphocytes). NK-cells have bright CD45 fluorescence but have slightly more side scattering properties than the majority of the lymphocytes.

    3. CD45/side scatter gates for lymphocytes are assumed to contain >95% lymphocytes, and no further correlations need be made to the percentage subset results.

    4. Lymphocyte recovery cannot be determined without using a panel of monoclonal antibodies that identify T-cells, B-cells, and NK-

cells. Validation of a CD45/side scatter gate is recommended when beginning to use CD45/side scatter gates to help determine the CD45 and side scatter characteristics of T-cells, B-cells, and NK-cells and to ensure their inclusion in the gate.

C. Set cursors using the isotype control so that <2% of cells are positive. If an isotype control is not used, set cursors based on the tube containing CD3 and CD4 so that the negative and positive cells in the histogram are clearly separated. These cursors may be used for the remaining tubes. If CD16 and/or CD56 are included in a monoclonal antibody panel, an isotype control may be needed to help identify negative cells.

D. Analyze the remaining samples with the cursors set. In some instances, the isotype-set cursors will not accurately separate positive and negative staining for another sample tube from the same specimen. In such cases, the cursors can be moved on that sample to more accurately separate these populations. The cursors should not be moved when fluorescence distributions are continuous with no clear demarcation between positively and negatively labeled cells.

E. Analyze each patient or control specimen with lymphocyte gates and cursors for positivity set for that particular patient or control.

F. When spectral compensation of a particular specimen appears to be inappropriate because FITC-labeled cells have been dragged into the PE-positive quadrant or vice-versa (when compensation on all other specimens is appropriate, repeat the sample preparation, prewashing the

specimen with phosphate-buffered saline pH 7.2 to remove plasma before monoclonal antibodies are added.

G. Include the following analytic reliability checks, when available:

1. Optimally, at least 95% lymphocyte recovery (proportion of lymphocytes within the lymphocyte gate) should be achieved. Minimally, at least 90% lymphocyte recovery should be achieved. These determinations can only be made when using either CD14 or CD45 to validate the gate or when using T, B, and NK reagents to validate a gate.

2. Optimally, ≥ 90% lymphocyte purity should be observed within the lymphocyte gate. Minimally, ≥ 85% purity should be observed within the gate.

3. Optimally, the sum of the percentage of CD4 and CD8 lymphocytes should equal the total percentage of CD3 lymphocytes within ± 5%, with a maximum variability of ≤ 10%. In specimens containing a considerable number of T δγ cells, this reliability check may exceed the maximum variability.

4. Optimally, the sum of the percentage of CD3 (T-cells), CD19 (B-cells), and CD16 and/or CD56 (NK-cells) should equal the purity of lymphocytes in the gate ± 5%, with a maximum variability of ≤ 10%. If the data are corrected for lymphocyte purity, the sum should ideally equal 95% -105% (or at minimum 90%-110%).

1.2.4 Alternative technologies for CD4 lymphocyte counts

1.2.4.1 Microscopic assay

Cytosphere assay: a simple, manual method utilizes latex beads coated with CD4 antibody. Anticoagulated whole blood is incubated with monocyte blocking beads followed by incubation with larger bead coated with CD4 antibodies. The red blood cells are lysed, counting of bead coated cells on a hemacytometer and then multiplied by a dilution factor to determine the absolute CD4 count (15,16).

1.2.4.2 Immunoenzymatic assay

TRAx™ (Total Receptor Assay) CD4 test kit: sandwich enzyme immunoassay (EIA) monoclonal antibodies for the quantitative measurement of total CD4 protein and for the enumeration of CD4 lymphocytes in human peripheral blood specimens. The assay is performed in microtitre wells that have been pre-coated with monoclonal antibody to human CD4 protein. A second anti-CD4 monoclonal antibody conjugated to horseradish peroxidase is pipetted into the wells followed by standards, control, or specimens. The solubilized CD4 protein present in the standards, controls, or specimens binds to the antibody coated on the plate while the conjugated antibody binds to a second epitope on the CD4 molecule completing the sandwich. After a 3-hour incubation the wells are washed thoroughly to remove unreacted components and an enzyme-specific chromogen solution is added. After an additional short incubation, the reaction is terminated by the addition of stop solution and absorbance at 490 nm is measured. The colored endpoint, which results, is directly proportional to the amount of CD4 protein present and is equivalent to the number of CD4 lymphocytes in the original specimen. A standard curve is prepared

from the 6 kit standards. Control and specimen values are determined from the standard curve and reported as cells/μl (17). This assay is best suited to laboratories with a large number of specimens to test but with few personnel to do the testing and also advantageous for specimens collected from a remote testing site, because it allows specimens to be lysed and frozen, and then shipped in batches for testing (18). The utility of this new test was being investigated further in longitudinal studies and in HIV-positive pediatric populations and was being examined for other disease areas, such as autoimmunity, leukemia or lymphoma and immune monitoring associated with transplantation (19).

Capcellia® CD4/CD8 immunoassay: the assay is based on T-cell separation from whole blood by use of an anti-CD2 magnetic bead suspension followed by reaction of the CD4 or CD8 molecules with the corresponding monoclonal antibody coupled to peroxidase. Blood sample or human plasma taken as control was mixed with anti-CD2 mAb-coated magnetic beads and then shaken for 2 minutes. The blood-bead mixture and the human plasma-bead control mixture were then placed in the microwells of a microtiter plate fitted with magnets, which separate the T cells coated with the magnetic beads from uncoated cells. After 2 minutes, the residual whole blood containing untrapped cells was rapidly removed from the microwells by aspiration. In the second step of the assay, the magnets were separated from the microwells and a solution containing the anti-CD4 or anti-CD8-mAb conjugated with peroxidase was added to the assay wells and the control wells (bead-immunoconjugate blanks). The plates were allowed to stand for 20 minutes at room temperature, after which the plate and magnets were once again juxtaposed (2 minutes), and excess conjugate was removed by multiple washing. The peroxidase

activity in the microwells was measured by addition of an enzyme-specific chromogen solution to each well. The plates were allowed to stand 20 minutes in the dark, and then the stop solution was added. The absorbance was measured on a microtiter plate reader at 450 nm. The number of CD4 or CD8 T-cells/L in the blood samples was determined from the calibration curves. This new ELISA for cell markers may represent an efficient alternative to flow cytometry. This method offers the following advantages: a) accuracy and good reproducibility of T-lymphocyte counts; b) high specificity of the evaluation of the CD4 lymphocyte population, excluding contamination by CD4 monocytes; c) absence of technical problems linked to incomplete lysis of red blood cells, especially encountered in blood samples from certain patients; d) internal standardization by means of a freeze-dried CD4 or CD8 T-cell preparation, which permits comparison of counts obtained from different laboratories; and e) rapid and easy performance in all laboratories without the need for expensive equipment (20).

1.2.4.3 Immunofluorescence assay

Zymmune™ assay: a magnetic bead based fluorescent immunoassay that provides absolute CD4 and CD8 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 rosetted 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 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 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 resuspended and the signal measured using a microplate fluorescence reader. The numbers of the CD4 and CD8 lymphocytes are then calculated using the calibrator curves (21-23). The Zymmune™ system is more cost effective than traditional flow cytometry (24).

FACSCount system: a system designed to provide automated absolute CD4, CD8 and total CD3 T-lymphocyte enumeration. FACSCount was designed to preserve the power and advantages of flow cytometry while systematically addressing the perceived limitation of that technology, including whole blood lysing, the requirement for separate hematology instrumentation and the need for highly skilled technicians. Reagents are premeasured and dispensed in sealed tubes ready for the operator to add a measured quantifies of unlysed anticoagulated whole blood. After a 60-minute incubation, the whole blood and reagent mixture (contains antibody reagents and beads) is fixed. The samples are then vortexed and placed on the FACSCount instrument where fluorescently labeled cells are quantitated and results for absolute CD4, CD8 and total CD3 T cells printed (16).

VCS: the method is based on Coulter's volume, conductivity and scatter technology and integrated the technology of hematology with flow cytometry. It provides data on the five populations of white cells, as well as an absolute CD4 count. To perform this test, 100 µl of whole blood is first aspirated by the analyzer. Red blood cells are lysed and white blood cells are stabilized and analyzed with 30 seconds. Whole blood is then mixed and incubated with either Coulter CD4 or CD8 cyto-sphere reagent (small latex particles coated with a specific monoclonal antibody that attaches to the corresponding antigenic site). During a two-minute incubation, the attached beads change the cell's properties as measured by the analyzer. The absolute number of CD4 and CD8 lymphocytes is displayed along with the WBC data and the CD4/CD8 ratio. As a result of the 2-minute incubation, this method can provide a single CD4 result more quickly than any of the other methods currently available (25).

Flow cytometer technology has a significant advantage, 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. This technology, however requires extensive training in running the instrument, troubleshooting and data analysis and costly for adaptation as a routine method in laboratories in developing countries. Therefore, alternative simple, inexpensive and reliable assays are urgently needed (16). The manual cytosphere assay appears to be simple, less expensive and have the potential application in the limited laboratory facilities (15). For performing large numbers of CD4 assays in a batch mode, the CDC study indicated that the TRAx™ CD4 is the preferred method. For performing single CD4 counts quickly, the Coulter VCS technology appears to be the optimal technique. The Zymmune™ assay offers the

advantage of reduced interference from monocytes and soluble CD4 as compared to TRAx™ CD4, requires less expensive equipment as well as Capcellia® and has a higher through put than VCS or FACSCount. FACSCount, while not as rapid as the other tests, is similar in performance to the gold standard flow cytometry method (20,25).

1.2.5 Red blood cell lysing solution

In the field of hematology it is often useful to distinguish and identify the various types of white blood cells which are present in the blood or bone marrow. Analysis of subpopulations of leukocytes is of particular interest for evaluation of immune system-related diseases such as AIDS. In particular, analysis of lymphocytes, a mononuclear leukocyte involved in the immune response has clinical significance for management of immune system disorders. For example, it may be useful to detect or quantify the proportions of T-cell and B-cell subsets. Monocytes, neutrophils, eosinophils and basophils are other leukocyte subpopulations of clinical interest in addition to lymphocytes. These cell types can be resolved and analyzed by flow cytometry. Flow cytometers, these instruments have means for detecting forward scattered and side scattered light as well as one or more means for detecting fluorescence. Forward and side light scatters are used to determine physical parameters such as cell size and granularity. The various fluorescence detectors are used to distinguish cells labeled with fluorochromes, which can be excited to emit light at different wavelengths. A particular subpopulations of interest is then further analyzed by "gating" based on the data collected for the entire population. To select an appropriate gate, the data is plotted so as to obtain the best separation of

subpopulations possible. This is typically done by plotting Forward light scatter (FSC) versus side (orthogonal) light scatter (SSC) on a two-dimensional dot plot. The flow cytometer operator then selects the desired subpopulation of cells (those cell within the gate) and excludes cells, which are not within the gate. Typically, the operator selects the gate by drawing a line around the desired subpopulation using a cursor on a computer screen. Only those cells within the gate then further analyzed by plotting the other parameters for these cells such as fluorescence. While gating is a powerful tool for removing data for cells which are not of interest and thereby improving the ability to analyze the desired subpopulation, it can be a source of substantial error if separation of the subpopulations is not optimal (26). Efficient detection of lymphocytes in peripheral blood depends on the elimination of interfering cells. Whole blood lysis has been shown to be as effective as density gradient centrifugation in the preparation of peripheral blood mononuclear cells (PBMC) for lymphocyte subset analysis (27-30) and shorter sample preparation time, less handling of whole blood and less loss of lymphocyte subsets when compared to Ficoll-Paque density gradient separation (31-33).

1.2.5.1 FACS™ lysing solution

This lysing reagent is available from Becton Dickinson Immunocytometry Systems; San Jose, California (U.S. Patent nos. 4,902,613 and 4,654,312) comprises diethylene glycol, heparin, citrate buffer and formaldehyde (1.4%-3%), pH 7.2. Because of the presence of formaldehyde, FACS™ lysing solution provides the advantage of reducing the biohazard from biological samples. However, its presence in a sample being analyzed by flow cytometry causes cell shrinkage and may result in
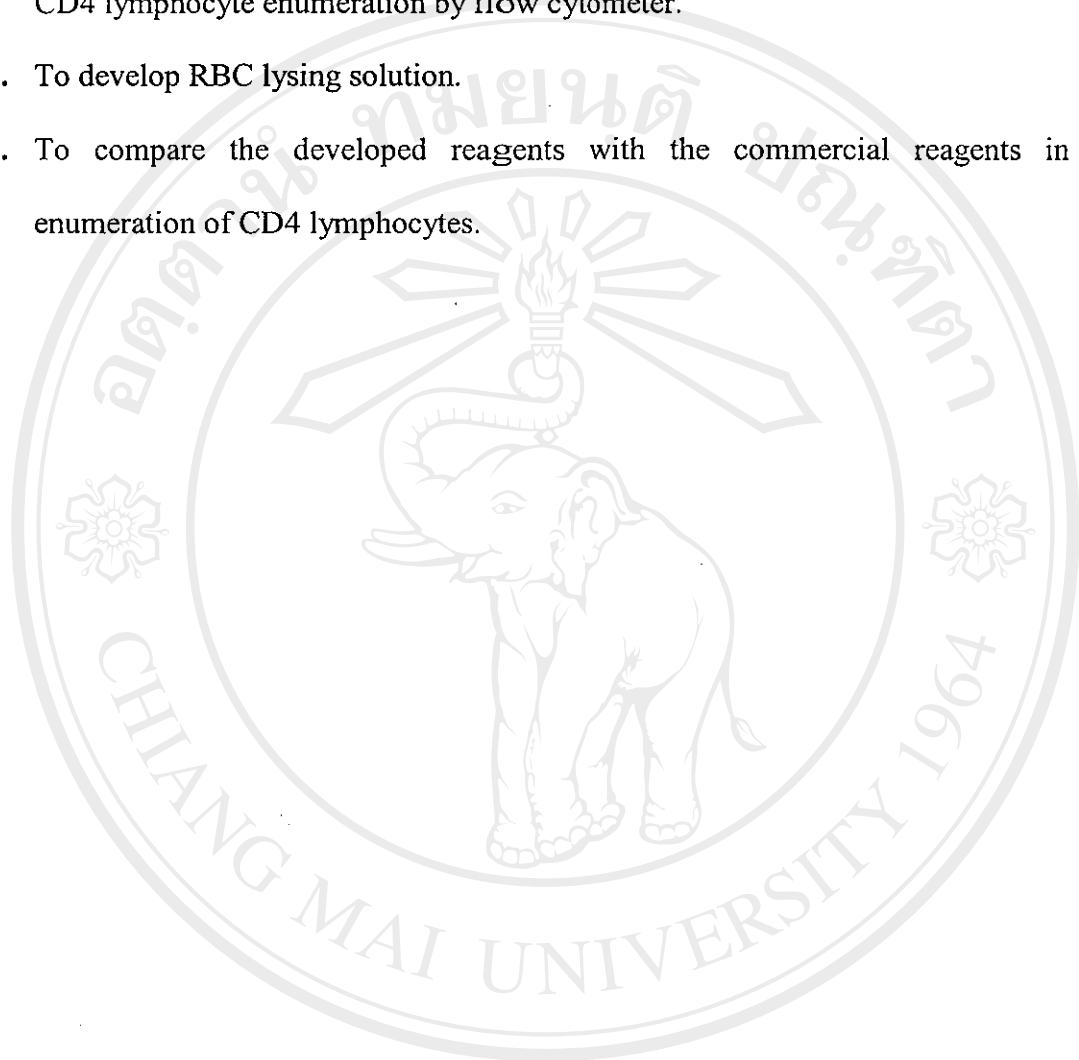
unsatisfactory levels of debris which can obscure the cell population to be gated, particularly lymphocytes. T o reduce the n egative e ffect of lysing reagents on light scattering parameters the lysed sample is typically centrifuged and washed to remove debris, unbound fluorochrome-labeled antibodies and the lysing solution itself, all of which contribute to poor light scatter analysis. While this method partially solves the problem of resolving leukocyte subpopulations based on light scattering characteristics, it is labor intensive, adds steps to the procedure, is difficult to automate and can result in a loss of cells which precludes making accurate cell counts on the sample. For example, to obtain accurate CD4 lymphocyte counts from lysed, washed blood samples, presently available methods require multiple steps and complex data analysis (26).

1.2.5.2 OptiLyse® B lysing solution

This lysing reagent is available from Immunotech, a Coulter Company, Marseille, France. OptiLyse B solution is a buffered solution containing 3.4% formaldehyde. This reagent intended for lysing human red blood cells following direct immunofluorescence staining of human peripheral blood cells with monoclonal antibodies in preparation for flow cytometric analysis. F ollowing i mmunostaining, the reagent fixes leukocytes and the erythrocytes are lysed resulting in a leukocyte suspension substantially free of red blood cells and suitable for flow cytomer without washing or centrifugation (34).

1.3 Objectives

1. To develop the three-color immunofluorescence monoclonal antibodies for CD4 lymphocyte enumeration by flow cytometer.

2. To develop RBC lysing solution.

3. To compare the developed reagents with the commercial reagents in enumeration of CD4 lymphocytes.