CHAPTER III

MATERIALS AND METHODS

1. Study Group

Sixty-one HIV-infected patients who came for regular follow-up, at least every 6 months for 2 or more years at the HIV/AIDS Clinic of Chulalongkorn Hospital from January 1986 to December 1990 were recruited into this longitudinal cohort study. HIV infection was identified by repeatedly (2x) reactive anti-HIV ELISA test which was subsequently confirmed by Western blot test (see below). They were not put on any antiviral or immunostimulating drugs because of financial constraints. This study was approved by the human ethical committee of the Faculty of Medicine, Chulalongkorn University. Patients were explained about the study objectives and informed consents were obtained. Patients were asked to return for clinical and laboratory follow-ups every 3-6 months or sooner if any symptoms ensued. Fifty-five were males and six were females. Their ages ranged from 16 to 51 with a mean age of 29.2 (SD = 7.2). They could be classified at entry into different stages according to CDC classificatian system [68], namely CDC stage II or asymptomatic HIV (N=18), CDC stage III or persistent generalized lymphadenopathy (PGL , N=34), CDC stage IV-A or HIV infection with constitutional symptoms or those conventionally called AIDS-related complex or ARC (N = 3) and CDC stage IV-C and IV-D or HIV infection with secondary infectious diseases and secondary cancers respectively or those conventionally called AIDS (N = 6). Diagnostic criteria for PGL , ARC and AIDS were based on CDC classification system (see , Appendix III)[68].

2. Specimen collection

Twenty milliliter of blood were collected by venepuncture and divided in half into 2 separate tubes, one was heparinized and the other one was for clotted blood. The heparinized blood was used to separate mononuclear cells by Ficoll-Hypaque density gradient centrifugation[98]. The mononuclear cells were used for quantitation of total T-cells and CD_4^+/CD_8^+ T cells by spontaneous E-rosette formation and immunofluorescent technique respectively[99,100,101]. Sera from clotted blood were used to assay β_2-microglobulin, p24 antigen and anti-p24. Sera were stored in aliquots at -70°C until use.

3. Separation of peripheral blood mononuclear cells (PBMC)

PBMC were obtained by Ficoll-Hypaque density gradient centrifugation[98]. Four ml of heparinized blood were overlayed on 3 ml of Ficoll - Hypaque solution (see , Appendix II) by aseptic technique and centrifuged at 220 G for 30 minutes at 4°C. The PBMC at Ficoll-Hypaque-Plasma interface were collected by sterile pasteur pipettes (Figure 2). The cells were washed three times with RPMI 1640 (Gibco, Grand Island, N.Y., USA), then counted in hemocytometer chamber and adjusted to $2x10^6$ cells/ml with RPMI 1640.
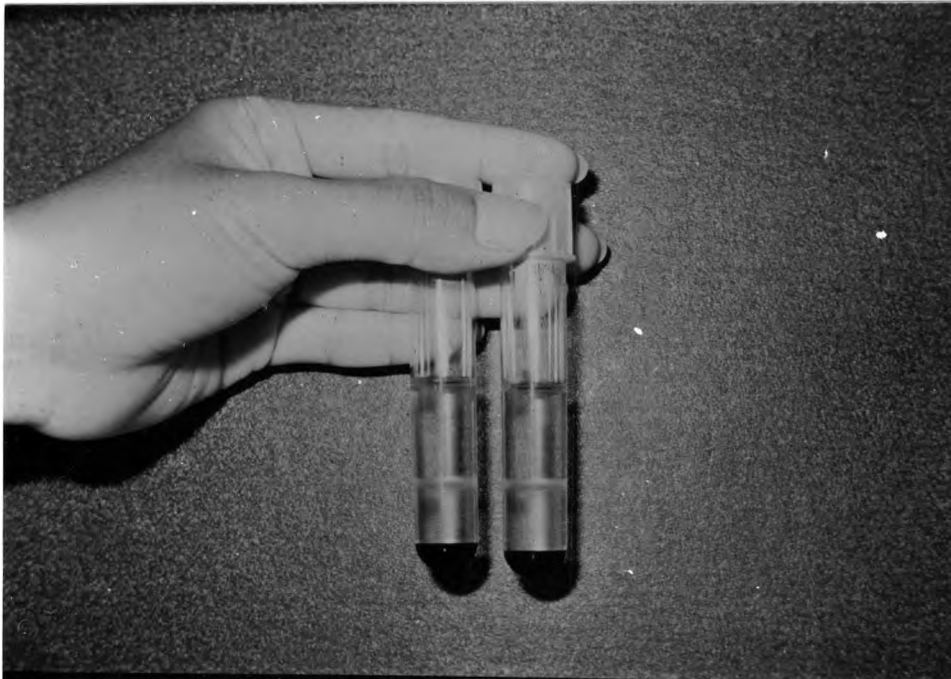
Figure 2. Ficoll-Hypaque density gradient separation of PBMC

4. Spontaneous E-rosette formation[99]

PBMC at 2×10^6 cells/ml were mixed in equal volume (0.5 ml each) with 1% sheep red blood cell suspension (SRBC) (see , Appendix II) in RPMI 1640 in a 12x75 mm test tube. The tubes were incubated at 37°C for 5 minutes and then centrifuged at 1000 rpm (approximately 150 G) for 5 minutes at 4°C. After overnight incubation in a refrigerator at 4°C , the cell pellets were gently resuspended by blowing in and out with pasteur pipette. The cell suspensions were transferred to a hemocytometer chamber and counted for E-rosette forming cells (E-RFC+). E-RFC+ was defined as mononuclear cells which were surrounded by three or more SRBC (Figure 3). Percentage of E-RFC+ was enumerated under light microscope (40x) from a total of 200 mononuclear cells.
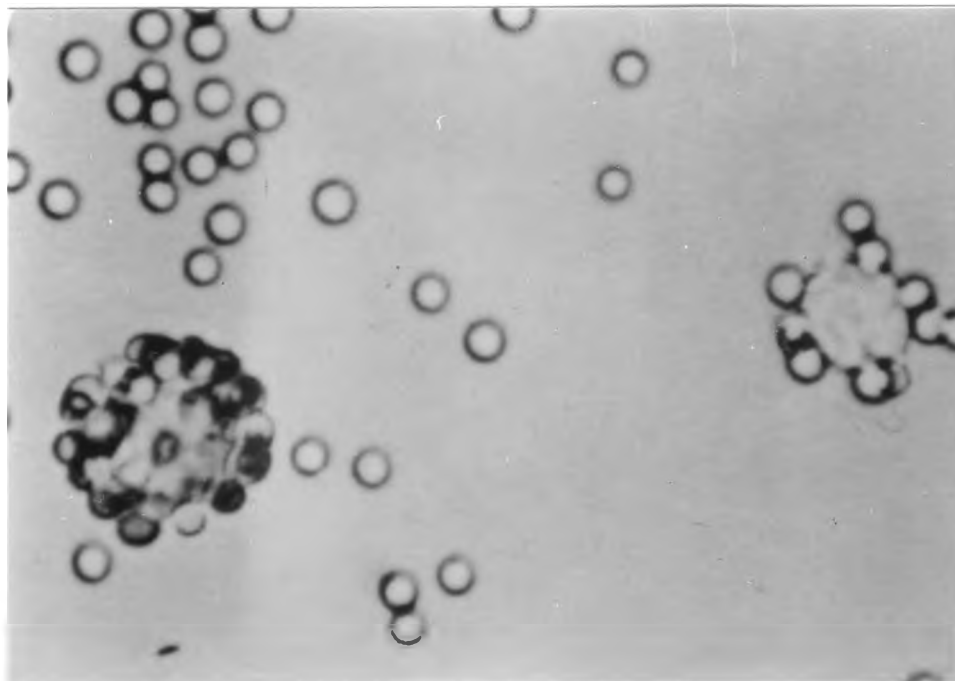
Figure 3. E-rosette forming cells

5. Enumeration of CD_4^+ and CD_8^+ T cells[100,101]

Two monoclonal antibodies directed to human helper/ inducer and suppressor/cytotoxic T cell surface antigens (OKT_4 and OKT_8 respectively) (Ortho Diagnostic, N.J., USA) were used to identify CD_4^+ and CD_8^+ T cells respectively by manual indirect immunofluorescent technique. $1x10^6$ PBMC suspensions were spun at 1,800 rpm (approximately 200 G) for 5 min to discard the supernatant. 5 μl of undiluted OKT_4 and OKT_8 monoclonal antibodies was added to the cell pellet, mixed and incubated at $4°C$ or in the ice bath for 30 min with gentle agitation every 10 min. After the first incubation, the cells were washed 2 times with RPMI 1640 by centrifugation at 1,800 rpm (200 G), for 5 min at $4°C$. The cell pellet was mixed with 100 μl of 1:20 dilution of fluorescein - conjugated rabbit anti - mouse immunoglobulin (Dako immunoglobulins, Copenhagen, Denmark) and incubated at $4°C$ for 30

min with gentle mixing every 10 min. The cells were washed 2 times as described above. The cell pellet was preserved in 0.5 ml of 1% paraformaldehyde (Sigma, Mo., USA) in PBS (see, Appendix II) and then kept at 4°C for at least 10 min. After the cell suspensions were spun down and supernatants were discarded, the packed cells were resuspended by 1 drop of mounting media (see, Appendix II) and mixed vigorously. The cells were mounted on a microscopic glass slide with cover slip for further fluorescent microscopic examination. The fluorescein-stained lymphocytes (Figure 4) were counted in a total of at least 300 lymphocytes visualized on bright light field and scored as the percentage of OKT_4^+ (CD_4^+) and OKT_8 (CD_8^+) cells.
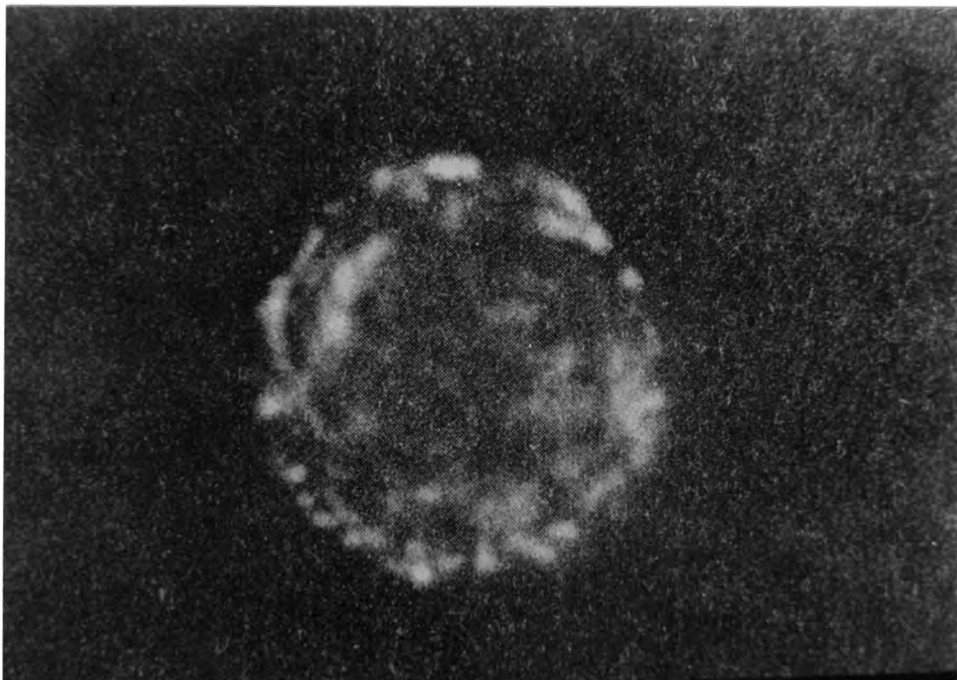

Figure 4. The fluorescein-stained CD_4^+ or CD_8^+ lymphocytes

6. Determination of β_2-microglobulin level[102]

By using competitive ELISA (Pharmacia Diagnostics AB , Uppsala , Sweden), β_2-microglobulin in serum specimens or standard β_2-microglobulin competes with a fixed amount of enzyme labelled β_2-microglobulin for the binding sites of murine monoclonal anti - β_2-microglobulin bound to microtiter wells via coated anti-mouse antibodies (as shown in Figure 5). Samples or standards are mixed with enzyme-β_2-microglobulin conjugate and allow to react with anti-β_2-microglobulin antibody in the anti-mouse antibody coated well. The microtiter wells are incubated on a shaker for one hour and the wells are washed three times to remove unbound material. Bound β_2-microglobulin conjugate is measured by addition of enzyme substrate to the wells. After 15 minutes of substrate incubation, the coloured product and the reaction are interrupted by addition of stop solution. The absorbance is measured at 405 nm by a microplate reader. The absorbance is inversely proportional to the concentration of β_2-microglobulin in the sample.
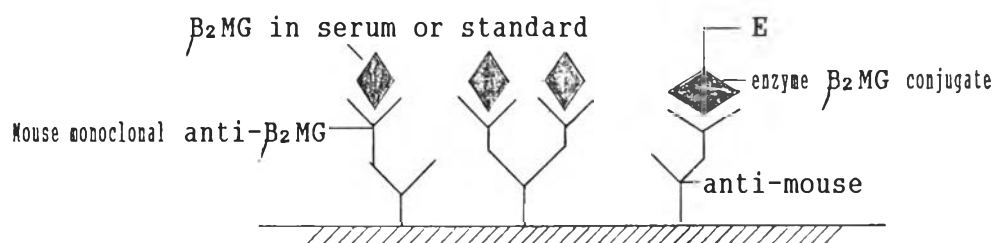

Figure 5. Principle of competitive ELISA for β_2-microglobulin

7. Determination of p24 antigen[103]

By using antigen capture EIA (Diagnostic Pasteur, Marnes La Coquette, France) polyclonal anti-HIV coated wells are incubated with serum samples or with known amount of standard p24 antigen. After unbound material is washed out, enzyme-anti-HIV core (anti-p24) conjugate is added and then incubated for a further period of time. The unbound material is washed out again and substrate is added. After 30 minutes of substrate incubation, stop solution is added to stop the enzyme-substrate reaction. The absorbance is measured at 492 nm by a microplate reader. The absorbance is proportional to the concentration of p24 antigen in the sample. In our hand, the test could detect as low as 5 pg/ml of standard p24 antigen.

8. Determination of anti-p24

Western blot assay (Diagnostic Biotechnology , Singapore) was used to determine anti-p24 level in patients' sera. Anti-p24 was graded as positive , weakly positive , very weakly positive or negative according to the intensity of color shown on the strip.(Figure 6)
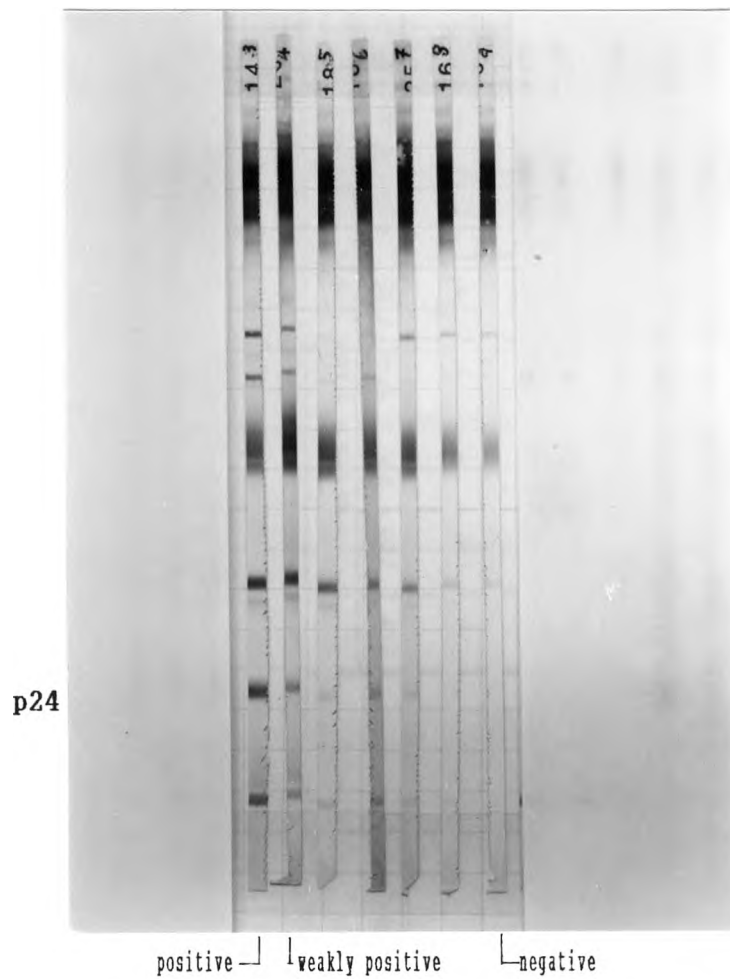
Figure 6. Grading of anti-p24 level by Western blot assay

For western blot assay, in brief, whole viral antigens were separated according to their molecular weights by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) and then transferred to nitrocellulose membrane. Anti-HIV in patient's sera reacts with various separated bands of viral antigen on nitrocellulose membrane.

After enzyme-anti-human Ig conjugate incubation, substrate was added to demonstrate the presence of bound antibodies on the strip which appeared as dark bands. (Figure 7)
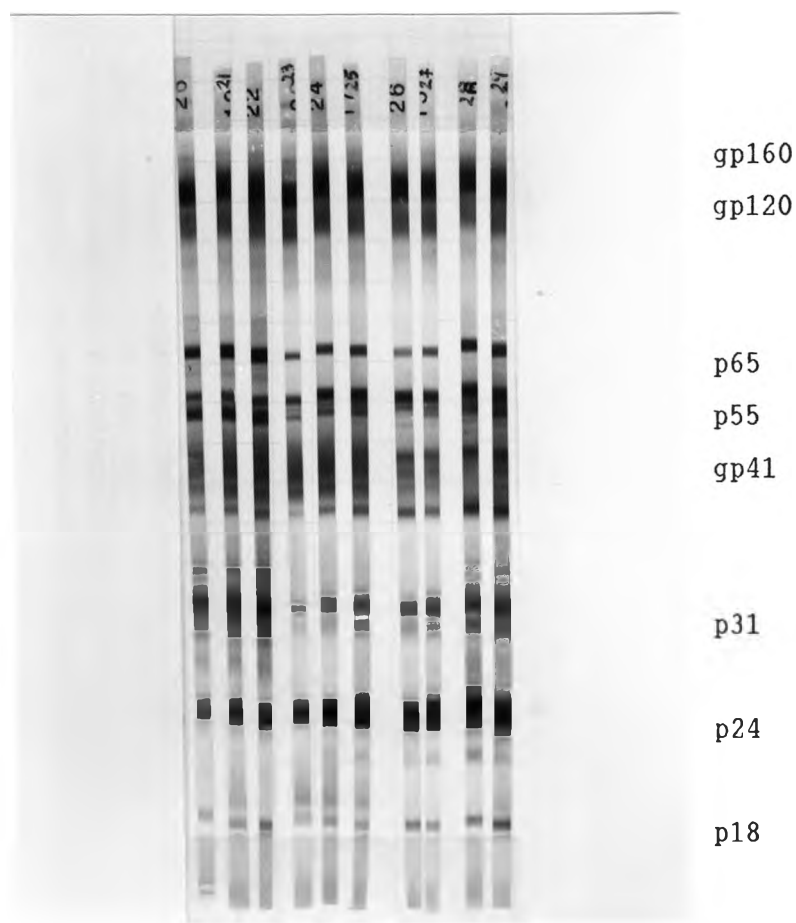

gp160
gp120

p65
p55
gp41

p31

p24

p18

Figure 7. Pattern of anti-HIV positive by Western blot assay

9. Statistical Analysis

Unpaired Student's t-test and Chi-square test were used to assesses the statistical significance of the variables and parameters under study. Any p value of less than 0.05 was considered statistically significant. Kaplan-Meier plots were

used to illustrate the development of AIDS or survival with time in different subsets of various immunologic marker[104]. The relative hazards were used to assess the relative relevance or predictive values of each subset of the immunologic markers based on the one which was almost near normal [105,106,107]. Relative hazard was calculated as the ratio of disease present in people with a specific factor or exposure to disease present in people without that particular factor or exposure according to the following formula

	Exposure	No-exposure
Outcome Cases	a	b
Controls	c	d

$$\text{Relative risk} = \frac{\text{Disease present/people with factor}}{\text{Disease present/people without factor}}$$

$$= \frac{a/a+c}{b/b+d} = \frac{a}{a+c} \times \frac{b+d}{b} = \frac{a}{b} \cdot \frac{d}{c} = \frac{ad}{bc}$$

$$b+d = d \ (\because d >>> b)$$
$$a+c = c \ (\because c >>> a \text{ in a rare disease})$$

Any relative hazards of 2.0 or more were considered statistically significant. The higher the value, the more significance is that parameter for that particular subdivision. 95% confidence intervals (95% CI) were also calculated for each relative hazard. Any values fall outside the 95% CI were considered statistically significant. The correlation between variables was assessed by Pearson correlation coefficient.