CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Population and Subjects.

Twenty asymptomatic HIV-1 infected patients who attended the Anonymous Clinic of the Thai Red Cross AIDS Research Centre and the Immune Clinic of the King Chulalongkorn Memorial Hospital were included according to the inclusion and exclusion criteria.

Inclusion Criteria

- Confirmed anti-HIV antibody positive
- CD4+ cells are equal or more than 300 cells/ cu.mm.

Exclusion Criteria

- Patients who have been or being treated by antiretroviral therapy.
- Patients who have active concurrent infection
  and/ or opportunistic infection.
- Patients who are on cytotoxic drugs and/ or irradiation therapy.
- Patients who are on immunosuppressive drugs.

All subjects have provided written informed consent.

1.2 Specimen Collection

40 mL of heparinized whole blood was collected from each subject.

2. Methods

2.1 Truncated Peptides Design and Synthesis

The sequences of Tat exon I were designed based on the sequences of viruses from 12 HIV-1 infected Thais. Six out of 12 patients showed the same amino acid sequence as Tat exon I amino acid sequence of CRF01_AE (CM240) accession number U54771. Thus, this consensus amino acid sequence was used for the Tat exon I truncated peptides design. The complign PPC MacMolly® Tetra, Version 3.5-March,

'97 was used to identify the consensus sequence from 42 sequences of Tat exon II HIV-1 CRF01_AE isolated from Thailand 1990-1995. The total of 101 amino acids of Tat protein (exon I 72 amino acids, and exon II 29 amino acids) were pasted in the Peptgen program on the Los Alamos website at http://hiv-web.lanl.gov/PEPTGEN/PeptGenSubmitForm.html. to subdivide protein sequences into shorter overlapping peptides. Ten truncated Tat peptides of 17 to 21 amino acids overlapped by 10 amino acids were given as a result. These peptides were then synthesized at Natural and Medical Sciences Institute at the University of Tuebingen (Germany)

2.2 Peptide Preparation

1. Stock peptide 1 mg/mL

   Peptide 1 mg was dissolved by 1% DMSO in PBS 1 mL
2. Diluted peptide 100 μg/mL

   Stock peptide 100 μl was diluted in sterile PBS 900 μl
3. Pooled peptides

   Five individual peptides were put into 1 microtube by adding 100 μl of each peptide. Each pool therefore containing 5 truncated peptides.

2.3 Mononuclear Cells were Separated from Whole Peripheral Blood Using Ficoll Hypaque.

1. Heparinize whole blood was diluted approximately 1:1 with RPMI-1640 medium.
2. The Ficoll Hypaque (density gradient=1.077g/l) was added to a 15 mL centrifuge tube. The ratio of diluted blood to the gradient was approximately 2:1
3. An equal volume of the blood sample was carefully overlain on the Ficoll Hypaque. The surface of the Ficoll Hypaque must not be disturbed.
4. The tube was place in centrifuge and the speed was slowly turn up to 1,800-2000 rpm, centrifuged for 20±5 minutes at 20°C, no break.
5. The tube was immediately removed from the centrifuge. Five distinct layers were formed, from top to bottom: plasma, PBMCs, the Ficoll-Hypaque, granulocytes, and erythrocytes.
6. The plasma layer was discarded and the mononuclear band at the interface between the plasma and Ficoll-Hypaque was harvested.

7. PBMCs were washed 2 times by RPMI-1640 medium and centrifuged at 1,500-1,600 rpm for 10 minutes.
8. PBMCs were resuspended in 5-10 mL RPMI-1640 medium supplemented with L-glutamine, penicillin 100 U/ mL, streptomycin 100 μg/ mL, and 10% fetal bovine serum (R-10).
9. Cell count was performed and adjusted to desired cell concentration and stored at 37°C, 5% CO_2 with humidity.

2.4 Elispot Assay for Gamma-interferon (IFN-γ) Detection.

1. Ninety six-well polyvinylidene difluoride (PVDF) backed plates (MAIP S 45; Millipore, Bedford, MA) were coated with 50 μl/ well of anti-IFN-γ mAb 1-D1K (Mabtech, Stockholm, Sweden) at 10 μg/mL concentration in sterile phosphate-buffered saline (PBS) pH 7.4 and were incubated for 3 hours at 37°C in 5% CO_2 with humidity.
2. Unbound antibody was washed for 6 times with 200 μl PBS per well. The plates were then blotted and blocked with R-10 for at least 1 hour at room temperature.
3. Plates were then washed 6 times with 200 μl PBS per well. PBMCs were added 100 μl/well to the precoated plates. Input cell numbers were 2.5×10^5cells/ well, in positive wells added PBMC 2.5×10^4 cells/ well. Peptides were added to each labeled-well in duplicate at a final concentration of 10 μg/ mL. For the positive control wells, phytoheamagglutinin (PHA) was added at a final concentration of 20 μg/ mL. Gently shaked the pletes. Incubation at 37°C in 5% CO_2 with humidity for 16 hours.
4. After incubation, plates were washed 6 times with 200 μl/ well of PBS+0.05% Tween20 (PBS-T) and one time with 200 μl/ well of PBS. Next, 50 μl of 1 μg/ mL of the biotinylated anti-IFN-γ mAb 7-B6-1 (Mabtech, Stockholm, Sweden) was added.
5. After 3 hours of incubation in room temperature, plates were washed 6 times with 200 μl/ well of PBS-T and one time with 200 μl/ well of PBS. Fifty μl of 1:1000 dilution of streptavidin-alkaline phosphatase conjugate (Mabtech, Stockholm,

Sweden) was added 50 µl to the wells and the plates were incubated at room temperature for 1 hour.

6. The wells were again washed 6 times with 200 µl/ well of PBS-T and one time with 200 µl/ well of PBS and 100 µl of chromogenic alkaline phosphatase substrate (Bio Rad Labs., Hercules, CA) was added. After 30-45 minutes when dark spots emerged, the wells were washed with tap waters terminate to the colorimetric reaction and plates were then air-dried.

2.5 Enumeration of IFN-γ SFU.

Spots were counted by using a stereomicroscope (Olympus, Japan). Responses were considered significant if the average number of established SFU count of duplicated wells subtracted by negative control value was equal to or more than 100 SFU/ 10^6 input cells and 2 folds higher than negative control. The negative controls were always less than 100 SFU/ 10^6 input cells.

2.6 Statistic Analysis

Tat-specific CTL responses were shown as a proportion of patients who have responses to at least one Tat peptide.