ความสามารถในการกระตุ้นภูมิคุ้มกันในหนูทดลองของดี-เอน-เอ วัคซีนที่จำเพาะต่อเปลือกนอกของไวรัสเอช-ไอ-วี ทัยป์ 1 ที่มีการสร้างลำดับเบสให้ใกล้เคียงกับดี-เอน-เอ ของคนโดยปฏิกิริยาลูกโซ่โพลีเมอเรส

นางสาว สุณี ศิริวิชยกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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IMMUNOGENICITY OF A PCR-BASED HUMANIZED HIV ENVELOPE DNA VACCINE IN MICE

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สุณี ศิริวิชยกุล : ความสามารถในการกระตุ้นภูมิคุ้มกันในหนูทดลองของดี-เอน-เอ วัคซีนที่จำเพาะต่อเปลือกนอกของไวรัส เอช-ไอ-วี ทัยป์1 ที่มีการสร้างลำดับเบสให้ใกล้เคียงกับดี-เอน-เอ ของคนโดยปฏิกิริยาลูกโซ่โพลีเมอเรส (IMMUNOGENICITY OF A PCR-BASED HUMANIZED HIV ENVELOPE DNA VACCINE IN MICE) อ. ที่ปรึกษา :ศาสตราจารย์นายแพทย์ประพันธ์ ภานุภาค, อ. ที่ปรึกษาร่วม : อ.ดร. ทวีศักดิ์ ตีระวัฒนพงษ์, 200 หน้า, ISBN 974-17-3430-1

โรคภูมิคุ้มกันบกพร่อง หรือโรคเอดส์ ซึ่งเกิดจากการติดเชื้อ human immunodeficiency virus type 1 (HIV-1)เป็นปัญหาสาธารณสุขที่สำคัญซึ่งกำลังแพร่กระจายไปทั่วโลก ตัวไวรัสสามารถเข้าสู่เซลล์เม็ดเลือดขาวชนิดซีดี 4 (CD4 + T cells) ซึ่งเป็นเซลล์สำคัญของระบบภูมิคุ้มกันของมนุษย์ และทำลายเซลล์ดังกล่าว ทำให้ภูมิคุ้มกันของผู้ติดเชื้อลดลงเรื่อยๆ และทำให้เกิดโรคภูมิคุ้มกันบกพร่องขึ้นในที่สุด ในปัจจุบัน ถึงแม้จะมีการค้นพบยาต้านไวรัสเอดส์ ซึ่งสามารถยับยั้งไวรัสที่ช่วงต่างๆในวงจรชีวิตของตัวไวรัสได้ แต่ยาดังกล่าวก็ยังมีราคาแพง ซึ่งผู้ติดเชื้อส่วนใหญ่ทั่วโลกโดยเฉพาะผู้ติดเชื้อในประเทศกำลังพัฒนาไม่สามารถเข้าถึงยาดังกล่าวได้ วัคซีนเป็นหนึ่งในหลายวิธีที่สามารถป้องกันการติดเชื้อได้ดีที่สุด ได้มีความพยายามจากนักวิทยาศาสตร์ทั่วโลกในการคิดค้นวัคซีนเอดส์ แต่จนถึงขณะนี้ก็ยังไม่มีอะไรที่ได้ผลดีชัดเจน

เพื่อเป็นการศึกษาถึงวัคซีนเอดส์ที่หวังว่าจะใช้ได้กับประชากรทั่วโลก คณะผู้วิจัยได้สร้างดี-เอน-เอวัคซีนที่จำเพาะต่อเปลือกนอกของไวรัสที่มีขนาด 297 คู่เบสที่ครอบคลุมส่วน V3 และบริเวณใกล้เคียงซึ่งมีลำดับเบสใกล้เคียงกับดี-เอน-เอ ของคนและเป็นตัวแทนของไวรัส 8 สายพันธุ์สำคัญของโลกโดยใช้ปฏิกิริยาลูกโซ่ และสร้างดี-เอน-เอวัคซีนที่จำเพาะต่อเปลือกนอกของตัวไวรัสที่มีขนาด 297 คู่เบสในบริเวณเดียวกันแต่มีลำดับเบสเป็นของตัวไวรัสเอง และดี-เอน-เอวัคซีนที่จำเพาะต่อเปลือกนอกทั้งชิ้นของตัวไวรัสที่มีขนาด 2.5 กิโลเบส โดยที่วัคซีนสองอย่างหลังได้มาจากการเพิ่มขยายจำนวนโดยใช้ดี-เอน-เอ ของตัวไวรัสที่แยกได้จากเซลล์เม็ดเลือดขาวของผู้ติดเชื้อไวรัสเอดส์สายพันธุ์ A/E ที่พบมากในประเทศไทยเป็นต้นแบบ

การศึกษาพบว่าวัคซีนที่จำเพาะต่อเปลือกนอกของตัวไวรัสที่มีขนาด 297 คู่เบสซึ่งมีลำดับเบสใกล้เคียงกับดี-เอน-เอ ของคน และวัคซีนที่จำเพาะต่อเปลือกนอกทั้งชิ้นของตัวไวรัสที่มีขนาด 2.5 กิโลเบสสามารถนำไปสู่การสร้างโปรตีนที่มีขนาด 13 กิโลดาลตัน และ 160, 120 และ 38 กิโลดาลตันตามลำดับได้ในหลอดทดลองโดยวิธี transfection ในขณะที่วัคซีนขนาด 297 คู่เบสที่มีลำดับเบสเป็นของตัวไวรัสเองไม่สามารถสร้างโปรตีนได้ เมื่อนำวัคซีนทั้ง 3 ชนิดมาฉีดในหนูทดลอง ก็พบว่าวัคซีนทั้ง 3 ชนิดสามารถกระตุ้นให้เกิดการตอบสนองทางภูมิคุ้มกันแบบ DTH ได้โดยวัดขนาดความบวมของอุ้งเท้าหนูหลังทดสอบด้วยแอนติเจนที่เป็นส่วนของ V3 นอกจากนี้ ยังสามารถตรวจพบว่าวัคซีนทั้ง 3 ชนิดมี immunogenicity ชัดเจนโดยวิธี ELISPOT และโดยวิธี ICCS ในกลุ่มที่ฉีดด้วยวัคซีนเปลือกนอกทั้งชิ้น และกระตุ้นด้วย rgp120 แต่ผลที่ได้ค่อนข้างต่ำ แต่ไม่สามารถตรวจพบแอนติบอดีย์ที่จำเพาะต่อเปลือกนอกของตัวไวรัส และไม่สามารถตรวจพบการตอบสนองต่อโปรตีนเปลือกนอกของตัวไวรัสโดยวิธี lymphoproliferation assay นอกจากนี้ การทดสอบปฏิกิริยาข้ามกลุ่มของวัคซีนที่มีลำดับเบสใกล้เคียงกับดี-เอน-เอ ของคนโดยการฉีดกระตุ้นซ้ำด้วย recombinant vaccinia gp160 ของสายพันธุ์อี ปรากฏว่าได้ผลเป็นลบ ซึ่งอาจเนื่องมาจาก ดี-เอน-เอ ที่ใช้มี immunogenicity ต่ำหรือเทคนิคที่ใช้ในการทดสอบไม่ไวพอ

กล่าวโดยสรุปคือ คณะผู้วิจัยสามารถพัฒนา ดี-เอน-เอวัคซีนที่จำเพาะต่อเปลือกนอกของไวรัสเอช-ไอ-วี ขึ้นมาทดสอบได้ รวมทั้งวัคซีนที่มีลำดับเบสใกล้เคียงกับคน ซึ่งแสดงออกได้ดีกว่าวัคซีนที่มีลำดับเบสของไวรัสเอง พบว่าวัคซีนทั้ง 3 ชนิดสามารถกระตุ้นภูมิต้านทานในหนูทดลองได้ในระดับหนึ่ง แต่คงต้องพัฒนาให้ดียิ่งขึ้นเพื่อจะได้นำไปทดสอบต่อไปทั้งในสัตว์ทดลองและในคน

สหสาขาวิชาจุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต.....
ปีการศึกษา 2546 ลายมือชื่ออาจารย์ที่ปรึกษา.....
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KEY WORD : HUMAN IMMUNODEFICIENCY VIRUS (HIV-1) / DNA VACCINE / FOOTPAD SWELLING / LYMPHOPROLIFERATION / INTRACELLULAR CYTOKINE STAINING / IFN-γ PRODUCTION

SUNEE SIRIVICHAYAKUL : IMMUNOGENICITY OF A PCR-BASED HUMANIZED HIV ENVELOPE DNA VACCINE IN MICE. THESIS ADVISOR : PROF. PRAPHAN PHANUPHAK, THESIS CO-ADVISOR : THAWEESAK TIRAWATNAPONG, Ph.D. 200 pp. ISBN 974-17-3430-1

Human immunodeficiency virus type 1 (HIV-1) infection is a major public health problem causing epidemic worldwide. It infects mainly CD4+ T lymphocyte and destroys the cells resulting in progressive immunodeficiency. Although many potent antiretroviral drugs acting at different stages of HIV life cycle are currently available, the majority of patients worldwide will not have easy access to these expensive and complicated therapeutic regimens. Protection including prophylactic vaccination represents one of the best and sustainable solutions to curb the worldwide epidemic. Many attempts to produce an effective HIV vaccine have been made without much success up to now.

To explore more about HIV vaccine, three HIV-1 DNA constructs, namely 297-bp humanized DNA encompassing the immunodominant epitopes of the V3 and its adjacent regions of multi-clade HIV-1 representing the last common ancestor consensus sequence of 8 HIV-1 subtypes circulating worldwide (297-bp hu V3 DNA), 297-bp non-humanized DNA and 2.5 kb full-length envelope DNA were studied. The 297-bp hu DNA was produced by a PCR-based method while the rests were directly amplified from DNA isolated from peripheral blood mononuclear cells (PBMC) of HIV-1 subtype A/E-infected individual. All 3 PCR products were successfully cloned into appropriate plasmid vectors to be used as HIV DNA vaccines.

The 297-bp multi-clade hu V3 DNA and the 2.5 kb full-length envelope DNA were well expressed *in vitro* by transfection experiments as 13 Kd and as 160, 120 and 38 Kd respectively but not the 297-bp non-humanized DNA. However, all 3 constructs were shown to be immunogenic in mice by *in vivo* DTH skin testing (footpad swelling) with specific V3 peptide. The *in vitro* correlates of the immunogenicity study was best demonstrated by Enzyme Linked Immunospot Assay (ELISPOT). Intracellular cytokine staining (ICCS) response could be well demonstrated only in 2.5 kb full-length envelope DNA primed / recombinant gp120 (rgp120) boosted group. Both the ELISPOT and ICCS responses were relatively weak but positive. No antibodies or antigen-stimulated lymphoproliferative response could be shown in this study. The attempt to demonstrate cross-reactivity of the hu V3 DNA by boosting with recombinant vaccinia gp160(E) yielded negative results which may be due to the low immunogenicity of the constructs or the techniques used.

In conclusion, we were able to produce DNA constructs including the humanized DNA of the envelope region to be tested as HIV-1 vaccine. Humanized DNA was better expressed than the non-humanized counterpart. The 3 DNA constructs were shown to have some immunogenicity in mice. Further improvements are needed to enhance their immunogenicity in order to allow for further pre-clinical and clinical testings.

FIELD OF STUDY : MEDICAL MICROBIOLOGY Student's signature…….

Academic year 2003 Advisor's signature…….

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LIST OF ABBREVIATIONS

HIV	=	Human immunodeficiency virus
RNA	=	Ribonucleic acid
CRFs	=	Circulating Recombinant Forms
CD	=	cluster of differentiation
cDNA	=	complementary deoxynucleic acid
LTRs	=	long terminal repeats
RT	=	reverse transcriptase
LTNP	=	long term non-progressor
HCW	=	health care worker
HEPS	=	highly exposed but persistently seronegative
CTL	=	cytotoxic T lymphocyte
LCA	=	last common ancestor
bps	=	base pairs
PCR	=	polymerase chain reaction
SI	=	syncytium inducing
NSI	=	non-syncytium inducing
ELISPOT	=	enzyme-linked immunospot assay
AIDS	=	acquired immunodeficiency syndrome
IVDU	=	intravenous drug user
ELISA	=	enzyme-linked immunoassay
STD	=	sexual transmitted disease
cpx	=	complex
ARC	=	AIDS related complex
CMV	=	cytomegalovirus
SDF-1	=	stromal cell-derived factor 1
ARV	=	antiretroviral
ZDV	=	zidovudine
ddI	=	didanosine
ddC	=	zalcitabine
d4T	=	stavudine
3TC	=	lamivudine

LIST OF ABBREVIATIONS (CONT.)

ABC	=	abacavir
NRTI	=	nucleoside reverse transcriptase inhibitor
NNRTI	=	non-nucleoside reverse transcriptase inhibitor
PI	=	protease inhibitor
HAART	=	highly active antiretroviral therapy
SIV	=	simian immunodeficiency virus
CNS	=	central nervous system
Th	=	T helper cell
mRNA	=	messenger RNA
APCs	=	antigen presenting cells
IFN-γ	=	interferon gamma
IL	=	interleukin
ODN	=	oligonucleotide
CRS	=	cis-repressor sequences
PBMC	=	peripheral blood mononuclear cell
PHA	=	phytohemagglutinin
IL-2	=	interleukin 2
IRB	=	Institutional Review Board
PBS	=	phosphate buffered saline
FBS	=	fetal bovine serum
TCM	=	tissue culture medium
Ag	=	antigen
UV	=	ultraviolet
ECL	=	electro chemiluminescense
IgG	=	immunoglobulin G
DTH	=	delayed type hypersensitivity
HRP	=	horse radish peroxidase
RPMI	=	Rosewell Park Memorial Institute
2-ME	=	2-Mercaptoethanol
µg	=	microgram

LIST OF ABBREVIATIONS (CONT.)

nm	=	nanometer
µl	=	microlitre
mg	=	milligram
^{3}H-Tdr	=	tritiated thymidine
Con A	=	Concanavalin A
S.I.	=	stimulation index
cpm	=	count per minute
PE	=	phycoerythrin
FITC	=	fluorescein isothiocyanate
NBT	=	nitroblue tetrazolium
BCIP	=	5-bromo-4-chloro-3-indolylphosphate
SFC	=	spot forming cell
rVV	=	recombinant vaccinia virus
WT	=	wild type
NBT	=	nitroblue tetrazolium
BCIP	=	5-bromo-4-chloro-3-indolylphosphate
kDa	=	kilodalton
SD	=	standard deviation

CHAPTER I

INTRODUCTION

BACKGROUND AND RATIONALE

Human immunodeficiency virus type 1 (HIV-1) infection is a major public health problem causing epidemic worldwide. HIV-1 is an RNA retrovirus within the lentivirus family[1]. Three groups of HIV-1 have been reported as

- Group M (Major) which includes 11 subtypes or sub-subtypes (A1, A2, B, C, D, F1, F2, G, H, J, K) and 14 Circulating Recombinant Forms (CRFs) (CRF01_AE, CRF02_AG, CRF03_AB, CRF04_cpx, CRF05_DF, CRF06_cpx, CRF07_BC, CRF08_BC, CRF09_cpx, CRF10_CD, CRF11_cpx, CRF12_BF, CRF13_cpx, CRF14_BG)[2-4]
- Group O (Outlier) which is endemic in Cameroon and neighboring countries in West Central Africa[5-6]
- Group N (New or Non-M, Non-O) which has been reported in a limited number of isolates from Cameroonian patients[7-8]

HIV preferentially infects a subset of T lymphocyte, the $CD4^+$ T cells. The HIV-1 genomic RNA is reversely transcribed into complementary DNA (cDNA) with two identical long terminal repeats (LTRs) containing enhancer and promoter sequences which is later integrated into host cell genomic DNA[9]. The provirus will persist in the cells and divide when cells divide. In addition, virus also replicates independently from cell replication. Mature virion buds out of the cell which eventually resulting in cell burst and death[9]. $CD4^+$ T cells in HIV-infected individuals will be gradually reduced.

The resultant T cell immunodeficiency is the prime cause of almost all clinical manifestations in acquired immunodeficiency syndrome (AIDS)[10].

There is no cure for HIV infection at present. In addition to psychosocial support as well as treatment and prevention of opportunistic infections, antiretroviral therapy is another but most important therapeutic modality that can significantly alter morbidity and mortality of HIV-infected patients[11-14]. Antiretroviral drugs reduce the viral burden in the body which in turn result in less destruction of $CD4^+$ cells and restoration of immunocompetence.

Although many potent antiretroviral drugs acting at different stages of HIV life cycle are currently available, the majority of patients worldwide will not have easy access to these expensive therapeutic regimens. Protection including prophylactic vaccination represents one of the best solutions to curb the worldwide epidemic like many other infectious diseases.

Still, there are so many obstacles in HIV-1 vaccine development both from the virus itself and from the testing procedures. Major obstacles are :

- the antigenic diversity of the HIV-1 itself which results from the error-prone reverse transcriptase (RT) enzyme. This can cause sequence divergence over time and even occurs in the same infected individual (intra-individual), the so called quasispecies of HIV-1[15-16]. It has been reported that the envelope protein could differ up to 20 % for HIV-1 in the same subtype. The difference could even increase up to 35 % between subtypes[17].

- the lack of consistent correlates of protection from natural or experimental infections even there are some evidences of protection from long term non-progressors (LTNP)[18-19], exposed health care workers (HCWs)[20], neonates born to HIV-infected mothers[21-22] and those highly exposed but persistently seronegatives (HEPS)[23-25].

- the sequestration of HIV in immune privileged sites such as central nervous system, which makes the immune system hardly get access to the HIV.

- the existence of immune suppressive factor from the virus such as tat (the protein encoded by *tat*, trans-activation). Tat also acts to increase gene expression in infected cells up to several hundred folds[26-28].

- immunological inaccessibility due to proviral DNA integration into host genome.

- the lack of satisfactory animal model for infection and disease since infection is not necessarily followed by disease.

Many attempts to produce HIV-1 vaccine have been made and the immune responses were varied. According to what Bagarazzi et al has reviewed in 1998[29], it seems that DNA vaccine is the most attractive form of vaccine. The DNA vaccine possesses many potential advantages as follow :[29-32]

- noninfectious / non-replicating but mimics aspects of live attenuated vaccines without the risk of infection with live virus

- proteins produced in native conformation, particularly those of viral proteins

- physicochemical homogeneity and stability, possibly easier to incorporate several components in a single vaccine

- easy to be produced (inexpensive)

- induction of both cellular and humoral immunity, especially cytotoxic T lymphocyte (CTL) response

- protection is supposed to be long-live and may require fewer booster doses.

It is hoped that a single vaccine that can induce both cell-mediated and antibody-mediated immune responses is feasible. The possibility to induce cross-reactivity is also important. Many attempts to overcome the heterogeneity and diversity of the HIV-1

subtypes have been established. One ideal alternative is to use the approximated last common ancestor (LCA) of all the available subtypes. The sequence has the advantage of being central and most similar to currently circulating strains of interest and may have enhanced potential for eliciting cross-reactive responses[17].

Codon optimization is another issue to be considered to increase the immunogenicity of DNA vaccine[33]. It has been postulated that for a DNA vaccine to be used in human, mammalian codons should be used to enhance the translation and protein expression. Better induction of immune responses has been reported with humanized DNA vaccination[34-35].

HIV envelope (env), especially the hypervariable region 3 (V3) is the first site of target recognition between the virus and host immune system. The envelope involves in receptor and co-receptor bindings, guides cell tropism and contains an epitope for antibody neutralization. The adjacent regions of the V3 are also important since they include CTL epitopes, T helper (Th) epitopes, CXCR4 binding site, CD4 binding and neutralizing epitopes[36-38].Thus, HIV-1 V3 region is an attractive vaccine candidate. Therefore, the V3 region of approximately 297-basepair (bp) in length (codon 291-391 of HIV envelope according to HXB2 strain and corresponds to codon 294-391 of CM240 reference strain, GenBank accession number U54771) was selected for optimized codon usage (humanized DNA) in this experiment.

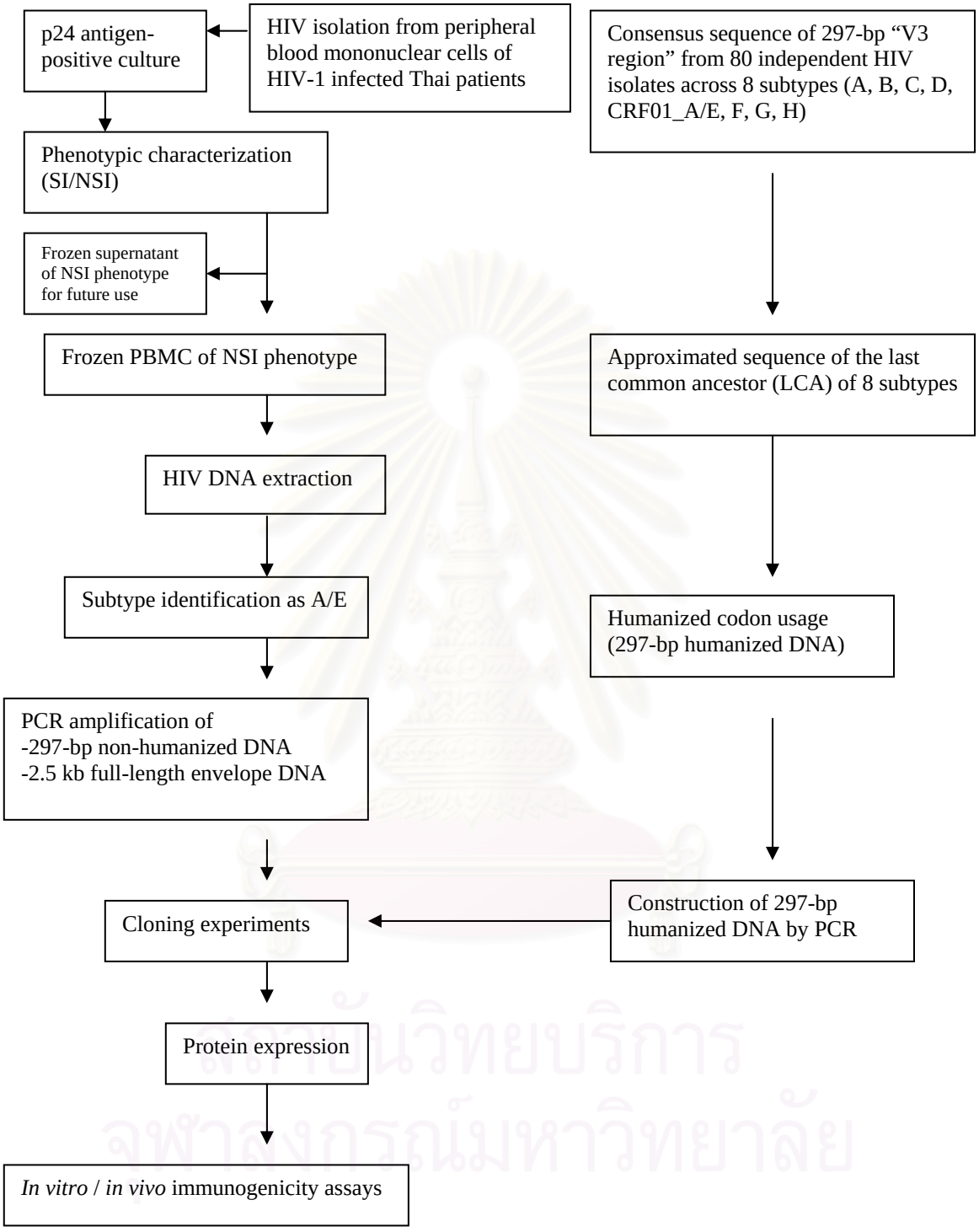
Hypothesis

Plasmid encoding humanized 297-bp HIV envelope protein derived from LCA is immunogenic and its immune response can cross-react with different HIV clades.

Objectives

1. To develop humanized HIV DNA of selected envelope epitopes (codon 291-391) using Polymerase Chain Reaction (PCR) technique.
2. To characterize its *in vitro* expression and its immunogenicity in mice

Conceptual Framework



Assumption

HIV-1 was isolated from peripheral blood mononuclear cells of HIV-1-infected individuals. The maximal volume of 10 ml blood which is enough for HIV-1 isolation was drawn from the infected individuals. Non-treatment related blood drawing from infected individuals was allowed.

Limitation

Six to eight weeks old female Balb/c mice were used in the experiments. Blood samples were collected from only a limited number of mice prior to immunization for the measurement of baseline antibodies. The unnecessary blood drawing from all mice could be avoided. Number of mice per group was kept at minimum. Number of samples and number of variables per experiment were kept at the maximally manageable level.

Operational Definition

HIV-1-infected individuals were defined by anti-HIV positive by 2 assays with different test principles. Successful *in vitro* protein expression was accepted only if the relevant proteins could be detected by anti-HIV positive serum / plasma. Immunogenicity was defined as *in vivo* and / or *in vitro* immune responses.

Expected benefit

1. Mastering the technique of producing humanized DNA of certain length using PCR technique which is cheaper than using DNA synthesizer.
2. Development of a prototype humanized DNA vaccine of 297 bp encoding V3 and the immunodominant regions of gp120 which can induce both the neutralizing antibody and CTL responses.
3. The prototype vaccine and the immunization approach can be further studied in higher mammals which will eventually result in the local production of an effective HIV vaccine in Thailand.

Research Methodology

1. HIV isolation from peripheral blood mononuclear cells of HIV-1-infected individuals
2. SI/NSI phenotypic characterization
3. Genotypic subtyping of HIV-1
4. Computerized derivation of 297-bp approximated sequence of the last common ancestor from an alignment of sequences from 80 independent HIV isolates across 8 subtypes
5. Humanization of this approximated sequence (297-bp humanized DNA)
6. PCR amplification of - the 297 bp humanized DNA
   - the corresponding 297-bp DNA from the isolated virus ("297-bp non-humanized DNA")
   - the 2.5 kb full-length envelope DNA from the isolated virus

7. Cloning experiments
8. *In vitro* protein expression
9. Mouse immunization
10. *In vivo* immunogenicity assay :- delayed type hypersensitivity skin testing by footpad swelling assay on day 40 or day 54
11. *In vitro* immunogenicity assays
    - humoral immune response (binding antibody assay) on day 0, day 42, day 56
    - cell-mediated immune response [lymphoproliferation assay, intracellular cytokine staining assay, enzyme linked immunospot (ELISPOT) assay] on day 42 or day 56
12. Data collection and analysis

    Blood was collected on day 0 (baseline), day 42 or day 56. Mice were sacrificed on day 42 or day 56 according to the immunization protocols. Nonparametric test was used to calculate the difference between pre- and post-immunizations, between before and after antigen challenge as well as between immunization groups.

CHAPTER II

REVIEW AND RELATED LITERATURES

Human immunodeficiency virus : Overview

Acquired immunodeficiency syndrome (AIDS) was first reported in 1981[39]. A group of previously healthy homosexual men and intravenous drug users (IVDU) in the big cities of the United States of America showed up with pneumocystis pneumonia, oral candidiasis and Kaposi's sarcoma[40-43]. These unusual clustering of clinical manifestations were indicative of some forms of cell-mediated immune deficiency which occurred in previously healthy adults. The syndrome was thus termed acquired immunodeficiency syndrome (AIDS). The same syndrome was later found in hemophiliacs[44-45].

The observation that the syndrome often clustered in certain groups of population supported the hypothesis that AIDS might be caused by an infectious agent transmitted by sex or by blood or blood products. Scientists in the early days were particularly interested in T lymphotropic virus since T cells were selectively impaired. This led to the discovery of human immunodeficiency virus (HIV), a human T cell lymphotropic virus, as the etiologic agent of AIDS in 1983[46-48].

Once the etiologic agent of the infectious disease is known and can be propagated, it is expected that scientists can soon discover its pathogenesis, serodiagnosis, treatment and prevention. It is true that we now know a lot about the biology of the virus and how the immune system responds to the virus. We know less about how the virus impairs the immune cells and even lesser, how the immune system

overcomes the virus. These latter informations are important in the development of an effective prophylactic and therapeutic vaccine against AIDS.

As anticipated, the first enzyme-linked immunosorbent assay (ELISA) kit was licensed for routine screening of donated blood in USA in March 1985[49]. Many more test kits and test devices have been developed and licensed since. The tests are based on the principle that HIV-infected individuals will produce antibodies against various protein components of the virus. This is also the principle of HIV-specific Western blot test which is frequently used as confirmatory test in population with low seroprevalence[50].

With the availability of relatively cheap, sensitive and specific diagnostic tests, the tests are being performed on patients with symptomatic HIV infection as well as on people at-risk for infection. The tests are also used in epidemiological survey both in general population and in defined risk groups. This results in the estimation of the size of the infected population within a community, a country as well as the global estimate.

Epidemiology of HIV infection : Global and regional

From its first description in USA in 1981, HIV infection has spread rapidly in all countries. Almost no country is spared of HIV infection. It was estimated that more than 60 million people throughout the world have been infected with the virus since the beginning of the epidemic. Over 20 millions have already died. This is left with 42 million people currently living with HIV/AIDS by the end of 2002 according to the estimates of UNAIDS and WHO[51].

Sub-Saharan Africa is the region hardest hit, with 29.4 million adults and children living with HIV/AIDS (Table 1). The epidemic started later in South and

South-East Asia but it is the region where the rate of increase is one of the fastest. With the size of its population, its economy and the current rate of spread, it is estimated that Asia will have the biggest number of HIV infection, outnumbering Africa by 2010. It should also be pointed out that although HIV epidemic started latest in Eastern Europe and Central Asia, the number of infection also climbs up rapidly (Table 1).

Table 1 Regional HIV/AIDS statistics and features, end of 2002[51]

Region	Epidemic started	Adults and children living with HIV/AIDS	Adults and children newly infected with HIV	Adult prevalence rate (*)	% of HIV-positive adults who are women	Main mode(s) of transmission(#) for adults living with HIV/AIDS
Sub-Saharan Africa	late ‘70s early ‘80s	29.4 million	3.5 million	8.8 %	58 %	Hetero
North Africa & Middle East	late ‘80s	550,000	83,000	0.3 %	55 %	Hetero, IDU
South & South-East Asia	late ‘80s	6.0 million	700,000	0.6 %	36 %	Hetero, IDU
East Asia & Pacific	late ‘80s	1.2 million	270,000	0.1 %	24 %	IDU, hetero, MSM
Latin America	late ‘70s early ‘80s	1.5 million	150,000	0.6 %	30 %	MSM, IDU, hetero
Caribbean	late ‘70s early ‘80s	440,000	60,000	2.4 %	50 %	Hetero, MSM
Eastern Europe & Central Asia	early ‘90s	1.2 million	250,000	0.6 %	27 %	IDU
Western Europe	late ‘70s early ‘80s	570,000	30,000	0.3 %	25 %	MSM, IDU
North America	late ‘70s early ‘80s	980,000	45,000	0.6 %	20 %	MSM, IDU, hetero
Australia & New Zealand	late ‘70s early ‘80s	15,000	500	0.1 %	7 %	MSM
TOTAL		**42 million**	**5 million**	**1.2 %**	**50 %**	

* The proportion of adults (15 to 49 years of age) living with HIV/AIDS in 2002, using 2002 population numbers.

Hetero (heterosexual transmission), IDU (transmission through injecting drug use), MSM (sexual transmission among men who have sex with men).

Five million new HIV infections were estimated to occur throughout the world during 2002[51]. Of these, 3.5 million occurred in Sub-Saharan Africa, 0.7 million occurred in South and South-East Asia and 0.8 million were children under 15 years. Estimate from the same source also indicated that 3.1 million throughout the world died during 2002; 2.4 million from Sub-Saharan Africa and 0.44 million from South and South-East Asia.

Within the Asia-Pacific region, India has the largest number of person living with HIV/AIDS, almost 4 million. China follows next, with 1 million people. Other countries in the region such as Thailand, Cambodia and Myanmar also have large number of people living with HIV. Many low epidemic countries in the region such as Indonesia, Vietnam, Malaysia, Nepal, Papua New Guinea are currently experiencing a rapid increase in HIV infection rate[51]. Table 2 lists the top 10 Asian countries with highest number of people living with HIV/AIDS by the end of 2001 together with the prevalence rate among general population as well as in selected populations with risk behaviors[52].

Table 2 Top ten countries in Asia with highest number of people living with HIV/AIDS, end 2001[52]

				Median HIV prevalence (%) : major urban areas**		
Country	**Estimated number of adults and children living with HIV/AIDS***	**Total population 2001 (millions)**	**HIV prevalence among adults (15-49) (%)**	**Women in ANC**	**Female sex workers**	**IDU**
1. India	3,970,000	1,025.1	0.8	2.0	5.3	3.5
2. China	850,000	1,285.0	0.1	0.0	0.0	0.2
3. Thailand	670,000	63.6	1.8	1.6	6.7	39.6
4. Cambodia	170,000	13.4	2.7	2.7	26.3	ND
5. Viet Nam	130,000	79.2	0.3	0.2	11.0	41.5
6. Indonesia	120,000	214.8	0.1	0.0	0.2	ND
7. Pakistan	78,000	145.0	0.1	0.0	0.0	0.0
8. Nepal	58,000	23.6	0.5	0.0	36.2	50.0
9. Malaysia	42,000	22.6	0.4	ND	6.3	16.8
10. Iran	20,000	71.4	< 0.1	0.0	0.0	ND

* No estimated figures from Myanmar

** Most recent HIV prevalence data were obtained from 1992 to 2000.

ND = no data

Modes of HIV transmission : Global and regional

Main modes of HIV transmission differ between continents and subcontinents and differ between countries in the same subcontinent as well as between regions of a

country. Sexual transmission among men who have sex with men (MSM) is the main mode of transmission in North America, Western Europe and Australia/New Zealand (Table 1). Heterosexual transmission is the main mode of transmission in Sub-Saharan Africa and countries like India, Thailand, Myanmar and Cambodia. Transmission through injecting drug user (IDU) is the main mode of transmission in Eastern Europe and Central Asia as well as in countries like Spain, Malaysia, Indonesia and Nepal. In certain eastern provinces in China, a country with 3 million paid blood donors, such as Henan, Anhui and Shanxi, HIV prevalence was found as high as 12 % among people who had donated plasma[51]. This was due to the contaminated blood and plasma donation equipments. It is important to point out that these risk behaviors are not mutually exclusive. They frequently mingle. For example, up to 20 % of Asian men having sex with men are bisexuals. Many intravenous drug users also visit female sex workers and vice versa. This will further widen the population at risk for HIV infection and makes the targeted intervention difficult. To be effective, preventive intervention has to be both targeted intervention (such as towards female sex workers and injecting drug users) and broad-based intervention (i.e., targeted at general population).

Number of reported AIDS cases : Global and regional

Almost all countries have a system to register or record all of their AIDS cases. The most common definition of AIDS is by clinical criteria (Appendix A). However, the number of reported AIDS cases is usually one-tenth to one-half of the actual or projected AIDS cases due to incomplete reporting and non-recognition. It can be projected that 5-6 % of HIV-infected individuals will develop AIDS annually[53-55].

HIV/AIDS epidemic in Thailand

Thailand experienced its first case of AIDS in late 1984 when a Thai patient with AIDS diagnosis returned home from USA (Limsuwan A, personnal communication). Thailand first 2 de novo AIDS cases were diagnosed in February 1985 at Chulalongkorn Hospital[56]. The diagnosis was made on clinical and immunological grounds as anti-HIV test was first available in Thailand in May 1985. HIV epidemic in Thailand has evolved considerably since then.

HIV epidemic in Thailand came in 5 different waves :

1985 : Men having sex with men

1988 : Intravenous drug users

1989 : Female sex workers

1990 : Male clients of female sex workers (i.e., male STD patients)

1991 : Housewives (general females) and newborns

The starting point for the large-scale spread of the virus was when the epidemic reached the female sex workers. A large portion of Thai men frequently visit prostitutes and consistent condom use is low. Once men are infected, their wives and casual sex partners can be infected. HIV was then finally settled in the Thai family.

HIV prevalence among the various risk groups in Thailand rose sharply during 1988 to 1994. For example, HIV prevalence among the intravenous drug users (IDU) attending the treatment clinic of Thanyarak Hospital in Pathumthani Province rose from 1 % in January 1988 to 35 % in August 1988. The sentinel surveillance in direct female sex workers, indirect female sex workers, male sex workers, male STD patients, military recruits, pregnant women and blood donors as carried out by the Thai Ministry of Public Health from June 1989 to June 2002 indicates that HIV infection rate among

male and female sex workers, military recruits (male general population at age 21) and blood donors (male general population, age 18-60) has started to decline since 1995. However, the infection rate among pregnant women (female general population, age 15-50) has just been leveled off. The HIV prevalence among IDU, on the other hand, shows a trend of increase since 1999.

The serosurveillance data from Thailand indicates that the government prevention effort, particularly, the large-scale condom campaign pays off. The prevention effort has to be continued to provide sustained control. More attention has to be given to the more hard-to-reach groups such as the IDU and men having sex with men (MSM).

From the beginning of the HIV epidemic in Thailand, it is estimated that a total of 1,033,500 Thais have been infected by the end of 2002. A total of 398,000 have died, leaving 635,500 still living with HIV/AIDS (Source : Ministry of Public Health, Thailand, December 2002). The Thai experts also estimated to have 23,700 new HIV infections and 51,800 new AIDS cases in the year 2002. The number of cumulative AIDS cases as reported to Ministry of Public Health by January 31, 2003 was 287,585. Of these, 65,861 have died, leaving a big gap between reported deaths and estimated deaths.

For Thai AIDS cases, 67 % were between 25-39 years old, the most productive period of life. Two-third of the AIDS cases were men and 83.7 % acquired HIV infection through sex (Source : Ministry of Public Health, Thailand, January 2003).

HIV-1 biology

Human immunodeficiency virus type 1 (HIV-1) is the major type of HIV causing epidemic worldwide. It is an RNA virus within the lentivirus family[1]. Three groups of HIV-1 have been described :

-Group M (Major) which includes 11 subtypes or sub-subtypes (A1, A2, B, C, D, F1, F2, G, H, J, K) and 13 Circulating Recombinant Forms (CRFs)(CRF01_AE, CRF02_AG, CRF03_AB, CRF04_cpx, CRF05_DF, CRF06_cpx, CRF07_BC, CRF08_BC, CRF09_cpx, CRF10_CD, CRF11_cpx, CRF12_BF, CRF13_cpx, CRF14_BG)[2-4].

-Group O (Outlier) which seems to be endemic to Cameroon and neighboring countries in West Central Africa[5-6]

-Group N (New or non-M, non-O) which has been reported in a limited number of isolates from Cameroonian patients[7-8]

The CRFs within group M represent certain isolates that clustered with different subtypes in different regions of their genomes when phylogenetic analyses were performed[55]. Members of a CRF should resemble each other over the entire genome, with similar breakpoints reflecting common ancestry from the same recombination event(s). There are currently several CRFs of HIV-1. Under new nomenclature proposals, each CRF will be designated by an identifying number, with letters indicating the subtypes involved[57-59]. If the genome contains sequences originating from more than two subtypes, the letters will be replaced by “cpx”, denoting “complex”. Some of the reported CRFs are shown in Table 3[58-62].

Table 3 Some defined Circulating Recombinant Forms (CRF) of HIV-1 group M[58]

NAME	SUBTYPES INVOLVED	GEOGRAPHIC DISTRIBUTION
CRF01_AE	A, E	Predominant in Southeast Asia, sporadic in Central Africa
CRF02_AG	A, G	Predominant in West and West Central Africa
CRF03_AB	A, B	Russia (Kaliningrad)
CRF04_cpx	A, G, H, K, U	Greece, Cyprus
CRF05_DF	D, F	Democratic Republic of Congo
CRF06_cpx	A, G, J, K	West Africa (Mali, Senegal, Nigeria, Burkina Faso, Niger)
CRF07_BC	B, C	Northwest China
CRF08_BC	B, C	Southeast China
CRF09_cpx	Unpublished	Senegal, US
CRF10_CD	C, D	Tanzania
CRF11_cpx	A, E, G, J	Central Africa (Cameroon, Central African Republic, Gabon

Joint United Nations Programme on HIV/AIDS (UNAIDS) has estimated that the global prevalence of HIV genetic subtypes based on envelope (*env*) gene as follow [51] :

- 48 % subtype C
- 25 % subtype A
- 16 % subtype B
- 4 % subtype D
- 4 % subtype E
- 3 % for the remaining subtypes combined

HIV-1 is an enveloped RNA retrovirus within the lentivirus family[1]. It penetrates into the lipid bilayer of host cells via interaction of viral gp120 envelope glycoprotein, especially hypervariable V3 region, and the cellular CD4 receptor along

with the relevant second receptors such as chemokine receptors CXCR-4 and CCR-5[63-69]. Once the HIV delivers its genetic materials into host cell, the viral life cycle starts as shown in Figure 1[9]. Briefly, the HIV uses its reverse transcriptase enzyme to generate the complementary DNA (cDNA) with two identical long terminal repeats (LTRs) containing enhancer and promoter sequences which is later integrated into host cell genomic DNA. The integrated viral DNA or provirus can stay dormantly in the host cell without doing any harm to its host which is the so-called latently infected stage. Once the host cell is activated, the DNA starts its replication cycle, together with the integrated viral genome. At some point when appropriate amounts of viral progeny are produced in infected cell, the newly produced viruses start budding out of infected cell which causes the lysis of infected cell, so-called lytic stage.

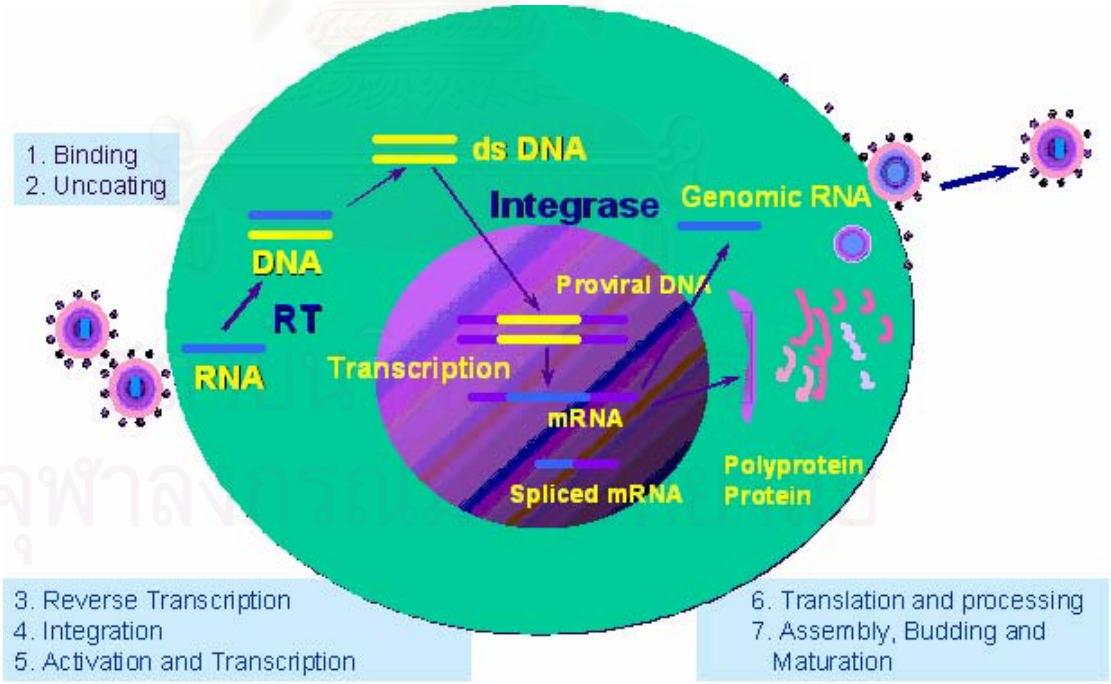


Figure 1 HIV-1 life cycle[9]

The HIV-1 genome is organized into three major structural genes (*gag*, *pol*, *env*) which are essential components of the retroviral particle, four accessory genes (*vif*, *vpr*, *vpu*, *nef*) and two regulatory genes (*tat*, *rev*), respectively.

Gag encodes for the core proteins which stay inside the viral particle. *Pol* encodes for the enzymatic proteins : reverse transcriptase (RT) which is used in reverse transcription step that makes cDNA, integrase (IN) which is important for the proviral integration step and protease (PR) which is used to process correct proteins of the virus. HIV-1 *env* encodes for the outer viral envelope glycoprotein 160 (gp160) which is glycosylated and is later cut into two smaller molecules, gp120 (outer surface membrane glycoprotein which acts as CD4 binding site) and gp41 (transmembrane glycoprotein) by a host protease as viral particles bud from the host cell membrane. More details of all the HIV genomes are shown in Table 4[70].

Table 4 Landmarks of the HIV Genome[70]

NAME (HIV proteins)	SIZE	FUNCTION	LOCALIZATION
Gag MA	p17	membrane anchoring; env interaction; nuclear transport of viral core. (myristylated protein)	virion
CA	p24	core capsid	virion
NC	p7	nucleocapsid, binds RNA	virion
	p6	binds Vpr	virion
Protease (PR)	p15	gag/pol cleavage and maturation	virion
Reverse transcriptase (RT), RNase H	p66 p51	Reverse transcription, RNase H activity	virion
Integrase (IN)		DNA provirus integration	virion
Env	gp120 gp41	external viral glycoproteins bind to CD4 and secondary receptors	plasma membrane, virion envelope
Tat	p16/p14	viral transcriptional transactivator	primarily in nucleolus/nucleus
Rev	p19	RNA transport, stability and utilization factor(phosphoprotein)	primarily in nucleolus/nucleus shuttling between nucleolus and cytoplasm
Vif	p23	promotes virion maturation and infectivity, inhibit CRMs	cytoplasm (cytosol, membranes), virion
Vpr	p10-15	promotes nuclear localization of preintegration complex, inhibits cell division, arrests infected cells at G2/M	virion, nucleus (nuclear membrane?)
Vpu	p16	promotes extracellular release of viral particles; degrades CD4 in the ER; (phosphoprotein only in HIV-1 and SIVcpz)	integral membrane protein
Nef	p12-p16	CD4 and class I downregulation (myristylated protein)	virion (nucleus?)

Clinical manifestations of HIV infection

HIV infection may result in a protean of manifestations. Three to 6 weeks after HIV entry, 10-20 % of the infected individuals may develop an ill-defined flu-like syndrome, called “acute HIV infection” or “primary HIV infection”. Symptoms in some patients may be too mild to be recognized. Acute HIV infection is characterized by acute onset of fever, headache, generalized lymphadenopathy, pharyngitis, myalgias, erythematous maculopapular rash, mucocutaneous ulceration, oral thrush, diarrhea,

nausea, vomiting, hepatosplenomegaly, weight loss and neurologic manifestations such as aseptic meningitis, peripheral neuropathy, facial palsy and cognitive impairment [71]. The symptoms may last 2-4 weeks, then resolve spontaneously.

HIV-infected individuals may remain asymptomatic for an average of 10 years. The only finding at the early asymptomatic phase is symmetrical generalized lymphadenopathy. The most prominent lymphatic chain is the posterior cervical chain, followed by axillar and inguinal chains. The size of the lymph node is usually 1-2 cm in diameter but some may be as large as 2-3 cm. They are multiple or in chains and are painless. Lymphadenopathy will spontaneously resolve after 1-2 years. Occasionally lymph node biopsy is performed to rule out underlining conditions and the histology shows reactive lymphoid hyperplasia.

Symptomatic HIV infection, formerly termed “AIDS Related Complex” or “ARC”

Table 5 lists the ARC symptoms commonly found in HIV-infected Thai patients[72]. Oral hairy leukoplakia was most commonly found (85.2 %), followed by pruritic papular eruptions, oral candidiasis, herpes zoster and others. Pruritic popular eruptions are uncommonly seen in Caucasian patients but are common in HIV-infected Asians and Africans. It is characterized by symmetrical papulonecrotic eruptions in the upper and lower extremities which is intensely pruritic. It rarely involves face or upper trunk. Lesions are healed by hyperpigmented macules and aggravated by insect bites.

Full-blown AIDS

Table 6 lists the AIDS-defining illnesses according to the United States Center for Diseases Control[71-73]

Table 5 AIDS-related complex in 236 untreated Thai patients[72]

	N	**%**
Oral hairy leukoplakia	201	85.2
Pruritic popular eruptions	120	50.8
Oral candidiasis	116	49.2
Herpes zoster	76	32.2
Severe weight loss	58	24.6
Chronic fever	46	19.5
Chronic diarrhea	40	16.9
Bacterial skin infections	36	15.3
Seborrheic dermatitis	27	11.4

Table 6 AIDS case definition (adults) : US CDC, 1997[71,73]

	N*
Candidiasis of esophagus, trachea, bronchi or lungs	16.0
Cervical cancer, invasive	0.6
Coccidioidomycosis, extrapulmonary	0.3
Cryptosporidiosis with diarrhea > 1 month	5.0
CMV of any organ other than liver, spleen or lymph node; eye	7.0
Herpes simplex with mucocutaneous ulcer > 1 month or bronchitis, pneumonitis, esophagitis	5.0
Histoplasmosis, extrapulmonary	0.9
HIV-associated dementia : Disabling cognitive and / or other dysfunction interfering with occupation or activities of daily living	5.0
HIV-associated wasting : Involuntary weight loss > 10 % of baseline plus chronic diarrhea ($\geq$ loose stools/day $\geq$ 30 days) or chronic weakness and documented enigmatic fever $\geq$ 30 days	18.0
Isosporosis with diarrhea > 1 month	0.1
Kaposi's sarcoma	7.0
Lymphoma, - Burkitt's	0.7
- immunoblastic	2.3
- primary CNS	0.7
Mycobacterium avium, disseminated	5.0
Mycobacterium tuberculosis, -pulmonary	7.0
- extrapulmonary	2.0
Pneumocystis carinii pneumonia	38.0
Pneumonia, recurrent bacterial ($\geq$ 2 episodes in 12 months)	5.0
Progressive multifocal leukoencephalopathy	1.0
Salmonella septicemia (nontyphoid), recurrent	0.3
Toxoplasmosis of internal organ	4.0

* indicates frequency of 23,527 reported adult AIDS cases in U.S.A. as of 1997[71-73]

In Thailand, disseminated penicilliosis has to be added on the list since it is common in South-East Asia[74]. Tuberculosis, pneumocystis pneumonia and cryptococcal meningitis are the 3 major presenting symptoms of Thai adult AIDS patients. The frequency in various hospitals was quite comparable except disseminated penicilliosis was much more common in the North. Table 7 lists the frequency of various AIDS manifestations of HIV-1 infected Thai patients collected during 1991-1993[75]. Larger nationwide collection of 101,945 cases from 1994 to 1998 also showed a similar pattern of AIDS manifestations throughout 5-year period[76].

Table 7 Spectrum (%) of AIDS manifestations in Thailand[75]

AIDS illnesses	**Chiang Mai Hospital (N = 866)**	**Bamrasnaradura Hospital (N = 1,444)**
Tuberculosis	32.0	34.0
Cryptococcosis	17.4	20.3
Pneumocystis pneumonia	13.0	13.4
Penicilliosis	14.0	4.5
Toxoplasma	5.4	4.0
Esophageal candidiasis	3.5	6.4
Cryptosporidiosis	2.5	1.1
Mycobacterium avium complex	-	0.2
Cytomegalovirus	-	0.1
AIDS dementia	-	2.5
Kaposi's sarcoma	-	0.1
Lymphoma	-	0.7
HIV wasting syndrome	0.7	-
Salmonella septicemia	8.2	-

Natural course of HIV-1 infection

After HIV infection, immune responses (both cell-mediated and humoral immune responses) are recruited in order to curb the infection. High levels of virus (HIV viral load) can be detected within 1 to 3 months after HIV infection. Cytotoxic T lymphocyte (CTL) response occurs later with the reduction of virus (HIV viral load) in the circulation[10]. Antibodies to HIV shows up later than the CTL response. Anti-HIV can be detected as early as 6-8 weeks after HIV infection but will be universally positive by 3-6 months.

The course of HIV-1 infection can be divided into 3 stages as[10] :

-primary infection, which is often associated with an acute flu- or mononucleosis-like clinical syndrome that lasts approximately 3-6 weeks following infection. The $CD4^+$ T lymphocytes which is the target of HIV-1 infection also declines during this first 2-8 weeks[77]. This is usually accompanied by a burst of viral replication (high viremia)[78-80]. Furthermore, infectious virus and viral proteins can be readily detected in the cell-free plasma as well as in cerebrospinal fluid. The number of virion could be as high as 10^6-10^7 per millitre. However, only a small percentage of the virions are infectious.

-clinical latency, which is relatively long period. Patients usually have a gradual deterioration of the immune system which is manifested particularly by the progressive depletion of $CD4^+$ T cells[81]. This stage is characterized by few, if any, clinical manifestations and the symptoms are usually mild.

-clinical apparent disease, which is accompanied by a steady decline of $CD4^+$ T lymphocytes and increasing viral replication. Opportunistic infections are usually found at this stage of disease.

The natural course of HIV-1 infection can be summarized as shown in Figure 2[10].

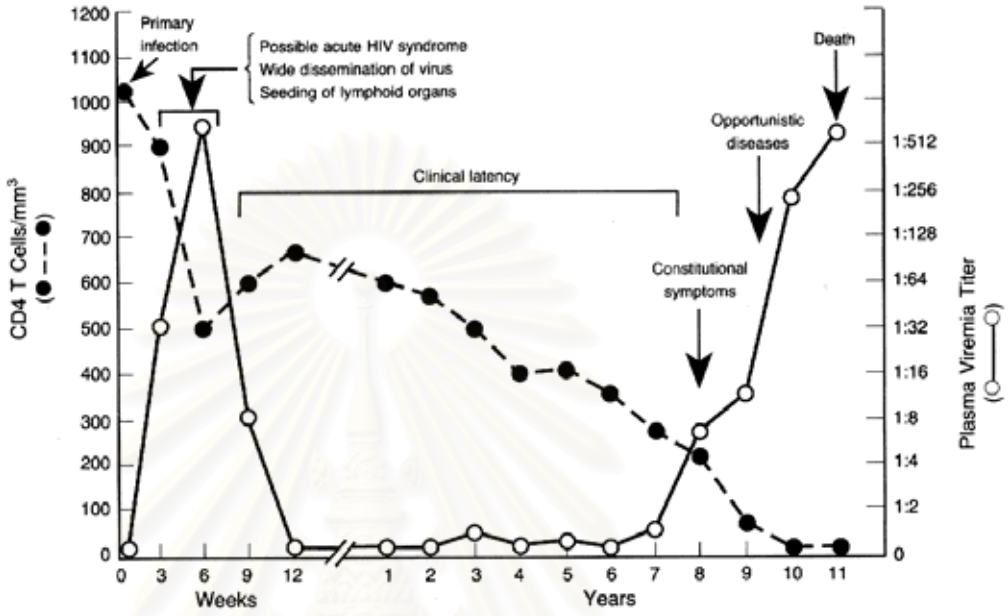


Figure 2 Course of HIV-1 infection showing primary HIV infection, clinical latency and symptomatic diseases in relation to the absolute number of $CD4^+$ T lymphocytes and the level of viremia[10]

HIV-infected individuals will develop AIDS within a mean interval of 8-10 years after HIV infection[82]. This is extrapolated from the observed mean annual progression rate of 5-6 %[83]. The study among female sex workers in Chiang Rai found that the median time from infection to having a CD4 count of < 200 x 10^6 cells/l was about 7 years after time of infection[84]. This is consistent with our findings in a smaller cohort of patients[54].

Clinical progression depends on the rate of decline of $CD4^+$ T cells which in turn is affected by the burden of HIV in the body[85]. $CD4^+$ T cells in HIV-1 infected individuals will decline at an average of 50 cells per annum. The higher the viral burden

in the body, the more rapid deterioration of $CD4^+$ T cells[86]. For example, CD4+ T cells will decline 80 cells per year in someone with a viral load over 30,000 copies/ml whereas only 40 cells per year in patients with viral load less than 500 copies/ml.

Viral burden can be boosted by vaccination, concurrent infection and other means of cellular or immune activation[87]. On the other hand, antiretroviral therapy is the effective mean in reducing the viral load in HIV-infected patients. As a result, concurrent infection such as tuberculosis, will enhance HIV disease progression whereas antiretroviral therapy will slow down clinical progression to AIDS.

The majority (80-90 %) of HIV-1 infected individuals will develop AIDS after a mean interval of 8-10 years, called typical progressors. About 5-10 % of HIV-1 infected individuals will develop AIDS within 3-4 years, called rapid progressors[88].The bases for rapid progressors may be due to the large inoculum of infection such as from blood transfusion or due to the aggressive viral phenotype such as syncytial inducing (SI) phenotype at infection or due to repeated concurrent infections as well as the weakened immune status at baseline. Another 5 % of HIV-1 infected individuals will maintain their $CD4^+$ T cell number over 500 cells/cu.mm. after 10-15 years of HIV-1 infection without any use of antiretroviral drugs, called slow progressors or “long-term non-progressors”[89-91]. These are patients with low viral set point and strong cytotoxic T lymphocyte (CTL) response to the virus[92]. Many slow progressors also have a genetic mutation of CCR5, a coreceptor of HIV on monocyte.macrophage and primary $CD4^+$ T cells. The mutation is a deletion of 32 base-pair from CCR5 gene, called CCR5-Delta(Δ)32 allele[93]. Individuals with homozygous Δ32 allele will be relatively resistant to HIV-1 infection whereas individuals with heterozygous Δ32 allele will have a slow clinical progression. Homozygous and heterozygous CCR5 Δ32 alleles are found in 1 % and 10-15 % of Caucasians, respectively[94]. The CCR5-Delta32 allele was not detected

in one study population of 200 healthy Thai blood donors[95]. However, there are also other genetic mutations of chemokine receptors which dictate the susceptibility and resistance to HIV-1 infections. These are CCR2 and SDF-1 (stromal cell-derived factor1)[96]. The frequency of CCR2-64I and SDF1-3A', markers of slow progressors, were found in 15.7 and 33.2 %, respectively in normal Thai individuals[95] suggesting that a small proportion of HIV-1 infected Thai individuals may also have a slow progression pattern.

Antiretroviral therapy

Although many potent antiretroviral (ARV) drugs acting at different stages of HIV life cycle are currently available, the majority of patients worldwide will not have easy access to these expensive therapeutic regimens. Induction of ARV drug resistance, short-term and long-term adverse side effects of ARV are other factors to be considered. Zidovudine (ZDV, azidothymidine, AZT) was the first antiretroviral agent approved for used in HIV infection[97]. It belongs to the class of nucleoside reverse transcriptase (RT) inhibitors. Many more drugs of the same class have been developed such as didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), and abacavir (ABC). There are also drugs from other classes such as nonnucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI) and entry inhibitors[98-104]. Combination of at least 3 of these drugs is the standard of care at present[105-106]. It offers the highest level of virologic suppression, termed, highly active antiretroviral therapy (HAART).

The objective of HAART is to maximally suppress viral replication. This will result in less destruction of $CD4^+$ T cell, accompanied by replenishment of the $CD4^+$ T cell pool[107-108]. The increased level of $CD4^+$ T cell will prevent the patient from

opportunistic infections. The final outcome is improved quality of life and prolonged survival[13-14]. The antiretroviral therapy guidelines as recommended by International AIDS Society (IAS)-USA Panel is shown in Table 8[109].

Table 8 Recommendations for Initiating Therapy in Treatment-Naïve Individuals[109]

Disease Type	Recommendation
Symptomatic HIV disease	Treatment recommended
Asymptomatic HIV disease, < 200 CD4 cells/µl	Treatment recommended
Asymptomatic HIV disease, > 200 CD4 cells/µl	Treatment decision should be individualized; recommendations are based on : CD4 cell count and rate of decline† HIV RNA level in the plasma ‡ Patient interest in and potential to adhere to therapy Individual risks of toxicity and drug-drug pharmacokinetic interaction

† Some clinicians and guidelines use a CD4 count threshold of 350 cells/µl to initiate therapy[110]; a high rate of CD4 cell count decline is > 100 cells/µl per annum[111].

‡ A high HIV RNA level is above 50,000-100,000 copies/ml[112]. The frequency of CD4 cell measurements before therapy is initiated may be guided by the plasma HIV RNA level.

Antiretroviral agents are expensive, as well as the laboratory monitorings of treatment results. Less than 10 % of HIV-infected individuals in developing countries worldwide can have access to antiretroviral therapy. Treatment is also faced with short-term and long-term side-effects, adherence problem and drug resistance[113-121]. In addition, drug therapy cannot eradicate the virus with present strategy. Therefore, although antiretroviral therapy can save millions of life, it is not the ultimate goal. We still need HIV vaccine to prevent the infection, alongside with condom and other preventive measures.

The Thai National Guidelines recommend the initiation of antiretroviral therapy when the patients are symptomatic or when $CD4^+$ T cells drop below 200 cells/mm^3 (Thailand National Guidelines for the Management of HIV-infected Adults and Children 2002, Ministry of Public Health). In concordant with the WHO Guidelines[122], viral load determination was not taken into account in deciding initiation of antiretroviral therapy in Thailand due to its cost and availability. Viral load is also not routinely recommended to monitor the outcomes of antiretroviral therapy. Therefore, it is anticipated that resistance may be a potential threat if adherence to therapy is not strictly observed[123]. This emphasizes the need to prepare the patients and the healthcare providers as well as the infrastructure of the healthcare facilities in order to implement a successful treatment program in resource-limited settings[122, 124].

HIV Vaccine

Like many other viral infections such as rabies and hepatitis B, vaccine to prevent HIV infection can be anticipated even there is no cure for the infection[125]. In fact, attempts to develop an AIDS vaccine started immediately after the discovery of AIDS virus in 1983[46-48]. Zagury et al reported the first HIV vaccine trial in man in 1988[126]. HIV vaccine development progresses parallely in animal and man. Many HIV-1 vaccine candidates have been developed in the laboratory and tested in animals. Much fewer number of candidate HIV-1 vaccines have advanced to human trials. Interest for HIV vaccine trials in man comes in waves depending on the success and failure stories of ARV therapy and of vaccine trials as well as on the push from international agencies or from US Administration.

In spite of nearly 2 decades of intensive researches, no effective HIV vaccine, either for therapeutic or prophylactic purposes, has yet emerged[127-131]. Main obstacles for the development of HIV vaccine are as follows :

1. Genetic variability

   There are 2 types of HIV, HIV-1 and HIV-2. HIV-1 is a big cluster of viruses currently spreading throughout the world whereas HIV-2 is mainly restricted to Western Africa [132-133]. HIV-2 is less pathogenic, less transmissible and closer to simian immunodeficiency virus (SIV) than HIV-1[134-136]. Within HIV-1, there are 3 main groups : M (major), O (outlier) and N (non-M and non-O)[1, 4-8]. And, within group M, there are at least 11 HIV-1 subtypes (clades) and 13 circulating recombinant forms (CRFs) which are antigenically different[4]. One or many subtypes may be endemic in one country or one region and one subtype may be endemic in many countries or many regions. This makes the selection of which HIV-1 subtypes as the prototype of HIV-1 vaccine for any country or any continent difficult. The solution to this problem is to select a subtype which is most prevalent for a particular population or to include all major subtypes in the candidate vaccine.

2. High rate of mutation

   HIV reverse transcriptase (RT) enzyme does not possess proof reading activity during the reverse transcription step. As a result, there is a high rate of mutation from the error-prone RT enzyme, approximately 1 nucleotide substitution per replication cycle/genome [1]. This can cause sequence divergence over time and even occurs in the same infected individuals (intra-individual), the so-called quasispecies of HIV-1[137-141]. It has been reported by Gashen et al[17] that the envelope proteins of the HIV-1 in the same subtype can differ up to 20 % and

can be as high as 35 % for HIV-1 in different subtypes. They even emphasized that the diversity was continually growing. So HIV that exists today may have different antigenic composition from the HIV 10 years from now. To catch up with the antigenic drift, especially the envelope protein, the antigenic composition of the vaccine may have to base on the most recent strains. The alternative is to use the conserved epitopes as the immunogens such as the gag proteins. Furthermore, broad cross-clade CTL recognition directed against gag-specific targets has also been well documented[142].

3. Nature of latent infection

HIV can co-exist with the host cell as a proviral DNA and the infection may be latent for several years[10]. Once HIV is integrated into host cell DNA, it is no longer susceptible to the immune attack by the antibody. The attack by cytotoxic T lymphocyte (CTL) may not be complete due to the latent nature of the infection. As a result, HIV vaccine may not be able to prevent infection if HIV rapidly integrates into the cells, i.e., not sterilizing immunity.

4. Lack of immune correlates for protection

It is desirable for vaccine development to know that which immune response correlates with disease protection. Scientists can then design a vaccine that will result in that beneficial immune response which can be either humoral or cellular immune response. They can then use that immune response to measure the effectiveness of their vaccines in addition to the clinical efficacy testing which takes time. Unfortunately, such immune correlates for HIV infection are not yet known since no one has been confirmed to recover from the infection. However, there are some collateral evidences that can be used as leads. These are highly exposed but persistently seronegative (HEPS) individuals such as

female commercial sex workers in Kenya[24], long-term non-progressors (LTNP)[18-19] and exposed but uninfected infants[21-22]. Evidences from these cohorts indicated that cytotoxic T lymphocyte (CTL) directed against various portions of HIV (gag-, pol-, and env-encoded products) might contribute to protection[143]. On the other hand, passive transfer of neutralizing antibody could protect chimpanzees from the infection with the identical challenged laboratory HIV-1 strains[144]. Broadly neutralizing antibody to primary HIV isolates was also detected in some LTNP but not in progressors[145].

These informations, together with the current knowledge on protective immunity in various viral infections, CTL response is the most desirable response. However, any HIV vaccine approach that can also result in broadly neutralizing antibodies against field isolates is also welcome. It is generally believed that HIV-1 vaccine that can induce strong and broadly reactive neutralizing antibodies will be able to prevent the infection (sterile immunity) whereas CTL can only attenuate the infection or at best, clear the settled infection[146-150]. Therefore, any HIV candidate vaccines or vaccine strategies that can induce both neutralizing antibodies and CTL are ideal.

5. Lack of appropriate animal model to study HIV vaccine

Chimpanzee is the only non-human primate species that can be infected by HIV. However, HIV does not replicate persistently in chimpanzees nor does HIV consistently cause diseases in this species[151]. In addition, the cost and the availability of chimpanzees are also another issues. As a result, scientists have been working on various simian immunodeficiency viruses (SIV), thought to be equivalent to HIV[152]. In addition, a chimeric virus of SIV/HIV hybrid (called SHIV) has been developed. It is composed of HIV-1 envelope, *tat*, *rev*

and *vpu* but SIV *gag-pol*[153]. The SIV backbone will overcome host range restrictions and allow for replication in monkeys. At the same time, HIV-1 envelope will allow efficacy testing of the envelope-based vaccine in monkeys. Scientists can then study whether rhesus monkeys immunized with envelope-based HIV-1 vaccine can be protected from SHIV challenge, i.e., sterile immunity.

6. Lack of knowledge of protective immunogens

Parallel to the lack of knowledge of protective immune correlates, protective immunogens are still unsettled. It is not known which parts (*env*, *gag*, *pol*, *tat*, *rev*, etc) of the virus will be the best to be used for candidate vaccine. Of the several components of HIV-1, which one should be incorporated into the candidate vaccine is still undetermined. Envelope and gag proteins are the most frequently tested components, either alone or in combination[154-157]. Whether pol-encoded proteins or the accessory genes-encoded proteins such as tat, nef and rev are also required is still uncertain. Even for envelope glycoproteins alone, some modifications are essential. Romoval of V1/V2 or carbohydrate residues from gp120 was shown to better expose the neutralizing epitopes of gp120[158-159]. Trimeric glycoprotein of recombinant gp120 was more efficient in inducing neutralizing antibody to primary isolates due to the more resemblance of the natural virus[160-161]. Therefore, although it is desirable to include as many essential components as possible in the candidate HIV-1 vaccine, one has to make sure not to include the interfering epitopes.

7. Multiplexity of route of infection

   Vaccination is frequently carried out by parenteral route but HIV infection can also be resulted from mucosal exposure; vaginal, anal and oral (such as breast feeding). Whether parenteral immunization will result in strong protective mucosal immunity or whether mucosal immunization is also required are topics under active investigations. It reflects the complexity of HIV vaccine development.

8. The sequestration of HIV in immune privileged sites such as CNS, lymphoid organs which makes the immune system hardly get access to the HIV.

9. The existence of immune suppressive factors from the virus such as tat (the protein encoded by *tat* or trans activation gene) that acts to increase viral gene expression in infected cells up to hundred folds[26-28].

10. Lack of incentives

    Vaccine research and development is costly. From the history of HIV-1 vaccine research and development, chance of success is small[162]. In addition, even it is successful, the chance for profit making is small since major demand comes from countries with limited resources. As a result, not very many private vaccine companies are actively doing HIV-1 vaccine research and development. In response, public funds such as those from the US-National Institutes of Health and International AIDS Vaccine Initiatives (IAVI) are being given to universities and pharmaceutical industry to develop and test HIV-1 candidate vaccines[163].

Forms of HIV vaccine delivery

There are many forms that an HIV vaccine can be delivered. Some of the approaches are classical vaccine approaches such as inactivated whole virus or recombinant proteins. Some are newer approaches such as live recombinant vector and DNA vaccines. Each form or each delivery system has its own advantages and disadvantages such as ease of production, safety and immunogenicity. Some of the HIV vaccines that have gone through clinical trials are[164-165] :

1. Live attenuated vaccine
2. Whole killed or inactivated vaccine
3. Virus-like particles
4. Synthetic peptide vaccine such as octameric V3 peptide (V3-MAPS), p17 (HGP-30), Ty.p17/p24 virus-like particles (VLP)
5. Recombinant subunit vaccine such as rgp160, rgp120 (IIIB, MN, SF2, E), rp24
6. Live recombinant vector vaccine such as vaccinia, canarypox, fowlpox, adenovirus, BCG with recombinant gp120/160, gag, pol
7. DNA vaccine encoding gp120, gp41, gag, pol, rev
8. Combination or prime-boost approach such as live recombinant vector followed by recombinant proteins, DNA followed by live recombinant vector

Many approaches have come to an end because of non-promising results. Few still receive active attention at present. The overall results of the completed and ongoing HIV vaccine trials can be summarized as follows :

(1) All candidate vaccines are safe and well tolerated even with live attenuated vaccine and inactivated whole virus vaccine. Nevertheless,

there are still some ethical concerns of using killed or live attenuated vaccine in normal volunteers.

(2) All vaccine candidates can uniformly induce antibody response. However, the antibody induced by the recombinant envelope vaccines can only neutralize the homologous laboratory strains used to make the vaccine. It can hardly neutralize the primary isolates from the patients, even it belongs to the same subtype with the vaccine[164,166-167]. Live recombinant vector vaccine can induce better antibody response and the antibodies can also cross neutralize field isolates of the same subtype[168].

(3) T helper cell response such as the lymphoproliferative response to the homologous antigen can also be readily demonstrated in almost all vaccine approaches[157, 169].

(4) Cytotoxic T lymphocyte (CTL) response, on the other hand, can hardly be detected by recombinant envelope immunization[170]. If detected, it is usually of CD4 phenotype, never of $CD8^+$ CTL. In contrast, live recombinant envelope vaccine could induce strong $CD4^+$ and $CD8^+$ CTL response[154]. However, the proportion of responder is usually under 50%.

(5) DNA immunization, the newest approach, was also shown to induce strong CTL[29, 32, 171-172]. However, it is inferior in term of antibody production. Therefore, it is hoped that boosting with recombinant proteins or live recombinant vector following DNA priming will offer an ideal approach in generating neutralizing antibodies and CTL.

History of HIV vaccine trials in Thailand

The first HIV vaccine trial in Thailand took place in 1994[173]. Many more trials have followed, ranging from phase I to phase III. Table 9 details the chronological sequence of the trials of HIV-1 candidate vaccines in Thailand, both completed and planned[165].

Table 9 Clinical trials of HIV candidate vaccines in Thailand[165]

Sequential number	Type of vaccine (Manufacturer)	Phase	Number of subjects	Launch-Finish date
1	MN synthetic V3 peptide (UBI)	I	30	6/6/94-23/1/95
2	MN gp120 (Genetech)-IDU	I/II	33	21/2/95-25/1/96
3	SF2 gp120 (Chiron)	I	54	29/8/95-29/11/97
4	HIV Immunogen (Immune Response)*	II	30	27/2/96-24/7/97
5	HIV Immunogen (Immune Response)*	II	297	27/8/97-99
6	HIV E/B gp120 (Chiron)	I/II	380	10/11/97-99
7	HIV E/B gp120 (Vaxgen)-IDU	II	92	16/2/98-99
8	HIV E/B gp120 (Vaxgen)-IDU	III	2,500	3/9-04
9	HIV E ALVAC (vcp1521) prime/gp120 or gp160 boost (PMC-Aventis/Chiron)	I/II	130	2000-2001
10	HIV E ALVAC (vcp1521) prime/gp120 or gp160 boost (PMC-Aventis/Vaxgen B/E)	I/II	130	2000-2001
11	HIV E ALVAC (vcp1521) prime/gp120 boost (Vaxgen) B/E	III	16,000	To be started in 2003
12	HIV B adenovirus (MSD)	I/II		To be started in 2003
13	HIV E DNA (env, gag, pol) prime/FPV boost (Australian-Thai Vaccine Initiatives)	I/II	100	To be started in 2004

* as therapeutic vaccine

Although the immune response from a particular vaccine approach may vary, Bagarazzi et al attempted to compare the strengths and weaknesses of various vaccine

approaches as summarized in Table 10[29]. It appears that DNA vaccine seems to be the most attractive approach.

Table 10 Immune responses induced by different vaccine approaches[29]

Vaccine approaches	Helper T cells		CTL	Antibody	Strengths	Weakness
	Th1	Th2				
Live attenuated	?	?	+	+	Cellular immunity Humoral immunity	Safety in immunocompromised host
Whole inactivated		+	-	+	Ease of preparation Cost	Limited immunity
Virus-like particles		+	-/+	+		Limited immunity Difficult preparation
Protein Subunit peptides		+	-/+	+	Humoral immunity	Poor cellular immunity Difficult preparation Cost
Plasmid DNA	+		+	+	Cellular immunity Humoral immunity Ease of preparation Cost	Limited experience

All of the HIV vaccine candidates being tested in Thailand up to now were developed by the foreign vaccine companies. Thai scientists as a group, felt that they need to develop or participate in the development of HIV-1 vaccine for Thailand's use. Several groups of Thai scientists are currently working on the various HIV-1 vaccine designs such as live recombinant BCG vaccine and DNA vaccine.

DNA VACCINE

The first genetic immunization or DNA vaccine approach was reported by Wolff JA in 1990[174]. They documented that intramuscular injection of plasmid DNA containing *LacZ* gene could result in β-galactosidase production in the surrounding muscle cells. This implied, for the first time, that naked DNA so injected could enter the cell without the help of any permeability agents like in the case of *in vitro* transfection and more importantly, could be expressed. These findings formed the rationales of using naked DNA as vaccine. Many studies of DNA vaccines soon followed and results showed that they were indeed immunogenic[175-177].

DNA vaccine represents a novel tool of expressing antigens (immunogens) *in vivo* for the generation of both humoral and cell-mediated immune responses. It has been shown that this can elicit immunotherapeutic effect for cancers[178-179] and protective immunity in a number of experimental diseases such as rabies, influenza, malaria, tuberculosis and allergic diseases[180-189]. DNA vaccine employs genes encoding proteins of pathogens or of tumors, rather than using the proteins themselves. It is composed of a bacterial plasmid with a strong viral promoter such as CMV (Cytomegalovirus) promoter, the gene of interest which is placed right after the viral promoter, and a polyadenylation/transcriptional termination sequence[190-192].

HIV DNA vaccine has received a lot of attention during the last 5-6 years. Plasmid DNA encoding env, gag and pol regions of HIV-1 subtypes A, B and A/E have been constructed and tested in animals as well as in men[169,171-172,193-196]. The studies showed that these HIV DNA constructs were safe, well tolerated and could stimulate strong humoral and cellular immune responses[29,32,157,169,171-172]. Repeated DNA immunizations could result in strong humoral and cellular immune responses even

without boosting with live recombinant vector vaccine (unpublished observation, Kent SJ, Australia). However, DNA vaccine-prime and vaccinia virus- or fowlpox virus-boost strategies can result in much enhanced immune responses[197-198].

HIV-1 DNA vaccine was first tested in normal human volunteers in 1999. The first published result showed that HIV-1 env/rev DNA vaccine was safe and well tolerated. Antigen-specific lymphocyte proliferation and beta-chemokines production could be detected in 100 % or 6/6 of immunized individuals after third immunization[169]. Many more HIV-1 DNA vaccine candidates using constructs of DNA from subtypes B, A/E, C have been tested in normal volunteers since [169,171-172,193-196].

As compared to other vaccine strategies, DNA vaccine is considered by many investigators to have the following advantages[29-32].

Potential advantages of DNA vaccine

(1) non-infectious / non-replicating but mimics aspects of live attenuated vaccines without the risk of infection with the microorganism

(2) proteins produced in native conformation, particularly the viral proteins

(3) physicochemical homogeneity and stability; the size of the nucleic acid insert can be as large as 9000 base pairs[199]

(4) easier to incorporate several fragmented components in a single vaccine

(5) only the genomic portion encoding the required immunodominant epitopes of the pathogen to be included in the plasmid DNA, not the unrequired portion

(6) simplified production (easily produced and potentially cheaper)

(7) induction of both cell-mediated and humoral immunity, especially cytotoxic T lymphocyte (CTL) response

(8) protection is supposed to be long-live and may require fewer booster doses

(9) DNA is heat-stable and can be lyophilized. Therefore, it is feasible to transport the vaccine in resource-limited setting where cold chain may not be available.

More specifically, as compared to recombinant proteins, DNA vaccines are more effective in inducing cell-mediated immunity to the immunizing antigen[32]. When compared to live attenuated vaccine which is also potent in generating cell-mediated immunity, DNA vaccine is much safer.

Disadvantages of DNA vaccine

Although DNA vaccine has many advantages, some disadvantages have been anticipated[200] such as :

(1) the potential of insertion into the genome that may cause the activation of oncogenes, inactivation of tumor suppressor genes or chromosomal instability. However, this risk has been reported as extremely rare[201]

(2) the induction of autoimmunity[202]. Injection of bacterial DNA into lupus-proned NZB/NZW mice could result in anti-DNA formation[203]. However, injection of HIV-1 DNA vaccine into non-lupus prone mice did not cause anti-DNA formation. Human study also did not reveal any anti-DNA formation. Autoimmune myositis is another safety concern since muscle cells harboring foreign antigens may be subjected to immune attack. This also was not found in the human study [172].

(3) the induced antibody, if any, is rather weak which may need a prime-boost strategy.

(4) the induction of immunologic tolerance, especially in fetal or germline cells[204]

Basic structure of vectors for DNA vaccine

Most plasmids used for vaccination purposes share the basic structures of vectors developed for *in vitro* expression of genes in transfected cell lines. The basic structures include:

(a) an origin of replication (ori) suitable for producing high yield of plasmid in host cell such as *E.coli*

(b) an antibiotic resistance gene to confer antibiotic-selected growth in *E.coli* which is helpful for the selection of the recombinant plasmids

(c) a strong enhancer/promoter to initiate transcription

(d) an mRNA transcript termination/polyadenylation sequence for directing expression in mammalian cells

(e) an intron sequence which helps to give higher level of antigen expression

Figure 3 depicts an example of the basic structure of plasmid vector as described above.

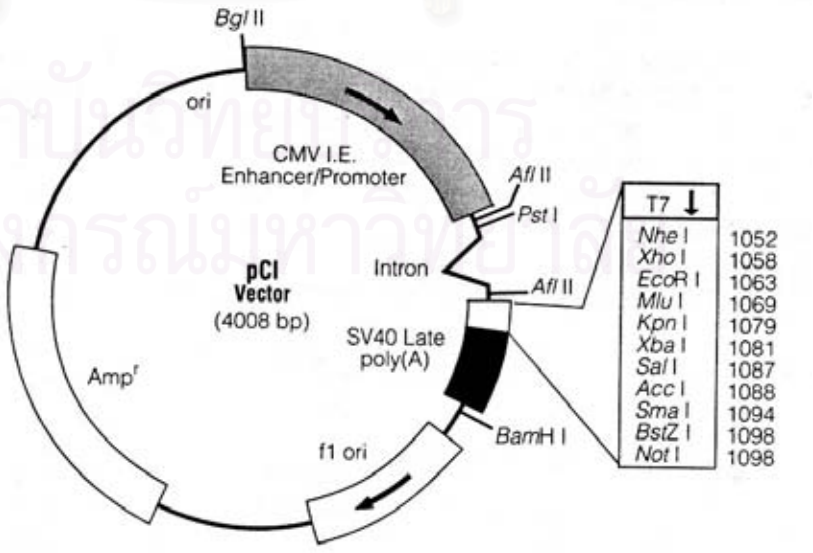


Figure 3 Basic structure of vectors for DNA vaccine

Mechanism of action of DNA immunization

When DNA is injected intramuscularly, myocytes appear to be the predominant cell type transfected[190]. Some injected DNA are also possibly taken up by antigen presenting cells[191]. Therefore, any cooperative mechanisms of antigen processing are of particular importance in immune responses induced by DNA immunization. Some suggested mechanisms that might explain the induction of MHC class I-restricted CTL are : (1) antigen presentation mediated directly by transfected myocytes, (2) transfer of antigen from transfected myocytes to professional APCs[192,205]. (3) professional antigen presenting cells (APCs) becoming transfected and serving as the APCs,

It has been shown that professional APCs present antigen in an MHC class I-restricted manner after DNA immunization and that muscle cells can produce the antigen that enters this pathways[206-207]. The synthesized proteins will be transported to the cell surface for excretion. One can document the expression of the inserted DNA by the detection of the specific proteins on the surface of muscle cell at the injection site or some related cells. Tadokoro et al reported detection of HIV envelope proteins in alveoli, lung, liver, spleen, regional lymph nodes and kidney of mice immunized either intranasally or intramuscularly with HIV-1 DNA plasmids by using fluorescence *in situ* hybridization (FISH) technique[208]. Furthermore, some evidences have shown that muscle cells were directly transfected with NP (nucleoprotein) DNA vaccines and the intramuscular route of administration of NP DNA yielded the best CMI-mediated protection[209-210].

The antibody response to the injected DNA is the result of the protein (antigen) being released from the muscle cells[190]. The release may be the result of cell death or the nature of the protein which can be secreted from the muscle cell. The released antigen is then processed by antigen presenting cell (dendritic cell) and presented to

CD4+ T helper cell via MHC class II receptors which will help B cell to produce antibody. The proposed mechanism of DNA vaccine is shown in Figure 4[205].

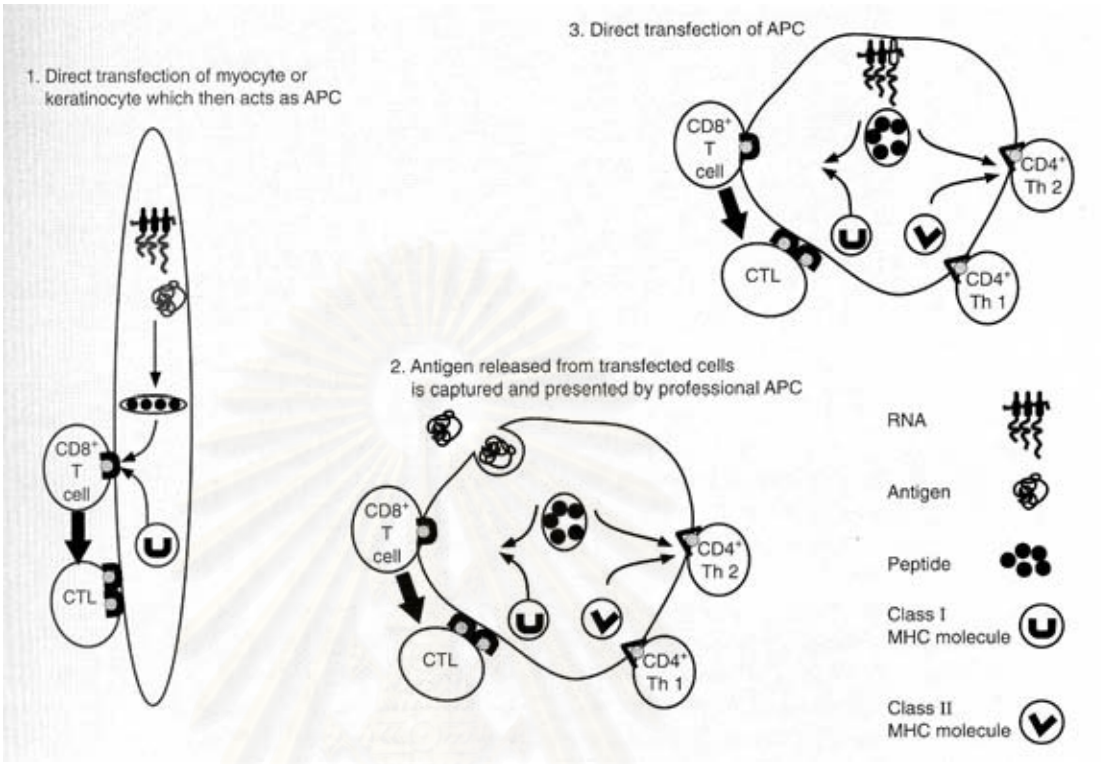


Figure 4 Mechanism of DNA vaccine[205]

DNA immunization has been tried in a variety of conditions. It ranges from prevention of infectious diseases to prevention of cancer and from treatment of allergic diseases to treatment of cancers[178-179]. DNA immunization has been studied to prevent rabies[180-181], malaria[182-184], influenza[185-186], tuberculosis[187] and allergic disease[188-189].

Adjuvant effect of CpG immunostimulatory motifs

The immunogenicity of DNA vaccines depends on the presence of immunostimulatory CpG motifs consisting of unmethylated CpG dinucleotides bounded by certain flanking sequences[211]. These CpG motifs are present as hexameric DNA

sequence motifs in bacterial DNA at a much higher frequency than in vertebrate DNA. The vertebrate immune system can recognize such sequences as a “danger signal” and respond by general immune activation. The CpG motifs are also known as immunostimulatory sequence (ISS)[212]. The maximally stimulatory CpG is TGACGTT. Preliminary evidence suggests that ISS induces cytokines such as IFN-γ, IL-12 and IL-18 which in turn is responsible for the Th1 type immune response following intramuscular delivery of naked DNA[213]. A typical plasmid used as a DNA vaccine vector backbone already contains many CpG motifs. Not all CpG motifs are immunostimulatory. That means not only the particular sequence context of the unmethylated CpG dinucleotide is important, but there are species-specific motifs. Many motifs which work well on mouse cells do not stimulate human cells, whereas those which do stimulate human cells will also stimulate, at least to some extent, mouse cells[214]. The best motif depends also on the backbone used, i.e., the best CpG motif with a phosphorothioate backbone of synthetic oligonucleotides (ODN) will not work the best with a phosphodiester backbone[212].

Humanized codon usage

Organisms use synonymous codons in a highly non-random fashion. These codon usage biases sometimes frustrate attempts to express high levels of exogenous genes in hosts of widely divergent species. It is remarkable to note that the synonymous codon-choice patterns among the vertebrates, or at least among the mammals, are very similar, but clearly different from the pattern of a taxonomically distant organism such as yeast[33].

The concept of humanized DNA was first introduced by Shiver in 1999[215]. It is well known that one amino acid may be encoded by several different codons. For

example, valine may be encoded by GTA, GTC, GTT or by GTG. Phenylalanine may be encoded by TTC or TTT. An organism, either virus, bacterium, mouse or man, may have codon bias for a particular set of codons to generate a particular amino acid[33]. In general, predominant codons with cytosine and guanine at the third codon position (G-C rich codons) are mainly found in human genes as compared to non-human genes[216-217]. For example, human prefers to use TTC to generate phenylalanine instead of TTT which is the predominant codon in non-human gene. This is termed "humanized codon usage". Therefore, it has been postulated that for a DNA vaccine to be used in human, mammalian codons should be used. It has been established that DNA plasmid with humanized codon will be better expressed and can induce higher immune response since the mRNA is more efficiently translated[218]. In addition, humanization of HIV-1 gene will disrupt cis-repressor sequences (CRS) or inhibitory/instability sequences (INS) which are present in almost all structural genes of HIV-1. This will allow better transportation of unspliced HIV-1 RNA into cytoplasm for protein synthesis without the help of *rev* gene which is normally required for non-humanized or native DNA[219-220].

By changing the viral DNA coding (A-T rich) sequence to those most commonly used in human (G-C rich) without changing the amino acid sequences of the protein that they encode, several groups have reported much better immune responses. These include :

(1) Shiver et al found that such humanized construct of *gag* DNA was more immunogenic than the native, unaltered DNA in mice[215]. In addition, they also reported longer lasting immune responses in monkeys immunized with humanized SIV DNA encoding gag.

(2) Fomsgaard et al[34-35] reported a successful use of mammalian high expression codons of HIV_{MN} gp160 and HIV_{MN} gp120 to induce both neutralizing antibody and CTL responses. The humanized gene-encoded secreted gp120 gave the highest neutralizing antibody response obtained by transferring the HIV_{MN} V3 loop to the secreted HBsAg as a fusion gene vaccine.

(3) Deml et al reported a better protein expression and immunogenicity of optimized codon usage of HIV-1 gag[221].

(4) Casimiro et al showed the maximizing expression of HIV-1 pol by optimized codon usage. A much improved T-cell immunogen was also elicited as compared to the wild-type gene. The humanized pol gene was capable of inducing both CTL and helper responses in mice and, more importantly, in monkeys[222].

HIV/SIV DNA Vaccines

Many DNA vaccines against HIV and SIV have been developed and tested in animals and man[223]. All HIV/SIV DNA vaccines were found safe and immunogenic. It could stimulate neutralizing antibodies and CTL[171-172,223-224]. However, in one study of SIV DNA vaccine, macaques challenged with pathogenic SIV after the decline of neutralizing antibody and CTL showed no protection of infection or protection from disease[225]. In another study in chimpanzee, protection from infection could be demonstrated with DNA immunization[226]. Similarly, rhesus monkeys immunized with DNA env vaccine and boosted with recombinant subunit proteins could be protected from SHIV challenge[227]. Similar challenge study in rhesus monkeys immunized with

SIV-gag and HIV-env DNA plasmid together with IL-2-Ig showed control of viremia and prevention of AIDS upon SHIV challenge[228-229].

HIV and SIV DNA vaccines have also been studied as therapeutic vaccines. Study in HIV-infected chimpanzees showed that injection of HIV-1 (gag, env, etc) plasmid DNA plus IL-12 plasmid could result in transient reduction of viremia[230]. HIV-1 plasmids were found safe and well tolerated in HIV-infected individuals. It could augment cell-mediated immune response to HIV-1 antigens[172, 231]. Whether this will be clinically useful needs further observations.

As mentioned earlier that protective vaccination represents one of the best solutions to curb the worldwide HIV epidemic like many other infectious diseases. In the context of HIV vaccine, envelope glycoprotein could be a critical part to be included in vaccine design. Here the 297-bp V3 region humanized DNA of HIV-1 is constructed and characterized for its in vitro expression and immunogenicity in mice.

CHAPTER III

MATERIALS AND METHODS

Study population

Approximately 50 six to eight weeks old Balb/c mice were immunized with the plasmid constructs namely, pCI (N = 8) , 297-bp humanized DNA-pCI (N = 10), 297-bp non-humanized DNA-pCI (N = 10) and 2.5 kb full-length envelope DNA-p1.1cI envE (N =20). Another 16 Balb/c mice were used for the DNA prime/recombinant vaccinia virus boost strategy.

Materials

1. Cell lines : COS-7 (African green monkey kidney cells), HEK293 (Human embryonic kidney cells)
2. *E. coli* (DH5α)
3. pCI mammalian expression vector (Promega, U.S.A.)
4. pCDNA3.1 mammalian expression vector (Invitrogen, U.S.A.)
5. PCR purification kit (QIAquick®, Qiagen, Germany)
6. PRISM Ready Reaction Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems, U.S.A.)
7. Lipofectamine (Invitrogen, U.S.A.)
8. MicroAmp PCR tibe (Axygen® Scientific, U.S.A.)
9. Microcentrifuge tube (Axygen® Scientific, U.S.A.)

10. Blood collection tube (Vacutainer®, Becton Dickinson, U.S.A.)
11. Polypropylene conical tubes : 15 and 50 ml (Costar®, U.S.A.)
12. Filtered pipette tips : 10, 20, 100, 200 µl (Axygen® Scientific, U.S.A.)
13. Sterile serological pipettes : 1, 2, 5, 10 ml (Costar®, U.S.A.)
14. Tissue Culture Flask, Culture Plates (Costar®, U.S.A.)

Equipments

1. Centrifuge (Beckman GS-6R, U.S.A.)
2. Refrigerated microcentrifuge (Eppendorf, U.S.A.)
3. -70^{o}C freezer (Revco, U.S.A.)
4. Light microscope (Olympus, U.S.A.)
5. Inverted microscope (Nikon, Japan)
6. DNA Thermal Cycler 2400, 9600, 9700 (Perkin Elmer, U.S.A.)
7. Gel Doc 1000 UV transilluminator (BioRad, U.S.A.)
8. Perkin Elmer 310 Genetic Analyser (Applied Biosystems, U.S.A.)
9. CO_2 humidified incubator (Forma, U.S.A.)
10. FACSort analyzer (Becton Dickinson, U.S.A.)
11. β scintillation counter (Beckman, U.S.A.)
12. Multiwell harvestor (Nunc, U.S.A.)
13. Peptide synthesizer (PSSM-8, Shimadzu, Japan)

Software and program for phylogenetic analysis

1. Backtranslate program (Program Manual for the Wisconsin Package, Version 8, 1994)
2. Clustal X program, version 1.4

Methods

1. Identification of HIV-1 infected Thai patients

   Ten HIV-infected Thai patients from the Immune Clinic of Chulalongkorn Hospital were recruited for virus isolation in this study. HIV-1 infection was diagnosed by positive anti-HIV antibody as confirmed by ELISA test (Abbott Diagnostics, Illinois, U.S.A.) and gel particle agglutination (Fujirebio, Kyowa, Japan).

2. Isolation of HIV-1 from HIV-infected patients by peripheral blood mononuclear cell (PBMC) co-cultivation technique[232]

   Ten milliliters of heparinized blood was obtained from each of these 10 HIV-infected Thai patients. Three patients were asymptomatic, 5 had symptomatic HIV or AIDS-related complex and 2 had full-blown AIDS. All were naïve to antiretroviral drugs. The study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University. Peripheral blood mononuclear cells (PBMC) were separated from the whole blood by Ficoll-Hypaque density gradient centrifugation. Briefly, heparinized whole blood was overlayed on top of Ficoll-Hypaque solution (Cat # 1070030,

Isoprep Solution, Robbins Scientific Corporation, Sunnyvale, CA, U.S.A.) followed by centrifugation at 2,000 rpm for 30 minutes at 25°C. The white monolayer of PBMC located at the interface between plasma and Ficoll-Hypaque was harvested and washed twice with sterile phosphate-buffered saline (PBS). The PBMC was adjusted to 10 $x10^6$ cells/ml with tissue culture medium (TCM) containing RPMI 1640 (Rosewell Park Memorial Institute 1640, Cat # 22400-071, Gibco™, Invitrogen Corporation, Carlsbad, CA, U.S.A.) supplemented with 10 % fetal bovine serum (FBS, Cat # 14-501F, BioWhittaker, Inc., Walkersville, MD, U.S.A.) plus 100 u/ml IL-2 (Interleukin-2, Chiron Corporation, Emeryville, CA, U.S.A.). The HIV-infected PBMC was co-cultured with equal number of 1-3-day PHA (Phytohemagglutinin, Cat # L9132, Sigma, U.S.A.)-stimulated normal donor PBMC in total volume of 10 ml of TCM in 25 mm^3 tissue culture flask(Costar, Corning, NY, U.S.A.). The culture was maintained at 37°C in 5 % CO_2 humidified incubator. Half the volume of culture supernate (5 ml) was removed and replaced with equal volume of fresh media twice a week with the replacement of 10 $x10^6$ cells of 1-3-day PHA-stimulated normal donor PBMC once a week. The culture supernatant was collected for p24 antigen (Ag) quantification (p24 Ag quantification kit, Coulter Corporation, Florida, U.S.A.). The co-culture was maintained for 28 days before being considered as negative. The culture was considered as positive if the amount of p24 Ag was more than or equal to 30 pg/ml for 2 consecutive time points. The positive culture supernate and the infected PBMC were frozen separately for future experiments.

3. <u>Phenotypic characterization of syncytium inducing (SI) and non-syncytium inducing (NSI) by MT-2 cells</u>[233]

Fifty microlitres of p24 Ag-positive culture supernatant were added to 5 x 10^4 MT-2 cells/well in total volume of 200 µl TCM (RPMI 1640 plus 10 % FBS) in 96-well sterile tissue culture plate (Costar, Corning, NY, U.S.A.). MT-2 cells were observed under inverted microscope for SI phenotype or ballooning of cells or multinucleated giant cells formation twice a week (Figure 5). On day 4, 7 and 11, the culture was re-suspended by pipeting up and down for 5-10 times and 130 µl of the culture was removed and replaced with 150 µl of fresh medium. The culture was maintained for 14 days before being considered as negative or NSI phenotype.

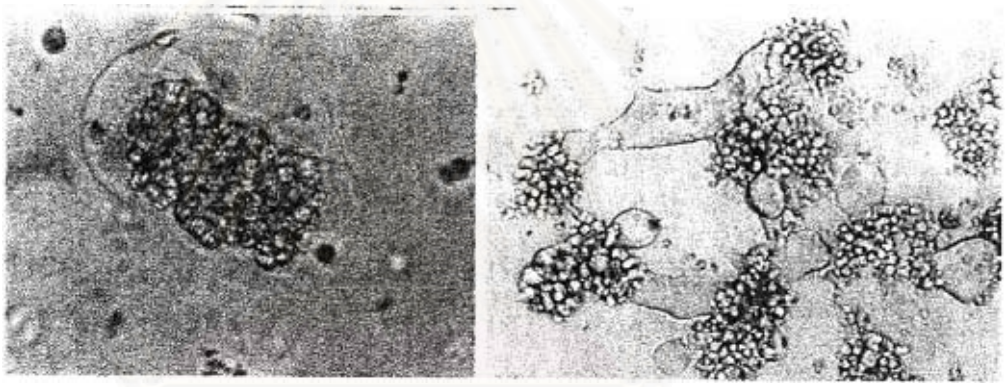


Figure 5 Syncytium inducing (SI) phenotype (Ref : http://hiv-web.lanl.gov)

4. HIV DNA extraction from p24 Ag-positive co-cultultivated PBMC[234]

The frozen PBMC from p24 Ag-positive culture was lysed with 1 ml of lysis buffer containing 10 mM Tris-Cl (pH 8), 0.1 M EDTA pH 8, 0.5 % SDS for 30 minutes at 56^{o}C. Twenty micrograms of pancreatic RNase A was added per 1 ml of lysis buffer and the lysate was incubated at 37^{o}C for another 1 hour. The lysate was finally treated with proteinase K at 50^{o}C for 3 hours. The cell lysate was extracted once with phenol/ chloroform and then precipitated with ethanol/sodium acetate at -70^{o}C for at least 1 hour. The precipitated DNA was

high-speed centrifuged at 13,000 rpm for 30 minutes at 4°C and then was washed once with 70 % ethanol. The DNA pellet was re-suspended in TE buffer. DNA concentration was assessed at 260 nm by UV spectrophotometer.

5. HIV-1 subtype identification

The 297-bp PCR product that covers the V3 region of the HIV genome was amplified from the HIV DNA using nested PCR. The outer primer sequences are as follow :

ss-env I : 5' GCGCTAATCTTAGCAATAGTAGTGTGAAC 3' (location 6013-6041 of HXB2)

ss-env II : 5' GCTCCATGTTTATCTAGATCTTGAGATAC 3' (location 8921-8892 of HXB2)

The PCR conditions are 94°C 5 min, 40 cycles of 94°C 1 min; 55°C 1 min and 72°C 5 min, then auto-extension at 72°C for 7 min and kept cool at 4°C. The inner primer sequences are :

env-6675 : 5' TAAA*GAATTC*GCCGCCACC**ATG**TCTGTAGAAATCAATTGTACC 3' (location 7091-7112 of HXB2)

env-6971 : 5' TATT*GTCGAC***CTA**AAACAGTTTTGTTGTATTGC 3' (location 7397-7378 of HXB2)

(The italic represents restriction sites, underline represents Kozak sequence, bold represents start or stop codons.)

The PCR conditions are 94°C 5 min, 35 cycles of 94°C 30 sec; 56°C 30 sec; 72°C 1 min, then auto-extension at 72°C for 7 min and kept cool at 4°C. The 297-bp amplified fragment was sequenced using the ABI PRISM Dideoxy Dye

Terminator Cycle Sequencing Kit (BigDye™, Applied Biosystem, Foster City, CA, U.S.A.) and then genotyped by BLAST search[235].

6. Humanization of 297-bp selected envelope region

The potential immunodominant HIV envelope epitopes encompassing 297-bp in length (codon 291-391 of HIV envelope according to HXB2 strain and corresponds to codon 294-391 of CM240 reference strain, GenBank accession number U54771) was selected for PCR construction. It includes the V3 region which is the principal neutralizing determinant (PND), CTL epitopes, T helper (Th) epitopes, CXCR4 and CD4 binding sites and neutralizing epitopes. A consensus sequence was calculated from an alignment of sequences from 80 independent HIV isolates across 8 subtypes (10 sequences from each subtype which included subtypes A, B, C, D, A/E, F, G, H) and translated to predicted amino acid sequence using DNAMAN version 4.15 (Lynnon Biosoft, Seattle, WA, U.S.A.). This sequence was back translated to an estimated preferred human codon sequence using Backtranslate (Program Manual for the Wisconsin Package, Version 8, 1994, Genetics Computer Group, Madison, WI, U.S.A.).

7. PCR amplification of 297-bp humanized DNA, 297-bp non-humanized DNA and 2.5 kb full-length envelope DNA

7.1 PCR amplification of 297-bp humanized DNA

Three primers of 25-mer overlapping regions with one another were synthesized as 134 (hu-5'), 138 (AS-73-210) and 125-mer (AS-186-297), respectively (Proligo Singapore Pte Ltd, Singapore). All of the oligos were purified by crude desalting only. The primer sequences are as follow :

hu-5' (134-mer) :

5'TATCGATAAGCTTGATATC*GAATTC*GCCGCCACC**ATG**AGCGTGGAGAT
CAACTGCACCAGGCCCAGCAACAACACCAGGACCAGCATCACCATCGG
CCCCGGCCAGGTG**TTCTACAGGACCGGCGACATCATCG** 3'

AS-73-210 (138-mer)

5'**CTGGAAGATGATGGTCTTGTTCTTG**AAGTGCTCCTTCAGCTTCTCGG
TCACCTGCTTCAGGGCCTCGTTCCACTTGGTGCCGTTGATCTCGCAGTAG
GCCTTCCTGATGTCGC**CGATGATGTCGCCGGTCCTGTAGAA** 3'

AS-186-297 (125-mer)

5'CTAG*TCTAGA***CTA**GAACAGCTTGGTGGTGTTGCAGTAGAAGAACTCGC
CCCTGCAGTTGAAGTGGTGCATGGTGATCTCCAGGTCGCCGCCGCTGGG
GGG**CTGGAAGATGATGGTCTTGTTCTTG** 3'

(The italic represents restriction sites, underline represents Kozak sequence, bold represents start or stop codons, bold and underline represent overlapping regions.)

The first primer of 134-mer in length, hu-5', includes an *Eco* RI restriction site, a Kozak sequence (to increase protein expression) and an ATG start codon. The third primer of 125-mer, AS-186-297, includes a *Xha* I restriction site and TAG stop codon. The three primers were mixed together in a standard master mix for polymerase chain reaction (PCR) without adding additional templates. Five microlitres each of 25 mM $MgCl_2$, 10 x PCR buffer and 10 mM dNTPs were mixed with 20 pmole of each primers and 1.5 u of *Taq* DNA polymerase (Promega Corporation, Madison, WI, U.S.A.) in a total volume of 50 µl. Nested PCR was performed with 2 µl of this PCR product with another pair of primers, small hu-5' and small hu-

3', to amplify and increase amount of the whole 297-bp humanized DNA. The sequences of the primers are as follow :

small hu-5' : 5' *GAATTC*GCCGCCACC**ATG**AG 3'

small hu-3' : 5' CTAG*TCTAGA***CTA**GAACAGCTTGGTG 3'

The PCR conditions for the first round PCR are 94^{o}C 5 min, 32 cycles of 94^{o}C 30 sec; 50^{o}C 30 sec; 72^{o}C 1 min, then autoextension at 72^{o}C 7 min and then kept cool at 4^{o}C. For the nested PCR, the conditions are the same as the first round but number of cycle was increased to 40 cycles for the 3-temperature PCR. First round PCR may yield 3 possible products (Figure 6). Only the longest first round PCR product (full-length or PCR product 3) will be nested by the 2 smaller primers. The sequence of the amplified 297-bp humanized DNA was verified by DNA sequencing analysis.

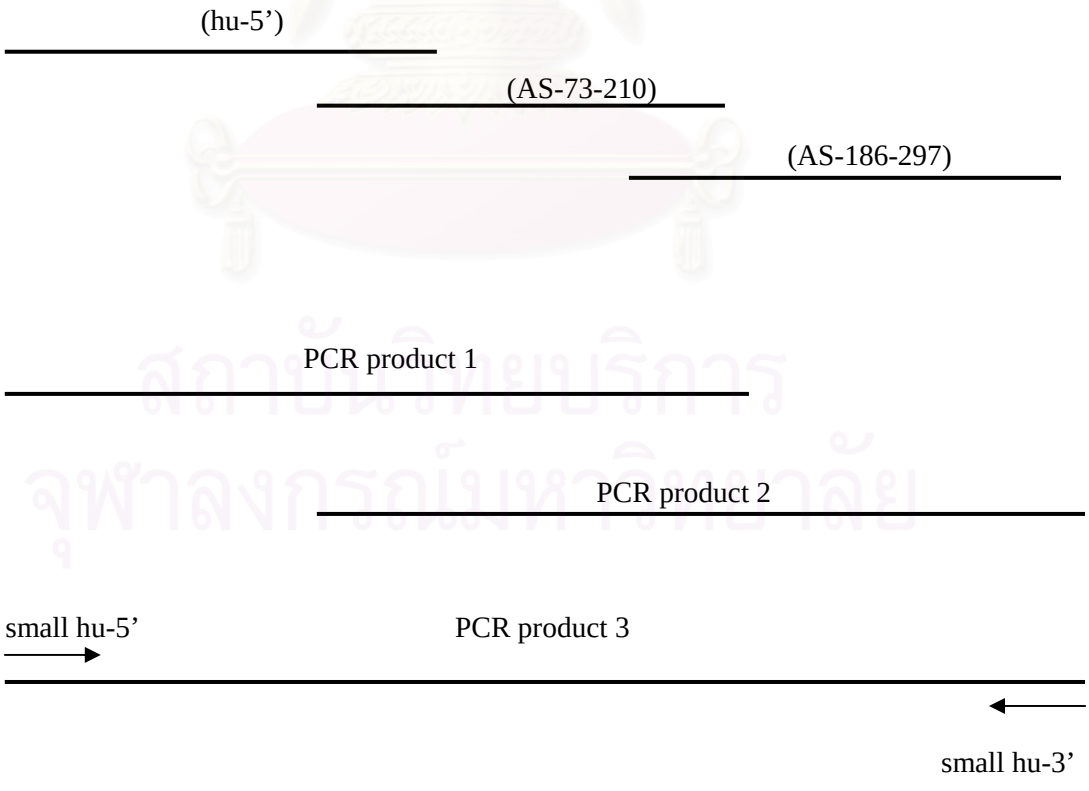


Figure 6 Primer orientation

7.2 PCR amplification of 297-bp non-humanized DNA

The 297-bp non-humanized DNA was amplified from HIV DNA extracted from HIV-1-infected cells from cocultivation using primers env-6675 and env-6971 (the same as those used in subtype identification as mentioned earlier). The *Eco* RI and *Sal* I restriction sites were added at 5'-end of the env-6675 and env-6971 primers, respectively.

7.3 PCR amplification of 2.5 kb full-length envelope DNA

The 2.5 kb full-length envelope DNA was amplified from HIV DNA (the same as 7.2) by nested PCR using outer primers ss-env I and ss-env II as mentioned earlier in subtype identification and then inner primers **env-E-Xho I-5'** (5' ATCG*CTCGAG*GCCGCCACC**ATG**AGAGTGAAGG 3', location 6213-6225 of HXB2) and **env-E-EcoR I-3'** (5' ATCG*GAATTC***CTA**GCAAAGTCCTTTCTAA 3', location 8776-8761 of HXB2). The PCR condition was 94^{o}C 5 min, 40 cycles of 94^{o}C 1 min; 56^{o}C 1 min; 72^{o}C 3 min, then autoextension at 72^{o}C for 7 min and then kept cool at 4^{o}C.

8. DNA sequencing analysis

DNA sequencing analysis was performed by ABI PRISM Dideoxy Dye Terminator Cycle Sequencing Kit (BigDye™, Applied Biosystems, Foster City, CA, U.S.A.). 3µl of BigDye reagent was mixed with 1 µl of primer (10 pmole) and 200 ng of DNA template in total volume of 10 µl. The mixture was subjected to thermal cycler for 25 cycles of 96^{o}C 10 sec, 50^{o}C 5 sec, 60^{o}C 4 min. The product was then precipitated with absolute ethanol plus 3 M sodium acetate (NaOAc) pH 4.6 and washed once with 70 % ethanol. The precipitated

sequencing product was re-suspended with template suppressor reagent (TSR) provided with the BigDye™ and then loaded into the ABI310 Genetic Analyser (Applied Biosystems, Foster City, CA, U.S.A.).

9. Cloning experiments

9.1 Cloning of 297-bp humanized DNA

The 297-bp humanized DNA was ligated to the pCI mammalian expression vector (Promega Corporation, Madison, WI, U.S.A.) at selected multiple cloning sites (*Eco* RI and *Xba* I at the 5' and 3'ends) using T4 DNA ligase (Boehringer Mannaheim, Germany) according to the manufacturer's protocol. The ligated product was then transformed in *E.coli* (DH5α). Transformed clones were selected by ampicillin resistance in ampicillin-containing LB plate and propagated in 5 ml ampicillin-containing LB medium. The correct clones were verified by specific restriction enzyme digestions and sequencing analysis. After the correct clones were verified, the clones were frozen at -70^{o}C as glycerol stock plasmid for future use.

9.2 Cloning of 297-bp non-humanized DNA

The 297-bp non-humanized DNA was ligated to the pCI mammalian expression vector (Promega) at selected multiple cloning sites (*Eco* RI and *Sal* I at the 5' and 3'-ends) and then transformed in *E.coli* as described above for 297-bp humanized DNA. The correct clones were also verified by specific restriction enzyme digestion and sequencing analysis.

9.3 Cloning of 2.5 kb full-length envelope DNA

The 2.5 kb full-length envelope DNA was directly cloned into pGEM-T Easy vector (Promega) by TA cloning procedure (Promega) according to the

manufacturer's protocol. The ligated product was then digested from the T Easy with *Eco* RI and was replaced into the EcoRI-1004-EcoRI-3575 sites of p1.1 cI IIIB IRESrev (kindly provided by Professor Kenji Okuda, Yokohama City University School of Medicine, Yokohama, Japan) as shown in Figure 7. The resulting p1.1 cI envE IRESrev (Figure 8) was then transformed in *E. coli* and verified as described above for 297-bp humanized DNA.

The correct clones were propagated to a total volume of up to 2,500 ml which contained approximately 10-15 mg of required DNA to be used for transfection and immunization experiments.

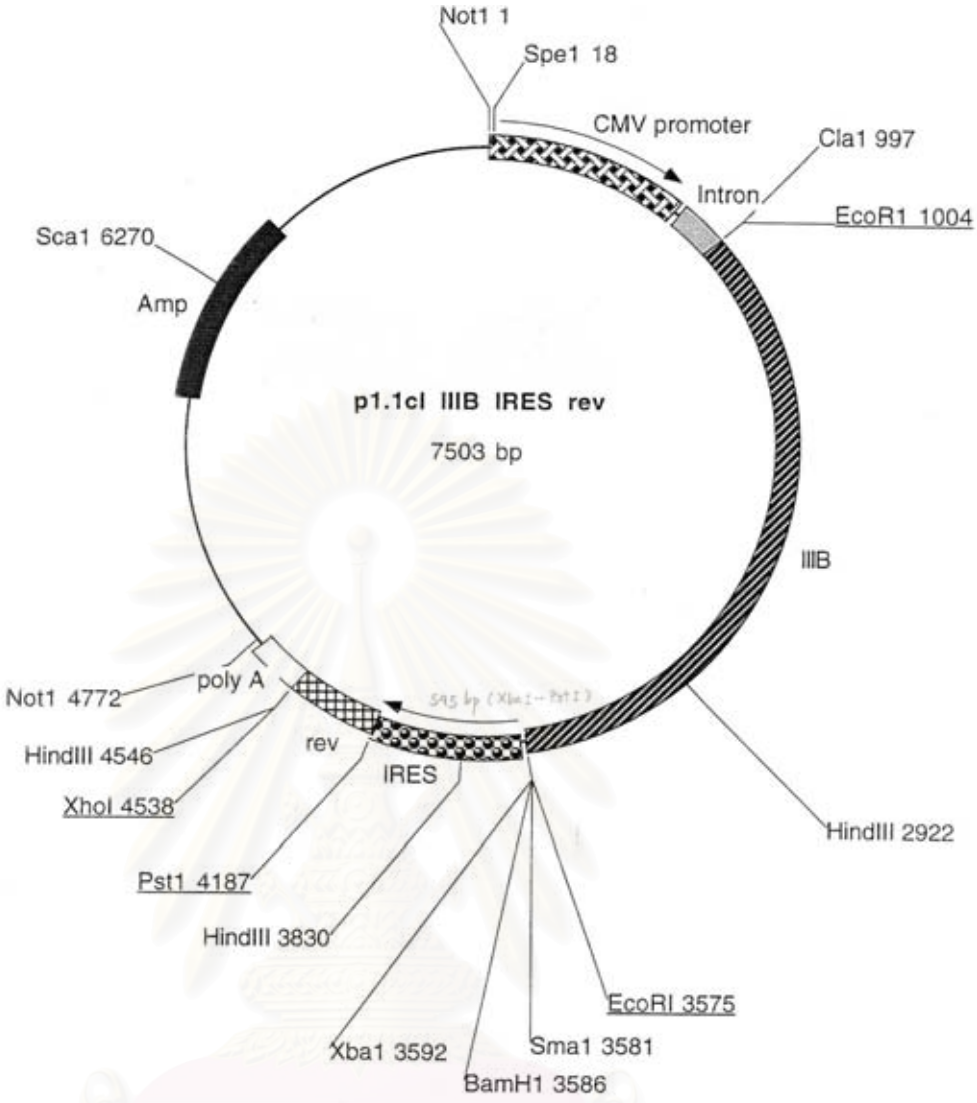


Figure 7 The original plasmid, p1.1 cI IIIB IRES rev

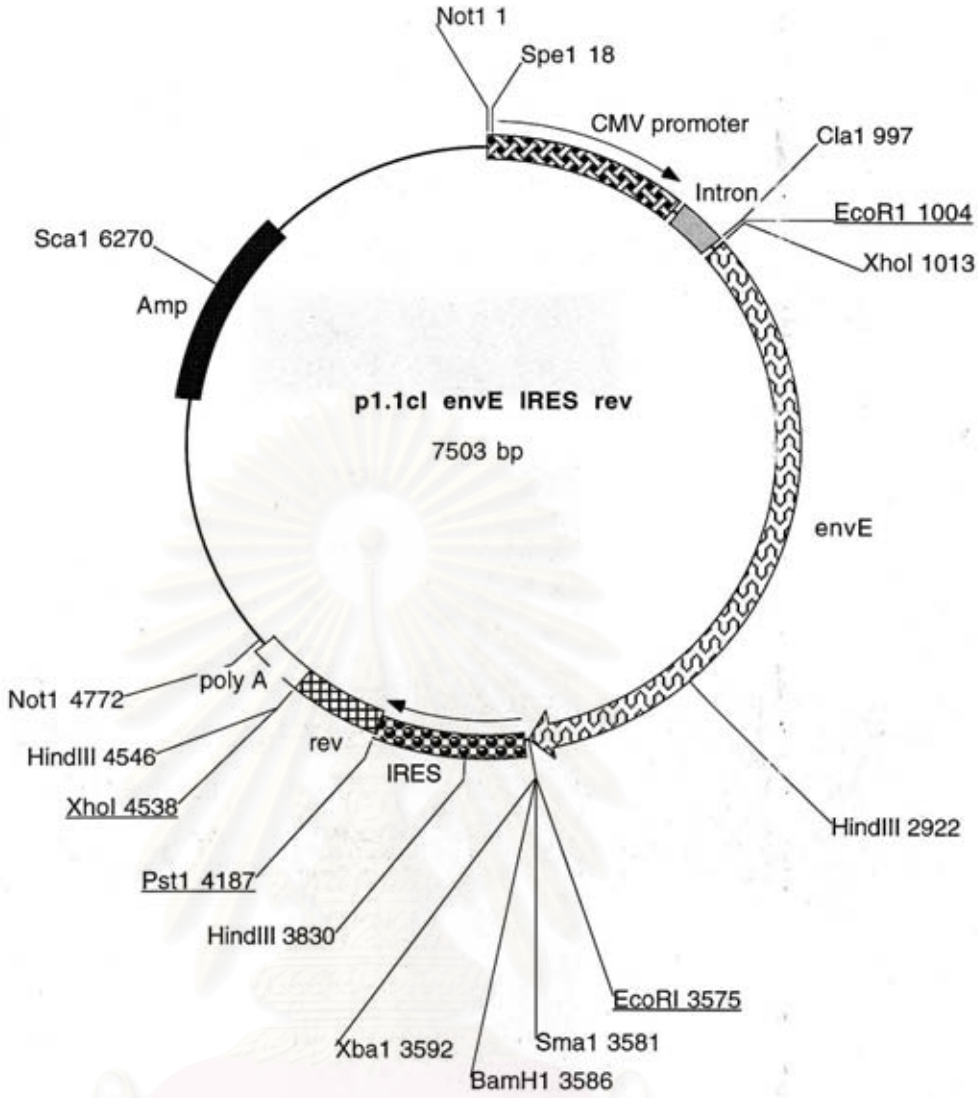


Figure 8 The envE inserted plasmid, p1.1 cI envE IRES rev

10. Transfection experiments for *in vitro* protein expression

All the DNA constructs were propagated in DH5α (Invitrogen, Carlsbad, CA, U.S.A.) and purified by using QIAGEN endotoxin-free Giga kit (QIAGEN, Hilden, Germany). Two micrograms of each plasmid DNA constructs were used to transfect HEK293 cells for 48 hours using Lipofectamine (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's protocol. Briefly, 2 µg of each plasmid DNA constructs was added into 250 µl of MEM (Minimum Essential Medium, Cat # 42360-032, Gibco™ Invitrogen Corporation, Carlsbad,

CA, U.S.A.) and 8 µl of Lipofectamine (Invitrogen) was separately diluted with 250 µl of MEM and was incubated at room temperature for 5 minutes. The diluted Lipofectamine was then added into diluted DNA and was left at room temperature for another 20 minutes. The Lipofectamine treated DNA was then added dropwise onto HEK293 cells. The cells were further incubated for 48 hours and then washed once with cold PBS and lysed with 100 µl NP-40 lysis buffer (50 mM Tris-Cl pH 7.6 with 1 % Nonidet P-40). Equal volume of 2 x SDS loading dye (125 mM Tris-Cl pH 6.8, 4 % SDS, 20 % glycerol, 0.01 % Bromphenol blue, 10 % β-mercaptoethanol) was mixed with the cell lysate and the mixture was boiled in boiling water for 10 minutes and then put on ice. Eighteen microlitres of the cell lysate were loaded into 4-12 % gradient polyacrylamide gel (Invitrogen) followed by transfer to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, England). The expected protein products were detected by using pooled anti-HIV positive plasma from HIV-1 CRF01_A/E-infected individuals. Rabbit anti-human-IgG-peroxidase (Cat # 309-035-003, Jackson ImmunoResearch Lab. Inc., PA, U.S.A.) was used as the second antibody. The β-actin band was used as a positive control.

11. Immunogenicity study in mice

A. Experimental design

Three experimental and one control groups were studied as follow :

- pCI empty plasmid control N = 8
- 297-bp humanized envelope DNA N = 10
- 297-bp non-humanized envelope DNA N = 10

- 2.5 kb full-length envelope DNA N = 20

B. Immunization protocol

Six to eight weeks old female Balb/c mice, weighed 20-25 grams, were used in the experiments. Number of mice per group was shown above. Mice were immunized intramuscularly with 100 µg of individual DNA Constructs in 100 µl PBS on day 0, 14 and 28. The intramuscular injection was done with insulin syringe needle gauge 27, ½ inch given at the gastrocnemius muscles of hind legs. Blood and spleen cells were collected on day 42 or 2 weeks after the third immunization. Half of the mice in each group, i.e., 4 mice from pCI control group, 5 mice each from 297-bp humanized and non-humanized groups and 10 mice from 2.5 kb full-length envelope group were boosted intramuscularly with 25 µg of recombinant gp120 (rgp120) of subtype A/E which was a gift from Dr. Sarngadharan MG (Advanced BioScience Laboratories, Inc. Kensington, MD, U.S.A.) on day 42. For the boosted group, blood was drawn on day 42 (before rgp120 boosting) and day 56 or 2 weeks after the boosting dose. Spleen cells were collected on day 56.

C. *In vivo* immunogenicity assay : Delayed type hypersensitivity (DTH) skin testing by footpad swelling

The DTH skin test was performed 48 hours before sacrifice for both the non-boosted (day 40) and the rgp120 boosted mice (day 54). Mice were challenged with 8 µg of relevant 15-residue V3 peptides on footpads. The V3 peptides, V3(E) : GVHMGPGRVFYRTGE and V3(hu) : SITIGPGQVFYRTGD [synthesized by Peptide synthesizer (PSSM-8,

Shimadzu Corporation, Kyoto, Japan) were prepared as 8 µg/50µl (160 µg/ml) in PBS and 25 µl was injected into each of the left and right hind footpads. The V3(hu) peptide was used in mice immunized with 297-bp humanized DNA. The V3(E) peptide was used in mice immunized with 297-bp non-humanized DNA, 2.5 kb full-length envelope DNA and pCI control. Footpad thickness was measured with a dial thickness gauge (Ozaki Seisakusho, Tokyo, Japan) both before and 24 hours after DTH skin testing. The difference of footpad swelling between pre- and post-injection was measured in unit of 10^{-2} mm.

D. *In vitro* immunogenicity assays

(1) Humoral immune response

Binding antibody assay using ELISA (Enzyme Linked Immunosorbent Assay)

Synthetic peptides [V3(E)-MAP or V3(hu)-MAP were used to coat 96-well flat-bottomed microplate (Maxisorp-Nunc, U.S.A.) at concentration of 2 µg/ml in 0.1 M carbonate buffer, pH 9.6 at 4^{o}C overnight. The plate was then processed as shown below.

(a) Coat plate with 100 µl/well of 2 µg/ml of MAP peptides, incubate at 4^{o}C overnight

(b) Wash plate 3 times with 0.05 % PBST (PBS + 0.05 % Tween-20)

(c) Block coated wells with 150 µl/well of 1 % BSA (Bovine serum albumin, Sigma, U.S.A.) in PBS and incubate at 37^{o}C for 2 hours

(d) Add 100µl/well of 2 fold serially diluted mouse sera, starting from 1 : 50, into individual wells and incubate at 4^{o}C overnight

(e) Wash plate 3 times with 0.05 % PBST

(f) Add 100 µl/well of second antibody (Goat anti-mouse-HRP) and incubate at 37^{o}C for 2 hours

(g) Wash plate 3 times with 0.05 % PBST

(h) Add 100 µl/well of substrate and incubate at room temperature for 30 minutes in the dark

(i) Add 50 µl/well of 1 M H_2SO_4 to stop the reaction

(j) Measure absorption at 492 nm

(2) Cell-mediated immune response

2.1 SPLEEN CELL PREPARATION

(a) Mice were sacrified by deep anesthesia with sterile technique and placed 1 ml sterile RPMI

(b) Press spleen with sterile spleen pressing apparatus

(c) Rinse the apparatus with 3 ml sterile PBS

(d) Pipet the spleen cell suspension up and down 5 times

(e) Pass the suspension through sterile mesh to get single cell suspension

(f) Centrifuge the suspension at 1,000 rpm for 4 minutes at room temperature

(g) Discard supernatant

(h) Add 2 ml of red blood cell lysis buffer (0.16 M NH_4Cl, 0.17 M Tris pH 7.65) and pipet the suspension up and down 5 times and pass the suspension through mesh again

(i) Centrifuge the suspension at 1,000 rpm for 4 minutes at room temperature

(j) Discard supernatant

(k) Re-suspend spleen cells with 1 ml RPMI supplemented with 10 % FBS + 50 µM 2-ME (β-mercaptoethanol)

(l) Proceed to cell count and adjust the cell concentration as needed with RPMI supplemented with 10 % FBS and 2-ME

2.2 LYMPHOPROLIFERATION ASSAY

One hundred microlitres of 2×10^6 spleen cells/ml were added in triplicate wells of 96-well tissue culture plate (Costar, Corning, NY, U.S.A.). One hundred microlitres of antigen [2 and 10 µg/ml V3(E) or V3(hu), 1 and 5 µg/ml rgp120] or mitogen (Concanavalin A, 5 µg/ml) were added to the wells with cells. The culture was incubated at 37^oC with 5 % CO_2 for 7 days. At 18 hours before the end of 7-day culture, the cells were pulsed with 0.5 µCi/well of ^3H-Tdr (tritiated thymidine) and cells were harvested using glass fiber filter at the end of the further 18 hours incubation. The glass fiber filter was left dry at room temperature and then subjected to liquid scintillation β-counter (Beckman, U.S.A.). The results were presented as counts per minute (cpm) and stimulation index (S.I.).

$$S.I. = \frac{\text{cpm with mitogen or antigen}}{\text{background cpm}}$$

2.3 INTRACELLULAR CYTOKINE ASSAY (ICCS)

1×10^7 isolated spleen cells were used for the ICCS according to the following procedure :

(a) Add 1 x 10^7 spleen cells in 1 ml of culture medium into the well of 24-well tissue culture plate (Costar)

(b) Stimulate with 20 µg/well of specific antigen, using V3(hu) for 297-bp humanized DNA immunized group and V3(E) for 297-bp non-humanized DNA and 2.5 kb full-length envelope E immunized groups as well as for pCI immunized group

(c) Incubate at 37^{o}C with 5 % CO_2 for 18 hours

(d) Add 1 µl/well of Golgi Plug (the Cytotox / Cytoperm, Cat # 555028, BD Bioscience Pharmingen, San Diego, CA, U.S.A.) and incubate for another 2 hours

(e) Collect cells in 1.5 ml Eppendorf tube and wash with 1 ml of staining buffer (3 % FBS , 0.09 % sodium azide in PBS)

(f) Add 4 % normal mouse serum in staining buffer/tube

(g) Incubate at 4^{o}C for 30 minutes

(h) Wash cells with 1 ml of staining buffer, 2 times

(i) Add 1 µl of PE-labelled anti-mouse CD8a (Cat # 01045A, BD Bioscience Pharmingen)

(j) Incubate at 4^{o}C for 30 minutes

(k) Wash cells with 1 ml of staining buffer, 2 times

(l) Add 250 µl Cytotox/Cytoperm solution (Cat # 555028, BD Bioscience Pharmingen)

(m) Incubate at 4^{o}C for 30 minutes

(n) Wash with 1 ml of Perm/Wash solution for 2 times

(o) Add 1 µl FITC-labelled anti-mouse interferon-gamma (IFN-γ) (Cat # 11-7311-82, BD Bioscience Pharmingen)

(p) Incubate at 4^{o}C for 30 minutes

(q) Wash with 1 ml of Perm/Wash solution for 2 times

(r) Add 700 µl staining buffer

(s) Analyse by Flow cytometer

The sample will be considered positive if the % positivity is more than or equal to the cut-off value which is the mean of % positivity of the control group (splenocytes from pCI immunized group stimulated with antigen) plus 2 SD.

2.4 ENZYME LINKED IMMUNOSPOT (ELISPOT) ASSAY

Spleen cells were adjusted as 1 x 10^6 cells/ml for the ELISPOT assay according to the following procedure :

(a) Pre-wet IP plate (MultiScreen® Immobilon^{TM}-P Filtration Plate, Millipore, U.S.A.) with 50 µl of 70 % ethanol, then decant and let the plate dry and wash again with 150 µl PBS

(b) Coat IP plate with 50 µl of 10 µg/ml anti-mouse IFN-γ (Mabtech AB, Nacka, Sweden)

(c) Incubate at 4^{o}C, overnight

(d) Discard anti-mouse IFN-γ and wash plate 3 times with PBS

(e) Block wells with 200µl of culture medium (RPMI + 10 % FBS) for at least 2 hours at 37^{o}C and then discard blocking medium and then wash plate 5 times with PBS

(f) Add 100 µl of 1 x 10^6 cells/ml adjusted spleen cells per well of IP plate, total of 6 wells (3 wells will be stimulated with

relevant antigens and 3 wells will be un-stimulated which are served as control)

(g) Add 1 µg/well of peptide antigen [V3(hu) for 297-bp humanized DNA and V3(E) for 297-bp non-humanized DNA and 2.5 kb full-length envelope DNA]

(h) Incubate at 37^{o}C overnight at least 18 hours

(i) Wash plate 6 times with 0.1 % PBST (0.1 % Tween-20 in PBS)

(j) Add 50 µl/well of 1 : 1000 biotinylated detection antibody (Mabtech AB) in 0.5 % BSA in PBS

(k) Incubate for at least 2 hours at 37^{o}C

(l) Wash plate 4 times with 0.1 % PBST

(m) Add 50 µl/well of 1 : 1000 streptavidine-alkaline phosphatase (Mabtech AB) in PBS

(n) Incubate at room temperature for 1.5 hours

(o) Wash plate 5 times with 0.1 % PBST

(p) Add 50 µl/well of BCIP/NBT membrane phosphatase substrate (BioRad, U.S.A.)

(q) Wash plate 4 times with 0.1 % PBST to stop color development reaction

(r) Remove all excess liquid from wells

(s) Dry back of wells thoroughly with paper towel

(t) Let the plate dry in dark

(u) Count spot by computer-assisted video image analysis[236]

(v) The result of the ELISPOT assay was determined by the difference between spot forming cells (SFC) per 10^6 cells of stimulated culture and the non-stimulated culture. If the result was minus, i.e., non-stimulated culture has more SFC than stimulated culture, the number of net SFC was considered as zero (0) for the purpose of statistical comparison with other experimental groups.

(w) Positive control used in the ELISPOT assay was splenocytes from mouse immunized with vPE16 which is HIV-1 IIIBgp160 recombinant vaccinia virus construct stimulated with HIV-1 IIIB peptide (NNTRKRIQRGPGRAFVTIGKIGN).

E. Testing cross-reactivity of the 297-bp humanized DNA with rVVgp160(E) boosting

Single dose of 297-bp humanized DNA was used to prime mice on day 0. Mice were boosted with 3 x 10^6 pfu (plaque forming unit) recombinant vaccinia construct of HIV-1 subtype E gp160 (rVVgp160) kindly provided by NIH AIDS Research and Reference Reagents Program, U.S.A. on day 21. Mice were sacrified on day 35 or 2 weeks after the boosted dose. The immunization was performed in 4 groups of mice as follow :

Group 1	pCI + rVVgp160-E	(N = 4)
Group 2	297-bp humanized DNA + rVVgp160-E	(N = 4)
Group 3	297-bp humanized DNA + wild type vaccinia	(N = 4)
Group 4	297-bp humanized DNA only	(N = 4)

ELISPOT and ICCS were performed using mouse splenocytes. For ELISPOT assay, mouse splenocytes were separately stimulated with wild type vaccinia virus and rVVgp160 of subtype A, B, C and E to investigate cross reactivity responses. For ICCS, mouse splenocytes were separately stimulated with rVVgp160 of subtype B and E. Stimulated splenocytes were separately stained with anti-CD4 and anti-CD8 to distinguish the cell type that responded to the stimulated antigens.

12. Institutional Review Board (IRB) approval

The study proposal was submitted for approval from the IRB of the Faculty of Medicine, Chulalongkorn University.

13. Statistical analyses

Nonparametric test was used to calculate the difference between pre- and post-immunizations, between before and after antigen challenge as well as between immunization groups.

CHAPTER IV

RESULTS

1. Isolation and phenotypic characterization of HIV-1 from HIV-infected patients by peripheral blood mononuclear cell (PBMC) co-cultivation technique

Ten HIV-1 isolates were successfully isolated from 10 HIV-infected Thai patients. Among these, 4 were syncytium inducing (SI) and 6 were non-syncytium inducing (NSI) phenotypes. The details of these patients are shown in Table 11 .

Table 11 Patient characteristics and the SI/NSI phenotype

Isolate number	Risk factor	Staging	HIV phenotype
01-99	Heterosexual	AIDS	NSI
02-99	Heterosexual	AIDS	NSI
03-99	Heterosexual	ARC	SI
04-99	Heterosexual	ARC	SI
05-99	Heterosexual	Asymptomatic	NSI
06-99	Heterosexual	Asymptomatic	NSI
07-99	Heterosexual	ARC	NSI
08-99	Heterosexual	ARC	SI
09-00	Heterosexual	ARC	SI
10-00	Heterosexual	Asymptomatic	NSI

N.B. : ARC = AIDS related complex

Only DNA from NSI phenotypes, especially from the asymptomatic patients (05-99, 06-99, 10-00) were used for further PCR amplification since this is the most frequently found phenotype in early HIV-infected patients.

2. HIV subtype identification

Frozen PBMC from the 3 patients with NSI phenotypes (05-99, 06-99, 10-00) were used to extract DNA and then proceeded to PCR amplification using primers that cover the V3 region (env-6675 and env-6971). The sequence of those amplified fragments were genotyped by BLAST search in the PubMed. The BLAST search revealed that the 2 isolates (05-99, 10-00) were subtype E or CRF01_AE, accession numbers AY366933 and AY366934 respectively (Appendix B).

3. PCR amplification of 297-bp humanized DNA, 297-bp non-humanized DNA and 2.5 kb full-length envelope DNA

The 297-bp humanized DNA, 297-bp non-humanized DNA and 2.5 kb full-length envelope DNA were successfully amplified using nested PCR technique according to the procedures described in Materials and Methods. The amplified products were shown by agarose gel electrophoresis to have the correct size of DNA, i.e., 297-bp and 2.5 kb as shown in Figure 9. The nucleotide sequence of the 297-bp humanized PCR product was verified by DNA sequencing analysis (Appendix C).

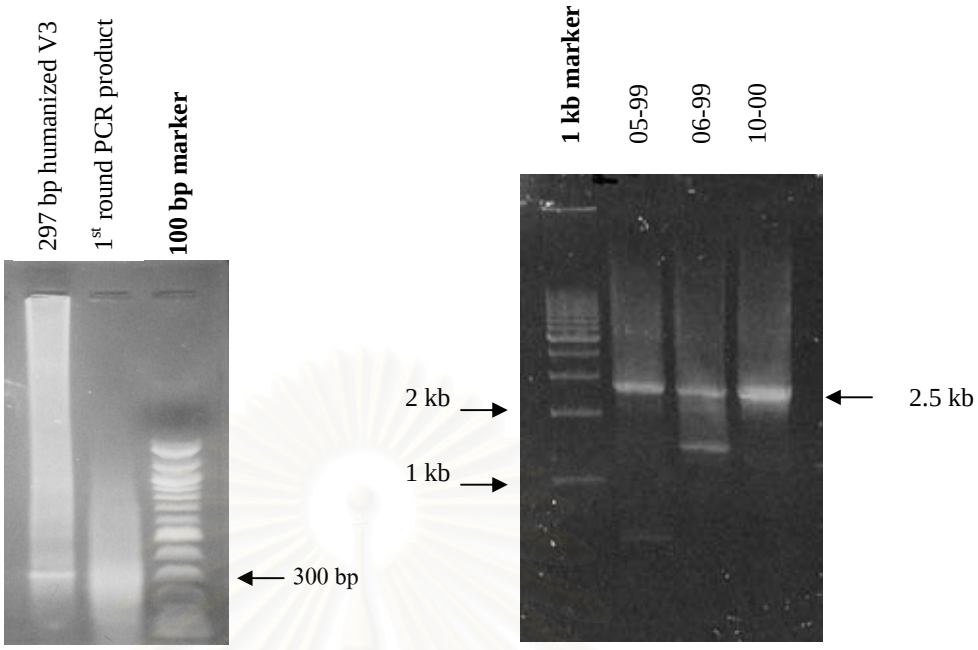


Figure 9 PCR products of 297-bp humanized DNA, 297-bp non-humanized DNA (A) and 2.5 kb full-length envelope DNA (B)

4. Cloning experiments of 297-bp humanized DNA, 297-bp non-humanized DNA and 2.5 kb full-length envelope DNA

The three amplified DNAs were digested with specific restriction enzymes according to the procedures mentioned in Materials and Methods. Briefly, the 297-bp humanized DNA was digested with *Eco* RI and *Xba* I, the 297-bp non-humanized DNA was digested with *Eco* RI and *Sal* I and the 2.5 kb full-length envelope DNA was digested with *Xho* I. The 3 digested DNAs were then successfully cloned into the pCI plasmid (for 297-bp humanized DNA and non-humanized DNA) and p1.1 cI plasmid (for 2.5 kb full-length envelope DNA). The ligated products were then verified by using corresponding restriction enzyme digestions showing the correct size of the inserted DNA by agarose gel electrophoresis (Figure 10) and the correct reading frame by sequencing analysis.

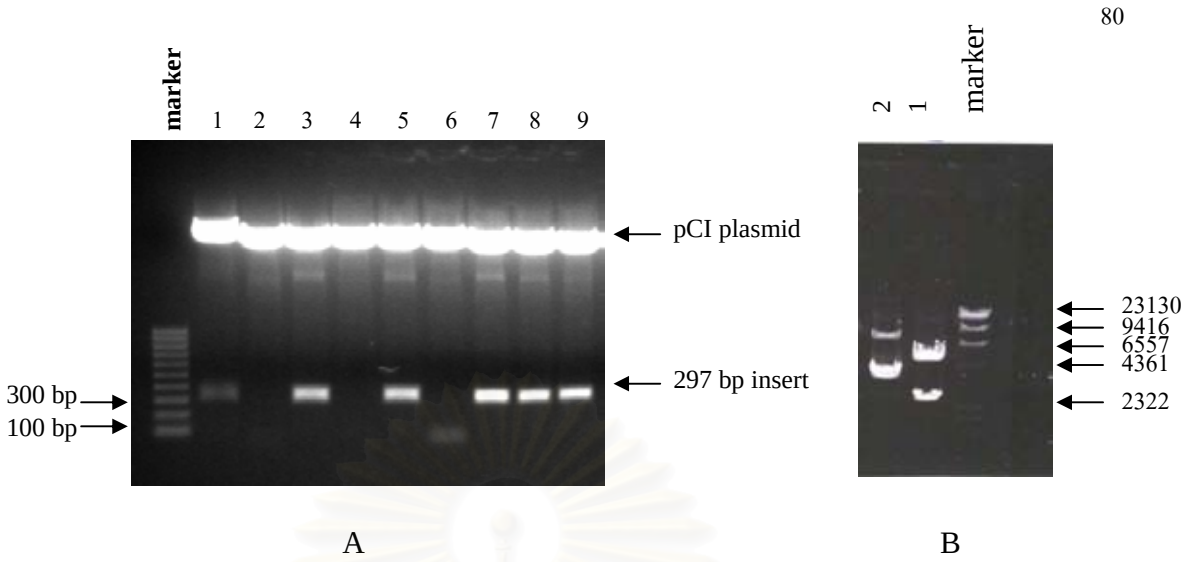


Figure 10 Restriction enzyme digestions of cloning experiments of 297-bp humanized DNA, 297-bp non-humanized DNA (A) in 1.5 % agarose gel and 2.5 kb full-length envelope DNA (B) in 0.8 % agarose gel (lane 1, digested with *Eco* RI yielded 2 bands of 4932, 2571; lane 2, digested with *Xho* I yielded 2 bands of 3969, 3534)

The comparison of the V3 region peptide between that encoded by the 297-bp humanized DNA which is the approximated last common ancestor and those encoded by the 8 subtypes (A, B, C, D, A/E, F, G, H) is shown in Figure 11. The pairwise comparisons of V3 region peptide encoded by 297-bp humanized DNA and individual subtypes are also shown in Figure12-19. The comparison of similarity and difference of the amino acid sequence was done by using Clustal X program, version 1.4.

Table 12 summarizes the level of amino acid difference in the V3 region peptide between individual subtype and the multiclade derived from the last common ancestor, i.e., 297-bp humanized DNA. It is interesting to note that subtype E has the smallest difference (6 amino acids) whereas subtype D has the biggest

difference (41 amino acids). The differences in other subtypes range from 27 to 35 amino acids.

Table 12 Number of amino acid difference in the V3 region peptide (99 amino acids) of the consensus HIV-1 8 subtypes and of the isolate 10-00 used for the amplification of the 297-bp non-humanized DNA from that encoded by the 297-bp humanized DNA

HIV-1 subtype	**Number of amino acid difference from a total of 99 amino acids**
A	28
B	30
C	27
D	41
A/E	6
F	35
G	34
H	28
10-00 (297-bp non-humanized)	16

```
F               SVQINCTRPNNNTRKRISLGPGRVFYTTGEIIGDIRKAHCNVSGTQWRNTLAKVKAKLGS
H               PVQINCTRTGNNTRKSIRIGPGQAFYATGDIIGDIRRAYCNISGKQWNETLHKVITKLGS
C               PVEIMCTRPDNNTRKSIRIGPGQTFYATGDIIGDIRQAHCNISEDKWNETLQNVSKKLAE
G               SIEINCIRPNNNTRKSIPIGPGQAFYATGDIIGDIRQAHCNVSRIKWREMLKNVTAQLRK
D               SVTINCTRPNNNTVQSIHMGPGRALFTT-KIIGKIRQAHCNISGTGWNKTLQQVATKLRD
Humanized       SVEINCTRPSNNTRTSITIGPGQVFYRTGDIIGDIRKAYCEINGTKWNEALKQVTEKLKE
A/E             SVEINCTRPSNNTRTSITIGPGRVFYRTGDIIGNIRKAYCEINGTKWNKVLKQVTEKLKE
A               SIEINCTRPFKKVRISARIGPGRVFHTTGNINGDIRKAYCEINKTKWKETLKQVTRKLRE
B               PVEINCTRPNNNTRKSIHIGPGRAFYATGDIIGNIRQAYCTLNRARWNDTLKQIAEKLGE
                .: * * *. ::.     :***:.:. * .* *.**:*:* :.   *.. * ::  :* .

F               YFPN-ATIKFNSSSGGDLEITRHNFNCMGEFFYCNTDELF
H               YFDN-KTIILQPPAGGDIEIITHSFNCGGEFFYCNTTKLF
C               HFPN-KTIIFNSSSGGDLEITTHSFNCRGEFFYCNTSGLF
G               IYNN-KNITFNSSAGGDLEITTHSFNCRGEFFYCNTSGLF
D               LYNR-TEINFKPSSGGDPEITTHSFNCGGEFFYCNTSGLF
Humanized       HFKN-KTIIFQPPSGGDLEITMHHFNCRGEFFYCNTTKLF
A/E             HFN--KTIIFQPPSGGDLEITMHHFNCRGEFFYCNTTKLF
A               HLNGTMTISFRPSSGGDPEITMHHFNCRGEFFYCNTTALF
B               QFKN-KTIVFNQSSGGDPEIVMHSFNCGGEFFYCNSTQLF
                      * :. .:*** **  * *** *******:  **
```

***** indicates complete similarity

: indicates minor difference

. indicates some similarity

(Not marked) indicates no conservation

Figure 11 Comparison of the consensus amino acid sequence of V3 region across multiple HIV-1 subtypes with the sequence encoded by the 297-bp humanized DNA derived from the approximated last common ancestor of the 8 subtypes

```
Humanized       SVEINCTRPSNNTRTSITIGPGQVFYRTGDIIGDIRKAYCEINGTKWNEALKQVTEKLKE
A               SIEINCTRPFKKVRISARIGPGRVFHTTGNINGDIRKAYCEINKTKWKETLKQVTRKLRE
                *:******* ::.* *  ****:**: **:* ********** ***:*:*****.**:*

Humanized       HFKN-KTIIFQPPSGGDLEITMHHFNCRGEFFYCNTTKLF
A               HLNGTMTISFRPSSGGDPEITMHHFNCRGEFFYCNTTALF
                *::.  ** *:*.**** ****************** **
```

Figure 12 Comparison of the consensus amino acid sequence of V3 region of subtype A with that encoded by the 297-bp humanized DNA derived from the approximated last common ancestor of the 8 subtypes

```
Humanized       SVEINCTRPSNNTRTSITIGPGQVFYRTGDIIGDIRKAYCEINGTKWNEALKQVTEKLKE
B               PVEINCTRPNNNTRKSIHIGPGRAFYATGDIIGNIRQAYCTLNRARWNDTLKQIAEKLGE
                .********.****.** ****:.** ******:**:*** :* ::**::***::*** *

Humanized       HFKNKTIIFQPPSGGDLEITMHHFNCRGEFFYCNTTKLF
B               QFKNKTIVFNQSSGGDPEIVMHSFNCGGEFFYCNSTQLF
                :******:*: .**** **.** *** *******:*:**
```

Figure 13 Comparison of the consensus amino acid sequence of V3 region of subtype B with that encoded by the 297-bp humanized DNA derived from the approximated last common ancestor of the 8 subtypes

```
Humanized       SVEINCTRPSNNTRTSITIGPGQVFYRTGDIIGDIRKAYCEINGTKWNEALKQVTEKLKE
C               PVEIMCTRPDNNTRKSIRIGPGQTFYATGDIIGDIRQAHCNISEDKWNETLQNVSKKLAE
                .*** ****.****.** *****.** *********:*:*:*.  ****:*::*::** *

Humanized       HFKNKTIIFQPPSGGDLEITMHHFNCRGEFFYCNTTKLF
C               HFPNKTIIFNSSSGGDLEITTHSFNCRGEFFYCNTSGLF
                ** ******:..******** * ************: **
```

Figure 14 Comparison of the consensus amino acid sequence of V3 region of subtype C with that encoded by the 297-bp humanized DNA derived from the approximated last common ancestor of the 8 subtypes

```
Humanized       SVEINCTRPSNNTRTSITIGPGQVFYRTGDIIGDIRKAYCEINGTKWNEALKQVTEKLKE
D               SVTINCTRPNNNTVQSIHMGPGRALFTT-KIIGKIRQAHCNISGTGWNKTLQQVATKLRD
                ** ******.***  ** :***:.:: * .***.**:*:*:*.** **::*:**: **::

Humanized       HFKNKTIIFQPPSGGDLEITMHHFNCRGEFFYCNTTKLF
D               LYNRTEINFKPSSGGDPEITTHSFNCGGEFFYCNTSGLF
                 ::.. * *:*.**** *** * *** *******: **
```

Figure 15 Comparison of the consensus amino acid sequence of V3 region of subtype D with that encoded by the 297-bp humanized DNA derived from the approximated last common ancestor of the 8 subtypes

```
Humanized       SVEINCTRPSNNTRTSITIGPGQVFYRTGDIIGDIRKAYCEINGTKWNEALKQVTEKLKE
A/E             SVEINCTRPSNNTRTSITIGPGRVFYRTGDIIGNIRKAYCEINGTKWNKVLKQVTEKLKE
                **********************:**********:**************:.**********

Humanized       HFKNKTIIFQPPSGGDLEITMHHFNCRGEFFYCNTTKLF
A/E             HFN-KTIIFQPPSGGDLEITMHHFNCRGEFFYCNTTKLF
                **: **********************************
```

Figure 16 Comparison of the consensus amino acid sequence of V3 region of subtype A/E with that encoded by the 297-bp humanized DNA derived from the approximated last common ancestor of the 8 subtypes

```
Humanized       SVEINCTRPSNNTRTSITIGPGQVFYRTGDIIGDIRKAYCEINGTKWNEALKQVTEKLKE
F               SVQINCTRPNNNTRKRISLGPGRVFYTTGEIIGDIRKAHCNVSGTQWRNTLAKVKAKLGS
                **:******.****. *::***:*** **:********:*::.**:*.::* :*. ** .

Humanized       HFKNKTIIFQPPSGGDLEITMHHFNCRGEFFYCNTTKLF
F               YFPNATIKFNSSSGGDLEITRHNFNCMGEFFYCNTDELF
                :* * ** *:..******** *:*** ******** :**
```

Figure 17 Comparison of the consensus amino acid sequence of V3 region of subtype F with that encoded by the 297-bp humanized DNA derived from the approximated last common ancestor of the 8 subtypes

```
Humanized       SVEINCTRPSNNTRTSITIGPGQVFYRTGDIIGDIRKAYCEINGTKWNEALKQVTEKLKE
G               SIEINCIRPNNNTRKSIPIGPGQAFYATGDIIGDIRQAHCNVSRIKWREMLKNVTAQLRK
                *:**** **.****.**.*****.** *********:*:*::.  **.* **:** :*::

Humanized       HFKNKTIIFQPPSGGDLEITMHHFNCRGEFFYCNTTKLF
G               IYNNKNITFNSSAGGDLEITTHSFNCRGEFFYCNTSGLF
                 ::**.* *:..:******* * ************: **
```

Figure 18 Comparison of the consensus amino acid sequence of V3 region of subtype G with that encoded by the 297-bp humanized DNA derived from the approximated last common ancestor of the 8 subtypes

```
Humanized       SVEINCTRPSNNTRTSITIGPGQVFYRTGDIIGDIRKAYCEINGTKWNEALKQVTEKLKE
H               PVQINCTRTGNNTRKSIRIGPGQAFYATGDIIGDIRRAYCNISGKQWNETLHKVITKLGS
                .*:*****..****.** *****.** *********:***:*.*.:***:*::*  ** .

Humanized       HFKNKTIIFQPPSGGDLEITMHHFNCRGEFFYCNTTKLF
H               YFDNKTIILQPPAGGDIEIITHSFNCGGEFFYCNTTKLF
                :*.*****:***:***:**  * *** ************
```

Figure 19 Comparison of the consensus amino acid sequence of V3 region of subtype H with that encoded by the 297-bp humanized DNA derived from the approximated last common ancestor of the 8 subtypes

```
Humanized       SVEINCTRPSNNTRTSITIGPGQVFYRTGDIIGDIRKAYCEINGTKWNEALKQVTEKLKE
10-00           SVEINCTRPSNNTRTGVHMGPGRVFYRTGEIIGNIRIAYCEINGTQWNKTLTQVAEKLKE
                ***************.: :***:******:***:** ********:**::*.**:*****

Humanized       HFKNKTIIFQP--PSGGDLEITMHHFNCRGEFFYCNTTKLF
10-00           HF-NKTIIFQPQPPSGGDLEITMHHFNCRGEFFYCNTTKLF
                ** ********  ****************************
```

Figure 20 Comparison of the consensus amino acid sequence of V3 region of 10-00 (297-bp non-humanized DNA) with that encoded by the 297-bp humanized DNA derived from the approximated last common ancestor of the 8 subtypes (Antigenic epitopes are shown in Appendix D)

5. Transfection experiments for *in vitro* protein expression

Each plasmid DNA construct was allowed to transfect HEK293 cells for 48 hours and cell lysate was loaded into 4-12 % gradient polyacrylamide gel for protein expression as described in Materials and Methods.

The expression of protein encoded by the 297-bp humanized DNA yielded a single protein of approximately 13 kilodalton (kDa) (Figure 21) which corresponded to the predicted molecular size of the protein. In contrast, we could not detect protein expression from the 297-bp non-humanized DNA transfected HEK293 cells even we increased the

amount of DNA to 4 µg (Figure 21). The expression of 2.5 kb full-length envelope DNA yielded 3 bands at 160, 120 and approximately 38 kDa (Figure 22) which corresponded to gp160, gp120 and gp41 of HIV envelope structural proteins respectively.

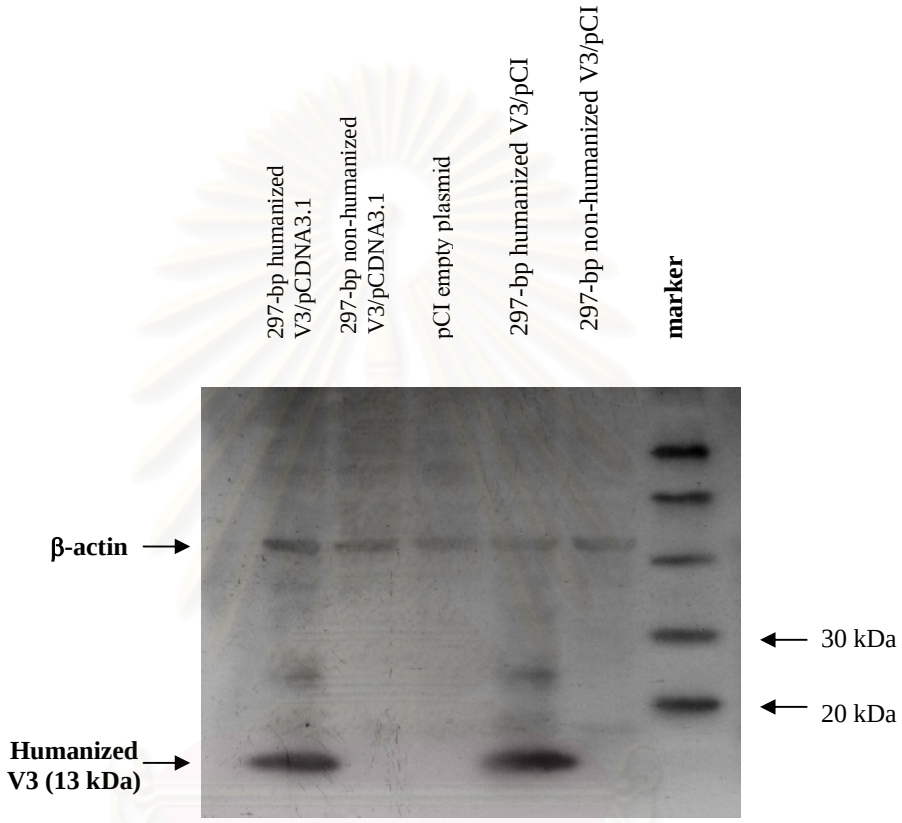


Figure 21 Protein expression of 297-bp humanized DNA (13 kilodalton protein) in 4-12 % gradient polyacrylamide gel electrophoresis

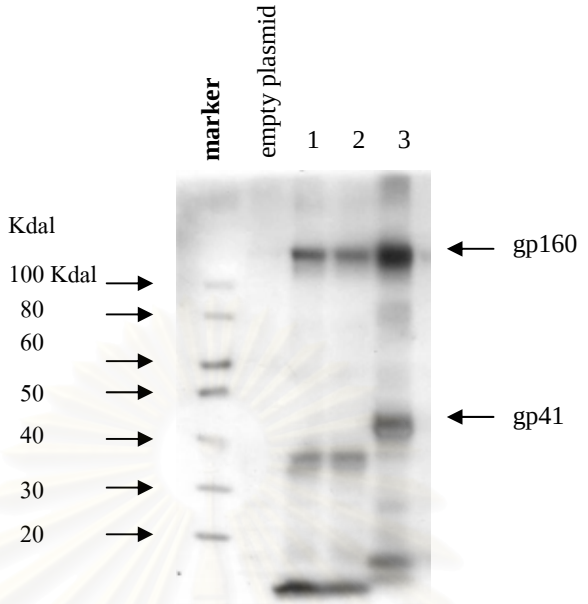


Figure 22 Protein expression of 2.5 kb full-length envelope DNA in 4-12 % gradient polyacrylamide gel electrophoresis, lane 1 : transfected with 2 μg of p1.1cI envE IRESrev, lane 2 : transfected with 4 μg of p1.1cI envE IRESrev, lane 3 : transfected with 2 μg of p1.1cI IIIB IRESrev as positive control.

6. *In vivo* immunogenicity assay

Delayed type hypersensitivity (DTH) skin testing by footpad swelling

Groups of 10-20 Balb/c mice were immunized with 3 intramuscular injections of each DNA construct on day 0, 14 and 28. A group of 8 mice served as negative control, immunized with empty plasmid or pCI (see Materials and Methods). Half of the mice in each group were boosted with recombinant gp120 of subtype A/E (or with pCI in the negative control group) on day 42. Delayed type hypersensitivity (DTH) skin testing was performed by footpad injection with the appropriate V3 peptide as described in Materials and Methods. Footpad thickness

was re-measured at 24 hours and the increment from baseline thickness was taken as DTH footpad swelling response.

The 24-hour footpad swelling measurements of the 297-bp humanized and 297-bp non-humanized DNA without rgp120 boosting were shown in Table 13. The groups with rgp120 boosting were shown in Table 14 including controls. Only the 297-bp humanized DNA immunized group without rgp120 boosting was able to induce significant footpad swelling response. The mean footpad thickness 24 hours after antigen challenge was 1.96 (0.03) mm, significantly thicker than the baseline (unchallenged) thickness of 1.87 (0.03), $p < 0.002$ (Table 13). Both the 297-bp humanized DNA and 297-bp non-humanized DNA immunized groups with rgp120 boosting also seemed to be able to induce footpad swelling (2.08 ± 0.08 vs 1.99 ± 0.04 and 2.03 ± 0.03 vs 1.95 ± 0.04, respectively), but the difference was not statistically significant which might be due to the small sample size (Table 14). It was evident that the values of baseline (pre-challenged) footpad thickness of mice in all groups were quite similar. Footpad thickness of immunized mice (either immunized with 297-bp humanized DNA or immunized with 297-bp non-humanized DNA) after appropriate antigen challenge was significantly higher than the non-immunized group but similarly challenged (Table 13 and Table 14). This was true for both boosted and non-boosted group although the difference in the boosted 297-bp humanized DNA group did not reach statistical significance (Table 14).

For the 2.5 kb full-length envelope DNA immunized group, no DTH or footpad swelling could be detected if mice were not boosted with rgp120 (Table 15). With rgp120 boosting, significant footpad swelling could be elicited with the

V3(E) peptide : GVHMGPGRVFYRTGE, (2.02 $\pm$ 0.04 vs 1.92 $\pm$ 0.03, $p < 0.025$) but not with the V3(hu) peptide : SITIGPGQVFYRTGD, (1.98 $\pm$ 0.04 vs 1.91 $\pm$ 0.02, $p < 0.078$) (Table 16). This difference of pre- and post-footpad challenge was also confirmed when the 24-hour footpad swelling of V3(E) challenged group was compared to negative controls challenged with V3(E) (2.02 $\pm$ 0.04 vs 1.91 $\pm$ 0.03, $p < 0.033$) (Table 16). The V3(hu) challenged group again did not reach statistically significant difference when the immunized group was compared to the non-immunized group (1.98 $\pm$ 0.04 vs 1.91 $\pm$ 0.03, $p = 0.1$).

Table 13 Delayed-type hypersensitivity (DTH) responses to 297-bp humanized and non-humanized envelope DNA vaccine in mice without boosting

GROUP	DNA Immunization	MOUSE #	Skin test Antigen	FOOTPAD THICKNESS (mm) Baseline R	Baseline L	24-hr R	24-hr L	FOOTPAD SWELLING (Δ thickness, x10^{-2} mm)	p VALUE Pre/Post	Compared to group A Baseline	Compared to group A 24-hr
		1		1.9	1.92	1.91	1.9	-0.5			
		2		1.89	1.88	1.93	1.91	4			
A	pCI	X (SD)	V3 (E)	1.90 (0.02)		1.92 (0.01)			0.5		
		1		1.89	1.88	1.98	1.99	10			
		2		1.83	1.85	1.93	1.93	9			
		3		1.89	1.91	1.96	1.97	6.5			
		4		1.87	1.87	1.98	1.93	8.5			
B	humanized	X (SD)	V3 (hu)	1.87 (0.03)		1.96 (0.03)			0.002*	0.348	0.027*
		1		1.91	1.91	1.97	1.96	5.5			
		2		2.05	2.02	1.98	1.95	-7			
		3		1.9	1.92	2.01	2	8.5			
C	non-humanized	X (SD)	V3 (E)	1.95 (0.08)		1.98 (0.02)			0.618	0.311	0.026*

* indicates statistically significant difference
V3 (E) peptide : GVHMGPGRVFYRTGE
V3 (hu) peptide : SITIGPGQVFYRTGE

Table 14 Delayed-type hypersensitivity (DTH) responses to 297-bp humanized and non-humanized envelope DNA vaccine in mice boosted with recombinant gp120

GROUP	DNA Immunization	MOUSE #	Skin test Antigen	FOOTPAD THICKNESS (mm) Baseline R	Baseline L	24-hr R	24-hr L	FOOTPAD SWELLING (Δ thickness, x 10^{-2} mm)	p VALUE Pre/Post	p VALUE Compared to group A Baseline	p VALUE Compared to group A 24-hr
		1		1.96	1.94	1.96	1.97	1.5			
		2		1.95	1.95	1.98	1.97	2.5			
A	pCI	X (SD)	V3 (E)	1.95 (0)		1.98 (0.01)			0.126		
		1		1.94	1.95	1.98	1.99	4			
		2		1.98	1.98	2.08	2.11	11.5			
		3		2.02	2.04	2.14	2.16	12			
B	humanized	X (SD)	V3 (hu)	1.99 (0.04)		2.08 (0.08)			0.073	0.257	0.155
		1		1.98	1.97	2.03	2.01	4.5			
		2		1.97	1.96	2.1	2.03	10			
		3		1.96	1.95	2.02	1.96	3.5			
		4		1.88	1.89	2.03	2.05	15.5			
C	non-humanized	X (SD)	V3 (E)	1.95 (0.04)		2.03 (0.03)			0.065	1	0.043 *

Table 15 Delayed-type hypersensitivity (DTH) responses to full-length subtype A/E envelope DNA vaccine in mice without boosting

GROUP	DNA Immunization	MOUSE#	Skin test Antigen	FOOTPAD THICKNESS (mm) Baseline R	Baseline L	24-hr R	24-hr L	FOOTPAD SWELLING (Δ thickness, x 10^{-2} mm)	p VALUE Pre/Post	Compared to group A Baseline	Compared to group A 24-hr
		1		1.86	1.87	1.96	1.88	5.5			
		2		1.87	1.85	1.87	1.86	0.5			
A	pCI	X (SD)	V3 (E)	1.87 (0.01)		1.9 (0.04)			0.374		
		1		1.89	1.9	1.86	1.85	-4			
		2		1.87	1.88	1.87	1.85	-1.5			
		3		1.85	1.86	1.87	1.86	1			
		4		1.86	1.87	1.93	1.91	5.5			
		5		1.88	1.89	1.89	1.88	0			
		X (SD)	V3 (E)	1.88 (0.02)		1.88 (0.03)			1	0.15	0.657
		1		1.88	1.89	1.86	1.86	-2.5			
		2		1.85	1.84	1.94	1.88	6.5			
		3		1.83	1.84	1.88	1.89	5			
D	env E	X (SD)	V3 (hu)	1.86 (0.03)		1.89 (0.03)			0.448	0.781	0.805

Table 16 Delayed-type hypersensitivity (DTH) responses to full-length subtype A/E envelope DNA vaccine in mice boosted with recombinant gp120

GROUP	DNA Immunization	MOUSE#	Skin test Antigen	FOOTPAD THICKNESS (mm) Baseline R	Baseline L	24-hr R	24-hr L	FOOTPAD SWELLING (Δthickness, x 10^{-2} mm)	p VALUE Pre/Post	Compared to group A Baseline	Compared to group A 24-hr
		1		1.96	1.94	1.92	1.93	-2.5			
		2		1.88	1.87	1.89	1.88	1			
A	pCI	X (SD)	V3 (E)	1.92 (0.05)		1.91 (0.03)			0.795		
		1		1.94	1.95	1.96	1.95	1			
		2		1.89	1.88	2.04	2.05	16			
		3		1.89	1.9	2.04	1.96	10.5			
		4		1.96	1.95	2.04	2.02	8.5			
		5		1.88	1.89	2.07	2.02	16			
		X (SD)	V3 (E)	1.92 (0.03)		2.02 (0.04)			0.025 *	0.947	0.033 *
		1		1.91	1.89	1.99	1.89	4			
		2		1.93	1.92	2	1.97	6			
		3		1.9	1.92	2.03	2.01	11			
D	env E	X (SD)	V3 (hu)	1.91 (0.02)		1.98 (0.04)			0.078	0.97	0.1

In order to have a larger sample size for comparison, footpad thickness of all 38 mice at baseline, i.e., before antigen challenge, was pooled. This baseline footpad thickness was then used to compare with post-challenge footpad thickness of various immunization groups. Results of such comparison are shown in Table 17. All immunization groups (297-bp humanized DNA, 297-bp non-humanized DNA and 2.5 kb full-length envelope DNA) with or without rgp120 boosting, could induce significant footpad swelling 24-hour after antigen challenge except the non-boosted 2.5 kb full-length envelope DNA group. With boosting, V3(E) and V3(hu) were equally effective in eliciting footpad swelling in mice immunized with 2.5 kb full-length subtype A/E envelope DNA (Table 17 and Figure 23). And, as expected, V3(E) challenge in negative control mice (immunized with pCI plasmid only) did not result in any significant DTH or footpad swelling. Table 17 also clearly shows that boosting in the full-length envelope DNA group resulted in significantly larger footpad swelling (DTH) than the non-boosted group. The effect of boosting was not or was less apparent in the 297-bp humanized DNA and the 297-bp non-humanized DNA groups (*p* value 0.123 and 0.082 respectively).

Table 17 Comparison of post-challenge footpad thickness of all immunization groups with the pre-challenge footpad thickness pooled from all mice

Mouse group	Skintest Ag	N*	Footpad thickness (mm)	p VALUE as compared to baseline thickness	p VALUE between boost and non-boost group
Baseline footpad thickness		38	1.91 (0.05)		
(A) pCI immunization	V3 (E)	8	1.91 (0.04)	NS	
(B) 297-bp humanized DNA	V3 (hu)	4	1.96 (0.03)	0.01**	
(C) 297-bp humanized DNA + rgp120 boosting	V3 (hu)	3	2.08 (0.08)	0.002**	0.123
(D) 297-bp non-humanized DNA	V3 (E)	3	1.98 (0.02)	0.002**	
(E) 297-bp non-humanized DNA + rgp120 boosting	V3 (E)	4	2.03 (0.03)	0.002**	0.082
(F) Full-length env DNA	V3 (E)	5	1.88 (0.03)	NS	
(G) Full-length env DNA + rgp120 boosting	V3 (E)	5	2.02 (0.04)	0.002**	0.001**
(H) Full-length env DNA	V3 (hu)	3	1.89 (0.03)	NS	
(I) Full-length env DNA + rgp120 boosting	V3 (hu)	3	1.98 (0.04)	0.002**	0.033**

* : Number of mouse in each group, each mouse had both right and left hindfeet measured.
** : Indicates statistically significant difference
NS = no statistical difference

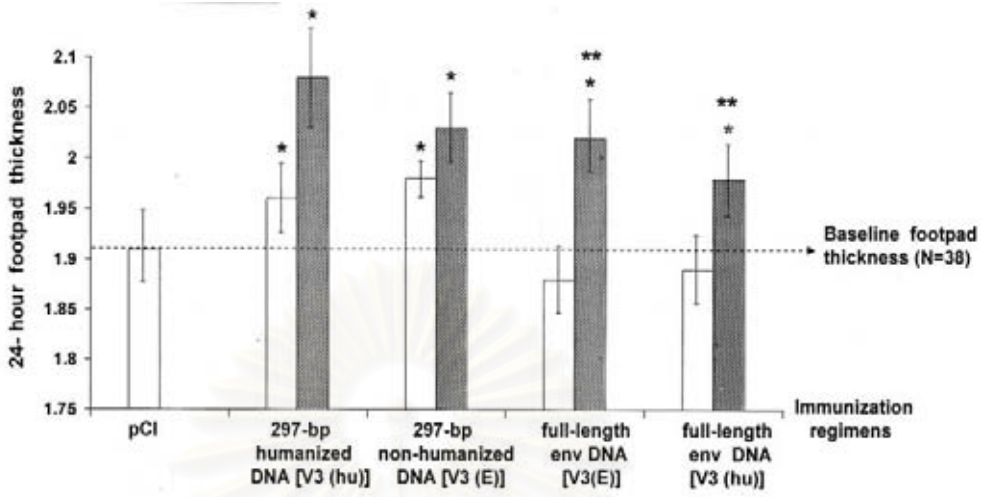


Figure 23 Mean 24-hour footpad thickness of mice immunized with pCI, 297-bp humanized DNA, 297-bp non-humanized DNA and 2.5 kb full-length envelope DNA and skin tested with V3(E) or V3(hu) 15-amino acid residue peptides injected into the footpads (☐ = without boosting, ▨ = with boosting). The horizontal dotted line represents the mean pre-injected footpad thickness of all 38 mice, servingas the baseline footpad thickness.

* indicates statistically significant difference as compared to baseline thickness

** indicates statistically significant difference between boost and non-boost group

7. *In vitro* immunogenicity assays

7.1 Humoral immune response

Serum specimens were obtained from each mouse in all experimental groups on day 42 for the non-boosted groups and on day 56 for the boosted groups. Pre-immunization sera were randomly obtained from 5 mice as negative control. All serum specimens were frozen at -20^{o}C until the antibody assay was done. The antibody to V3(E) and V3(hu) was measured by ELISA test according to the method described in Materials and Methods.

None of the DNA constructs (297-bp humanized DNA, 297-bp non-humanized DNA and 2.5 kb full-length envelope DNA) could induce any antibody response as shown in Figure 24, even after rgp120 boosting. The mean (SD) OD readings of each DNA immunization group ranged from 0.150 (0.016) to 0.318 (0.092) while the OD of the negative control (pCI) group ranged from 0.128 (0.004) to 0.232 (0.065), and of the pre-immunization sera was 0.116 (0.021) to 0.284 (0.074). When the sera were tested by gel particle agglutination test (every specimen) and Western blot (10 % randomly selected specimen), no antibody was detected.

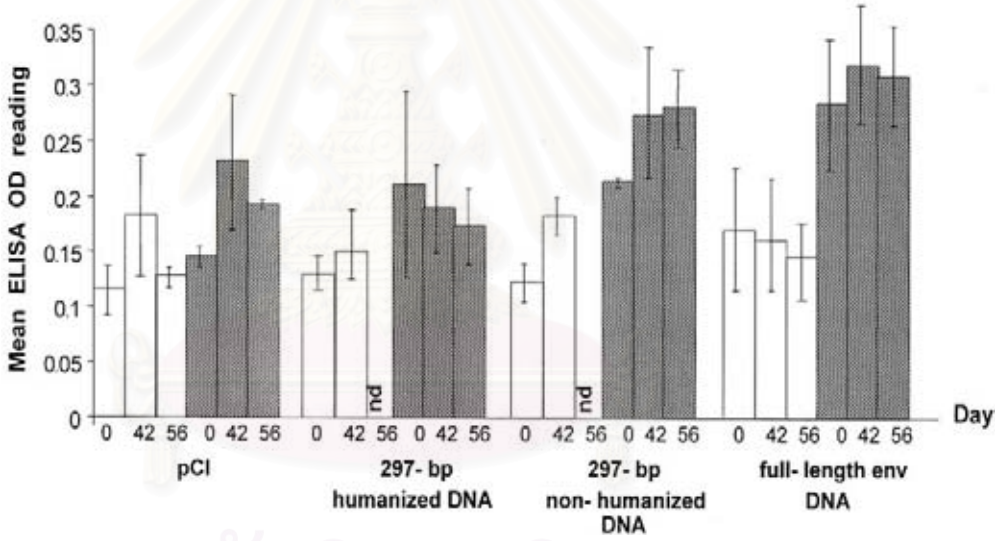


Figure 24 Mean ELISA OD reading of antibodies to envelope proteins [V3(hu), V3(E), rgp120(E)] in mice immunized with pCI, 297-bp humanized DNA, 297-bp non-humanized DNA and 2.5 kb full-length envelope DNA without rgp120 boosting (D42) and with rgp120 boosting (D56) detected by rgp120-coated well (▨) or by 15-amino acid residue peptide, V3(hu) or V3(E)-coated well (☐)

7.2 Lymphoproliferation assay

Lymphoproliferative response was set up for Balb/c mouse system using phytohemagglutinin (PHA) and concanavalin A (Con A) as positive mitogen controls. Spleen cells were obtained from 2 normal Balb/c mice and adjusted to a cell concentration of 1×10^6 splenocytes/ml as described in Materials and Methods. One hundred microlitres of the splenocyte suspension ($= 1 \times 10^5$ splenocytes/well) were added in triplicate wells of 96-well tissue culture plate (Costar). One hundred microlitres of mitogens was added in the wells with cells. Cells were cultured for 3 days in 37^oC 5 % CO_2 incubator. Tritiated thymidine (^{3}H-Tdr) was added during the last 18 hours of culture. Cells were harvested and the radioactivity incorporated into the extracted DNA, an index of cell proliferation, was counted as described in Materials and Methods. The results of mitogen-induced proliferation were shown in Table 18 . The results indicated that the culture system for mouse spleen cells was properly set up to measure mitogen-induced lymphoproliferation.

Table 18 : Lymphoproliferative response to mitogens (PHA and Con A) of mouse splenocytes

Mouse #	Mitogen used	Average cpm (a)	Δ cpm (b)	S.I. (c)
1	-	452	-	-
	PHA 100 μg/ml	5013	4561	11.09
	PHA 20 μg/ml	2597	2145	5.75
	Con A 10 μg/ml	26832	26380	59.36
	Con A 5 μg/ml	44374	43922	98.17
2	-	966	-	-
	PHA 100 μg/ml	3217	2251	3.33
	PHA 20 μg/ml	7476	6510	7.74
	Con A 10 μg/ml	15935	14969	16.50
	Con A 5 μg/ml	20750	19784	21.48

(a) average of triplicate cultures

(b) Δ cpm = cpm with mitogen – cpm without mitogen

(c) SI = stimulation index = $\frac{\text{cpm with mitogen}}{\text{cpm without mitogen}}$

The established splenocyte culture system for mitogen-induced lymphoproliferation was then applied to measure antigen-induced lymphoproliferation of spleen cells from mice immunized with various DNA plasmid constructs. Spleen cells of the non-boosted group were obtained on day 42 or 2 weeks after the last (3rd) DNA immunization. For the boosted group, spleen cells were obtained on day 56 or 2 weeks after boosting.

2×10^5 spleen cells were cultured with mitogen (Con A, 5 μg/ml) or specific antigen in 96-well tissue culture plate. Antigens used were either V3(hu) peptide for 297-bp humanized DNA immunized group or V3(E) peptide for 297-bp non-humanized DNA, 2.5 kb full-length envelope and pCI immunized groups. Two concentrations of V3(hu) and V3(E) peptides were used, 2 and 10 μg/ml. In addition, recombinant gp120 (rgp120) of subtype E at concentration of 1 and 5 μg/ml was used as *in vitro* antigen stimulation for all immunization groups. Culture was maintained for 7 days and pulsed with ^{3}H-Tdr during the last 18 hours of culture (see Materials and Methods). Results are shown in Table 19-22.

As a group, no significant antigen-stimulated lymphoproliferation could be demonstrated in any of the DNA immunization groups, either with or without rgp120 boosting. This was true either using specific V3 peptide [V3(hu), V3(E)] or using rgp120 (E) as stimulating antigens in the culture system. Occasionally, one can see significant antigen stimulation, i.e., stimulation index (S.I.) over 2.0 (see Materials and Methods) in certain mice in group immunized with full-length envelope (E) DNA without boosting, stimulated with peptide (E) (mouse # 3 & 5) or with rgp120 (E) (mouse # 2 & 3, Table 21). In the same immunization group but boosted, significant antigen stimulation could be seen in only 1 out of 5 mice stimulated with rgp120 in *in vitro* (mouse # 3, Table 22). In the same culture set-up (Table 22), rgp120-stimulated proliferation could be seen in 1 / 2 mice immunized with 297-bp humanized DNA and boosted (mouse#2) and in 1/3 mice immunized with 297-bp non-humanized DNA and boosted

(mouse #1). In one culture set-up (Table 20), S.I. over 2.0 was observed in many mice of all groups including that injected with empty pCI plasmid in response to many concentrations of peptide (E) or peptide (hu) and rgp120 *in vitro*. In this particular culture set-up, mitogen contamination was evident since there was a very high background proliferation (background cpm in the range of 2,500-6,000). It is also important to point out that in all of the 5 culture set-ups in this experiment, 7-day concanavalin A stimulation which served as cell survival control did not consistently give significant stimulation and if any, the S.I. and the Δ cpm were usually low as compared to the 3-day culture system.

Table 19 Antigen-stimulated lymphocyte proliferative response in mice immunized with 297-bp humanized DNA and 297-bp non-humanized DNA without boosting

Immunization	Mouse #	Background cpm	Con A (5 µg/ml)		Peptide (10 µg/ml)		Peptide (2 µg/ml)		rgp120 (5 µg/ml)		rgp120 (1 µg/ml)	
			Δ cpm	S.I.	Δ cpm	S.I.	Δ cpm	S.I.	Δ cpm	S.I.	Δ cpm	S.I.
	1	607	942	2.55	185	1.3	160	1.26	-63	0.9	244	1.4
	2	1165	427	1.37	-204	0.82	-134	0.88	-503	0.57	-73	0.94
pCI	X (SD)	886 ± 394	684 ± 364	1.96 ± 0.83	-10 ± 275	1.06 ± 0.34	13 ± 207	1.07 ± 0.27	-283 ± 311	0.74 ± 0.23	86 ± 224	1.17 ± 0.33
	1	819	700	1.85	97	1.12	-10	0.99	-451	0.45	-163	0.8
	2	1224	436	1.36	-506	0.59	-557	0.54	-796	0.35	-718	0.41
	3	449	428	1.95	308	1.69	60	1.13	226	1.5	44	1.1
	4	1077	1010	1.94	-213	0.8	-466	0.57	-551	0.49	-205	0.81
	5	861	337	1.39	183	1.21	-203	0.76	-350	0.59	-132	0.85
humanized	X (SD)	886 ± 294	582 ± 274	1.70 ± 0.30	-26.2 ± 330	1.08 ± 0.42	-235 ± 272	0.80 ± 0.26	-384 ± 379	0.68 ± 0.47	-235 ± 286	0.79 ± 0.25
	1	846	572	1.68	-84	0.9	-449	0.47	-279	0.67	-370	0.56
	2	533	399	1.75	117	1.22	276	1.52	-226	0.58	353	1.66
	3	869	1944	3.24	-208	0.76	-7	0.99	-500	0.42	91	1.1
	4	582	1194	3.05	-11	0.98	284	1.49	-188	0.68	324	1.56
	5	721	1821	3.53	89	1.12	343	1.48	-139	0.81	272	1.38
non-humanized	X (SD)	710 ± 151	1186 ± 702	2.65 ± 0.87	-19 ± 132	1.00 ± 0.18	89 ± 330	1.19 ± 0.46	-266 ± 140	0.63 ± 0.14	134 ± 299	1.25 ± 0.44

Table 20 Antigen-stimulated lymphocyte proliferative response in mice immunized with 297-bp humanized DNA and 297-bp non-humanized DNA with rgp120 boosting

Immunization	Mouse #	Background cpm	Con A (5 µg/ml)		Peptide (10 µg/ml)		Peptide (2 µg/ml)		rgp120 (5 µg/ml)		rgp120 (1 µg/ml)	
			Δ cpm	S.I.	Δ cpm	S.I.	Δ cpm	S.I.	Δ cpm	S.I.	Δ cpm	S.I.
	1	2459	20	1.01	1660	1.68	1120	1.46	565	1.23	3467	2.41
	2	2693	2771	2.03	1435	1.53	8131	4.02	11651	5.33	8916	4.31
pCI	X (SD)	2576 ± 165	1395 ± 1945	1.52 ± 0.72	1547 ± 157	1.61 ± 0.11	4625 ± 4957	2.74 ± 1.81	6108 ± 7838	3.28 ± 2.90	6191 ± 3853	3.36 ± 1.34
	1	3862	-398	0.9	4253	2.1	2491	1.65	12860	4.33	8019	3.08
	2	10479	1134	1.11	-1841	0.82	-375	0.96	15902	2.52	12332	2.18
	3	9321	-1728	0.81	8065	1.87	-795	0.91	-1000	0.89	5966	1.64
	4	5623	-308	0.95	3977	1.71	-172	0.97	12257	3.18	-1109	0.8
	5	5376	-2569	0.52	-824	0.85	214	1.04	8328	2.55	336	1.06
humanized	X (SD)	6932 ± 2821	-773 ± 1425	0.86 ± 0.22	2726 ± 4057	1.47 ± 0.60	272 ± 1292	1.00 ± 0.31	9669 ± 6545	2.69 ± 1.25	5108 ± 5541	1.75 ± 0.91
	1	4484	23	1.01	1728	1.39	-572	0.87	1552	1.35	3010	1.67
	2	4767	-2794	0.41	3423	1.72	4798	2.01	13289	3.79	6921	2.45
	3	2904	977	1.34	978	1.34	3604	2.24	6870	3.37	1587	1.55
	4	2407	1745	1.42	6198	3.57	4279	2.78	5200	3.16	7755	4.22
	5	5130	-1960	0.62	-583	0.89	-1401	0.73	17461	4.4	124	1.02
non-humanized	X (SD)	3938 ± 1206	-401 ± 1926	0.96 ± 0.44	2348 ± 2589	1.78 ± 1.04	2141 ± 2901	1.73 ± 0.89	8874 ± 6410	3.21 ± 1.14	3879 ± 3331	2.18 ± 1.25

Table 21 Antigen-stimulated lymphocyte proliferative response in mice immunized with 297-bp humanized DNA, 297-bp non-humanized DNA and 2.5 kb full-length envelope DNA without boosting

Immunization	Mouse #	Background cpm	Con A (5 µg/ml)		Peptide(E) (5 µg/ml)		Peptide(hu) (5 µg/ml)		rgp120 (2.5 µg/ml)		rgp120 (0.5 µg/ml)	
			Δ cpm	S.I.	Δ cpm	S.I.	Δ cpm	S.I.	Δ cpm	S.I.	Δ cpm	S.I.
	1	2025	-834	0.59	-845	0.58	-1094	0.46	-1591	0.21	-1306	0.36
	2	558	423	1.76	22	1.04	138	1.25	-88	0.84	118	1.21
pCI	X (SD)	1291 ± 1037	205 ± 888	1.18 ± 0.83	411 ± 613	0.81 ± 0.33	478 ± 871	0.86 ± 0.56	839 ± 1062	0.53 ± 0.45	594 ± 1006	0.79 ± 0.60
	1	1013	953	1.94	-332	0.67	-504	0.5	-432	0.57	-412	0.59
	2	491	566	2.15	95	1.19	113	1.23	490	2	543	2.11
	3	557	1957	4.51	1227	3.2	376	1.68	354	1.64	1000	2.8
	4	1076	773	1.72	-292	0.73	-438	0.59	-553	0.49	-568	0.47
	5	473	718	2.52	664	2.4	141	1.3	177	1.37	319	1.67
full-length env	X (SD)	722 ± 296	993 ± 556	2.57 ± 1.12	272 ± 667	1.64 ± 1.12	62 ± 387	1.06 ± 0.50	7 ± 471	1.21 ± 0.66	403 ± 518	1.53 ± 1.00
	1	510	533	2.05	189	1.37	196	1.38	272	1.53	263	1.52
	2	875	2535	3.9	-189	0.78	75	1.09	-282	0.68	-137	0.84
	3	431	481	2.12	249	1.58	40	1.09	-150	0.65	106	1.2
humanized	X (SD)	605 ± 236	1183 ± 1171	2.69 ± 1.05	83 ± 237	1.24 ± 0.41	103 ± 81	1.19 ± 0.17	53 ± 289	0.95 ± 0.50	77 ± 201	1.19 ± 0.34
	1	654	373	0.57	-108	0.83	20	1.03	-9	0.99	298	1.46
	2	453	268	1.59	354	1.78	143	1.32	371	1.82	-74	0.84
	3	279	2328	9.34	-101	0.64	62	1.22	-88	0.68	86	1.31
non-humanized	X (SD)	462 ± 187	989 ± 1160	3.83 ± 4.80	48 ± 264	1.08 ± 0.61	75 ± 62	1.19 ± 0.15	9 ± 245	1.16 ± 0.59	103 ± 186	1.20 ± 0.32

Table 22 Antigen-stimulated lymphocyte proliferative response in mice immunized with 297-bp humanized DNA, 297-bp non-humanized DNA and 2.5 kb full-length envelope DNA with rgp120 boosting

Immunization	Mouse #	Background cpm	Con A (5 µg/ml)		Peptide(E) (5 µg/ml)		Peptide(hu) (5 µg/ml)		rgp120 (2.5 µg/ml)		rgp120 (0.5 µg/ml)	
			Δ cpm	S.I.	Δ cpm	S.I.	Δ cpm	S.I.	Δ cpm	S.I.	Δ cpm	S.I.
	1	366	202	1.55	956	3.61	1173	4.2	140	1.38	222	1.61
	2	1391	-175	0.87	-802	0.42	-813	0.42	-677	0.51	-744	0.47
pCI	X (SD)	878 ± 724	13 ± 266	1.21 ± 0.48	77 ± 1243	2.02 ± 2.26	180 ± 1404	2.31 ± 2.67	268 ± 577	0.95 ± 0.62	261 ± 683	1.04 ± 0.81
	1	794	707	1.89	92	1.12	402	1.51	-5	0.99	53	1.07
	2	873	2457	3.81	-154	0.82	123	1.14	661	1.76	195	1.22
	3	542	800	2.48	-46	0.92	25	1.05	11	1.02	2390	5.41
	4	671	569	1.85	157	1.23	-125	0.81	23	1.03	94	1.14
	5	560	379	1.68	360	1.64	295	1.53	-189	0.66	170	1.3
full-length env	X (SD)	688 ± 144	982 ± 839	2.34 ± 0.87	81 ± 196	1.15 ± 0.32	144 ± 209	1.21 ± 0.31	100 ± 325	1.09 ± 0.40	580 ± 1013	2.03 ± 1.89
	1	760	929	2.22	-426	0.44	110	1.14	-57	0.93	-4	0.99
	2	320	439	2.37	309	1.97	82	1.26	203	1.63	638	2.99
humanized	X (SD)	540 ± 311	684 ± 346	2.30 ± 0.11	58 ± 519	1.21 ± 1.08	96 ± 19	1.20 ± 0.08	73 ± 183	1.28 ± 0.49	317 ± 453	1.99 ± 1.41
	1	371	536	2.44	155	1.42	181	1.49	178	1.48	374	2.01
	2	577	3510	7.08	50	1.09	-96	0.83	-51	0.91	38	1.07
	3	608	1041	2.71	-51	0.92	145	1.24	53	1.09	-82	0.87
non-humanized	X (SD)	518 ± 128	1695 ± 1591	4.08 ± 2.60	51 ± 103	1.14 ± 0.25	76 ± 150	1.19 ± 0.33	60 ± 114	1.16 ± 0.29	110 ± 236	1.32 ± 0.61

7.3 Intracellular cytokine staining assay

For intracellular cytokine staining (ICCS) assay, spleen cells from mice Immunized with 297-bp humanized DNA and with 297-bp non-humanized DNA and 2.5 kb full-length envelope (E) DNA were stimulated *in vitro* with V3(hu) and V3(E) peptides, respectively for 18 hours. Stimulated $CD8^+$ T Cells producing interferon-gamma intracellularly were stained and counted in flow cytometer (Figure 25 for 297-bp humanized and non-humanized DNA and Figure 26 for 2.5 kb full-length envelope DNA) as described in Materials and Methods. Results were shown in Table 23-24 and Figure 25-27.

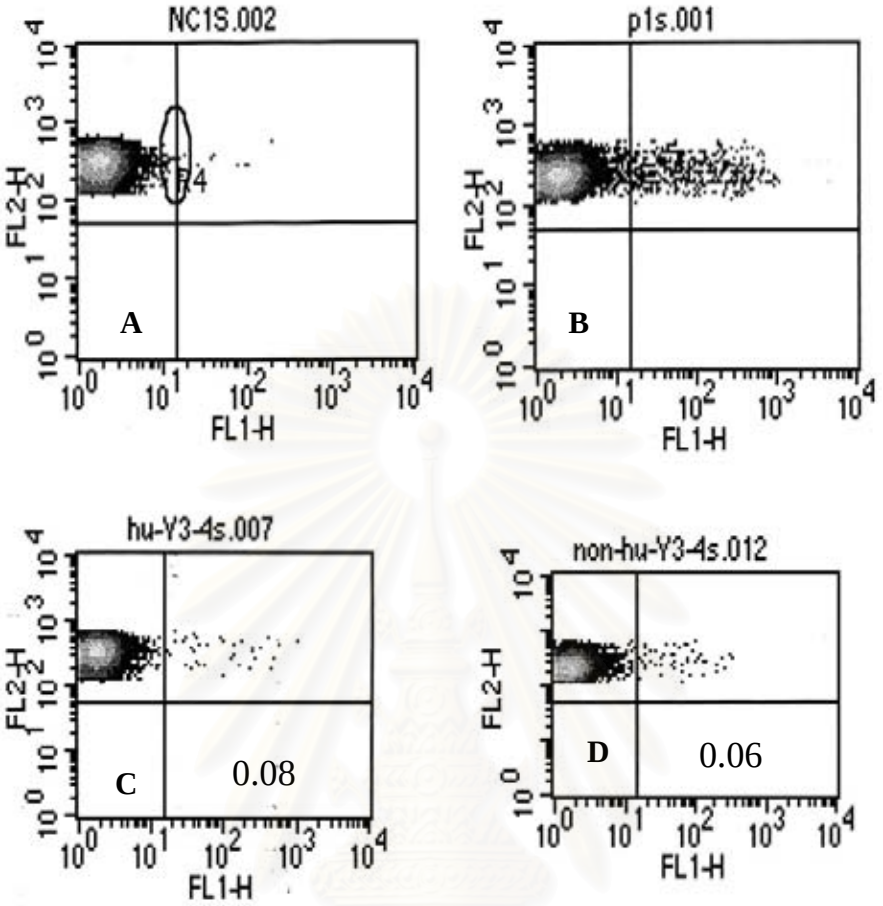


Figure 25 ICCS as detected by Flow cytometer

A : mouse immunized with pCI = negative control

B : mouse immunized with rVVgp160(B) = positive control

C : mouse immunized with 297-bp humanized DNA

D : mouse immunized with 297-bp non-humanized DNA

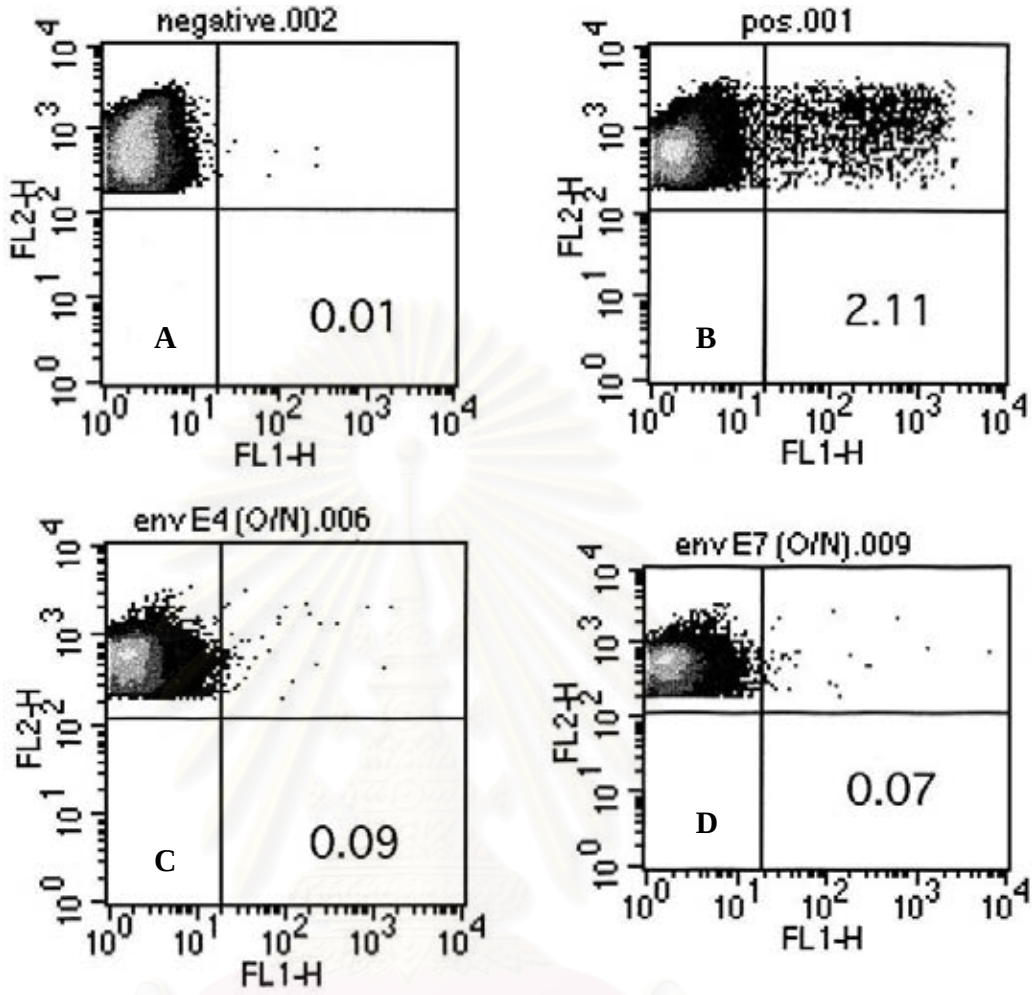
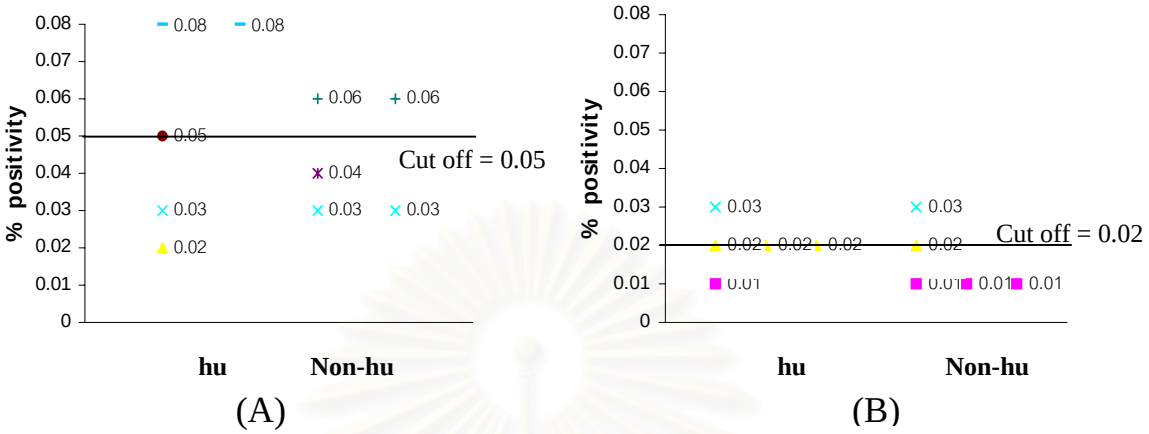


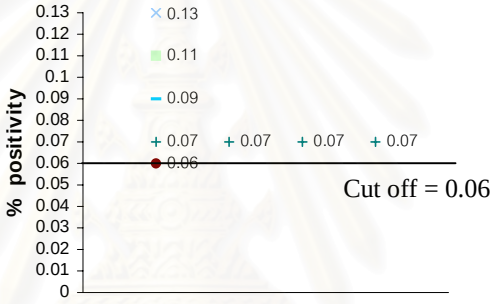
Figure 26 ICCS as detected by Flow cytometer

A : mouse immunized with pCI = negative control

B : mouse immunized with rVVgp160(B) = positive control

C, D : mice immunized with 2.5 kb full-length envelope DNA





(C)

Figure 27 ICCS of

(A) mice immunized with 297-bp humanized and non-humanized DNA vaccine without rgp120(E) boosting (out-off = 0.05)

(B) mice immunized with 297-bp humanized and non-humanized DNA vaccine with rgp120(E) boosting (out-off = 0.02)

(C) mice immunized with 2.5 kb full-length subtype A/E envelope DNA vaccine with rgp120(E) boosting (out-off = 0.06)

Table 23 Intracellular cytokine staining of mice immunized with 297-bp humanized and non-humanized DNA vaccine with and without rgp120 boosting

Group	DNA	MOUSE#	STIMULATED Ag	% positive (ICCS)	
				without rgp120 boosting	With rgp120 boosting
A	pCI	1	V3(E) + rgp120	0.02	0.01
		2		0.04	0.01
		mean (SD)		0.03 (0.01)	0.01 (0)
B	humanized	1	V3(hu) + rgp120	0.08*	0.01
		2		0.02	0.02*
		3		0.05*	0.02*
		4		0.08*	0.02*
		5		0.03	0.03*
		mean (SD)		0.05 (0.03)	0.02 (0.01)
		No.+* / No. tested (%)		3/5 (60 %)	4/5 (80 %)
C	non-humanized	1	V3(E) + rgp120	0.03	0.01
		2		0.04	0.01
		3		0.06*	0.02*
		4		0.06*	0.01
		5		0.03	0.03*
		mean (SD)		0.04 (0.02)	0.02 (0.01)
		No.+* / No. tested (%)		2/5 (40 %)	2/5 (40 %)

Cut-off = X + 2SD = 0.05 (without boosting), 0.02 (with boosting)

NB* = No. positive ICCS $\geq$ X + 2SD

Table 24 Intracellular cytokine staining of mice immunized with full-length subtype A/E envelope DNA vaccine with rgp120 boosting

GROUP	DNA	MOUSE#	STIMULATED Ag	% positive ICCS with rgp120 boosting
A	**pCI**	1	V3(E) + rgp120	0.01
		2		0.04
		mean (SD)		0.02 (0.02)
D	**envE**	1	V3(E) + rgp120	0.11*
		2		0.06*
		3		0.13*
		4		0.09*
		5		nd
		6		0.07*
		7		0.07*
		8		0.07*
		9		0.06*
		10		0.07*
		mean (SD)		0.08 (0.02)
		No.+* / No. tested (%)		9/9 (100 %)

Cut-off = X + 2SD = 0.06

NB* = No. positive ICCS $\geq$ X + 2SD

For the 297-bp humanized and non-humanized DNA vaccinated groups, the responses were quite low (Table 23 and Figure 27 A, B). Using the standard criteria of positive ICCS as described in Materials and Methods, namely any % positivity more than or equal to the mean of % positivity of negative control (pCI immunized group) plus 2 SD as the cut-off value, only 3/5 mice in the 297-bp humanized DNA immunized group gave positive responses (% positivity = 0.08, 0.05, 0.08, cut-off = 0.05). Another 2/5 mice in the 297-bp non-humanized DNA immunized group gave positive responses (% positivity = 0.06, 0.06, cut-off = 0.05). The ICCS responses were not increased after boosting with rgp120 (Table 23).

For the 2.5 kb full-length envelope DNA immunized group, the intracellular cytokine response seemed to be higher (Table 24 and Figure 27 C). All immunized mice had responded to the stimulated antigens. Unfortunately, only the results from those immunized with 3 doses of DNA plus rgp120 boosting could be analysed. The % positivity were 0.11, 0.06, 0.13, 0.09, 0.07, 0.07, 0.07, 0.06, 0.07, cut-off = 0.06. The results from the non-boosted group could not be analysed due to cell death as seen from flow cytometry.

7.4 Enzyme linked immunospot assay (ELISPOT)

Number of spleen cells secreting interferon-gamma after antigen challenge was measured by ELISPOT assay as described in Materials and Methods. The spot forming cells (SFC) per 10^6 cells were counted by computer-assisted video image analysis. Results of ELISPOT assay were shown in Tablle 25-26 and Figure 28.

Table 25 ELISPOT results of 297-bp humanized and non-humanized DNA immunized mice with and without rgp120 boosting

	SFC / 10^6 cells (stimulated *– unstimulated)	
Mice	**Without rgp120 boosting**	**With boosted rgp120**
(A) Negative control 1	20	-20
Negative control 2	0	-50
Negative control 3	70	10
X (SD)	30 (36)	3 (6)
(B) Humanized 1	70	10
Humanized 2	230	-40
Humanized 3	90	-60
Humanized 4	70	-11
Humanized 5	130	-10
X (SD)	118 (67) ($p < 0.05$) **	2 (4) (NS)
(C) Non-humanized 1	190	-90
Non-humanized 2	100	-10
Non-humanized 3	90	-10
Non-humanized 4	80	80
Non-humanized 5	140	60
X (SD)	120 (45) ($p < 0.025$) **	28 (39) (NS)
Positive control	750	-200

NS = non statistical difference

* : stimulated with V3(hu) for humanized group or with V3(E) for non-humanized group and negative control group

** : Statistical significant as compared to the negative control group (group A)

For the 297-bp humanized and non-humanized groups without boosting, both groups had significantly higher ELISPOT than the negative control (pCI immunized) group. The 297-bp humanized and non-humanized V3 groups had 118 (67) and 120 (45) SFC/ 10^6 cells respectively whereas the negative control group had 30 (36) SFC/ 10^6 cells, $p < 0.05$ and < 0.025 respectively (Table 25). However, with boosting, no ELISPOT could be demonstrated in the humanized DNA group but 2/5 mice in non-humanized DNA immunized group. This was due to survival problem in that particular experiment since positive control also did not show any SFCs whereas in the other experiment, the ELISPOT in the positive control was 750 SFC/10^6 cells (Table 25).

For the 2.5 kb full-length envelope DNA immunized group, the mean SFC in the non-boosted group was significantly higher than the negative control group (123 ± 36 vs 30 ± 36, $p < 0.01$). Again, the culture system for the boosted group was not satisfactory since no SFC could be detected either in the full-length envelope DNA immunized group or in positive control (Table 26).

Table 26 ELISPOT results of mice immunized with 2.5 kb full-length envelope DNA

	SFC / 10^6 cells (stimulated *– unstimulated)	
Mice	**without rgp120 boosting**	**with rgp120 boosting**
(A) Negative control 1	20	-20
Negative control 2	0	-50
Negative control 3	70	10
X (SD)	30 (36)	3 (6)
(D) Envelope 1	140	-50
Envelope 2	100	-50
Envelope 3	80	50
Envelope 4	140	-130
Envelope 5	110	-150
Envelope 6	100	-70
Envelope 7	140	-10
Envelope 8	200	-50
Envelope 9	100	-50
Envelope 10	nd	-80
X (SD)	123 (36) ($p < 0.01$) **	5 (16) (NS)
Positive control	750	-200

NS = non statistical difference

* : stimulated with V3(E)

** : statistical significance as compared to the negative control group (group A)

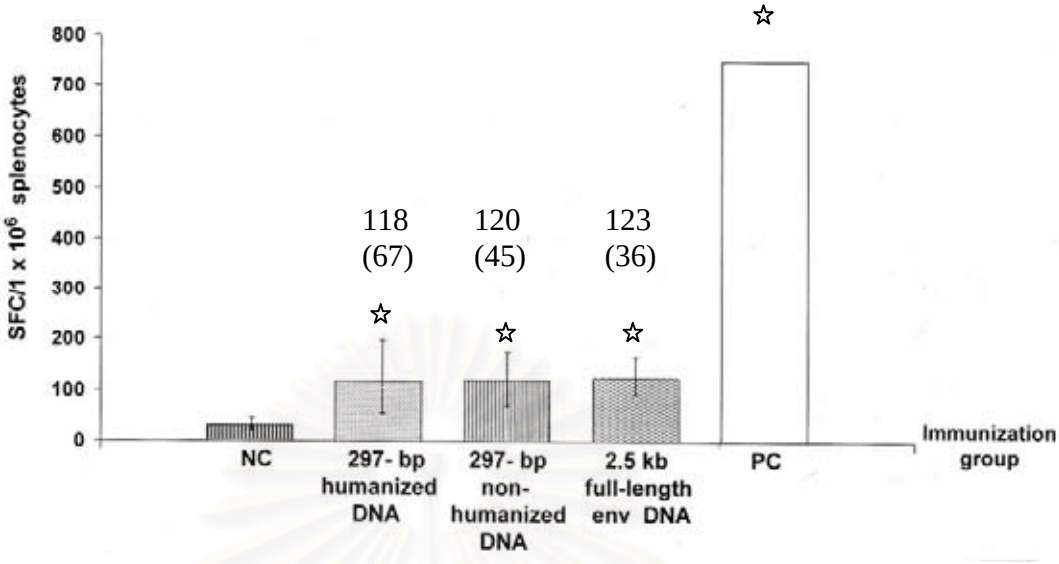


Figure 28 Mean ELISPOT results of mice immunized with 297-bp humanized DNA, 297-bp non-humanized DNA and 2.5 kb full-length envelope DNA without rgp120 (E) boosting. NC = negative control, injected with pCI. PC = positive control, immunized with rVVgp160(B). ☆ indicates statistical significance as compared to negative control.

7.5 Testing cross-reactivity of the 297-bp humanized DNA with rVVgp160(E) boosting

To test whether the multi-clade 297-bp humanized DNA can cross-react with other HIV-1 subtypes, mice were primed with a single injection of the 297-bp humanized DNA and boosted with recombinant vaccinia containing gp160 of subtype E [rVVgp160(E)]. Two weeks later, mouse splenocytes were stimulated *in vitro* with rVVgp160 of subtypes A, B, C and A/E and cytokine responses were measured by ELISPOT and ICCS assays. Results are shown in Table 27 and Figure 29 for ELISPOT assay and Table 28 for ICCS assay. For

the ELISPOT assay, it is apparent that the IFN-γ cytokine was released in response to stimulation with vaccinia virus. Mice primed with empty plasmid (pCI) and boosted with rVVgp160-E 2 weeks before sacrifice gave a mean ELISPOT of 75.5 and 44.9 SFC/ 5 x 10^5 splenocytes upon stimulation with wild-type vaccinia virus and rVVgp160-E respectively (group A, Table 27) suggesting that the response was vaccinia virus-specific, not HIV-specific. Mice immunized with 297-bp humanized DNA alone (group D) gave only 7.3 and 5 SFC/5 x 10^5 splenocytes with similar stimulations respectively suggesting that single immunization with DNA vaccine could not result in any ELISPOT response. ELISPOT response became positive when 297-bp humanized DNA primed mice were boosted with wild-type vaccinia virus (group C), again suggesting vaccinia virus-specific response. *In vitro* stimulation of splenocytes from this group of mice (group C) with various recombinant vaccinia constructs did not result in higher ELISPOT response as compared to stimulation with wild-type vaccinia virus alone suggesting that boosting with wild-type vaccinia virus in 297-bp humanized DNA primed mice did not result in any HIV-induced IFN-γ release *in vitro*. Similarly, no HIV-specific ELISPOT could be demonstrated in mice primed with 297-bp humanized DNA and boosted with rVVgp160-E (group B). The mean SFC / 5 x 10^5 splenocytes in response to stimulation with wild-type vaccinia virus, rVV-A, rVV-B, rVV-C and rVV-E in this group of mice were 91.3, 55.3, 94.3, 60.8 and 72.5 respectively (group B, Table 27). It is interesting to note that 297-bp humanized DNA primed/wild-type vaccinia boosted group (group C) gave higher ELISPOT than the 297-bp

humanized DNA primed/rVVgp160-E boosted group (group B) across all stimulating antigens used *in vitro* but significant difference was seen only when wtVV was used as *in vitro* stimulating antigen (Table 27).

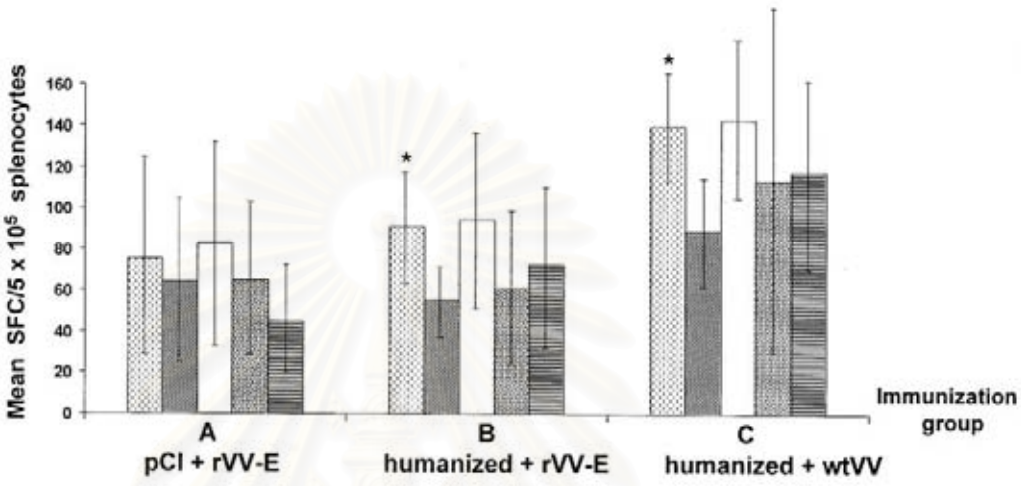


Figure 29 Mean ELISPOT results (Mean SFC / 5 x 10^5 splenocytes) in mice primed with 297-bp humanized DNA and boosted with recombinant vaccinia virus(E). (Splenocytes were stimulated with wtVV ⊡ , rVV-A ▨ , rVV-B □ , rVV-C ▩ , rVV-E ▤ , N = 4 in each group.) Immunization with 297-bp humanized DNA only (Group D) had < 10 SFC / 5 x 10^5 splenocytes)

* : indicates statistical significance as compared between group B and group C

Table 27 ELISPOT results of mice primed with 297-bp humanized DNA and boosted with recombinant vaccinia virus (E)

Immunization group	**Mouse #**	**SFC / 5 x 10^5 splenocytes**				
		WT	**rVV-A**	**rVV-B**	**rVV-C**	**rVV-E**
(A) pCI + rVV-E	1	65	55	80	66.5	37.5
	2	16	13	18	17	12.5
	3	107	98	100	93.5	58
	4	114	90	132.5	83	71.5
mean (SD) group A		75.5 (45.2)	64 (38.8)	82.6 (48.2)	65 (33.9)	44.9 (25.7)
(B) humanized + rVV-E	1	70	38.5	75	44.5	59
	2	90	73.5	101	57.5	60
	3	70	46	56	23.5	45
	4	135	63	145	117.5	126
mean (SD) group B		91.3 (30.7)*	55.3 (15.9)	94.3 (38.5)	60.8 (40.3)	72.5 (36.3)
(C) humanized + WT	1	169	85	200.5	245	163.5
	2	116	54.5	99.5	55	100
	3	140	121.5	169	77.5	141
	4	134	96.5	122.5	77	66.5
mean (SD) group C		139.8 (22)*	89.4 (27.8)	147.9 (45.5)	113.6 (88.2)	117.8 (43.1)
(D) humanized	1	28	19.5	32	19	19
	2	0	0	0	0	0
	3	0	0.5	1	1	0
	4	1	0	0.5	0	1
mean (SD) group D		7.3 (13.8)	5 (9.7)	8.4 (15.8)	5 (9.3)	5 (9.3)

* = $p < 0.05$ when group B and C were compared

For ICCS assay, the laboratory set up seemed to work properly as shown in Figure 30. Unstimulated splenocytes of all immunization groups gave the lowest ICCS results (mean ranged from 0.02-0.05, Table 28 and Figure 31). Con A stimulation gave ICCS results between 0.03-0.16 in both $CD4^+$ and $CD8^+$ cells.

As compared to unstimulated culture, rVV-B and rVV-E stimulations Gave a much higher ICCS response in mice primed/boosted with pCI/rVV-E (group A), as well as with 297-bp humanized DNA/rVV-E (group B) and with 297-bp humanized DNA/wtVV (group C). The response was seen primarily in the $CD8^+$ T cell population. The recombinant vaccinia B and E-stimulated $CD8^+$ ICCS responses in pCI primed / rVV-E boosted mice (group A) were 0.19 and 0.16 respectively. These responses were highest in humanized DNA primed/wtVV boosted group (group C), mean ICCS of 0.57 and 0.57 respectively. These were much higher than similar responses in humanized DNA primed/rVV-E boosted group (group B) (mean ICCS of 0.25 and 0.28 respectively) but the difference was statistically significant only when the splenocytes were stimulated with rVV-B *in vitro* (Table 28).

297-bp humanized DNA immunized mice without vaccinia boosting (group D) gave only weak ICCS response upon *in vitro* recombinant vaccinia stimulation (mean of 0.04 for both rVV-B and rVV-E stimulation as compared to 0.02 for unstimulation, Table 28).

However, when wild-type vaccinia virus was used in *in vitro* stimulation, the ICCS responses were in the same ranges as those obtained

with rVV-B and rVV-E stimulations in all immunization groups (Table 28) indicating that the $CD8^+$-ICCS response was vaccinia-specific. Or other word, no HIV-related ICCS response could be observed in this study.

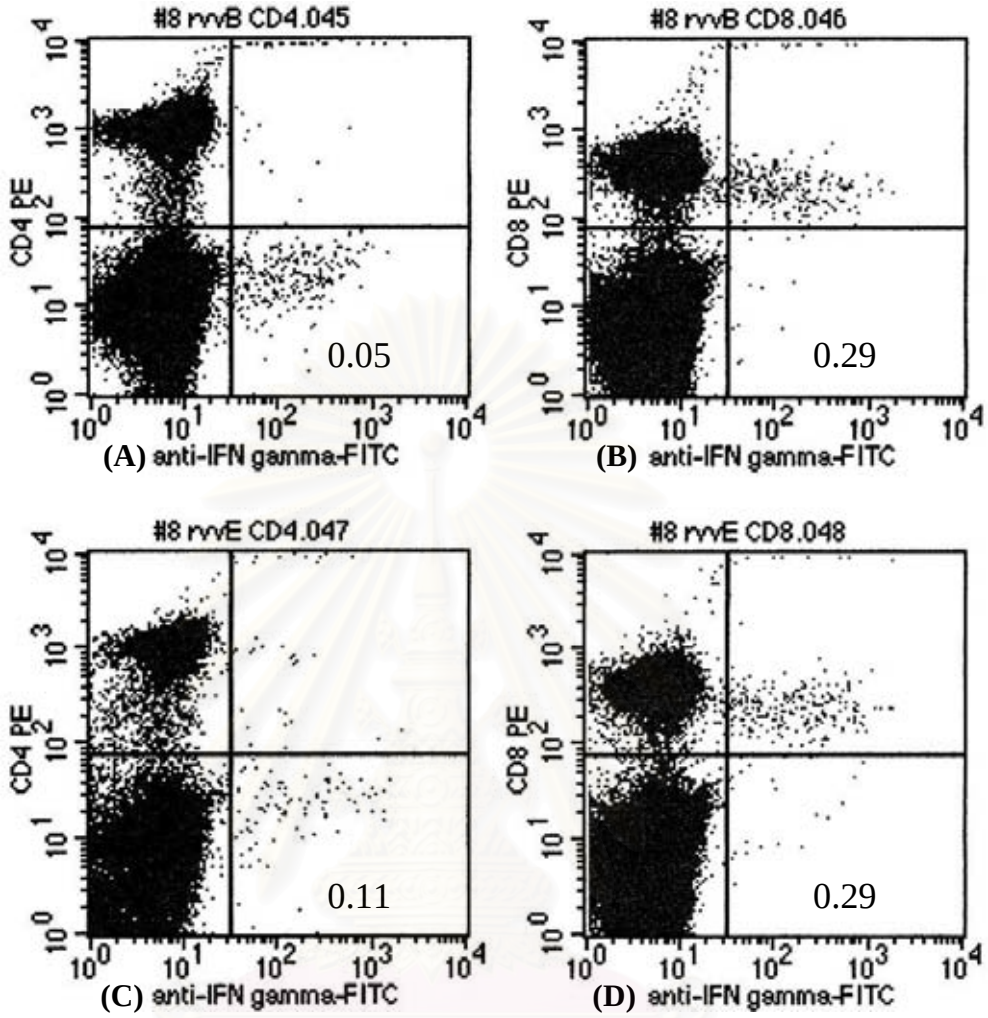


Figure 30 ICCS of humanized DNA primed / recombinant vaccinia virus (E) boosted

A, C : mouse splenocytes stimulated with rVV-E and stained with anti-CD4 / anti-IFNγ antibodies

B, D : mouse splenocytes stimulated with rVV-E and stained with anti-CD8 / anti-IFNγ antibodies

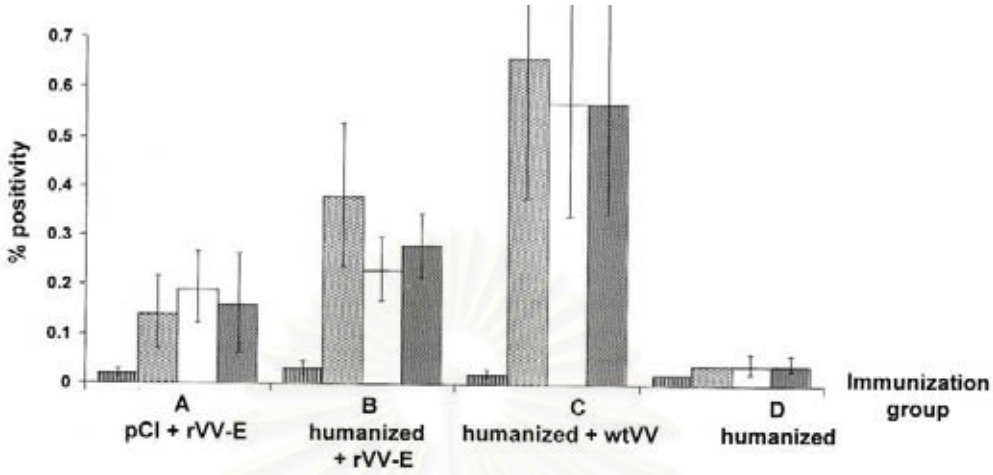


Figure 31 ICCS results of IFNγ^+ / CD8$^+$ cells in mice primed with 297-bp humanized DNA and boosted with recombinant vaccinia virus (E)

(▥ = unstimulated, ▨ = stimulated with wtVV,

□ = stimulated with rVV-B, ▨ = stimulated with rVV-E)

Table 28 ICCS results of mice primed with 297-bp humanized DNA and boosted with recombinant vaccinia virus (E)

Immunization group	stimulating antigens	% positivity									
		mouse #1		mouse #2		mouse #3		mouse #4		X (SD)	
		CD4/IFN-γ	CD8/IFN-γ	CD4/IFN-γ	CD8/IFN-γ	CD4/IFN-γ	CD8/IFN-γ	CD4/IFN-γ	CD8/IFN-γ	CD4/IFN-γ	CD8/IFN-γ
	unstimulated	0.04	0.02	0.07	0.03	0.04	0.02	0.04	0.02	0.05 (0.02)	0.02 (0.01)
	wtVV	0.13	0.1	0.04	0.18	nd	nd	nd	nd	0.09 (0.06)	0.14 (0.06)
	rVV-B	0.05	0.12	0.11	0.17	0.07	0.18	0.05	0.27	0.07 (0.03)	0.19 (0.06)
(A) pCI + rVV-E	rVV-E	0.06	0.09	0.04	0.09	0.06	0.19	0.07	0.27	0.06 (0.01)	0.16 (0.09)
	unstimulated	0.04	0.02	0.03	0.03	0.05	0.03	0.04	0.02	0.04 (0.01)	0.03 (0.01)
	wtVV	0.06	0.47	0.04	0.29	nd	nd	nd	nd	0.05 (0.01)	0.38 (0.13)
	rVV-B	0.07	0.31	0.06	0.24	0.04	0.16	0.05	0.29	0.06 (0.01)	0.25 (0.07)*****
(B) humanized + rVV-E	rVV-E	0.05	0.3	0.07	0.35	0.06	0.18	0.11	0.29	0.07 (0.03)	0.28 (0.07)
	unstimulated	0.06	0.02	0.02	0.03	0.03	0.02	0.03	0.01	0.04 (0.02)	0.02 (0.01)
	wtVV	0.05	0.46	0.09	0.86	nd	nd	nd	nd	0.07 (0.03)	0.66 (0.28)
	rVV-B	0.06	0.33	0.07	0.86	0.06	0.6	0.07	0.47	0.07 (0.01)	0.57 (0.23)*****
(C) humanized + wt	rVV-E	0.05	0.22	0.05	0.68	0.24	0.82	0.08	0.54	0.11 (0.09)	0.57 (0.26)
	unstimulated	0.03	0.02	0.02	0.02	nd	nd	nd	nd	0.03 (0.01)	0.02 (0)
	wtVV	0.02	0.06	0.02	0.01	nd	nd	nd	nd	0.02 (0)	0.04 (0.04)
	rVV-B	0.04	0.06	0.04	0.02	nd	nd	nd	nd	0.04 (0)	0.04 (0.03)
(D) humanized	rVV-E	0.02	0.05	0.03	0.02	nd	nd	nd	nd	0.03 (0.01)	0.04 (0.02)
Con A (positive control)	Con A	0.06	0.06	0.1	0.13	0.06	0.16	0.05	0.03	0.07 (0.02)	0.1 (0.06)

* = statistically significant difference between group B and group C

CHAPTER V

DISCUSSION AND CONCLUSION

Ten isolates of human immunodeficiency virus (HIV) were successfully obtained from 10 HIV-infected untreated Thai individuals with different clinical stagings; 3 asymptomatic, 5 AIDS related complex (ARC) and 2 full-blown AIDS. Out of these 10 isolates, 6 were identified as non-syncytial inducing (NSI) phenotype and 4 as syncytial inducing (SI) phenotype (Table 11). The isolates from the 3 asymptomatic individuals were all NSI which is consistent with the notion that less virulent (i.e., NSI) phenotype, is generally found in early stage of infection[237]. It is the phenotype that is most frequently transmitted among individuals. Therefore, it is the phenotype of interest to be used as prototype of preventive vaccine. All of the 3 NSI isolates from the asymptomatic patients were identified as subtype A/E by sequencing analysis. As a consequence, one of these 3 isolates (isolate 10-00) was randomly selected as PCR template for the amplification of the required DNA constructs. No prior history of antiretroviral treatment is also essential in order to avoid any drug-pressured mutations. It is interesting to see 2 NSI phenotype from 2 patients with AIDS. This is still possible[238] but unfortunately, we did not have the result of CD4 count in these patients to tell how advanced they were.

The region of HIV-1 genome which was of interest in this study was the envelope (*env*) region. The envelope protein, especially the V3 region of gp120 is the part of the virus that has the first contact with the infected cell. Neutralizing antibodies to V3 and its surrounding region are anticipated to confer protective or sterile immunity

if they are present before viral entry. Therefore, in addition to amplifying the 2.5 kb full-length envelope from the selected viral template, a 297 basepair (bp) DNA encompassing V3 and the adjacent region was also attempted. The region (codon 291-391 of HXB2 strain, codon 294-391 of CM240 strain) covers V3 which is the principal neutralizing determinant (PND), CTL epitopes, T helper (Th) epitopes, CXCR4 and CD4 binding sites and neutralizing epitopes. Once this region of HIV-1 genome was defined, a consensus sequence (approximated last common ancestor) was generated from the alignment of sequences from 80 independent HIV isolates across 8 HIV-1 subtypes circulating worldwide. This hypothetical, computerized sequence was aimed to represent a consensus sequence of the V3 region which covers all 8 HIV-1 subtypes. It is hoped that this consensus multi-clade V3 sequence will give cross-protective immunity against all subtypes included.

Once this hypothetical consensus multi-clade V3 sequence was generated, it was translated into corresponding amino acid sequence using DNAMAN version 4.15. When compared to the similar consensus V3 sequence from subtype A, B, C, D, A/E, F, G and H, our multi-clade V3 sequence is closest to subtype A/E (only 6 amino acid difference or 6 % , 6/99 difference) (Figure 16 & Table 12). The greatest difference is from subtype D, a 41 amino acid difference (Figure 15 & Table 12). This consensus multi-clade V3 sequence was then back translated to an estimated preferred human codon sequence using Backtranslate (Program Manual for the Wisconsin Package, Version 8, 1994, Genetics Computer Group, Madison, WI, U.S.A.). It consisted of 297 base pair and was referred to as “297-bp humanized V3 DNA” throughout this study. Humanized DNA does not result in different peptide from the non-humanized DNA but will give better protein expression and better immunogenicity. However, it needs to point that the term “297-bp non-humanized V3” used in this study refers to the

corresponding V3 region of subtype A/E. It has a 16 amino acid difference from the V3 encoded by the "297-bp humanized V3 DNA" which is multi-clade as mentioned above.

It has been well described that humanized DNA vaccine is better expressed in mammalian cells and more immunogenic in rodent and non-human primate[221-222]. Deml et al[221] demonstrated that their humanized *gag* construct could be better expressed in H1299, COS-7 and C2C12 cell lines than the non-humanized *gag*. In addition, they could also detect p24 antigen secreted in the supernatant of the transfection system while p24 antigen could hardly be detected in the supernatant of cell transfected with non-humanized *gag*. Moreover, their humanized *gag* construct could also induce much better anti-gag IgG and CTL in Balb/c mice[221]. Casimiro et al have shown that their humanized *pol* construct could induce much better CTL responses in Balb/c mice as detected by both ^{51}Cr-release CTL assay and ELISPOT assay. When they used the same construct to immunize rhesus macaques (N = 3), they found that all 3 macaques had better CTL response as detected by ^{51}Cr-release CTL assay than the non-humanized *pol* construct[222].

The 2.5 kb full-length envelope DNA and the 297-bp non-humanized V3 DNA were successfully amplified from the selected DNA template extracted from isolate 10-00 (subtype A/E) using nested PCR technique. Both had the correct molecular size (Figure 9) and were confirmed to have the right nucleotide sequence.

The approach to construct the 297-bp humanized V3 in this study is a challenge. It does not have full DNA template for PCR amplification since it is in fact a hypothetical gene. From the computerized sequence, 3 overlapping primers of 134, 138 and 125-mer long which covered the entire sequence were mixed together in the polymerase chain reaction without any additional template. As described in the Materials and Methods, first round PCR proceeds randomly which may result in 3

different PCR products as shown in Figure 6. Only the longest (full-length) product will be nested by the 2 smaller primers located at each end. The molecular size and the DNA sequence of this “297-bp humanized V3 DNA” were correctly confirmed by agarose gel electrophoresis (Figure 9) and DNA sequencing analysis, respectively.

Such construction of DNA by amplification from small pieces of overlapping primers has previously been described. It was described in the construction of HIV-1 gp120 by Haas et al[218]. Although the DNA successfully constructed in this study was only 297 bp, our laboratory was also successful in constructing a 700 bp *nef* humanized DNA construct using the same multiple overlapping primers (Lorenzen SI et al, unpublished results). Therefore, the PCR technique can be reliably used to construct DNA of variable sizes. It is particularly useful if the full DNA template is not available but only the full sequence is known. It is approximately 10 times cheaper than that obtained from direct DNA synthesizer. It is the method that should be first attempted in working with any humanized DNA vaccines at the laboratory scale before ordering it from commercial DNA synthesizing companies. This is particularly true for developing countries such as Thailand.

Both of the 297-bp V3 DNA constructs (humanized and non-humanized) were successfully cloned into the pCI mammalian expression vector whereas the 2.5 kb full-length A/E envelope DNA was successfully used to replace the IIIB envelope of p1.1cI IIIB IRES rev. The reason that a different vector was used with the 2.5 kb full-length envelope DNA was because the 2.5 kb full-length envelope requires *rev* (which already exists in p1.1 cI IIIB IRES rev) for expression. The cloned vector was allowed to propagate in DH5α and was proven by restriction enzyme digestions and sequencing analysis to verify the correct size (Figure 10) and the correct nucleotide sequences indicating successful ligation (cloning).

The 297-bp humanized V3 DNA was proved to be well expressed in HEK293 cells as a protein of approximately 13 kilodalton (kDa) in size when cell lysate was subjected to polyacrylamide gel electrophoresis (Figure 21). In contrast, the 297-bp non-humanized V3 DNA could not be expressed in the same cell. When the other cell type, COS-7, was used for transfection, again no protein expression of the 297-bp non-humanized V3 DNA could be detected (data not shown). These findings may support the proposition that humanized codon usage will result in better translation or protein expression in mammalian cells as described by several investigators[221-222]. However, these two DNA constructs cannot be directly compared based on humanization alone since they encode two V3 peptides which have 16 amino acid difference out of a total of 99 amino acids as discussed earlier.

For the 2.5 kb full-length envelope DNA, its expression in HEK293 cells upon transfection was well documented. The cell lysate yielded 3 HIV-related proteins of 160, 120 and approximately 38 kDa on 4-12 % gradient polyacrylamide gel which corresponded to gp160, gp120 and gp41 of HIV envelope protein respectively (Figure 22). The reason of getting the 38 kDa rather than the 41 kDa protein may be possibly explained by the presence of cellular protease that further cleaved the 41 kDa protein into 2 smaller proteins that compose of a 38 kDa protein and another much smaller size protein.

To detect the expression of a gene once it gets into the cell, one may detect the transcribed mRNA within the cell by reverse transcriptase polymerase chain reaction (RT-PCR) or by Northern blot assay. One may detect the synthesized proteins within the cell with the method similar to the one used in this experiment. Intracellular proteins have to be separated by the difference in their molecular size and counterstained by monospecific or polyspecific antibodies. We used pooled serum from HIV-1 infected

Thai individuals to detect the HIV-specific proteins from the transfected cell lysate. Strong HIV-specific protein bands could be demonstrated and their identities were confirmed by their known molecular sizes, i.e., gp160, gp120 or gp41. In addition to the polyclonal antibodies from the infected patients, monoclonal antibodies can also be used to detect specific proteins.

One may also look at the secreted proteins in the tissue culture media by method such as ELISA or look at the proteins on the cell surface by immunofluorescent microscope or by flow cytometer[208]. It is generally assumed that if the required proteins show up on the surface of transfected cells or being secreted into culture supernatant, it will imply that there is considerable protein expression as compared to just intracellular proteins being detected. More gene expression, more proteins will be produced and more proteins will be detected on the cell surface. The immunofluorescent technique is also simpler and quicker to perform, thus, it is the ideal screening test for gene expression *in vitro*. We were able to detect HIV-specific proteins on the surface of HIV-infected cell line (MT-4^{IIIB}) by immunofluorescent technique using pooled serum from HIV-infected individuals (results not shown). However, even the fluorescent signal was quite strong, there was considerable background fluorescent staining with normal (HIV-negative) sera. Such background staining could be overcome by serum dilution. At a cut-off point of 1 : 100 serum dilution, HIV-infected cell line (MT-4^{IIIB}) could be clearly stained by HIV-positive serum without any interfering background from the HIV-negative serum.

When using the three DNA constructs to immunize Balb/c mice, both *in vivo* and *in vitro* immunogenicity could be demonstrated to some extent by all constructs including the 297-bp non-humanized V3 DNA whose gene expression could not be

shown in *in vitro* transfection experiment. Overall, immunogenicity of these three DNA constructs could be better demonstrated *in vivo* than *in vitro*.

The *in vivo* immunogenicity study using footpad swelling as a measure of delayed type hypersensitivity (DTH) response was most rewarding in this study. The same technique was used to nicely demonstrate the DTH response in mice immunized with PPD[239]. It has the same principle as using increased ear thickness upon antigen challenge on the ear as a measure of DTH in allergic contact dermatitis[240]. This *in vivo* DTH response is better correlated with IFN-γ-secreting CD4+ cells in ICCS assay but unfortunately we only studied IFN-γ-secreting CD8+ cells which was thought to represent CTL[241] and we found only very little response.

In our *in vivo* DTH study, 2 different skin test antigens were used. V3(E) peptide : GVHMGPGRVFYRTGE was used to skin test mice immunized with 297-bp non-humanized V3 DNA and mice immunized with 2.5 kb full-length envelope DNA since both of these 2 DNA constructs were derived from HIV-1 subtype A/E. Negative control mice (mice immunized with pCI empty plasmid) were also skin tested with V3(E) peptide. V3(hu) peptide : SITIGPGQVFYRTGD was used to skin test mice immunized with 297-bp humanized V3 DNA which was derived from consensus multi-clade V3. These two 15-amino acid residue peptides were derived from the sequence at the tip of V3 loop, position 315 to 329 of the corresponding V3 peptides intended as immunogens. The similar V3 peptide derived from subtype B HIV-1 was found to be a good DTH skin test antigen in experimental HIV-1 subtype B DNA vaccine in mice (Okuda et al, unpublished results).

It should be noted that 9 out of the 15-amino acid residues of the 2 peptides are identical. As a result, V3(hu) was also used to skin test mice immunized with the full-

length envelope DNA to see whether there was any cross-reactivity between these 2 skin test antigens.

Documenting significant DTH footpad swelling response can be obtained by 2 different approaches. One is to compare the mean footpad thickness 24 hours after antigen challenge with the mean footpad thickness before antigen challenge, i.e., pre- and post-challenge comparision. The other approach is to compare the mean 24-hour footpad thickness of the immunized mice with the negative control mice similarly challenged, i.e., immunized and control comparison. Due to the small number of mice in each group, inter-mouse variation and the over sensitivity of the dial thickness gauge (measuring in unit of mm), statistical significance was difficult to achieve in certain groups or in certain comparisons. To avoid the problem of small sample size, baseline footpad thickness of all 38 mice (76 feet) used in this experiment was pooled. This mean baseline footpad thickness with smaller standard deviation was used as overall comparator for the post-challenge footpad thickness of all immunization groups, i.e., pre- and post-challenge comparison. Such approach turns out to be quite satisfactory and simpler to interprete the experimental results.

As shown in Table 17, control mice immunized with pCI empty plasmid did not give any significant footpad swelling 24 hours after challenged with V3(E) peptide. Mice immunized with all 3 DNA constructs, with and without rgp120 boosting, showed significant footpad swelling 24 hours after antigen challenge except the non-boosted full-length envelope DNA group. The 297-bp humanized and non-humanized V3 DNAs were equally immunogenic in inducing DTH footpad swelling response. V3(E) and V3(hu) were equally effective in eliciting DTH in mice primed/boosted with full-length envelope DNA and rgp120 respectively. This finding suggested that there was cross-reactivity between V3(E) and V3(hu). It would be of interest to test V3(B) as the skin

test antigen. If V3(B) cannot elicit DTH in full-length envelope A/E DNA-immunized and rgp120(E) boosted mice while V3(hu) can, it will imply that the multi-clade V3, i.e., V3(hu) is cross-reactive with V3(E). If V3(B) can also elicit DTH in A/E-immunized mice, cross-reactivity between multi-clade V3 and V3(E) cannot be assumed since it may just imply that the 15-amino acid V3 peptide, no matter derived from B, A/E or multi-clade, is small enough to be used as “universal” DTH skin antigen.

Except for the full-length envelope DNA immunized group, the effect of rgp120(E) boosting in other groups was not evident. As compared to the non-boosted group, rgp120(E) boosting in the 297-bp humanized and the 297-bp non-humanized V3 DNA groups resulted in only slight but non-significant increase in footpad thickness (*p* value 0.123 and 0.082 respectively, Table 17). The lack of boosting effect in these two DNA primed groups may be due to the small sample size or due to the non-crossreactivity between the 297-bp humanized V3 DNA used in priming and the rgp120(E) used for boosting. The latter explanation cannot be applied for the 297-bp non-humanized V3(E) DNA prime/rgp120(E) boost group.

Why rgp120 boosting is essential for the immunogenicity of full-length envelope DNA but not for the 297-bp humanized and non-humanized V3 DNA is at present unknown. It may be that the full-length envelope DNA is less immunogenic than the 297-bp V3 DNA or may contain some immunosuppressive epitopes, thus, requires boosting. This interpretation is consistent with the report of Denner et al. that envelope glycoprotein contains immunosuppressive epitopes[242]. Therefore, removing that immunosuppressive epitopes or using the shortened envelope peptide such as V3 peptides used in this study may enhance the immunogenicity of the envelope vaccine[158,159].

Up to this point, we have shown the correlation between the *in vitro* DNA expression (protein expression by the transfected cell) and the *in vivo* DNA expression (immunogenicity in inducing DTH response) of the 297-bp humanized V3 DNA and the 2.5 kb full-length envelope DNA constructs. However, discordance was seen with the 297-bp non-humanized V3 DNA construct. Although *in vitro* expression could not be shown, the 297-bp non-humanized V3 DNA was immunogenic in mice in inducing DTH response. The discordance may be due to the difference in antigen presentation. For *in vivo* expression, the immunized DNA might get better processed and presented on the antigen presenting cells which later was able to stimulate the immune response. While the *in vitro* expression was just the result of transcription and translation in certain selected protein expression systems after DNA transfection to get the whole protein. The other explanation is that one might want to use other *in vitro* expression systems which are more sensitive to allow the expression of the weaker genes.

In this study, the *in vitro* immunogenicity of the DNA constructs was looked at by several assays. Serum was collected 2 weeks after last DNA injection or 2 weeks after rgp120 boosting and assayed for anti-HIV envelope antibodies by ELISA. Spleen cells were collected at the same time and assayed for antigen-stimulated lymphoproliferative response and for antigen-induced IFN-γ secretion by intracellular cytokine staining (ICCS) and by enzyme-linked immunospot (ELISPOT) assay. The overall humoral and cell-mediated immune responses of all DNA constructs were either weak or absent.

To measure humoral immune response to the 3 DNA constructs, the same V3(E) and V3(hu) peptides as used for DTH skin test were used to coat 96-well microtiter plate and sera from the immunized mice as well as selected pre-immunized sera were tested for antibodies to V3(E) or to V3(hu) by ELISA technique. No antibodies could be

detected in any mice from any immunization groups either boosted or non-boosted. Similarly, no antibodies could be detected when sera were tested by gel particle agglutination assay or by Western blot. The lack of antibody response to DNA immunization is not unexpected since it was known that DNA vaccine could hardly induce any antibody response. Boosting with recombinant proteins was found to be able to induce antibody response in mice immunized with DNA vaccine, the prime-boost approach[243]. Boosting with rgp120 in this study was not immunogenic enough to stimulate antibody response. It will be of interest to study the antibody response if live recombinant vector (such as recombinant gp120 vaccinia) is used as booster in our system.

For the lymphoproliferative assay, the laboratory technique with 3-day mitogen stimulation was properly set up. Con A gave a better stimulation than PHA (Table 18). With 7-day culture, cell survival was a problem as evidenced by modest or very low Con A-induced stimulation index (S.I.) (Table 19-22). Antigen-stimulated lymphoproliferative response was also performed in a 7-day culture system, using V3(E), V3(hu) and rgp120(E) as stimulating antigens *in vitro*. As a group, no significant antigen-induced lymphoproliferation was seen in any of the immunization groups, boosted or unboosted. Only certain mice in some culture set ups might have an S.I. of over 2.0 but this was not restricted to any particular immunization groups (Table 22). On the other hand, mitogen contamination was seen in one culture set-up where high S.I. was seen in all groups of mice including the control group as well as high endogenous turnover, i.e., high background (unstimulated) count (Table 20).

Many groups of investigators were able to show antigen-stimulated lymphoproliferative response in mice immunized with HIV-1 DNA vaccines[244-245]. It is

usually accepted as a correlate of cell-mediated immune response to the vaccine. For a positive lymphoproliferation assay, a S.I. of more than 3 is usually seen[246]

We do not believe that failure to demonstrate antigen-induced lymphoproliferation in this study is because our DNA constructs are entirely non-immunogenic. We believe that the 7-day cell culture condition for antigen-induced lymphoproliferation was not properly set up in our laboratory. Unfortunately, we did not include a positive control in our culture system. Mice immunized with a protein antigen or microbial antigen which is known to be able to induce strong *in vitro* proliferative response such as ovalbumin or PPD (purified protein derivative) should be used as a positive control in the culture system. In addition, one may try 5-day culture or modify the culture condition such as replacing half of the culture media with fresh media after first 3 days. In our laboratory, 3-day culture with Con A stimulation gave a good stimulation, S.I. of 59.4-98.1 (Table 18). It is also possible that we were not using the right antigens for *in vitro* stimulation. The full (99 amino acid) V3 peptides may be better than the 15 amino acid residue peptides [V3(E), V3(hu)] used in the experiment. Nevertheless, full-length rgp120(E) was not able to consistently induce cell proliferation in mice immunized / boosted with full-length envelope DNA in our study. In addition, the culture system for antigen-stimulation is highly susceptible to technical errors such as mitogen contamination or cell death due to inappropriate temperature and CO_2 controls.

With intracellular cytokine (IFN-γ) staining (ICCS) assay, only mice primed/boosted with full-length envelope DNA/rgp120 respectively consistently showed positive ICCS response (9 out of 9 mice, Table 24). Only 40-60 % of 297-bp humanized and non-humanized V3 DNA immunized mice had positive ICCS response (Table 23). The response was not enhanced by rgp120 boosting in these groups of mice,

however. Nevertheless, it should be pointed out that the level of ICCS response in our study was relatively low. With the universally accepted criteria that any ICCS over mean + 2SD of the negative control (in our case, the pCI-immunized group) is considered positive, our positive results were never more than twice of the cut-off point.

It is evident from other studies mentioned above that the ICCS response in our study was a weak response. Reasons may be that all DNA constructs tested are not very immunogenic or the techniques used need further optimization. For example, a larger collection of overlapping envelope peptides of various sizes have to be tested for the maximal stimulation of intracellular IFN-γ production. However, we have used the same technique to measure the ICCS response in mice immunized with a single dose of recombinant vaccinia containing gp160(B) and observed a ICCS of 2.11 % with a cut-off value of 0.03 % when cells were stimulated with V3(B) peptide (Figure 26).

In addition, the same technique could detect vaccinia-specific ICCS response which gave a response as high as 0.14 (Table 28). Such an ICCS response will be considered adequate by most investigators. Therefore, data suggested more to us that the weak ICCS response in our experiments was due to the weak immunogenicity of our DNA constructs rather than the technique. Nevertheless, among the 3 DNA constructs tested, full-length envelope DNA was most immunogenic in ICCS response when the construct was given with rgp120 boosting.

As discussed earlier, our ICCS response was measured on antigen-stimulated $CD8^+$ T cells (see Materials and Methods). It might be of interest to measure $CD4^+$ T cell ICCS response which might give a stronger response than the $CD8^+$ ICCS response. The $CD4^+$ ICCS response will correlate better with the DTH skin test response seen in our experiments. In such a case, ConA may serve as a good positive control for $CD4^+$ ICCS response.

Lastly, with ELISPOT response, the response could be seen in mice immunized with all 3 DNA constructs (Table 25, 26). The response was significantly higher than the negative control mice, i.e., mice injected with pCI. However, the response was far lower than that obtained from mice immunized with single-dose recombinant vaccinia containing gp160(B) gene and stimulated *in vitro* with HIV-1IIIB V3 peptide (positive control, Table 25). However, due to some unexplained technical reasons, the ELISPOT response after rgp120 boosting failed either with 297-bp humanized and non-humanized V3 DNA priming or with full-length envelope DNA priming (Table 25, 26). The negative result in the boosting experiment was most likely due to the cell death since no ELISPOT response was seen even in the positive control group.

To calculate spot forming cells (SFC) in the ELISPOT response, one looks at excess of the SFC with antigen stimulation over that without antigen stimulation, i.e., stimulated minus unstimulated SFC per 10^6 spleen cells (see Materials and Methods). Theoretically, the antigen-stimulated culture should have more SFC. However, in certain mice in certain groups, the unstimulated culture had higher SFC than the stimulated culture. The minus value of ELISPOT was considered in this study as equivalent to zero which was equal to no ELISPOT response. This is of particular importance in statistical calculation of the mean ELISPOT value of the entire group.

The reason for the minus value in ELISPOT calculation is not clearly understood. When one looked at the ELISPOT plate (IP plate) in our experiments, one might see a confluent purple well which might be just the background without any SFC but it might be counted as many spots by the reader. One might also question about the accuracy of such computer device in counting a dense well with large number of spots. Therefore, if the difference in stimulated and unstimulated spots is small, it is possible to see a minus value of ELISPOT. We predict that this might be the case in our

experiments with rgp120 boosting (Table 25, 26). If the above explanation is true, one has to reduce the number of cells to be put into each well. And even this explanation is true, it is difficult to explain why the unstimulated spot in one experiment was low, thus SFC could be read while in another experiment it was high, resulting in negative value. It should be pointed out that the computer-assisted video image analysis device was newly acquired in our laboratory, its appropriate and optimal operation needs to be further refined.

Our ELISPOT set-up did not differentiate between CD4 and CD8 SFC. The readings were therefore a combined result of CD4 and CD8 SFC. It might be better to just look at CD8 SFC which is a better correlate of protective cytotoxic T lymphocyte (CTL) response[241]. This should help reduce the number of spots so that visual or computer-assisted count will become more reliable.

The last part of our approach was to investigate whether our 297-bp humanized multi-clade V3 DNA could cross-react with other HIV-1 subtypes. The approach was to prime mice with a single injection of the 297-bp humanized V3 DNA. Twenty-one days later, mice were boosted with recombinant vaccinia containing the whole envelope genome of subtype A/E (rVVgp160(E)). Fourteen days after boosting, splenocytes were assayed for intracellular production (ICCS) and extracellular release (ELISPOT) of IFN-γ upon brief *in vitro* stimulation with wtVV as well as rVVgp160 of subtypes A, B, C and A/E. Several appropriate control groups were included. We did not use rgp120(E) or V3(E), V3(hu) as the *in vitro* stimulating antigens as we did previously in this last experiment since we aimed to get maximal stimulation *in vitro*. As a result of using recombinant vaccinia as booster and as *in vitro* stimulating antigen, vaccinia-specific response was anticipated. We also anticipated to see enhanced response over the background vaccinia-specific response when appropriate HIV-1 antigens were

added in culture, i.e., rVVgp160 (A, B, C and A/E). Table 29 summarized the various prime/boost groups included in this experiment and the expected outcomes. The group that was of interest when the experiment was being set up was group B, primed with 297-bp humanized V3 DNA and boosted with rVVgp160(E). The answer that we were interested to know was that whether priming with 297-bp humanized (multi-clade) V3 DNA would yield stronger E-specific responses as compared to group A which received no priming (prediction B-1 in Table 29) and whether such E-specific response would also apply to other subtypes used in *in vitro* stimulation as well (prediction B-2 in Table 29). If the first prediction (B-1) is correct, it implies that the humanized multi-clade V3 could cross-react with subtype A/E envelope, namely, able to prime for rVVgp160(E) boost. If the second prediction (B-2) is correct, it implies that the humanized multi-clade V3 could cross-react with many subtypes besides subtype A/E.

The results of this experiment were quite complicated. It is best summarized in Table 30 so that the actual outcome can be compared with the expected outcome in Table 29. As shown in Table 30, single dose of 297-bp humanized V3 DNA (group D) could not result in any ELISPOT or ICCS response no matter which antigen was used to stimulate spleen cells *in vitro*. However, when the same group of mice was boosted with wtVV 2 weeks before ELISPOT and ICCS assays, strong vaccinia-specific ELISPOT and ICCS responses were seen (group C). No HIV-specific response was seen in this group. 297-bp humanized V3 DNA priming and followed by rVVgp160(E) boosting (group B) did not result in any higher HIV-specific response as compared to mice without priming but received only one rVVgp160(E) injection 2 weeks before assays (group A). Within group B, there was no difference in ELISPOT and ICCS responses among the various subtypes of rVVgp160 used *in vitro*. Taken together, our results indicated that the 297-bp humanized multi-clade V3 DNA did not cross-react with the

gp160 of any subtypes (i.e., A, B, C and A/E). Therefore, the prediction B-3 in Table 29 was correct. These results weaken our hope to use the 297-bp humanized multi-clade V3 DNA as universal priming worldwide for a prime-boost HIV immunization strategy.

Table 29 Expected outcome of 297-bp humanized DNA prime/rgp120(E) boost experiment to look for cross reactivity

	Immunization		ELISPOT					ICCS		
				rVVgp160					rVVgp160	
GROUP	Prime	Boost	wtVV	A	B	C	A/E	wtVV	B	A/E
A	pCI	rVVgp160(E)	+	+	+	+	++	+	+	+
	297-bp hu DNA	rVVgp160(E)	+	?	?	?	?	+	?	?
B	B-1 if x-react to E only			+	+	+	++++		+	++++
	B-2 if x-react to all			+++	+++	+++	++++		+++	++++
	B-3 if not x-react			+	+	+	++		+	++
C	297-bp hu DNA	wtVV	+	+	+	+	+		+	+
D	297-bp hu DNA	none	-	-	-	-	-	-	-	-

Table 30 Actual outcome of 297-bp humanized DNA prime/rVVgp160(E) boost experiment to look for cross-reactivity

	Immunization		Mean ELISPOT					Mean ICCS (CD8+)		
		Boost		rgp160					rgp160	
GROUP	Prime		wtVV	A	B	C	A/E	wtVV	B	A/E
A	pCI	rVVgp160(E)	75.5	64	82.6	65	44.9	0.14	0.19	0.16
B	297-bp hu DNA	rVVgp160(E)	91.3	55.3	94.3	60.8	72.5	0.38	0.25	0.28
C	297-bp hu DNA	wtVV	139.8	89.4	147.9	113.6	117.8	0.66	0.57	0.57
D	297-bp hu DNA	none	7.3	5	8.4	5	5	0.04	0.04	0.04

In this cross-reactivity study, it is evident that the ICCS response was limited to CD8+ T cell population (Table 28). However, the response was vaccinia-specific since the vaccinia virus was well known as strong CTL inducer. We are disappointed in not observing any envelope protein-stimulated ICCS or ELISPOT responses (Table 27, 28, 30). Typically, mice immunized with rVVgp160 (group A and B) should give a strong gp160-specific ICCS and ELISPOT responses. The response should be elicited by rgp160 stimulation *in vitro*. The reason that we could not demonstrate any gp160-specific response may be because rVVgp160 was used as *in vitro* stimulating antigen. The resultant vaccinia-specific response was too strong to detect any added effect of gp160. Or other word, the high vaccinia-specific response may overshadow the added but low gp160-specific response. It is essential to add rgp160 or truncated peptides as the stimulating antigen in future such experiments with comparison to unstimulated culture. We hope that such approach will be able to elicit envelope-specific response and the question of cross-recativity of our humanized multi-clade V3 DNA can be answered more definitely.

To put the immunogenicity results together, although our 297-bp humanized V3 DNA and 2.5 kb full-length envelope (E) DNA could be strongly expressed in *in vitro* transfection experiments, their *in vivo* immunogenicity was relatively weak. The most promising immunogenicity was shown by delayed type hypersensitivity response, the footpad swelling response. Even the *in vitro*-non-expressed DNA construct, the 297-bp non-humanized V3(E) DNA, could also induce significant DTH response indicating that the construct itself can indeed be expressed. The non-expression *in vitro* may be a technical problem that we may need a more sensitive expression system for a weaker DNA construct. The *in vitro* correlates of immunogenicity study could be shown, at least, by ELISPOT assay in mice immunized with any of the 3 DNA constructs. In

addition, weak but significant ICCS response could be observed in mice immunized with full-length envelope (E) DNA and boosted with rgp120(E).

Taken together, we believe that all of our 3 DNA constructs, the 297-bp humanized multi-clade V3 DNA, the 297-bp non-humanized V3(E) DNA and the 2.5 kb full-length gp160(E) DNA can be expressed both *in vitro* and *in vivo*. However, the immunogenicity of these constructs is relatively weak as compared to other HIV-1 vaccine candidates. Nevertheless, the good immunogenicity in mice does not always guarantee the same response in primate since there are evidences that DNA is good in mice but is not good in primate.

There are many reasons to explain the low immunogenicity of our DNA constructs as shown in this study. They can be divided into specific or technical issues and the general or conceptual issues. One of the important technical issues encountered during this study was the wide variation of some laboratory results. This can be improved by better standardization of the laboratory techniques and by increasing the sample size. For example, the 15-amino acid peptides used in this study may be too short and/or contain too few appropriate epitopes for immunologic stimulation. Statistical calculation in this study was greatly hampered by the small sample size. When number of mice per group is increased, one has to simplify the studies or simply the tests to be done or has to subdivide the study into many experiments or otherwise more helping hands are needed.

For the general or conceptual issues, the low immunogenicity of our DNA constructs can be explained by the following reasons :

(1) the poor expression of the introduced DNA

(2) the inappropriate route of DNA administration

(3) the poor immunogenicity of the protein encoded by the DNA construct

(4) the inappropriate tertiary structure of the protein antigen

(5) the wrong antigen used for *in vivo* and *in vitro* measurements of immunogenicity

(6) adjuvant may be needed

(7) the inappropriate immunization protocol

The poor expression of the introduced DNA

As was shown in Results that we could not detect any protein expression of the 297-bp non-humanized DNA whereas the 297-bp humanized DNA was well expressed as a protein of approximately 13 kilodalton (kDa) in size. The expression of 2.5 kb full-length envelope DNA also yielded 3 bands at 160, 120 and approximately 38 kDa which corresponded to gp160, gp120 and gp41, respectively of HIV envelope protein. Even we considered that the protein expression was quite high by *in vitro* protein expression, but still, it might not be high enough to be highly immunogenic once it gets into the *in vivo* system.

The inappropriate route of DNA administration

Many routes of DNA vaccination have been reported. Sometimes the immunogenicity of the DNA vaccine can be increased by changing the route of administration. For example, it was reported that immunization by gene gun was able to induce more Th1 type response and required fewer vaccine[247].

The poor immunogenicity of the protein encoded by the DNA construct

The immunogenicity of the protein is the first thing to be considered. The immunodominant regions that cover all the important epitopes needed to stimulate host immune responses should be included. For our 297-bp DNA

construct, the V3 region of HIV-1 which is the principal neutralizing determinant, CTL epitopes, T helper (Th) epitopes, CXCR4 and CD4 binding sites and neutralizing epitopes are already included. It may be necessary to include more broader immunodominant epitopes.

The inappropriate tertiary structure of the protein antigen

It has been reported that the gp120 glycoprotein, which can be shed from the envelope complex, elicits both virus-neutralizing and non-neutralizing antibodies during natural infection. Neutralizing antibodies must access the functional envelope glycoprotein complex[248] and typically recognize conserved or variable epitopes near the receptor-binding regions[249-250]. The outcome of our protein structure encoded by the 297-bp humanized and non-humanized DNA has to be taken into account that whether they are presented the way as they are supposed to even neutralizing epitoipes have been included. X-ray crystallography study may be needed to study the tertiary structure of the proteins encoded by our DNA constructs.

The wrong antigen used for *in vivo* and *in vitro* measurements of immunogenicity

The antigens used for *in vitro* stimulation are quite critical. Peptide antigens that were used in our experiments were 15-residue peptide that were selected based on their reported immunogenicity in some published articles[251-252]. Using similar peptide, Ahlers et al[251] reported induction of strong neutralizing antibody responses in mice after two immunizations with their V3 loop peptide 18 construct. This also indicated that the peptide 18 which was a 15-residue peptide was immunogenic with this special construct. It has also been reported that the peptide 18 is a B cell epitope located within the hypervariable

V3 loop region of the HIV-1 IIIB envelope known as PND (principal neutralizing determinant) , and is the major immunodominant CTL epitope in mice[253], as well as being recognized by human CTL[254]. Anchour et al[252] also reported generation of CTLs in humans immunized against HIV-1 gp160. The CTL response was directed against P18 (residues 315-329 : RIQRGPGRAFVTIGK) which corresponded to our 15-residue peptides. Our peptide sequences are V3(E) : GVHMGPGRVFYRTGE and V3(hu) : SITIGPGQVFYRTGD. Even the immunogenicity of the peptides has been elicited, some may argue that it may not always be true. Therefore, it may be necessary to use the whole gp160 or gp120 proteins or even the gp160 recombinant vaccinia virus for the *in vitro* stimulation assays to ensure proper antigen presentation.

Adjuvant may be needed

Adjuvants are widely used to increase the immunogenicity of the DNA vaccine. Okuda et al have extensively reviewed the effect of various cytokines and costimulatory factors-encoding plasmids as adjuvants for DNA immunization as shown in Table 31[255]. It is appropriate that future HIV DNA vaccine studies should also include cytokines or others as adjuvants.

Table 31 Summary of effects of cytokines and expression plasmids on conventional and DNA immunization[255]

Immunomodulatory molecules	Effect
A. Cytokine proteins	
IL-1	Antibody (Ab) ↑
IL-2	Antibody (Ab) ↑
IL-12	Th1 (DTH) ↑
IFN-γ	Ab, DTH ↑
GM-CSF	Ab ↑
B. Expression plasmids	
IL-12	CTL (i.m. and i.n.) ↑ DTH (i.m. and i.n.) ↑ Ab (i.m. and i.n.) →
GM-CSF	Ab (i.m.) ↑ CTL (i.m.) ↑
TCA3	CTL (i.m.) → DTH (i.m.) ↑
B7-2	CTL (i.m.) ↑ DTH (i.m.) ↑
CD40(L)	Ab (i.m.) ↑ CTL (i.m.) ↑

i.m. = intramuscular immunization

i.n. = intranasal immunization

<u>The inappropriate immunization protocol</u>

Immunization protocol is another important issue to be considered. The DNA vaccine, even has been proved to be immunogenic, it needs multiple injections. More than that, most DNA vaccines need boosting, the so called prime-boost strategy[243]. Here, we used only 3 immunization doses of DNA vaccine without any immunologic adjuvants. More frequent DNA immunization may be needed (Kent et al, personnal communication). We also used

recombinant gp120 as our boosting. It is important to test live recombinant vector such as recombinant vaccinia virus as booster. The schedule of immunization is also important. In our study, we immunized and boosted the mice every 2 weeks and sacrified the mice 2 weeks after last immunization. Both an accelerated and a slower immunization schedules have to be tested.

With all of the aforementioned conceptual discussion, let's see how we can further improve the immunogenicity of our future HIV-1 DNA vaccine candidates.

(a) Promoter selection and promoter stimulation

Selecting a good promoter is an important step in the construction of a DNA vaccine. Promoter will initiate the transcription of mRNA. Cytomegalovirus (CMV) immediate/early promoter is an example of a good promoter that is widely used[244]. In addition, Arai et al reported that simultaneous administration of 8Br-cAMP with the DNA vaccine could enhance the humoral and cell-mediated responses to an HIV-1 DNA vaccine in mice[256]. The increased immune response with cAMP co-administration could be explained by the fact that cAMP response element (CRE) consensus sequence, TGACGTCA, is similar to one of the maximally immunostimulatory sequence (ISS), TGACGTT. Therefore, it may elicit the CpG effect[256].

(b) Rev-dependent expression

HIV-1 genome is unique in terms that there is only 1 promoter site and only 1 mRNA sequence produced. The mRNA produced is unspliced or incompletely spliced which is responsible for the production of the structural proteins of HIV-1, namely gag, pol, and env. These intron-containing RNAs will

not be able to travel from nucleus to cytoplasm to have translation and protein production. The virus has to use Rev, a viral protein which binds to a stem-loop secondary structure on the unspliced or incompletely spliced RNA, called Rev-responsive element (RRE) in order to transport that RNA out to the cytoplasm[257]. Therefore, *Rev* gene is an essential part of the HIV-1 DNA vaccine for the DNA to be well expressed. This is particularly true for a complete structural protein such as full-length env protein. Whether Rev is needed for a smaller structural protein such as the 99-amino acid segment of the gp120 encoded by our 297-bp non-humanized env DNAs is uncertain. Our findings that the non-humanized 297-bp env DNA could not be expressed in either COS-7 cells or HEK293 cells may imply that it is Rev-dependent. The reason that the 297-bp humanized DNA could be expressed is due to the disruption of the CRS/INS inhibitory sequences on the structural genes as a result of genetic synthesis according to human codon usage.

We are surprised to see that our full-length envelope gene could be expressed even without the *Rev* gene insert (result not shown). However, we found that when the *Rev* gene was constructed into the same plasmid DNA as the full-length env gene, the expression of envelope proteins was much stronger as shown in Figure 21 and Okuda et al[244]. The 2.5 kb full-length envelope DNA used throughout our study did have *rev* in the construct (see Materials and Methods and Figure 8).

(c) Humanized codon usage

It was well established that if the HIV-1 codon usage was changed to humanized codon usage, gene translation and protein synthesis are much more efficient[218, 221-222]. Our results with the 297-bp humanized *env* DNA also

confirms this. The mechanism of how humanized DNA is better expressed has been discussed earlier, i.e., possible disruption of the CRS/INS inhibitory sequences.

(d) Cytokine adjuvants

Many cytokines are known to modulate the immune response. Therefore, cytokines have been extensively studied in the field of vaccinology. Cytokines can either be given together with the DNA vaccine or a particular cytokine gene can be inserted into the DNA vaccine construct. Many cytokine genes can be inserted simultaneously. Cytokine gene has the advantage over soluble cytokines in terms of stability and cost. The most commonly used cytokines or cytokine genes in the field of DNA vaccine are interferon-gamma, IL-2, IL-12 and IL-18[258-261]. These are the cytokines that will enhance the Th1 or cell-mediated immune responses. Another set of cytokines; IL-4, IL-13 enhances antibody production through the stimulation of Th2 cells[258,261]. Granulocyte-macrophage colony stimulating factor (GM-CSF) is another group of cytokine which stimulates the differentiation of hematopoietic progenitor cells, particularly macrophages and dendritic cells which are antigen presenting cells. This in turn results in enhanced immune responses to DNA vaccines[262-263]. In addition, beta-chemokines such as RANTES and MIP-1α as well as adhesion molecules have also been shown to enhance immune responses to HIV-1 DNA vaccines[264-266].

(e) Conventional adjuvants

DNA vaccine may be injected alone or may be mixed adjuvants. The conventional adjuvants that have been co-administered with DNA vaccines include alum, monophosphoryl lipid A (MPL), cholera toxin, liposome, QS-21

and polyvinyl pyrollidone[267]. Some of the conventional adjuvants are widely available and have been used in human for long time such as alum.

(f) Methods to enhance cellular uptake

Similar to *in vitro* transfection, there are methods to enhance cellular entry after *in vivo* administration of DNA. Examples are electroporation and the incorporation of DNA into cationic liposome. These methods have been shown to enhance the immunogenicity of the DNA vaccines[268-269].

(g) CpG motif

Bacterial DNA sequence containing unmethylated CpG has immunostimulatory activity[270]. It directly stimulates NK cell, monocyte, macrophage and B cell resulting in production of various Th1 cytokines. It has been shown that injecting CpG motif together with DNA vaccine or incorporating CpG motif in the same plasmid DNA vaccine can enhance cell-mediated immune response to the DNA vaccine[271].

(h) DNA vaccine delivery system

To further improve the immunogenicity of DNA vaccine given by intramuscular injection, other vaccine delivery systems have been studied. Mucosal immunization such as intranasal immunization was found effective in generating both mucosal and systemic immunity[263]. In addition, liposome and many adjuvants such as cholera toxin, carboxymethylcellulose, QS-21 saponin could further potentiate the immunogenicity of DNA vaccines administered intranasally[272-273]. Intradermal and gene gun injections also offer the potent methods in delivering DNA vaccines. Gene gun technology can also save considerable amount of the immunizing DNA[274].

(i) Prime-boost vaccination approach

DNA vaccine alone may not be very potent in stimulating antibody production[275]. It was found that boosting with recombinant protein antigens or with live recombinant vaccine vectors could result in high antibody production[276]. Therefore, DNA priming / protein or live recombinant vector boosting may offer the ultimate goal in future vaccination. Nevertheless, there are preliminary evidences that repeated DNA immunization (more than 3 injections) may also offer a potent approach in stimulating both humoral and cell-mediated immunities (Kent et al, unpublished results, Kasinrerk et al, personal communication).

In conclusion, we were able to produce three DNA constructs encoding the envelope region of HIV-1 with the aim to have DNA candidate vaccine for human trial. One construct was the 2.5 kb full-length gp160 DNA amplified from HIV-1 subtype A/E-infected Thai patient with NSI phenotype. A 297-bp DNA segment surrounding the V3 region with many of the important epitopes was also amplified from the same virus using PCR technique. The same 297-bp V3 region was also constructed by computer-assisted approximation of the sequence of the last common ancestor (LCA) using 10 isolates from each of the 8 HIV-1 subtypes circulating worldwide (subtype A, B, C, D, A/E, F, G, H). The approximated consensus LCA V3 sequence was translated into amino acids which were then back translated into nucleotide sequence using human codon usage. This so-called “297-bp humanized V3 DNA” was constructed by PCR technique using multiple overlapping small primers without any added DNA template. This 297-bp humanized DNA was postulated that it might cross-react with other HIV-1 subtypes since it was constructed in such a way from many subtypes, i.e., multi-clade V3 DNA.

The 297-bp humanized multi-clade V3 DNA and the 2.5 kb full-length gp120(E) DNA were well expressed *in vitro* by transfection experiment but the 297-bp non-humanized V3(E) DNA could not be expressed. However, all 3 constructs were shown to be immunogenic when used to immunize mice. The immunogenicity was best seen by *in vivo* DTH skin testing. Boosting with rgp120(E) was necessary only for the 2.5 kb full-length gp160(E) DNA-immunized group. The *in vitro* correlates of the immunogenicity study was best demonstrated by ELISPOT assay. ICCS response could be demonstrated in 2.5 kb full-length gp160(E) DNA primed/rgp120(E) boosted group. Both the ELISPOT and ICCS responses were rather weak but positive response. No antibodies or antigen-stimulated lymphoproliferative response could be shown in the immunized mice. The attempt to demonstrate the cross immunogenicity of the 297-bp humanized multi-clade V3 DNA yielded negative results. The reasons for therelatively low immunogenicity of our DNA constructs were discussed as well as the means to further improve their immunogenicity.

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APPENDICES

Appendix A

Clinical criteria of AIDS definition

1993 Revised Classification System for HIV Infection and Expanded Surveillance Case Definition for AIDS Among Adolescents and Adults

Clinical Categories

The clinical categories of HIV infection are defined as follows :

Category A

Category A consists of one or more of the conditions listed below in an adolescent or adult (greater than or equal to 13 years) with documented HIV infection. Conditions listed in Categories B and C must not have occurred.

- Asymptomatic HIV infection
- Persistent generalized lymphadenopathy
- Acute (primary) HIV infection with accompanying illness or history of acute HIV infection

Category B

Category B consists of symptomatic conditions in an HIV-infected adolescent or adult that are not included among conditions listed in clinical Category C and that meet at least one of the following criteria : a) the conditions are attributed to HIV infection or are indicative of a defect in cell-mediated immunity; or b) the conditions are considered by physicians to have a clinical course or to require management that is complicated by HIV infection. Examples of conditions in clinical Category B include, but are not limited to :

- Bacillary angiomatosis
- Candidiasis, oropharyngeal (thrush)

- Candidiasis, vulvovaginal; persistent, frequent, or poorly responsive to therapy
- Cervical dysplasia (moderate or severe) / cervical carcinoma in situ
- Constitutional symptoms, such as fever (38.5^{o} C) or diarrhea lasting greater than 1 month
- Hairy leukoplakia, oral
- Herpes zoster (shingles), involving at least two distinct episodes or more than one dermatome
- Idiopathic thrombocytopenia purpura
- Listeriosis
- Pelvic inflammatory disease, particularly if complicated by tubo-ovarian abscess
- Peripheral neuropathy

Category C

Category C includes the clinical conditions listed in the AIDS surveillance case definition (Appendix B). For classification purposes, once a Category C condition has occurred, the person will remain in Category C.

Appendix B : Conditions included in the 1993 AIDS Surveillance case definition

- Candidiasis of bronchi, trachea, or lungs
- Candidiasis, esophageal
- Cervical cancer, invasive *
- Coccidioidomycosis, disseminated or extrapulmonary
- Cryptococcosis, extrapulmonary
- Cryptosporidiosis, chronic intestinal (greater than 1 month's duration)
- Cytomegalovirus disease (other than liver, spleen, or nodes)

- Cytomegalovirus retinitis (with loss of vision)
- Encephalopathy, HIV-related
- Herpes simplex : chronic ulcer(s) (greater than 1 month’s duration); or bronchitis, pneumonitis, or esophagitis
- Histoplasmosis, disseminated or extrapulmonary
- Isosporiasis, chronic intestinal (greater than 1 month’s duration)
- Kaposi’s sarcoma
- Lymphoma, Burkitt’s (or equivalent term)
- Lymphoma, immunoblastic (or equivalent term)
- Lymphoma, primary, or brain
- Mycobacterium avium complex or M. kansasii, disseminated or extrapulmonary
- Mycobacterium tuberculosis, any site (pulmonary * or extrapulmonary)
- Mycobacterium, other species or unidentified species, disseminated or extrapulmonary
- Pneumocystis carinii pneumonia
- Pneumonia, recurrent *
- Progressive multifocal leukoencephalopathy
- Salmonella septicemia, recurrent
- Toxoplasmosis of brain
- Wasting syndrome due to HIV

APPENDIX B

Submitted HIV-1 sequences of isolate number 05-99 and 10-00

LOCUS AY366933 303 bp DNA linear SYN 27-AUG-2003
DEFINITION Synthetic construct HIV-1 isolate 05-99 envelope glycoprotein gene, complete cds.
ACCESSION AY366933
VERSION AY366933.1 GI:34106197
KEYWORDS .
SOURCE synthetic construct
ORGANISM synthetic construct
artificial sequences.
REFERENCE 1 (bases 1 to 303)
AUTHORS Sirivichayakul,S., Phanuphak,P., Tirawatnapong,T., Ruxrungtham,K., Oelrichs,R. and Lorenzen,S.
TITLE Immunogenicity of 297-bp humanized DNA
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 303)
AUTHORS Sirivichayakul,S., Phanuphak,P., Tirawatnapong,T., Ruxrungtham,K., Oelrichs,R. and Lorenzen,S.
TITLE Direct Submission
JOURNAL Submitted (27-JUL-2003) Department of Medicine, Faculty of Medicine, Chulalongkorn University, Rama IV Road, Bangkok 10330, Thailand
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61 ggaccaggac aagcattatt atataaaaca ggagacataa taggagatat aagaagagca
121 tattgtgaga ttaatggaac aaaatggaat aaagtgttaa gacaggtagc tgaaaaatta
181 aaagagcact tcagtaagaa tatatccttt caaccaccct caggaggaga tctagaaatt
241 acaacgcatc attttaattg tagaggggaa tttttctatt gcaatacaac aaaactgttt
301 tag
//

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DEFINITION Synthetic construct HIV-1 isolate 10-00 envelope glycoprotein gene, complete cds.
ACCESSION AY366934
VERSION AY366934.1 GI:34106199
KEYWORDS .
SOURCE synthetic construct
ORGANISM synthetic construct
artificial sequences.
REFERENCE 1 (bases 1 to 306)
AUTHORS Sirivichayakul,S., Phanuphak,P., Tirawatnapong,T., Ruxrungtham,K., Oelrichs,R. and Lorenzen,S.
TITLE Immunogenicity of 297-bp humanized DNA
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 306)
AUTHORS Sirivichayakul,S., Phanuphak,P., Tirawatnapong,T., Ruxrungtham,K., Oelrichs,R. and Lorenzen,S.
TITLE Direct Submission
JOURNAL Submitted (27-JUL-2003) Department of Medicine, Faculty of Medicine, Chulalongkorn University, Rama IV Road, Bangkok 10330, Thailand
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121 tgtgagatta atggaacaca atggaataaa actttaacac aagtagctga aaaattaaaa
181 gagcacttta ataagacaat aatctttcaa ccacaaccac cctcaggagg agatctagaa
241 attacaatgc atcattttaa ttgtagaggg gaatttttct attgcaatac aacaaaactg
301 ttttag
//

APPENDIX C

Nucleotide sequences of 297-bp humanized and non-humanized DNA

Nucleotide sequence of 297-bp humanized DNA

Atgagcgtggagatcaactgcaccaggcccagcaacaacaccaggaccagcatcaccatcggccccggccaggtgttcta
caggaccggcgacatcatcggcgacatcaggaaggcctactgcgagatcaacggcaccaagtggaacgaggccctgaag
caggtgaccgagaagctgaaggagcacttcaagaacaagaccatcatcttccagccccccagcggcggcgacctggagat
caccatgcaccacttcaactgcaggggcgagttcttctactgcaacaccaccaagctgttctag

Nucleotide sequence of 297-bp non-humanized DNA

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acaggagaaataataggaaatataagaatagcatattgtgagattaatggaacacaatggaataaaactttaacacaagtagct
gaaaaattaaaagagcactttaataagacaataatctttcaaccacaaccaccctcaggaggagatctagaaattacaatgcatc
attttaattgtagaggggaatttttctattgcaatacaacaaaactgttttag

APPENDIX D

Antigenic epitopes within the 99-amino acid peptide

```
Humanized       SVEINCTRPSNNTRTSITIGPGQVFYRTGDIIGDIRKAYCEINGTKWNEALKQVTEKLKE
10-00           SVEINCTRPSNNTRTGVHMGPGRVFYRTGEIIGNIRIAYCEINGTQWNKTLTQVAEKLKE
                ***************.: :***:******:***:** ********:**::*.**:*****

Humanized       HFKNKTIIFQP--PSGGDLEITMHHFNCRGEFFYCNTTKLF
10-00           HF-NKTIIFQPQPPSGGDLEITMHHFNCRGEFFYCNTTKLF
                ** ********  ***************************
```

Antigenic epitopes within the 99-amino acid peptide that have been reported (corresponding to the underlined region)

: Th-epitope (in murine) = NTRKRIRIQRGPGR

: Balb/c CTL epitope = NTRKRIRIQRGPGRAFVTIGK

= RGPGRAFVTI

: B cell epitope of HIV_{IIIB} = SVEINCTRPSNNTRT

: linear neutralizing epitopes (Sutthent R)

= RTSITIGPGQVFYRTGDIIG

= CRGEFFYCNTTKLFNNTCIGN

BIOGRAPHY

Miss Sunee Sirivichayakul was born on June 3, 1962 in Bangkok, Thailand. She graduated with the Bachelor degree of Science in Medical Technology from the Faculty of Medicine, Chulalongkorn University in 1985. She got her Master degree of Science in Medical Microbiology from the Graduate School of the same University in 1991. Her academic position is the senior scientist at Division of Allergy and Clinical Immunology, Department of Medicine, Faculty of Medicine, Chulalongkorn University. During Sep 15, 1994-Oct 30, 1996, she worked as a research fellow of Fogarty Program for the HIV immunologic and virologic studies at Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, U.S.A. During Sep 5-8, 2001, she got Graduate Student and Fellow Travel Grant Award to attend and present part of her thesis work at the AIDS Vaccine 2001, Philadelphia, PA, U.S.A. During her occupational experience, she has had 16 publications. The most recent 2 papers since 2001 are as follow :

1. **Sirivichayakul S**, Chantratita W, Sutthent R, Ruxrungtham K, Phanuphak P, Oelrichs RB. Survey of reverse transcriptase from the heterosexual epidemic of human immunodeficiency virus type 1 CRF01_AE in Thailand from 1990-2000. AIDS Res Hum Retroviruses 2001; 17 : 1077-1081.
2. **Sirivichayakul S,** Ruxrungtham K, Ungsedhapand C, Techasathit W, Ubolyam S, Chuenyam T, Emery S, Cooper D, Lange J, Phanuphak P. Nucleoside analogue mutations and Q151M in HIV-1 subtype A/E infection treated with nucleoside reverse transcriptase inhibitors. AIDS 2003; 17 : 1889-1896.