CHAPTER III

MATERIALS AND METHODS

Part I : Optimization for serotyping

Materials

Study group

Sixty HIV-infected individuals who came for regular follow-up at the Immune Clinic of Chulalongkorn Hospital were selected on the basis of different risk factors. The subjects were derived from 3 different risk/groups of each, namely intravenous drug user (IVDUs), homosexual or bisexual, and heterosexual. HIV infection was identified by repeatly reactive anti-HIV by ELISA test which was confirmed by western blot test or serodiagnostic kit. Fifty-two were males and eight were females. Each group could be classified into different stages according to the 1987 CDC classification system, namely CDC stage II or asymptomatic HIV (n=10), CDC stage III or persistent generalized lymphadenopathy (PGL, n=9), CDC stage IV-A or HIV infection with constitutional symptoms or AIDS-related complex (ARC, n=26), and CDC stage IV-C and IV-D or HIV infection with secondary infections and secondary cancers respectively or those conventionally called AIDS (n=15). Diagnostic criteria for PGL, ARC and AIDS were based on CDC classfication system.[139]

Normal Control group

Sera obtained from healthy 50 HIV sero-negative blood donors at the National Blood Center of the Thai Red Cross Society were used as normal controls.

Positive Control group

Five sera from HIV sero-positive individuals with known subtypes E (3 samples) and B (2 samples) by sequencing analysis of V3 region were used as positive controls.[45]

Specimen

Twenty milliliters of blood were collected by venipuncture from each subject and divided in half into 2 separate tubes, one for heparinized blood and the other one for clotted blood. The heparinized blood was used to separate mononuclear cells by Ficoll-Hypaque density gradient centrifugation.[140] This tube is stored for later HIV-1 genotyping analysis. Sera from clotted blood were used for serotyping of HIV-1 subtypes. Sera were stored in aliquots at -70°C until use.

Synthetic peptides

Two peptides, each of 26 amino acid in length, designated PND-A (subtypeE: TRPSNNTRTSITIGRGQVFYRTGDII) and PND-B (subtype B : TRPNNNTRKSIHLGPGQAWYTTGQII) were compared with the others two peptides, each of 16 amino acid lenght PND-E (subtype E : TSITIGPGQVFYRTGD) and PND-B_{Thai} (subtype B: KSIHLGPGQAWYTTQE) from the principal neutralizing determinant (PND) on V3 loop of HIV-1 gp120. Peptides PND-A/PND-B and PND-E/PND-B_{Thai} were derived from the consensus sequences of Thai subtype E

and B, respectively. Both peptides were kindly provided by Dr. Kenji Okuda, Department of Bacteriology, Yokohama City University, School of Medicine, Yokohama, Japan.

Method

Serotyping by Indirect Enzyme Link Immunosorbent Assay (ELISA)

1. Setting up the optimal conditions for peptide serotyping by indirect ELISA test using 26 amino acid peptide

1. 1 Determination of the optimal concentration of V3 peptide

A 26 amino acid V3-peptide concentrate was dissolved in coating buffer to the concentrations of 5 and 10 µg/ml. 200 µl of each concentration was used to coat the microtitre plate at 37° C for 1 hour. The remaining solution was then aspirated . Next, 200 µl of blocking buffer was added and the plate was incubated at 37° C for 1 hour. After incubation, the plate was washed with PBS-tween 4 times and dried at 37° C for 1 hour. 200 µl of the positive and negative control sera, diluted at 1:100 and 1: 200 in diluent buffer, was added and the plate was again incubated at 37° C for 30 minutes. Each peptide concentration was assayed in duplicate. After 4 washes with PBS-tween, 200 µl of rabbit anti-human Ig G peroxidase conjugate, diluted at 1:1000 and 1:3000, was added followed by incubation at 37° C for 30 minutes. The plate was washed with PBS-tween 6 times. After 100 µl of substrate solution was added, the plate was incubated at room temperature for 10 minutes in the dark. The color development was stopped by the addition of 50 µl of 2 N H_2SO_4 . The absorbance was measured with a spectrophometer at 492 nm.

1.2 Determination of the optimal temperature for antigen incubation

Microtiter plates were coated with 10 μg/ml of PND-A and 5 μg/ml of PND-B at 37°C for 1 hour compared to at 4°C overnight. The plates were then processed in the similar manner as that described above.

2. Setting up the optimal conditions for peptide serotyping by indirect ELISA using the 16 amino acid peptides

2.1 Determination of the optimal concentration of V3 peptide

PND-E and PND-B_{Thai} was diluted in coating buffer to the concentration of 5 and 10 μg/ml. The plates were then processed in the similar manner as that described earlier.

2.2 Optimization for background reduction in peptide serotyping

2.2.1 Determination of the optimal serum dilution

Serum dilutions at 1:100, 1:200, and 1:400 were used in the assay. The plate was processed in a similar manner to described above.

2.2.2 Determination of the optimal conjugate dilution

Conjugate dilutions at 1:1000, and 1:2000 were used in the assay. The plate was processed in a similar manner as described above.

Results Readings

The absorbance of negative control sera was used to calculate the cut-off O.D of each run by adding 7 S.D. to the mean O.D of the negative controls. The cut-off O.D was approximated at 0.3. If the reaction to both PND-A and PND-B was positive, the rule of 3(ratio) would be applied, i.e., the subtype was assigned to the one with higher absorbance and ratio was 3 times greater than the other. Dual reactive was assigned if the absorbance of both peptides was greater than 0.3 but the ratio of the absorbance of both subtypes was less than

3.0 and non-rective was assigned to the serum specimen which was negative to both peptides.

II Validation of Subtyping by Genotyping

Genotyping of HIV-1 subtype was done by Nested Polymerase chain Reaction (PCR). Setting up of PCR conditions was described below .

Sample Preparation

PBMC were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation.[140] Four ml of heparinized blood was overlayed on 3 ml of Ficoll-Hypaque solution (see, Appendix II) by sterile technique and centrifuged at 220 G for 30 minutes at 4 °C. The PBMC at Ficoll-Hypaque-Plasma interface were collected and washed three times with RPMI 1640 (Gibco, Grand Island, N.Y., USA), then centrifuged at 14,000 rpm 1 minute. The supernatant was discarded and dried packed PBMC were collected. Ten microlitres of the cells were suspended and lysed in 50 µl of lysis buffer (see, Appendix II). The lysed cells were digested with proteinase K for 1 hour at 55 °C at which time the enzyme was inactivated for 15 minutes at 94 °C. The lysates were stored at -20 °C until used.

Primers

DNA Olionucleotides were synthesized in a oligosynthesizer (see, Appendix I). The nucleotide sequences of the primers and their locations in the HIV-1 genome are shown in the figure 1. The primer set BNI 5 and BNI 8 were used in first round PCR, the primer set BNI 6 and 7 were used in nested or second round PCR which were specific for HIV-1

infection, the primer set SA1/SA2 and BNI 7 were used in nested which were specific for subtype E whereas the primer set SB and BNI 7 were used for subtype B. The specific primer sets SA1, SA2 and SB were synthesized based on the sequence of all Thai isolates (Table III and figure 1). The designing of the primers was based on the single mutation principle, similar to the detection of AZT resistant mutant gene. The primer set SA1/ SA2 and SB were specifically designed to amplify the nucleotide which code for the V3 region of the HIV-1 subtype E and B respectively.

Amplification technique

The PCR was performed in a two-step reaction, first with a pair of outer primers (BNI 5 and 8) and then with a pair of nested primers (BNI 6 and 7 for detection of HIV-1 gene V3, SA_1/ SA_2 or SB and BNI 7 for detection HIV-1 subtype E and B_{Thai}, respectively). The PCRs were performed in 0.5 ml microfuge tubes (Biomed group) with 45 µl of the reaction mixtures (see, Appendix II) and 5 µl of cell lysate. Lysis buffer was used as a negative control to check the reagents for contamination. The samples were first denatured for 5 minutes at 94 °C in a Thermal cycler (Ericomb), then cycled 40 times at 94 °C for 30 seconds, annealed at 52 °C for 30 seconds, and extended at 72 °C for 30 seconds, and finally incubated at 72 °C for 5 minutes. After the first PCR, 2 µl of the product was amplified for 30 cycles with specific primers (SA1, SA2 and SB) and BNI 7 and the annealing temperature was varied for appropriate conditions. 10 µl of product from the second PCR was analyzed by electrophoresis on a 1.5 % agarose gel (Gibco) stained with ethidium bromide (Gibco, Grand Island, N.Y., USA).

Positive control group

PBMCs from HIV-positive individuals with known subtypes E and B by sequencing analysis of V3 region were used as positive controls.[45]

III. Natural History Analysis

An initially asymptomatic HIV-1 infected individuals who had been followed-up at Chulalongkorn Hospital and had complete clinical record at least or more than 3 years and had regularly CD4 cell count in each year for at least 3 years were selected for HIV-1 serotyping. The natural courses were compared by the CD4 declining rate and disease progression between each subtype.

Statistical analysis

Unpaired student t-test and Mann-whittney and Chi-square test were used for statistical analysis. Any p value of less than 0.05 was considered statistically significant.