Chapter III

Materials and Methods

Subjects

A total of 45 subjects were included in this study. They were consisted of 3 different groups, fifteen of each were HIV seronegative blood donors (group A), HIV-infected persons with CD4+ T cell count $\geq$ 200 cells/μl (group B), and HIV-infected persons with CD4+ T cell count < 200 cells /μl (group C).

The followings were the inclusion criteria of each group:

Group A:

1. Voluntary blood donors
2. Anti-HIV seronegative
3. No any active illness

Group B:

1. Asymptomatic HIV-infected person
2. Documented HIV infection: anti-HIV positive and confirmed a second EIA test or western blot or any equivalent test
3. CD4+ T cell count $\geq$ 200 cells/μl
4. No any active illness
5. No history of pre-treated with antiretroviral therapy
6. No history of immunosuppressive or immune-based therapy

Group C:

1. Symptomatic stage of HIV infection
2. Documented HIV infection: anti-HIV positive and confirmed by a second EIA test or western blot or any equivalent test
3. CD4+ T cell count < 200 cells/μl

4. No any active illness
5. No history of pre-treated with antiretroviral therapy
6. No history of immunosuppressive or immune-based therapy

Subjects in group A were referred to A1-A15, respectively. HIV-1-infected individuals were categorized as group B and group C. Subjects in group B were referred to HIV-infected asymptomatic patients who were defined as individuals with no AIDS - defining symptoms and whose CD4 cell counts falling at least 200 cells/μl at the time of study. The subjects were referred to B1-B15, respectively. Subjects in group C were referred to HIV-infected symptomatic patients with CD4 cell counts falling below 200 cells/μl at the time of study. They were referred to C1- C15, respectively.

Study Sites

HIV-seronegative voluntary blood donors were from National Blood Bank, Thai Red Cross Society. HIV-infected persons were obtained from Immune Clinic at the King Chulalongkorn Memorial Hospital and Anonymous Clinic, Thai Red Cross AIDS Research Centre. The laboratory analyses were performed at the Laboratory of Cellular Immunology, Division Immunology, Department of Microbiology; and the Division of Allergy and Clinical Immunology, Department of Medicine, Faculty of Medicine, Chulalongkorn University.

Sample size

According to the previous report revealed that IL-18 (IGIF) mRNA could be detected in total RNA of unstimulated PBMC from human. (9) If the expected rate of IL-18 gene expression in PBMC from HIV infected patients was 50% lower as compared to HIV negative controls. Sample size for each group has been calculated to give 90% power to detect a significant reduction of 0.5 with $P = 0.05$ and $\alpha = 0.05$ (two tailed). The estimated sample size is 15 subjects per group.

Sample Specimens

Ten ml of whole blood was collected from each subject in a vacutainer tube containing preservative-free heparin. Peripheral blood mononuclear cells (PBMCs) were separated from the blood samples by Ficoll-Hypaque gradient. The separated PBMCs were washed twice in phosphate-buffered saline (PBS), and the number of viable leukocytes was determined by trypan blue exclusion and hemocytometer. PBMC pellets were collected at approximately $4x10^6$ cells/tube. The PBMC samples were stored in duplicated dry pellets at -70°C until RNA extraction and RT-PCR analysis were performed. Of note, the heparinized whloe blood samples, especially HIV-infected whole blood with CD4 < 200 cells/μl unless they can be harvested for enough yield of PBMC from 10 ml whole blood, collected more volume of the whole blood or required collecting a new blood sample from another subjects.

RNA extraction from PBMC pellet by Guanidine thiocyanate/phenol/chloroform extraction

Amount of 500 μl of chilled working solution of lysis GTC buffer was added to a 1.5 ml eppendorf tube containing of each PBMC pellet approximately 4 x 10^6 PBMCs, then homogenized the lysis mixture by a vortex mixer. If the pellet was not well resuspened, heat the mixture in 65°C water bath for 10 minutes and then remixed it by the mixer. Added 50 μl of 2 M NaAcetate and mixed. Pre-warmed phenol-Tris of 500 μl was added, and then added 100μl of chloroform/isoamyl alcohol (49:1), mixed it well by inverting and vortexing. Cooled it on ice for 5 minutes. Then centrifuged at 14,000 rpm for 15 minutes at 4 °C. The acid-condition phenol extracts the RNA, while the chloroform provided layering for simple removal of aqueous RNA. Carefully remove eppendorf tubes so as not to disturb layers. The upper RNA-containing aqueous phase was tranfered to a fresh sterile RNase-free microcentrifuge tube. Chilled isopropanol (stored at 4 °C) of 500 μl was added and well mixed, then it was frozen at -70 °C for an hour to precipitate RNA. The tubes can be kept overnight at this stage if necessary. The RNA sample was then centrifuged at 14,000 rpm at 4 °C for 20 minutes to precipitate the total RNA pellet. Carefully discarded supernatant, and 1 ml of chilled 70% EtOH (4 °C) was added to wash the RNA pellet without disturbing pellet and then it was centrifuged at 14,000 rpm at 4 °C for 15 minutes. The supernatant was carefully discarded and then pellet was washed at second time with the same amount of chilled 70% EtOH (4 °C) and was

centrifuged at 14,000 rpm at 4 ^{0}C for 15 minutes. The supernatant was carefully discarded. The excess fluid at lip and sides of the tube was removed by kimwipe paper while tube is still inverted. A parafilm was placed on top of tube. Three small holes were punched at the top and RNA was dried in the SpeedVac® for a few minutes. RNA pellet was reconstituted in 1U/µl of RNase inhibitor containing diethylpyrocarbonate (DEPC) - treated H_2O. The RNA concentration was determined in µg/µl at 260 nm.

cDNA Synthesis

To prepare cDNA, 1µg of total RNA was reverse transcribed using oligo-dT_{12-18} (Pharmacia Corp.) as a primer for reverse transcription. The reaction took place in a total volume of 20 µl containing the following components (purchased from Promega Corp., Madison, USA) at the final concentrations as indicated : 5 mM $MgCl_2$, 1x PCR buffer, 1mM each of dNTPs, 0.3 µM Oligo-dT_{12-18}, 2.5 U/µl M-MLV RT, 1.2 U/µl RNase inhibitor and total RNA 1µg, and adjusted to the final volume using DEPC-H_2O. The RT reaction was carried out in one cycle at 42^{O}C for 15 min, then at 99^{O}C for 5 min (to inactivate RT enzyme) and terminated at 5^{O}C for 5 min. The cDNA was then used for PCR or stored at –20^{O}C for further use.

To detect possible contaminations of genomic DNA or cDNA in the RNA samples, or reagents or during all steps of laboratory reactions, non-reverse transcribed control (NRT) in parallel with each set of reaction was always performed. NRT control was done by the used of DEPC-H_2O instead of the reverse transcriptase in an equal volume.

Polymerase Chain Reaction

To insure that the extracted samples have the total RNA messages in the similar amounts, a RT-PCR of an ubiquitous housekeeping gene,β-actin was performed as a control in each of the samples. The β-actin PCR was carried out with a total volume of 25 µl containing the following components (Promega Corp.) at the final concentrations as indicated : 2 mM $MgCl_2$, 1xPCR buffer, 0.2 mM each of dNTPs, 0.4 µM 3′β-actin primer, 0.4 µM 5′β-actin primer, 1.25 U Taq DNA polymerase and 2.5 µl of cDNA mixture, and adjusted to final volume by DEPC-H_2O. Each PCR cycle included at 95^{O}C for 2 min for 1

cycle, at 95 ^{O}C for 1 min and then at 60^{O}C for 1 min for 25 cycles. The final extension phase was at 60 ^{O}C for 7 min and stored at 4 ^{O}C until further analysis. The human β-actin primers were as the following : the sense primer (5′) was 5′ GTG GGG CGC CCC AGG CAC CA 3′and the antisense primer (3′) was 5′ CTC CTT AAT GTC ACG CAC GAT TTC 3′. The size of β-actin PCR product is 548 bp.

Each cDNA sample obtained from 1μg of total RNA was subjected for PCR analysis of the specific primers of IL-18, MIP-1α and RANTES, by which the product sizes were 342 bp, 229 bp and 195 bp, respectively. The nucleotide sequences of these specific primers were as the followings :IL-18 (5′) : 5′ GCT TGA ATC TAA ATT ATC AGT C 3′ , IL-18 (3′) : 5′ GAA GAT TCA AAT TGC ATC TTA T 3′ ; MIP-1α (5′) : 5′ AAC ATA TGG CAT CAC TTG CTG CTG ACA CGC CG 3′, MIP-1α (3′) : 5′ AAG GAT CCT CAG GCA CTC AGC TCT AGG TCG CT 3′ ; RANTES (5′) : 5′ CCT CGC TGT CAT CCT CAT TG 3′ , RANTES (3′) : 5′ ACT TGG CGG TTC TTT CGG GT 3′. For PCR reaction : 2.5 μl of RT product was used in a total volume of 25 μl containing the following components (Promega Corp.) in final concentrations : 2 mM $MgCl_2$, 1x PCR buffer, 0.2 mM each of dNTPs, 0.4 μM of 3′ specific primer, 0.4 μM of 5′ specific primer, 1.25 U Taq DNA polymerase and cDNA mixture and adjusted to final volume by DEPC-H_2O. The PCR condition was carried out as follows : at 95 ^{0}C, 2 min for 1 cycle, then at 94 ^{0}C, 0.5 min at 60 ^{0}C, 2 min at 72 ^{0}C, 3 min for 33 cycles. The products were stored at 4 ^{0}C until further analysis.

The reverse transcriptase and PCR reactions were performed in DNA thermal cycles (Perkin-Elmer Gene Amp PCR system 9600). To test the linearity of amplification, a two-fold dilution series, starting at 1:2 of undiluted cDNA mixture from the HIV seronegative sample, were amplified under conditions identical to those described above by the specific primers for β-actin, IL-18, MIP-1α and RANTES.

Amplified Products Analysis

Ten- microliter PCR products and the 100-bp DNA ladder (Promega Corp.) were electrophoresed through a 1.5% agarose gel containing 1x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8) with 0.2 μg/ml ethidium bromide, and visualized by UV illumination and polaroid photographed.

Statistical Analysis

The differences in the numbers of subjects with cytokine or chemokines gene expressions in PBMCs from the different clinical stages of HIV-infected groups and HIV control group were statistically compared using the Fisher's exact test and StatView® version. Values of $p \leq 0.05$ were considered to be statistically significant.