CHAPTER III

MATERIALS AND METHODS

1. Study group, sample size and specimen collections

1.1 Study group were taken from HIV-1 infected patients attended the Anonymous Clinic of Thai Red Cross AIDS Research Centre

Inclusion Criteria

- Confirmed anti-HIV positive by ELISA and gelatin particle agglutination (GPA) assay.
- CD4+ T cell counts more than or equal to 300 cells/cu.mm. by flow cytometry.

Exclusion Criteria

- On antiretroviral drugs
- Active concurrent infection and/or opportunistic infection
- On cytotoxic drugs and/or irradiation infection
- On immunosuppressive drugs

1.2 Sample size

As a pilot study, 25 asymptomatic HIV-1 infected Thai patients were enrolled.

1.3 Specimen collection

40 ml of heparinized blood and 7 milliliter (ml) EDTA blood were collected from each volunteer.

2. Dried pack cell preparation

PBMCs preparation from EDTA blood samples (7 ml) by density gradient centrifugation method. The EDTA blood was diluted with RPMI1640 at 1 : 1 and layer on 4 ml of ficoll-hypaque reagent (density gradient = 1.077 g/l) and was centrifuged at 1500 round per minute (rpm) for 30 minutes (min) at room temperature. The PBMC layer was harvested and washed 2 times with RPMI1640. Adjusted cell at 5 x 10^6 cells/ml and aliquoted 1 ml of each for 2 cryotubes. The samples were centrifuged at 14000 rpm for 1 min. Discarded the supernatant and then dried pack cells

were kept at –70°C up until being sent to tested for HIV-1 genotyping at The Cellular Immunology Laboratory, Division of Allergy and Clinical Immunology, Chulalongkorn University.

3. HIV-1 genotyping assay[141]

HIV envelope region was amplified by triple nested polymerase chain reaction (PCR) from proviral DNA extracted from infected PBMCs. The PCR products of approximately 600 base pair (bp) were purified by QIAGEN PCR purification kit. The optimal amount of DNA were subjected to Bigdye dideoxy dye terminator sequencing kit and then sequenced by ABI PRISM 310 Genetic analyzer (PE Applied Biosystems, CA, U.S.A.) The env sequences were then aligned and clustered according to update HIV-1 subtype sequences as described in the Human Retrovirus and AIDS 1998 Compendium.

4. Peripheral blood mononuclear cells (PBMC) preparation

Ten ml of heparinized blood was subjected for PBMCs separation by density gradient centrifugation method as described previously. The harvested PBMCs was resuspensed with R10 (see appendix II). Then PBMCs samples were used for establishing BLCL as target cells and also for effector cells preparation.

5. Culture of B95-8 cells

B95-8 cells (kindly provided by NIH, Thailand) were grown in culture media R20 (see appendix II) in plastic tissue culture flasks at concentrations 1 x 10^6 cells/ml in a 37°C incubator with a humid atmosphere of 5% carbon dioxide (CO_2) in air. Healthy cultures of B95-8 contain floating clumps of cells, while some cells adhere to the plastic surface so that flasks are usually kept flat to give the maximum surface area for adherence. To grow the cells for virus production, culture up to 250 ml in a 175 squre centimeter (cm^2) flask was expanded.

6. Harvesting Epstein-Barr Virus (EBV) from B95-8 cultures

The B95-8 culture supernatant was centrifuged at 2000 rpm for 20 min. at room temperature to remove most of the cells. Store the B95-8 culture supernatant stocks in aliquots (1 ml of each) at –70°C, until used.

7. B lymphoblastoid cell lines (BLCL) preparation

PBMCs 10 x 10^6 cells/ml were centrifuged at 1500 rpm for 10 min. Discarded the supernatant and resuspensed the pellets in 1 ml of undiluted B95-8 culture supernatant and incubate at 37 °C with a humid atmosphere of 5% CO_2 in air for 1 hour, loosen caps slightly to allow for air exchange.

Following incubation with EBV, cells were washed once with RPMI1640 then resuspend in culture medium R20 at 1 x 10^6 cells/ml and aliquots to 24-well flat-bottom tissue culture plates. Cyclosporin A (CSA), which inhibits the T-cell receptor signal transduction pathway via the formation of CSA-cycloplilin complex that inhibits calcineurin, was added to the culture medium to a final concentration of 1 μg/ml.

Initially, the cells were fed at weekly intervals by removing half of the supernatant and replacing it with culture media R20 without disturbing the cell layer. Proliferating foci of B cells were usually microscopically visible 1-2 weeks after infection with EBV and these continued to proliferate on sub-culturing.

After 1-2 weeks of culture, proliferating foci of B lymphocytes were observed microscopically in all wells.

Once immortalized, proliferating B cells were transferred from 2 ml cultures in 24-well plates to 25 cm^2 tissue culture flasks, which were kept upright initially. Thereafter cultures were expanded into larger vessels, usually diluting the cells 1:2 every 3-7 days depending on their growth rate with culture media R20.

8. Sample preparation for HLA genotyping assay

BLCL 10 x 10^6 cells/ml were centrifuged at 1500 rpm for 10 min. Dicarded the supernatant and 600 μl of cell lysis buffer (Puregene, U.S.A.) was added and transferred to cryotube. The samples were kept at room temperature until being sent for HLA genotyping at MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, OX3 9DS, UK.

9. HLA genotyping assay

In all patients, HLA types were molecularly defined by PCR using sequence specific primers (SSP). The system used was originally set up by M.Bunce together with P.Krausa and M. Browning[142]. All HLA typings were performed by Tim Rostron in the Human Immunology Unit in Oxford. A total of 144 SSP reactions were used to detect simultaneously all known HLA-A, B, C, DRB3, DRB4, DRB5 and DRBQ1 specialities in an allele-specific or group specific manner using the same methods, reagents, PCR parameters and protocols for all loci. The results from this integrated class I and II method can be visualized on a single photograph and is described as 'phototyping'. Briefly, 10 microlitre of each 144 SSP primer set (each contains a positive control primer for β_2m) into V bottom 96 well PCR plates, overlaying with mineral oil, and then storing them at –20°C until needed. The TDMH bufffer for the final PCR reaction was prepared using 2.0 mM $MgCl_2$, 17mM ammonium sulphate, 67 mM Tris HCL (pH8), 6.7 M EDTA, 0.017% bovine serum albumin, 200 μM of each dNTPs, 2 mM $MgCl_2$, and stored at -20°C at 850μl/aliquot. For each DNA sample, one vial of TDMH buffer as thawed, and 6.4 μl of Taq polymerase, 26 μl of diluted DNA (200-300 μg/ml), and 480 μl of water were added, and mixed gently. Then 8 μl of the mixture was put into each well of the 96 well primer plate. The plate was spun at 500 rpm for 20 seconds and was then ready for the PCR which was performed through 30 cycles on an MJ-research PTC-200 thermal Cycles. Ten μl of each reaction was run out on a 2% agarose gel and the resulting gel was photographed. The tissue type was determined by the presence or absence of the appropriate-sized bands on the gel.

10. Recombinant vaccinia virus and HIV-1 clade A gag peptides

HIV-1 rVV clade B (HIV-1 gag B 1.5 kb and HIV-1 pol B 3.0 kb) and vaccnia virus (wild type: WT) control were obtained from Dr. Jean-Claude Chermann, I.N.S.E.R.M., U-322, Marseille, France.

HIV-1 rVV clade A (HIV-1 gag A 1.5 kb and HIV-1 pol A 3.0 kb) and HIV-1 clade A gag truncated peptide from a consensus sequence of 92UG037, 1-499 amino acids. Each peptide is 20 amino acids in length, with 10 amino acids overlap between sequential peptides. A total of 49 peptides which were obtained from the NIH AIDS Research and Reference Reagent

Program, USA. Each peptides were pooled as 5 peptides/pool. Thus, 10 pools of peptides were tested in this study (see appendix III).

The sequence of peptide gag A (92UG037) which were obtained from the NIH has a homology closed to the sequence of gag A/E (CM240) from Chiangmai, Thailand. The comparison of the sequence of CM240 and 92UG037 by program Multiple Aligment Legend showed that the homologous is shown identity of 87.58% (437/499) and similar residues of 10.02% (50/499) between the 2 subtypes (see appendix IV).

11. Effectors cells preparation by *in vitro* stimulation (IVS) assay

One fifth of each patient PBMC sample was dispensed into a 15 ml centrifuge tube. Pelleted the cells and discarded the supernatant. rVV pooled (gag, pol) clade A and B at a concentration of 5 plaque-forming units/cell (pfu/cell) were added. Incubated at 37°C with a humid atmosphere of 5% CO_2 in air for 1.30 hours, loosen caps slightly to allow for air exchange.

After one and a half hours of infection, cells were washed 2 times with 5 ml of RPMI1640 and resuspended. These cells were mixed with the remaining four fifth responder PBMCs. Put in 24-well plate and added 330 units/ml of human interleukin-7 (IL-7) on day 0. On day 3, 7 and 10, 100 units/ml of human recombinant interleukin-2 (IL-2) was added to culture media R10. Media was added as needed until the CTL assay was done on day 14.

12. Cross-clade CTL activity by classical chromium release assay

Day 1 : Autologous target cells preparation

Approximately 10 ml of the patient's BLCL was centrifuged. The pellet was resuspend in 5 ml of culture media R20 and number of viable cells was counted. The viability the EBV transformed cells of 80% is considered as a satisfactory yield.

Dispensed 5 x 10^5 BLCL into a 15 ml centrifuge tube (one tube should be set up for each target cell eg. rVV gag A, pol A, gag B, pol B and WT as a control). Pelleted the cells, discharge the supernatant and add rVV at concentration of 5 pfu/cell. Incubate at 37°C with a humid atmosphere of 5%

CO_2 in air for one and a half hours, loosen caps slightly to allow for air exchange.

After one and a half hours, cells were washed for 2 times with 5 ml of RPMI1640, and then resuspend with 1 ml of culture media R20 and put in 24-well plate. Incubated at 37°C with a humid atmosphere of 5% CO_2 in air for 16-18 hours.

Day 2 : CTL assay

The target cells were added in 15 ml centrifuge tube (one tube should be set up for each target cell). Pelleted the cells, discarded the supernatant. Eighty μCi of chromium-51 was added to each target cell. Incubated at 37°C with a humid atmosphere of 5% CO_2 in air for 2 hours.

During the time, effector cells were adjusted at the concentration 5 x 10^6 cells/ml. with culture media R10. And then, put it in 37°C incubator with a humid atmosphere of 5% CO_2 in air, loosen caps slightly to allow for air exchange.

After 2 hours, each target cells was washed for 2 times with 5 ml of RPMI1640 and then resuspend with 10 ml of culture media R20 (final dilution 5 x 10^4 cells/ml)

Effector and target cells are plated in a 96 well, U-bottomed microtiter plate, 200 μl final volume of each 100 μl of effector cells concentration in triplicate and 100 μl of target cells. The ratios of effector to target cells were at 100:1 and 50:1. For the total release (TR) or maximum release 5% triton-X 100 (TX 100) was added to the target cells. The chromium-51 release from the wells with culture media R10 only added is used to calculate the spontaneuos release (SR) or minimum release. Plates were centrifuged at 500 rpm for 5 min. Incubated at 37°C with a humid atmosphere of 5% CO_2 in air for 4 hours.

After 4 hours, removed the plate from incubator and centrifuged at 500 rpm for 5 min. 100 μl of supernatant put into a microtube which have 100 μl of 5% TX 100 and gamma ray was counted with a gamma counter.

13. CTL epitope mapping by classical chromium release assay

CTL assays were performed similar to previous description, except the target cells were pulsed with each of HIV-1 gag A 10 pooled peptides.

14. HLA-restriction of CTL activity

The process is the same as of CTL assay but different HLA class I single allele matched target cells were used. HIV-1 gag-specific CTL mapping was performed with autologous or partially HLA-matched target cells pulsed with 20 amino acids overlapping peptides and no pulsed peptide as the control. Classical chromium-51 assay was then performed at two ratio of effector to target cells i.e., 30 : 1 and 15 : 1[14].

15. Specific killing calculation

The level of CTL activity as denoted by the percent specific lysis (% specific lysis) of labeled BLCL targets is determined by the following formula.

$$\% \text{ spontaneous lysis} = \frac{\text{cpm. Spontaneous Release (SR)}}{\text{cpm. Total Release (TR)}} \times 100$$

$$\% \text{ specific lysis} = \frac{\text{cpm. Test} - \text{cpm. SR}}{\text{cpm. TR} - \text{cpm. SR}} \times 100$$

16. The criteria of positive CTL activity are following[14,15]

(1) % Spontaneous release is less than or equal to 30%
(2) % specific cytotoxicity of the test is more than 10% lysis of the WT vaccinia at the both effector to target ratio.
(3) % specific cytotoxicity of the test is value subtracted with the result of WT

17. Statistic analysis

Descriptive analysis was used.