GENOTYPIC AND BIOTYPIC CHARACTERIZATION OF HIV-1 SUBTYPE E ISOLATED FROM DISCORDANT AND CONCORDANT COUPLES

APASARA MUDNGOEN

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE (MICROBIOLOGY)
FACULTY OF GRADUATED STUDIES
MAHIDOL UNIVERSITY
2000

ISBN 974-664-142-2

Thesis
entitled

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Apasara Mudngoen
Miss Apasara Mudngoen
Candidate

Assoc.Prof. Ruengpung Sutthent, M.D., Ph.D.
Major-advisor

Asst. Prasert Auewarakul, M.D., Dr. Med.
Co-advisor

Wannee Kantakamalakul
Asst. Wannee Kantakamalakul, Ph.D.
Co-advisor

Prof. Liangchai Limlomwongse, Ph.D.
Dean
Faculty of Graduate studies

Uraiwan Kositanont
Assoc.Prof. Uraiwan Kositanont, Ph.D.
Chairman
Master of Science Program
In Microbiology
Faculty of Medicine, Siriraj Hospital

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GENOTYPIC AND BIOTYPIC CHARACTERIZATION OF HIV-1 SUBTYPE E ISOLATED FROM HIV-1 INFECTED DISCORDANT AND CONCORDANT COUPLES

was submitted to the Faculty of Graduate Studies, Mahidol University
For the degree of Master of Science (Microbiology)

on

May 30, 2000

Apasara Mudngoen
.....
Miss Apasara Mudngoen
Candidate

.....
Assoc.Prof. Ruengpung Sutthent, M.D., Ph.D.
Chairman

.....
Asst. Prasert Auewarakul, M.D., Dr. Med.
Member

Busarawan Sriwanthana
.....
Busarawan Sriwanthana, Ph.D.
Member

Wannee Kantakamalakul
.....
Asst. Wannee Kantakamalakul, Ph.D.
Member

.....
Prof. Liangchai Limlomwongse, Ph.D.
Dean
Faculty of Graduate studies
Mahidol University

.....
Prof. Chanika Tuchinda
Dean
Faculty of Medicine, Siriraj Hospital
Mahidol University

ACKNOWLEDGEMENTS

First of all, I would like to express my deepest gratitude to my advisor, Dr. Reungpeung Sutthent, for her great supervision, suggestion, and encouragement throughtout my study. I also gratefully appreciate all of my co-advisor, Dr. Prasert Auewarakul, Dr. Wannee Kantakamalakul, and the external committee, Dr. Busarawan Sriwanthana, for their excellent instruction and critical reading of my thesis.

I am also grateful to the staff of the Division of Virology, Department of Microbiology, Faculty of Medicine Siriraj Hospital for their helpful and encouragement, the staff of the Department of Obsteric-Gynecology, Faculty of Medicine Siriraj Hospital for the specimen collection, the staff of the Blood bank, Siriraj Hospital for their kind support of leucopaque.

Very special thanks to Dr. Victoria R. Polonis for her kind advice and technical support about the neutralization assay.

I am particularly indebted to all of the patients who donated specimens for this study. I especially thank to all of them.

Finally, I would like to express my heartiest gratitude to my mother for her patience, sympathy, and cheerfulness throughout my study. My special thank also expresses to my relative sisters and James Troescher for their helpful in my thesis writing.

Apasara Mudngoen

4037137 SIMI/M: MAJOR: MICROBIOLOGY; M.Sc. (MICROBIOLOGY)
KEY WORDS : HIV-1 SUBTYPE E, HETEROSEXUAL TRANSMISSION, HIGHLY EXPOSED PERSISTENTLY SERONEGATIVE

APASARA MUDNGOEN: GENOTYPIC AND BIOTYPIC CHARACTERIZATION OF HIV-1 SUBTYPE E ISOLATED FROM HIV-1 INFECTED DISCORDANT AND CONCORDANT COUPLES. THESIS ADVISOR: RUENGPUNG SUTTENT, Ph.D., M.D., PRASERT AUEWARAKUL, M.D., Dr. Med., WANNEE KANTAKAMALAKUL, Ph.D. 177 p.
ISBN 974-664-412-2

HIV-1 subtype E was rapidly expanded thoughout the century, becoming the predominant subtype in Thailand. Several groups reported cases or series of persons either presumptively or definitely exposed to HIV-1 in whom subsequent evidence of sustained HIV-1 infection did not occur. The present study investigated viral factors and host factors that influenced lower transmission in Highly Exposed Persistently Seronegative (HEPS) groups. HEPS subjects consisted of 15 males (HH01, HH02, HH11, HH13, HH16, HH17, HH18, HH31, HH33, HH35, HH39, HH43, HH45, HH49, and HH55) and 2 females (HW48 and HW53). The HIV-1 infected partners of HEPS consisted of 15 female (PW11, PW35, PW45, PW49, PW55, PW01, PW02, PW13, PW16, PW17, PW18, PW31, PW33, PW39, and PW43) and 2 male (PH48 and PH53) and 5 HIV-1 infected concordant couples (CH06, CW06, CH07, CW07, CH34, CW34, CH57, CW57, CH59 and CW59). All subjects were asymptomatic at the time of study. EDTA blood was collected from both HIV-1 infected – and HEPS subjects. HEPS confirmed eligibility by HIV-1/2 ELISA and PBMC DNA PCR with *gag/pol* gene. None of them were infected. Mean viral load of HIV-1 infected partners of HEPS was log 4.20 ± 0.88 copies/ml and that of concordant couples was log 4.32 ± 0.45 copies/ml. No significant difference was found between these two groups.

To access any HIV-1 genetic abnormality presented in HIV-1 infected partners of HEPS, nucleotide sequencing of *nef* gene and LTR was performed on HIV-1 proviral DNA and genomic RNA of 17 HIV-1 infected partners of HEPS and 5 HIV-1 infected concordant couples. Four deletion patterns of *nef* gene were found in this experiment. Point mutations in *nef* gene and LTR region were found.

HIV-1 biological phenotypes were studied from 6 HIV-1 isolates from partners of HEPS and 7 isolates from concordant couples. Of 6 isolates from HIV-1 infected partners of HEPS, 3 isolates (PW33, PW49 and PH53) were M-tropic viruses, one (PW55) was T-tropic virus and 2 isolates (PW11 and PW45) retained both M-tropic and T-tropic (dual tropic) viruses. Of 7 isolates from concordant couples, 3 isolates (CW07, CH57 and CH59) were M-tropic viruses, two (CW33 and CW56) were T-tropic viruses and the last two isolates (CW34 and CH34) were dual tropic viruses.

The capability of viral infection was compared between HEPS PBMC and normal donor PBMC. Neither PBMC from HEPS nor those from normal donors were different in supportive infection of the viruses.

Neutralizing activity of NPO3 HIV-1 subtype E lab strain was relatively sensitive to neutralization by multiple plasma. All plasma from partners of HEPS except PH48 and PH53 contained neutralizing activities against lab strain subtype E (NP03) less than those of plasma from concordant couples and pool plasma. Viruses from partners of HEPS and discordant couples were not found any difference of the sensitivity to neutralization. However, PW55 isolates from partner of HEPS was resistance of neutralization by all of tested plasma. Plasma of these two concordant couples showed more broadly neutralization against primary isolates than those of partners of HEPS.

To determine nucleotide sequence of R5 gene, whose protein act as the secondary receptor of HIV-1 to enter the cell. No mutation sequence was detected in these HEPS R5 gene.

In conclusion, No significant difference of mean viral load was found between HIV-1 infected partners of HEPS and concordant couples. Viral factors found in partner of HEPS were *nef* gene deletion and point mutations in *nef* gene and LTR region. There was no difference in replication pattern of HIV-1 isolates in each kind of biotype between these two groups and NT activity was not different between these groups. No mutation sequence was detected in R5 gene of their HEPS.

These results suggest that the *nef* deletion and point mutations in *nef* gene and LTR region in HIV-1 infected partners of HEPS seem to be non-transmitted to the partner, who has repeated unprotected sexual relation for several years. These cases might have low inoculum level or too defective virus to transmit to their partners. The finding of HIV-1 variants with *nef* deletion in HIV-1-infected partners of HEPS provides additional impetus for consideration of the vaccine approach.

4037137 SIMI/M : สาขาวิชา : จุลชีววิทยา; วท.ม. (จุลชีววิทยา)

อภัสรา หมุดเงิน : ลักษณะทางยีโนมและชีวภาพของเชื้อ เอชไอวี-1 สัปทัยป์ อี ที่แยกได้จากคู่สามี-ภรรยาที่มีผลทางน้ำเหลืองตรงกันและไม่ตรงกัน (GENOTYPIC AND BIOTYOIC CHARACTERIZATION OF HIV-1 SUBTYPE E ISOLATED FROM HIV-1 INFECTED DISCORDANT AND CONCORDANT COUPLES) คณะกรรมการควบคุมวิทยานิพนธ์: รวงผึ้ง สุทเธนทร์, Ph.D., M.D., ประเสริฐ เอื้อวรากุล, M.D., Dr. Med., วรรณี กัณฐกมาลากุล, Ph.D. 177 หน้า ISBN 974-664-412-2

เอชไอวี-1 สัปทัยป์ E มีการระบาดอย่างมากจนกลายเป็นสัปทัยป์หลักในประเทศไทย จากการรายงานในหลายกลุ่มเกี่ยวกับกลุ่มผู้ที่มีความเสี่ยงสูงต่อการติดเชื้อเอชไอวี-1 แต่ไม่มีการติดเชื้อเกิดขึ้น การทดลองนี้เป็นการศึกษาปัจจัยของเชื้อเอชไอวี-1 และปัจจัยของโฮสต์ที่มีผลต่อการติดเชื้อในกลุ่มผู้ที่มีความเสี่ยงสูง (HEPS) HEPS เป็นชาย 15 คน (HH01, HH02, HH11, HH13, HH16, HH17, HH18, HH31, HH33, HH35, HH39, HH43, HH45, HH49, and HH55) และหญิง 2 คน (HW48 และ HW53) ผู้ที่ติดเชื้อคู่ของ HEPS เป็นหญิง 15 คน (PW11, PW35, PW45, PW49, PW55, PW01, PW02, PW13, PW16, PW17, PW18, PW31, PW33, PW39, และ PW43) และชาย 2 คน (PH48 และ PH53) และคู่สามี-ภรรยาที่ติดเชื้อเอชไอวี-1 จำนวน 5 คู่ (CH06, CW06, CH07, CW07, CH34, CW34, CH57, CW57, CH59 และ CW59) เก็บเลือดไม่แข็งตัวทั้งจากผู้ที่ติดเชื้อและผู้ที่มีความเสี่ยงสูงแต่ไม่ติดเชื้อ โดย HEPS จะถูกตรวจสอบยืนยันอีกครั้งโดย HIV-1/2 ELISA และ PBMC DNA PCR ตรงตำแหน่งของ gag/pol gene ไม่มีผู้ใดมีการติดเชื้อ ค่าเฉลี่ยของปริมาณไวรัสในเลือดของคู่ของ HEPS เท่ากับ log 4.20 ± 0.88 copies / ml และของคู่สามีภรรยาที่ติดเชื้อเท่ากับ log 4.32 ± 0.45 copies / ml ไม่พบว่ามีความแตกต่างอย่างมีนัยสำคัญของทั้งสองกลุ่ม

เพื่อศึกษาความผิดปกติทางยีโนมของเชื้อเอชไอวี-1ของคู่ของ HEPS จึงได้ทำการศึกษาการเรียงตัวของนิวคลีโอไทด์ของยีน nef และส่วนของ LTR จากเอชไอวี-1 DNA และ RNA จากคู่ของ HEPS จำนวน 17 คน และคู่สามี-ภรรยาที่ติดเชื้อจำนวน 5 คู่พบ point mutation และ deletion ในยีน nef และพบ point mutation ในส่วนของ NF-*k*B และ SP1 binding site ของ LTR

จากการศึกษาลักษณะทางชีวภาพของเชื้อเอชไอวี-1 ที่แยกได้จากผู้ที่ติดเชื้อคู่ของ HEPS จำนวน 6 สายพันธุ์ และ 7 สายพันธุ์ จากคู่สามี-ภรรยาที่ติดเชื้อ พบว่า จาก 6 สายพันธุ์ที่แยกได้จากคู่ของ HEPS 3 สายพันธุ์ (PW33, PW49, และ PH53) เป็นสายพันธุ์ที่มีความสามารถในการติดเชื้อในแมคโคฝาจ 1 สายพันธุ์ (PW55) มีความสามารถในการติดเชื้อใน T เซลล์และอีก 2 สายพันธุ์ (PW11 และ PW45) สามารถติดเชื้อได้ทั้งในแมคโครฝาจและ T เซลล์

เมื่อเปรียบเทียบความสามารถในการติดเชื้อในเซลล์เม็ดเลือดขาวจาก HEPS และกลุ่มปกติ ไม่พบว่ามีความแตกต่างของการติดเชื้อในเซลล์จากทั้งสองกลุ่ม

Neutralizing activity ของ NPO3 เอชไอวี-1 สัปทัยป์ E สายพันธุ์ทางห้องปฏิบัติการ มีความไวต่อการถูก neutralize โดยพลาสมาต่าง ๆ พลาสมาจากผู้ที่ติดเชื้อคู่ของ HEPS ยกเว้นจาก PH48 และ PH53 สามารถ neutralize ต่อ NPO3 ได้ต่ำกว่าพลาสมาจากคู่สามี-ภรรยาและจากพลาสมารวม ไวรัสที่แยกได้จากคู่ของ HEPS และคู่สามี-ภรรยาที่ติดเชื้อไม่พบว่ามีความแตกต่างของความไวต่อการถูก neutralization อย่างไรก็ตาม ไวรัสที่แยกได้จาก PW55 ไม่ถูก neutralize จากทุกพลาสมา พลาสมาจากคู่สามี-ภรรยาที่ติดเชื้อมีความสามารถในการ neutralize เชื้อไวรัสที่แยกได้มากกว่าพลาสมาจากคู่ของ HEPS

จากการศึกษาการเรียงตัวของนิวคลีโอไทด์ ของยีน R5 ไม่พบความผิดปกติบนยีนนี้

สรุป ค่าเฉลี่ยของไวรัสในเลือดทั้งในกลุ่มคู่ของ HEPS และคู่สามี-ภรรยาที่ติดเชื้อพบว่าไม่มีความแตกต่างกันอย่างมีนัยสำคัญ ปัจจัยของเชื้อเอชไอวี-1 ที่พบคือ มี point mutation ในส่วนของยีน *nef* และ LTR และ ยีน *nef* เกิดการ deletion ไม่พบความแตกต่างของลักษณะทางชีวภาพและ neutralizing activity ในทั้ง 2 กลุ่ม และไม่พบความผิดปกติในส่วนของยีน R5 ของ HEPS

จากผลการศึกษาชี้แนะว่า nef deletion และ point mutation บนยีน nef และส่วนของ LTR ของผู้ที่ติดเชื้อคู่ของ HEPS ดูเหมือนว่าจะมีบทบาทสำคัญในการไม่ส่งผ่านเชื้อไปยัง HEPS ซึ่งอาจจะเนื่องมาจากมีปริมาณไวรัสน้อย หรือว่าไวรัสมีความผิดปกติมากจนไม่สามารถติดเชื้อต่อไปอีกคนหนึ่งได้ ดังนั้น nef deletion และ point mutation บนยีน nef และ LTR น่าจะเป็นแนวทางสำคัญในการพัฒนาวัคซีนต่อไป

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Figure 15. Alignment of amino acid sequence of R5 gene 108

LIST OF ABBREVIATIONS

Abbreviation or symbol	
Ab	antibody
AIDS	acquired immunodeficiency syndrome
AMV	avian myeloblastosis virus
AP	adapter protein
APC	antigen presenting cell
bp	base pair
^{0}C	degree Celsius
CCPs	clathin coated pits
CPE	cytopathic effect
CSW	commercial sex worker
CTF	CCAAT-binding transcription factor
CTL	cytotoxic T lymphocytes
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNTP	deoxynucleotide triphosphate
ECL	electrochemiluminescence
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
g	gravity
FBS	fetal bovine serum
GALT	gut-associated lymphoreticular tissue

LIST OF ABBREVIATIONS (Cont.)

GI	gastrointestinal
HEPS	highly exposed persistently seronegative
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
HLA	human leukocyte antigen
hr	hour
IL-2	Interleukin-2
l	liter
IDU	intravenous drug user
IEF	iso-electro-focusing
Ig	immunoglobulin
IP	infectious particle
LBP-1	leader binding protein 1
LTR	long terminal repeat
Kb	kilobase
kDa	kilodalton
M	Molar
MALT	mucosa-associated lymphoid tissue
mg	milligram
MHC class I	major histocompatibility complex class I
min	minute
ml	milliliter

LIST OF ABBREVIATIONS (Cont.)

mM	millimolar
mRNA	messenger RNA
NF-*k*B	nuclear factor-*k*B
ng	nanogram
NHP	normal human plasma
nm	nanometer
NSI	non-syncytium inducing
PAK	p21-activated kinase
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PHA-P	phytohemagglutinin P
pg	picogram
pmol	picomolar
RIPA	radio-immuno-precipitation assay
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
sec	second
Ser	serine
SI	syncytium inducing
STD	sexual transmitted disease

LIST OF ABBREVIATIONS (Cont.)

TAR	transactivation response
TBE	Tris-borate-EDTA
TCR	T cell receptor
Thr	threonine
TGN	trans-Golgi network
U	unit
μg	microgram
μl	microliter
USF-1	upstream stimulatory factor 1
UV	ultraviolet
WB	western blot
WT	wild type

CHAPTER I

INTRODUCTION

Human immunodeficiency virus (HIV) is the most significant emerging infectious pathogen of this century. Since recognition of the acquired immunodeficiency syndrome (AIDS) in 1981, HIV has produced a worldwide epidemic.

Based on *env* sequences, at least twelve subtypes (alternatively termed "clades" or "genotypes") of HIV-1 isolates are classified. These subtypes designated A through J, constitute the major group of HIV-1, group M. In addition, a ninth and tenth subtypes, I and J, has recently been described. Divergent or "out lying" strains of HIV-1 outside group M was reported and provisionally categorized as group O and group N. HIV-2 is phylogenetically classified into subtypes, although the number of sequenced isolates remain limited (1).

In Thailand, the first case of AIDS was reported in male homosexual who had a foreign partner in 1984; however, there was no evidence of substantial epidemic spread of HIV within the Thai population from this or other sporadic early cases through 1987 (2). However, In early 1988 a dramatic increase in HIV prevalence was documented among drug users. HIV seroprevalence among drug users seeking treatment rose from 0% in late 1987 to 32 to 43% in late 1988 (2, 3). This was the first clear epidemiologic evidence of extensive spread of HIV in Thailand. Subsequently, two major subtypes of HIV, B and E were found in infected individual (4). HIV-1

subtype B', which was similar to the predominant HIV-1 circulating in North America and Europe, was first spread rapidly among intravenous drug users (IDUs) (5). Until 1990, HIV-1 epidemic was observed among males who had sexual contact with female sex worker in Northern Thailand (6). The virus, later found to be subtype E, rapidly expanded throughout the country, becoming the predominant subtype in Thailand (7, 8). Approximately 98% of heterosexually acquired cases in Thailand are subtype E. Although HIV-1 subtype B was first found to be the common subtype among IDU group, recently infected cases among IDU group in Thailand was reported to be infected by HIV-1 subtype E for 75%.

Several groups reported cases or series of persons either presumptively or definitively exposed to HIV-1 in whom subsequent evidence of sustained HIV-1 infection did not occur. These highly exposed persistently seronegative (HEPS) derived from a variety of populations: female commercial sex workers (CSWs); sex partners of HIV-infected subjects (heterosexual and male homosexual HIV seronegative status-discordant couples); infants born to HIV-infected mothers; health care workers with needle stick exposure; and intravenous drug users who shared needle with infected persons (9). Each of these studies documented findings that suggested a relationship between certain host factors or immunologic responses and protective immunity in HIV-1; however, no uniform or consistent finding emerged.

HIV-1 is a highly pathogenic lentivirus that requires transcription of its provirus genome of the viral life cycle and the production of progeny virions. Since the first genetic analysis of HIV-1 in 1985, much has been learned about the transcriptional regulation of the HIV-1 genome in infected cells. It has been demonstrated that HIV-1 transcription depends on a varied and complex interaction of

host cell transcription factors with the *cis*–acting DNA sequences within U3 region of the long terminal repeat (LTR) promoter. The U3 region comprises the 5' part of the LTR in the proviral HIV DNA genome and overlap the *nef* gene in the HIV-1 RNA genome. U3 contains most of the important cis-acting elements, such as the NF-AT, TCF-1α, NF-*k*B, and SP1 sites. Viral or cellular proteins recognize these sites and thereby regulate several steps in the viral replication cycle. The R-U5 region contains elements such as the TAR hairpin and the poly(A) hairpin. Besides the *cis*-acting binding sites, other elements inside and just downstream of the LTR are important for integration and reverse transcription. Whereas the primer binding site (PBS), located just downstream of U5 in the untranslated leader sequence, is of importance for initiation of reverse transcription by binding of the tRNAlys primer. Thus, the truncations or even point mutations in functional sites of the LTR might be associated with low viral replication and attenuated pathogenesis in HIV-1 infected person (10).

Furthermore, the *nef* gene is considered to play a crucial role in the development of acquired immunodeficiency syndrome. The virus accessory gene nef encodes a multifuntional protein that modulates cell activation pathways, downregulates the CD4 receptor for the virus and major histocompatibility complex class I molecules, and the enhances virion infectivity. Many studies reported that HIV-1 infected individuals who are LTNPs appear to be infected with defective virus. One patient from central Massachusetts (11) and nine from Australia (12) are infected with Nef-deleted forms of HIV-1. Infection with this replication–competent, attenuated form of HIV-1 is clearly responsible for slow progression or nonprogression in these individuals. This is particularly evident for eight of the individuals in the Australia cohort, who were all infected by a single blood donor, who all have the Nef-defective

form of the virus, and who all have been slow progressors or nonprogressors (13, 14). Moreover, several pathogenesis and vaccine studies have been performed with derivatives of the molecular clone SIVmac239, which is pathogenic in adult rhesus macaques. Although Nef is dispensable for viral replication in cell cultures in vitro, Nef is important for the infection of high virus loads and progression to fatal simian AIDS (SAIDS). Adult macaques infected with a clone containing a large deletion in *nef* (SIVmac239Δnef) exhibited low virus loads and did not display clinical signs of disease for an observation period of 2 years. These findings indicated that *nef* was important for both high viemia and pathogenesis in juvenile and adult macaques and provided the basis for designing live attenuated SIV vaccines, which are based on viral clones with deletions in accessory genes rather than on viral clone with premature stop codons in accessory genes. However, more recent studies have demonstrated that a derivative of SIVmac239, also with a large deletion in *nef*, produced a fatal AIDS-like disease both in newborn macaques and, with low efficiency, in adult macaques. Although this latter observation raised concern about the safety of live attenuated primate lentivirus vaccines, the potential for viral genetic changes was not explored in animals displaying disease after infection with viral clones containing deletions in nef. Previous studies showed that primate immunodeficiency viruses containing large deletion in the *nef* gene or substitutions of cytokine genes in place of *nef* were attenuated for virulence in juvenile and adult macaques. Thus, such viruses could serve as live attenuated vaccines to prevent viral infection and AIDS (15).

CHAPTER II

OBJECTIVES

This study was conducted according to these following objectives:

1. To characterize HIV-1 isolated from partners of HEPS and HIV-1 infected concordant couples genetically by comparison of LTR and *nef* genes nucleotide sequences
2. To determine mutation of R5 gene in HEPS.
3. To determine biotype of HIV-1 subtype E isolated from partners of HEPS in comparison with HIV-1 infected concordant couples.
4. To determine neutralization activity of plasma from partner of HEPS, concordant, and pool positive plasma against primary isolates from partner of HEPS.

Information of HIV-1 subtype E from this study will provide a better understanding of the factors influence the lower transmission in Highly Exposed Persistently Seronegative (HEPS) group.

CHAPTER III

LITERATURE REVIEW

HIV-1 genome organization

The genome size of HIV-1 is about 9.8 kilobases, with ten open reading frames coding for several viral proteins (Figure 1). In common with other retroviruses, it contains two long terminal repeat (LTR) and three major genes: *gag* (group-specific antigen), *pol* (RT, protease and integrase) and *env* (envelope) genes. The genome also encodes three regulatory proteins: Rev (regulator of virion protein), Tat (transactivator of transcription) and Nef (negative regulatory factor). Three other accessory proteins that are probably involved in virus maturation and release are encoded: Vif (virion infectivity factor), Vpu (viral protein U), and Vpr (viral protein R) (Table 1).

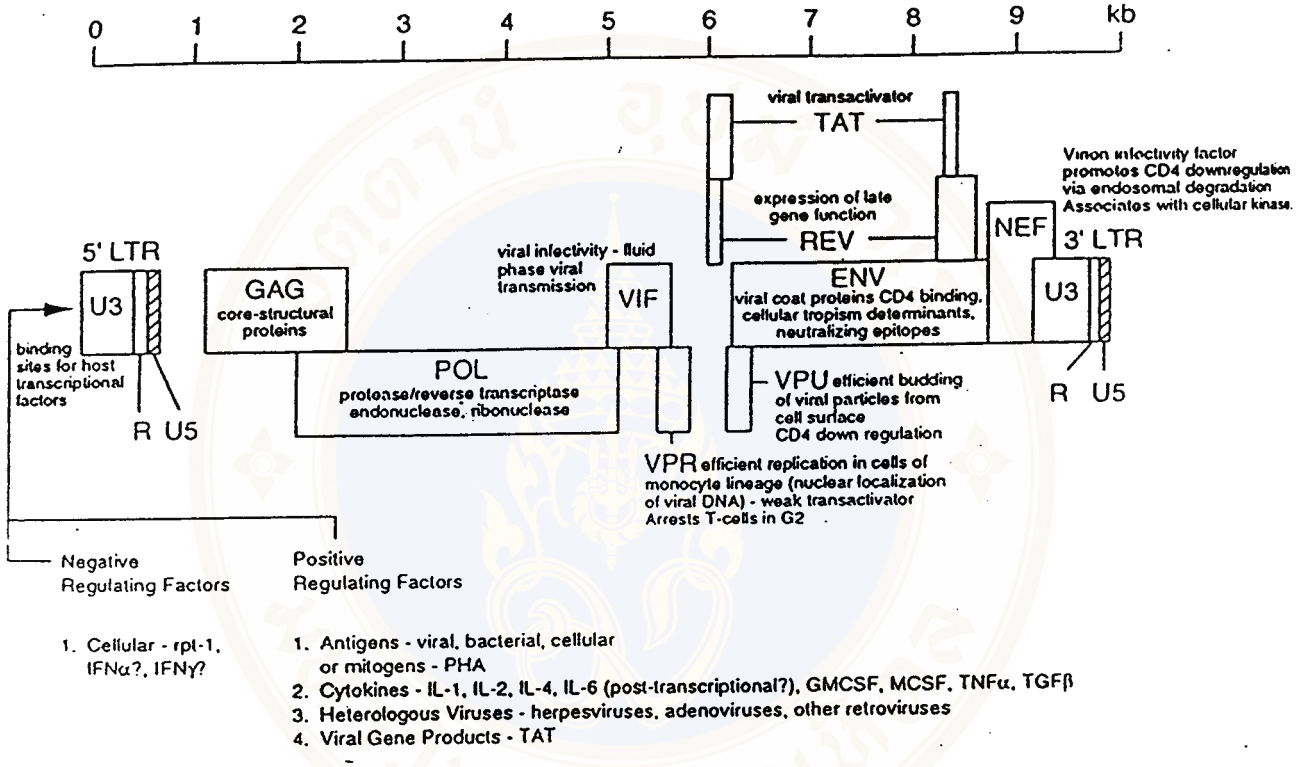

Figure 1. Genomic organization of HIV-1 and transcription. HIV-1 genome is flanked by long terminal repeats (LTRs). The proviral genes and the size and location of the open reading frames are shown. The genome contains the structural *env*, *pol* and *gag* genes and several accessory genes whose identity and function are outlined. (16)

Table 1. HIV protein and their function

Protein	Size (kDa)	Function	Additional features
Gag	p24	Capsid (CA) structural protein	Myristoylated, preintegration component
	p17	Matrix (MA) protein-myristoylated	facilitates nuclear localization of viral DNA
	p9	RNA binding and encapsidation	annealing of RNA lys, dimerization of RNA genome
	p6/7	viral maturation and detachment	required for Vpr encapsidation
Polymerase (Pol)	p66, p51	Reverse transcriptase (RT); RNase H-inside core	
Protease (PR)	p10	Post-translation processing of viral proteins	
Integrase (IN)	p32	Viral cDNA integration	
Envelope	gp120	Envelope surface (SU) protein receptor binding	CD4 downregulation, SI, cytopathic determinant
	gp41(gp36)	Envelope transmembrane (TM) protein	
Tat	p14	Transactivation	
Rev	p19	Regulation of viral mRNA splicing nuclear export, stability of viral mRNA	
Nef	p27	Pleiotropic, can increase or decrease viral replication	CD4 downregulation
Vif	p23	Increase viral infectivity and cell-to-cell transmission; helps in proviral DNA synthesis and/or in virion assembly	Env processing
Vpr	p15	Helps in virus replication; transactivation	facilitates nuclear localization of viral DNA
Vpu	p16	Helps in virus release; disrupts gp160-CD4 complexes	CD4 downmodulation
Tev	p26	Tat/Rev activities	

The *env* gene of HIV-1 is first encoded for the 88 kDa precursor on membrane-bound ribosome from a singly spliced mRNA. Then, N-linked glycosylation occurs at the site that contains Asn-X-Ser/Thr, at yield the glycosylated envelope precursor of 160kDa (gp160), which is further cleaved in the Golgi apparatus by a cellular protease to produce a 120 kDa surface virion envelope glycoprotein (gp120) and a noncovalently associated transmembrane glycoprotein (gp4). Both gp120 and gp41 are transported to the plasma membrane for incorporation into virion particles, while most of the uncleaved gp160 is delivered to lysosome and is degraded there. The gp120 is exposed to the extracellular environment and is responsible for tropism of the virus; it contains the determinants that interact with the host receptor $CD4^+$ as well as co-receptor and confers the ability of the virus to infect cells of monocyte/macrophage lineage. One of the features of the HIV-1 envelope-$CD4^+$ interaction is the capacity to induce fusion of membranes. This results in the production of multinucleated giant or syncytial cells. The gp41 contains complex sugar side chains and stretch of hydrophobic amino acids that serve to anchor the protein in the cell membrane and to catalyze fusion between the virus membrane and the membrane of the target cell (10).

The effector functions of Nef

nef gene was first identified as an open reading frame near the 3' end of the genome, overlapping a portion of the 3' long terminal repeat and the C-terminus of the envelope. Thus, the *nef* gene appears secondary to essential genomic segments that are universal to all retroviruses. After viral infection, Nef protein is expressed from the most abundant, multiply spliced mRNA, up to 80% of the total early viral transcripts,

and is found predominantly in the cytoplasm associated with the plasma membrane (17). However, the gene product is not necessary for viral replication, either *in vitro* or *in vivo*. Thus, understanding its function has not been straightforward. Because in vitro infection did not require Nef, experimentalists characterized the protein through Nef-mediated cellular phenotypes. These include surface cellular receptor modulation, physical association with cellular moieties, and altered cellular activation pathways. Nef also effects the efficiency of *in vitro* and *in vivo* viral infection.

Downregulation of cell surface CD4

The first clear activity to be assigned to Nef was down-regulation of cell surface CD4 expression. CD4 is the primary receptor for HIV-1, and downregulation would therefore be predicted to reduce the formation of complexes between CD4 and newly synthesized HIV-1 envelope protein on the infected cell surface. It would also facilitate the release of HIV-1 virions (18). The Nef protein is post-translationally modified by myristoylation of its amino terminus, and this modification targets Nef to the inner surface of the plasma membrane and to the trans-Golgi network (TGN). It is believed that Nef binds the cytoplasmic tail of CD4 directly, although this remains to be fully proven. Downregulation of cell surface CD4 is a specific event requiring a cluster of leucine and isoleucine residues in the CD4 cytoplasmic tail and results from the targeting of CD4 into clarthin coated pits (CCPs) followed by internalization and transport to lysosomes, where CD4 is degraded (19). Direct fusion of Nef to the extracellular and transmembrane domains of CD4 recapitulates the phenotype seen when Nef is expressed in *trans*, i.e. the CD4-Nef fusion protein is also internalized via

CCPs and degraded. This observation raised the possibility that Nef was acting as a connector between CD4 and the cellular endocytic machinery.

Internalization of cell surface receptors via CCPs requires the action of adaptor protein (AP) complexes that bind to both clathin and either directly to the receptor itself or indirectly, via a connector protein. AP complexes exist in two major varieties term, AP-1 is found in the TGN, and the other, AP-2, found at the plasma membrane. While both AP complexes are heterotetramers of similar size and structure, there are no subunits in common. Analysis of the ability of Nef to interact with the various subunits of AP-1 and AP-2 demonstrated that Nef can directly interact with both the μ1 component of AP-1 and the related (40% identity) μ2 component of AP-2 (20). As AP-2 is the adaptor present at the plasma membrane, and as internalization from the plasma membrane is the major mechanism for CD4 downregulation, it appears probable that CD4 internalization primarily results from the recruitment of CD4 to CCPs due to the direct interaction of Nef with both CD4 and the μ2 subunit of AP-2.

Downregulation of cell surface MHC class I

In addition to CD4, Nef also induces the specific downregulation of cell surface MHC I receptors, albeit with somewhat lower efficiency (20). MHC class I downregulation results from both the internalization of cell surface MHC class I receptors and from the sorting of MHC class I molecules from the TGN into AP-1 containing clathin coated vesicles. This downregulation requires specific sequences in the MHC class I cytoplasmic tail including a key tyrosine residue (20, 21). No leucine motif similar to that required for CD4 downregulation exists in MHC class I.

Because MHC class I is required to present viral peptide epitopes to cytotoxic T lymphocytes (CTL), downregulation of cell surface MHC class I could inhibit CTL-mediated lysis of HIV-1-infected cells. This was indeed demonstrated (22). But this result might suggest that MHC class I downregulation was the major contributor to the *in vivo* Nef phenotype. This may not be the case as a clear positive effect of Nef on viral replication *in vivo* seen in SIV-infected macaques by two weeks postinfection, i.e., before an effective CTL response can be mounted by the infected animal. The fact that a readily detectable anti-HIV-1 CTL response was observed in most infected individuals (23) demonstrated that the Nef-mediated inhibition of antigen presentation via MHC class I is clearly incomplete.

Analysis of Nef protein demonstrated that CD4 and MHC class I downregulation can be, at least in part, mutationally segregated (20). However, as the cytoplasmic tails of CD4 and MHC class I display no sequence homology, this segregation could simply reflect the selective disruption of the MHC class I and CD4 targeting functions of Nef. The simplest interpretation of the available data was that Nef connects both CD4 and MHC class I to the intracellular protein sorting machinery by binding to CD4 or MHC class I on the one hand and either AP-1 or AP-2 on the other. Regardless of whether AP-1 or AP-2 is recruited, which may largely depend on whether recruitment occurs at the TGN or at the plasma membrane, these target proteins are then sorted into clathin coated vesicles that deliver them for degradation in lysosomes.

Enhanced virion infectivity

In addition to a possible enhancement in virion infectivity due to CD4 downregulation, Nef also entirely enhances virion infectivity by the CD4 independent mechanism (24). This enhancement is conferred by Nef during the process of virion assembly and cannot be complemented by expression of Nef in target cells. While the effect of Nef on virion infectivity is quite modest in most culture settings, this effect may increase to 10-fold or more when primary T cells are infected with HIV-1 while quiescent and then subsequently activated or if certain highly susceptible target cells, such as $CD4^+$-Hela cells, are used.

While HIV-1 virions produced in the presence and absence of Nef did not differ in terms of cell-free reverse transcriptase activity or in terms of their ability to bind to and enter target cells, they were less able to complete proviral DNA synthesis. While Nef did not cause any major change in the morphology or composition of virions, two differences were reported. First, Nef itself was packaged into virions at low efficiency ($\leq$ 10 molecules per virion) and even underwent specific processing by the HIV-1 protease. While this finding may be important, it could also simply reflect a low level of nonspecific packaging of the membrane-associated Nef protein into virions during viral budding from the cell membrane. It was reported that serine phosphorylation of the matrix component of HIV-1 Gag was modified in Nef-expressing cells. Nef was reported to associate specifically with a serine kinase related to p21-activated kinase (PAK), and it was therefore possible that Nef may recruit a PAK-like kinase to sites of virion assembly (25). While a Nef-induced change in the phosphorylation state of matrix could certainly affect virion infectivity, this hypothesis remains to be proven.

Effects on cellular signal transduction and activation

While there were number of reports documenting the effects of Nef on signal transduction pathways and on the activation state of both lymphoid and nonlymphoid cells, no clear consensus existed as to cellular targets or as to whether the effect of Nef was positive or negative. Nef-induced degradation of CD4 results in the release of the normally CD4-bound tyrosine kinase lck, and this could have a marked effect on signaling via the TCR/CD3 complex. In addition, Nef recruits to the plasma membrane a serine/threonine kinase related to PAK and also contains an SH3-binding motif that could interact with membrane-bound tyrosine kinases or other signaling molecules. By juxtaposing enzymes and proteins that are not normally in contact, Nef may either inadvertently or intentionally modulate the activation state of the cell (26).

Long Terminal Repeat (LTR)

LTR sites are at the 5' and 3' ends of the HIV proviral genome integrated into the host cell chromosome, each with identical viral sequences of DNA (Figure 2). The HIV LTR can be divided into the three subregions U3, R and U5, which play role in viral integration and transcription. The 5' LTR contains *cis*-acting DNA target sequences which conduct a variety of cellular transcription factors. A series of *cis*-acting core elements that consist of three SP1 binding sites, a TATA element, and upstream promoter. Several DNA elements required for stimulation of transcription in activated lymphocytes present in this region are two binding sites for nuclear factor, NF-*k*B and NF-AT, and the upstream stimulatory factor-1 (USF-1). Consensus sites for leader binding protein-1 (LBP-1) and CCAAT-binding transcription factor (CTF)/ nuclear factor (NF)-1 are also present 3' to the TATA box (Figure 2). The variety of

binding sites in the HIV-1 LTR for constitutive and inducible transcription factors is sufficient to stimulate low levels of transcription. However, the HIV LTR also contains a unique region named the transactivation response (TAR) for Tat protein, which is a potent transactivator of transcription and is required for virus infectivity. The integrated provirus contains a complete LTR at the 3'end of the genome. The 3' LTR function as a transcriptional terminator and contains the appropriate signals for the polyadenylation of viral mRNA (27)

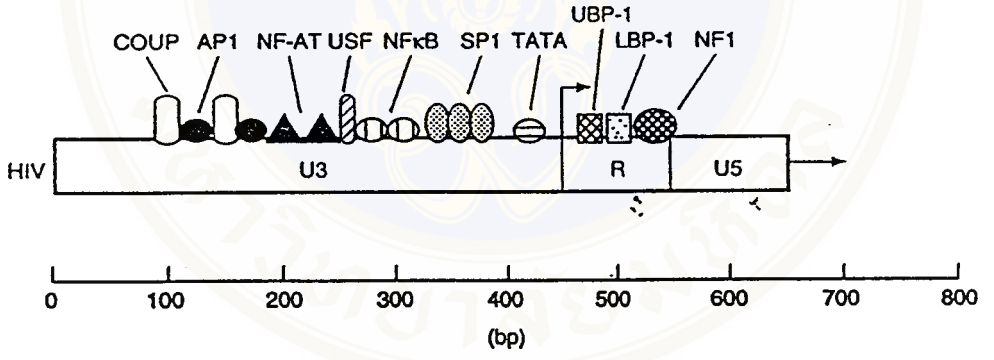

Figure 2. *cis*-acting sequences of the HIV-1 LTR. HIV-1 LTR contains a myriad of transcriptional promoter elements, some of which are presented: activator protein (AP)-1 binding sites, the nuclear factor (NF)-AT binding site, the upstream stimulatory factor (USF)-1 binding site, and nuclear factor (NF)-*k*B, SP1 and TATA binding site. (27)

The HIV-1 Replication Cycle

General features of the HIV replication cycle are shown in Figure 3. The early phase 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.

The host cell receptor is the CD4 molecule, which is a glycoprotein with four extracellular immunoglobulin-like domains that CD4 lymphocytes and some macrophages express on their surfaces. CD4 alone is not sufficient to permit HIV-1 entry into cells. Other human-specific accessory factors, X4 and R5, have been identified that collaborate with CD4 molecules. X4, the co-receptor for T-tropic HIV-1 strains, is a member of the seven–transmembrane G-protein-coupled receptor family. R5, a second co-receptor for macrophage-tropic HIV-1 strains, is a receptor for the β-chemokines. This leads to fusion of viral and cell membranes so that viral nucleoprotein complexes enter the target cell cytoplasm. Within these complexes, reverse transcriptase directs the synthesis of a DNA copy of the viral RNA genome. The viral DNA containing complexes then migrates to the nucleus, where the integrase protein directs integration of viral DNA into the cell's chromosomal DNA to form a provirus.

Expression of the integrated provirus produces spliced and unspliced viral mRNA transcripts that encode the regulatory and structural viral proteins. The precursor *gag* and *gag-pol* viral polyprotein precursors along with genomic-length viral RNA are assembled into new virus particles at the cell surface. As HIV-1

particles bud through the cell membrane, they acquire a lipid bilayer that contains the envelope proteins. During or shortly after budding, the viral protease cleaves *gag* and *gag-pol* precursor polyproteins to the mature individual proteins, which generates infectious virus (10).

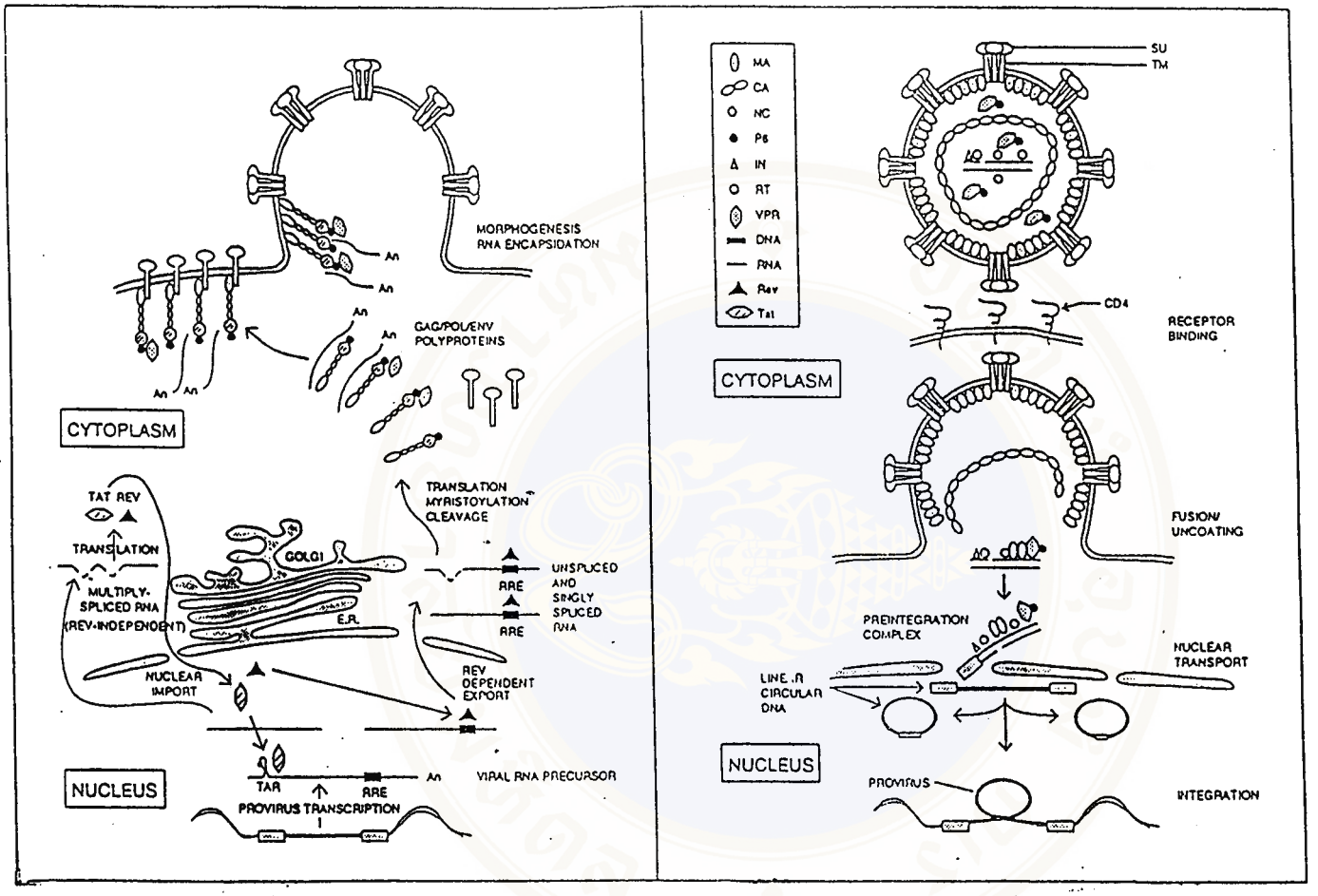

Figure 3. General feature of the HIV-1 replication cycle. The early phase begins with CD4 recognition and involves events up to and including integration of the proviral DNA are indicated on the right-hand side of the figure; and the late phase includes all events from transcription of the integrated DNA to virus budding and maturation is presented on the left-hand side of the figure. (28)

Features of HIV Transmission

The transmission frequency of a virus is influenced by the amount of infectious virus in a body fluid and the contact time of each individual with that body fluid. Established infection depends on three points of a classic epidemiologic: characteristics of the infectious agent (e.g., virulence and infectiousness), host-related factors (e.g., susceptibility, contagiousness, and immune response), and environment factors (e.g., social, culture, and political) (29). The routes of the transmission are blood and blood product, sexual contact, and mother to child. The virus load observed during acute HIV infection or the symptomatic period might be related to present the greatest risk of HIV transmission.

HIV in Blood

The several studies indicated that both free infectious virus and infected cells were present in blood. And HIV-1 infected cells appeared to be more numerous than the infectious virus. In blood as well as other body fluids, noninfectious viruses and cells containing defective viral genomes detected by polymerase chain reaction (PCR) can be present in quantities but do not present sources of transmission.

Infectious virus

The blood samples contain circulating infectious virus, whether or not the HIV-infected individual was asymptomatic or had AIDS (30-34). The quantity can reach 100 to 1,000 infectious particles (IP) per ml of blood. Importantly, the level was very low in healthy subjects and often undetectable in long-term survivors. Infectious HIV was readily found during acute (primary) infection, but within weeks the level of

free virus detected in the blood was markedly reduced (30-33, 35-37). The reason for this decrease in viremia was most probably due to an active antiviral cellular immune response. Then, as the disease develops with its characteristic loss of $CD4^+$ cells, the concentration of infectious HIV in blood rises substantially, reflecting the increased virus load in peripheral blood mononuclear cells (PBMC) and lymphoid tissues (30-33). Thus, the risk of transmission of infectious HIV would seem to be highest in the early stage of infection and during the symptomatic periods.

Virus-infected cells

In many cases, levels of free virus, even following antiviral therapy, did not consistently show a correlation to virus numbers in PBMC (38) or in the lymph node (39, 40). The total number of HIV-infected cells was estimated to be several hundred billion (41), a much greater number than those of the free virions were found in blood. Moreover, studies indicated that one infected cell can produce 200 to 1,000 particles per day (42-45). Thus, the total number of infected cells responsible for the high viral RNA level in the blood could represent only a small fraction (10 to 20 million) of cells to be infected (41, 44). Several studies indicated that virus-infected cells are more common in blood than are infectious virions. As with the free virus, the number of infected cells was increased with symptomatic disease and reduced $CD4^+$ cell counts. Most of the infected cells (50 to 90%) contained HIV latent state (46).

The majority of cells showing HIV infection detected by molecular techniques were $CD4^+$ lymphocytes (46-48). Not many circulating macrophages were found to be infected. When the virus is latent, the factor that activates virus production in infected cells is important in HIV pathogenesis. Close to 50% of the virus-infected

cells in lymphoid tissue from some infected individuals appeared to contain infectious virus (49). For the transmission and spread of the virus in the host, the virus-infected cells became more important than the infectious viral load in the blood (Table 2). During symptomatic infection, especially AIDS, the number of infectious viruses and infected cells was usually much larger (e.g., 1,000 IP/ml; up to 1:10 $CD4^+$ cells/ml) (48, 50-52). Thus the chance of transmitting infection, particularly by cells containing HIV, could be even greater (44). Furthermore, even if a latently infected cell was transferred, it could serve as a source of HIV transmission after its activation in the new host (53).

Table 2. HIV pathogenesis: Importance of the virus-infected cell with respect to free virus (54)

1. Present at higher levels in the body (>200 billion cells) than free virus (1-10 billion particles)
2. Reservoir for persistent virus production (1,000 particles/day)
3. Transfer HIV to new cells more effectively than infection by free virus
4. Can induce apoptosis by cell-to-cell contact
5. Release viral products that are toxic to the host (e.g., g120, gp41, and Tat)
6. Release toxic cellular products (e.g., tumor necrosis factor α and interleukin-6)
7. Present at higher levels (5-50 times) than free virus in genital fluids and blood
8. Remains viable in genital fluids and blood longer than free virus

HIV in genital fluids

In the case of sexual transmission, the amount of virus in genital fluids is important. Generally, seminal and vaginal fluids showed the presence of free infectious virus and/or virus-infected cells in 10 to 30% of specimens. Measurements of viral RNA levels shown a greater frequency of free virus in these fluids, but the risk of transmission would depend on the number of infectious virions (55).

HIV in seminal fluid

The largest amount of infectious virus in genital fluids might be expected in the acute phase of infection and during the symptomatic periods. Transmission was found to be increased when the male partners with disease engaged in heterosexual or homosexual activity (56). In some reports, recovery of the infectious virus from semen was highest from men with low $CD4^+$ cell counts (57). However, only a small number of subjects were studied, and the virus was recovered primarily from infected cells.

Most studies suggested that the presence of infectious virus in seminal fluid does not correlate with clinical state (58, 59). Both syncytium-inducing (SI) and non-syncytium-inducing (NSI) strains could be isolated from semen (60). The quantity of HIV in semen also did not necessarily reflect the level of plasma viremia. In addition, the virus found in semen did not always show the same biologic phenotype as that in the blood (NSI versus SI) (61). Direct infection (62) or local inflammation in these various body compartments appeared to be the important factor in determining the level of virus produced. Finally, in limited studies, the virus was detected in semen by cell culture or PCR techniques despite antiviral therapy (57, 59, 63). These findings probably reflected the inability of some drugs to penetrate the blood-testes barrier.

The prevalence of virus-infected cells appeared to be an important variable in genital fluids (64). In seminal fluid, this number could range from 0.01% to 5%. Semen usually had over 1 million leukocytes/ejaculate (65, 66), but levels and subsets of cells could vary widely from day to day in the same individual. Nevertheless, HIV-infected cells ($>10^4$ in some cases) seemed to be the grater source of transmission than

free infectious virus. T cells appeared to be most commonly infected followed by macrophages.

The cellular source of HIV in seminal fluid was not well defined. By culture and *in situ* PCR hybridization procedures, HIV was detected in large quantities in the testes of infected individuals. It was found in urethral cells (67), spermatogonia, spermatocytes, and occasionally in spermatids, but not in Sertoli cells. Since only the head enters the egg during fertilization, a relevance of this finding to possible germ line transmission of HIV is unlikely. Nevertheless, a glycolipid resembling galactosyl ceramide on the middle portion of the sperm tail (68) could serve as an attachment site for HIV, as it did not some brain and bowel cells.

HIV was isolated from semen from vasectomized men (69), and after vasectomy, many lymphocytes could be found in the ejaculate. Infectious virus was recovered from preejaculatory fluid from infected men (70, 71). Thus, the virus and virus-infected cells must come from the urethra, prostate, and other secretory glands, as well as the testes. HIV was not detected in the epithelia of the prostate, epididymus, seminal vesicles, or penis of men with AIDS by *in situ* PCR hybridization procedures.

HIV in vaginal fluid

The vagina canal was found to contain the free-infectious virus only rarely. Generally, infected cells were detected, but the quantity was not reported. Even by PCR analysis, HIV was found in cervicovaginal secretions of only 28% of infected women. As with seminal fluid plasma viremia did not correlate with virus shedding. The prevalence of the virus was found to be significantly higher in pregnant women

than in nonpregnant women (72). It was also higher in women with cervico ectopy, abnormal vaginal discharge, or serve vitamin A deficiency (73) and in those taking oral contraceptive pills. One report of recently infected Kenya women revealed viruses in cervical secretions that differed genotypically from those found in the peripheral blood (42). This recognition of tissue-specific variants showed a different distribution (or evolution) of viruses can be found in the blood and genital tissues.

The source of HIV in vaginal fluid is not known but is most probably the secretory glands in the vagina or cervix, leukocytes in the uterine cavity, and, in some cases, menstrual blood. Using *in situ* PCR hybridization procedures, HIV was detected in the cervix, it was found primarily in monocytes and macrophages and in the glandular epithelium in the zone of transformation between columnar and squamous cells. The cervix is a more frequent source of virus than the vagina.

In these studies, detection of infectious virus was probably limited, since culturing of HIV could be technically difficult. These body fluids can be cytotoxic in culture, and survival of the virus can depend on the pH and potential antiviral factors present.

Mucosal Immunity and AIDS

The critical pathogenic event in AIDS is a marked decrease in $CD4^+$ T-helper cell (74). $CD4^+$ T cells have a pivotal role in generating virtually all immune responses (75) and depletion of these cells produces a profound immunodeficiency syndrome that leaves the infected individual vulnerable to opportunistic infectious pathogens. IgM anti-HIV antibodies occur within 2 weeks of infection and reach peak titers by 5

weeks (76, 77). IgG anti-HIV antibodies appear later and reach sustained levels when IgM responses have waned (76-78). HIV infection induces a strong antibody and cellular immune response to external envelope (gp120), transmembrane protein (gp41) major core protein (p24), and other proteins in most infected people. However, this vigorous response does not eliminate the virus or prevent the ultimate progression of HIV disease (79, 80).

The mucosal immune system differs in several fundamental ways from its counterpart, the systemic lymphoid system. These unique attributes contribute to the efficient protection of the > 400 m^2 of mucosal exposed to and at risk from the environment. First, the major antibody isotype in mucosal secretion is immunoglobulin A (IgA), which is normally dimeric IgA. In contrast, IgG is the most common immunoglobulin isotype in the systemic immune system. Second, most of the antibody producing plasma cells in the body occur in the mucosa-associated lymphoid tissues (MALT), not in systemic lymphoid tissue. The concept of a common mucosal immune system explains the observation that an immune response originating at one mucosal surface results in a strong immune response at distant mucosal surfaces, and this response is not necessarily coupled with a strong systemic humoral immune response (81).

Mucosal immune system of the female reproductive tract

The female reproductive tract has the capacity to mount an immune response to environmental stimuli. Immunoglobulins are present in the cervico-vaginal secretions of a number of species. The immune system in the female reproductive tract is of central importance for protection against spread of sexually transmitted diseases,

including AIDS. However, it is the least well understood arm of the immune system in the terms of origin of immune cells, the role of local CTL responses, and the induction of local antibody responses, including the contribution of serum-derived versus locally produced antibodies (82).

The mucosa of the vagina consists of a nonkeratinized, stratified squamous epithelium and an underlying vascular submucosa. The ectocervix has a similar architecture, whereas the endocervix consists of a simple columnar epithelium covering a vascular submucosa. M cells have not been described in the vagina or cervix, but Langerhans cells and macrophages are present in the vaginal mucosa. Langerhans cells are MHC class II, $CD4^+$ dendritic cells located within squamous epithelia throughout the body. Langerhans cells are common in the skin where they can bind antigen and migrate to the draining lymph node. Langerhans cells transform into interdigitating dendritic cells of the T cell rich paracortex (83-86).

The mucosal immune system in the genital tract of the female rhesus macaque consists of a resident population of monocyte/macrophages and T cells in the submucosa of the vagina and cervix (87). These cells are specifically localized in the superficial submucosa, just beneath the vaginal epithelium. A similar population of lymphocytes and macrophages are in the submucosa of the human cervix where they have been called MALT (88). Langerhans cells are abundant in the vaginal and ectocervical mucosa of women (88-92).

By analog to a primary immune response in the gut-associated lymphoreticular tissues (GALT), antigen that reaches the submucosa of the vagina is taken up by antigen presenting cells, which then migrate to draining lymph nodes. Once in the lymph node, the antigen presenting cells stimulate B and T lymphocytes,

including memory subpopulations that enter the bloodstream via the efferent lymph and thoracic duct. The T and B lymphocytes (including memory cells) migrate to the genital tract. Upon exposure to the antigen, these cells participate in a secondary immune response. In the vagina, both Langerhans cells and mononuclear phagocytes are capable of acting as APCs and in initiating an immune response.

Several studies shed light on the cellular origin of Ig isotypes present in reproductive tract secretions of women. The lamina propria of the endo- and ectocervix contain the highest numbers of Ig-producing plasma cells, with significant numbers also present in the fallopian tube and in the vagina (93, 94). The major isotype present in these tissues was IgA, with small numbers of IgM^+ and IgG^+ plasma cells. Furthermore, most of the IgA^+ plasma cells were also shown to contain J chain and this clearly suggested that the IgA produced in this region is largely polymeric (93-95). Interestingly, an approximately equal distribution of IgA1 and IgA2 plasma cells were noted, and this pattern is most similar to that seen in the lower region of GI tract. Women with a variety of sexually transmitted diseases have increased numbers of plasma cells (especially IgA plasma cells) in the submucosa of the endocervix (96). Finally, the epithelial cells that line the above mentioned portions of the female reproductive tract produce secretory component, which is required for transport of polymeric IgA1 and polymeric IgA2 into the reproductive tract secretions.

In summary, these studies clearly indicated that certain regions, e.g., the fallopian tubes, ecto- and endocervix and perhaps the vagina, exhibit characteristics of mucosal effector sites.

Heterosexual transmission of HIV

Heterosexual contact is by far the most common method of HIV transmission. However, epidemiologic data indicated that HIV is not efficiently transmitted by sexual contact. In fact many other sexually transmitted diseases (STDs) are more efficiently transmitted. In contrast, less than 15% of those monogamous individuals repeatedly exposed to an infected sexual partner become infected with HIV (97). It is estimated that for a single sexual contact, the infectivity of HIV is 0.3% (98, 99). However, some individuals become infected after a single or a few sexual contacts (99, 100). Whereas others remain uninfected despite hundred of contacts. HIV is apparently more efficiently transmitted from men to women than from women to men two-to-fivefold (101). Several cofactors were identified which increase the risk of an individual acquiring HIV through heterosexual contact. Cervical ectopy, receptive and intercourse, genital ulcer disease and infection with other STDs are the most significant factors associated with HIV infection of women (102, 103). Whereas the presence of an intact foreskin and genital ulcer disease of the penis are the risk factors most often associated with HIV infection in men (104, 105).

Three possible explanations exist to explain the variability in the sexual transmission of HIV. First, unique factors in the HIV-infected individual (stage of disease, immune response, presence of other STDs) may influence infectivity. Some HIV-positive persons are highly infectious and others are not (100). The degree to which an individual is infectious may be related to the stage of disease. During primary HIV infection, the levels of antigen and virus in plasma are high (106, 107) and this initial viremia may be associated with high levels of HIV in genital secretions. HIV is more common in the semen of healthy chronically infected individuals (108).

Second, unique factors in specific HIV strains may be inherently more likely than other strains to be shed in the secretions of infected persons or some strain may, by virtue of their cellular tropism, have an increased affinity for target cells in the reproductive tract. Third, unique factors (stage of menstrual cycle, nutritional status or the presence or absence of the risk factors) may influence the susceptibility of individuals to HIV infection after exposure. Some people may be highly susceptible to HIV infection, while others may be more resistant.

Both cell-associated and cell-free viruses are present in genital secretions and, it is not clear if one or both forms of virus are involved in sexual transmission. Cell free and cell associated HIV and SIV could be isolated from cervico-vaginal secretions at any stage of menstrual cycle (109-112). HIV was present in semen in both cell free and cell-associated form and could be isolated from asymptomatic individuals and AIDS patients (108, 113). HIV genome in semen was widely divergent. In one study HIV RNA and DNA was detected in the spermatozoal mononuclear cells in 74% (17/23) and in the seminal fluid of 65% (15/23) of the semen samples tested (114).

The fate of HIV infected cells in the vagina is unknown. It is unlikely that infected cells can move across an intact genital mucosa, but after lysis of the seminal monocytes, virions would be released and could bind to the target cells in the genital mucosa. Recently completed SIV transmission studies in monkeys suggested that cell-associated HIV may contribute significantly to the sexual transmission of the virus in humans. Intravenous inoculation of 100 SIV-infected rhesus peripheral blood mononuclear cells infected 2 of 2 animals, but none of 6 mature female rhesus macaques intravaginally inoculated with 10^4 or 10^3 infected allogenic peripheral blood

mononuclear cells became infected. The difficulty in transmitting cell-associated SIV may be related to the low pH (3.5 to 6.0) in the vagina (115).

In an ejaculation, lymphocytes may be able to survive somewhat longer than in the vagina. The pH of seminal plasma ranges from 7 to 8, and lymphocytes and cell-free virus could survive for extended periods in these conditions. After receiving an ejaculated semen, the pH of the vagina rose to 7.0 or higher (116). This rise in pH was presumably due to the buffering effect of seminal plasma and explains the observation that 90% of autologous lymphocytes, suspended in seminal plasma are alive 30 minutes after infusion in to a woman's vagina. These findings suggested that cells and virions infused into the vagina are much more stable if they are in seminal plasma. The lifespan of foreign lymphocytes in the vagina is still limited due to the presence of bacteria, protease, lysozyme and the fact that the cells are washed from the vagina by the action of vagina secretion. Therefore, it seems likely that only cells producing infectious HIV particle at the time of inoculation can contribute to sexual transmission. It seems unlikely that latently infected cells in the ejaculate can survive long enough in the vagina to produce infectious virions from activated integrated genome. However, infected cells may be capable of causing transmission if they gain access to other sites in the reproductive tract.

Since only a few $CD4^+$ T cells were present in the submucosa of the vagina (87), the most likely target cells in the vagina mucosa were macrophages or Langerhans cells (87, 117). In chronically infected female rhesus macaques, SIV-infected cells were present in the uterus, cervix, and vagina (117). The majorities of the SIV-infected cells were located in the submucosa of the ectocervix and vagina, and had morphology consistent with T lymphocytes and monocytes/macrophages. SIV-

infected cells were also found within the stratified squamous epithelium of the vagina. Some of the infected cells in this location had a dendritic morphology consistent with Langerhans cells (117).

In cervical biopsy, T cells and macrophages were determined to be the cell types infected with HIV (118). All the cells that were found to be infected in the genital tract of both human and rhesus presumably express the CD4 molecule. There was no evidence that non-$CD4^+$ cell types (epithelial cells) were infected in the reproductive tracts of either the monkeys or humans. These finding suggested that Langerhans cells may have a role as target cells in the sexual transmission of HIV or SIV. These antigen-presenting cells were potentially efficient disseminators of these viruses from the genital mucosa to draining lympnode (83-86, 119, 120). Blood dendritic cells (of which Langerhans cell precursors are a subset) could be infected with HIV (121-124). Furthermore, when infected *in vitro* the cells produced much higher levels of virus than T cells, but they did not exhibit the usual cytopathic effects associated with HIV infection (124). Thus, Langerhans cells in the vagina and ectocervix (88-92) may be especially well suited as target cells for the sexual transmission of HIV and SIV (117, 125).

Hypothetical routes of virus dissemination during the genital transmission (Figure 4) of HIV virus contact the genital mucosa and infects a target cell, presumably a macrophage or Langerhans cell. The infected target cell moves through lymphatic vessels to the draining lymph node. Both macrophage and Langerhans cells are $CD4^+$, MHC class II^+ antigen presenting cells. Normally these cells enter the $CD4^+$ T cell rich paracortex of the lymph node and present processed antigen to the $CD4^+$ cells, initiating an immune response. The infected antigen presenting cells pass the

virus to the $CD4^+$ T cell. The virus replicates in the lymph node and both cell-free and cell-associated viruses leave the lymph node via the efferent lymph node and migrate to proximal lymph nodes. They eventually pass through the thoracic duct into the blood stream. After entering the bloodstream, the virus homes to and infects systemic lymphoid tissue (spleen, thymus, bone marrow, and lymph nodes). Infection of these tissues results in systemic infection.

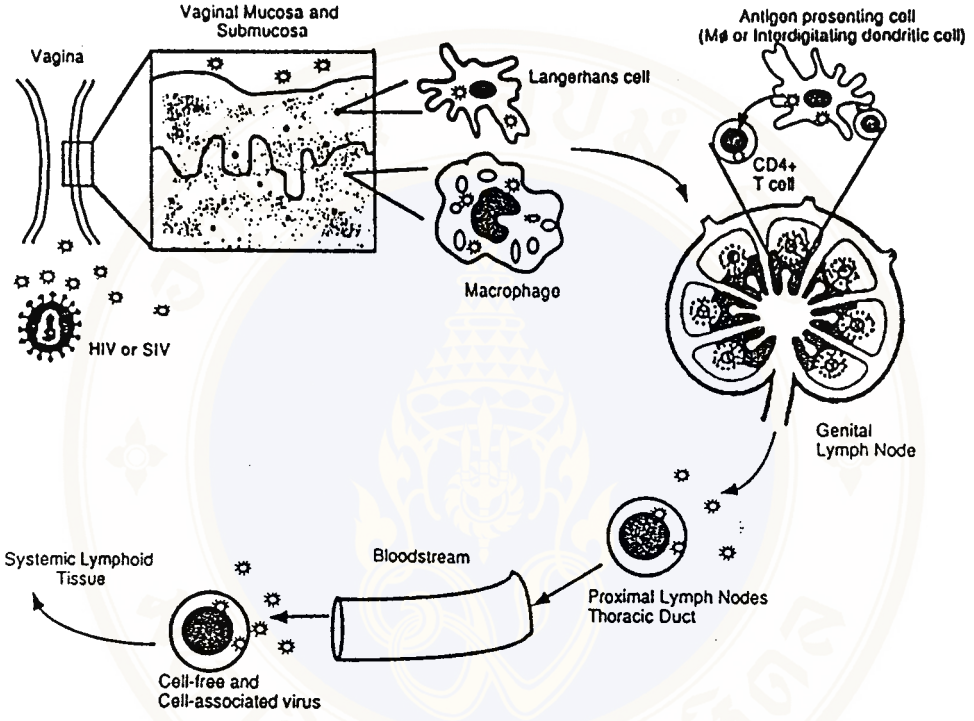

Figure 4. Hypothetical route of virus dissemination during the genital transmission of HIV (116).

There is some speculation that trauma to tissues during intercourse might be necessary for heterosexual transmission of HIV, and that virus can gain direct access to the bloodstream through these wounds. The hemodynamic pressures in the peripheral vasculature do not permit the movement of particles (cells or viruses) from tissues directly into blood vessels. This is the same pathway that vaginal macrophages and Langerhans cells would take after infection with HIV (125). It is reasonable to assume that trauma would increase the amount of virus that crosses the mucosa and that the hemorrhage associated with the trauma would increase the number of $CD4^+$ target cells in the submucosa of the vagina. The increased number of virions and target cells might increase the efficiency of HIV transmission, but probably would not affect the route of virus dissemination from the genital tract to systemic lymphoid tissue (116).

Heterogeneity in exposed uninfected individuals

In the last few years, documentary evidence produced by a number of research groups showed the existence of subjects who, despite multiple exposures to HIV-1, remain seronegative for HIV-specific antibodies and apparently uninfected (HEPS: Highly Exposed Persistently Seronegative). Until now, it has been unknown as or whether these individuals: 1) are simply fortunate to have encountered insufficient virus to establish infection (or defective virus particles) and have been inadvertently "vaccinated" by such exposure, or 2) have successfully cleared the virus after having been infected, developing a still undetermined state of protection, or 3) are indeed infected, but have established with the virus an unconventional equilibrium

that makes them unsusceptible to a regular course of infection in spite of repeated exposures.

Several studies documented a range of HIV-specific cellular and humoral immune responses in seronegative people with a history of HIV exposure (126, 127). Moreover, there were some evidences of the existences of genetic factors that influences HIV-1 infection (128, 129)

implication of these findings was that exposure to HIV could lead to the generation of specific cell-mediated immunity in the absence of antibody production. It remains unclear whether exposure to viral antigens is sufficient to prime the CD4-positive cell responses, or whether these responses are simply markers of exposure, or rather of prolonged protective immunity.

HIV-specific cytotoxic T lymphocytes (CTLs) in the blood of exposed, seronegative individuals was reported by several groups (138-142). Most of these CTLs were $CD8^+$ and MHC class I-restricted (139, 140, 143, 144). In some cases, these responses were specific for regulatory protein antigens, and were demonstrated in different cohorts of seronegative exposed individuals, including participants in unprotected sexual (138, 140, 143), newborns of HIV^+ mothers (139, 141) and healthy care workers (144). These results were suggestive that at least one round of viral replication took place in the HEPS subjects, to prime them to antigens that are absent from virions. Rowland-Jones and colleagues reported the presence of CTLs recognizing epitopes that are cross-reactive between HIV-1 and HIV-2 in a group of repeatedly HIV-exposed but uninfected prostitutes (143). Furci *et al* (145) reported the evidence of an oligonal T cell response mediated by helper T cells specific for C5, a conserved region of the HIV-1 envelope. Clones derived from these cells, following antigen stimulation, produced very high levels of C-C chemokines, the natural ligands of the major coreceptor of macrophage-tropic strains of HIV. The chemokine-producing helper cells suppressed selectively the replication of M-tropic, non-syncytia inducing strains of HIV-1.

Humoral immunity

Reportedly, antibodies to HIV could be elicited in at least some exposed uninfected individuals by *in vitro* stimulation of B-lymphocytes in both adult individuals (146, 147) and in children born to infected mothers. A study of an American cohort of seronegative intravenous drug users (IDU) demonstrated the presence of antibodies cross-reactive between HLA class I and gp 120 in 33% of individuals tested (135). The molecular mimicry was mapped to the C5 region of gp 120 by monoclonal antibody M38 (148). Most anti-HLA Ab-positive IDU also exhibited T cells specific for enveloped peptides. This suggested that the presence of anti-HLA Abs was directly linked to exposure to envelope antigens (135). Vaccination of healthy volunteers with recombinant envelope also reportedly induced anti-HLA class I antibodies (149).

In cohort of Italian couples with discordant HIV status, Lopalco *et al* (150) identified the same type of antibodies in 16 out of 27 seronegative partners using a cytofluorimetric competition assay that measures serum inhibition of Mab M38 binding to cell surface HLA class I. The specificity of the anti-HLA antibodies present in sera from HEPS was further analyzed by two different biochemical methods: RIPA (radio-immuno-precipitation assay) and IEF (iso-electro-focusing). Furthermore, the presence of HIV neutralizing activity was observed in 5 out of 27 sera of the same subjects. In 4 of the 5 neutralizing sera showed that the HIV-suppressive activity is associated uniquely with the purified Ig fraction.

Parallel studies on the same cohort demonstrated the presence of anti-CD4 antibodies in 6 out of 18 individuals (151) and a high frequency of CD4-positve T cells specific for a peptide derived from gp120 C5 region (145). In some HEPS, these

unconventional immune responses were persistent, lasting up to two years after the last possible exposure. These subjects may be constantly exposed to antigenic stimuli derived from a latent form of infection because or antibody response to HIV is highly correlated with persistent infection (127).

Lopalco *et al* (150) studied the possible correlation between the different parameters analyzed (anti-cell Abs, neutralizing Abs and T cell clones specific for gp 120). They proved to be independent from each other, even if almost all HEPS were positive in at least one assay. The hypothesis of the heterogeneity of these immune responses was the exposure to HIV may induce neutralizing antibodies that are not directed against viral antigens (given the seronegativity of HEPS), but may be directed against cell surface antigens involved in the infection/entry process.

HIV specific mucosal and cellular immunity was analyzed in another cohort of heterosexual couples discordant for HIV serostatus and characterized by having persisted in their unprotected sex habits. Mazzoli et al (152) detected HIV-specific IgA in urine and vaginal washes of EU individuals, and their PBMC produced IL-2 following stimulation by env-peptides. Moreover, Belec et al (153) observed the cervicovaginal IgA and IgG anti-gp 160 antibodies in cervicovaginal secretions from twelve HIV-discordant heterosexual couples, matched with twelve HIV-concordant heterosexual couples at similar stage of HIV disease. The mean reciprocal end-point titers of cervicovaginal IgA or IgG to gp 160 were similar in cases and in controls. In comparison with HIV-concordant couples these observations suggested that cervicovaginal antibodies to HIV do not appear as biological indicators sufficiently relevant to explain a possible reduced infectivity of the female index case in HIV-discordant couples.

Genetic resistance to HIV

The MHC class I and class II genes play a major role in determining the specificity of T and B cell antiviral immune responses. A number of MHC alleles as well as other host factors were reported to influence predisposition or protection against HIV infection or disease (128). Some researchers found that specific HLA alleles could predispose an individual to immune responses against viral epitopes in certain tissues such as the central nervous system, lungs, or against certain HIV-infected cell types such as mono-macrophages and dendritic cells; therefore, these alleles may determine protection from infection or condition disease progression. Data suggested that combinations of MHC-encoded TAP and class I genes may synergize in providing salutary anti-HIV responses.

In different cohorts of people, heterogeneity in the susceptibility to HIV infection *in vitro* of peripheral blood cells. Ometto *et al* (154) suggested that mother to child transmission is also influenced by the individually variable susceptibility of the children target cells, in particular monocyte-macrophages. The heterogeneity of host cells to sustain the growth of some HIV strains was evaluated in two different labs: the heterogeneity was analyzed by Spira and Ho in healthy donors (155) and by Lederman *et al* (156) in a cohort of hemophiliacs.

The strongest evidence of a genetic factor that influences HIV-1 infection came from the observation that the cells of rare individuals homozygous for a deletion (delta 32) within the C-C chemokine receptor gene R5 (Figure 5) were highly resistant to M-tropic strains of HIV-1 (129). M-tropic viruses are considered to be preferentially involved in sexual transmission, while the T cell tropic viruses appear later in the course of the infection and are associated with disease progression. It is

estimated that R5 Δ32 homozygosity is present in 1% of the Caucasian population (of northern origin) (157). In the HEPS cohort studies by Dean et al the frequency of homozygotes was increased to 2.8% (158), thus the R5 deletion is not the only mechanism of protection and the HEPS population is heterogeneous. In the Italian population, the frequency of the mutant alleles is 5.5% (159). Thus homozygotes are expected to be less than 0.3%.

In contrast, some documentary evidences suggest that Δ32/Δ32 homozygosity for the R5 gene does not confer absolute protection against HIV-1 infection (160-162). Either macrophage tropic viral strains could use coreceptors other than R5 or infect independently of the presence of a functional R5 coreceptor. Alternately, there may be an occurrence though exceptional, of primary infection sustained by T cell tropic isolates. Moreover, in an effort to identify an immunological basis for natural resistance to HIV-1 infection, Luscher *et al* (163) examined serum antibody responses to HLA class I antigens in female prostitutes of the Nairobi Sex Workers Study. Anti-HLA antibodies are known to block HIV infectivity *in vitro* and can be protective against SIV challenge in macaques immunized with purified class I HLA. Thus, it was postulated that broadly cross-reactive alloantibodies recognizing common HLA alleles in the client population might contribute to the prevention of heterosexual transmission of HIV. In fact, 12% of the women were found to have serum IgG antibodies against class I alloantigens. Comparisons of the proportions of PRA-positive women between the different groups showed no statistically significant differences at any value of the assay parameters. These were no differences between HIV infected and HIV-resistant women, suggesting that naturally occurring anti-HLA antibodies do not account for protection against sexually acquired HIV infection.

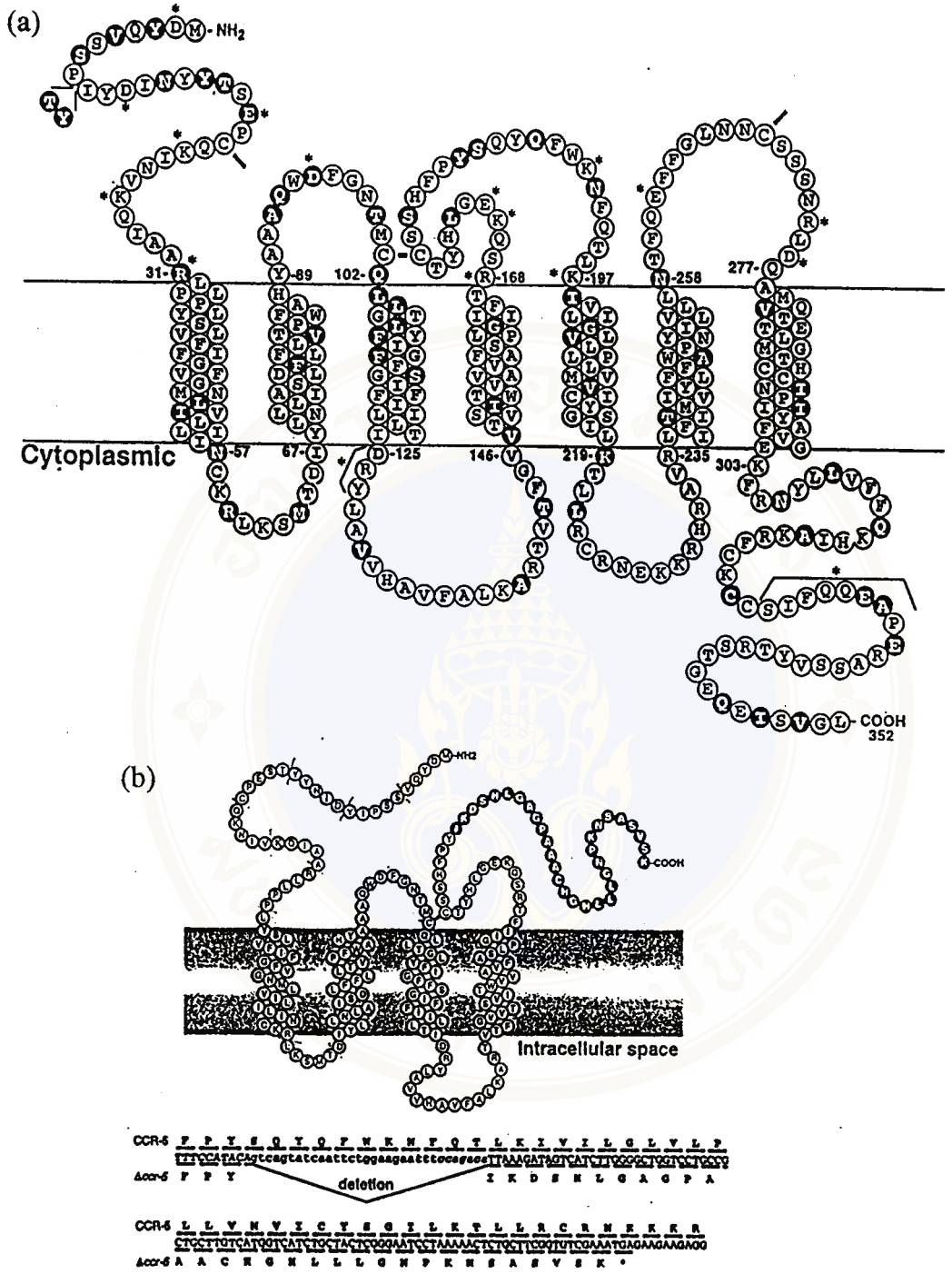

Figure 5. Structure feature of R5 (a) Normal human R5 is shown. Shaded residues indicate human residues that are different from those in the mouse R5 homolog (b) structure of mutant form of human R5, Amino acid represented in black correspond to unnatural residues resulting from the frame shift caused by the deletion (157).

A similar result in the child cohort implied that serum IgG anti-HLA antibodies were not correlated with protection against mother-to-child transmission of HIV.

Long Term Nonprogressor

Although AIDS develops within 10 years after HIV-1 infection, some individuals remain symptom-free for prolonged periods (164, 165). Most long-term asymptomatic survivors of HIV-1 infection still have evidence of disease progression in the form of declining $CD4^+$ lymphocyte concentrations. However, some rare cases not only are asymptomatic but also maintain stable levels of $CD4^+$ lymphocytes in the normal or near-normal range. Although the definition of nonprogression may vary, approximately 5 percent of seropositive persons have shown on HIV-related disease or declines in $CD4^+$ cell counts despite 10 or more year of documented HIV-1 infection (164).

Viral factors, host factors, or both may account for the absence of progression in some persons. Host factors may include the inherent susceptibility of a person's cells to HIV-1 replication (166) or an HLA-determined ability to mount an adequate immune response (167, 168). Defects in viral genes are linked to nonprogressive infection. Rhesus monkeys experimentally inoculated with simian immunodeficiency virus (SIV) carrying deletions in the *nef* gene have low viral loads and normal $CD4^+$ T cell counts. They show no signs of disease progression, deletion of Vpx and Vpr produces a similar outcome (169).

SIVmac Nef contained two N-terminal tyrosines that were proposed to be part of an SH2-ligand domain and/or a tyrosine-based endocytosis signal and a putative SH3-ligand domain (P(104)xxP(107)). The result showed that the mutation of

Y(28)F, Y(39)F, P(104)A, and P(107)A (FFAA-Nef) had little effect on Nef functions such as the association with the cellular tyrosine kinase Src, downregulation of cell surface expression of CD4 and class I major histocompatibility complex, and enhancement of virion infectivity. However, mutations in the PxxP sequence reduced the ability of Nef to stimulate viral replication in primary lymphocytes. Three macaques infected with the SIVmac239 FFAA-Nef variant showed high viral loads during the acute phase of infection.

In conclusion, mutations in both the tyrosine residues and the putative SH3 ligand domain apparently did not disrupt major aspects of SIV Nef function *in vivo*.

Great variability in the course of HIV-1 infection results from complex interplay between host and viral factors. Some of the patients with prolonged nonprogressive infection were reported to harbor virus variants with gross deletions in the accessory *nef* gene that was implicated in *in vivo* pathogenicity in simian and mouse models. An early study documented one long-term nonprogressor infected only with *nef* deleted HIV-1 since 1983 (170). More recently, six Australian long-term nonprogressors were shown to have been infected with *nef* deleted HIV-1 from a single blood donor (171). Three LTNP drug addicts in Italy cohort were shown small (2-12 amino acids) in frame deletions and insertions in the N-terminal polymorphic and variable regions. A 36-bp deletion close to the 5' end of NEF that impaired Nef function was found in 1 LTNP. Forms containing an asjacent duplication of 33 bp were also found. The duplication showed no homology to the deleted region but retroed the overall length of the first variable loop of Nef. NEF alleles carrying the duplication were active in class I major histocompatibility complex down-modulation nd enhancement of virus infectivity. However, they showed little effect activity in

CD4 down regulation and were unable to stimulate viral replication in PBMCs. These results indicated that the capacity for repair of attenuating deletions in HIV-1 infection and a selective pressure for Nef-mediated MHC-1 down-modulation and/or enhancement of virion infectivity exists. Moreover, Mendelia and coworkers found that 2 LTNP shown homozygous mutations in the Sp1 and NF-*k*B binding sites. The non-progression of HIV-1 infection in some LTNP seemed to be due to single mutations in the viral genome resulting in a less replication or to a mutant chemokine receptor leading to a reduced HIV-1 entry in to $CD4^+$ cells. Members of the Sydney Blood Bank (SBBC) were infected with attenuated strain of HIV-1 with a natural *nef*/LTR mutation and maintained relatively stable $CD4^+$ T lymphocyte counts for 14-18 years. These patients not only had normal levels of naïve $CD4^+$ and $CD8^+$ T lymphocytes, but also had primed $CD45RO^+$ $CD4^+$ T lymphocytes at or above normal levels. Furthermore, these primed cells expressed markers suggesting recent exposure to specific antigen. SBBC members exhibited variable activation of $CD8^+$ T lymphocytes. In particular, SBBC members with undetectable plasma HIV-1 RNA had normal levels of activated $CD8^+$ T lymphocytes. Therefore, the results of LTNP infection with natural *nef*/LTR mutant HIV-1 in these subjects suggested a decreased cytopathic effect of attenuated HIV-1 on susceptible activated $CD4^+$ T lymphocyte subsets *in vivo*, and minimal activation of $CD8^+$ T lymphocytes. These results demonstrated that viral defects can be response for, or at the very least contribute to, the absence of disease progression in SIV-infected rhesus monkeys and HIV-1 infected humans.

During the past few years definite progress was made in the field of HIV-1 vaccines. Initial attempts using envelope gp120 or gp41 from T-cell line-adapted

(TCLA) HIV-1 strains to vaccinate chimpanzees showed that neutralizing antibody-based immune responses was protective against challenge with homologous TCLA virus strains or strains with low replicative capacity. But these neutralizing antibodies remained inactive when tested on primary HIV-1 isolates, casting doubts on the efficacy of gp120-based vaccines in the natural setting. Development of a live attenuated SIV vaccine was undertaken in the macaque model using whole live SIV bearing multiple deletions in the *nef*, *vpr* and *vpx* genes. This vaccine provided remarkable protective efficacy against wild type SIV challenge, but the deletion mutants remain pathogenic, notably in neonate monkeys.

Rhesus macaque infected with SIV containing either a large nef deletion (SIVmac239Delta(152)nef) or IL-2 in place of nef developed high viral load and progress to simian AIDS. Viruses recovered form both juvenile and neonatal macaques with disease produced a novel truncated Nef protein, tNef. Viruses recovered from juvenile macaques infected with serial passage-virus expressing tNef exhibited a pathogenic phenotype. These finding demonstrated strong selective pressure to restore expression of a truncated Nef protein. This reversion was linked to increased pathogenic potential in live attenuated SIV vaccines.

From this evidence there is a suggestion that loss of function alone is not always sufficient to prevent the emergence of virulent mutants. New strategies that attenuate via mechanism distinct from loss of function are needed for enhancing the safety phenotype of viral genome. Quinto and coworkers constructed an HIV-1 genome carrying the cDNA of a proteolysis-resistant NF-*k*B inhibitor in the *nef* region. HIV-1 expressing this mutation down-regulated viral expression and was highly attenuated in both Jurkat and PBMCs. They provided formal proof that the

phenotypic and attenuating characteristics of this mutant permitted its stable maintenance in a live, replicating HIV-1 despite 180 days of forced *ex vivo* passaging in tissue culture. As compared with other open reading frames embedded into HIV/SIV genome, this degree of stability was unprecedented. Thus, this mutant offered proof of principle that artifactually gained functions, when used to attenuate the replication of live HIV-1, can be stable. These finding illustrate gain of function as a feasible strategy for developing safer live-attenuated HIV to be tested as candidates for AIDS vaccine.

Live, attenuated viruses were the most successful vaccines in monkey models of HIV-1 infection. However, there are several safety concerns about using such as anti-HIV vaccine in humans, including reversion of the vaccine strain to virulence and recombination with endogenous retroviral sequences to produce new infectious and potentially pathogenic viruses. Because testing in humans would inevitably carry a substantial risk.

CHAPTER IV

MATERIALS AND METHODS

Subjects

Unclotted EDTA blood was collected with informed consents from HEPS and their HIV-1 infected partners and HIV-1 infected concordant couples attended at STD clinic, Siriraj Hospital.

HEPS, who fit standardized criteria for repeated high-risk sexual exposure to HIV-1 with their HIV-1 infected partners at least 2 years, had no evidence of condom use and had no challenge with other risk. Their HIV-1 infected partners were interviewed for a history of exposure to sexual transmitted disease (STD). HEPS were assigned eligibility by evidence of HIV-1/2 ELISA and peripheral blood mononuclear cells (PBMC) DNA PCR with *gag/pol* gene. There were 15 HEPS male (HH01, HH02, HH11, HH13, HH16, HH17, HH18, HH31, HH33, HH35, HH39, HH43, HH45, HH49, HH55) and 2 HEPS female (HW48 and HW53). The HIV-1 infected partners of HEPS consisted of 15 female (PW11, PW35, PW45, PW49, PW55, PW01, PW02, PW13, PW16, PW17, PW18, PW31, PW33, PW39, and PW43) and 2 male (PH48 and PH53).

Five HIV-1 infected concordant couples (CH06, CW06, CH07, CW07, CH34, CW34, CH57, CW57, CH59 and CW59) were enrolled as the control group. All couples were also interviewed after written consent was obtained and, had STD screening and no event of exposure to other risk.

None of the subjects in this study had HIV-associated diseases. Ten to twenty milliliters of blood was collected in EDTA vacutainer tube, from both HIV-infected subjects and HEPS.

Sample preparation

Unclotted EDTA blood was collected from each subject via a vacutainer tube. The tube was centrifuged at 1,200 rpm for 10 minutes to separate blood cells from plasma. The plasma was removed and stored at –70°C, which kept for viral RNA quantification, RT-PCR, and neutralization assays. The blood cell was diluted with on equal volume of 0.2M phosphate buffer saline (PBS). The diluted blood cell was placed on a one-third volume of Isoprep^{R} (Robbins Scientific, CA, USA) in a 15-ml centrifuge tube and centrifuged at 1,500 rpm for 20 min without brake. PBMC fraction, which was seen as an opaque band located on the gradient interface, was carefully removed to a new centrifuge tube. PBMC was washed twice with 5 ml of 0.2M PBS and the third was washed by RPMI-1640, which containing 10U/ml of penicillin and 10 μg/ml of streptomycin. All washing were centrifuged at 1,200 rpm for 10 min. The supernatant was discarded, and the cell pellet was resuspended and counted with RPMI-1640. $2x10^6$ PBMCs were used for co-culture method, and $1x10^6$ cells were kept for PCR amplification at –20°C. The remaining cells were transferred to pre-cool DMSO medium (appendix) and kept at –70°C. The cell density was around $10x10^6$ cells per ml DMSO medium.

Amplification of HIV-1 *env*, *nef* and LTR regions

DNA was lysed from PBMCs by proteinase K lysis buffer. The nested PCR was performed in a two-steps reaction. First, with an outer primer pair and then with inner primer pair. In this study, three sets of primers specific to *env* (ENV B-AO_2 / AI_1-AI_2), *nef* (NEF1F – NEF1R / NEF2F – NEF2R) and LTR (PH5'KPN - PH3'LTR / PH5'KPN – PH56) regions of HIV-1 were used (Table 3).

Table 3. Sequence of primers and location in the HIV-1 genome

Primer	Sequence (5'->3')	Location
***env* gene**		
ENV B	AGA AAG AGC AGA AGA CAG TGG CAA	6191-6214
AO_2	GGA ATT CAA AGG TGA GTA TCC CTG	8335-8358
AI_1	GGG ATC CTT ATT ATG GGG TTC CTG TGT GG	6317-6345
AI_2	GGA ATT CTT TCC TCC TCC AGG TCT GAA	7609-7635
***nef* gene**		
NEF1F	GCC ATT CTC CAC ATA CCT AG	8746-8765
NEF1R	GTT AAC CAC TCC CCA ACT CC	9479-9498
NEF2F	GCT ATA ACA TGG GAG GCA AG	8796-8815
NEF2R	CCA GCG GAA GTC CTA GTT AG	9429-9448
LTR region		
PH5'-KPN	CTC AGG TAC CTT TAA GAC C	9012-9030
PH3'-LTR	CTG AGG GAT CTC TAG TTA CCA GAG	9665-9688
PH56	ATT GAG GCT TAA GCA GTG GG	9595-9615

For the amplification of the *env, nef* and LTR region, the PCR was performed by using ENV B – AO_2, NEF1F – NEF1R and PH5'KPN – PH3'LTR primers, respectively, in the first round of amplification. Thus 5 µl of first round PCR product was amplified further by using AI_1 – AI_2, NEF2F–NEF2R, PH5'KPN – PH56 primers, respectively. To avoid contamination of the PCR product, preparation of reaction reagents for primary and secondary PCR was done in separate areas and a plugged-tip was used for the aspirating primary PCR product.

The first round PCR reaction mixture for *env*, *nef* and LTR regions were similar and consisted of 5.0 µl of 10x PCR buffer, 3.0 µl of 25 mM $MgCl_2$, 4.0 µl of 10 mM each of dNTP, 2.0 µl of 10 pmol/µl of outer primers (ENV B – AO_2, NEF1F – NEF1R and PH5'KPN – PH3' LTR for amplify *env*, *nef* and LTR, respectively), 23.7 µl of sterile deionized water, 0.3 of 5U/µl of *Tag* polymerase enzyme, and 10 µl of cell lysate.

The second round PCR reaction mixture consisted of 5.0 µl of 10x PCR buffer, 3.0 µl of 25 mM $MgCl_2$, 4.0 µl of 10 mM each of dNTP, 2.0 µl of 10 pmol/µl of inner primers (AI_1 – AI_2, NEF2F – NEF2R and PH5'KPN – PH56 for amplify *env*, *nef* and LTR, respectively), 28.7 µl of sterile deionized water, 0.3 of 5U/µl of *Tag* polymerase enzyme, and 5 µl of first round PCR product for each region.

PCR was performed by using the automated Gene Amp PCR System 9700 and 2400 DNA Thermal Cycler (Perkin Elmer Cetus, CT, USA). It was programmed for each of primer pairs and each region. The reaction cycles for amplifications are as follow:

For the *env* gene, the first round reaction cycles are 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min. After the last cycle, the final extension was

performed at 72°C for 5 min. The second round PCR was programmed as the first round PCR cycles but the annealing temperature was changed from 50°C to 55°C. Nested PCR product of *env* gene was 1,115 bases.

For the *nef* gene, the first and the second round reaction cycles are 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min and the final extension at 72°C for 5 min. Amplified product of *nef* gene was 700 bases.

For the LTR region, the first and the second round reaction cycles are the same as 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 45 sec and the final extension at 72°C for 7 min. The PCR product was 615 bases.

The PCR product was examined by agarose gel electrophoresis.

Amplification of *nef* gene from HIV-1 genomic RNA

The one tube RT-PCR was performed into 2 steps. First, cDNA was performed by using RT-AMV. Then cDNA was amplified by *Taq* polymerase, the same primer pair, NEF1F – NEF1R, was used in both steps. The DNA product was amplified further by the second round PCR.

For the amplification of *nef* gene from HIV-1 genomic RNA, purified by Nuclisens nucleotide isolation (Organon Teknika, WI, USA), the RT-PCR was performed by using NEF1F – NEF1R for reverse transcribed from RNA into DNA. Then the cDNA was amplified by first round PCR by using the same primer set. 5 µl of the first round PCR product was amplified further by using NEF2F – NEF2R, as described previously.

The one tube RT-PCR reaction mixture consisted of master mix 1 and master mix 2. Master mix 1 consisted of 3.2 µl of sterile RNase free water (Ameresco, OH,

USA), 10.0 µl of 10 mM each for dNTP, 2.5 µl of 100 mM DTT solution (supplied), 0.3 µl of 40U/µl RNase inhibitor (Promega, WI, USA). Master mix 2 consisted of 8 µl of sterile RNase free water, 10 µl of 5x RT-PCR buffer (supplied), 6 µl of 25 mM $MgCl_2$ (supplied), 1 µl of enzyme mix. Sixteen µl of master mix 1 and 25 µl of mater mix 2 were added to a 0.2-ml thin walled PCR tube on ice. The tube was mixed properly and centrifuged briefly in order to collect the mixture at the bottom of the tube.

Five µl of RNA template was added to a 0.2 ml thin walled PCR tube which containing 2 µl of 10 pmol/µl of each primer, NEF1F – NEF1R. A template was denatured prior to initiation of the reverse transcription at 94°C for 5 min and then rapidly cooled on ice. A fourty-one µl of reaction mixture was transferred to the denatured RNA tube.

One tube RT-PCR was performed in Gene Amp System 2400 Thermal Cycler. It was programmed for 50°C for 45 min, 94°C for 5 min for inactivated reverse transcriptase activity in the reaction and then cDNA was amplified by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min. The extension was performed at 72°C for 5 min. The first round PCR product was then amplified further by the second round PCR.

Examination of amplified DNA by agarose gel electrophoresis

The amplified product was normally verified on agarose gel electrophoresis to see whether the certain DNA fragment was amplified and obtained as expected. The 2% agarose gel (GIBGO BRL, NY, USA) was used to detect the amplified DNA

fragment of *nef* and LTR region, and 1% agarose gel was used for *env* gene. The gel was prepared by boiling the agarose powder in 0.5x TBE buffer (appendix) until it was completely dissolved and allowed to cool down. Then the gel was poured in a gel-casting platform with a well former. The gel was left for 30 min to hardening at room temperature, and the well former was then carefully taken off. The gel and casting platform were transferred into an electrophoresis chamber containing 0.5x TBE buffer sufficient volume to cover the gel surface. The solution of DNA marker (100 bp DNA ladder) (BioLab) was mixed with 1 µl of loading dye (appendix) and applied into the first well. Five microliters of PCR product was mixed with 1 µl of loading dye and then applied into the well. The electrophoresis was carried out at constant voltage at about 100 volts. The running gel was stopped when the dye migrated to the bottom of the gel. Then the gel was stained in ethidium bromide solution (appendix) for 5-10 min and destained in distilled water. The DNA fragment in the gel was observed on an UV-transluminator and photographed by using Polaroid camera fitted with a red-orange filter.

Gel extraction by using QIA quick gel extraction kit (QIAGEN Inc, CA, USA)

The positive band of DNA fragment from the agarose gel was excised. The size of the gel slice was minimized by removing extra agarose, and weighed in a 1.5-ml eppendorf tube. The gel was dissolved by using buffer QG incubated at 50°C until completely dissolved. The sample was applied to the QIAquick spin column, and centrifuged for 1 min at 10,000 rpm. Buffer QG was used for removing all traces of agarose and additional of buffer PE was used for removing the salt. Residual ethanol

from buffer PE was completely removed. To elute DNA from DNA-binding column would use buffer EB or sterile distilled water. The extracted DNA was used for direct sequencing and cloned.

Ligation of purified DNA product with pGEM-T easy (Promega, WI, USA)

The purified DNA of each region was ligated by using pGEM-t easy ligation kit. The ligation reaction consisted of 5 µl of 2x rapid ligation buffer (supplied), 1 µl of pGEM-t easy vector, 1 µl of T4 DNA ligase, 3 µl of purified DNA. The reaction was mixed by a pipette, and incubated for 1 hr at room temperature. The incubation period may be extended to increase the number of colonies after transformation. An overnight incubation at 4°C will generally produce the maximum number in a transformation.

After the incubation period, 30 µl of x-gal (Promega, WI, USA) was plated on the amplicillin–LB plate (appendix). JM109 competent cell was removed from –70°C storage and placed in an ice bath until just thawed about 5 min. The ligation reaction tube was centrifuged to collect contents at the bottom of the tube and transferred carefully to thaw the JM109 tube. The cells were mixed by gently flicking the tube, and it was placed it on ice for 30 min. The cells were then heat-inactivated for 45 sec in a 42°C water bath. The tube was then immediately returned to the ice for 2 min. A 300 µl of SOC medium (appendix) was added to the tube. It contained cells transformed with ligation reaction, incubated for 1 hr at 37°C with a shaking at approximate 200 rpm. Next the tube was centrifuged at 10,000 rpm, and a 200 µl of

supernatant was discarded. Cells and the remaining supernatant were mixed and plated on the amplicillin-LB plate as prepared above. The plate was incubated overnight (16-24 hr) at 37°C. White colonies generally contained insert DNA. Longer incubation or storage of plate at 4°C, after 37°C overnight, may be used to facilitate blue/white screening. White colonies were collected into the amplicillin-LB broth (appendix), and shaken at 37°C overnight.

Plasmid extraction

The bacterial suspension was poured into a 1.5-ml eppendorf tube. The tube was spun at 10,000 rpm for 10 sec, and the supernatant was decanted. A 150 µl of GTE solution (appendix) was then added to the tube, and it was mixed. This tube was allowed to stand at room temperature for 5 min. After that, 200 µl of fresh solution 2 (appendix) was added and gently mixed by inverting it 4-6 times. The tube was plated on ice for 5 min.150 µl of KoAc (appendix) was then added to the tube and it was again mixed. The tube was incubated on ice for 5 min and spun for 10 min. A 400 µl of supernatant was carefully removed to a new 1.5-ml eppendorf tube containing 800 µl of 95% alcohol for precipitating plasmid. The tube stood for 5 min and was then spun for 5 min. To wash out the remaining salts in the pellet, 1,000 µl of 70% alcohol was added and centrifuged for 1 min. Alcohol was decanted. The pellet was dried at 37°C for 1 hr then pellet was resuspended by using 30 µl of buffer or deionized water.

The extracted plasmid was normally cut by EcoRI restriction enzyme (Promega, WI, USA) and verified on agarose gel to see whether the certain DNA fragment was ligated and obtained as expected. The cutting reaction consisted of 2 µl

of 10x buffer H (supplied), 0.2 µl of acetylated BSA (supplied), 3 µl of extracted plasmid, 0.4 µl of 12 U/µl EcoRI enzyme, 14.4 µl of deionized water. The reaction was incubated at 37°C, 95% humidity for 1 hr. The product was verified by agarose gel.

Big dye terminator cycle sequencing reaction

Purified PCR product (direct sequencing) and extracted plasmid DNA (cloning and sequencing) were used as templates in a nucleotide sequencing reaction. The sequencing reaction consisted of 4.0 µl of terminator ready reaction mix, 3.2 µl of 1 pmol/µl of sequencing specific primer for each region as using in PCR amplification, NEF2R for *nef* gene, PH56 for LTR, and 4.0 µl of 30-90 ng of purified PCR product or 2.0 µl of extracted plasmid and 8.8 µl of sterile deionized water. The cycle sequencing reaction was performed by using DNA thermal Cycler programmed as follows: 25 cycles of 96°C for 10 min, 50°C for 5 min, 60°C for 4 min then soaked at 4°C.

The cycle sequencing product was then precipitated by 64 µl of 95% alcohol mixed with 16 µl of sterile deionized water. It was allowed to stand for 20 min and then spun at 10,000 rpm for an additional 20 min. The supernatant was carefully removed. Another 250 µl of 70% alcohol was added to the tube and spun at 10,000 rpm for 10 min. The supernatant was removed carefully without disturbing the DNA pellet. The remaining supernatant was dried at 37°C for 1 hr in upright position. The dried sequencing product was mixed with template suppression reagent and incubated

at 95°C for 2 min and rapidly cooled on ice. The sequencing reaction was detected by the Genetic Analyzer in Automate DNA Sequencer (ABI 310, Perkin)

Nucleotide sequence reported from the automated genetic analyzer was then analyzed by computer software as DNASIS, ESEE and MEGA.

Phytohemagglutination (PHA) -stimulated of normal donor PBMC

Donor leukocyte fraction was transferred into a sterile bottle, and diluted with equal volume of 0.2M PBS. The diluted donor cells was transferred onto the top of one-third volume of Isoprep in a 50 ml centrifuge tube, which was spun at 2,000 rpm for 30 min without cessation. The PBMC fraction was carefully removed to a new centrifuge tube containing 30 ml of 0.2M PBS. After centrifugation at 1,500 rpm for 10 min, the supernatant was discarded and washed once. RPMI-1640 was used for a final wash. Then cells were counted and resuspended at $2x10^6$ cells per ml in stimulated medium (appendix). The $15\text{-}20x10^6$ cells were put in a T-25 flask. The culture flask was stood upright in the CO_2 incubator for 3-7 days. After the stimulation period, cells were put in a 15-ml polypropylene tube and spun at 1,200 rpm for 10 min. Then the supernatant was decanted and the pellet was resuspended at about $2\text{-}3x10^6$ cells per ml in the culture medium (appendix). Stimulated cells could be used for the experiments.

Co-culture method

Two million cells of PBMCs from HIV-1 infected subject were placed in a T-25 tissue culture flask containing 10 ml of the culture medium and co-cultured with $8x10^6$ PHA-stimulated donor PBMC. The flask was incubated at 37°C in a 5% CO_2

atmosphere for up to 42 days. Approximately 3 ml of the culture medium above the settle PBMC was removed twice weekly. One tube was kept for testing and the other was discarded and replaced with an equal volume of fresh medium. The settled PBMCs was removed out approximately $3x10^6$ cells and additional equal number of PHA-stimulated donor PBMCs weekly to maintain the viable cell concentration.

HIV replication was detected by testing of the culture supernatant in a p24 antigen-capture enzyme-linked immunosorbent assay (Organon Teknika, WI, USA) once a week. A culture was considered positive if two serial supernatant fluid sampling were p24 antigen positive. The positive culture was transferred into a 15-ml centrifuge tube and spun at 1,200 rpm for 10 minutes. Next the cell-free virus supernatant was collected and kept at $-70^{\circ}C$ for biotyping, TCID50 determination and NT assay. The infected cells were parted into 2 tubes; one for PCR amplification and one other kept in DMSO medium.

Biotype determination

Macrophage preparation and infection

Approximately 2 ml of normal human serum (NHS) was added into a T-25 culture flask (corning, NY, USA) and incubated for 30 min in a laid down position. Subsequently $2.5x10^7$ cells of normal donor PBMCs, which were suspended with 3-ml macrophage attachment medium (appendix), were added into the incubated flask. The flask was laid down in an incubator for 3-7 days at $37^{\circ}C$, among the incubation period macrophage would attach onto the flask. Then the PBMC residues were washed out by gently blowing 4 ml of 0.2M PBS 4-6 times. 4 ml of macrophage medium

(appendix) was added into the flask and placed into the CO_2 incubator until used. Total incubated time from beginning to infected day was not over than 7 days.

An approximately cell-free supernatant containing p24 antigen 1 ng was added to the macrophage's flask and placed in a CO_2 incubator overnight (16-18 hours). After infection, 5 ml of 0.2M PBS was added to the flask and allowed to sit briefly in order to washed out unbound viruses. The PBS was carefully removed, and the solution was washed 3 times. Then 5 ml of macrophage medium was added into the flask and placed in an incubator. HIV replication was determined by testing p24 antigen for day 7, day 14 and day 21.

T-cell line preparation and infection

MT2 and SupT1 were rapidly thawed in the 37°C-water bath. Cells were then added to each 15-ml tube containing 10 ml of wash medium (appendix) and then centrifuged at 1,200 rpm for 10 min. Supernatant was decanted and cell pellets were taken to each T-25 flask which contained a 10 ml cell-line medium (appendix). The flasks were placed in a CO_2 incubator for 3 days.

$2.5x10^5$ cells of MT2 and SupT1 were dispensed at 100 µl of cell-line medium into each 15-ml tube. Approximately 1 ng p24 of cell-free virus supernatant was added to each tube with cells and then incubated overnight. After infection, 10 ml of 0.2M PBS was added to the tubes to wash unbound viruses. The tubes were centrifuged for 10 min at 1,200 rpm and washed at once. Then MT2 and SupT1 were plated in a 24-well plate with 2 ml of cell-line medium. MT2 and SupT1 cells were sub-cultured twice a week. HIV replication in each cell was detected by testing p24 antigen once a week.

Determination of $TCID_{50}$

Infection tubes set up

PHA-stimulated donor PBMC was counted, resuspended at $10x10^6$ per ml, and dispensed at 100 µl into 6 tubes for each virus to be titered. Six tubes of cells were prepared for each virus. NHP was filtered and heat inactivated before it was used. NHP was diluted at 1:6.7 with a culture medium and then 100 µl of diluted plasma was added to each tube containing cells. Virus titration was performed at five-fold dilutions of the virus stock, depending on the range of dilutions needed to achieve an endpoint. Usually six dilutions per virus were performed. The usual virus dilution series was 5^{-2} (1:25) to 5^{-7}.

Viral dilutions were prepared in separate tubes at 3 times more concentration than the final desired dilution. To start, the final 1:20 was prepared at 1:8.3. Thus, the dilutions were prepared by mixing 100 µl of virus stock with 730 µl of the culture medium. 800 µl media was put in five tubes and subsequent five-fold serial dilutions of the initial 1:8.3 were prepared by transferring 200 µl of previous dilution into 800 µ l of the next tube. Then a 100-µl aliquot of each virus dilution was added to one tube of cells and the NHP inputted for a final volume of 300 µl in the infection assay.

Infection and wash step

The tubes were placed in an incubator overnight at 37°C in CO_2 incubator. This was day 0. After infection, 10 ml of washed medium was added to each tube and centrifuged for 10 minutes at 1,200 rpm. Supernatant was gently decanted from the tubes. Cells were gently vortexed once, and another 10 ml of wash medium was added

to all tubes. Tubes were repeatedly spun and gently decanted, and allowed to stand 5 min. The residual medium was carefully removed by using a pipette. This did not disturb the cell pellet. 1 ml of culture medium was added to tubes and each 0.2 ml aliquots of washed cells were transferred to each well of a U-bottom 96-well plate. 200 µl of PBS was added to the outer wells of microtiter culture plate to minimize evaporation loss from inner wells. The U-bottomed plates were placed in a CO_2 incubator.

Culture and harvest the plates

On day 4, 100 µl supernatant was removed carefully with multi-channel pipette and put in another plate. It was re-fed with 100 µl of fresh medium in all wells. At day 8, the original culture plate was harvested and 100 µl of supernatant transferred to a 96-well plate for p24 antigen capture analysis.

Determine the $TCID_{50}$ of the virus

The number of total negative wells were counted and the $TCID_{50}$ was calculated based on the Spearman-karber formula as follows:

$$1/TCID50 = B^e$$

$$\text{and } e = E + d\,(0.5 - N/r)$$

e = exponent for reciprocal titer

B = base for reciprocal titer, B is the fold-dilution used in the dilution series, usually B = 5

r = the number of repetition for each virus dilution, usually 5

E = the highest exponent used in the dilution series, so if the series 5^{-2} to 5^{-7} is used, E = 7

N = sum of negative wells in the titration

d = spacing or distance between dilution, usually 1

Neutralization assay

Preparation of the test sera or plasma

The same normal donor PBMCs were used for each panel of serum or plasma. Stimulated normal PBMCs were resuspended at $10x10^6$ cells per ml. All sera and plasma were heat inactivated at 56°C for 30 minutes and centrifuged at 4,000 rpm for 15 minutes. The supernatant was then transferred to a new tube. Heat inactivated sera or plasma were diluted at 1:6.7, three-fold the desired final concentration of 1:20, with culture medium. Triplicate controls were set up with HIV negative plasma (NHP), and test sera or plasma was duplicated.

Prepare the neutralization test tubes and plates

Two hundred µl of PBS was added to the outer rows of a 96-well U-bottom culture plate in order to minimize evaporation loss from inner wells. Two tubes were used for each plasma tested. Forty µl of the diluted plasma was added to corresponding tubes. And virus dilutions were prepared at a three-fold great concentration than the final desired dilution. For 100 $TCID_{50}$, the reciprocal titer was divided by 100 and then by three. The culture medium was used to dilute the virus. A 40 µl aliquot of the diluted virus was added to all duplicate tubes for each plasma

being tested against that virus. Tips were changed between tubes and mixed well. After adding the virus, tubes were incubated at 37°C for 30 minutes. Then cells were dispensed at 40 µl for $4x10^5$ cells and then added to each tube of plasma. The virus was then mixed. There were three component in the assay, cells, plasma and virus. The virus stock dilution was at 100 $TCID_{50}$ when all components were mixed together. The same was true for the plasma at 1:20.

Infection and wash step

The tubes were closed loosely, mixed carefully and placed in a CO_2 incubator for one hour or overnight (16-18 hours), depending on the desired infection time. The same infection period was used when the virus $TCID_{50}$ was determined. The set-up day was day 0. After the infection period, 10 ml of wash medium was added to each tube. For overnight infection, this was day 1. The tubes were then centrifuged for 10 min at 1,200 rpm and the wash medium was gently removed. Another 10 ml of wash medium was added to the tubes. The tubes were centrifuged and the medium removed. It sat for 5 min, and then the residual medium was removed carefully with a pipette. If the same virus was used in all tubes, the same pipette was used as there was very little serum or virus left in the supernatant at thar point. 400 µl of appropriate culture medium was added to each tube of washed cells by using a 1-ml pipette. It was transferred to two wells of a U-bottom 96-well plate. These plates were placed in an incubator.

Culture and harvest the plates

On day 4, 100 µl supernatant was carefully removed and re-fed with 100 µl of fresh culture medium. At day 8, 100 µl supernatant was harvested from each well of the original culture plate for a p24 antigen capture analysis.

Analysis of neutralization assay

One hundred $TCID_{50}$ were used. All 4 of the virus and the NHP wells must be p24 positive or greater than the 100 pg/ml. The percentage of p24 reduction by neutralizing activity was determined by calculating the average p24 produced in the test samples and dividing this number by the control average of p24. This would give percent reduction of virus growth or percent neutralization number.

$[1 - (S / C)] \times 100$ = percent neutralization

S = average p24 produced in duplicate experimental wells

C = average p24 produced in the control (NHP) wells

Quantification of HIV-1 RNA by using NucliSens HIV-1 QT (Organon Teknika, WI, USA)

The NucliSens HIV-1 QT assay is based on four separate stages: nucleic acid release, isolation, amplification, and detection.

Nucleic acid release

The sample was added to NucliSens Lysis Buffer containing guanidine thiocyanate and Triton X-100. Any viral particles and cells presented in the sample

were disintegrated and any RNase and DNase presented in the sample were inactivated. Nucleic acid was released.

Nucleic acid isolation

Three synthetic RNAs (Qa, Qb, Qc) of high, medium and low concentration, respectively, were added to the lysis buffer containing the released nucleic acid. These RNAs serve as internal calibrators, each differing from the HIV-1 wild-type (WT) RNA by only a small sequence. Under high salt conditions, all nucleic acid in the buffer, including the calibrators, was bound to silicon dioxide particles. Three particles, acting as the solid phase, were washed several times. Finally, the nucleic acid was eluted from the solid phase. It was used in the detection step and the remaining solution was kept at –70°C for RT-PCR amplification.

Amplification

Any WT HIV-1 RNA presented in the eluted nucleic acid was co-amplified with three internal calibrators. Amplification is based on repeated transcription. That is, multiple copies of each WT and calibrators RNA target sequence is synthesized by T7-RNA polymerase by means of an intermediate DNA molecule, which contains the double stranded T7-RNA polymerase promoter. Each transcribed RNA copy entered a new amplification cycle. The DNA intermediate was generated by binding a primer to the RNA template, extending the primer by AMV-RT (Avian Myeloblastosis Virus Reverse Transcriptase) to form an RNA-DNA duplex, degrading the RNA strand of the duplex by RNase-H, binding a second primer to the remaining DNA strand and extending the second primer to form the double-stranded T7-RNA polymerase

promoter needed for transcription. The primers were complementary to two different parts of the HIV-1 RNA. Together they defined the amplified sequence within the HIV-1 *gag* region. Since the NASBA process required no strand separation, amplification was isothermal and continuous.

Detection

Detection of HIV-1 RNA to a sample is based on the NucliSens Reader electrochemiluminescence principle. To differentiate between the amplificates (WT, Qa, Qb and Qc) aliquots of the amplified sample were added to four hybridization solutions, each specific for one of the amplificates. The respective amplificates were hybridized with a bead-oligo and a ruthedium-labeled probe. The paramagnetic beads carrying the hybridized amplificate/probe complex were captured on the surface of an electrode by means of a magnet. Voltage applied to this electrode triggered the electrochemiluminescence (ECL) reaction. The light emitted by the hybridized ruthedium-labeled probe was proportional to the amount of the four amplificate. Calculation based on the relative amounts of the four amplificates revealed the original amount of WT HIV-1 RNA in the sample.

Quantification of HIV-1 RNA by using AMPLICOR HIV-1 MONITOR test.

The AMPLICOR HIV-1 MONITOR Test (Roche Diagnostic System, NJ, USA) is based on five major processes: specimen separation, reverse transcription of the target RNA to generate cDNA, PCR amplification of target cDNA using HIV-1 specific complimentary primers, hybridization of the amplified products to

oligonucleotide probes specific to the targets, and detection of the probe-bound amplified products by colorimetric determination.

The quantification of HIV-1 viral RNA was performed by using a Quantification Standard (QS) RNA which is a non-infectious RNA transcript that contains the identical primer binding site as the HIV-1 target and a unique probe binding region. The QS RNA was simultaneously amplified with HIV-1 target RNA.

The HIV-1 viral RNA was extracted from plasma of HIV-1 infected subjects by using a lytic reagent containing guanidine thiocyanate and the known amount of quantification standard (QS). HIV-1 viral RNA and QS RNA molecules were precipitated with isopropanol (Sigma, MO, USA) and resuspended in a buffer-containing carrier RNA. A 142 bp sequence with 5' end biotinylated primer SK431 and SK462 was used. Reverse transcription and PCR amplification were carried out in a single reaction by using thermostable recombinant enzyme, rTth DNA polymerase from *Thermus Thermophilus* that possesses efficient reverse transcriptase and DNA polymerase activities. The use of rTth polymerase allows incorporation of uracil-N-glycosylase for the prevention of DNA carryover contamination of previously amplified material. To measure the HIV-1 and QS amplicons over a large dynamic range fivefold serial dilutions of the amplification product were made. The biotinylated HIV-1 and QS amplicons were captured in separate wells of a microtiter plate coated with HIV-1 specific and QS specific oligonucleotide probes, respectively. The bound biotinylated amplicons were detected with an avidin-horseradish peroxidase conjugate and a chromogenic substance mixture. Absorbance was measured at 450 nm. The HIV-1 RNA copy number was calculated from the known input copy number of the QS RNA.

Determination of CCR5 region

PBMCs of HEPS were resuspended in a 200 µl of PCR lysis buffer (appendix) containing 10 mg/ml proteinase K (Ameresco, Ohio, USA). Cell suspension was incubated at 56°C for 1 hr, and then proteinase K was inactivated by heating the suspension at 95°C for 10 min. The cell lysate was used for PCR amplification.

The amplification of CCR5 region was performed in one step reaction by using F2 (5'-GGTGGAACAAGATGGATTAT) and R2 (5'-CATGATCAGAACTGTGACTG) primers.

The PCR amplification mixture consisted of 5 µl of 10x buffer (supplied), 3 µl of 25 mM $MgCl_2$ (supplied), 4 µl of 10 mM each of dNTP, 1 µl each of 12.5 pmol/µl F2&R2 primers, 23.75 µl of sterile deionized water, 0.25 µl of *Taq* polymerase (Promega, WI, USA) and 10 µl of cell lysate.

CCR5 gene amplification was performed by using Gene Amp PCR System 2400 Thermal Cycler. It was programmed for 30 cycles of 93°C for 1 min, 60°C for 1 min, 72°C for 1 min and the final extension at 72°C for 6 min. The amplified product was 1,000 bases.

The PCR product was examined by agarose gel electrophoresis and extracted for direct sequencing.

CHAPTER V

RESULTS

1. Clinical status of the subjects

The criteria of HEPS were individuals, who repeated high-risk sexual exposure to HIV-1 with their partners for at least 2 years and had no evidence of condom use. They had no challenge with other risks. Laboratory identification of HEPS was HIV 1/2 ELISA and peripheral blood mononuclear cells (PBMC) DNA PCR of *gag/pol* gene.

Twenty-seven HIV-1 infected subjects, consisting of seventeen partners of HEPS and five concordant couples, were asymptomatic at the time of the sample collection. There were no sign or any symptom of sexually transmitted disease. They were heterosexual transmitted individuals and had no event of intravenous drug abuse and had never been partner with the intravenous drug users.

Fifteen HIV-1 infected partners of HEPS were women, and two were men (Table 4 and Figure 6). The mean age and the mean viral load of HIV-1 infected individuals were 28.1 ± 9.2 years and 4.20 ± 0.88 log copies/ml., respectively. HEPS were confirmed eligibility by peripheral blood mononuclear cells (PBMC) DNA PCR with *gag/pol* gene. None of them were infected.

The mean age and mean viral load of five HIV-1 infected concordant couples were 29.9 ± 6.3 years and 4.32 ± 0.45 log copies/ml., respectively.

There were no differences in the mean age group and viral load from partners of HEPS and concordant couples.

Mean viral load in four partners of HEPS with *nef* deletion was 4.54 ± 1.05 log copies/ml that was not significantly different from the viral load of the wild type *nef* (4.18 ± 0.84 log copies/ml).

HIV-1 subtype was determined by peptide ELISA and/or *env* gene nucleotide sequencing of HIV-1 proviral DNA in PBMC specimens.

Table 4. Epidemiologic characteristic of HIV-1 infected partners of HEPS and concordant couples

	Partners of HEPS (n = 17)	Concordant couples (n = 10)
Gender		
Male	2	5
Female	15	5
Mean age (years)	28.1 ± 9.2	29.9 ± 6.3
Mean viral load (log copies/ml)*	4.20 ± 0.88	4.32 ± 0.45
Mechanism of HIV-1 infection	Heterosexual contact	Heterosexual contact

*performed by Nuclisens (Organon Teknika, WI, USA)

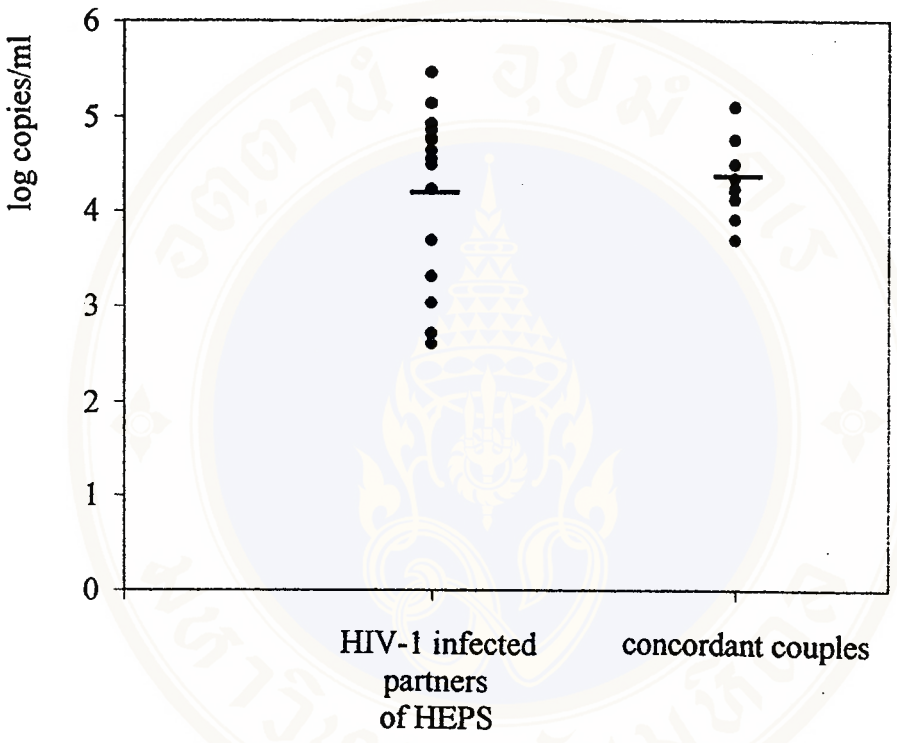

Figure 6. Viral load distribution of HIV-1 infected partners of HEPS and concordant couples. The horizontal lines indicated the mean values.

2. Nucleotide sequence of HIV-1 *env*, *nef* gene and LTR

To access any HIV-1 genetic abnormality presented in the HIV-1 infected partner of HEPS, nucleotide sequencing of *env*, *nef* gene and LTR was performed on HIV-1 proviral DNA and genomic RNA of 17 HIV-1 infected partners of HEPS (PW01, PW02, PW11, PW13, PW16, PW17, PW18, PW31, PW33, PW35, PW39, PW43, PW45, PH48, PW49, PH53 and PW55) and 5 HIV-1 infected concordant couples (CH06, CW06, CH07, CW07, CH34, CW34, CH57, CW57, CH59 and CW59).

2.1 Amplification of *env* V3 region

The deduced amino acid sequence alignments of env V3 of HIV-1 of partners of HEPS and concordant couples were shown in Figure 7. There were some differences in V3 amino acid sequences of HIV-1 infected subjects.

2.2 Amplification of *nef* gene

Amplification of *nef* gene from proviral DNA

HIV-1 *nef* gene in proviral DNA of subjects: HIV-1 infected partner of HEPS and HIV-1 infected concordant couples were detected by nested PCR with NEF1F&NEF1R, as outer primers and NEF2F&NEF2R, as inner primers. The 615-bp fragment of the amplified *nef* gene product was directly detected on the agarose gel after ethidium bromide staining.

Amplification of *nef* gene from genomic RNA

The AMV reverse transcriptase and NEF1R primer, the reverse primer, were used for reverse transcriptase of the cDNA for the first round of the nested PCR in the RT-PCR mixture. Then the nested PCR with NEF1F&NEF1R and NEF2F&NEF2R were also used for amplify the *nef* gene from cDNA.

Figure 8 showed the amino acid sequence of the Nef protein from HIV-1 isolates of partners of HEPS with deletion position within the *nef* gene of four isolates (PW01, PW13, PW16, and PW43). Four deletion patterns of *nef* gene were found in this experiment (Figure 8). First, four amino acids deletions were found in clone derived from PW16 (11.11%). Second, the deletion pattern was 86 amino acids in length and was located in the *nef*-unique portion from amino acid 39 to 124 in clone derived from PW13 (66.67%). Third, two regions, the 47 amino acids and 31 amino acids, were deleted and located in the *nef*-unique portion from amino acid 62 to 108 and 134 to 164 in clone derived from PW43 (11.11%). The last pattern was deleted 26 amino acids (at amino acid position 80-105) in clone derived from PW01 (10%). Although a number of deletions were present in the region of *nef* gene that overlaps U3 in the long terminal repeat, none of the deletions affected *cis*-acting sequences.

Point mutations were found in PxxP region at amino acid position 75 to 78 in clone derived from PH53 (100%), PW01 (10%) and CW59 (100%) and at position 145 to 148 in clone derived from PW01 (80%), PW11 (100%), PW18 (100%), PW33 (23.07%) and CW07 (100%).

Figure 9 showed the phylogenetic tree obtained with the various *nef* sequences derived from HIV-1 infected partners of HEPS and concordant couples with

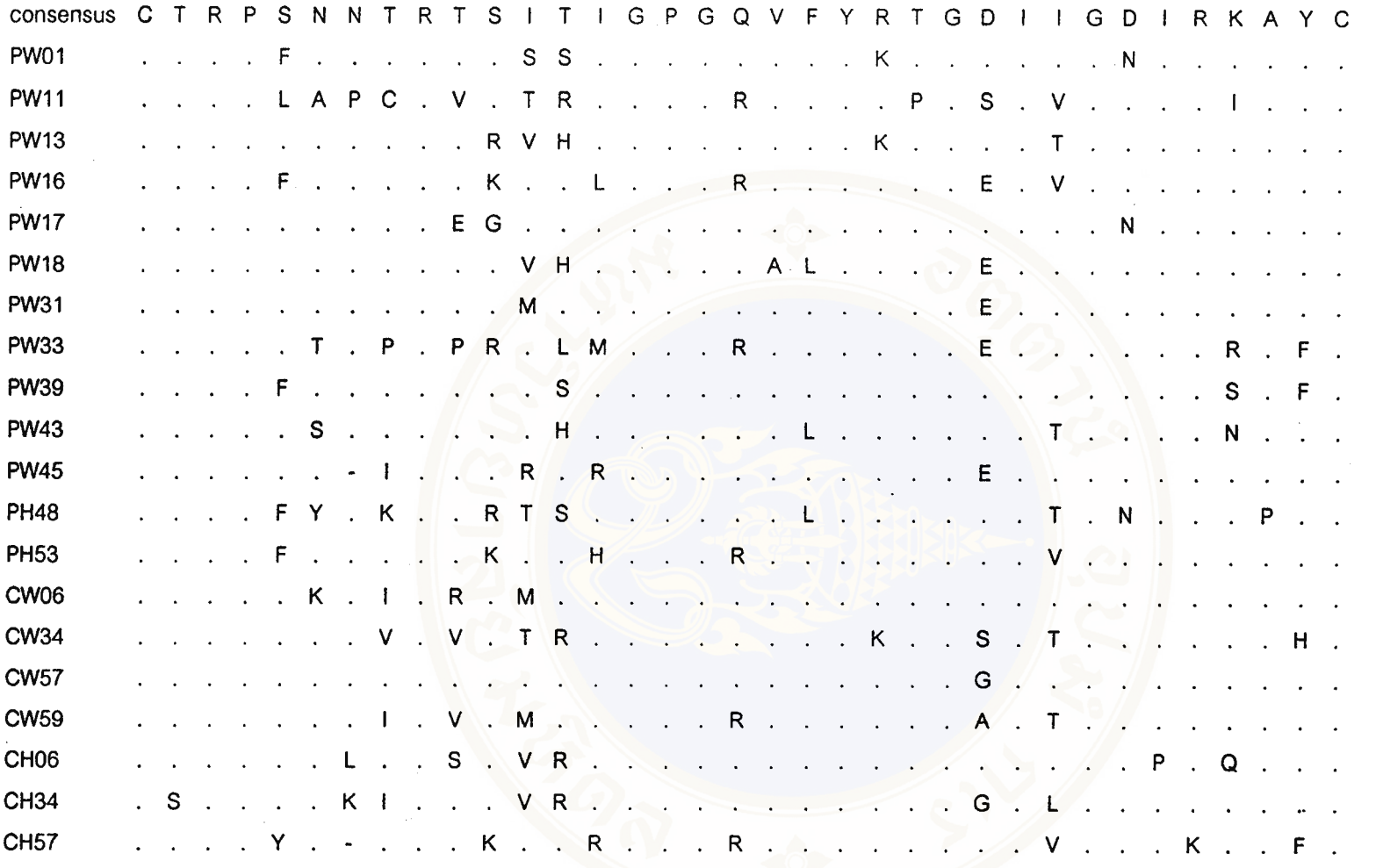

Figure 7. Alignment of predicted amino acid sequences (single letter) of V3 region of the HIV-1 isolates of partners of HEPS and concordant couples. A consensus sequence of V3 region of HIV-1 subtype E was shown. Predicted amino acid different from the consensus sequence were shown. The nucleotide similarity and deletion were indicated by dot and dash, respectively.

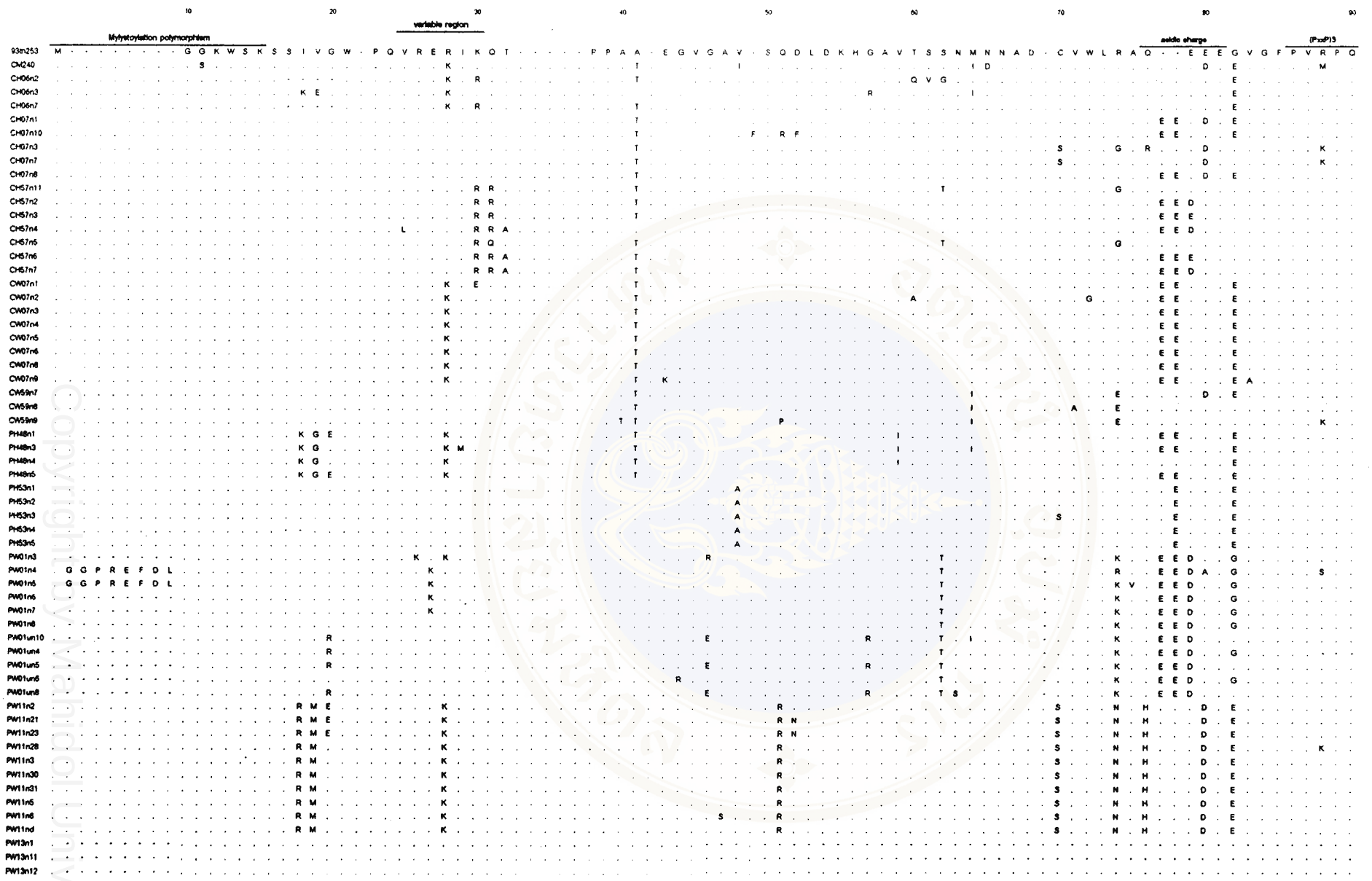

Figure 7. Alignment of predicted amino acid sequences (single letter) of Nef of the HIV-1 infected partners of HEPS and concordant couples was created by DNASTAR. A consensus sequence, 93th253, was shown. The number above the sequence indicated the position of amino acids. The critical regions within the Nef protein were shown above the consensus sequence. The amino acids similarity and deletion were indicated by dots and dashes, respectively.

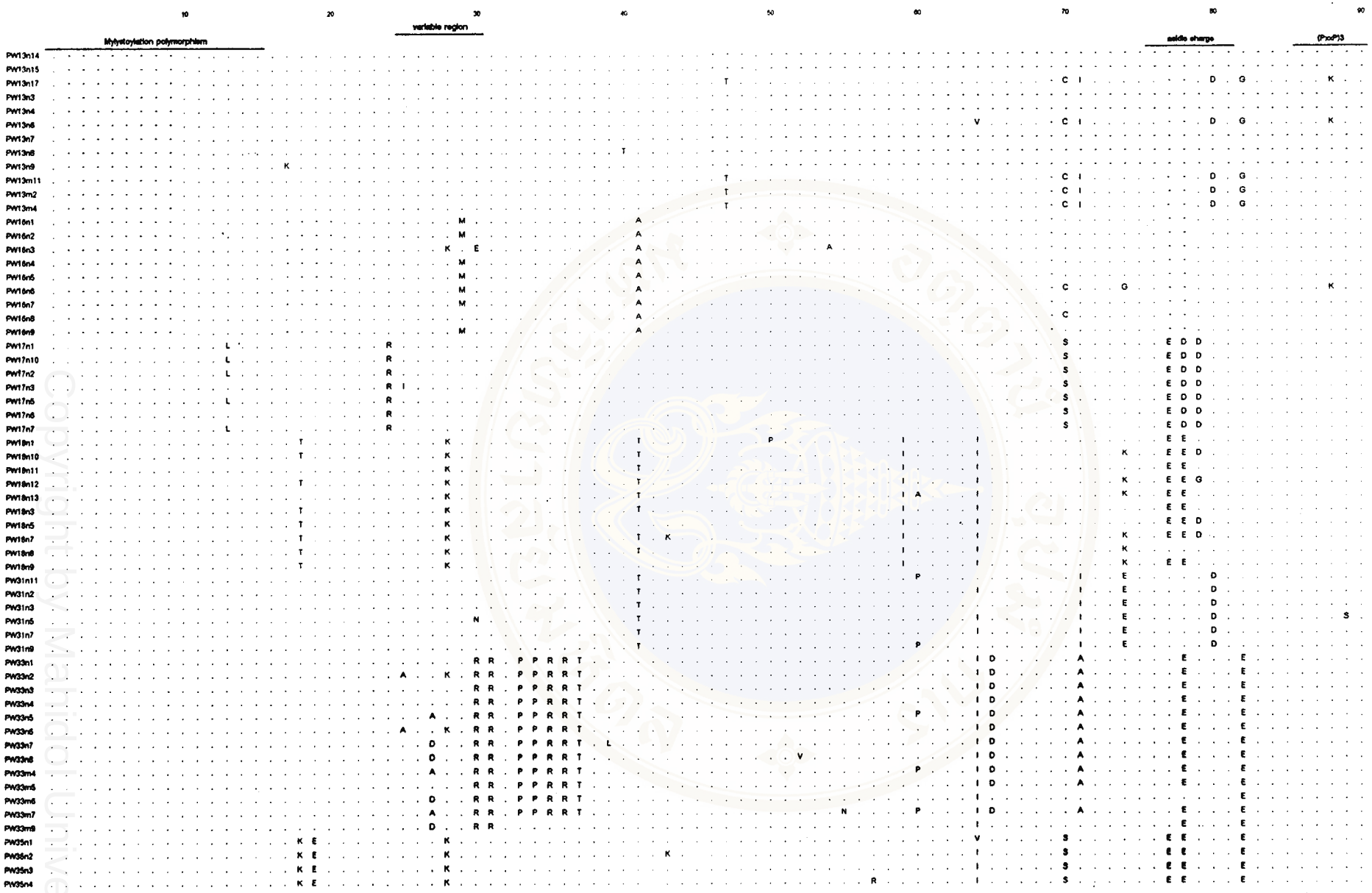

Figure 7. Alignment of predicted amino acid sequences (single letter) of Nef of the HIV-1 infected partners of HEPS and concordant couples was created by DNASTAR. A consensus sequence, 93th253, was shown. The number above the sequence indicated the position of amino acids. The critical regions within the Nef protein were shown above the consensus sequence. The amino acids similarity and deletion were indicated by dots and dashes, respectively.

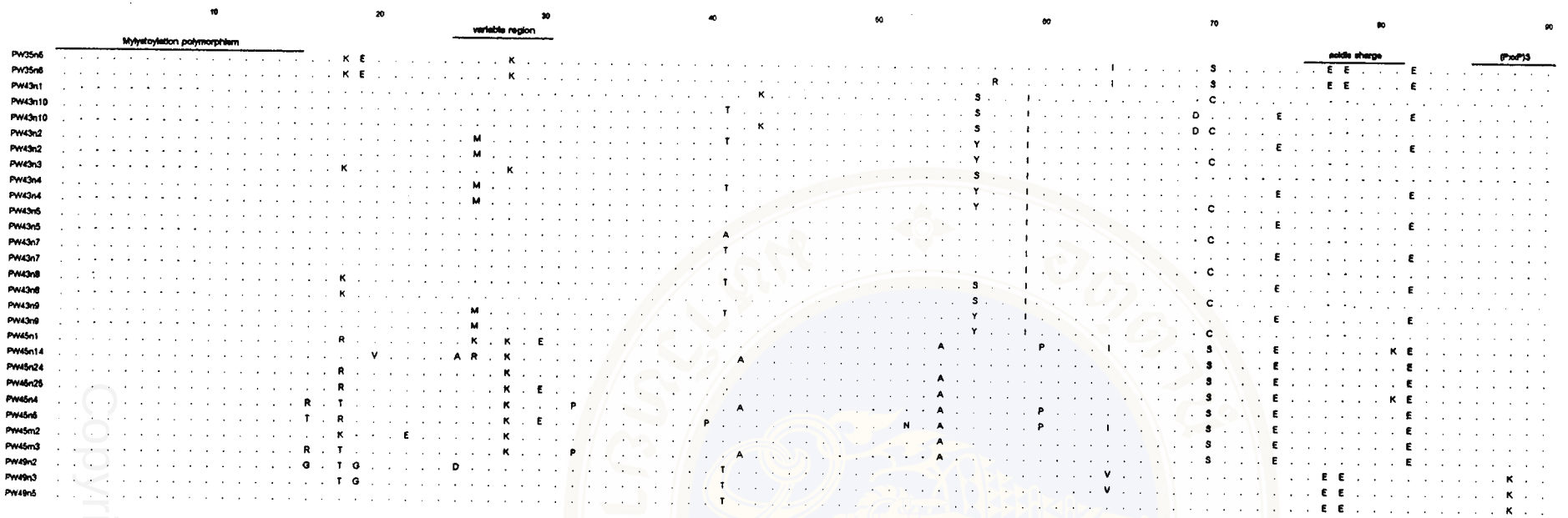

Figure 7. Alignment of predicted amino acid sequences (single letter) of Nef of the HIV-1 infected partners of HEPS and concordant couples was created by DNASTAR. A consensus sequence, 93th253, was shown. The number above the sequence indicated the position of amino acids. The critical regions within the Nef protein were shown above the consensus sequence. The amino acids similarity and deletion were indicated by dots and dashes, respectively.

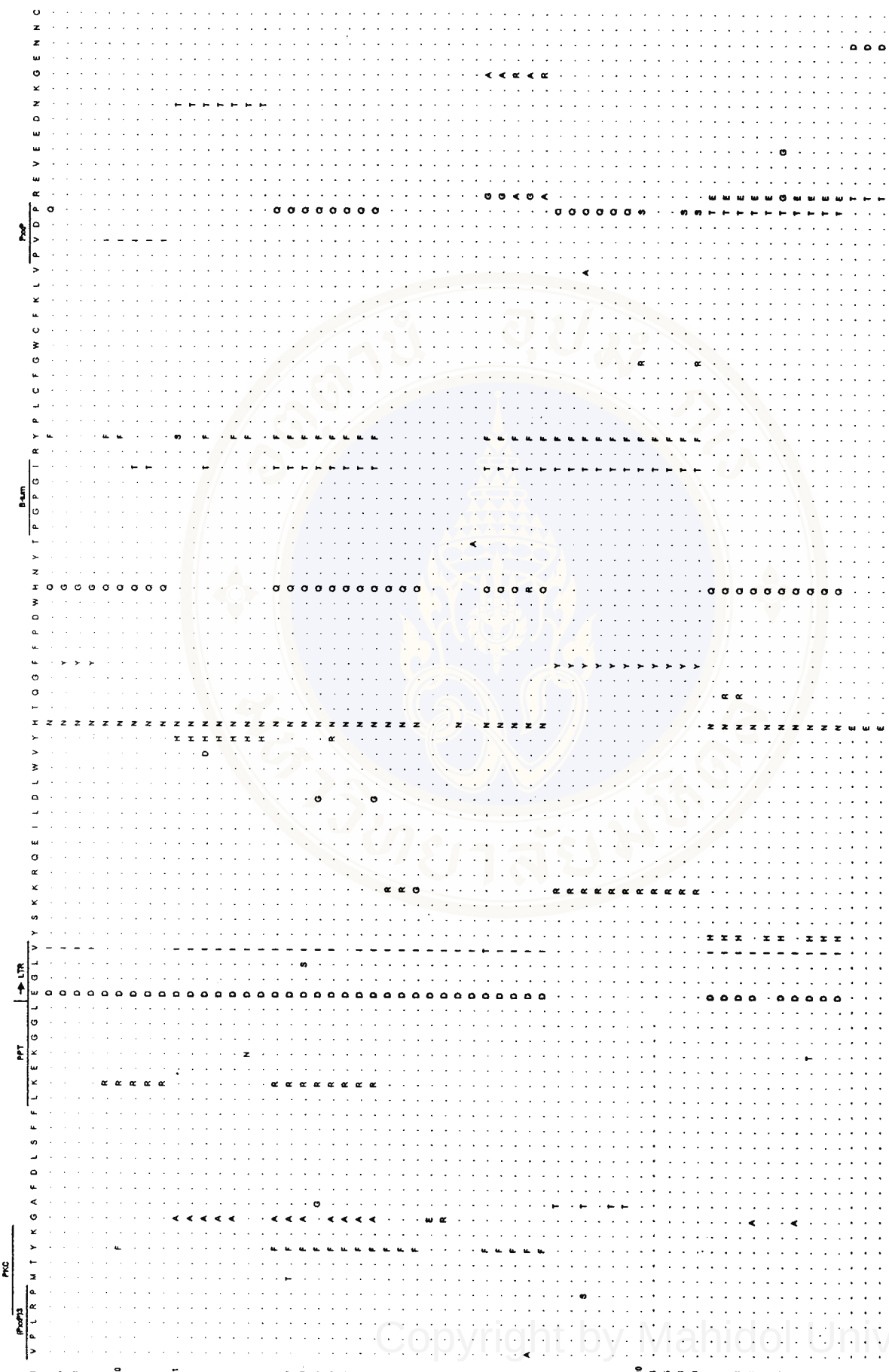

Figure 7. Alignment of predicted amino acid sequences (single letter) of Nef of the HIV-1 infected partners of HEPS and concordant couples (cont.).

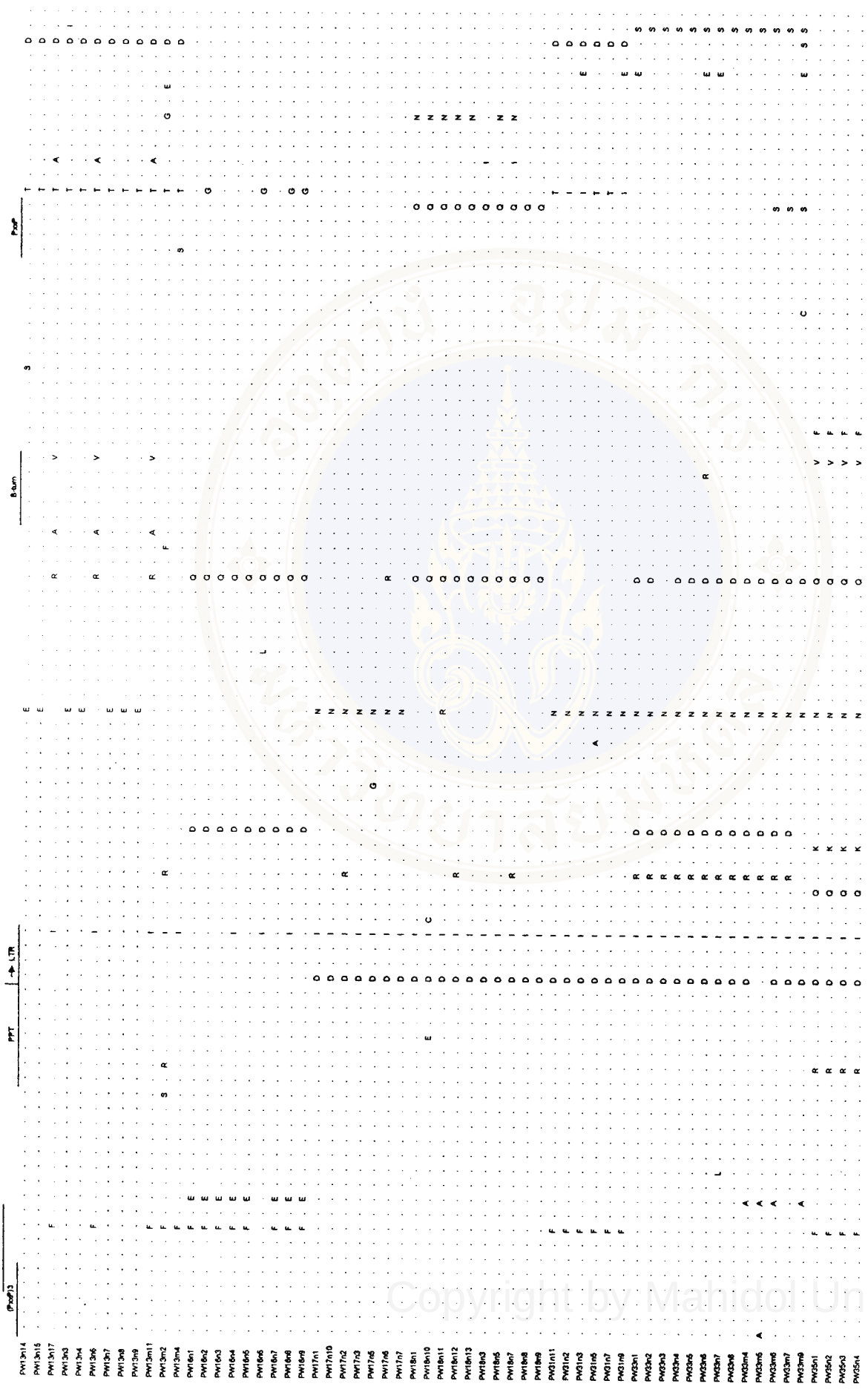

Figure 7. Alignment of predicted amino acid sequences (single letter) of Nef of the HIV-1 infected partners of HEPS and concordant couples (cont.).

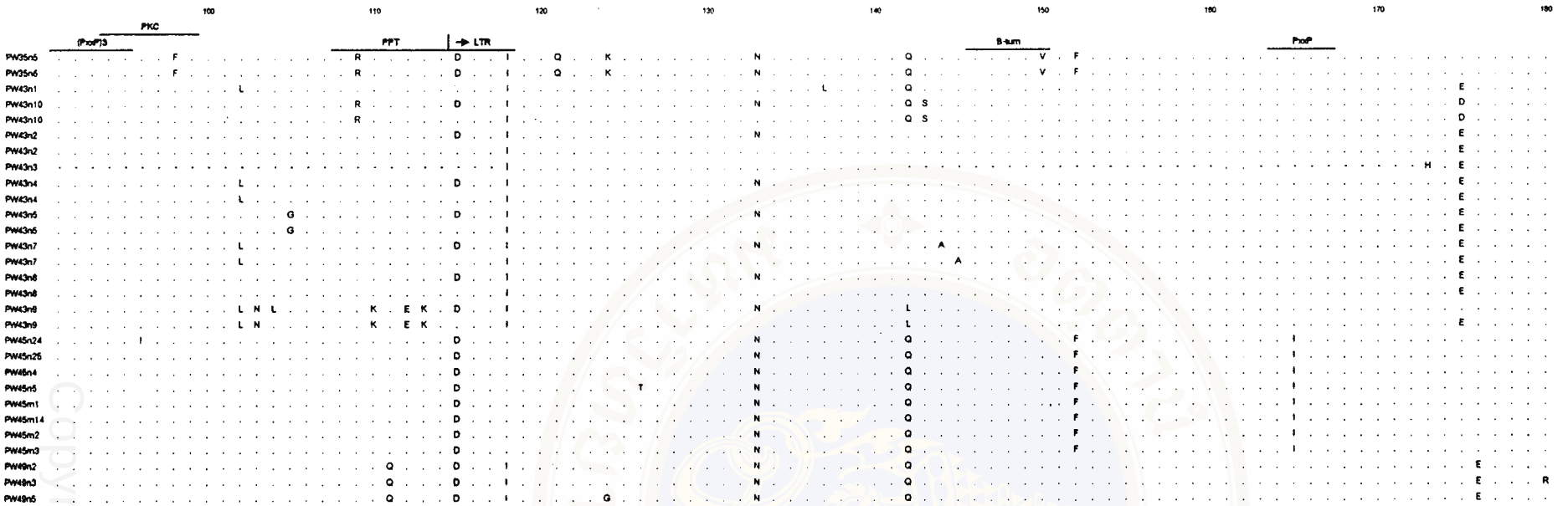

Figure 7. Alignment of predicted amino acid sequences (single letter) of Nef of the HIV-1 infected partners of HEPS and concordant couples (cont.).

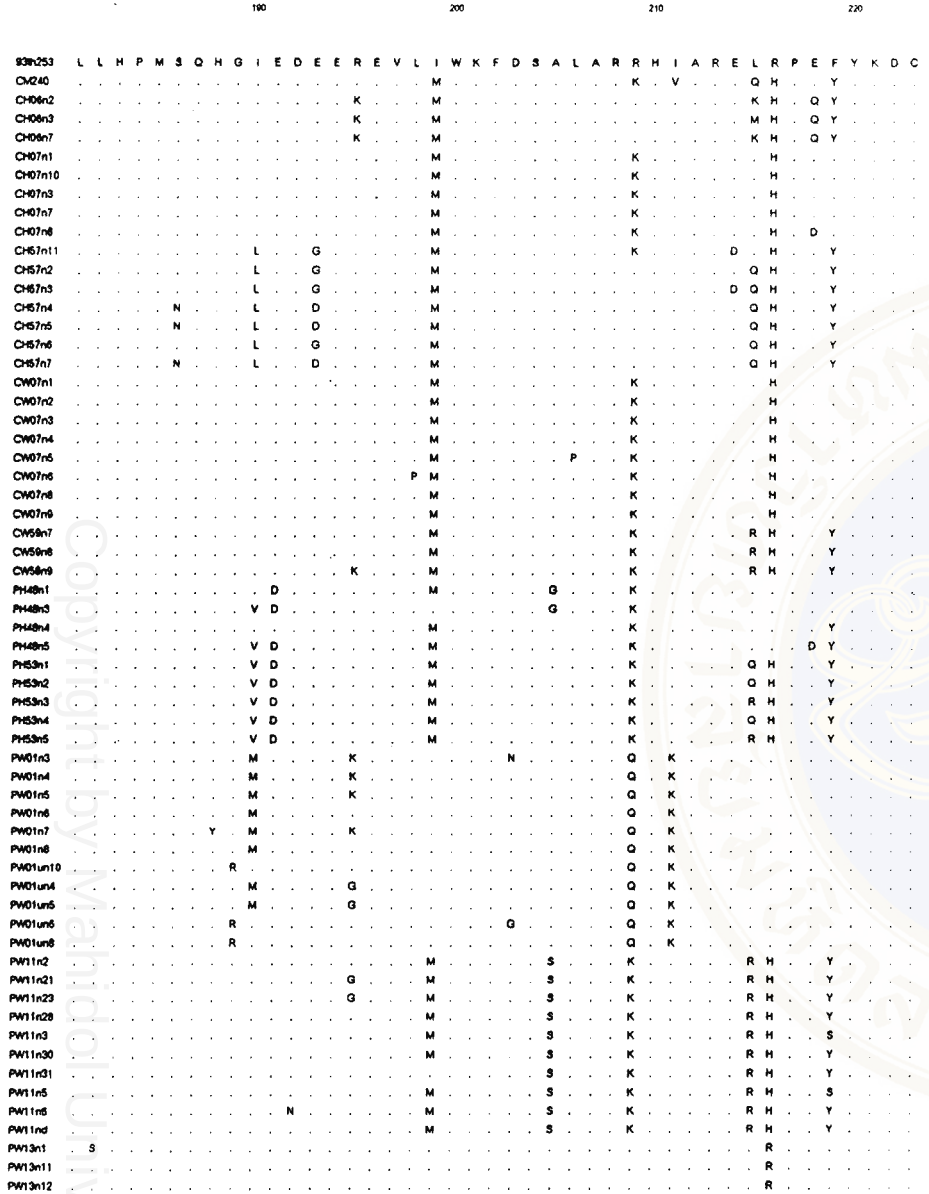

Figure 7. Alignment of predicted amino acid sequences (single letter) of Nef of the HIV-1 infected partners of HEPS and concordant couples (cont.).

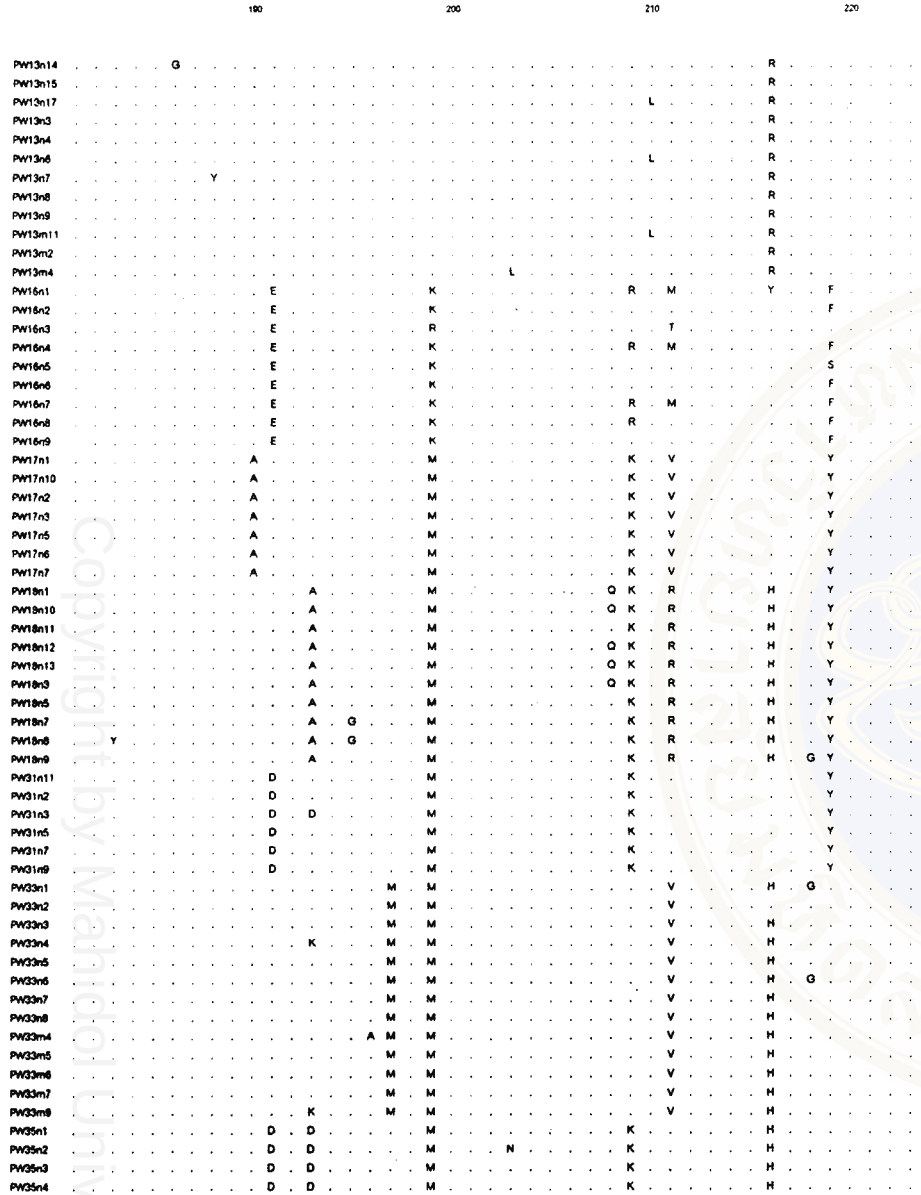

Figure 7. Alignment of predicted amino acid sequences (single letter) of Nef of the HIV-1 infected partners of HEPS and concordant couples (cont.).

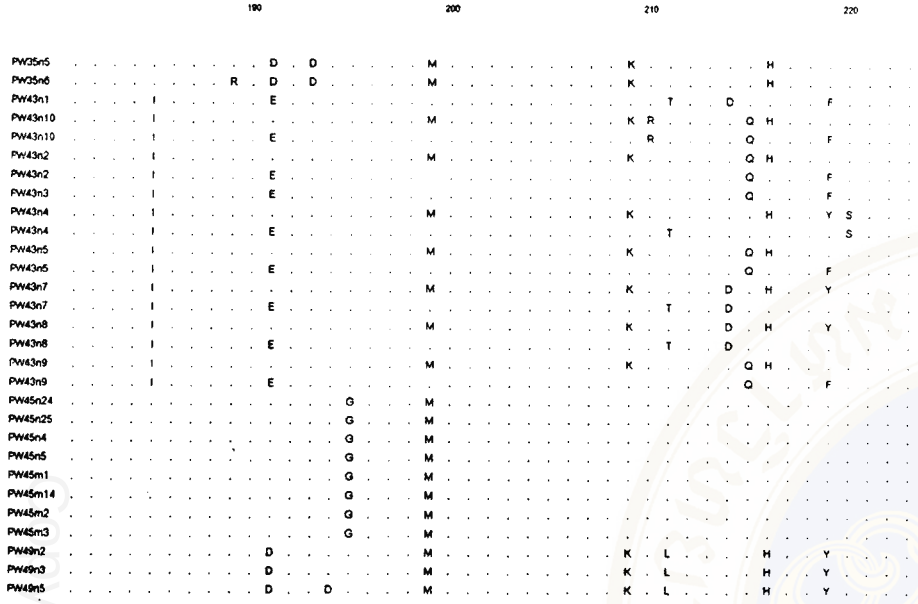

Figure 7. Alignment of predicted amino acid sequences (single letter) of Nef of the HIV-1 infected partners of HEPS and concordant couples (cont.).

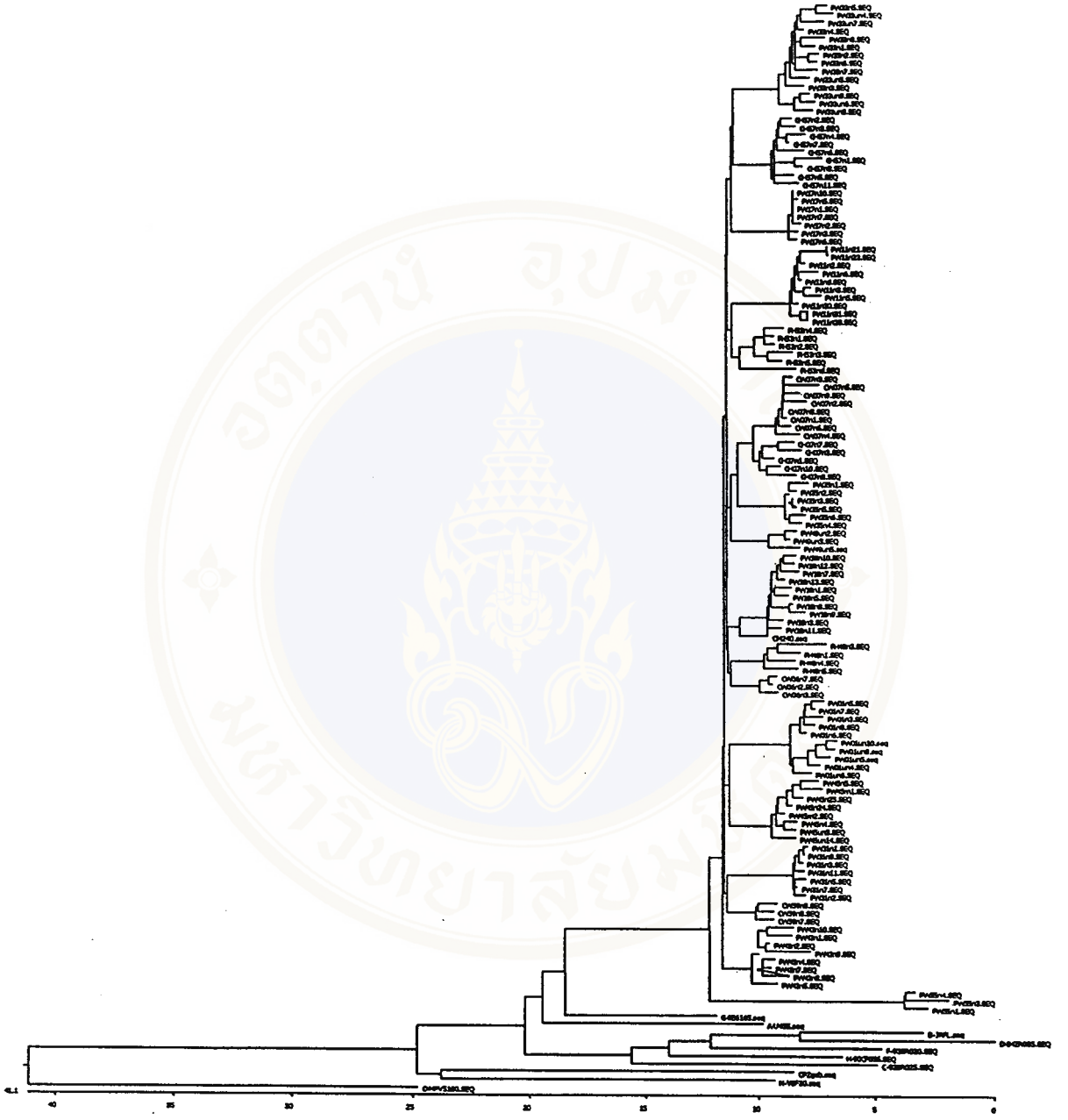

Figure 10. Phylogenetic analysis of the *nef* gene of HIV-1 infected partners of HEPS and concordant couples. Tree was constructed by Clustral and Neighbor Joining methods in DNASTAR software. The code of the HIV-1 isolates had been described in materials and methods. The reference subtypes were included, which letter A, B, C, D, E, F, G and H referred to HIV-1 subtypes followed by the name of reference isolates. N means Minor group of HIV-1 and CPZgab means SIV strain. The scale bar represented the percentage of nucleotide sequence divergence. The tree was unrooted and branch length was proportional to the number of nucleotide change.

2.3 Amplification of HIV-1 LTR

Amplification of LTR region from proviral DNA

The LTR region was amplified by nested PCR using specific oligonucleotide primers, PH5'KPN&PH3'LTR and PH5'KPN&PH56 as outer- and inner primers. The products of LTR region, 633 bp, were purified and used for direct sequencing and cloning.

Amplified of LTR region from genomic RNA

The PH5'KPN primer was used for reverse transcribing from RNA to cDNA. Then cDNA was further amplified by nested PCR. No deletion was found in the LTR region of HIV-1 infected partner of HEPS and concordant couples. Point mutations in the LTR region and phylogenetic tree were shown in Figure 10 and 11, respectively. Clones derived from PW01 and PW13 were found point mutations in the NF-*k*B I and SP1 II binding sites. There were found point mutations from clones derived from PW16 and PW39 in the SP1 I binding site, equal to 100% and 16.67%, respectively. Clones derived from PW31 were found the mutations in SP1 I (33.33%) and SP1 II (33.33%). Clones derived from PW43 were found the point mutations in the NF-kB I (57.14%), SP1 I (57.14%), SP1 II (57.14%) and SP1 III (14.29%). All LTR sequences were clustered into subtype E (Figure 11).

From above, PW01 was found *nef* deletion, point mutations in *nef* gene and LTR region. There were found *nef* deletion and point mutations in LTR from clones derived from PW13, PW16 and PW43. The point mutations in only *nef* gene found in clones derived from PW11, PW18, PW33, PH53, CW07 and CW59. Clones derived from PW31 and PW39 were found only point mutations in LTR region.

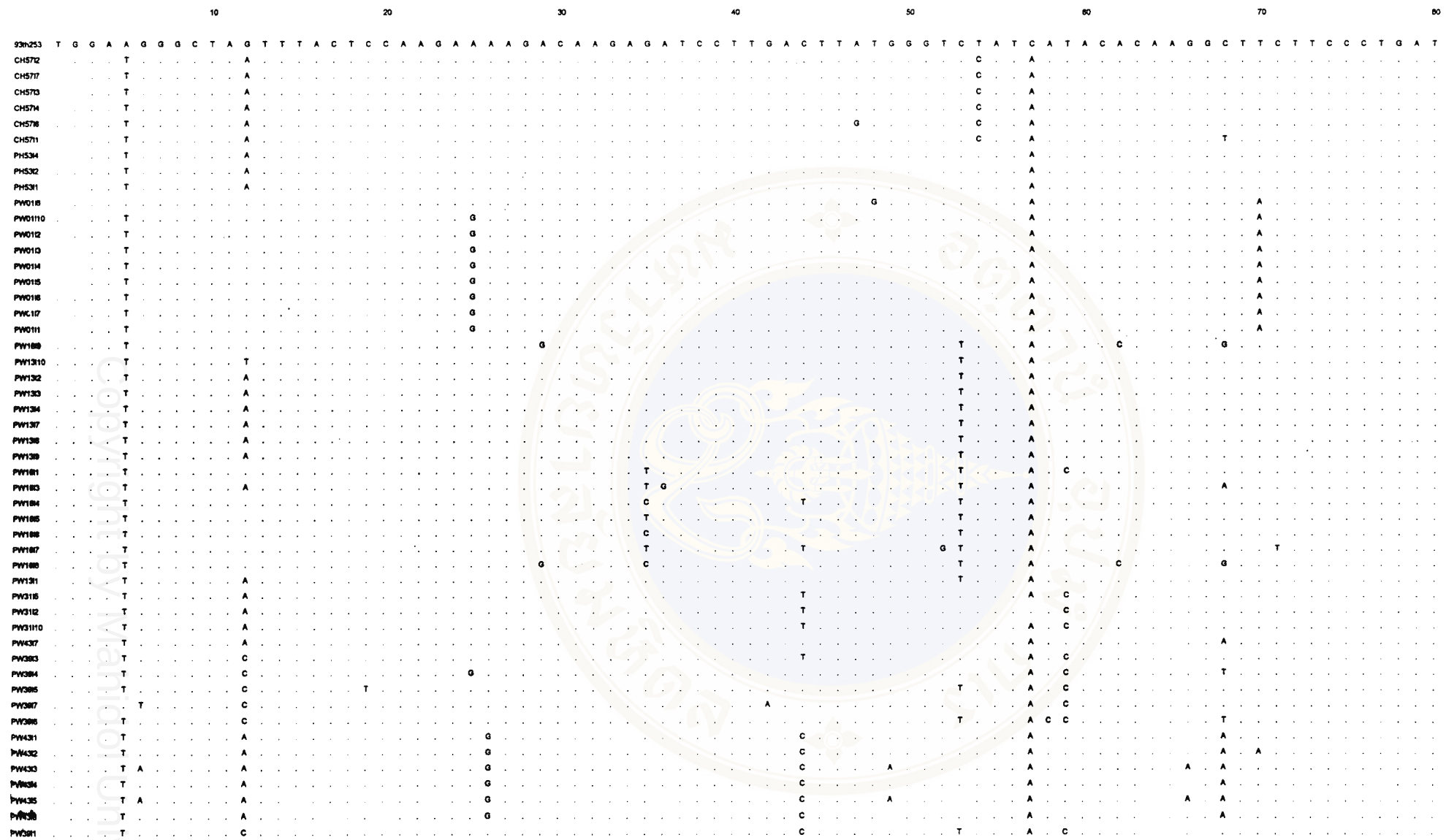

Figure 9. Alignment of nucleotide sequences of LTR region of the HIV-1 infected partners of HEPS and concordant couples was created by DNASTAR. A consensus sequence, 93th253, was shown. The number above the sequence indicated the position of nucleotides. The binding sites within the LTR region were shown above the consensus sequence. The nucleotide similarity and deletion were indicated by dots and dashes, respectively.

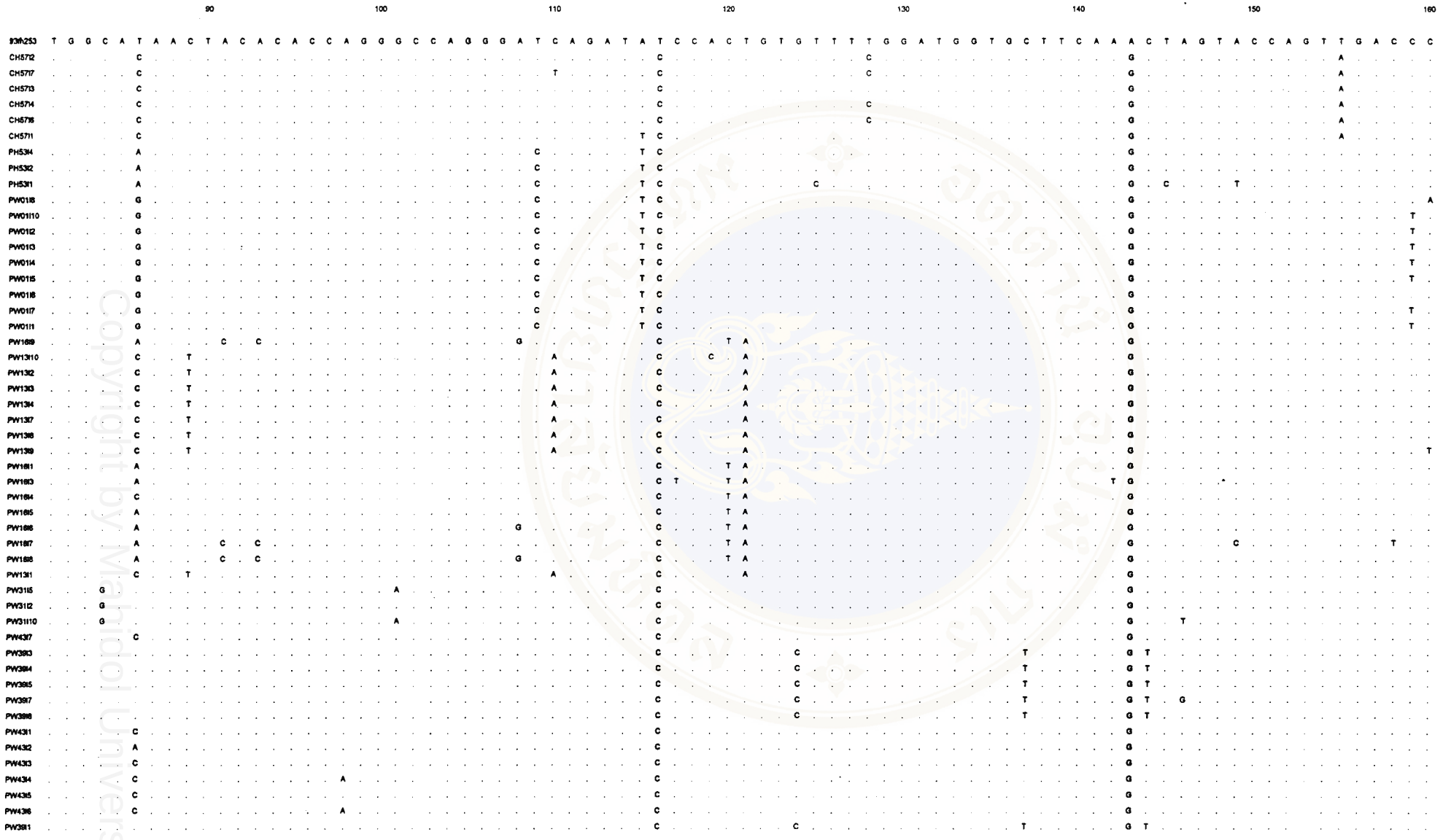

Figure 9. Alignment of nucleotide sequences of LTR region of the HIV-1 infected partners of HEPS and concordant couples (cont.).

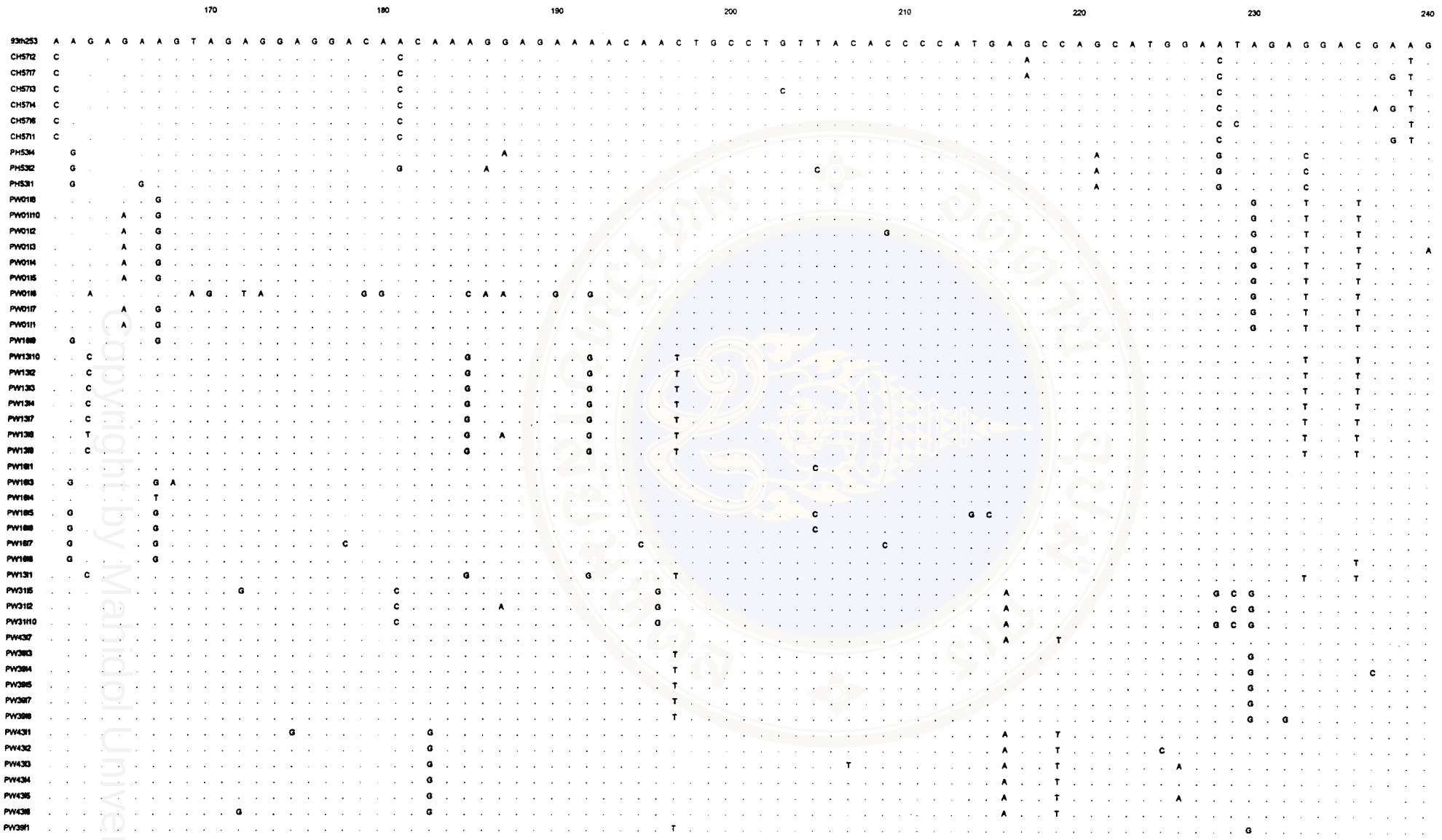

Figure 9. Alignment of nucleotide sequences of LTR region of the HIV-1 infected partners of HEPS and concordant couples (cont.)

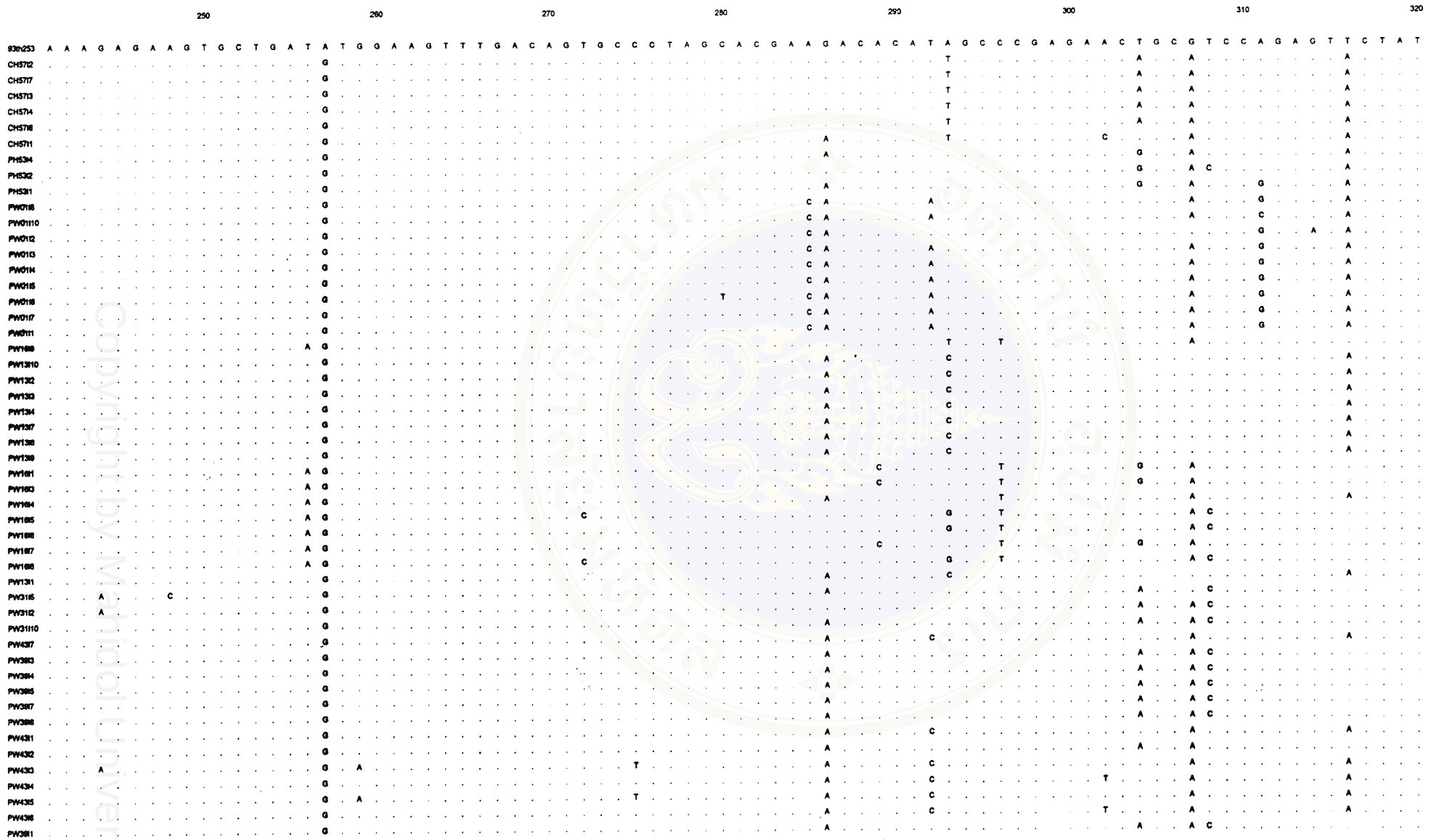

Figure 9. Alignment of nucleotide sequences of LTR region of the HIV-1 infected partners of HEPS and concordant couples (cont.).

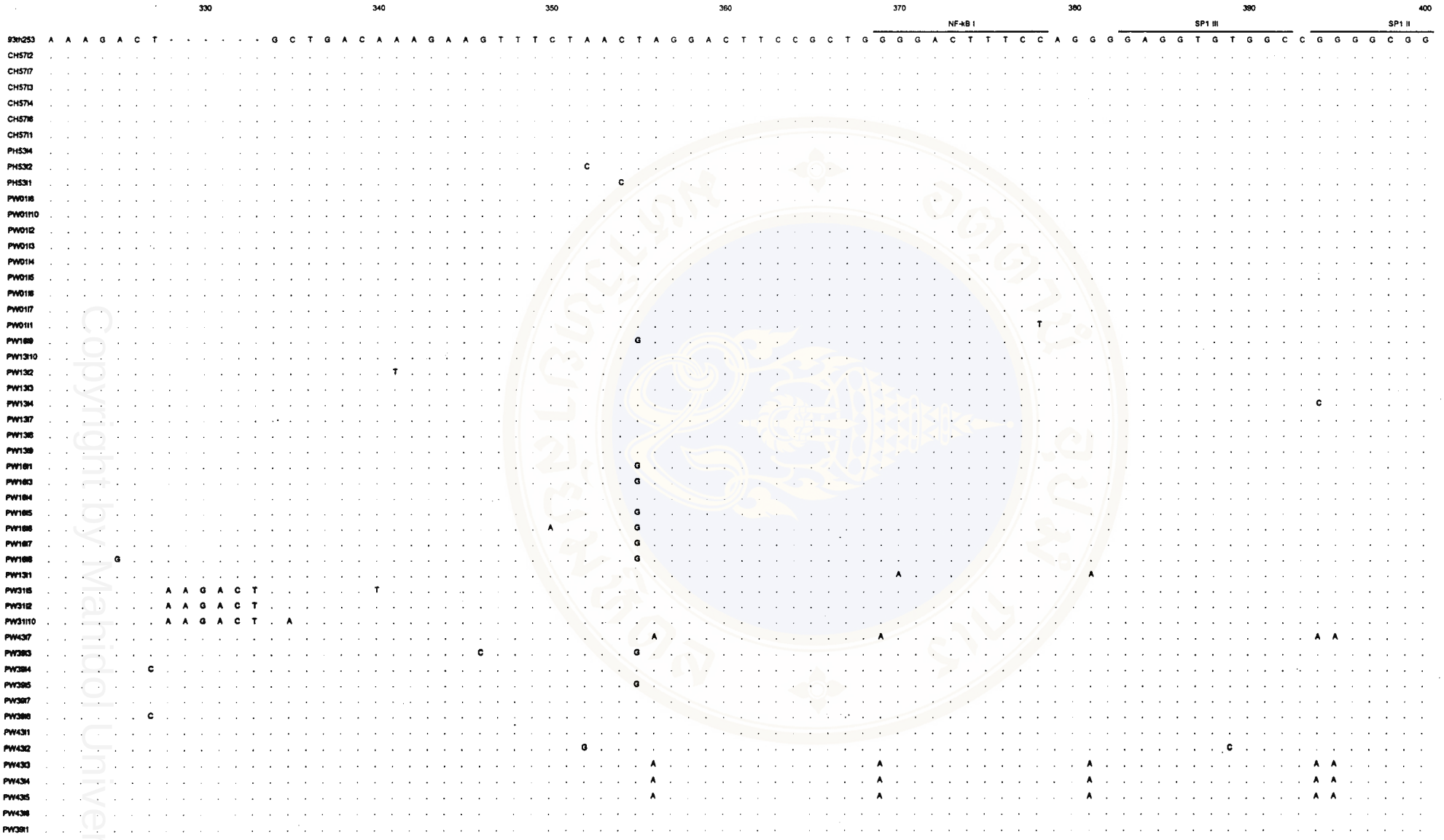

Figure 9. Alignment of nucleotide sequences of LTR region of the HIV-1 infected partners of HEPS and concordant couples (cont.).

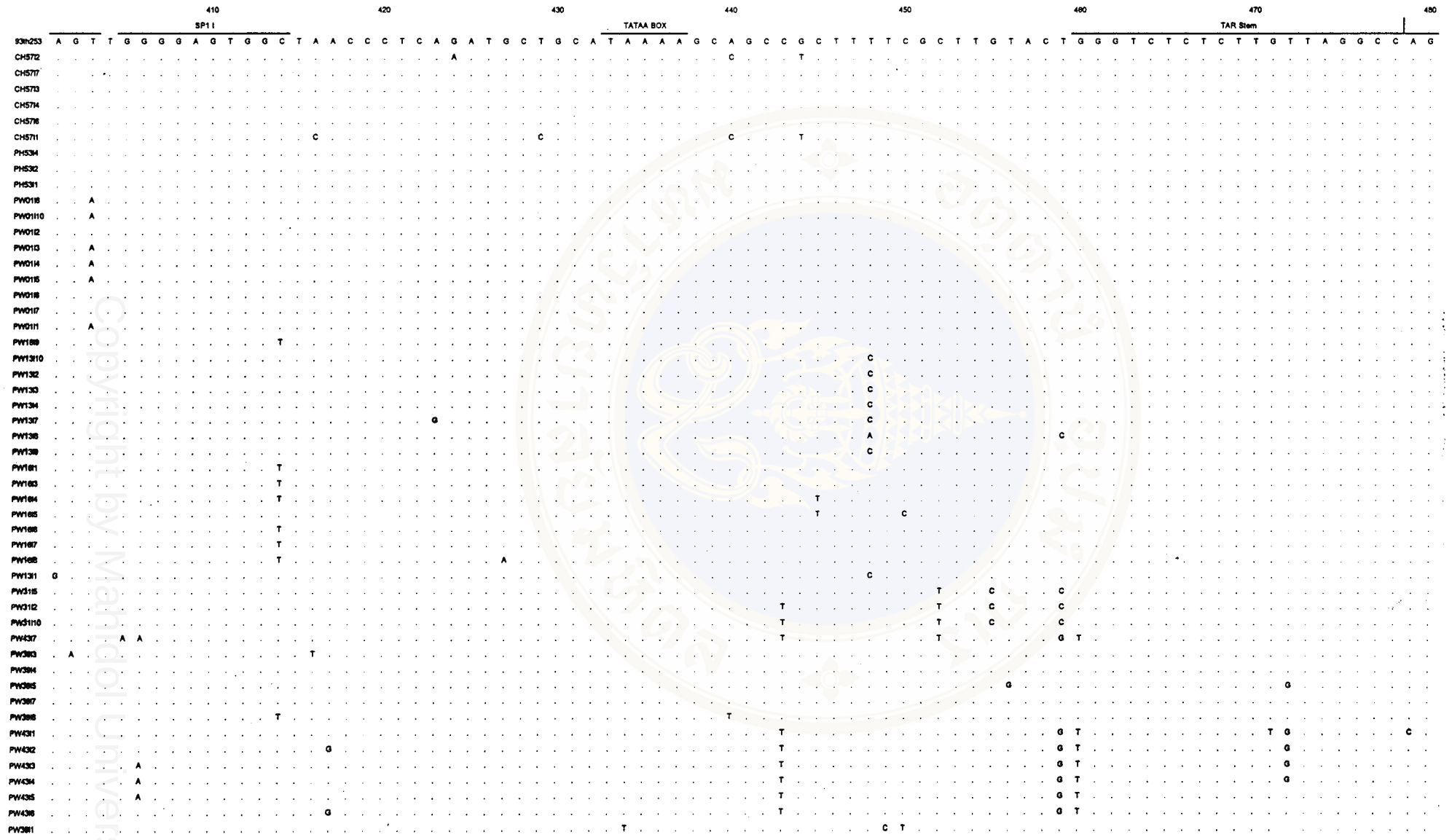

Figure 9. Alignment of nucleotide sequences of LTR region of the HIV-1 infected partners of HEPS and concordant couples (cont.).

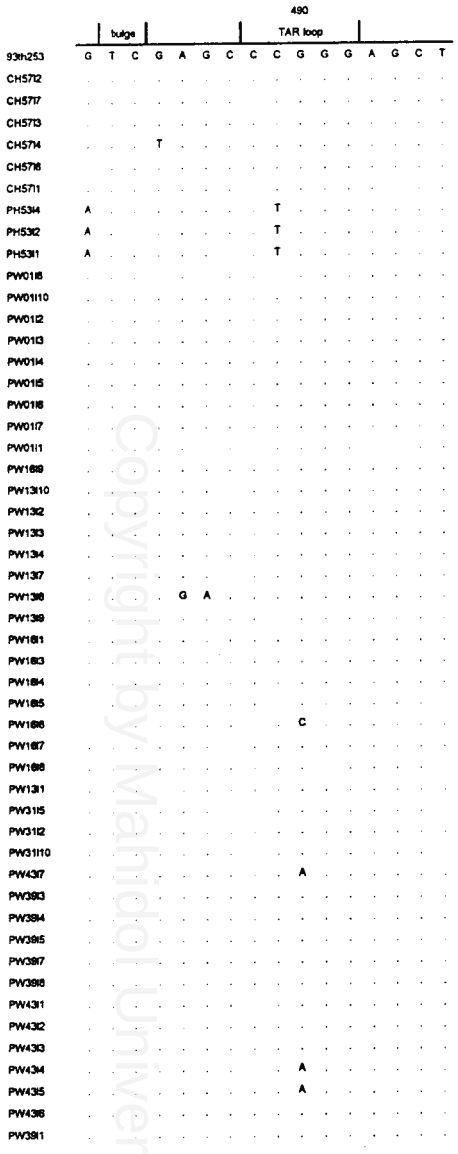

Figure 9. Alignment of nucleotide sequences of LTR region of the HIV-1 infected partners of HEPS and concordant couples (cont.).

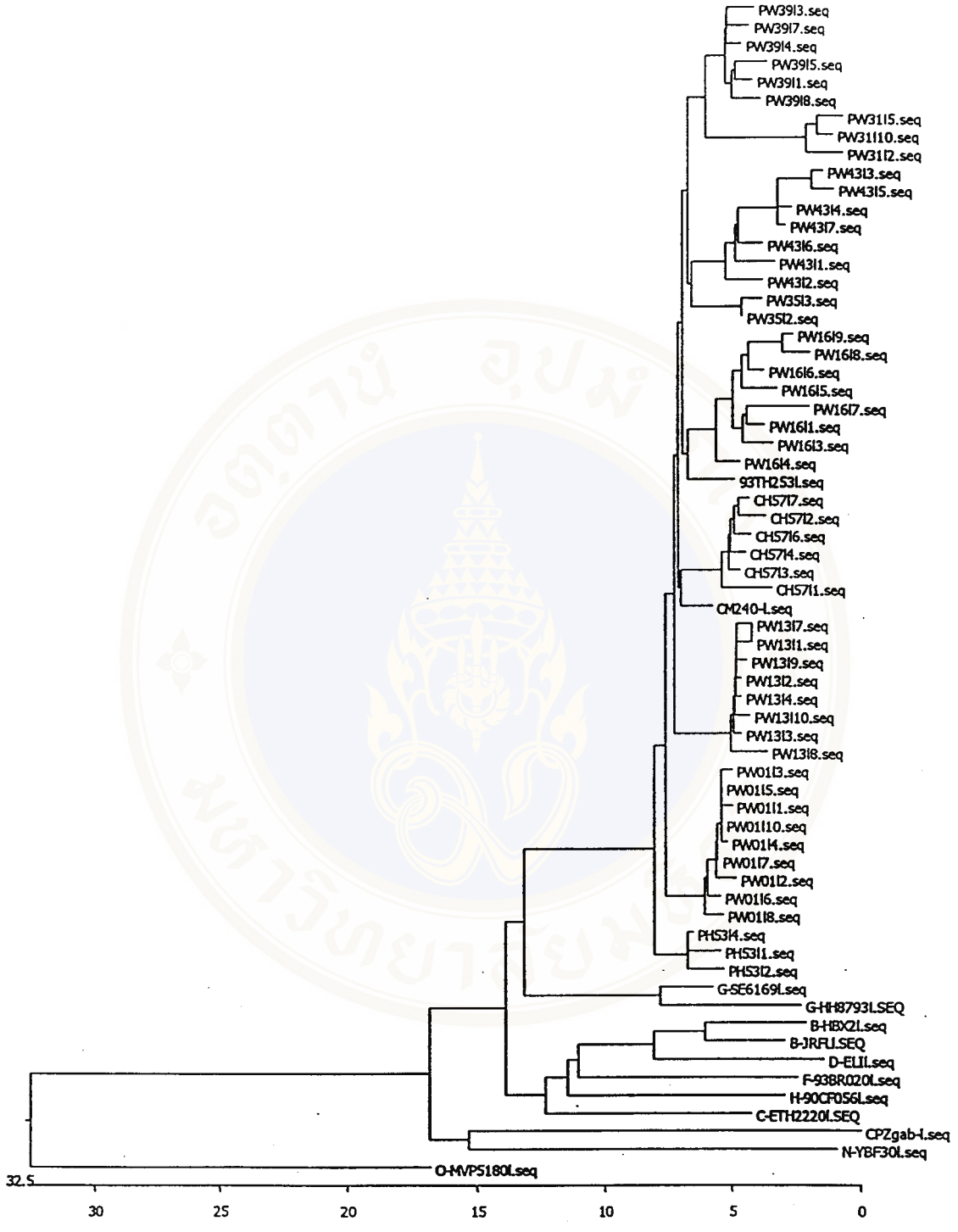

Figure 11. Phylogenetic analysis of the LTR region of HIV-1 infected partners of HEPS and concordant couples. Tree was constructed by Clustral and Neighbor Joining methods in DNASTAR software. The code of the HIV-1 isolates had been described in materials and methods. The reference subtypes were included, which letter A, B, C, D, E, F, G and H referred to HIV-1 subtypes followed by the name of reference isolates. N means Minor group of HIV-1 and CPZgab means SIV strain. The scale bar represented the percentage of nucleotide sequence divergence. The tree was unrooted and branch length was proportional to the number of nucleotide change.

3. HIV-1 phenotype

HIV-1 biological phenotypes were studied from 6 HIV-1 isolates. These were from partners of HEPS and from 7 isolates of concordant couples. All primary isolates were tested for tropism and SI activity.

Biotype of HIV-1 isolates from partner of HEPS and concordant couples

Syncytial inducing capacity was tested in the MT2 cell line. The criteria of SI phenotype was the ability of p24 production and syncytial formation in the MT2 cell line. The results were shown in Figure 12 and 13. All of the HIV-1 infected partners of HEPS were NSI, whereas 2/7 (28.57%) (CW33 and CW56) of HIV-1 isolates from the concordant couple were of SI phenotype.

T cell line tropism was performed in the SupT1 cell line and determined by p24 antigen production in SupT1 culture supernatant. The results were shown in Figure 13 and Table 5. Three out of six isolates from partners of HEPS (PW11, PW45 and PW55) and 4 of 7 isolates from concordant couples (CW33, CW34, CH34, and CW56) were T cell tropic virus.

The criteria of macrophage tropism was the ability of p24 production in primary macrophage culture. The results were shown in Figure 13 and Table 5. Five out of six isolates from partners of HEPS (PW11, PW33, PW45, PW49 and PH53) and 5/7 (71.43%) isolates from concordant couples (CW07, CW34, CH34, CH57 and CH59,) were macrophage tropic.

Of 6 isolates from HIV-1 infected partners of HEPS, 3 isolates (PW33, PW49 and PH53) were M-tropic viruses, one (PW55) was a T-tropic virus and 2 isolates (PW11 and PW45) retained both M-tropic and T-tropic (Dual tropic) viruses. Of 7

isolates from the concordant couples, 3 isolates (CW07, CH57 and CH59) were M-tropic viruses, two (CW33 and CW56) were T-tropic viruses and the last two isolates (CW34 and CH34) were Dual tropic viruses.

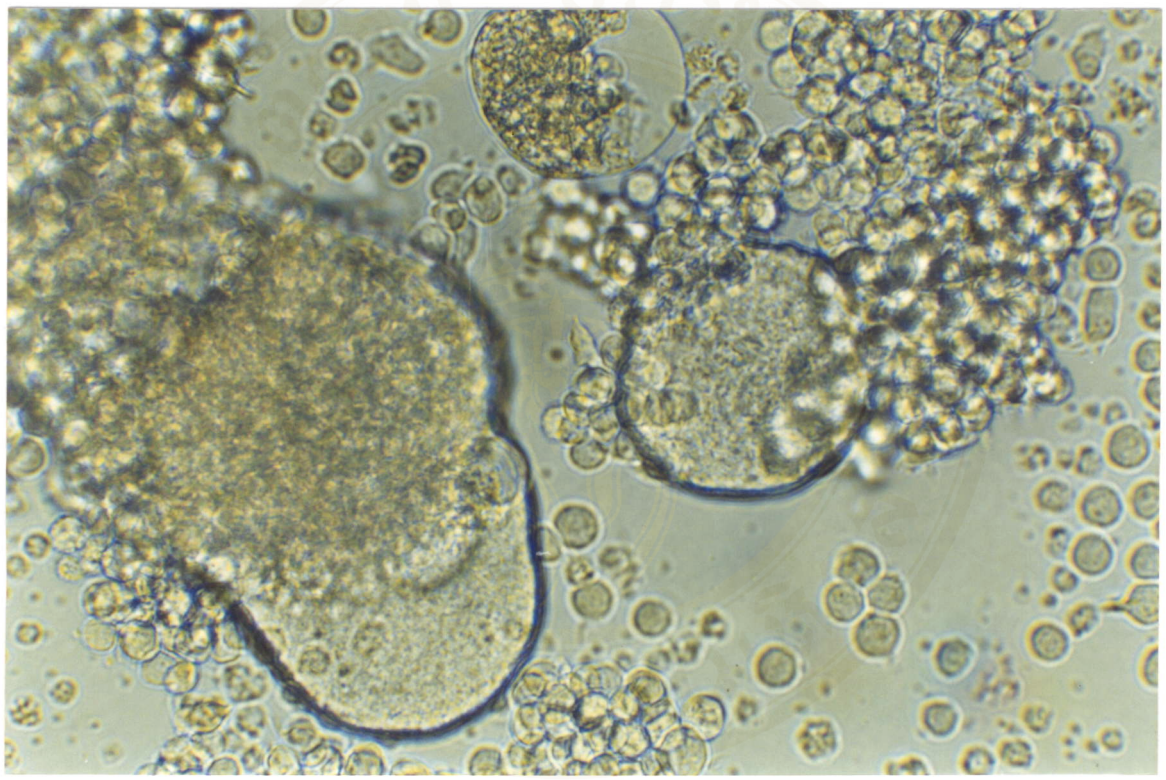

Figure 12. Cytopathic effect (syncytium formation) of HIV-1 subtype E in MT2 cell line. Multinucleated giant cells with large cytoplasm and cell membrane fusion are seen.

Table 5. Ability of p24 production of viruses of HIV-1 infected partners of HEPS and concordant couples. Each HIV-1 isolate was inoculated into each cell types and measured p24 production at day 7, 14, and 21. The value of p24 antigen more than 20 pg/ml was considered to be positive. SI/NSI ability was tested in MT2 cell line. T-cell tropism was tested in SupT1 cell line. M-tropic was tested in primary macrophage. SI was considered as ability to induce syncytial formation in MT2 cell line.

Patient code	macrophage			MT2			SupT1			NSI/SI	phenotype
	D7	D14	D21	D7	D14	D21	D7	D14	D21		
PW11	265.66	2918.64	>2918.64	241.06	-	-	182.63	>217.54	>217.54	NSI	D
PW33	58.38	75.41	>183.77	0	0	0	0	0	0	NSI	M
PW45	3.5	11.7	16.89	29.03	264.82	271.08	75.01	253.21	253.74	NSI	D
PW49	0	0	68.6	0	0	0	0	0	0	NSI	M
PH53	61.19	-	>217.54	0	0	0	0	0	0	NSI	M
PW55	0	3.83	1.82	69.13	711.29	1755.87	88.99	236.4	1933.35	NSI	T
CW07	243.35	220.52	246.26	0	0	0	0	0	0	NSI	M
CW33	0	0	0	92.06	>217.54	-	>217.54	>217.54	>217.54	SI	T
CW34	-	122.83	>217.54	-	38.9	92.46	-	81.27	123.6	NSI	D
CH34	2.74	77.76	252.29	24.91	55.77	69.36	48.69	59.51	85.71	NSI	D
CW56	0	0	0	122.83	>217.54	-	>217.54	>217.54	>217.54	SI	T
CH57	1.69	13.76	28.12	0	0	0	0	0	0	NSI	M
CH59	253.51	-	234.42	0	0	0	0	3.6	6.43	NSI	M

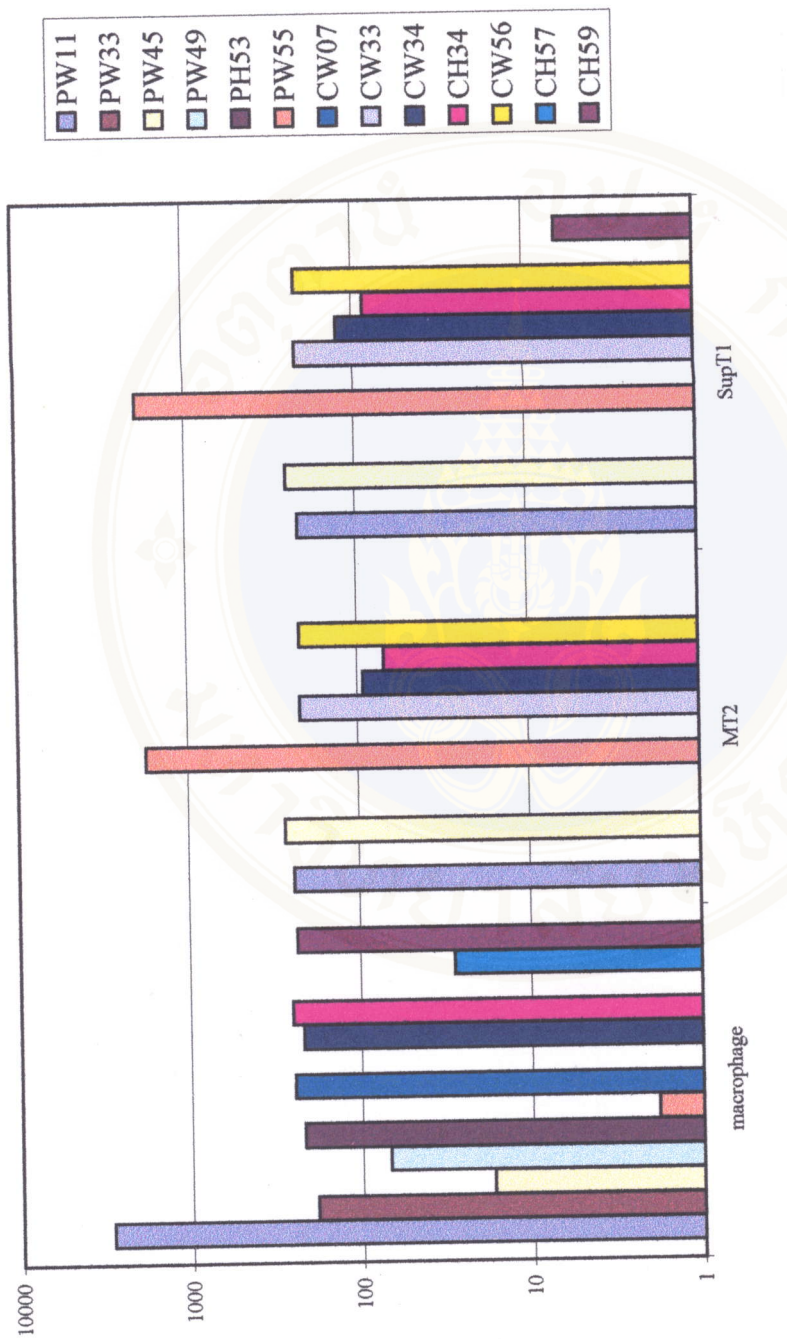

Figure 14. Replication capacity of viruses from HIV-1 infected partners of HEPS and concordant couples in MT2, SupT1 and primary macrophage at day 21. The p24 values were plotted in log scale.

4.Infection ability of HIV-1 primary isolates from partners of HEPS in PHA activated PBMC separated from HEPS

The peripheral blood mononuclear cell (PBMC) was separated from the blood of five HEPS (HH11, HH45, HH49, HW53 and HH55) and cultivated in RPMI-1640, which contains 15% FBS, antibiotic and PHA-P. They were infected by HIV-1 isolates from partners of HEPS. The capability of viral infection was compared between HEPS PBMC and normal donor PBMC. The results were shown in Figure 14. Neither PBMC from HEPS nor those from normal donors were different in supportive infection of the viruses.

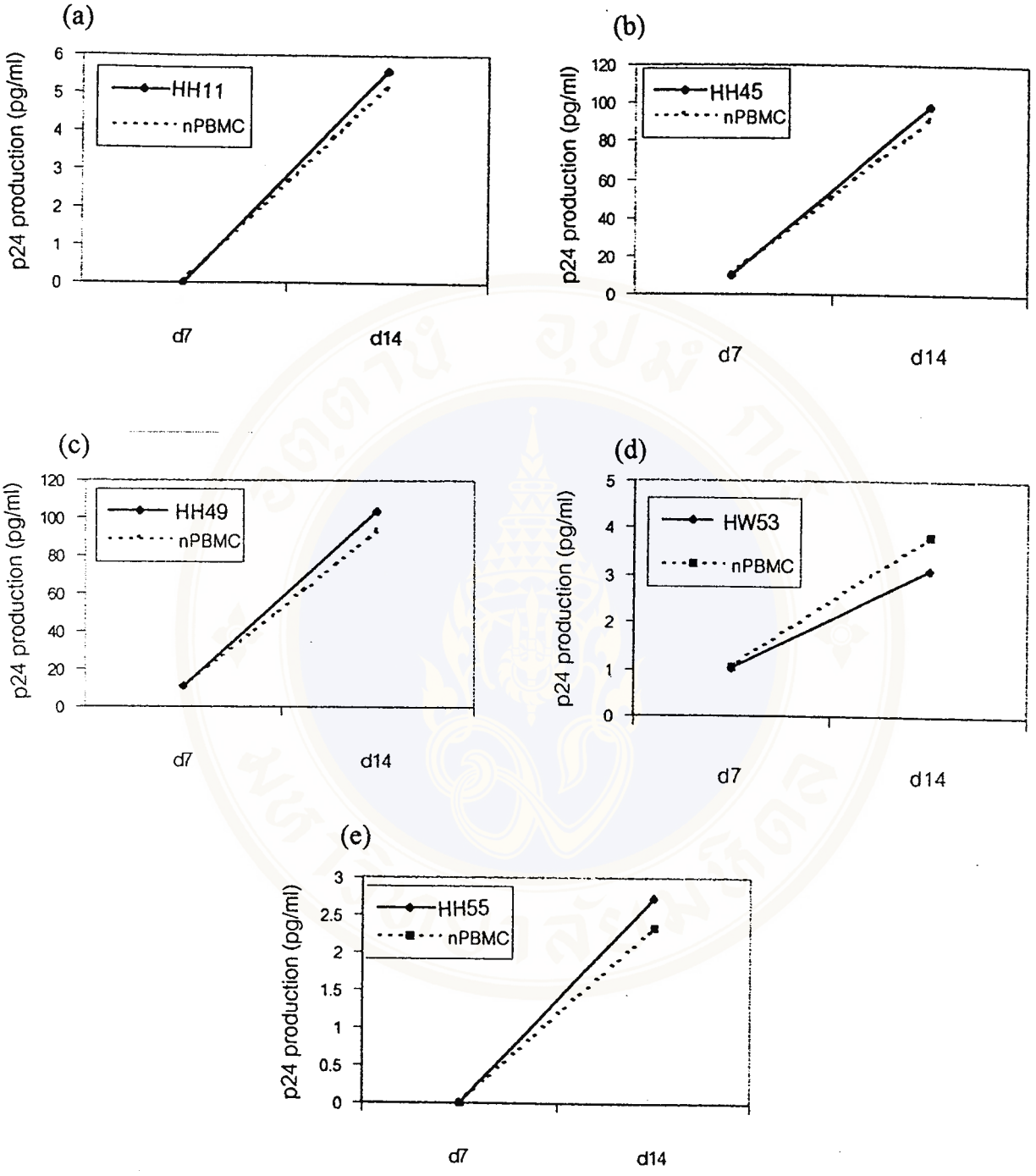

Figure 14. Infection ability of HIV-1 primary isolates of HIV-1 infected partners of HEPS in PBMC separated from HEPS. HIV-1 replication was detected by p24 detection at day 7 and 14. The line (-) referred PBMC from HEPS, dots (...) referred normal PBMC.

5. Titration of virus stock

Six primary HIV-1 isolates from partner of HEPS (PW11, PW33, PW45, PW49, PH53 and PW55), 5 isolates from concordant couples (CW07, CW34, CH34, CH57 and CH59), lab strain subtype B (IIIB), lab strain subtype B' (SL14) and lab strain subtype E (NPO3) were titrated in PHA-stimulated donor PBMC as described in materials and methods. The $TCID_{50}$ were shown in table 6. HIV-1 primary isolates had less $TCID_{50}$ than those of lab strains.

Table 6. Titration of the virus stock in PHA-P stimulated PBMC, the TCID50 values were calculated according to Spearman-Karber formula.

Viral strain	TCID50
Lab strains	
NPO3	6,988
IIIB	34,939
SL14	34,939
Viruses from partners of HEPS	
PW11	2660
PW45	3670
PW49	56
PH53	77
PW55	532
PW33	386
Concordant viruses	
PW07	40
PH34	3,670
PW34	1,012
PH57	77
PH59	77

6. Detection of neutralizing activities

The virus stock that was used in the neutralization assay, its $TCID_{50}$ must be more than 300. Therefore, only four of six isolates from the partners of HEPS (PW11, PW33, PW45 and PW55) and 2 of 6 isolates from concordant couples (CW34 and CH34) were selected (Table 6).

6.1 Neutralizing activities of lab strains by positive pool plasma

Three-pool plasma (P1, P2 and P3) was used for neutralization with lab strains (NPO3, IIIB and SL14). No pool plasma can neutralize SL14 (Table 7). NPO3 and IIIB isolates can be better neutralized by pool plasma P1 more than by the other two pool plasma.

Therefore, P1 was used for test neutralizing activity against the primary isolates (Table 7). All HIV-1 primary isolates from both partners of HEPS and from the concordant couple were neutralized by pool plasma P1 at ≥ 50% P24 reduction at dilution 1:20.

6.3 Neutralizing activities of plasma against HIV-1 isolates

Virus replication was accessed by measuring p24 antigen production, with appropriate controls being performed to ensure that there was no interference with p24 detection. Each plasma sample was titrated 1:20 and 1:200 and the ID_{50} values for each serum were determined (Table 7). If 50% neutralization was not achieved at a dilution of 1:20, it was scored as negative. No clear pattern of neutralization could be observed on the inspection of the tabulated ID_{50}.

The neutralizing activities of 11 plasma from HIV-1 infected partners of HEPS (PW01, PW11, PW13, PW16, PW35, PW43, PW45, PH48, PW49, PH53 and PW55), 2 concordant couples (CH07 & CW07 and CH57 & CW57), and pool plasma P1 against 4 HIV-1 primary isolates from partners of HEPS (PW11, PW43, PW45 and PW55), 2 primary isolates from concordant couples (CH34 and CW34) and 3 lab strains (NP03, SL14, and IIIB) were tested. Viruses from four out of eleven plasma of partner of HEPS (PW01, PW13, PW16 and PW43) showed *nef* deletion.

From the checkerboard, NPO3 HIV-1 subtype E lab strain was relatively sensitive to neutralization by multiple plasma. All plasma from partners of HEPS except PH48 and PH53 contained neutralizing activities against lab strain subtype E (NP03) less than those of plasma from concordant couples and pool plasma. Viruses from partners of HEPS and discordant couples were not found to show any difference in sensitivity to neutralization. However, the PW55 isolates from the partner of HEPS was resistant to neutralization by all of tested plasma. Plasma of these two concordant couples showed more broad neutralization against primary isolates than those of partners of HEPS.

Table 7. Reciprocal titer of ID_{50} values. Neutralizing activity of HIV-1 subtype E primary isolates (PW11, PW33, PW45, PW55, CH34 and CW34), lab strain subtype B (III B), lab strain subtype B' (SL14) and lab strain subtype E (NPO3) against pool plasma (P1, P2 and P3); plasma of HIV-1 infected partners of HEPS (PW11, PW35, PW45, PH48, PW49, PH53 and PW55); plasma of HIV-1 infected partners of HEPS who showed nef deletion (PW01, PW13, PW16 and PW13) and plasma of concordant couples (CH07, CW07, CH57 and CH57). The results were shown as the titer that reduced the p24 antigen production at least 50%.

	Reciprocal of ID50 for indicated HIV-1 isolates																
viruses	Pool Plasma			Plasma of HIV-1 infected partner of HEPS							Plasma of nef deletion subjects				plasma of concordant couples			
	P1	P2	P3	DW11	DW35	DW45	DH48	DW49	DH53	DW55	DPS01	DPS13	DPS16	DPS43	CH07	CW07	CH57	CW57
NPO3	200	20	20	20	20	20	200	20	200	20	20	20	20	20	200	200	200	200
IIIB	20	-	-	-	-	-	-	20	-	-	-	20	-	20	200	20	20	-
SL14	-	-	-	-	-	20	-	20	-	-	20	20	20	-	20	-	-	-
PW11	20	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	20	20	-	20
PW33	20	ND	ND	ND	ND	ND	ND	ND	ND	ND	20	200	20	-	20	-	20	-
PW45	20	ND	ND	ND	ND	ND	ND	ND	ND	ND	20	20	20	20	20	20	200	20
PW55	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-	-
CH34	20	ND	ND	ND	ND	ND	ND	ND	ND	ND	20	20	20	20	200	20	-	20
CW34	20	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-	-	-	200	-	200

7. Sequence of R5 gene from HIV-1 negative partners

To determine nucleotide sequence of the R5 gene, whose protein act as the secondary receptor of HIV-1 to enter the cell. Cell lysate from PBMC of 17 HEPS was extracted and used for PCR amplification. The R5 gene was amplified by a single round PCR using specific oligonucleotide primers (R2/F2). The amplified products, 1,810 bp, were visualized by agarose gel staining with ethidium bromide and purified by using a gel extraction kit (QIAGEN). The entire R-5 coding region was sequenced by automated sequencer ABI prism 310 (Perkin Elmer Cetus, CI, USA). No mutation sequence was detected in these HEPS's R5 gene (Figure 15).

```
  1  P I Y D I N Y Y T S E P C Q K I N V K Q I A A R L L P P L Y
 31  S L V F I F G F V G N M L V I L I L I N C K R L K S M T D I
 61  Y L L N L A I S D L F F L L T V P F W A H Y A A A Q W D F G
 91  N T M C Q L L T G L Y F I G F F S G I F F I I L L T I D R Y
121  L A V V H A V F A L K A R T V T F G V V T S V I T W V V A V
151  F A S L P G I I F T R S Q K E G L H Y T C S S H F P Y S Q Y
181  Q F W K N E Q T L K I V I L G L V L P L L V M V I C Y S G I
211  L K T L L R C R N E K K R H R A V R L I F T I M I V Y F L F
241  W A P Y N I V L L L N T F Q E F F G L N N C S S S N R L D Q
271  A M Q V T E T L G M T H C C I N P I I Y A F V G E K F R N Y
301  L L V F F Q K H I A K R F C K C C S I F Q Q E A P E R A S S
331  V Y T R S T G E Q E I S V G L
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Figure 15. Amino acid sequence (Single letter) of R5 gene. The number beside the sequence indicated the position of amino acids.

CHAPTER VI

DISCUSSION

Two phenomena have recently been the subject of extensive study in human immunodeficiency virus (HIV) infection: the absence of clinical progression in some HIV-infected patients (172), and the ability of some individuals to resist to HIV infection despite multiple and repeated exposure to HIV (126, 127). Substantial variation in the rates of disease progression has been observed among individuals infected with HIV-1. The median time to development of AIDS in most cohort is approximately 10 years following initial exposure to the virus (173). However, a small fraction of HIV-1-infected people remain clinically healthy and show no decline in CD4+ T cell counts even though they have been seropositive for 10 or more years (164, 168, 174). The mechanisms involved in the establishment of such long-term nonprogressive infection with HIV-1 are not clear. The immune response mounted by the host and the ability of the virus to replicate in the face of this response are two important factors that probably determine the course of the disease (22, 170, 174-176).

Resistance to HIV infection in HIV-exposed individuals is a relatively frequent condition that is observed in several different categories of individuals at high risk of infection. This condition is likely to depend on features of both the pathogen and the host, and has been repeatedly linked to the activation of HIV-specific cell-mediated immunity in the absence of detectable HIV-specific serum humoral immunity (174).

All HIV infected subjects in this study were heterosexually infected with HIV-1 subtype E, which is the most common subtype, spread in Thailand. They had no sign nor any symptom of AIDS at the time of sampling. Age of both HIV-1 infected partners of HEPS and concordant couples was 28.1 ± 9.2 years and 29.9 ± 6.3 years, respectively. Mean viral load of HIV-1 infected partners of HEPS was 4.20 ± 0.88 log copies/ml that was not significantly different from that of concordant couples (4.32 ± 0.45 log copies/ml). However, LTNP subjects who had *nef*-deleted viruses exhibited a lower median plasma viral load than the controls (3.78 vs 4.60 log copies/ml, respectively). These subjects also exhibited a lower virus isolation rate (65%) than in control (100%).

In this experiment four deletion patterns of *nef* gene were found in HIV-1 isolates from partner of HEPS. First, four amino acid deletions were found in clone derived from PW16. The deletion in this region has no effect in viral infectivity and replication. Second, the deletion pattern was 86 amino acids in length and was located in the *nef*-unique portion from amino acid 39 to 124 in clones derived from PW13. Third pattern found in clones derived from PW43, two regions, 47 amino acids and 31 amino acids, were deleted and located in the *nef*-unique portion from amino acid 62 to 108 and 134 to 164, respectively. These two deletion patterns removed a highly conserve domain and a highly conserved $(PXXP)_3$ motif. The last pattern was the deleted of 26 amino acids (80-105) in clones derived from PW01. It lacked a highly conserved polyproline tract repeated. Plasma HIV-1 RNA quantity of PW01, PW13, PW16 and PW43 was 3.03, 5.45, 4.90 and 4.76 log copies/ml, respectively, which were not significantly different from those of non-deleted *nef*. However, these *nef*-

deleted viruses could not be isolated by coculture. These productive viruses might be too defective or less ability of infection.

Defect in viral genes were linked to nonprogressive infection. Rhesus monkeys experimentally inoculated with simian immunodeficiency virus (SIV) carrying deletions in the *nef* gene had low viral load and normal $CD4^+$ T cell counts and showed no signs of disease progression (22). These characteristics were very similar to those of individuals with long-term nonprogressive HIV-1 infection (175-177). An early study documented one long-term nonprogressor infected only with *nef* deleted HIV-1 since 1983 (170). Deacon *et al* (171) reported that six Australian long-term nonprogressors were infected with *nef* deleted HIV-1 from a single blood donor. Moreover, Mariani *et al,* showed that one individual with nonprogressive HIV-1 infection yielded an unusually high frequency of disrupted *nef* open reading frames and Nef proteins defective for CD4 downregulation. These results demonstrate that viral defects can be responsible for, or at least contribute to, the absence of disease progression in SIV-infected rhesus monkeys and HIV-1-infected humans.

The mutation rate of HIV-1 depends on the number of replication cycles, the growth rate of the viral population and the infidelity of the viral polymerase. The evolution rate is dependent not only on this mutation rate, but also on the positive selection for variation of environmental conditions and the negative selection against variation imposed by the functional constraints of the virus. The molecular basis of the variation is the viral polymerase error occurring in the absence of exonuclease activity (proof reading). Error frequency rates originally determined on DNA template range from 1/1,700 to 1/7,400 nucleotides (178-180). In addition to single-base substitutions,

minus-one frame and sequence changes involving more than one nucleotide have been reported to occur frequently during DNA synthesis by RT (181).

Recombination adds further to the complexity of genetic variation by spreading variation from one genome to another. Retrovirus recombination, which requires a heterozygous virion (182), can be highly efficient. Minus-strand recombination may increase the virus variability by producing a normal recombination from two RNA molecules with different deleterious mutations (183).

Furthermore, point mutations were also found in the *nef* gene and in the LTR region of HIV-1 infected partners of HEPS and concordant couple. These mutations resulted in removed of the PxxP at amino acid position 75 to 78 in clone derived from PH53 (100%), PW01 (10%) and CW59 (100%) and at position 145 to 148 in clone derived from PW01 (80%), PW11 (100%), PW18 (100%), PW33 (23.07%) and CW07 (100%).

The type II polyproline helix is formed by residues that comprise a consensus sequence for SH3 binding (PXXP), where X is any amino acid residue. These residues are essential for the enhancement of viral infectivity. Thus, in the absence of the polyproline repeat, downregulation of the CD4 receptor was observed by Goldsmith *et al* (184), indicating that the polyproline repeat play no role in the reduction of CD4 counts. The PXXP motif is essential for maximal proliferation of the HIV-1 virus in primary cell cultures, and it is thus likely that interaction of Nef with the Src family of protein may be responsible for the enhancement of viral replication and infectivity (185).

Rhesus monkeys inoculated with a derivative of the pathogenic SIV_{mac239} clone containing a 182-bp deletion in *nef* became infected and persistently antibody

positive. However, they had extremely low viral burdens, normal $CD4^+$ lymphocyte concentrations and no sign of disease progression (22, 186, 187). In these monkeys, additional deletions accumulate over time in the region of *nef* that overlap U3, without affecting the critical *cis*-acting sequences. The *nef* sequence that overlap U3 are apparently not advantageous to the virus in the absence of an intact *nef* gene and are selectively lost.

Huang *et al.* (188), suggested that deletion in *nef* may not be a common explanation for the absence of progression, and that different factors are likely to contribute in other patients. Viral factors that could contribute include different type of mutations in a wide variety of viral genetic elements. Viral and host factors cannot be dissociated from each other, since an effective immune response is an essential feature of non-transmission. Disease outcome of the seropositive partner is likely to be determined by a delicate balance between the ability of the virus to replicate and the host's ability to mount an adequate immune response.

The *nef* deletion and point mutations in the *nef* gene and in the LTR region in HIV-1 subtype E infected partners of HEPS seemed to be non-transmitted to the partner, who had repeated unprotected sexual relation for several years. Sexual transmission of HIV-1 in humans is a highly complex process. Among the variables involved are the precise transmission route and the interactions between multiple viral and host factors. In some of these cases there might be a low inoculum level or a too defective virus to transmit to their partners. The finding of HIV-1 variants with *nef* deletion in HIV-1-infected partners of HEPS provides additional impetus for consideration of the vaccine approach.

The main selection pressures are preservation of function and host immune response. The mutated nef gene might have fewer epitopes, and as such be less visible to the specific immune response, but it will lose some function. The advantage of the mutation might help the attenuated viruses escape the immune response.

Nef is a myristoylated, membrane-associated protein, and is one of the earliest viral gene products expressed in newly infected target cells. Although Nef is clearly important for viral replication *in vivo*, it is difficult to determine the basis for this. Several claims were made on the function of the *nef* gene product, many of which were disputed or unconfirmed, and yet the precise role of Nef was not completely clarified. However, several consistent consequences of Nef expression were defined: enhancement of viral replication, downregulation of cell surface CD4 and HLA class I molecules, and signal transduction in cells (184, 189-194).

Multiple HLA-restricted cytotoxic T lymphocyte (CTL) epitopes were identified in Nef, and CTLs targeted against these epitopes were postulated to play a role in protection from infection in HIV-1 exposed subjects (195, 196). Nevertheless, impaired CTL recognition due to genetic variations was observed in the main immunogenic region of the HIV-1 Nef protein (197, 198).

Furthermore, NF-kB and SP1 binding sites were found the point mutation. Clones derived from PW01 and PW13 were found point mutations in the NF-kB I and SP1 II binding sites. There were found point mutations from clones derived from PW16 and PW39 in the SP1 I binding site, equal to 100% and 16.67%, respectively. Clones derived from PW31 were found the mutations in SP1 I (33.33%) and SP1 II (33.33%). Clones derived from PW43 were found the point mutations in the NF-kB I (57.14%), SP1 I (57.14%), SP1 II (57.14%) and SP1 III (14.29%).

NF-*k*B recognizes a 10-bp stretch of DNA with the consensus sequences 5'-GGGPuNNPyPyCC-3' (199). The NF-kB binding sites in the HIV-1 LTR are highly conserved among different viral isolates, suggests an essential role in virus replication. Mutation of the NF-*k*B site resulted in a dramatic loss of LTR promoter activity in transient transfection studies with LTR-CAT reporter constructs (200, 201). Mutations of the NF-*k*B binding sites in infectious HIV-1 clones yielded conflicting results regarding their contribution to virus replication. Initial studies indicated that the NF-kB enhancer elements are dispensable for virus growth (202, 203), but more recently analyses demonstrated the importance of these elements for optimal HIV-1 replication (204, 205). Interestingly, a direct correlation was observed between the severity of the replication defect of NF-*k*B site-mutated viruses and the NF-*k*B protein level of the cell type used for infection (206, 207)

In the LTR, primate lentiviruses typically possess three SP1 binding site, and isolated CM240 (HIV-1 subtype E from Thailand) is normal in this regard. In the HIV-1 lineage, available sequences from subtypes A, B, and D have two sites for NF-κB binding sites emerged with the first sequence from clade C, which possesses an additional site. In contrast to clades A, B, C and D, subtype E has only one canonical NF-κB binding site. Additional atypical features were observed in the transactivation response (TAR) stem-loop structure of subtype E. This structure, located at the 5' end of the genomic RNA, regulates transcription by forming a binding site for the viral protein Tat and cellular proteins (26). It was shown that the critical regions for binding are the bulge on the side of TAR and the tip of the loop. Subtype E differs from the B, C and D clades in having a two-base bulge instead of a three-base bulge. The tip of the

TAR loop also differs by one base from those of HIV-1 clade A through G, being CCGGG instead of CTGGG (208).

In this study, all sequences of LTR were clustered into subtype E. Naghari *et al.* (209, 210) and others (211, 212) reported that the HIV-1 long terminal repeat can be used for phylogenetic classification of HIV-1 into subtypes. The genetic diversity of the LTR may result in HIV-1 subtypes with different replicative properties. Transcription of HIV-1 genome is regulated by the interaction of viral LTR *cis*-acting sequence with a complex of a viral regulatory protein and cellular transcription factors (213). Several cellular transcription factors have been identified that bind to the HIV-1 LTR. The significance of these factors, as well as their type and extent of interaction with the LTR of different HIV-1 subtypes, are still not fully understood. Besides the general transcription factors-binding sites, the HIV-1 LTR promoter contains several cell type of stimuli such as cytokine and mitogens (213). Subtype-specific differences in the HIV-1 enhancer, as well as divergent LTR-mediated transcriptional regulation, have been suggested for Thai subtype E viruses (211, 214).

In a previous study by Jones *et al.* (215), it was shown that SP1-I and/or SP1-II are responsible for LTR promoter activity in *in vitro* transcription assay, whereas the third SP1-III site had minimal effects on viral transcription. The SP1-III site was also the most variable site, suggesting that it might be less important than the SP1-I and SP1-II sites. The sequence surrounding and including the TATA box (-43 to+80) seems to be sufficient for basal promoter activity and mutations in this region have been correlated with alterations in the Tat-induced activation of HIV-1.

The most of primary HIV-1 isolates in this study were NSI, equally to 6 and 5 in HIV-1 infected partners of HEPS and concordant couples, respectively. Only two of viruses that isolated from concordant group were SI (CW33 and CW56).

HIV variants were shown to display variation in their biological properties. Isolates can be distinguished on the basis of their replication rate, their capacity to infect different cell types and their capacity to induce syncytia. Isolates recovered from individuals with AIDS or AIDS related complex showed higher replication rates than isolates recovered from asymptomatic individual. In contrast to virus isolated from asymptomatic individuals, they were able to establish persistent infection in T cell lines. The transition from non-syncytium-inducing (NSI) to syncytium inducing (SI) viral isolates correlated with disease progression. NSI isolates, however, appeared to be much more monocytotropic than SI isolates and were not transmissible to T cell lines. Moreover, the emergence of subtype E SI variants is associated with the development of AIDS, as it is for subtype B HIV-1.

HIV-1 subtype E isolates in this experiment were classified into 3 groups: M-, T- and Dual tropic. Three isolates (PW33, PW49 and PH53) from HIV-1 infected partners of HEPS and three isolates (CW07, CH57 and CH59) from concordant subjects were M-tropic. The dual tropic group consisted of two isolates (PW11 and PW45) from HIV-1 infected partners of HEPS and two (CH34 and CW34) from the concordant group. One isolate (PW55) from HIV-1 infected partners of HEPS and two (CW33 and CW56) form the concordant were T-tropic.

There was no difference of any virus, to grow in HEPS PBMC and the donor PBMC. This showed that HEPS PBMCs were able to support HIV-1 replication as well as the donor PBMC did. Moreover, the high-risk seronegative subjects were

determined if the HIV-coreceptor is defect. R5 deletion was not found in this study. CD4 is the major receptor protein, but by itself is not sufficient for HIV infectivity. Two species-specific cell-surface coreceptors are required; R5 is the elusive second receptor for M-tropic strain and X4 for T-tropic strain. A 32-bp deletion was found in the gene coding R5 in the region corresponding to the second extracellular loop, results in severely truncated molecule that fails to reach the cell surface. Two mutants R5 genes (homozygous) appear to provide powerful genetic protection against HIV even after repeated exposure, one mutant R5 allele and one normal allele (heterozygous) could slow progression to AIDS in infected individual. However, the HIV-resistance allele, or deletion mutant, of the R5 gene is not distributed equally among the world's peoples. It is virtually absent in Africa and eastern Asian populations, and in Native Americans, and is rare in African-Americans. It is fairly prevalent among Caucasians (descendants of the early settlers of Europe and Western Asia)

The NPO3 HIV-1 subtype E lab strain was relatively sensitive to neutralization by multiple plasma. Viruses from partners of HEPS and concordant couples were not found to have any differences in their sensitivity to neutralization. Actually, four viruses from partners of HEPS with *nef* deletion could not be isolated by coculture method. However, PW55 isolate from the partner of HEPS was resistant to neutralization by all of the tested plasma. Plasma of the concordant couple showed more broad neutralization than those of partners of HEPS.

The study showed that the viral factors, which are *nef* deletions (4 isolates), the point mutation in *nef* gene and LTR region might be the cause of non-transmission from HIV-1 infected partner of HEPS. Moreover, other possibilities may be: first, they

had a low viral load, as reflected by the relative by small number of infected $CD4^+$ cells and free infectious virus in the peripheral blood and lymphoid tissues. Second, viruses from many HIV-1 infected partners of HEPS could not be isolated even they had high viral load. These may be less infectious virions. Third, the viruses recovered from these patients were nonvirulent HIV-1 strains that grow to low titer and are not cytopathic, and some do not replicate in established T-cell lines.

The host factors that may influence or disrupt the transmission were also determined. No R5 mutation sequence was detected in these HEPS. HIV-1 neutralizing activities seemed not to be the cause of non-transmission. However, the trend of the neutralizing activity seemed to be stronger in the concordant plasma against NPO3 HIV-1 subtype E. In this study, we didn't determine CTL activity and mucosal IgA in HEPS.

Neutralization by HIV-1 positive sera both within and cross clade tends to be sporadic in nature: some isolates are fairly sensitive to neutralization, and others are resistant, some sera possess relatively broad and potent neutralizing activity, but most lack it. An additional complication is that some sera enhance rather than neutralize HIV-1 infection *in vitro*, and some isolates are particularly susceptible to infectivity enhancement (216).

Most sera from individuals infected with clade B and E strains did not contain high titers of clade-specific neutralizing antibodies (217, 218). The genetic clade did not directly correspond to neutralization serotypes. The clades of HIV-1 are defined on the basis of primary sequence, whereas virus neutralization is a much more complex process, which depends on antibody interactions with epitopes that are influenced by the tertiary and quaternary structures of glycoprotein oligomers (219).

The env sequence-derived genetic subtypes can be represented by peptides representing V3 consensus sequences. Sera from HIV-1 subjects bind to their V3 peptide representing subtypes A to E in a type specific manner (220). As binding can be shown to be at least in part subtype specific, the presence of cross-neutralization rather than type specific neutralization suggested that the V3 peptide does not contribute significantly to the capacity of human HIV^+ sera or plasma to neutralize isolates of HIV-1 in PBMCs. The cross neutralizing in human sera appeared to be directed to epitopes outside gp120 (221).

The main difference between primary isolates and laboratory strains is the ability of laboratory strains to replicate in T cell lines, since these strains had been adapted to growth in T cell lines as a result of being cultures in T cell line for many years. But primary isolates had been grown only in PBMC and was of low passages number. Primary isolates were resistant to neutralization by soluble forms of viral receptor protein CD4 (222-223), and were relatively resistance to neutralization by anti-gp120 monoclonal antibodies and serum samples from HIV-1 infected individuals (224-225) compared with laboratory strains. Nonetheless, these primary isolates could be potently neutralized by a subset of human polyclonal or monoclonal antibodies. Although the mechanism responsible for neutralization resistance is unknown, adaptation in T cell lines rendered primary isolates sensitive to soluble CD4-mediated (226) and antibody-mediated neutralization (227, 228). Therefore, Moore and coworkers (222) suggested that the conformational structure of the glycoprotein, which might differ between primary isolates and T cell line-adapted viruses, control sensitivity or resistance to neutralization. The adaptation to T cell line probably results in subtle alterations in the structure of the oligomeric envelope glycoprotein complex

present on virions of adapted variants. These alterations might improve the accessibility or antibody binding sites present in the glycoprotein complex of the T-cell line–adapted virus variants, while primary isolates had naturally less accessible antibody binding sites (229). Orloff and coworkers showed that adaptation of primary isolates to T cell line made virus sensitive to neutralization by soluble CD4 associated with mutations in the C1 region of gp120 (230).

The selective cellular tropism of different strains of HIV-1 may be determined in part by coreceptor usage. Laboratory-adapted variants of the virus that use X4 as a coreceptor were highly sensitive to neutralization by sera from HIV-1 infected individuals, whereas primary isolates that use R5 instead of, or in addition to, X4 were neutralized poorly. Montefiori *et al* (231), suggested that X4 did not render SI primary isolates highly sensitive to neutralization, and R5 usage did not explain why primary isolates were less sensitive to neutralization than laboratory-adapted strains.

SI and NSI primary isolates were equally difficult to neutralization in PBMC despite their differential use of X4 and R5 (175, 216, 217, 232, 233). Other factors were more likely to explain why laboratory-adapted HIV-1 was highly sensitive to neutralization compared to primary isolates. One possibility was that envelope glycoprotein folding, subunit-subunit interactions, or positioning of glycosylation sites differently (234, 235). Antibodies to these epitopes may be able to neutralize the virus regardless of which coreceptors are used, as long as the epitope is exposed for antibody to bind. The V3 loop of gp120 contains multiple neutralization epitopes that were readily exposed on laboratory-adapted viruses (236, 237), but were hidden in the native structure of gp120 on primary isolates (238). The manner in which the envelope

glycoproteins interact with their coreceptors might be another factor that effects virus neutralization by an antibody. Different strains of HIV-1 used different binding motifs on both X4 (239) and R5 (240, 241). It possible that virus neutralization depend on which motifs are used in the neutralization process.

CHAPTER VII

CONCLUSION

In summary of identification of the viral factors and host factors involving in resistance infection of HIV-1 infected discordant couples, the study found that.

1. Mean viral load of HIV-1 infected partners of HEPS was 4.20 ± 0.88 log copies/ml and that of concordant couples was 4.32 ± 0.45 log copies/ml.
2. Viral factors found in HEPS were four deletion patterns of *nef* gene in clone derived from PW01 (10%, 1/10), PW16 (11.11%, 1/9), PW13 (66.67%, 10/15) and PW43 (11.11%, 1/9). Point mutations in *nef* gene were found in PxxP region at amino acid position 75 to 78 in clone derived from PH53 (100%, 5/5), PW01 (10%, 1/10) and at position 145 to 148 in clone derived from PW01 (80%, 8/10), PW11 (100%, 9/9), PW18 (100%, 10/10) and PW33 (23.07%, 3/13). Point mutations in LTR region were also found in clone derived partners of HEPS. PW01 (11.11%, 1/9) and PW13 (12.5%, 1/8) were found point mutations in the NF-*k*B I and SP1 II binding sites. There were found point mutations from clones derived from PW16 (100%, 8/8) and PW39 (16.67%, 1/6) in the SP1 I binding site. Clones derived from PW31 were found the mutations in SP1 I (33.33%, 1/3) and SP1 II (33.33%, 1/3). Clones derived from PW43 were found the point mutations in the NF-*k*B I (57.14%, 4/7), SP1 I (57.14%, 4/7), SP1 II (57.14%, 4/7) and SP1 III (14.29%, 1/7).
3. There was no difference in replication pattern of HIV-1 isolates in each kind of biotype between HIV-1 infected partner of HEPS and concordant couples.

4. No mutation sequence was detected in R5 gene of their HEPS.
5. NT activity is not different between HIV-1 infected partners of HEPS and concordant couples

The *nef* deletion and point mutations in *nef* gene and LTR region in HIV-1 infected partner of HEPS seemed to be non-transmitted to the partner, who has repeated unprotected sexual relation for several years. Sexual transmission of HIV-1 in humans is a highly complex process. Among the variables involved are the precise transmission route and the interactions between multiple viral and host factors. In these cases might have low inoculum level or too defective virus to transmit to their partners. The finding of HIV-1 variants with *nef* deletion in HIV-1-infected partners of HEPS provides additional impetus for consideration of the vaccine approach.

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APPENDIX

Stock solution

1. Tris-HCl 1M pH 8.3

Tris hydroxymethyl aminomethane	12.1	g
Distilled water	80	ml

Adjust to pH 8.3 with concentrated HCl

Mix and add deionized H_2O to 100 ml

Sterile by autoclaving at 121°C with pressure 15 lb/inch^2 for 15 min.

2. $MgCl_2$ 1 M

$MgCl_2$-6.H_2O	20.3	g

H_2O to 100 ml

3. KCl 1 M

KCl	7.46	g

Distilled water to 100 ml

4. NaOH 10 N

NaOH	400	g

Distilled water to 100 ml

5. EDTA (ethylenediamine tetraacetic acid) 0.5 M

Dissolve 18.61 g Na_2EDTA-2.H_2O in 60 ml H_2O

Adjust pH to 8.0 with NaOH pellet

Add H_2O to 100 ml and sterile by autoclaving

Reagent for PCR technique

1. PCR lysis buffer

1 M Tris-HCl (pH 8.3)	1	ml
1 M KCl	5	ml
1 M $MgCl_2$	0.25	ml
NP-40 (Nonidet-P N3516, Sigma)	0.45	ml
Tween-20 (Sigma)	0.45	ml

Adjust to volume with distilled water to 100 ml

Sterile by autoclaving at 121°C with pressure 15 lb/inch^2 for 15 min

and store at 4°C

2. Proteinase K (10 mg/ml)

proteinase K	10	mg
Distilled water	1	ml

Store at –20°C

To prepare working solution, 8 µl of proteinase K solution was added to 1 ml of PCR lysis buffer before used

3. Deoxynucleotide triphosphate (dNTPs) mixture

Each dATP, dCTP, dGTP, dTTP was supplied as individual 100 mM stock from Pharmacia LKB

dATP	25	µl
dCTP	25	µl
dGTP	25	µl
dTTP	25	µl
Distilled water	900	µl

4. Loading dye for agarose gel electrophoresis (6x solution)

Bromphenol blue	100	g
Sucrose	20	g
0.5xTBE buffer	50	ml

5. Tris Borate EDTA (TBE) electrophoresis buffer (10x stock solution)

TBE	175	g
H_2O to 1 liter		

6. Agarose gel

About 1% or 2% agarose gel in 0.5x TBE (w/v)

Melting, be sure all agarose particles are completely melted

7. Ethidium bromide solution (10 mg/ml)

Ethidium bromide	1	g
Distilled water	100	ml

Working solution, 0.5 g/ml in distilled water

Reagent for DNA cloning

1. Solution I (GTE buffer)

Glucose	0.9	g
1 M Tris-HCl pH 8.0	2.5	ml
0.5 M EDTA pH 8.0	2.0	ml

2. Solution II

5 M NaOH	4.0	ml
10% SDS	10.0	ml

Adjust the volume to 100 ml with distilled water

3. Solution III (KoAc)

Potassium acetate	29.4	g
Cracial acetic acid (CH_3COOH)	40.0	ml

4. Luria-Bertani (LB) broth

Tryptone	10	g
Yeast extract	5	g
NaCl	10	g
10 N NaOH	200	µl

Add distilled water to 1 liter, sterile by autoclaving 15 min

Cool at 50°C and add 1 ml of 50 mg/ml of amplicillin

Store at 4°C

5. Luria-Bertani (LB) agar

Prepare LB broth as above, and add 15 g/L agar before autoclaving

Cool at 50°C and add 1 ml of 50 mg/ml of amplicillin

Pour 25 ml per plate, lid open slightly in laminar air flow 30 min

Store at 4°C

6. SOC medium

2% tryptone

0.5% yeast extract

10 mM NaCl

2.5 mM KCl

10 mM $MgSO_4$

20 mM Glucose

Reagent for HIV-1 isolation

1. Stimulated medium

RPMI-1640	85	ml
FBS (heat inactivation at 56°C, 30 min)	15	ml
50,000 U/ml penicillin	200	µl
50,000 µg/ml streptomycin	200	µl
L-glutamine	1	ml
PHA	50	µl

2. Culture medium

RPMI-1640	85	ml
FBS (heat inactivation at 56°C, 30 min)	15	ml
50,000 U/ml penicillin	200	µl
50,000 µg/ml streptomycin	200	µl
L-glutamine	1	ml
10,000 U/ml IL-2	100	µl

3. Cell line medium

RPMI-1640	90	ml
FBS (heat inactivation at 56°C, 30 min)	10	ml
50,000 U/ml penicillin	200	µl
50,000 µg/ml streptomycin	200	µl
L-glutamine	1	ml

4. Macrophage attachment medium

RPMI-1640	80	ml
Normal Human Serum	20	ml

(heat inactivation at 56°C, 30 min)		
50,000 U/ml penicillin	200	µl
50,000 µg/ml streptomycin	200	µl
L-glutamine	1	ml

5. Macrophage medium

RPMI-1640	80	ml
FBS (heat inactivation at 56°C, 30 min)	20	ml
50,000 U/ml penicillin	200	µl
50,000 µg/ml streptomycin	200	µl
L-glutamine	1	ml

6. Wash medium

RPMI-1640	98	ml
FBS (heat inactivation at 56°C, 30 min)	2	ml
50,000 U/ml penicillin	200	µl
50,000 µg/ml streptomycin	200	µl

7. DMSO medium

RPMI-1640	50	ml
FBS (heat inactivation at 56°C, 30 min)	40	ml
50,000 U/ml penicillin	200	µl
50,000 µg/ml streptomycin	200	µl
DMSO	10	ml

8. Trypan blue (0.4%)

Trypan blue	0.4	g
0.2 M PBS	100	ml

Dissolve thoroughly and filtrate through filter, store at room temperature

BIOGRAPHY

NAME	Miss Apasara Mudngoen
DATE OF BIRHT	29 May 1976
PLACE OF BIRTH	Bangkok, Thailand
INSTITUE ATTENDED	Khonkaen University, 1993-1996: Bachelor of Science (Microbiology second class honored) Mahidol University, 1997-1999: Master of Science (Microbiology)
RESEARCH GRANT	National Science and Technology Development Agency (NSTDA)