การศึกษาซีทีแอล อีพิโทป ในคนไทยที่ติดเชื้อเอชไอวี-1 ที่มีซีดี 4 มากกว่าหรือเท่ากับ 300 เซลล์ต่อลูกบาศก์มิลลิเมตร ต่อโปรตีนวีพียูและวีพีอาร์ของเชื้อเอชไอวี-1 โดยวิธีอินเตอเฟอรอนแกมม่า อีไลสปอท

นางสาวสุธิดา เกิดสันติ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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HIV-1 VPU AND VPR CTL EPITOPE MAPPING BY IFN-γ ELISPOT ASSAY IN HIV-1 INFECTED THAI INDIVIDUALS WITH CD4+ COUNT ≥ 300 CELLS/MM^3

MISS SUTHIDA KERDSAN

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	HIV-1 Vpu and Vpr CTL Epitope Mapping by IFN-γ ELISPOT Assay in HIV-1 Infected Thai Individuals with CD4+ Count ≥ 300 cells/mm^3
By	Miss Suthida Kerdsan
Field of study	Medical Science
Thesis Advisor	Associate Professor Kiat Ruxrungtham, M.D.

Accepted by the Faculty of Medicine, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

……..…….Dean of Faculty of Medicine
(Professor Pirom Kamolratanakul, M.D.)

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……...Thesis Advisor
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……..Member
(Pokrath Hansasuta , M.D.,D.Phil.)
……...…Member
(Associate Professor Apiwat Mutirangura, M.D.,Ph.D.)

สุธิดา เกิดสันติ์: การศึกษาซีทีแอลอีพิโทปในคนไทยที่ติดเชื้อเอชไอวี-1 ที่มีซีดี4 มากกว่าหรือเท่ากับ 300 เซลล์ต่อลูกบาศก์มิลลิเมตร ต่อโปรตีนวีพียูและวีพีอาร์ของเชื้อเอชไอวี-1 โดยวิธีอินเตอเฟอรอนแกมม่า อีไลสปอท. (HIV-1 Vpu and Vpr CTL Epitope Mapping by IFN-γ ELISPOT Assay in HIV-1 Infected Thai Individuals with CD4+ Count ≥ 300 cells/mm^3) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ นายแพทย์เกียรติ รักษ์รุ่งธรรม ; 73 หน้า. ISBN 974-17-3420-4

สายพันธุ์ของเชื้อไวรัสเอชไอวีที่พบระบาดอยู่ในประเทศไทย คือ สายพันธุ์ CRF01_AE ซึ่งมีความแตกต่างจากสายพันธุ์ B ที่มีการระบาดอยู่ในกลุ่มประเทศที่พัฒนาแล้ว ไซโตทอกสิก ที ลิมป์โฟซัยท์ (Cytotoxic T Lymphocytes หรือ CTLs) เป็นเซลล์ที่มีบทบาทสำคัญในการควบคุมการติดเชื้อเอชไอวี โปรตีนวีพีอาร์เป็นโปรตีนซึ่งมีบทบาทสำคัญในการยับยั้งการแบ่งตัวของเซลล์โดยหยุด cell cycle ไว้ที่ G2/M phase ทำให้เชื้อเพิ่มจำนวนได้มากขึ้น โปรตีนวีพียูเหนี่ยวนำให้เกิดการลดจำนวนของซีดี 4โมเลกุล และช่วยในการปลดปล่อยอนุภาคไวรัสออกจากเซลล์ ยังไม่มีข้อมูลการศึกษาหาการตอบสนองของไซโตทอกสิก ที ลิมป์โฟซัยท์ ต่อโปรตีนทั้งสองที่พบได้บ่อยและ/หรือที่พบใหม่ และการทำ epitope mapping ในสายพันธุ์ CRF01_AE ยังมีผู้สนใจศึกษาเป็นจำนวนน้อยมาก ดังนั้นการศึกษาอาจนำมาซึ่งข้อมูลที่มีประโยชน์ต่องานวิจัยและพัฒนาวัคซีนสำหรับการป้องกัน และรักษาโรคเอดส์ที่เหมาะสำหรับประเทศไทย

การศึกษานี้ได้ตรวจหาการตอบสนองของไซโตทอกสิก ที ลิมป์โฟซัยท์ ต่อโปรตีนวีพีอาร์และวีพียู และการทำ epitope mapping ในอาสาสมัครคนไทยที่ติดเชื้อเอชไอวี-1 โดยใช้เทคนิคอีไลสปอท (ELISPOT) และใช้โปรตีนวีพีอาร์และวีพียูท่อนสั้นๆ ของเชื้อเอชไอวีสายพันธุ์ CRF01_AE (ความยาว 15-20 amino acids แต่ละเส้นมีความเหลื่อมกัน 10 amino acids) ในการกระตุ้นเซลล์สำหรับการตรวจคัดกรองการตอบสนองของไซโตทอกสิก ที ลิมป์โฟซัยท์ ในเบื้องต้นนั้นใช้สายโปรตีนหรือเปปไทด์จำนวน 4 ชุด แต่ละชุดประกอบด้วย 5 เปปไทด์ ยกเว้นชุดสุดท้ายมีเพียง 3 เปปไทด์ หลักเกณฑ์ที่ถือว่าให้ผลบวก คือ ผลการตรวจที่มีจำนวน spot หลังจากลบ background แล้วมีค่า ≥ 100 SFU/10^6 PBMCs และสูงมากกว่า 2.5 เท่าของ negative control อาสาสมัครที่ให้ผลบวกต่อชุดเปปไทด์จะได้รับการตรวจหาการตอบสนองโดยใช้แต่ละเส้นเปปไทด์ที่จำเพาะของ Vpu และ Vpr ต่อไป

จากการแยกชนิดของการติดเชื้อพบว่า อาสาสมัครจำนวน 18 รายจาก 20 ราย มีการติดเชื้อเอชไอวีสายพันธุ์ CRF01_AE ซึ่งอาสาสมัครกลุ่มนี้จำนวน 9 ราย จาก 18 ราย (50%) แสดงผลบวกต่อชุดเปปไทด์วีพีอาร์ ผลการตอบสนองอยู่ระหว่าง 132-1,800 SFU/10^6PBMCs (ค่ามัธยฐาน 470 SFU/10^6PBMCs) และอาสาสมัคร 5 รายจาก 18 ราย (28%) แสดงผลบวกต่อชุดเปปไทด์วีพียู ผลการตอบสนองอยู่ระหว่าง 124-908 SFU/10^6PBMCs (ค่ามัธยฐาน 394 SFU/10^6PBMCs) อาสาสมัครที่ให้ผลบวกต่อชุดเปปไทด์วีพีอาร์แสดงผลบวกต่อแต่ละเปปไทด์ที่จำเพาะจำนวน 7 ราย (7/18 = 39%) ผลการตอบสนองอยู่ระหว่าง 114-1,572 SFU/10^6PBMCs (ค่ามัธยฐาน 880 SFU/10^6PBMCs) และ 3 ราย (3/17 = 18%) แสดงผลบวกต่อเปปไทด์ที่จำเพาะของวีพียู ผลการตอบสนองอยู่ระหว่าง 136-680 SFU/10^6PBMCs (ค่ามัธยฐาน 306 SFU/10^6PBMCs) ในการศึกษานี้มี 2 epitopes ที่อาจเป็นการค้นพบใหม่ โดยมีอย่างละ 1 epitope ในโปรตีนวีพีอาร์และในโปรตีนวีพียูที่ยังไม่เคยมีการรายงานมาก่อน ทั้งนี้ควรมีการศึกษารายละเอียดเพื่อการยืนยันต่อไป

หลักสูตร วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต.....

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2545

##4375270930 : MAJOR MEDICAL SCIENCE

KEY WORD : / CTL EPITOPE MAPPING / ELISPOT/ IFN-γ / HIV-1 / Vpr / Vpu

SUTHIDA KERDSAN : HIV-1 Vpu and Vpr CTL Epitope Mapping by IFN-γ ELISPOT Assay in HIV-1 Infected Thai Individuals with CD4+ Count ≥ 300 cells/mm^3. THESIS ADVISOR : ASSOC.PROF. KIAT RUXRUNGTHAM, M.D., 73 pp. ISBN 974-17-3420-4

Circulating Recombinant Form (CRF) AE is the most common HIV-1 subtype found in Thailand which causes AIDS epidemic in Thailand. The virus sequence of AE subtype is different from subtype B which commonly found in developed countries. Cytotoxic T Lymphocytes (CTLs) are considered as an important protective immunity against HIV infection. Vpr arrests cell cycle at the G2/M phase that can increase virus production. Vpu facilitates the budding of new virus particles from infected cells and also enhances the degradation of CD4. There are no CTL studies of HIV-1 CRF01_AE-specific CTL epitope mapping directed against both Vpu and Vpr. So this study may provide additional useful data for the research and development HIV/AIDS vaccine in Thailand.

This study is to demonstrate Vpu and Vpr-specific CTL responses and to map the corresponding epitopes in 20 untreated HIV-1 infected Thais by using IFN-γ ELISPOT assay. HIV-1 CRF01_AE based Vpu and Vpr peptides 15-20 amino acids in length, and overlapped by 10 amino acids were used for this study. There are 4 pools of Vpr and Vpu peptides, each contains 5 truncated peptides except the last pool contains 3 Vpu peptides. The positive cut-off of ELISPOT was defined as the IFN-γ spot-forming unit (SFU)/10^6PBMCs after the background subtracted is ≥ 100 SFU/10^6PBMCs and > 2.5 folds higher than negative control. Patients who showed positive results were then further identified for Vpr and Vpu specific epitope (s) recognition.

Eighteen out of 20 showed CRF01_AE infection by subtyping. Nine out of 18 (50%) AE-infected patients showed IFN-γ ELISPOT positive responses to Vpr pooled peptides. The magnitude of responses ranged from 132-1,800 SFU/10^6PBMCs (median 470 SFU/10^6PBMCs). Five out of 18 AE-infected patients (28%) showed positive responses to Vpu pooled peptides. The magnitude of responses ranged from 124-908 SFU/10^6PBMCs (median 394 SFU/10^6PBMCs). 7/18 HIV-1 CRF01_AE patients (39%) who showed positive responses to Vpr pooled peptides showed positive results to Vpr individual peptides. The magnitude of responses ranged from 114-1,572 SFU/10^6PBMCs (median 880 SFU/10^6PBMCs). 3/17 HIV-1 CRF01_AE patients (18%) showed positive results to Vpu individual peptides with the magnitude of responses ranged from 136-680 SFU/10^6PBMCs (median 306 SFU/10^6PBMCs). However, in this study there are 2 possible novel epitopes : one in Vpr and the other in Vpu were found. Further characterization of these epitope is warranted.

Program Medical Science Student's signature.....

Field of study Medical Science Advisor's signature.....

Academic year 2002

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LIST OF ABBREVIATIONS

AIDS	=	Acquired Immunodeficiency Syndrome
β-TrCP	=	Beta-transducin repeats containing protein
CA	=	Capsid
CD	=	Cluster of Differentiation
CMI	=	Cell-Mediated Immunity
CO_2	=	Carbon Dioxide
CRF	=	Circulating Recombinant Form
CTL	=	Cytotoxic T lymphocyte
CyA	=	Cyclophillin A
^{o}C	=	Degree Celsius
DMSO	=	Dimethysulphoxide
DNA	=	Deoxy Nucleic Acid
ELISPOT	=	Enzyme-linked Immunospot
Env	=	Envelope
ER	=	Endoplasmic Reticulum
et al.	=	et alii
FBS	=	Fetal Bovine Serum
mg	=	Milligram
Gag	=	Group antigen gene
gp	=	Glycoprotein
Group M	=	Major group
Group N	=	Non-M and non-O group
Group O	=	Outlier group
HIV	=	Human Immunodeficiency Virus
HLA	=	Human Leukocyte Antigen
ICS	=	Intracellular Cytokine Staining
IFN-γ	=	Gamma interferon
IN	=	Integrase
kb	=	kilobase

kD	=	kilodalton
LDA	=	Limiting Dilution Assay
LTNP	=	Long-Term Nonprogressor
LTRs	=	Long Terminal Repeats
MA	=	Matrix
MHC	=	Major Histocompatibility Complex
MHR	=	Major Homology Region
ml	=	milliliter
mRNA	=	messenger Ribonucleic Nucleic Acid
μg	=	microgram
μl	=	microliter
NC	=	Nucleocapsid
Nef	=	Negative factor
p	=	protein
PBMCs	=	Peripheral Blood Mononuclear Cells
PBS	=	Phosphate Buffer Saline
PHA	=	Phytohemagglutinin
Pol	=	Polymerase
PR	=	Protease
Rev	=	Regulatory of Expression of Viral protein
RNA	=	Ribonucleic Acid
rpm	=	round per minute
RPMI 1640	=	Rosewell Park Memorial Institute formular 1640
RT	=	Reverse Transcriptase
SFU	=	Spot-Forming Unit
SIV	=	Simian Immunodeficiency Virus
SU	=	Surface glycoprotein
T cell	=	Thymus-derived lymphocyte
TAR	=	Trans-activation response element
Tat	=	Transactivator of transcription
TCR	=	T Cell Receptor

TM	=	Transmembrane glycoprotein
TNF-β	=	Tumor Necrosis Factor Beta
Vif	=	Viral Infectivity Factor
Vpr	=	Viral protein R
Vpu	=	Viral protein U

CHAPTER I

INTRODUCTION

1. Background and Rational

The global HIV/AIDS epidemic update as of December 2002 showed approximately 42 million and with that approximately 5 million newly infected cases with HIV. Over 3.1 million people died from AIDS worldwide in 2002.[1] Almost 1 million people in Asia and Pacific acquired HIV infection in 2002, by which has led to an estimated 7.2 million people currently living with the virus.

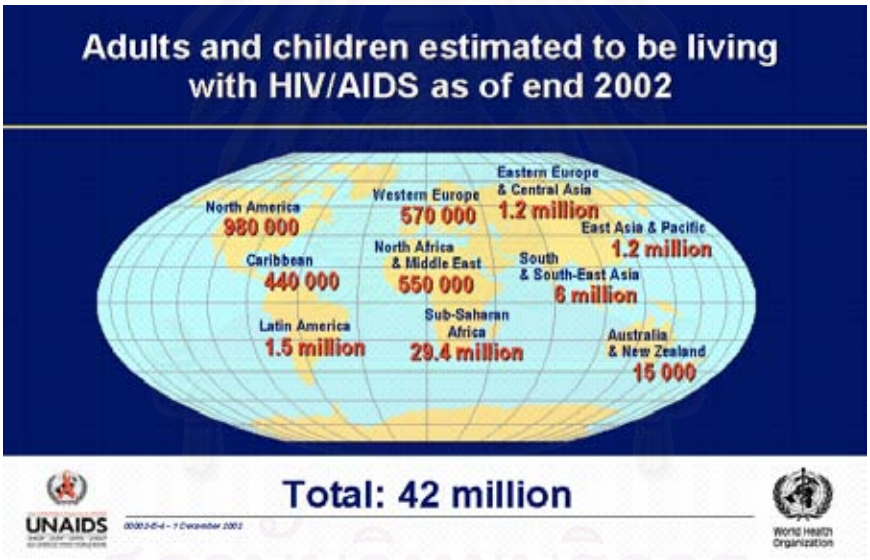


Figure 1 Global epidemic of HIV infection in year 2002. (www.unaids.org)

In Thailand, the HIV/AIDS epidemiological update in February 2002 showed that a total of 189,884 cases reported to be living with HIV/AIDS.[2] However, approximately 800,000 HIV-seropositive people infected with HIV in year 2002.[2]

Over 95% of HIV-1-infected individuals live in developing countries and have no access to antiretroviral treatment. The need for a vaccine that protects against HIV-1 infection has been more urgent. Understanding the correlates of protective immunity is a

first step in vaccine development. Vaccine strategies aimed at blocking virus entry have so far failed to induce protection against the heterologous viruses because of the HIV-1 envelope strain variability.[3-5] Thus, the control of viral infection and the prevention of disease progression may represent a more achievable goal of HIV-1 vaccine strategies.

The human immunodeficiency virus stimulates strong immune response by cytotoxic T lymphocytes (CTLs) in infected people despite causing profound immunodeficiency. In the acute phase of the infection, the CTL response initially follow the rise in HIV in the blood and when that response reaches a peak the virus level falls [6], after that there is an inverse relationship between CTL response and viral load.[7]
(Figure 2).

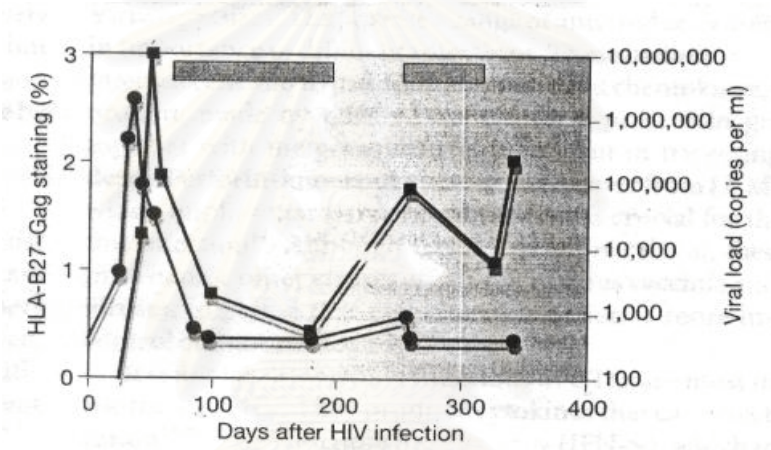


Figure 2 An inverse relationship between CTL response and HIV viral load.

In addition, Schmitz,et al 1999 [8], studied the control of viremia in SIV infection by CD8+ lymphocytes. An animal model of AIDS, SIV-infected rhesus monkey, was used to show that virus replication is not controlled in monkey depleted of CD8+ lymphocytes during primary SIV infection. Eliminating CD8+ lymphocytes from monkey during chronic SIV infection resulted in a rapid and marked increase in viremia that was again suppressed coincident with the reappearance of SIV-specific CD8+ T cells.These results confirm the importance of cell-mediated immunity in controlling HIV-1 infection. These data imply that CTLs are important in control of the virus.

The central role of CTLs in controlling the virus is also emphasized by the influence of human leukocyte antigen (HLA) type on the rate of progress on HIV infection towards AIDS.[9,10] CTLs recognized virus peptides presented by HLA class I molecules,

and different HLA types present different peptides and thus affect the quality of the immune response. HLA is polymorphic in humans. HLA allele frequencies vary greatly among different ethnic groups. For the common HLA class I molecules around the world, a 17% prevalence of HLA-A2 and 11% of HLA-A28 are found in North America , 30% HLA-A35 is found in South America and 17% of HLA-A2 is found in Africa. In Southeast Asia and Thailand , the polymorphism frequency is found HLA-A11 33%, HLA-A2 25% and HLA-A24 14%.[11] Phenotypic and genotypic diversity of HIV-1 have also been widely observed, as evidenced by clade B is found commonly in North America, clade CRF01_AE is the most common subtype found in Thailand and Southeast Asia, whereas clade A is the most common virus found in Subsahara Africa.

There are very little recent data about the role of cellular immune response directing against the accessory proteins. The relative small accessory HIV-1 protein Vpu and Vpr have important functions in G2/M cell cycle arrest, nuclear transport of the preintegration complex (PIC), viral assembly, and down-regulation of CD4 on the cell surface.[12-18] Thus these proteins appear to be essential for viral replication. So Vpu and Vpr proteins may be a potential target for future vaccine design by using Vpr and/or Vpu combined with other viral proteins.

In this study, HIV-1 CRF01_AE Vpu and Vpr specific cell mediated immune responses were measured by IFN-γ ELISPOT assay using truncated and overlapping synthetic peptides of HIV-1 CRF01_AE Vpu and Vpr. IFN-γ ELISPOT is currently a method of choice in the preliminary screening of dominant CTL responses in population studies. Because there are several advantages of this assay, for example it is highly sensitive, simple, efficient and rapid.[19] In addition, the IFN-γ ELISPOT assay has been shown to be a reliable method to map optimal CTL epitopes.[20] It makes testing of a wide spectrum of truncated synthetic peptides and screening of a large number of HIV-infected individuals can be performed easily and efficiently.[20,21]

As mentioned above, there is no studies on HIV-1 CRF01_AE CTL epitope mapping directing against Vpu and Vpr proteins. The identification and characterization of HIV-1 CRF01_AE Vpu and Vpr-specific CTL response by using IFN-γ ELISPOT assay might provide new data and additional useful data for the HIV/AIDS vaccine development in Thailand.

Research Questions

Primary Question

: Do HIV-1 infected Thai individuals with high T lymphocytes CD4+ count of more than ≥ 300 cells/mm^3 have IFN-γ HIV-specific CD8+ T lymphocytes responses ELISPOT positive against HIV-1 CRF01_AE Vpu and Vpr proteins, and with what proportion and magnitude ?

Secondary Question

: What is the most common ELISPOT specific-Vpu and Vpr epitope that found in this study group ?

Tertiary Question

: Any new epitopes of Vpu and Vpr protein can be identified ?

Research Objectives

: To find the prevalence of HIV-1 infected Thai Individuals who have CD8+ T cells response against HIV-1 CRF01_AE Vpu and Vpr proteins.

: To study HIV-1 CRF01_AE Vpu and Vpr epitopes that recognized by HIV-1 infected Thai.

Hypotheses

: The prevalence of HIV-1 infected Thais who have IFN-γ ELISPOT response against HIV-1 CRF01_AE Vpu and Vpr proteins is different from that of other population.

: Novel HIV-1 CRF01_AE Vpr and/or Vpu epitopes can be identified among HIV-1 infected Thais with CD4+ T cell count ≥ 300 cells/mm^3.

Key Words

CTL Epitope Mapping

ELISPOT

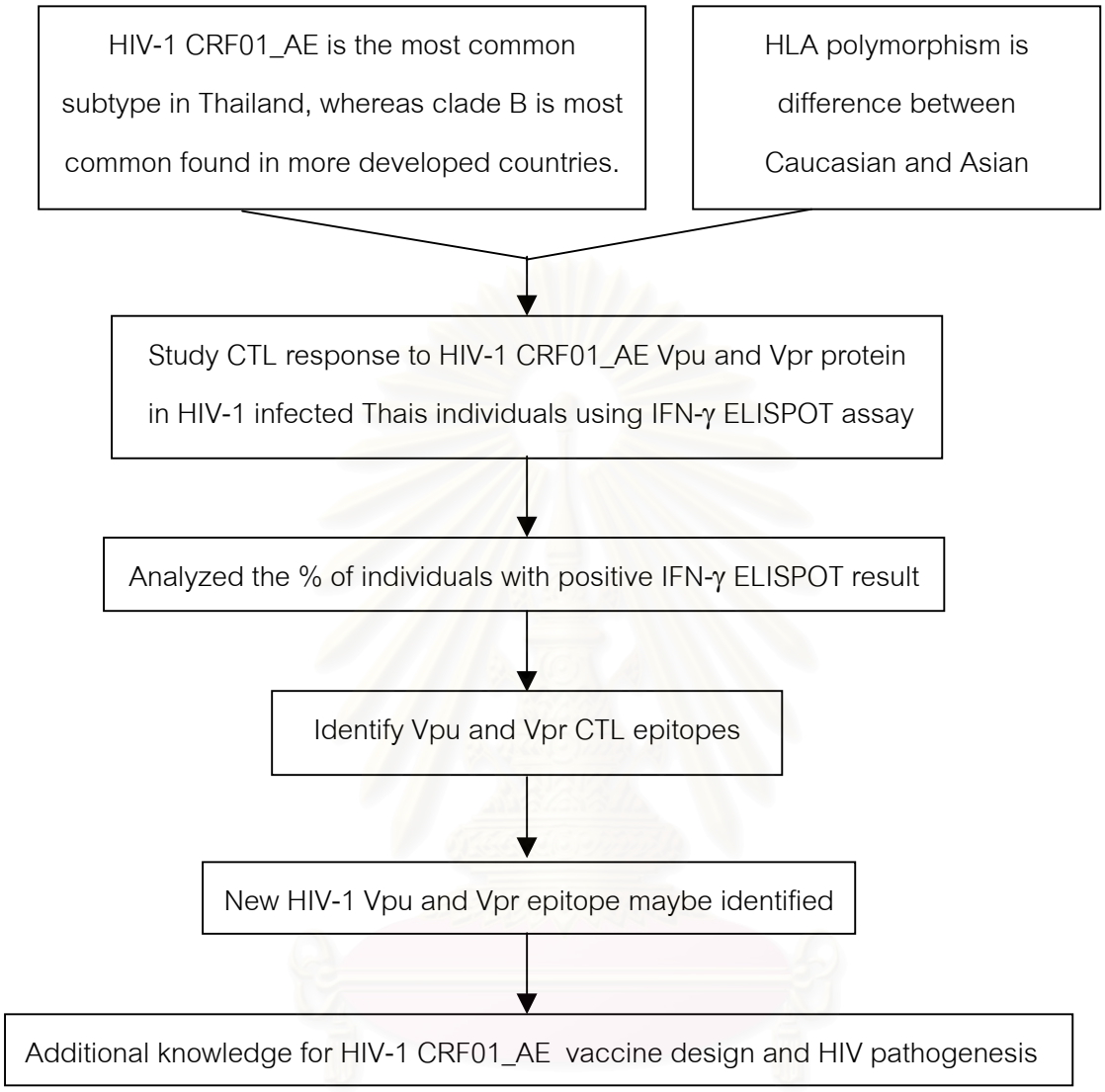
IFN-γ

HIV-1

Vpu

Vpr

Conceptual Framework



Expected Benefits & Applications

1. This study will generate information of how common Vpu and/or Vpr-specific CTL responses in HIV-1 infected Thai.

2. Be able to identify the most common HIV-1 Vpu and Vpr CTL epitopes among HIV-1 infected Thai.

3. Identification of novel CTL epitopes of HIV-1 CRF01_AE Vpu and Vpr.

4. The data may be helpful for further development in HIV-1 vaccine that are suitable for Thai individuals.

Research Methodology

Study Subjects

Twenty antiretroviral treatment naïve HIV-1 infected Thais, who were more than 20 years old with CD4+ T lymphocytes count ≥ 300 cells/mm^3 were included. All were obtained written consent form. The study was approved by the Chulalongkorn Medical Institutional Review Board (IRB), Faculty of Medicine, Chulalongkorn University.

Study Methods

1. Truncated and overlapping Vpr and Vpu peptides Design and Synthesis

In this study, we designed and synthesized 10 truncated HIV-1 CRF01_AE Vpr peptides and 8 truncated HIV-1 CRF01_AE Vpu peptides. These peptides are 15 to 20 amino acids in length with 10 amino acids overlapping between sequential peptides. The Vpr peptide sequence was based on the sequence of HIV-1 isolate CM_240 from Thailand 1990, complete genome (accession number U54771). The complign PPC MacMolly®Tetra ,Version 3.5 March'97 program was used to find the consensus sequence of these peptides. The truncated Vpu and Vpr peptides were synthesized at the Natural and Medical Sciences Institute at the University of Tuebingen ,Germany.

2. Seperation of Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs was seperated from 10 ml of heparinized peripheral blood by the Ficoll-Hypaque density gradient centrifugation.

3. Enzyme-linked Immunospot Assay (ELISPOT) for the Detection of Human Gamma-Interferon (IFN-γ)

3.1 For the Vpr and Vpu IFN-γ ELISPOT screening : Each subject was screened by pooled Vpr and Vpu peptides (4 pools of peptides, each pool contain 5 peptides except the last pool of Vpu peptide contains 3 peptides.)

3.2 Identification of Vpr and Vpu-specific response : The pooled peptide that showed IFN-γ ELISPOT positive responses from screening was then further tested with each individual peptide.

4. Enumeration of IFN-γ responses and Data Collection

The IFN-γ ELISPOT positive spots were counted by a stereomicroscope and are expressed as an average spot-forming units (SFU) per million PBMCs (SFU/10^6PBMCs) of the duplicate wells.

5. Statistical Analysis

The number of specific IFN-γ-secreting T cells was calculated by subtracting the negative control value from the established SFU count. Results of ≥ 100 SFU/10^6 PBMCs input cells[22] and more than 2.5 folds of negative control were considered as a positive responses. The negative control has to be < 100 SFU/10^6PBMCs. The Vpr and Vpu-specific ELISPOT responses were shown as a proportion of the patients who have response to at least one of the Vpr and/or Vpu peptides.

Administration and Time Schedule

Order	Procedure	Time (month)											
		1	2	3	4	5	6	7	8	9	10	11	12
1	Prepare materials & Reagent	◄	►										
2	Design peptide		◄	—	►								
3	Sample collection & Experiment				◄	—	—	—	—	►			
4	Data collection & Analysis						◄	—	—	—	►		
5	Thesis writing								◄	—	—	—	►

CHAPTER II

REVIEW OF THE RELATED LITERATURE

HIV-1 Biology [23-25]

Human Immunodeficiency Virus type I (HIV-1) is a member of the lentivirus genus, which includes retroviruses that possess complex genomes and exhibit cone-shaped capsid core particles. Like all retrovirus, HIV's genome encoded by RNA,which is reverse-transcribed to viral DNA by the viral reverse transcriptase (RT) upon entering a new host cell. The general features of the mature HIV virion and the structurally characterized of viral proteins are shown in Figure 3.

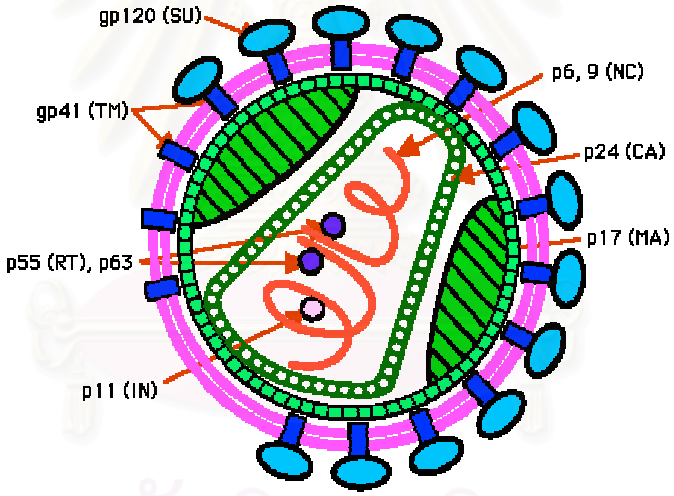


Figure 3 The mature HIV virion and general structure.

Particles of HIV are enveloped by a lipid bilayer that is derived from the membrane of the host cell. Exposed surface glycoproteins (SU,gp120) are anchored to the virus via interaction with transmembrane protein (TM,gp41). The capsid particle encapsidated two copies of the unsplicing viral genome, and also contains three essential virally encoded enzymes protease (PR), reverse transcriptase (RT), and integrase (IN). Virus particles also package the accessory proteins.

The HIV-1 Replication Cycle [23]

General features of the HIV-1 replication cycle are shown in Figure 4. The early phage begins with the recognition of the target cell by the mature virion and involves all processes leading to and including integration of the genomic DNA into the chromosome of the host cell. The late phase begins with the regulated expression of the integrated proviral genome, and involves all processes up to and including virus budding and maturation.

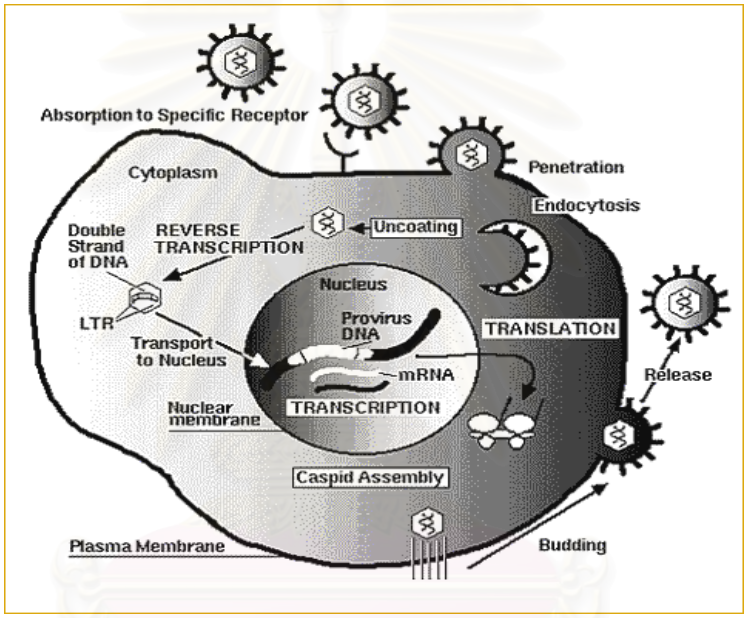


Figure 4 The HIV-1 replication cycle.

Viral Proteins and Their Functions [24-35]

The integrated form of the HIV-1 also known as the provirus, is approximately 9.8 kilobases in length. Both ends of the provirus are flanked by the repeated sequence known as the long terminal repeats (LTRs). The gene of HIV are located in the central region of the proviral DNA and encoded at least 9 proteins. These proteins are divided into 3 classes :

1.The major structural proteins :

-Gag (group antigen gene)

-Pol (polymerase)

-Env (envelope)

2.The regulatory proteins :

-Tat (trans-activator of transcription)

-Rev (regulator of expression of virion protein)

3.The accessory proteins

-Vpu (viral protein U)

-Vpr (viral protein R)

-Vif (virion infectivity factor)

-Nef (negative factor)

Structural proteins

1. Gag proteins [24]

The *gag* gene gives rise to the 55-kD Gag precursor protein, also called p55, which is expressed from the unspliced viral mRNA. The p55 forms the core viral particle and interacts with other viral and cellular components, including RNA, Pol and Env proteins, and the plasma membrane to facilitate their incorporation into the budding viral particle. The self-associating properties of the Gag portion also direct the incorporation of some molecules of the Gag-Pol fusion protein into the forming particle. During or shortly after assembly, the protease is activated and the Gag precursor is cleaved into four proteins : matrix ($p17^{MA}$), Capsid ($p24^{CA}$), nucleocapsid ($p7^{NC}$) and $p6^{gag}$.

Matrix domain (MA) [24]

The MA polypeptide is derived from the N-terminal,myristoylated end of p55. Most MA molecules remain attached to the inner surface of the virion lipid bilayer, stabilizing the particle. In addition, MA play a role in the transport of the preintegration complex to the nucleus.[26] A nuclear localization signal had been proposed within the MA protein.[26]

Capsid domain (CA)

The CA of all retroviruses contains a major homology region (MHR) that is required for efficient viral replication and particle production.[25] CA also interacts with the cellular protein, cyclophilin A (CyA), which is incorporated in virions and which is required for viral infectivity.[27]

Nucleocapsid domain (NC)

NC is a basic protein that bind (nonspecifically) to viral RNA. It may help condense the viral RNA for packaging into the nucleocapsid. NC also binds specifically to the major packaging signal on the HIV-1 genome.

$P6^{gag}$ protein

The p6 polypeptide region mediates interactions between p55 Gag and the accessory protein Vpr, leading to the incorporation of Vpr into assembly virions.[28]

2. Pol (polymerase) [24]

The *pol* gene encodes reverse transcriptase. Pol has RNA-dependent and DNA-dependent polymerase activities. During the process of reverse transcription, the polymerase makes a double-strand DNA copy of the dimer of single-stranded genomic RNA present in the virion. All of the *pol* gene products can be found within the capsid of free HIV-1 virions.

3. Env glycoprotein [24]

The HIV-1 envelope glycoproteins are the product of the *env* gene. The 160 kD Env (gp160) is expressed from singly spliced mRNA. A cellular protease cleaves gp160 to generate gp41 and gp120. gp41 contains the transmembrane domain of Env,while gp120 is located on the surface of the infected cell and of the virion through noncovalent interactions with gp41.

Regulatory proteins

1. Tat (trans-activator of transcription)

Tat is a transcriptional transactivator that is essential for HIV-1 replication.[29] Tat binds to a short-stem loop structure, known as a trans-activation response element (TAR).[30,31] The binding of Tat to TAR activates transcription from the HIV LTR at least 1000-folds.

2. Rev (regulator of expression of virion protein)

Rev is a 13-kD sequence-specific RNA binding protein. Rev acts to induce the transition from the early to the late phase of HIV gene expression.[32] Rev is absolutely requires for HIV-1 replication : proviruses that lack Rev function are transcriptionally active but do not express viral late gene and thus do not produce virions.

Accessory proteins

1. Nef (negative factor) [33,34]

Nef is a 27 kD myristoylated protein that is encoded by a single exon that extends into the 3' LTR. Nef has been shown to have multiple activities including the down regulation of the cell surface expression of CD4, the perturbation of T-cell activation, and the stimulation of HIV infectivity.

2. Vif (virion infectivity factor)

Vif is essential for the replication of HIV in peripheral blood lymphocytes, macrophages, and cell line.[35] Vif has been shown to influence the infectivity but not the production of virus particles.

3. Vpu (viral protein U, viral protein Unknown) [36-40]

Vpu ,one of four accessory proteins encoded in the HIV-1 genome ,is an 81-residue membrane proteins. Vpu have two well described functions: CD4 degradation in the Endoplasmic Reticulum (ER) and enhancement of viral particle release.[36,37] The evidence from biological, functional, and structural studies indicated that its principal biological activities are associated with different structural domains of the protein. Vpu facilitates the budding of new viral particles from infected cells and also enhances the

degradation of CD4, an activity associated with interactions between the cytoplasmic domains of Vpu and CD4. Willy,R.L.,et al in 1992 [17] shown in the absence of Vpu, processing of gp160 to form the gp41 and gp120 polypeptides is impaired because of the formation of stable complexes with CD4 in the ER of infected cells. Thus Vpu indirectly regulates processing of gp160 in the Golgi complex into gp120 and gp41 proteins needed for the assembly of infectious virus particles.

These two biological activities of Vpu have been correlated with two distinct structural domains in the Vpu protein, an N-terminal transmembrane helix and a C-terminal cytoplasmic domain. The interesting evidence implicating the cytoplasmic domain of Vpu in CD4 degradation. In the ER, Vpu bind to CD4 and targets it for proteolysis in the cytosolic ubiquitin-proteosome pathway. Vpu mediates this process by binding to β-TrCP (β-transducin repeats containing protein), which in turn bind to the proteosome-targeting factor Skp-1p.[38] In deed, a complex of CD4-Vpu-β-TrCP has been identified, and binding of Vpu to β-TrCP requires the two phosphoserine residues 52 to 56 that are essential for CD4 degradation.

4. Vpr (viral protein R, viral protein Regulatory) [41-48]

Viral protein R (Vpr), a polypeptide of 96 amino acids, is a major virion-associated accessory protein of HIV-1. This protein contains four structural regions[41] :the N-terminal region, the arginine-rich C-terminal region,and one central domain containing two putative α helices (Hα1 and Hα2)

Vpr play a role in early events in the viral cycle. Vpr was proposed to participate in the nuclear translocation Hα1 and Hα2 of the HIV-1 preintegration complex.[41] In addition, Vpr efficiently arrests cell cycle at the G2/M phase.[12,42] Vpr delays infected cell in the G2 stage of the cell cycle where the LTR is more active. The increase in expression in G2 results in higher virus production over a single round of replication.[13] In fact, despite limiting amounts of Vpr in the virion, there are sufficient quantities of packaged Vpr to induce cell cycle arrest in the infected T cell.[43,44] Its action as a transcription activitor of a several viral and cellular promoters. Recent result have indicated that the function of Vpr as a transcription activator is affected by nuclear coactivators. In year 1998 Felzien.L.,et.al [45] show the ability to activate HIV transcription

correlates with its ability to induce G2/M growth arrest, and this effect is mediated by the p300 transcription activator, which promotes cooperative interation between the RelA subunit of NF-κB and cyclin B1.Cdc2 lead to increase HIV gene expression. These data suggest that p300, through its interaction with NF-κB, basal transcriptional components and Cdk2, is modulated by Vpr and regulates HIV replication. The regulation of p300 by Vpr provides a mechanism to enhance viral transcription.

Previous study show the presence of Vpr can increase virus production.[13] They compared the amount of viral antigen secreted into the medium by using Jurkat T cells transfected with a replication-defective provirus that expressed Vpr to one that did not express Vpr. They found that virion production from the transfected cells was higher in the presence of Vpr than in the absence of Vpr. Morever,cells infected with a replication-defective virus that was Vpr+ secreted more virus than did cells infected with a replication-defective virus that was Vpr- during the first 48 hours after infection. This evidence indicate that Vpr increases viral production in a single round of viral replication, and this increase is correlated with increased expression in G2 phase.

Other studies suggest that the prolonged G2 arrest induce by Vpr may ultimately lead to apoptosis of the infected cell, possibly leading to increased virion production.[46-48]

Classification of HIV

The extraordinary genetic diversity of HIV-1 was recognized as soon as the first strains from the United States, Europe, and Africa were sequenced in the mid-1980s.[49,50] In 1989, strains in the global epidemic were subdivided into "North America" and Zairean.[51] By the early 1990s, several genetic subtypes of HIV-1 had been recognized each with a geographic distribution.[52-54]

Phylogenetic analyses of numerous strains of HIV-1 isolated from diverse geographic origins, have revealed three distinct clades of viruses,which have been termed group M (main or major), N (new or non-M, non-O) and O (outlier). Within group M, there is further phylogenetic structure which has allowed the classification of strains into numerous subtypes. Group O isolates were first described around 1990. These viruses are highly divergent from group M, exhibiting only about 50% amino acid

sequence homology to group M in the *env* gene.[55,56] Group O seems to be endemic to Cameroon and neighboring countries in West Central Africa.

Since 1992, phylogenetic analyses of *env* and *gag* sequences have been used to classify the prevalent viruses observed in the global AIDS epidemic. [52,54] Subtypes were supposed because most sequences were found to fall into a limited number of discrete clades. Initially, five subtypes were identified, but more extensive global sampling has revealed additional subtyped : at the latest count, there are 11, designated A - K.[57] The subtypes are approximately equidistantly related, exhibiting 25-35% amino acid sequence difference in their Env proteins, and up to 20% difference within subtypes.[58] In year 1998, new subtypes have been described, previously identified subtypes have been re-classified as CRF (Circulating Recombinant Forms), and subtypes have been divided into sub-subtypes.[59]

Worldwide distribution of HIV-1 variants [60-65]

Figure 5 Worldwide distribution of HIV-1

The greatest genetic diversity of HIV-1 has been found in Africa. Group M subtypes A and C are most common, but all groups and subtypes are found, consistent with this continent being the source of the epidemic.[60]

In North America, Europe and Australia, subtype B is by far the most common. However, various other group M subtype, and even group O viruses, have been reported in the United States[61] and several European countries.[62]

Across Asia, different forms of HIV-1 have taken hold in different regions. In india, subtype C predominates, but subtype A and B co-circulate, and A/C recombinants are also found.[63,64] In Thailand, first report indicated two seperated epidemics,of subtype B and subtype E (CRF01_AE) viruses, spreading in different sections of the population : IVDU were infected with subtype B viruses whereas those infected heterosexually predominantly carried CRF01_AE. CRF01_AE is also increasing in frequency in other Southeast Asian countries.[65]

Cytotoxic T Lymphocytes (CTLs) [66]

The Cytotoxic T Lymphocytes (CTLs) are a subset of T cells that kill target cells expressing MHC-assaociated peptide antigens. The majority of CTLs express the CD8 molecule and specifically recognize foreign peptides antigens (9-12 amino acids) derived from antigens degraded in the cytosol (the endogenous pathway of antigen presentation) and then presented in the groove of an major histocompatibility complex (MHC) class I molecules. Rare CTLs express the CD4 molecule and recognize peptides associated with class II MHC molecules. Target peptides for CTLs are fragment of virus or tumor antigens. The T cell antigen receptor genes utilized by CD8+ CTLs are indistinguishable from those utilized by CD4+ helper T cells. The preference of CD8+ T cells for class I MHC molecules is instead related to the direct binding of the CD8 molecule to non-polymorphic regions of the class I MHC molecules. So the induction of a CTL response is dependent upon CD4+ T lymphocytes to provide help, in the form of activation stimuli.

The process of CTL-mediated lysis consists of five steps (Figure 6)

1. Recognition of antigen and conjugate formation.

The CTLs bind to the target cell, using its specific antigen receptor and other accessory molecules such as CD8, CD2, and LFA-1. target cell recognition, therefore, involves class I MHC molecules complexed to specific peptide (the complex serving as

the ligand for TCR), LFA-3 (the ligand for CD2), and ICAM-1 or ICAM-2 (the ligands for LFA-1). The may be that transient conjugate formation can occur via CD2- and LFA-1-mediated adhesion in the absence of or prior to specific antigen recognition, or that antigen recognition may enhance the ability to form conjugates by augmenting the binding function of the adhesion molecules. CTL-mediated killing of target cells that do not express ICAM-1 or (in humans) LFA-1 is very inefficient.

2. Activation of the CTL.

The CTL is activated by cluster of its antigen receptor, initialed by recognition by MHC molecules-peptides complexes on the target cell. The intracellular signals generated by the TCR may be augmented by signals delivered through the various accessory molecules.

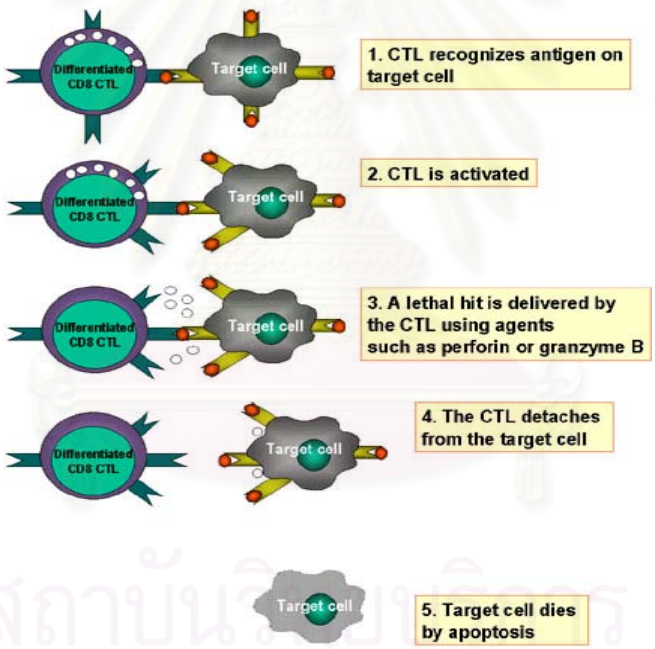


Figure 6 Steps in CTL-mediated lysis of target cells

3.Delivery of a “lethal hit” by the activated CTL to its conjugated target

Studies of CTL-mediated killing of target cells in vitro had suggested that granule exocytosis-dependent (i.e. perforin/granzyme-mediated) killing and granule exocytosis-independent (i.e. Fas ligand-mediated) killing were redundant. Perforin and granzyme are the key mediator of CTL function in immune responses to intracellular

microbes. In contrast, the Fas pathway appears to be more important for regulation than for CTL function, i.e., for controlling excessive lymphocyte activation, especially against self antigens.

4. Release of the CTL.

The CTL is released from its target cell, a process that may be facilitated by decreases in the affinity of accessory molecules for their ligands.

5. Programmed death of the target cells as a consequence of receiving a lethal hit by a combination of apoptosis and osmotic lysis.

Control of HIV-1 infection by CTLs [67]

There are increasing evidences that cytotoxic T-lymphocytes (CTLs) play a central role in the immune response to HIV. They are activated in vivo to high level at the same time as the initial clearance of the primary viremia.[68,69] It is also clear that they are important in the maintainance of the asymptomatic phase of infection before the development of AIDS.[70-72] In addition, high level of CTL are typically observed in asymptomic infected adults, whereas CTL numbers decline in association with progression to AIDS.[72,73] In acute infection, the apperance of CTL is temporally linked with the reduction in viremia which occurs both in HIV and SIV infection. [68,69] Chronic HIV-infected adults naïve to antiretroviral therapy also show a strong negative association between CTL numbers and viral load. [7]

In 1999, experiments using anti-CD8 monoclonal antibody (MAb) infusions in SIV-infected-macaques have demonstrated directed that plasma virus levels show strong negative associations with CTL numbers, in both acute and chronic infection.[74,75] Macaques whose CTL response was delayed or abrogated altogether progressed significantly more rapidly to disease and death. Thus, take together these data suggest that HIV-specific CTL responses constitute an essential component of an effective HIV vaccine.

Techniques for Measurement of CTL Activities[76,77]

There are 5 assay systems that are currently employed measuring the activity of CTLs: Classical Chromium-release assay, Limiting Dilution Assay (LDA), HLA-peptide Tetrameric Complexes Staining, Intracellular Cytokine Staining, and Enzyme-linked Immunospot Assay (ELISPOT). The choice of assay technique will depend on the research question posed, the technical skill and experience of the operator, the types of samples available and the number of assays required of each study.

Classical Chromium-release Assay [76]

This assay measures the ability of cells removed from whole blood to kill target cells expressing the specific antigen. The assay involves the radiolabelling of target cells with sodium chromate ($Na_2{}^{51}CrO_4$). The radiolabelled target cells are then incubated with the test effector-cell population for a short period (4-6 hours). The amount of Cr^{51} released into the supernatant is then quantified, to provide a measure of target-cell lysis. The advantage of this assay is that it measures a clear, important T-cell function. However , it is cumbersome, relatively insensitive, difficult to quantitate reproducibly, radioactive hazard, and not very quantitative.

Limiting Dilution Assay (LDA) [76]

To provide quantitative estimates of the number or frequency of T cells present in a given PBMCs population that are specific for a particular antigen, LDAs are required. This assay provide an estimate of the precursor frequency of a given cell type. Positive result in this assay indicates the presence of antigen-specific precursor cells in the PBMCs population at the start, which have become activated and have subsequently divided during the period of cell culture. The function of these cells is then measured in the assay by either proliferation, cytokine production or cytotoxicity.

HLA-peptide Tetrameric Complexes [77]

HLA-peptide complexes allow the direct visualisation of antigen-specific CD8+ T-cells, independent of their functional characteristics. Four biotinylated, refolded HLA-

peptide complexes are attached to a streptavidin molecules, creating a stable tetrameric structure which will specifically binds to T cells expressing an appropiate T-cell receptor (TCR).[7,78] Although this technique does not rely on a functional endpoint, the antigen-specific CD8+ T-cells frequencies measured by flow cytometry have been shown to correlate with functional cell polulations by a number of methods, including direct CTL lysis, CTL cloning, ELISPOT and LDA.[7,79] It directly measures cells without receptors for a specific epitope without prior antigen stimulation. It is highly quantitative and sensitive. But this technique require knowing of the HLA type in each person being sampled and having a specific epitope known to be recognized by that HLA type. Therefore, it is not suitable as a screening assay for CTLs study.

Intracellular Cytokine Staining (ICS) [76]

Cytokine production at the level of single T cell analysed using flow cytometry to detect pools of intracellular cytokines. T cells are stimulated *in vitro* with a specific antigen, and for at least some of the stimulation period. Monensin or brefeldin A are present to block the transport of cytokines through the Golgi apparatus, and therefore prevent the secretion of cytokines. The T cells are then fixed, permeabilised, and stained for the presence of intracellular cytokines using directly conjugated anti-cytokine antibodies. In this method, large numbers of T cells can be analysed in a short period of time.

Enzyme-linked Immunospot Assay (ELISPOT) [77]

The ELISPOT is based on the same principles as an ELISA, but the technique has been modified to permit the detection of cytokine release at the single cell level in response to a given stimulus [80,81] and has recently been applied to the enumeration of antigen-specific CD8+ T-cells.[82-84]

ELISPOT is currently method of choice in the preliminary screening of dominant CTL responses in population studies. Because there are several advantages of this assay for example highly sensitive,quantitative,simple,efficient and low-technique setting. The advantages of the ELISPOT assay also include its speed (an overnight assay) ; the ability to screen for responses against multiple CTL epitopes and/or gene

products in a single assay ; and the need for relatively low cell input numbers (generally 2-4x10^5 per epitope/gene product tested) In addition, the ELISPOT assay has shown to be a reliable method to map optimal CTL epitopes.[20] It makes testing of a wide spectrum of truncated synthetic peptides and screening of a large number of HIV-infected individuals can be performed easily and efficiently.

Table 1 Comparison of each technique for detect CTL activity.

Measurement of CTL	Cell type measurement (function)	Time (date)	Price	Radio labelled	Sensitivity
1. Cr^{51} assay	Target (Lysis)	14	Low	Yes	$1/10^4$
2. ELISPOT	Effector (IFN-γ secretion)	2	High	No	1/10-5x10^5
3. Tetramer	Effector (Binding)	2	High	No	1/10-5x10^5
4. Intracellular IFN-γ assay	Effector (IFN-γ expression)	2	High	No	Not available

Vpr IFN-γ ELISPOT response in HIV-1 subtype B and C

To better understand immune control of viremia and for the design of potential HIV-1 vaccines, those region of HIV that induce strong virus-specific immune responses should be identified. To date, the analysis of HIV-1 specific immunity was largely focus on assessment of immune response directed against the structural HIV-1 proteins. However, very little is known about the role of cellular immune responses directed against the other accessory protein except Tat and Rev. In year 2001, Altfeld.M.,et al.[85] provides evidence that the Vpr and Vif proteins represent important targets of cellular defenses whereas Vpu is infrequently recognized. They analyzed in 60 HIV-1-infected individuals and 10 negative controls using overlapping peptides spanning the entire

Vpr, Vpu and Vif proteins. Peptide-specific IFN-γ production was measured by ELISPOT assay and flow-based intracellular cytokine quantification. CD8+ T cell response against Vpr, Vpu and Vif was found in 45%, 2%, and 33% of HIV-1-infected individuals, respectively. Multiple CTL epitope were identified in functionally important region of HIV-1 Vpr and Vif. In addition, the Vpr protein is one of the most frequently targeted HIV-1 proteins by CTL relative to the length of the protein. These finding will be important in evaluating the specificity and the breadth of immune response, and in the design and testing of candidate HIV-1 vaccines.

Vpu IFN-γ ELISPOT response in HIV-1 subtype B and C

In a previous report investigating CTL responses against all accessory protein found detectable CTL responses against HIV-1 Vpu in one out of 60 (2%) individuals screened.[85] In an extension of that study, The same researcher group have characterized HIV-1 Vpu-specific CTL responses in a total of 85 HIV-1-infected individuals at different stages of HIV-1 infection,[86] including

- 45 individuals with treated primary infection
- 2 individuals with untreated acute HIV-1 infection
- 18 individuals with treated chronic infection
- 20 individuals with untreated chronic infection (14 long-term non progressors and 6 progressors)

They found only two out of 85 screened subject had direct against HIV-1 Vpu. Both individuals, a long-term nonprogressor and one individual with primary infection, had well-controlled viremia in the absence of antiretroviral therapy. Furthermore, they also report the epitope EYRKILRQR (ER9, Vpu 29-37) was shown to be highly conserved among HIV clade B sequences and restricted by HLA-A*3303 which is also commonly seen in Southeast Asia and west African populations.

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Population and Subjects

Twenty asymptomatic and treat naïve HIV-1 infected Thai individuals from the Anonymous Clinic of the Thai Red Cross AIDS Research Center and the Immune Clinic of the King Chulalongkorn Memorial Hospital were included according to the inclusion and exclusion criteria.

Inclusion Criteria

- Confirmed anti-HIV positive.
- CD4+ T lymphocytes count $\geq$ 300 cells/mm^3.
- No antiretroviral therapy

Exclusion Criteria

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

All of these subjects have provided written informed consent.

1.2 Specimen Collection

Ten ml of heparinized whole blood was collected from each subject.

2. Methods

2.1 Truncated and overlapping Vpr and Vpu peptides Design and Synthesis

We designed and synthesized 10 truncated HIV-1 CRF01_AE Vpr peptides and 8 truncated HIV-1 CRF01_AE Vpu peptides of 15 to 20 amino acids in length with 10 amino acids overlapping between sequential peptides. Vpr peptides sequence was based on the isolate virus from the sequence of HIV-1 isolate CM_240 from Thailand 1990, complete genome (accession number U54771). The complign PPC MacMolly® Tetra, Version 3.5 March'97 program was used to find the consensus sequence of these peptides. The total of 97 amino acids of Vpr protein and 78 amino acids of Vpu protein 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 Vpr peptides and 8 truncated Vpu peptides of 15 to 20 amino acids in length overlapped by 10 amino acids were given as a result. These peptides were then synthesized at the Natural and Medical Sciences Institute at the University of Tuebingen ,Germany.

Summary of the peptides were showed in Table 2 and Table 3.

Table 2 Vpr peptides

Peptide No.	Region	Length	Vpr Amino acid Sequence
1	1-18	18	MEQAPEDQGPQREPYNEW
2	9-27	19	GPQREPYNEWTLELLEELK
3	18-36	19	WTLELLEELKNEAVRHFPR
4	27-45	19	KNEAVRHFPRPWLHGLGQH
5	36-54	19	RPWLHGLGQHIYNNYGDTW
6	45-63	19	HIYNNYGDTWEGVEAIIRI
7	54-72	19	WEGVEAIIRILQQLLFVHF
8	63-81	19	ILQQLLFVHFRIGCQHSRI
9	72-90	19	FRIGCQHSRIGIIPGRRGR
10	81-96	16	IGIIPGRRGRNGAGRS

Length : 19 mers = 8 peptides , 18 mers = 1 peptide , 16 mers = 1 peptide

Table 3 Vpu peptides

Peptide No.	Region	Length	Vpu Amino acid Sequence
1	1-19	19	MTPLEISAIVGLIVALILA
2	10-28	19	VGLIVALILAIVVWTIVAI
3	19-37	19	AIVVWTIVAIEFKKILRQR
4	28-46	19	IEFKKILRQRKIDRLVKRI
5	37-55	19	RKIDRLVKRIRERAEDSGN
6	46-64	19	IRERAEDSGNESEGDTDEL
7	55-73	19	NESEGDTDELAKLVEMGDF
8	63-78	16	ELAKLVEMGDFDPWVG

Length : 19 mers = 7 peptides , 16 mers = 1 peptide

2.2 Peptide Preparation

2.2.1 Stock peptide 1 mg/ml: 1 mg/ml of peptide was dissolved by 1 ml of 1% DMSO in 1 ml of PBS.

2.2.2 Diluted peptide 100 μg/ml: 100 μl of the stock peptide was diluted in sterile PBS 900 μl

2.2.3 Pooled peptide: five individual peptides were put into 1 microtube by adding 100 μl of each of the peptide. Each pool therefore containing 5 truncated peptides, except the last pool has only 3 Vpu peptides. The final concentration of each peptide in the pool is 20 μg/ml.

Summary of the pooled peptides :

pooled peptide No.1 = Vpr peptide no. 1-5

pooled peptide No.2 = Vpr peptide no. 6-10

pooled peptide No.3 = Vpu peptide no. 1-5

pooled peptide No.4 = Vpu peptide no. 6-8

2.3 Seperation of Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were seperated from 10 ml of heparinized peripheral blood samples by the Ficoll-Hypaque density gradient centrifugation.

1. Ten ml of heparinized blood were diluted approximately 1:1 with RPMI-1640 medium.
2. 4 ml of the Ficoll-Hypaque (density gradient = 1.077 g/l) was added to a 15 ml centrifuge tube.
3. The diluted heparinized blood was carefully overlayed on the Ficoll-Hypaque. The surface of the Ficoll-Hypaque must not be disturbed.
4. Centrifuge at 2,200 rpm for 20 minutes at room temperature (20-25 $^{\circ}$C), without break.
5. The tube was carefully removed from the centrifuge. Four distinct layers were formed,from top to bottom : plasma, PBMCs, the Ficoll-Hypaque, and Red Blood Cells.
6. The plasma layer was discarded.
7. The PBMCs band at Ficoll-Hypaque interface was collected by a sterile pipette.
8. The PBMCs were washed 2 times with RPMI-1640 medium and centrifuge at 1,800 rpm for 10 minutes.
9. Resuspended PBMCs in 5-10 ml R-10 (RPMI-1640 medium supplemented with L-glutamine, penicillin 100 U/ml, streptomycin 100 μg/ml, and 10% Fetal Bovine Serum).
10. The PBMCs were counted and adjusted to a desired concentration, i.e., 2.5 x 10^6 cells/ml, then store at 37 $^{\circ}$C, 5% CO_2 with humidity until being used.

2.4 ELISPOT Assay for Detecting Gamma-interferon (IFN-γ)

1. Ninety six-well polyvinylidene difluoride (PVDF) backed plates (MAIP S45 ; 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 for 6 times of 200 μl PBS per well. PBMCs were added 100 μl/well to the precoated plates. Input cell numbers were $2.5x10^5$ cells/well, in positive wells were added PBMCs $2.5x10^4$ cells/well. Peptides were added to each labeled-well in duplicate at a final concentration of 10 μg/ml. For the positive control wells, phytohemagglutinin (PHA) was added at a final concentration of 20 μg/ml. Gently shaked the plates. Incubation at 37^oC in 5% CO_2 with humidity for 16 hours.
4. After incubation, plates were washed for 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 at room temperature, plates were washed for 6 times with 200 μl/well of PBS-T and one time with 200 μl/well of PBS. Next, 50 μl of 1:1000 dilution of streptavidin-alkaline phosphatase conjugate (Mabtech,Stockholm, Sweden) was added to the wells and the plates were incubated 1 hour at room temperature.
6. The plates were again washed for 6 times with 200 μl/well of PBS-T and one time with 200 μl/well of PBS. And then 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 to terminate the colorimetric reaction and plates were then air-dried.

2.5 Enumeration of IFN-γ SFU

The IFN-γ ELISPOT positive spots were counted by a stereomicroscope (Olympus, Japan). Responses were considered significant if the average number of established SFU count of duplicate wells subtracted by the negative control value was more than or equal to 100 SFU/10^6PBMCs input cells and more than 2.5 folds higher than negative control. The negative controls were always less than 100 SFU/10^6PBMCs.

2.6 Statistical Analysis

Vpr and Vpu-specific IFN-γ ELISPOT responses were shown following these equation

% Individuals who have IFN-γ ELISPOT positive results to Vpr peptide

$$= \frac{\text{the number of individuals who have Vpr-specific responses}}{\text{the number of target population}}$$

% Individuals who have IFN-γ ELISPOT positive results to Vpu peptide

$$= \frac{\text{the number of individuals who have Vpu-specific responses}}{\text{the number of target population}}$$

CHAPTER IV

RESULTS

1. Clinical characteristics

The clinical characteristics were summerized in Table 4. All 20 patients were asymptomatic and antiretroviral naïve. Eighteen were infected with CRF01_AE and 2 patients were infected with B'. The CD4+ T cell were ranged from 303 to 968 cells/mm^3 with mean of 513 cells/mm^3. The plasma HIV-RNA were ranged from 2,335 to > 500,000 copies/ml with the median of 6,755 copies/ml. (In one patient whose plasma HIV-RNA of > 500,000 copies/ml was treated as 500,000 for the calculation purposes).

Table 4 Clinical characteristics of HIV-1 infected patients

Patients no.	Initial name	Gender	Age (years)	HIV-1 subtype	Risk factor	CD4+T cells (cells/mm^3)	HIV-RNA (copies/ml)
1	OK	M	27	B'	Homosexual	327	5,207
2	TB	F	27	CRF01_AE	Heterosexual	386	6,602
3	PA	F	25	CRF01_AE	Heterosexual	968	4,878
4	DK	M	29	CRF01_AE	Homosexual	909	26,309
5	TW	F	45	CRF01_AE	Heterosexual	406	10,176
6	PK	F	49	B'	Heterosexual	332	49,565
7	AP	F	34	CRF01_AE	Heterosexual	321	2,738
8	KK	F	24	CRF01_AE	Heterosexual	482	10,268
9	TS	M	45	CRF01_AE	Heterosexual	435	14,638
10	KP	F	31	CRF01_AE	Heterosexual	809	8,976
11	JL	F	35	CRF01_AE	Heterosexual	315	24,333
12	PC	F	36	CRF01_AE	Heterosexual	471	5,269
13	NI	F	29	CRF01_AE	Heterosexual	563	2,335
14	PS	M	37	CRF01_AE	Heterosexual	381	8,360
15	CY	F	32	CRF01_AE	Heterosexual	303	>500,000
16	ST	F	35	CRF01_AE	Heterosexual	487	4,954
17	RM	F	32	CRF01_AE	Heterosexual	454	3,761
18	PN	F	35	CRF01_AE	Heterosexual	564	6,755
19	HN	M	20	CRF01_AE	Heterosexual	636	3,779
20	MB	M	25	CRF01_AE	Heterosexual	707	99,338

M = Male

F = Female

2. HIV-1 CRF_01AE-specific ELISPOT-based responses against Vpr and Vpu pooled peptides.

As shown in Table 5 ,Figure 7 and Figure 8 ; 10/20 (50%) of all patients or 9/18 (50%) of HIV-1 CRF_01AE patients showed IFN-γ ELISPOT responses to Vpr pooled peptides. The magnitude of responses range from 132 to 1,800 SFU/10^6PBMCs. (Mean 686, Median 470 SFU/10^6PBMCs). 5/20 (25%) of all patients or 5/18 (28%) of HIV-1 CRF_01AE patients showed IFN-γ ELISPOT responses to Vpu pooled peptides. The magnitude of responses range from 124 to 908 SFU/10^6PBMCs. (Mean 421, Median 394 SFU/10^6PBMCs).

Seven out of 18 patients (39%) showed IFN-γ ELISPOT responses to Vpr peptide pool no.1 (Figure 9). The magnitude of responses range from 132 to 1,800 SFU/10^6PBMCs. (Mean 639, Median 444 SFU/10^6PBMCs). 2/18 patients (11%) showed IFN-γ ELISPOT responses to Vpr peptide pool no.2 (Figure 10) . The magnitude of responses were 576 and 1,368 SFU/10^6PBMCs.

Four out of 17 patients (24%) showed IFN-γ ELISPOT responses to Vpu peptide pool no.1 (Figure 11). The magnitude of responses range from 152 to 908 SFU/10^6PBMCs. (Mean 502, Median 474 SFU/10^6PBMCs). 2/17 patients (12%) showed IFN-γ ELISPOT response to Vpu peptide pool no.2 (Figure 12). The magnitude of responses were 124 and 392 SFU/10^6PBMCs.

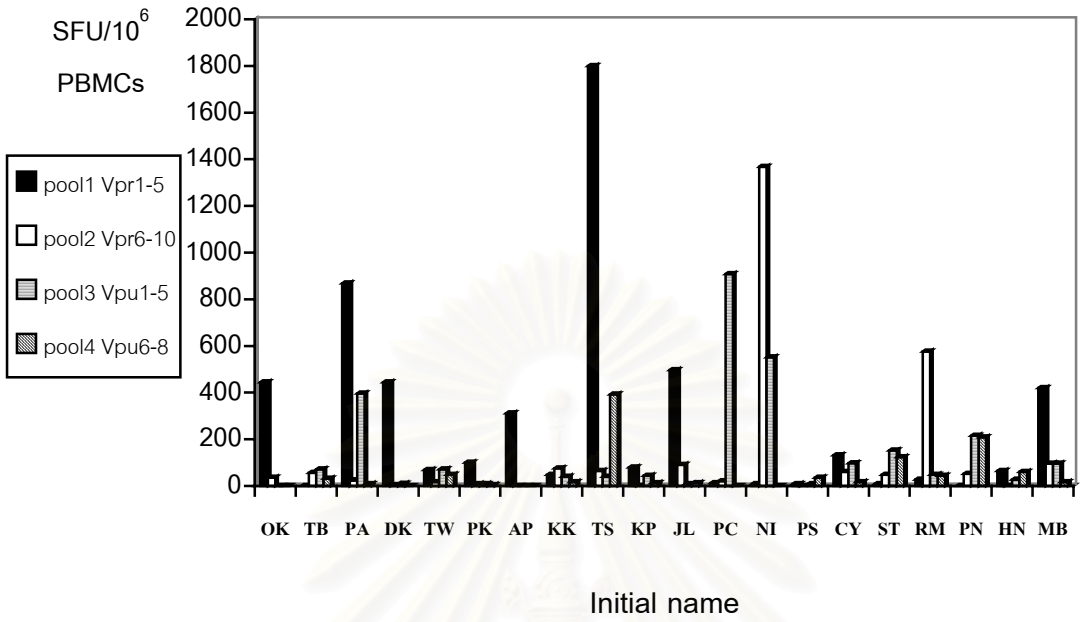
The magnitude of response of IFN-γ ELISPOT results in all positive patients were shown in Figure 13. The magnitude of response of IFN-γ ELISPOT results in HIV-1 CRF01_AE patients only were shown in Figure 14.

Percentages of IFN-γ ELISPOT positive patients to Vpr and Vpu pool peptide in all patients were shown in Figure 15 and the percentages of IFN-γ ELISPOT positive patients to Vpr and Vpu pool peptide in 18 HIV-1 CRF01_AE patients were shown in Figure 16.

Table 5 IFN-γ ELISPOT positive responses to Vpr and Vpu pooled peptides (N=20)

Patients no.	Initial name	IFN-γ ELISPOT positive responses (SFU/10^6PBMCs)				
		neg	Vpr pool no.1	Vpr pool no.2	Vpu pool no.1	Vpu pool no.2
1	OK	72	444	36	0	0
2	TB	68	0	56	72	32
3	PA	8	868	24	396	8
4	DK	24	444	4	10	0
5	TW	20	68	16	72	48
6	PK	16	100	8	8	4
7	AP	52	312	0	0	0
8	KK	52	48	76	40	16
9	TS	20	1,800	64	40	392
10	KP	4	80	0	44	12
11	JL	8	496	90	8	12
12	PC	12	12	20	908	0
13	NI	104	8	1,368	552	0
14	PS	68	8	0	8	36
15	CY	8	132	60	98	16
16	ST	28	8	48	152	124
17	RM	20	28	576	48	44
18	PN	90	0	52	216	208
19	HN	48	64	0	28	60
20	MB	24	420	96	96	16

Figure 7 IFN-γ ELISPOT positive responses to pooled peptide in all 20 patients



Twenty patients (X-axis) were screened in the IFN-γ ELISPOT assay by using 4 pools of HIV-1 CRF01_AE based, 15-20 amino acids synthetic truncated Vpr and Vpu peptides overlapping by 10 amino acids. The responses were shown as SFU/10^6PBMCs (Y-axis).

Figure 8 Number of patients who have IFN-γ ELISPOT positive responses to pooled peptides in HIV-1 CRF01_AE patients only. (N=18)

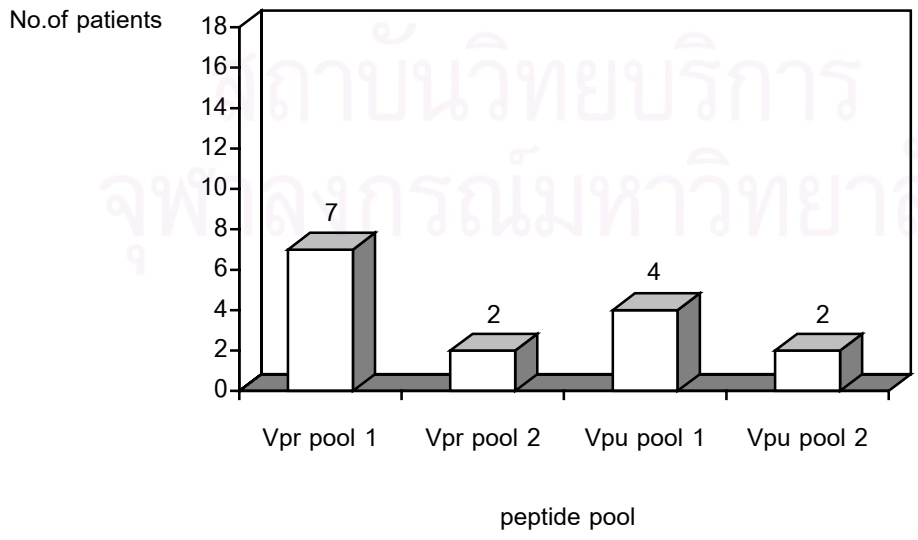
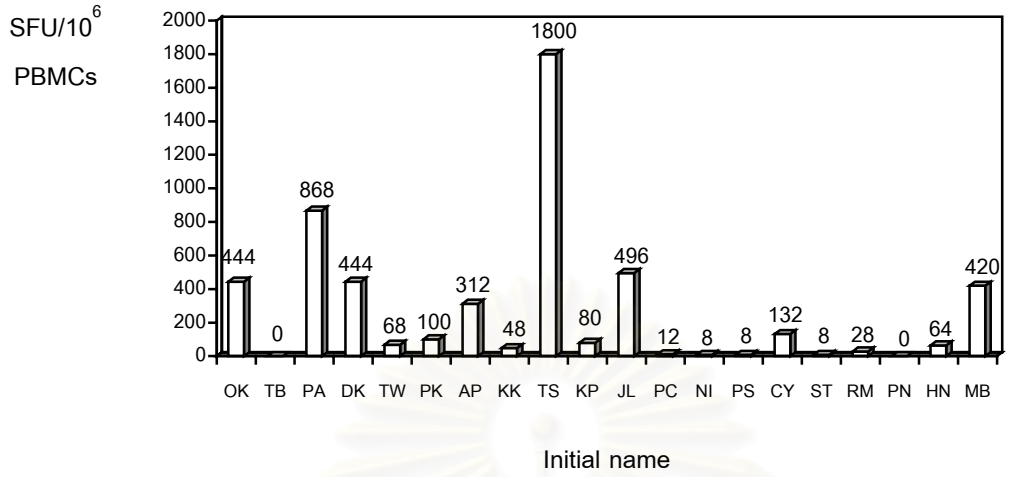
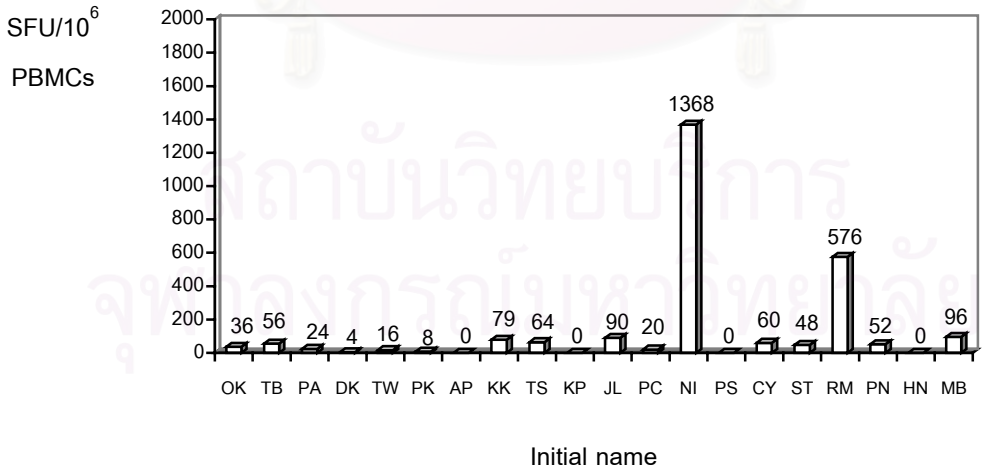


Figure 9 IFN-γ ELISPOT positive responses to Vpr peptide pool no.1 (background subtracted)



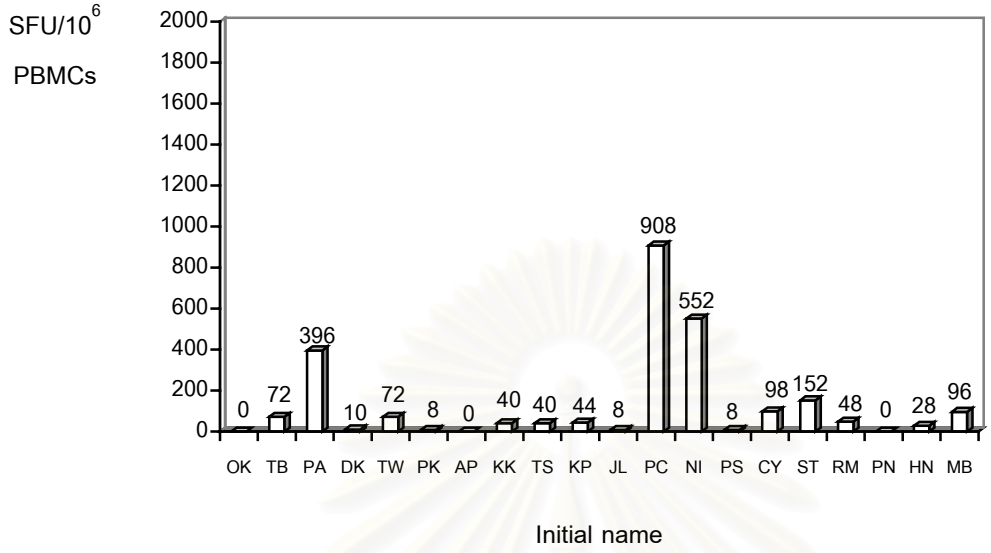
There are 8 patients (OK,PA,DK,AP,TS,JL,CY,and MB) showed IFN-γ ELISPOT positive cells to Vpr peptide pool no.1. However, 1 out of 8 patients (OK) was HIV-1 subtype B'. So seven patients (39%) showed IFN-γ ELISPOT positive responses to Vpr peptide pool no.1. The response was considered as a positive result if the number of established SFU/10^6PBMCs subtracted by negative control is ≥ 100 and >2.5 folds more than background.

Figure 10 IFN-γ ELISPOT positive responses to Vpr peptide pool no.2 (background subtracted)



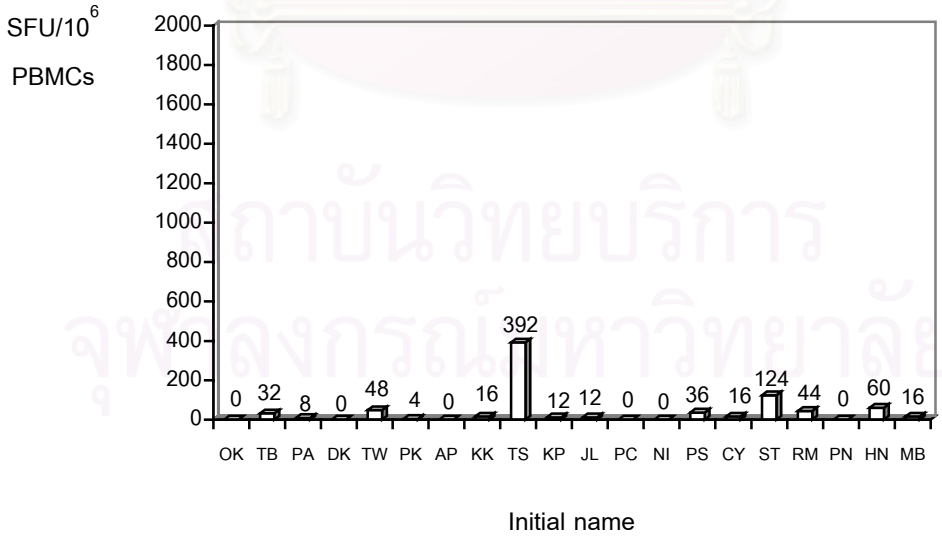
There are 2 patients (NI and RM) showed IFN-γ ELISPOT positive responses to Vpr peptide pool no.2. The response was considered as a positive result if the number of established SFU/10^6PBMCs subtracted by negative control is ≥ 100 and >2.5 folds more than background.

Figure 11 IFN-γ ELISPOT positive responses to Vpu peptide pool no.1 (background subtracted)



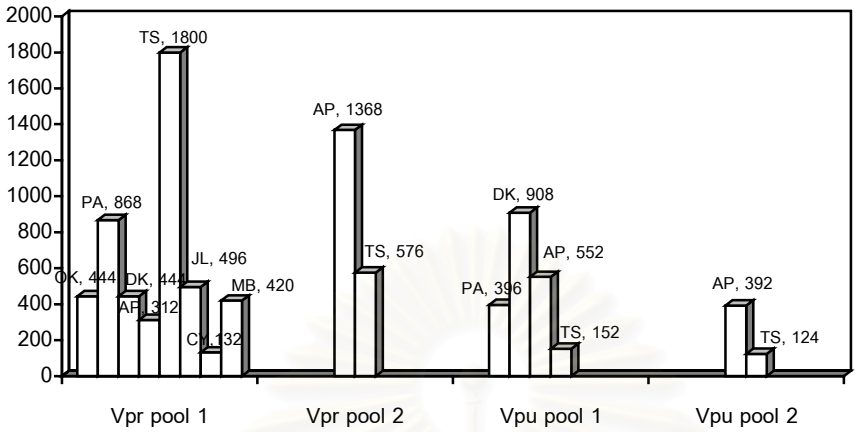
There are 4 patients (PA,PC, NI and ST) showed IFN-γ ELISPOT positive responses to Vpu peptide pool no.1. The response was considered as a positive result if the number of established SFU/10^6PBMCs subtracted by negative control is ≥ 100 and > 2.5 folds more than background.

Figure 12 IFN-γ ELISPOT positive responses to Vpu peptide pool No.2 (background subtracted)



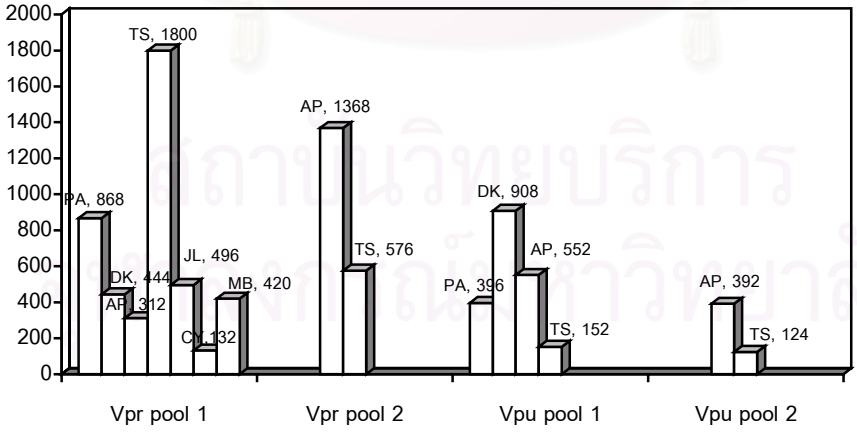
There are 2 patients (TS and ST) showed IFN-γ ELISPOT positive responses to Vpu peptide pool no.2. The response was considered as a positive result if the number of established SFU/10^6PBMCs subtracted by negative control is ≥ 100 and > 2.5 folds more than background.

Figure 13 The magnitude of responses of IFN-γ ELISPOT positive results in all positive patients. (N=20)



There are 8 patients showed IFN-γ ELISPOT positive responses to Vpr peptide pool no.1 and 2 patients showed IFN-γ ELISPOT positive responses to Vpr peptide pool no.2. For Vpu peptide, there are 4 patients showed IFN-γ ELISPOT positive responses to Vpu peptide pool no.1 and 2 patients showed IFN-γ ELISPOT positive responses to Vpu peptide pool no.2.

Figure 14 The magnitude of responses of IFN-γ ELISPOT positive results in HIV-1 CRF01_AE patients only. (N=18)



In HIV-1 CRF01_AE patients, There are 7 patients showed IFN-γ ELISPOT positive responses to Vpr peptide pool no.1 and there are 2 patients showed positive responses to Vpr peptide pool no.2.

Figure 15 Percentage of IFN-γ ELISPOT positive patients to Vpr and Vpu pooled peptide in all 20 patients.

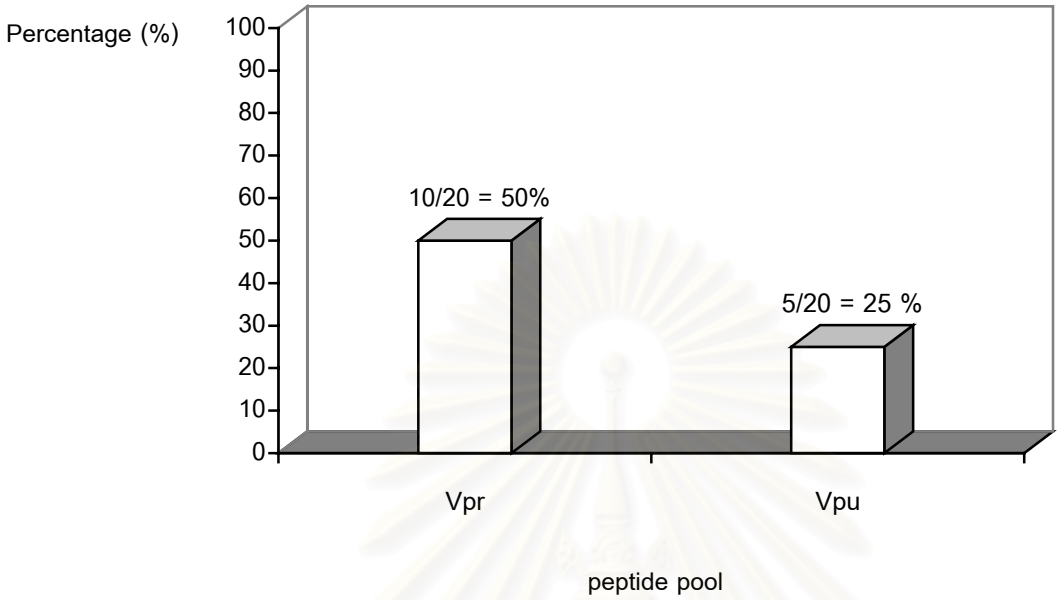
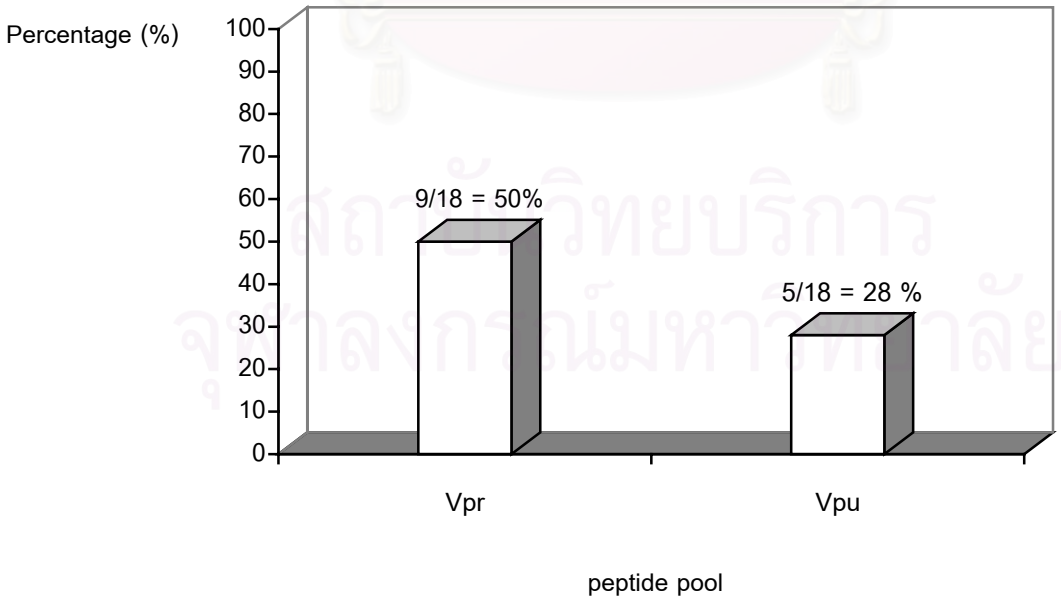


Figure 16 Percentage of IFN-γ ELISPOT positive patients to Vpr and Vpu pooled peptide in 18 HIV-1 CRF01_AE patients only.



3. HIV-1 CRF_01AE-specific ELISPOT-based responses against Vpr and Vpu individual peptides.

There are seven out of nine patients (78%) showed IFN-γ ELISPOT positive responses to Vpr individual peptides. The magnitude of responses range from 114 to 1,572 SFU/10^6PBMCs (Mean 823, Median 880 SFU/10^6PBMCs ,Table 6) Overall, there are 7/18 in HIV-1 CRF01_AE patients (39%) were showed IFN-γ ELISPOT positive responses to Vpr individual peptides. In addition we found two out of nine patients (RM and MB) can response more than one regions of Vpr peptides as showed in Table 6 and Figure 20. Patients RM showed responses against Vpr no.7 (Vpr 54-72, WEGVEAIIRILQQLLFVHF) and Vpr no.8 (Vpr 63-81, ILQQLLFVHFRIGCQHSRI). Patient MB showed responses against Vpr no.3 (Vpr 18-36, WTLELLEELKNEAVRHFPR) and Vpr no.4 (Vpr 27-45, KNEAVRHFPRPWLHGLGQH).

The number of patients who showed IFN-γ ELISPOT positive responses against each regions of Vpr and Vpu peptides were shown in Figure 17 and Figure 18.

Three out of five patients (60%) showed IFN-γ ELISPOT positive responses to Vpu individual peptides (Table 7). The magnitude of responses range from 136 to 680 SFU/10^6PBMCs (Mean 327, Median 306 SFU/10^6PBMCs). On the other hand, there are 3/17 patients (18%) were shown IFN-γ ELISPOT positive responses against Vpu individual peptides.

The percentage of IFN-γ ELISPOT positive patients against Vpr and Vpu individual peptides were shown in Figure 19 : % individuals who have IFN-γ ELISPOT positive results to Vpr peptides was 39 % and 18% in Vpu peptides .

The IFN-γ ELISPOT positive responses to individual Vpr and Vpu peptides in each subjects were shown in Figure 20.1- 20.7 and 21.1- 21.3, respectively.

The IFN-γ ELISPOT positive responses to Vpr and Vpu pooled and individual peptides in each subject. were shown Figure 22 and Figure 23.

The regions of Vpr and Vpu overlapping peptides which showed IFN-γ ELISPOT positive responses in each subject were shown in Table 8.

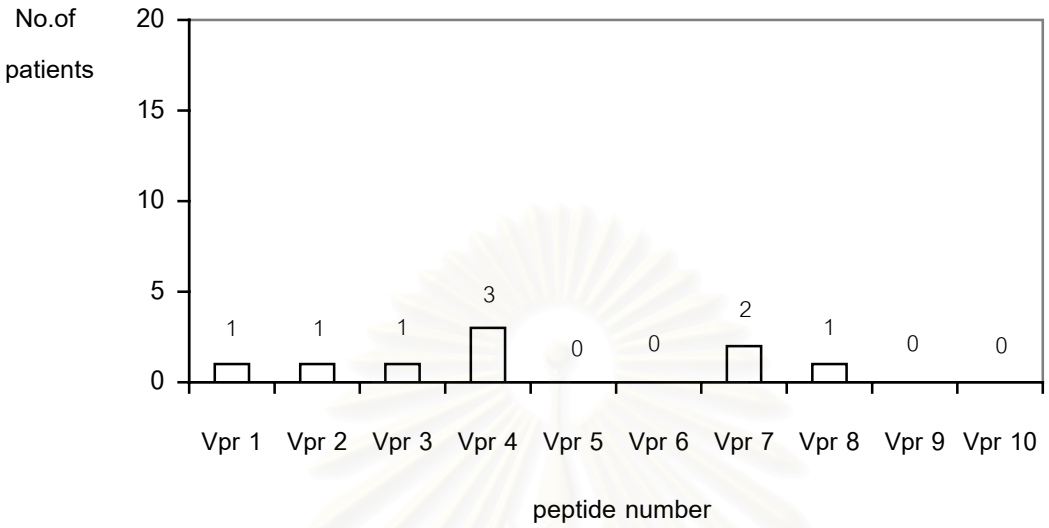
The summary results of CTL epitope mapping based on IFN-γ ELISPOT assay in HIV-1 CRF01_AE Vpr and Vpu peptides were shown in Figure 24 and Figure 25.

Table 6 IFN-γ ELISPOT positive responses to Vpr individual peptides in HIV-1 CRF01_AE infected patients. (background subtracted)

Patients no.	Initial name	IFN-γ ELISPOT positive responses (SFU/10^6PBMCs)										
		neg	no.1	no.2	no.3	no.4	no.5	no.6	no.7	no.8	no.9	no.10
1	PA	40	40	40	20	1344	28	0	0	0	0	0
2	DK	96	0	0	0	176	0	0	0	0	0	0
3	AP	50	9	20	14	82	24	0	0	0	0	0
4	TS	0	20	1572	44	16	20	40	20	12	20	20
5	JL	260	696	0	0	0	32	0	0	0	0	0
6	NI	104	0	0	0	0	0	0	920	24	0	0
7	CY	48	52	28	28	114	60	0	0	0	0	0
8	RM	12	0	0	0	0	0	16	1192	880	8	8
9	MB	8	8	4	480	212	56	0	0	0	0	0

There are seven out of eighteen patients (39%) showed IFN-γ ELISPOT positive responses to Vpr individual peptides. The magnitude of responses range from 114 to 1,572 SFU/10^6PBMCs (Mean 823, Median 880 SFU/10^6PBMCs). Patients RM and MB can responses against more than one of Vpr individual peptides.

Figure 17 Number of HIV-1 CRF01_AE-infected patients who showed IFN-γ ELISPOT positive responses against each regions of Vpr peptides. (N = 9/18)



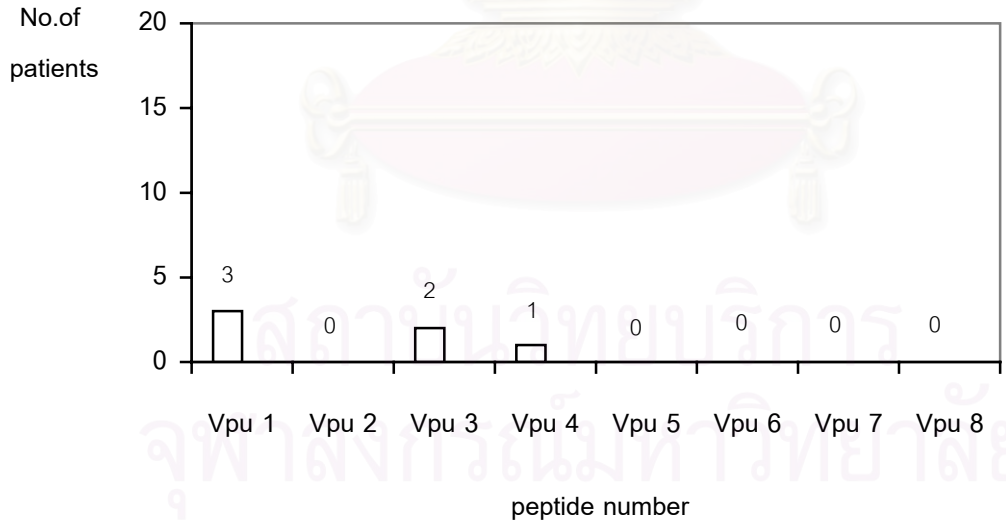
Most of subjects (4/18 = 22%) showed responses only one region against Vpr peptides whereas 3 out of 18 patients (17%) showed responses against Vpr peptide region 4 (Vpr 27-45, KNEAVRHFPRPWLHGLGQH) and 2 out of 18 patients (11%) showed responses against Vpr peptide region 7 (Vpr 54-72, WEGVEAIIRILQQLLFVHF). So we can conclude that Vpr peptide region 4 (Vpr 27-45, KNEAVRHFPRPWLHGLGQH) should be the most common epitope that found in ths study.

Table 7 IFN-γ ELISPOT positive responses to Vpu individual peptides HIV-1 CRF01_AE Infected patients. (background subtracted)

Patients no.	Initial name	IFN-γ ELISPOT positive responses (SFU/10^6PBMCs)								
		neg	no.1	no.2	no.3	no.4	no.5	no.6	no.7	no.8
1	PA	40	164	20	292	320	32	0	0	0
2	TS	0	0	0	0	0	0	16	20	12
3	PC	0	136	24	680	8	0	0	0	0
4	NI	104	372	72	88	60	36	0	0	0

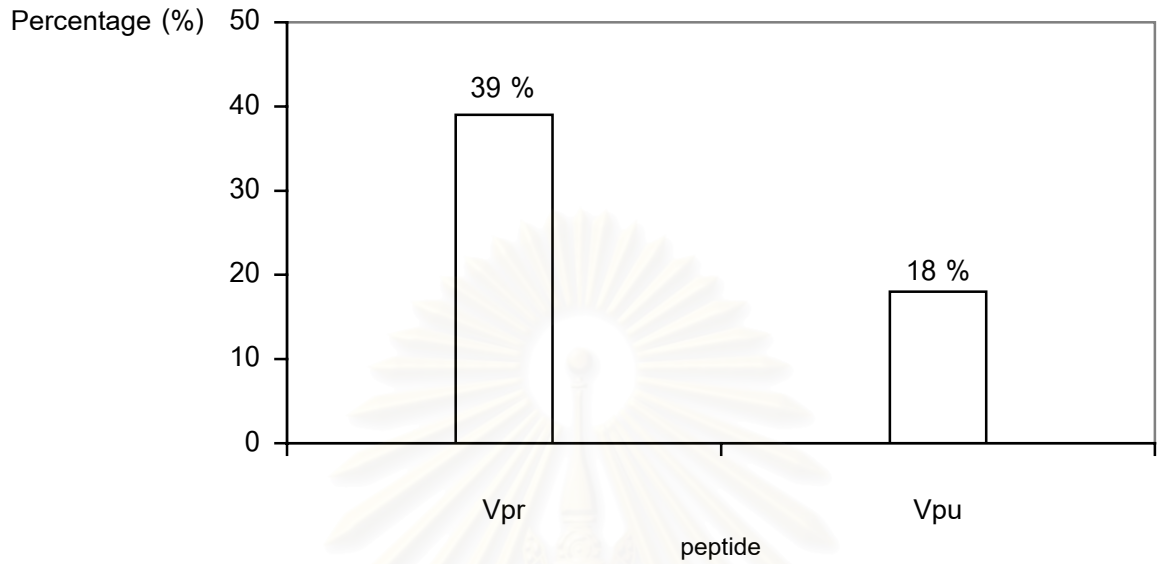
Three out of seventeen patients (18%) showed IFN-γ ELISPOT positive responses to Vpu individual peptides. The magnitude of responses range from 136 to 680 SFU/10^6PBMCs (Mean 327, Median 306 SFU/10^6PBMCs).

Figure 18 Number of HIV-1 CRF01_AE-infected patients who showed IFN-γ ELISPOT positive responses against each regions of Vpu peptides (N = 5/18)



Most of the subjects (3/17 = 18%) showed responses against Vpu peptide region 1 (Vpu 1-19, MTPLEISAIVGLIVALILA) and 2/17 (12%) showed responses against Vpu peptide region 3 (Vpu 19-37, AIVVWTIVAIEFKKILRQR). So we can conclude that Vpu peptide region 1 (Vpu 1-19, MTPLEISAIVGLIVALILA) should be the most common epitope that found in ths study.

Figure 19 Percentage of IFN-γ ELISPOT positive patients against Vpr and Vpu individual peptides



% individuals who have IFN-γ ELISPOT positive results to Vpr peptides

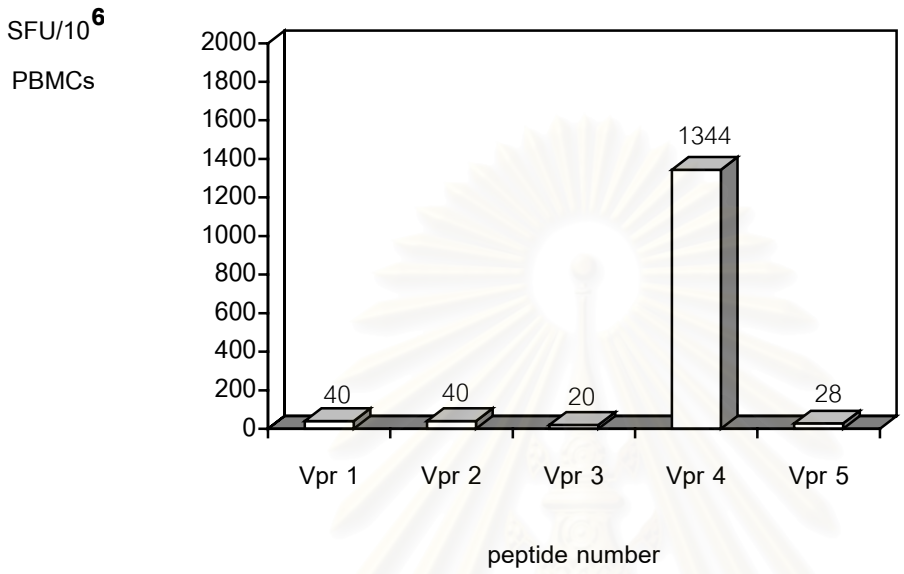
$$= \frac{\text{number of individuals who have Vpr-specific response}}{\text{number of target population}} = 7 / 18 = 39\ \%$$

% individuals who have IFN-γ ELISPOT positive results to Vpu peptides

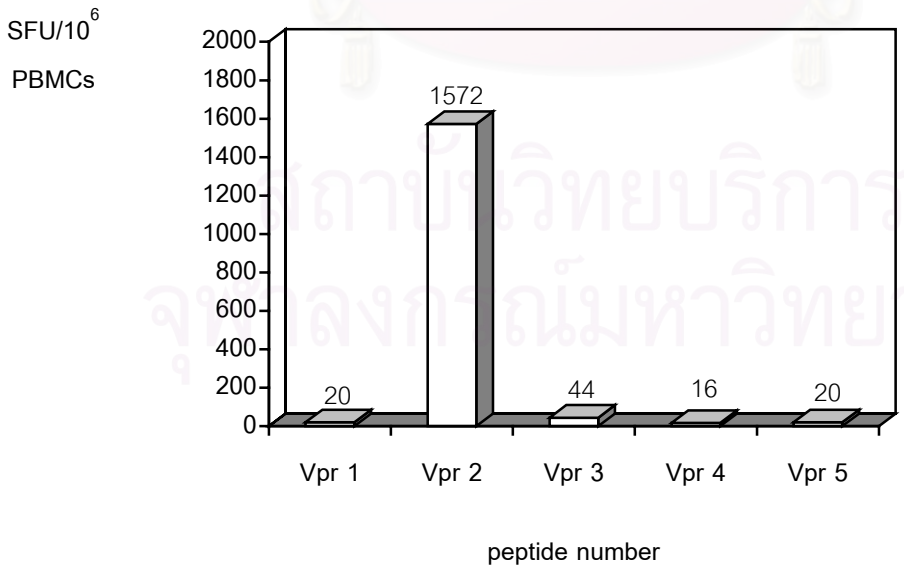
$$= \frac{\text{number of individuals who have Vpu-specific response}}{\text{number of target population}} = 3 / 17 = 18\ \%$$

Figure 20 IFN-γ ELISPOT positive responses to individual Vpr peptides in each subject. (background subtracted)

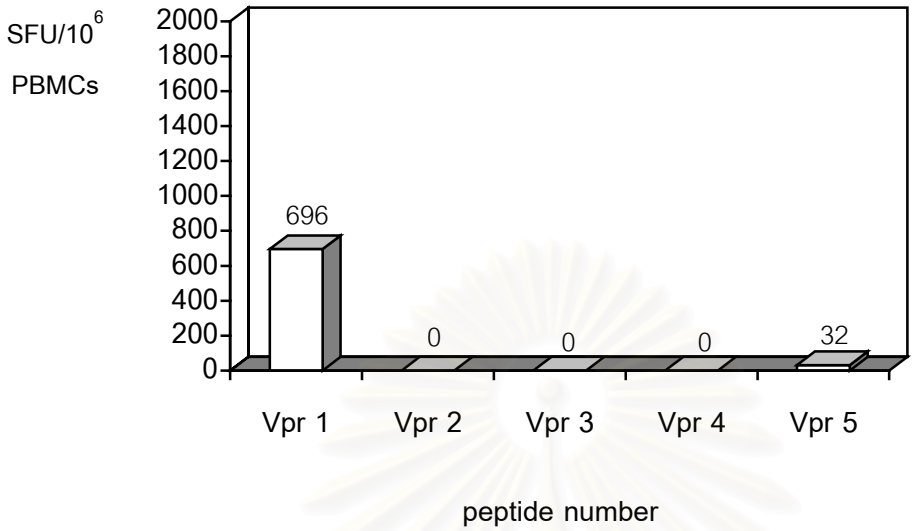
20.1 Subject PA (CD4+ T cell 968 cells/mm^3 , HIV-RNA 4,878 copies/ml)



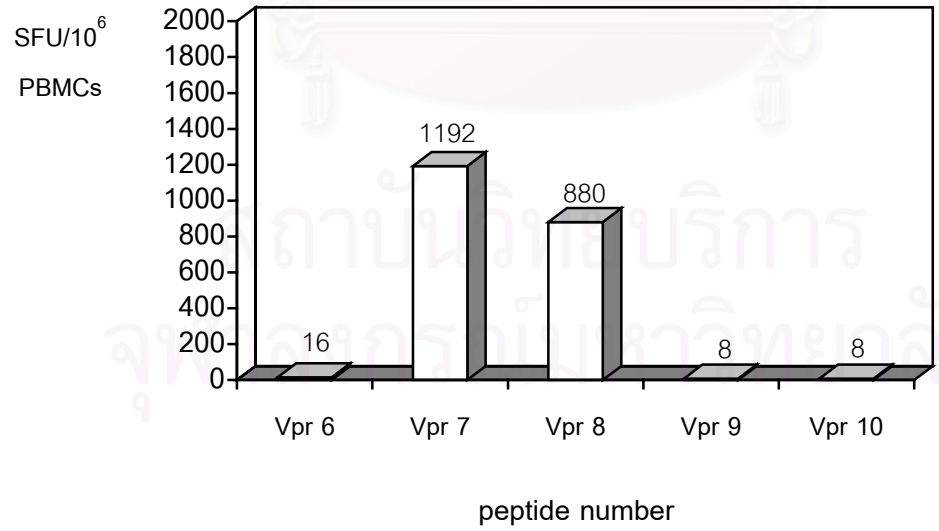
20.2 Subject TS (CD4+ T cell 435 cells/mm^3 , HIV-RNA 14,638 copies/ml)



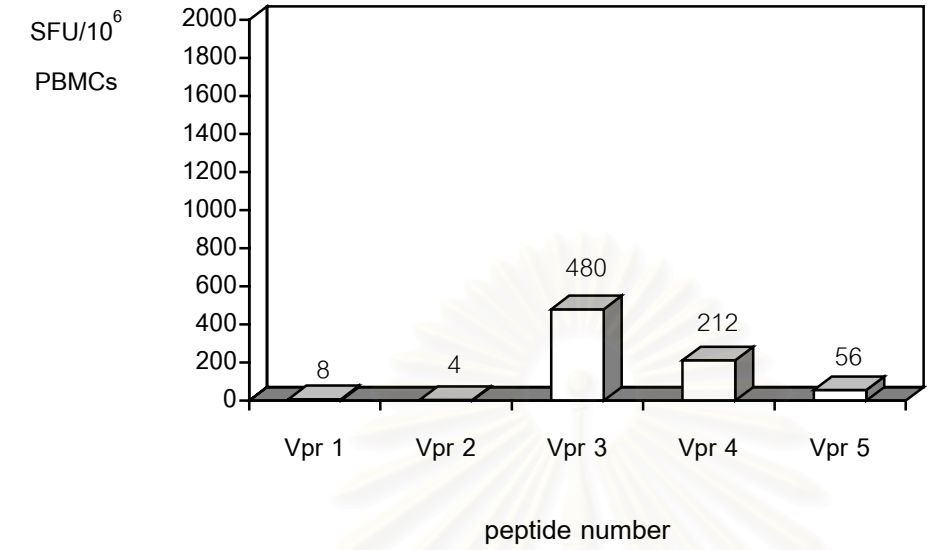
20.3 Subject JL (CD4+ T cell 315 cells/mm^3 , HIV-RNA 24,333 copies/ml)



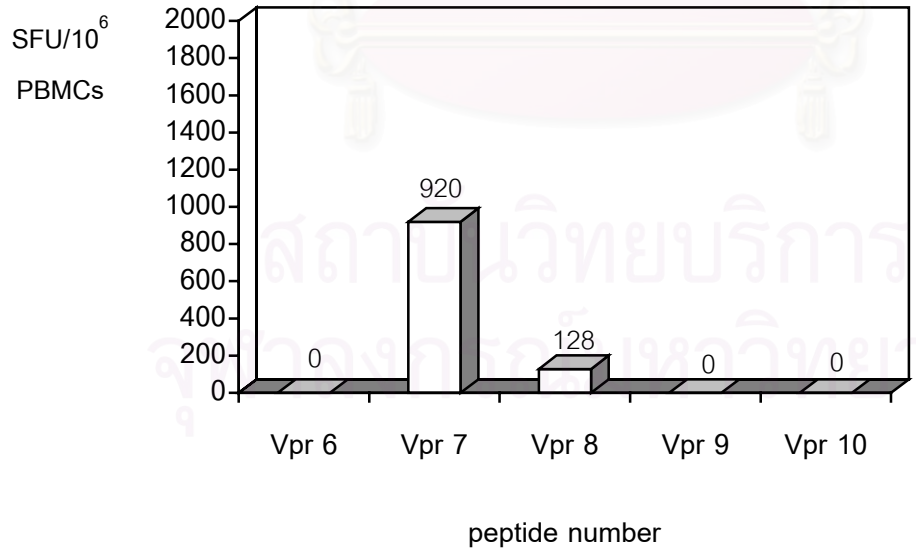
20.4 Subject RM (CD4+ T cell 454 cells/mm^3 , HIV-RNA 3,761 copies/ml)



20.5 Subject MB (CD4+ T cell 707 cells/mm^3 , HIV-RNA 99,338 copies/ml)



20.6 Subject NI (CD4+ T cell 563 cells/mm^3 , HIV-RNA 2,335 copies/ml)



20.7 Subject CY (CD4+ T cell 303 cells/mm^3 , HIV-RNA >500,000 copies/ml)

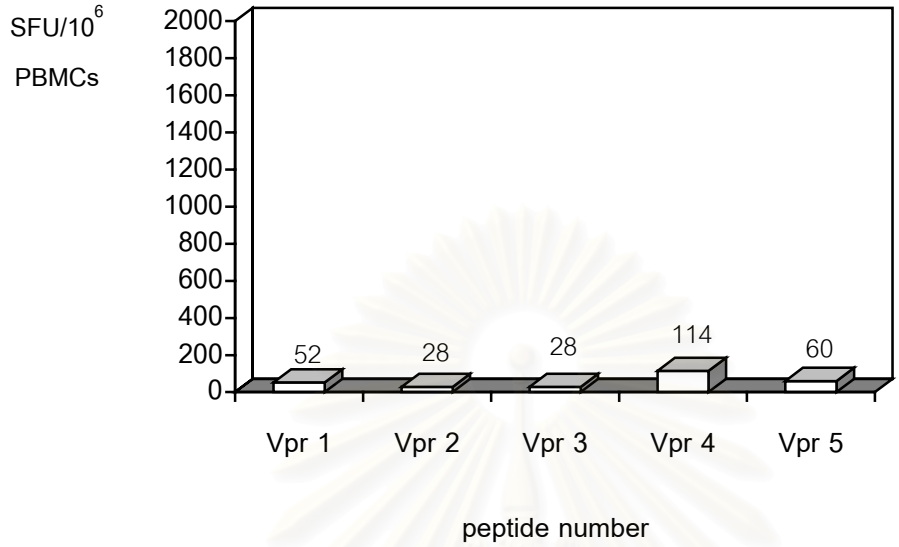
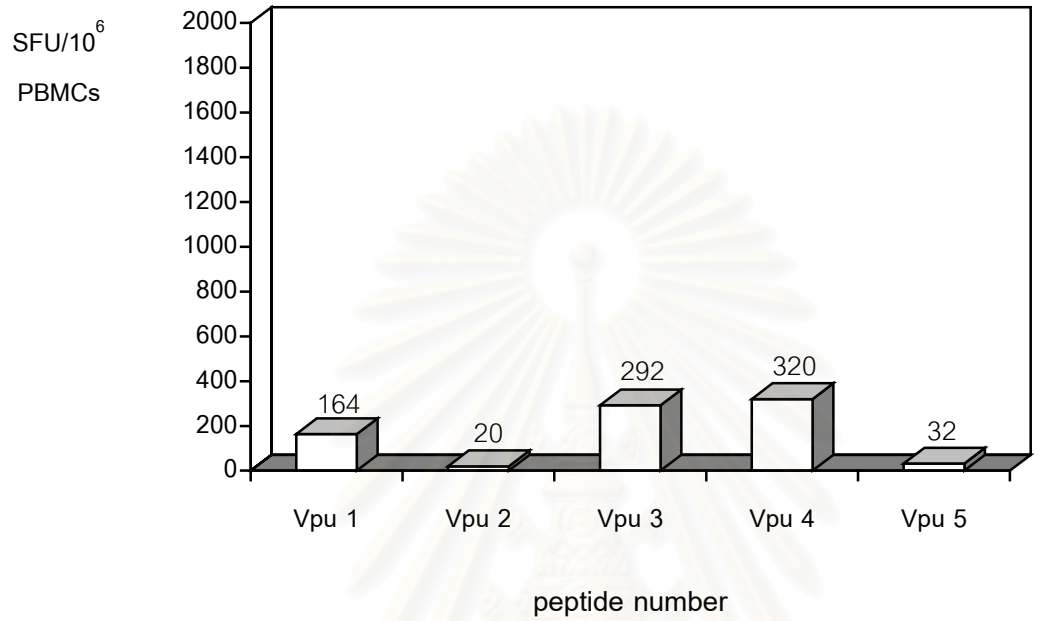
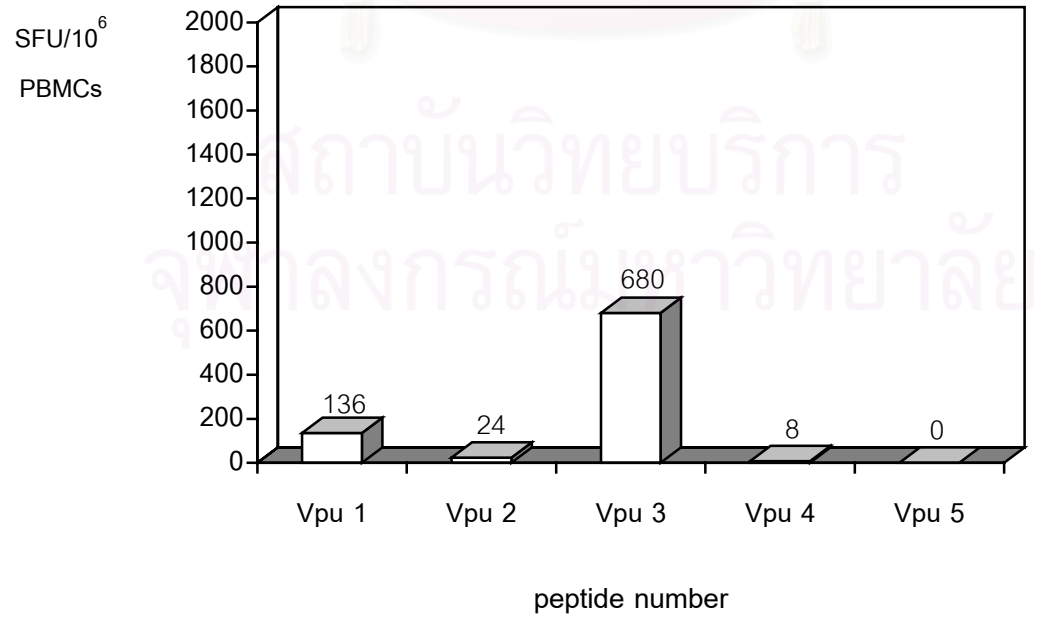


Figure 21 IFN-γ ELISPOT positive cells to individual Vpu peptides in each subject. (background subtracted)

21.1 Subject PA (CD4+ T cells 968 cells/mm^3 , HIV-RNA 4,878 copies/ml)



21.2 Subject PC (CD4+ T cells 471 cells/mm^3 , HIV-RNA 5,269 copies/ml)



21.3 Subject NI (CD4+ T cells 563 cells/mm^3 , HIV-RNA 2,335 copies/ml)

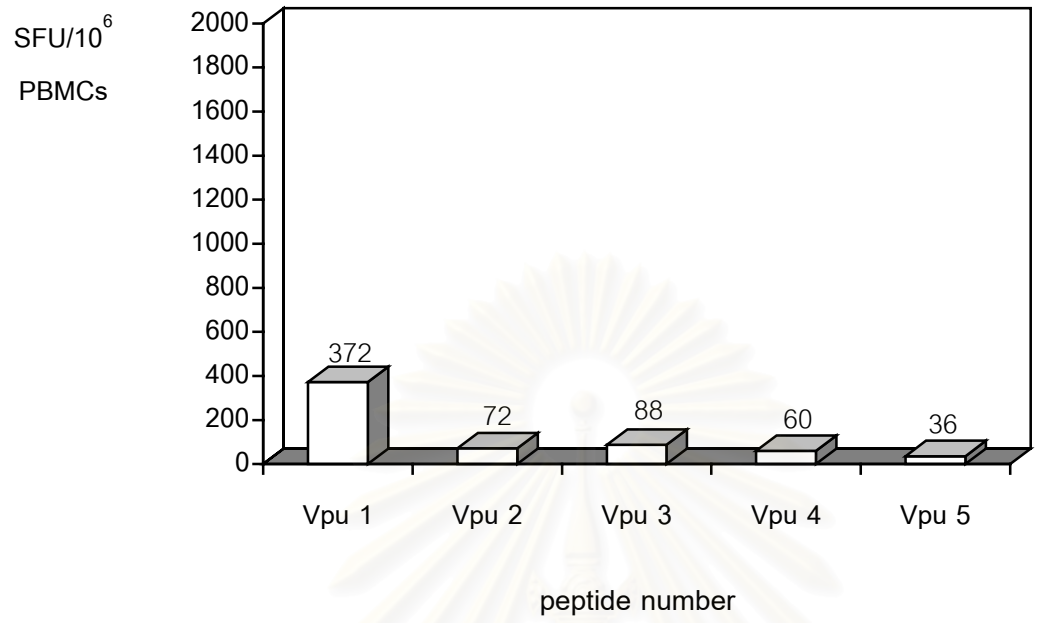


Figure 22 IFN-γ ELISPOT positive responses to Vpr pooled and individual peptides in each subjected. (N=9)

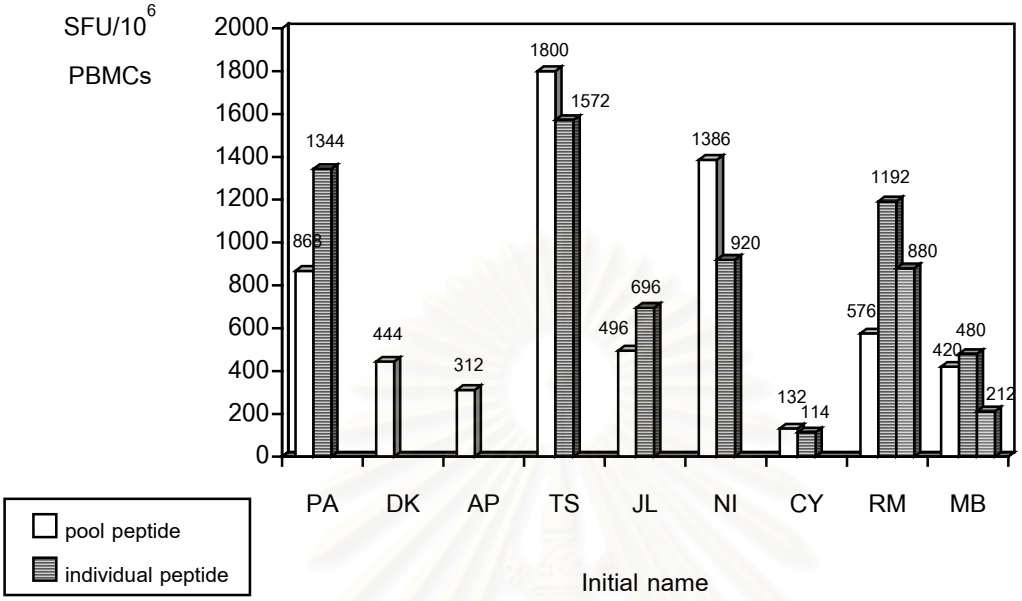


Figure 23 IFN-γ ELISPOT positive responses to Vpu pooled and individual peptides in each subjected. (N=4)

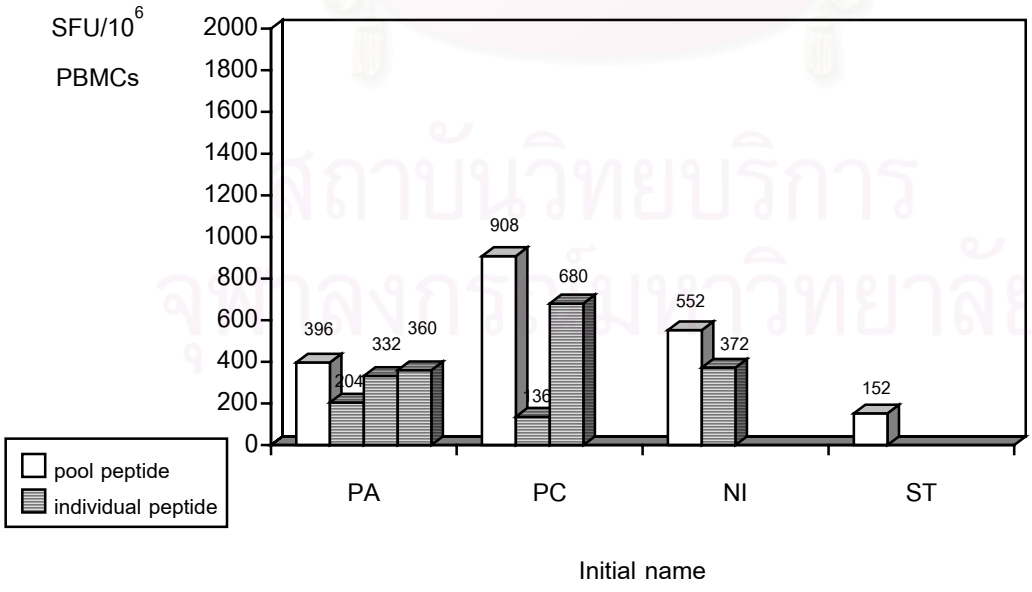


Table 8 The regions of Vpr and Vpu overlapping peptides which showed IFN-γ ELISPOT positive responses in each subject.

Initial name	Peptide no.	Region	Amino acid sequence of overlapping peptide	Length	The magnitude of response (SFU/10^6PBMCs)
PA	Vpr 4	27-45	KNEAVRHFPRPWLHGLGQH	19	1,344
	Vpu 1	1-19	MTPLEISAIVGLIVALILA	19	164
	Vpu 3	19-37	AIVVWTIVAIEFKKILRQR	19	292
	Vpu 4	28-46	IEFKKILRQRKIDRLVKRI	19	320
TS	Vpr 2	9-27	GPQREPYNEWTLELLEELK	19	1,572
JL	Vpr 1	1-18	MEQAPEDQGPQREPYNEW	18	696
RM	Vpr 7	54-72	WEGVEAIIRILQQLLFVHF	19	1,192
	Vpr 8	63-81	ILQQLLFVHFRIGCQHSRI	19	880
MB	Vpr 3	18-36	WTLELLEELKNEAVRHFPR	19	480
	Vpr 4	27-45	KNEAVRHFPRPWLHGLGQH	19	212
NI	Vpr 7	54-72	WEGVEAIIRILQQLLFVHF	19	920
	Vpu 1	1-19	MTPLEISAIVGLIVALILA	19	372
PC	Vpu 1	1-19	MTPLEISAIVGLIVALILA	19	136
	Vpu 3	19-37	AIVVWTIVAIEFKKILRQR	19	680

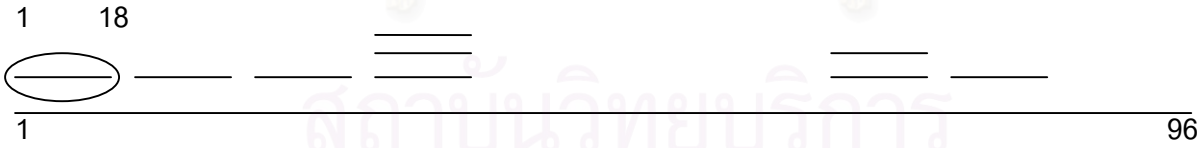
Figure 24 Summary results of CTL epitope mapping based on IFN-γ ELISPOT assay in HIV-1 CRF01_AE Vpr protein. ("____" represents the CTL epitope which responded by one patient)

Figure 24 A

MEQAPEDQGP$_{10}$QREPYNEWTL$_{20}$ELLEELKNEA$_{30}$VRHFPRPWLH$_{40}$GLGQYIYNNY$_{50}$GDTW

EGVEAI$_{60}$IRILQQLLFV$_{70}$HFRIGCQHSR$_{80}$IGIIPGRRGR$_{90}$NGAGRS

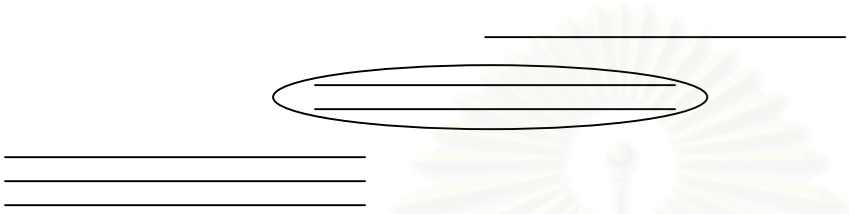
Figure 24 B



Circle line represent possible new epitope that have not yet been reported in the Los Alomos Database.

Figure 25 Summary results of CTL epitope mapping based on IFN-γ ELISPOT assay in HIV-1 CRF01_AE Vpu protein. ("____" represents the CTL epitope which responded by one patient)

Figure 25 A



MTPLEISAIV$_{10}$ GLIVALILAI$_{20}$VVWTIVAIEF$_{30}$KKILRQRKID$_{40}$RLVKRIRERA$_{50}$EDSGNESEGD$_{60}$

TDELAKLVEM$_{70}$GDFDPWVG

Figure 25 B

Circle line represent possible new epitope that have not yet been reported in the Los Alomos Database.

CHAPTER V

CONCLUSION AND DISCUSSION

1.Clinical characteristics of the study subjects.

The clinical characteristics were summarized in Table 4 . All 20 patients were asymptomatic and antiretroviral treatment naïve. The majority (18/20 or 90%) were heterosexuals and 2/20 (10%) were homosexuals. Based on HIV-1 genotyping and genotyping analysis, 18 donors infected with subtype A/E (CRF01_AE), and 2 donors (OK and PK) were infected with subtype B'. The CD4+ T lymphocytes were ranged from 303 to 968 cells/mm^3 with median of 463 cells/mm^3. The plasma HIV-RNA were ranged from 2,335 to > 500,000 copies/ml with the median of 6,755 copies/ml.

2. Vpr and Vpu –specific IFN-γ ELISPOT responses.

In this study, 10 out of 20 (50%) patients showed IFN-γ ELISPOT positive response to Vpr pooled peptides and 5 out of 20 (25%) patients showed IFN-γ ELISPOT positive responses to Vpu pooled peptides. We found that patient OK who was HIV-1 subtype B' infection can response to Vpr peptide pool no.1 which designed based on HIV-1 subtype CRF01_AE. The magnitude of responses was 444 SFU/10^6PBMCs. The possible reason should be cross-clade HIV-specific response in Vpr protein. Because of the consensus sequence of CRF01_AE subtype was showed 85.72% homology compared with subtype B. It could be conclude that this region of Vpr protein (region 1-54) has been conserve between subtypes.

consensus CRF01_AE :

1 MEQAPEDQGPQREPYNEWTLELLEELKNEAVRHFPRPWLHGLGQHIYNNYGDTW 54

55 EGVEAIIRILQQLLFVHFRIGCQHSRIGIIPGRRGRNGAGRS 96

consensus B :

1 MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHGLGQHIYETYGDTW 54

55 AGVEAIIRILQQLLFIHFRIGCQHSRIGITRQRRARNGASRS 96

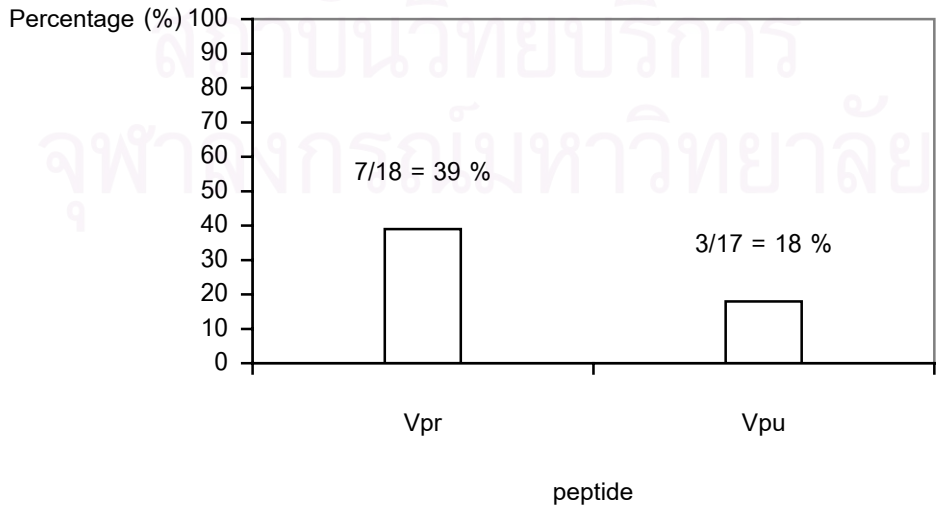
All of patients in subtype CRF01_AE who showed positive results were then further identified for Vpr and Vpu-specific response. We found 7 out of 18 patients (39%) showed IFN-γ ELISPOT positive responses to Vpr individuals peptides with the magnitude of responses ranged from 114 to 1,572 SFU/10^6PBMCs (Mean 823, Median 880 SFU/10^6PBMCs) but 2 showed negative result although their response for pooled peptide was positive. In addition, we found 3 out of 17 patients (18%) showed IFN-γ ELISPOT positive responses to Vpu individuals peptides with magnitude of response ranged from 136 to 680 SFU/10^6PBMCs (Mean 327, Median 306 SFU/10^6PBMCs). However, we are lost follow up in one of patient (ST) who showed positive responses to Vpu individuals peptides. So we can report only 17 patients in the responses to Vpu protein.

We found the magnitude of response in pooled peptide was different from the magnitude of response in individual peptide. We also found 2 patients (DK,AP) have no responses to Vpr individual peptide and 1 patient (ST) has no responses to Vpu individual peptide. The possible explanation may be because of the assays were based on specimen from different time points. The screening for pooled peptides were done in the first visit of patients but the test for Vpu- and Vpr-specific individual peptide and epitope mapping were performed on the samples of the second visit which was approximately 3-6 months later. The other possibilities are viral mutation or escape mutation in some of amino acid sequences of these proteins or caused by the lower frequency of CTL clones in the second visit. To avoid this discrepancy and to reduce workload and cost, the MATRIX assay, which is the new method for screening and find map CTL epitope in only one time of blood sample collection, is the more useful option.

Of note, patients with the magnitude of response higher than 500 SFU/10^6PBMCs to pooled peptides always showed the positive results against Vpr and Vpu-specific individual peptides. This observation suggests that the magnitude of response higher than 500 SFU/10^6PBMCs may be a predicted cut-off to showed individual peptides positive result for Vpr and Vpu proteins. Our results indicate that 39% (7/18) of patients showed IFN-γ ELISPOT positive responses to HIV-1 CRF01_AE Vpr peptides whereas 18% (3/17) of patients showed positive responses to HIV-1 CRF01_AE Vpu peptides as shown in Figure 26. In this study, the Vpr IFN-γ ELISPOT response is much more common than that of Vpu.

The study of Buranapraditkun, et al 2000 [87] showed % frequency of gag-specific cross clade CTL response (ELISPOT) directed against GAG E and B proteins in HIV-1 CRF01_AE infected patients were higher than that of Vpr / Vpu-specific CTL response in our study (90% in GAG E and 62% in GAG B with the magnitude of responses range from 100 to 2,952 SFU/10^6PBMCs and 52 to 1,176 SFU/10^6PBMCs,respectively) . This study indicates that the *gag* IFN-γ ELISPOT responses are much more common than that of Vpr and Vpu proteins.

Figure 26 Percentage of IFN-γ ELISPOT positive patients to Vpr and Vpu individual peptides in HIV-1CRF01_AE infection only. (N=18)



3. Vpr and Vpu-specific IFN-γ ELISPOT responses in different subtypes.

Table 9 The IFN-γ ELISPOT of responses directed against Vpr and Vpu proteins between this study and others.

Study Group	HIV-1 Sub Type	N	% frequency of IFN-γ ELISPOT Positive responses	Magnitude of Response (SFU/10^6PBMCs)	Amino Acid position	Most frequently target peptides
1.Novitsky et al. 2002						
-Vpr	C	56	21.4%	196 (range 101-403)	31-50	VRHFPRPWLHSLGQYIYETY
- Vpu	C	58	-	122 (range 100-269)	not shown	Data not shown
2.Addo et.al. 2002						
- Vpr	B	70	40%	245 (range 50-1,400)	55-70	AGVEAIIRILQQLLFI
-Vpu	B	70	2%	153 (range 170-210)	25-40	IVFIEYRKILRQRKID
3.This study,2002						
- Vpr	CRF01_AE	18	39%	880 (range114-1,572)	27-45 54-72	KNEAVRHFPRPWLHGLGQH WEGVEAIIRILQQLLFVHF
- Vpu	CRF01_AE	17	18%	306 (range 136-680)	1-9 19-37	MTPLEISAIVGLIVALILA AIVVWTIVAIEFKKILRQR

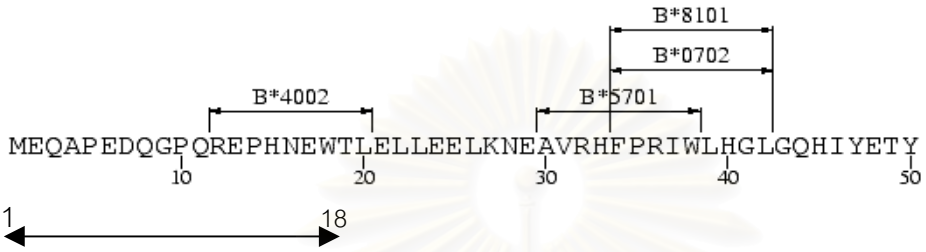
The results from this study are similar to the studies in HIV-1 subtype B and subtype C as showed in Table 9. We found the of % frequency of IFN-γ ELISPOT positive responses directed against Vpr was higher than that of Vpu protein. We also found the magnitude of response in Vpr and Vpu proteins was similar in each subtypes.

The most common Vpr epitope that found in this study (Vpr peptide no.4 region 27-45 : KNEAVRHFPRPWLHGLGQH) was the same region that most frequently target in the study of HIV-1 subtype C by Novitsky et al. 2002 (Vpr peptide region 31-50 : VRHFPRPWLHSLGQYIYETY). The most common Vpr epitope that found in this study (response in 3 patients) was different from most frequently targeted of subtype C in only 2 amino acid. In addition, the second common Vpr epitope that found in this study (Vpr peptide no.7 region 54-72 : WEGVEAIIRILQQLLFVHF) was included in the most frequently target peptide region in the study of HIV-1 subtype B by Addo et.al. 2002 (Vpr peptide region 55-70 : AGVEAIIRILQQLLFI). There are 2 amino acid different between most common Vpr epitope of subtype B and CRF01_AE. This observation suggest that the amino acid sequence of common Vpr epitope was mostly conserve between subtypes.

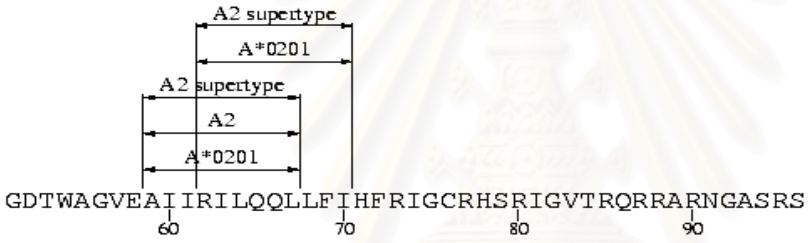
Morever, the most common epitope of Vpu subtype B (region 25-40 : IVFIEYRKILRQRKID) was included part of the second common epitope that found in the study. (region 19-37 : AIVVWTIVAIEFKKILRQR). We found 3 amino acid different between the most frequently targeted of subtype B and CRF01_AE. The percentage of IFN-γ ELISPOT responses in Vpu protein of this study was higher than that of the subtype B study. Possible explanations may be because of the difference in the number of sample size and the clinical characteristic of study subjects. The subtype B study was done by 70-HIV-1-infected individuals at different stage of infection (33 subjects: treated with HARRT, 21 individuals with chronic treated, and 16 individuals : LTNPs) whereas in this study was done in 20-HIV-1-infected individuals with asymptomatic, no antiretroviral treatment, and CD4+ T lymphocytes count $\geq$ 300 cells/mm^3. Another factor influencing the recognition of viral protein by CTL may be the amount of expression of these proteins during viral infection, with proteins expressed at high frequencies being more frequently targeted by CTL. However, a potential relationship between frequency of recognition by CTL and expression levels and other factors such as antigen processing and presentation of the corresponding protein remain to be determined. This data indicated that Vpr is frequently targeted HIV-1-specific CD8+ T cells, whereas Vpu is not.

4. HIV-1 CRF01_AE Vpr and Vpu epitope mapping

Figure 27 **Vpr CTL Epitope Map** (Los Alamos Database 2002, Last modified: Tue Jun 25 , 14:27:42 MDT 2002)

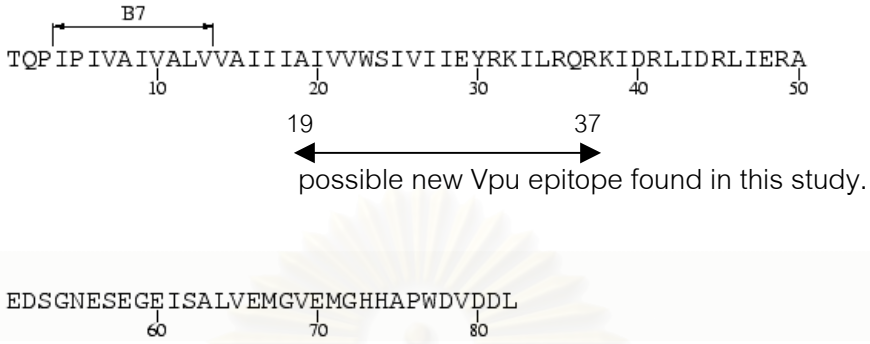


possible new Vpr epitope found in this study.



In this study, most of the Vpr epitopes observed have already been reported in Los Alamos Database 2002. Interestingly , the amino acid sequence of Vpr residues 1-18 has not yet been reported in the Los Alamos Database 2002 or other recent publications. It might be a Vpr novel epitope of which further characterization is needed. However, this epitope may also include part of the clade B restricted Vpr epitope (Table 9) at residue 12-20.

Figure 28 **Vpu CTL Epitope Map** (Los Alamos Database 2002, Last modified: Wed Mar 6 , 11:39:33 MST 2002)



Following the Los Alamos Database Epitope Map 2002, they report only one of the amino acid sequence of Vpu residues 4-13 was a CTL epitope. But from our result, HIV-1 CRF01_AE Vpu peptide residue 19-37 has not yet been reported. Also it might be a novel Vpu CTL epitope.

In conclusion, this study demonstrates that the HIV-1 CRF01_AE Vpr protein is a more frequent target recognized by IFN-γ producing T cells presumably CTLs compared to Vpu . Despite the small size of these proteins, multiple CTL epitopes within Vpr and one epitope within Vpu were defined. Several of them located within functionally important sites of these proteins. Our study as well as others have also found that the IFN-γ ELISPOT responses against the two HIV-1 proteins Vpr and Vpu were relatively low compared to those of other HIV-1 proteins, such as, gag ,nef, in particular.[88] The results may indicate its lower in immunogenicity of Vpr and Vpu compare to others. Two possible novel CTL epitopes identified, further epitope characterization and HLA restriction studies are warranted. Further studies are also needed to evaluate the exact role of accessory protein on HIV-1 disease progression and their potential for use in HIV vaccine. In addition, further studies will thus be needed to assess whether this infrequent response of HIV-1 Vpu reflects low immunogenicity of the protein *in vivo*, a lack of HLA class I processing of Vpu, or an underestimation of CTL responses directed against this highly variable protein using peptides based on HIV-1 consensus sequences or other factors.

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APPENDICES

APPENDIX I

CHEMICAL AGENTS AND INSTRUMENTS

A. Chemical Agents

Alkaline phosphatase substrate (Bio Rad Labs., Hercules, CA)

Anti-IFN-γ mAb 1-D1K (Mabtech, Stockholm, Sweden)

Anti-IFN-γ mAb 7-B6-1 (Mabtech, Stockholm, Sweden)

Clorox

DMSO (Sigma, UK)

Fetal Bovine Serum (Bio Whittaker, Maryland, USA)

Glutamine (Sigma, UK)

Isoprep (Robbins Scientific, Norway)

PBS (Sigma, UK)

Penicillin (General Drugs House, Thailand)

RPMI Medium 1640 (GIBCO, USA)

Streptavidin-alkaline phosphatase conjugate (Mabtech, Stockholm, Sweden)

Streptomycin (General Drugs House, Thailand)

Truncated Vpr peptide (Natural and Medical Sciences Institute at the University of Tuebingen, Germany)

Truncated Vpu peptide (Natural and Medical Sciences Institute at the University of Tuebingen, Germany)

Trypan blue (Sigma, UK)

B. Instruments

6-well flat plate (Costar, USA)

24- well flat plate (Costar, USA)

96-well polyvinylidene difluoride backed plates (Millipore, Bedford, MA)

Analytical balance (Precise, Swiss)

Automatic pipette (Gilson, USA)

Centrifuge

Conical tube 15, 50 mL (Falcon, USA)

Counting chamber

Cover slip

Cryotube (Sarstedt, Germany)

Freezer -70^{0}C

Glove

Heparin tube (Becton-Dickinson, USA)

Incubator (Forma Scientific, USA)

Microcentrifuge (Eppendrof, USA)

Microtube 250 μl

Mixer-Vortex-Genic (Scientific Industries, USA)

Multichannel pipette

Pipette tip

Serological pipette 1, 2, 5, 10, 25 mL (Costar, USA)

Stereomicroscope (Olympus, Japan)

Water bath (Shel-lab, USA)

APPENDIX II

AMINO ACID SEQUENCE OF VPR AND VPU PEPTIDES

1. Abbreviation for Amino Acids

A	alanine	L	leucine
R	arginine	K	lysine
N	asparagine	M	methionine
D	aspatic acid	F	phenylalanine
C	cysteine	P	proline
Q	glutamine	S	serine
E	glutamic acid	T	threonine
G	glycine	W	tryptophan
H	histidine	Y	tyrosine
I	isoleucine	V	valine

2. HIV-1 CRF01_AE Vpr peptides

Peptide No.	Region	Amino Acid Length	Vpr Amino acid Sequence
Pool 1			
1	1-18	18	MEQAPEDQGPQREPYNEW
2	9-27	19	GPQREPYNEWTLELLEELK
3	18-36	19	WTLELLEELKNEAVRHFPR
4	27-45	19	KNEAVRHFPRPWLHGLGQH
5	36-54	19	RPWLHGLGQHIYNNYGDTW

Peptide No.	Region	Amino Acid Length	Vpr Amino acid Sequence
Pool 2			
6	45-63	19	HIYNNYGDTWEGVEAIIRI
7	54-72	19	WEGVEAIIRILQQLLFVHF
8	63-81	19	ILQQLLFVHFRIGCQHSRI
9	72-90	19	FRIGCQHSRIGIIPGRRGR
10	81-96	16	IGIIPGRRGRNGAGRS

3. HIV-1 CRF01_AE Vpu peptides

Peptide No.	Region	Amino Acid Length	Vpu Amino acid Sequence
Pool 1			
1	1-19	19	MTPLEISAIVGLIVALILA
2	10-28	19	VGLIVALILAIVVWTIVAI
3	19-37	19	AIVVWTIVAIEFKKILRQR
4	28-46	19	IEFKKILRQRKIDRLVKRI
5	37-55	19	RKIDRLVKRIRERAEDSGN
Pool 2			
6	46-64	19	IRERAEDSGNESEGDTDEL
7	55-73	19	NESEGDTDELAKLVEMGDF
8	63-78	16	ELAKLVEMGDFDPWVG

BIOGRAPHY

Miss Suthida Kerdsan was born on September 7, 1978 in Bangkok , Thailand. She received a Bachelors' degree in Biology in 1999 From the Faculty of Science, Srinakarinwirot University, Bangkok, Thailand. She enrolled at Chulalongkorn University in a gratuate program for a Masters' Degree in Medical Science in 2000.