CHAPTER III

MATERIALS AND METHODS

Part I Standardization of RT-PCR analysis for IL-2, IL-18, RANTES and MIP-1α mRNA production

1. Study group

Five HIV-seronegative donors from the National Blood Centre, Thai Red Cross Society were included in the study.

2. Preparation of PBMC

Ten milliliters of fresh whole blood was collected from each donor into heparinized tubes. PBMC were separated by Ficoll-Hypaque gradient centrifugation. Cells were recovered, washed, and resuspended in RPMI 1640 with 10% FCS, at $1x10^6$ cells/ml concentration. The resuspended cells were stimulated with 5 μg/ml PHA for IL-2 and IL-18 and 10 μg/ml LPS for RANTES and MIP-1α analysis. After incubated at 37 ^{0}C in 5% CO_2 for 8 and 19 hours, respectively, cells were harvested, washed and divided into aliquots of $4x10^6$ cells per tube and then stored as dry-packed cells at -70 ^{0}C.

3. RNA isolation

Cells were lysed by guanidinium-isothiocyanate (4M) in sodium citrate (25mM) buffer, pH 7.0, with 0.5% sarcosyl and 0.1 M β_2-ME; 0.1 vol of 2 M sodium acetate was added together with 1 vol of water-saturated phenol and 0.2 vol of 49:1 chloroform: isoamyl alcohol. After centrifugation, RNA was extracted in the aqueous phase and the phenol/chloroform extraction was repeated once more. The RNA was then precipitated with isopropanol at –70 ^{0}C for 1 h or overnight.[130] After centrifugation, the pellet was twice washed with 70% ethanol, dried in the SpeedVac dryer and dissolved in diethylpyrocarbonate (DEPC)-treated water containing 1 U/μl RNase inhibitor.

4. cDNA synthesis

A reverse transcription (RT) reaction was performed on each mRNA sample, using up to an estimated 1 μg of total mRNA per tube, to produce a single batch of cDNA from each RNA sample. The RT reaction was carried out as a single tube reaction with uninterrupted thermal cycling by using the Gene Amp PCR System 9600 (Perkin-Elmer, Norwalk, Conn.). The final reaction mixture contained 5 mM $MgCl_2$, 1xPCR buffer, 1 mM dNTPs, 0.3 μM oligo dT, 2.5 U/μl M-MLV reverse transcriptase (Promega, USA), 1.2 U/μl RNase inhibitor and 1 μg of total RNA in a total volume of 20 μl. The reaction was incubated at 42 ^{0}C for 15 min, 99 ^{0}C for 5 min, and soaked at 5 ^{0}C for 5 min.

At each run of RT-PCR, negative and positive controls were included beginning at the RT step.

1. Non-reverse transcribed (NRT) negative control

The 0.25 μl of reverse transcriptase enzyme in the RT master mix was replaced with DEPC-H_2O. This control was to check for cDNA or DNA contamination from external sources, as well as for the purity of the extracted mRNA. NRT control was run in a β-actin RT-PCR in each mRNA sample.

2. Negative water control

This control consists of water which is run as a sample through the entire procedure. It was to monitor external contamination of reagents, i.e., contaminated primers, reagents or the specimen, etc., with specific RNA prior to RT step, as well as DNA contamination picked up at any time.

3. Positive control mRNA(s)

Positive controls for RT and PCR steps derived from donor mRNA which are positive for the IL-2, IL-18, RANTES and MIP-1α expression in Part I.

5. PCR amplification

cDNA obtained from reverse transcription was then amplified using PCR technique. Three microliters of cDNA were amplified in a 25 μl reaction mix [2 mM $MgCl_2$ (2.5 mM for IL-2), 1x PCR buffer, 1mM dNTPs, 0.4 M 5' and 3' primers (0.2 M for IL-2)]. *Taq* DNA polymerase (Promega, USA) was added to the master mix at 2.5 U per reaction and 22 μl of the mixture were dispensed into a thin-walled

MicroAmp tube. Three microliters of cDNA were added to each tube. The PCR reactions were amplified for a total of 33 cycles of denaturation at 94 ^{0}C for 0.5 min, annealing at 60 ^{0}C for 2 min, and extension at 72 ^{0}C for 3 min. The nucleotide sequences of the cytokine and chemokine primers are shown in Table I.

6. Analysis of PCR products

A 1.5% agarose gel was prepared in TAE buffer containing 1 μg of ethidium bromide per ml. Upon electrophoretically separating the 10 μl PCR products in TAE buffer, the bromphenol blue indicator should migrate about half the length of the gel. The amplified products were visualized on a UV light transilluminator. The 100 base pair DNA ladder was used as a marker for the size estimation of the products.

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Table I. Cytokine and chemokine primers used for PCR

Cytokines	Product size (bases)	Primer sequence
β-actin 5'	548[a]	5'-GTGGGGCGCCCCAGGCACCA-3'
β-actin 3'		5'-CTCCTTAATGTCACGCACGCATTTC-3'
β-actin 5'	218[b]	5'-AAGAGAGGCATCCTCACCCT-3'
β-actin 3'		5'-TACATGGCTGGGGTGTTGAA-3'
IL-2-5'	229	5'-GAATGGAATTAATAATTACAAGAATCCC-3'
IL-2-3'		5'-TGTTTCAGATCCCTTTAGTTCCAG-3'
IL-18-5'	474[c]	5'-AACATATGTACTTTGGCAAGCTTGAATCT-3'
IL-18-3'		5'-TTGGATCCCTAGTCTTCGTTTTGAACAGT-3'
MIP-1α-5'	229[d]	5'-AACATATGGCATCACTTGCTGCTGACACGCCG-3'
MIP-1α-3'		5'-AAGGATCCTCAGGCACTCAGCTCTAGGTCGCT-3'
RANTES-5'	195	5'-CCTCGCTGTCATCCTCATTG-3'
RANTES-3'		5'-ACTTGGCGGTTCTTTCGGGT-3'

[a] Primer used for Part I

[b] Primer used for Part II & III

[c] and [d] actually are cloning primer sets, the upper primers of c (IL-18-5') and d (MIP-1α-5') are Nde I site (CATATG) and the lower primers of those are Bam HI site (GGATCC)

Part II Reproducibility of RT-PCR amplification

To validate a reproducibility of RT-PCR amplification, two-fold serial dilutions of β–actin and RANTES cDNA containing samples were subjected to PCR. The amount of each band was determined by density scanning (Vilber Lourmat, France).

Part III Study of IL-2, IL-18 RANTES and MIP-1α mRNA production by PBMC of HIV-infected patients.

1. Patient population

A total of 48 eligible were recruited in this protocol. The eligible patients were asymptomatic HIV-infected individuals with a CD4+ cell count of at least 350 cells/mm^3 . The inclusion and exclusion criteria were described in Appendix III. Patients were randomly assigned to receive trial medication in accordance with the treatment arm summarized in Table II.

Table II. Randomization schedule and treatment assignments.

Treatment arm	Recruitment (n)	Study drugs
I. Antiretroviral control group (ART)	24	ddI and d4T*
II. Antiretroviral group plus sc. IL-2 (ART plus IL-2)	8	1.5 MIU bid + ddI and d4T*
	7	4.5 MIU bid + ddI and d4T*
	9	7.5 MIU bid + ddI and d4T*

* If intolerated to ddI or d4T, the treatment was replaced with AZT plus 3TC

IL-2 will be delivered by twice daily subcutaneous injection for 5 days every 8 weeks. A cycle of therapy is defined as five days of IL-2 plus antiretrovirals followed by 7 weeks of antiretroviral therapy alone.

Dosing will commence at 1.5 MIU bid x 5 days every eight weeks. This will escalate to 4.5 MIU bid and then to 7.5 MIU bid when a minimum of nine patients have completed a cycle with IL-2 at a lower dose level in the absence of dose limiting toxicities.

IL-2, IL-18, RANTES and MIP-1α mRNA production were evaluated from the PBMC of 10 patients at baseline and week 8 in the ART group and at the third cycle (precycle 3, day 4 and day 29) of IL-2 administration in ART+IL-2 group. PBMC collected at baseline and week 16 in both groups were analysed to compare the IL-2 treatment on IL-2 gene expression. To assess those effects in a dose response relation, PBMC collected from all members of the ART+IL-2 at baseline and day 4 of cycle 1 were analysed.

2. Specimen collection and preparation of PBMC

Ten milliliters of fresh blood were collected from patients by venepuncture into heparinized tubes at each time point. PBMC were separated by centrifugation on Ficoll-Hypaque. Cells were recovered, washed, and resuspened in RPMI 1640 with 10% FCS, at $4x10^6$ cells/ml concentrations and stored as dry-packed cells at $-70\ ^0C$ until the analysis were performed.

3. RNA isolation, cDNA synthesis and PCR amplification

PBMC specimens were thawed and subjected for RNA isolation, cDNA synthesis and PCR amplification as described in **Part I (3, 4,5).**

4. Analysis of PCR products

Ten microliters of the PCR products were analysed as described in **Part I (6).**

5. Statistical analysis

Fisher's exact test (using a statistical software, SPSS, USA) was used to analyse the proportion of patients with cytokine or chemokine mRNA expression between the groups and analyse paired values in the same group. Results were considered statistically significant when $p < 0.05$.

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