CHAPTER IV

RESULTS

Part I Optimization of Serotyping

1. Patient Population

Sixty HIV-1 infected individuals were the subjects of the first part of this study. They were divided into 3 groups of 20 each, namely, the heterosexual group (13 males, 7 females), the IVDU group (19 male, 1 female), homo/bisexual (20 males). Clinical staging of these 60 patients is shown in Table IV, according to the 1987 CDC Classification.[139]

2. Setting up the optimal conditions for peptide serotyping by indirect ELISA test using V3 26 amino acid peptide

2.1 Determination of the optimal concentration of V3 peptide

A 26 amino acid V3-peptide concentrate was dissolved in coating buffer to the concentrations of 5 and 10 μg/ml. 200 μl of each concentration was used to coat the microtitre plate at 37° C for 1 hour. The remaining solution was then aspirated. Next, 200 μl of blocking buffer was added and the plate was incubated at 37 °C for 1 hour. After incubation, the plate was washed with PBS-tween 4 times and dried at 37 °C for 1 hour. 200 μl of the positive and negative control sera, diluted at 1:100 and 1: 200 in diluent buffer, was added and the plate was again incubated at 37 °C for 30 minutes. Each peptide concentration was assayed in duplicate. After 4 washes with PBS-tween, 200 μl of rabbit anti-human Ig G peroxidase conjugate, diluted at 1:1000 and 1:3000, was added followed by incubation at 37 °C for 30 minutes. The

plate was washed with PBS-tween 6 times. After 100 µl of substrate solution was added, the plate was incubated at room temperature for 10 minutes in the dark. The color development was stopped by the addition of 50 µl of 2 N H_2SO_4 . The absorbance was measured with a spectrophometer at 492 nm.

The results indicated that the optimal concentration of PND-A was 10 µg/ml and PND-B was 5 µg/ml. The optimal serum and the dilution of conjugate was 1: 200 and 1 : 1000 respectively.

2.2 Determination of the optimal temperature for coating antigen

Microtiter plates were coated with 10 µg/ml of PND-A and 5 µg/ml of PND-B at 37°C for 1 hour compared to at 4°C overnight. The plates were then processed in the similar manner as that described above.

The result indicated that the optimal temperature for coating antigen was 37 °C. Although antigen incubation at 4 °C also gave the absorbance values as high as the incubation at 37 °C, the antigen incubation time was shorter at 37 °C which is 1 hour. The incubation temperature of 37 °C 1 hour was then selected for use in the subsequent experiments.

After optimizing peptide serotyping conditions, 5 sera from individuals with known genotyping by sequencing analysis were used as standard sera. The result showed that all known sequence B sera (N = 3) were positive with peptide PND-B but all known sequence A sera (N = 2) were positive with both peptide PND-A and PND-B. These results may imply that sera from patients with subtype A/E cross-reacted with 26 a.a V3 peptide of subtype. After reviewing the peptide sequences, it was found that a 10 amino acids sequence within the 26 a.a V3 region in both peptides were identical which could be responsibility for the cross-reactivity. For this reason, the new peptides were synthesized by deleting the identical 10 amino acid sequence in

peptides A and B so the new PND-A (PND-E) and new PND-B (PND-B_{Thai}) were 16 amino acids in length (Table V).

3. Setting up the optimal conditions for peptide serotyping by indirect ELISA using the 16 amino acid peptides

3.1 Determination of the optimal concentration

The 16 amino acid peptide PND-E and PND-B_{Thai} were diluted in coating buffer to the concentration of 5 and 10 μg/ml. The plates were then processed in the similar manner as that described earlier.

The results showed that the optimal concentration of PND-E and PND-B_{Thai} were 5 μg/ml and all procedures of the assay were the same as setting up condition of the 26 amino acid assay. When the assay was tested with known sequence sera, the result showed that known sequence A sera reacted only with PND-E peptide and known sequence B sera reacted only with PND-B_{Thai} peptide (Table V). There was no cross-reactivity but there was a high background interference in the assay.

3.2 Determination of the optimal serum dilution

Serum dilutions at 1:100, 1:200, and 1:400 were used in the assay. The plate was processed in a similar manner to described above.

3.3 Determination of the optimal conjugate dilution

Conjugate dilutions at 1:1000, and 1:2000 were used in the assay. The plate was processed in a similar manner as described above.

The result indicated that the optimal serum dilution was 1:400 and conjugate dilution was 1: 2000. Therefore, serum dilution at 1:400 and conjugate dilution at 1:2000 were chosen in the subsequent experiments.

After optimizing all conditions, the serum specimens from HIV-infected individuals were assayed by peptide serotyping with PND-E and

PND-B_{Thai} peptide (the 16 a.a peptide) but the results showed that all samples were negative. This problem was solved by diluting serum from 1:5, 1:10, 1:20, 1:30, 1:40, 1:50, 1:100 and 1:200. It was found that at dilution of 1:10, all samples were positive (O.D >0.5). Whereas at dilution 1:20 , the O.D of some samples were reduced by 50% . As a consequence, the dilution of 1:10 was chosen in the subsequence experiments but the high background must be solved.

3.4 Optimization to reduce the back ground of peptide serotyping

3.4.1 Determination of optimal conjugate dilution

Conjugate dilutions of 1:2000 and 1:3000 were used in the assay. The plate was then processed in the similar manner as that described earlier.

The results indicate that a conjugate dilution of 1:3000 is the optimal dilution which can reduce background to O.D of 0.4

3.4.2 Determination of appropriate washing buffer to reduce non-specific binding

PBS-tween washing buffer and PBS-tween high salts (appendixII) were compared in the washing step of the assay. The plate was then processed in the similar manner as that described above.

The result showed that PBS-tween high salts was better than PBS-tween because the background was only around 0.3-0.4 as compared to with PBS-tween washing buffer.

3.4.3 Determination of the optimal diluent buffer to reduce non-specific binding

Various concentrations of bovine serum albumin and normal rabbit serum were compared in order to get the optimal concentration of the diluent buffer which will reduce the non-specific binding. Commercial

serum and conjugate diluent of ABBOTT were also used to compared with inhouse diluent.

The result indicated that 3% BSA and 5% normal rabbit serum gave the best results but the commercial diluent from ABBOTT gave lower O.D background than the inhouse diluent (O.D<0.1). Therefore, in subsequent experiments, the commercial diluent from ABBOTT was used as sample diluent and conjugate diluent because this diluent was used in a routine ELISA test in the laboratory.

In Summary, the optimal peptide serotyping method is described as following.

Reagent	Concentration	Temperature	Duration
PND-E & PND-B	5 µg/ml (200 µl)	37 °C	1 Hour
Blocking buffer	200 µl	37 °C	90 minutes
Washing			4 times
Serum	1 : 10	37 °C	30 minutes
Washing			5 times
Peroxidase conjugate	1 : 3000	37 °C	30 minutes
Washing			5 times
OPD substrate		room temperature	10 minutes
H_2SO_4	2 normal	room temperature	

The reaction is read in the spectrophotometer with an absorbance at 492 nm. The results of 60 patients were summarized in Table VI.

II. Validation by Genotyping using Nested Polymerase Chain Reaction (PCR)

Genotyping of HIV-1 subtypes using nested PCR was developed to validate the serotyping method. Genotyping is more sensitive than serotyping due to its amplifying effect of the HIV-1 genome. Cell lysates from the samples used in the initial set up of serotyping were randomized for genotyping. All samples each from serotype E and B, all nontypable and dual reactive serotype genotyped by nested PCR with specific primers for E (SA1/SA2) and B (SB) subtype. The samples were first denatured for 5 minutes at 94 °C, then cycled 40 times at 94 °C 30 seconds, annealed at 52 °C for 30 seconds, and at 72 °C for 45 seconds for extension, and finally incubated at 72 °C for 5 minutes. After the first round PCR, 2 µl of the product was amplified for 30 cycles with specific primers. The results indicate that the annealing temperature at 97 °C is the optimal temperature. At first, the nested reaction was performed separately with specific primers for each subtype (SA1-7, SA2-7, SB-7) but the result showed non-specific binding between primer SA1, SA2 and SB (positive band with both E and B subtype). The problem was solved by using specific primer SA1, SA2 and SB in the same tube to reduce non-specific binding because of competitive binding for each primer. Then the result showed that for subtype E, primer SA1 and SA2 could be used but SA1 gave a more distinct band than SA2. Therefore, SA1 primer was selected in the following studies.

In summary, the optimal genotyping method is as follows: The PCR reactions were performed in a 0.5 ml microfuge tubes with 45 µl of the reaction mixtures and 5 µl of cell lysate. The samples were first denatured for 5 minutes at 94 °C in a Thermal cycler, then cycled 40 times at 94 °C for 30 seconds, annealed at 52 °C for 30 seconds, and at 72 °C for 30 seconds for extension.

The reaction was completed after 5 minute incubation 72 °C. After the first PCR, 2 µl of the product was amplified for 30 cycles with specific primers SA1, SB and P 7 in the same tube and the annealing temperature was 47 °C for 30 seconds 10 µl of product from the second PCR was analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide. Subtype E sample was determined at the band position of 239 base pairs whereas subtype B was determined at the 191 base pairs. The positive control band for HIV infection was identified at 333 base pairs after amplifying with specific primers P6 and P7 (Figure 2 and 3).

The results of genotyping from 18 nonserotypable sera, the only dual reactive serum and all specimens serotype E and B were summarized in TableVIII and IX. Genotyping confirmed the results of all known B_{Thai} and E subtypes Table VII). For the 18 nonserotypable patients, genotyping revealed 9 with subtype E, 2 with subtype B_{Thai} and 7 still remained nontypable.

Part II. Natural History of HIV-1 Subtype

1. Patient population

A total 94 initially asymptomatic patients with baseline CD4 equal to or greater than 200 cells/cu.mm. who had been followed for more than 3 years were included for analysis the study. They were 49 asymptomatic and 45 PGL patients. The majority were male (89 males, 5 females). The major risk was heterosexual as summarized in Table X. Mean CD counts in each year were summarized in Table XII.

2. HIV-1 subtype analysis

By the use of HIV-1 peptide serotyping and/or PCR-based genotyping, it revealed that the majority carried HIV-1 subtype E (57%). The rest were

infected with subtype B_{Thai} (10%), B_{MN} (16%) , dual serotypable (1%) and nontypable (16%) for all risk groups. By distinction in each risk group, all data of subtype analysis were summarized in Table XI.

3. Analysis of CD4 cell counts in different HIV-1 subtypes and risk groups

3.1 For different risk groups, the mean CD4 cell count at entry of the heterosexual group was significantly lower than that of the homo/bisexual and IVDU groups, i.e., 493 ± 32 cells/cu.mm ass compared to 950 ± 108 cells/cu.mm (p =.0001) and 824 ± 154 cells/cu.mm (p=0.003), respectively. (Table XII and figure 4).

The annual changes of CD4 cell count at 1, 2 and 3 years of follow-up were however, comparable among of the groups (Table XIII).

3.2 For different HIV-1 subtypes, patients who carried subtype E also showed a lower mean CD4 count at baseline as compared to subtype Non-E at base line (p=0.0001) (Figure 5). There was no significant difference in the annual CD4 declining rates of the subtype E and the Non-E groups up to 3 years of follow-up.

3.3 When the analysis was done within the heterosexual group only (which was the majority) , it was found that the mean CD4 cell count of patient who carried subtype E was comparable to that of subtype Non-E, i.e., 474 ± 39 cells/cu.mm and 553 ± 57 cells/cu.mm , respectively (p= 0.3) (Table XIV and figure 6). The subtype E carriers had a comparable declining rate of CD4 cells to the subtype Non-E group at the first and the third year of follow-up, but at the second year, the Non-E group showed a trend of greater CD4

cells decline than the subtype E group (p= 0.07), as shown in Table XV . The mean CD4 change per year was in between 50 and 100 cells/cu.mm.

4. Analysis of disease progression

4.1 Disease progression among risk groups

There was no significant difference among 3 risk groups through 3 years of follow-up in the disease progression and progression to AIDS (Figure 7).

4.2 Disease progression among subtype E and Non-E in the heterosexual groups (Table XVI and figure 8)

In the heterosexual group, it was presumed by the comparable CD4 cell count at baseline that both the subtype E and the Non-E groups had been infected with HIV-1 for a comparable length of time. The disease progression to either ARC or AIDS in both groups was analysed. The subtype E group progressed at the rate of 27%, 49% and 65%, while the Non-E group progressed at the rates of 13%, 33% and 47%, at the first , second and third year of follow up, respectively.

Although there was a trend of more rapid clinical progression in the subtype E group throughout the 3-year study but significant difference was found only at the third year (p=0.028).

Table III : Summary of Selected Primer Sequence for Genotyping

PRIMER	SEQUENCE
P6	5' GAA TGG CAG TCT AGC AGA AG 3'
P7	5' ACA ATT TCT GGG TCC CCT CC 3'
SA1	5' GAA ATT TGT ACA AGA CCC TC 3'
SA2	5' ATA GGA GAT ATA AGA AAA GCA T 3'
SB	5' CTA GGA CCA GGG CAA GCA TGG 3'

Table IV : Summary of Pateints in The Cross-sectional Study of Serotyping Optimization

Risk factor	Sex		Stage			
	M	F	Asymptomatic	PGL	ARC	AIDS
Heterosexual(n=20)	13	7	6(30%)	5(25%)	9(45%)	0
IVDUs(n=20)	19	1	1(30%)	4(20%)	8(40%)	7(35%)
Homo/bisexual(n=20)	20	0	3(15%)	0	10(50%)	7(35%)

Table V : Comparison of O.D value between PND-A/ PND-B and PND-E / PND-B_{Thai} of the known sequence sera showing cross-reactivity of the 26 a.a V3-peptide

	ThaiA/E	Thai B	Cut off
26mers Peptide			
P1	0.125	2.217	0.3
P2	0.096	2.863	0.3
P3	0.051	2.050	0.3
P4	1.512	1.630	0.3
P5	1.659	1.862	0.3
16mers Peptide			
P1	0.105	2.996	0.3
P2	0.167	2.916	0.3
P3	0.109	2.994	0.3
P4	0.936	0.050	0.3
P5	2.953	0.111	0.3

Table VI : Serotyping Results by the Use of Peptide PND-E and PND-B_{Thai} (N=60)

Risk factor	Serotype E	B	Nontype*	Dual-reactive**
Heterosexual(n=20)	14(70%)	1(5%)	3(15%)	0
IVDUs(n=20)	3(15%)	14(70%)	2(10%)	1(5%)
Homo/bisexual(n=20)	4(20%)	3(15%)	13(65%)	0

*The consequent immunoassay using PND-B_{MN} (YNKRKRIHIGPGRAFYTTKN) were carrying HIV-1 subtype B_{MN} (1/3 in Hetero, 2/2 in IVDUs and 8/13 in Homo/bisexual)

**The genotyping showed subtype B_{Thai}

Table VII : Comparison of the results between Serotyping and Genotyping from Known subtype

Isolate	Known sequence	Serotyping	Genotyping
1	E	E	E
2	E	E	E
3	B	B	B
4	B	B	B
5	B	B	B

Table VIII : Comparisons of Serotyping and Genotyping Results from 60 patients[a]

	Results		
Type of Assay	E	B_{Thai}	Nontypable[b]
Genotyping	31	20	7
Serotyping	22	18	18

[a]Two cases required further study by sequencing analysis, as one showed discrepancy results and the other showed dual serotypic reaction.

[b]Needs further study by sequencing analysis.

Table IX : Summary of 18 Sero-Nontypable Results by the Subsequent Genotyping

	Subtype		
	E	B_{Thai}	Nontypable
Genotyping	9/18(50%)	2/18(11%)	7/18(39%)

Table X : Summary of Pateint population at entry in Natural History Analysis (n=94)

	Asymptomatic (n=49)	PGL (n=45)
M/F	47/2	42/3
IVDUs	4(8%)	4(9%)
Male Homo/bisexual	7(14%)	12 (27%)
Male Heterosexual	34(70%)	25(55%)
Female Hetrosexual	2(4%)	3(7%)
Blood Transfusion	2(4%)	1(2%)

Table XI : Summary of HIV-1 Subtypes in the Natural History Cohort* (N=94)

	HIV-1 Subtypes				
Risk group	E	B	MN	Nontypable[a]	Dual-reactive
Heterosexual(n=64)	49(77%)	3(5%)	6(9%)	6(9%)	0
IVDUs(n=8)	0	4(50%)	1(13%)	3(37%)	0
Homo/bisexual(n=19)	3(16%)	2(11%)	8(42%)	6(32%)	0
Blood Transfusion(n=3)	1(33.3%)	0	0	1(33.3%)	1(33.3%)

*Peptide : PND-E , PND-B_{Thai} and PND-B_{MN} were used for the immunoassay

[a]Genotyping still remained untypable

Table XII : Summary of CD4 cell counts in the Natural History Cohort (N=91)*

	Mean ± S.E of CD4 cell counts (cells/cu.mm.)			
Risk group	Yr0	Yr1	Yr2	Yr3
Heterosexual(n=64)	493±32	431±29	358±22	276±25
IVDUs(n=8)	824±154[a]	752±159[a]	526±70[a]	540±77[a]
Homo/bisexual(n=19)	950±108[b]	734±118[a]	523±83[b]	519±80[b]

(*The transfusion acquired patients, n=3, were excluded from the analysis, due to too small sample size)

[a]$p < 0.005$, [b]$p < 0.0005$ as compare to that of the heterosexual group at the corresponding year .

Table XIII : Summary of CD4 Changes in Different Risk Groups (N=91)

Risk group	Change of CD4 mean ± S.E (cells/cu.mm.) Year 1	Year 2	Year 3
Heterosexual (n=64)	61±31	74±25	82±24
IVDUs (n=8)	72±168	226±144[a]	-14±68
Homo/bisexual (n=19)	216±92	211±84[a]	4±66

[a] $p < 0.05$ as compared to the heterosexual group (unpaired t-test)

Table XIV : Summary of CD4 cell counts in different subtypes in Asymptomatic or PGL patients with CD4 ≥ 200 cells/cu.mm. at entry. (Heterosexuals only, N =64)

	Mean ± S.E of CD4 counts (cells/cu.mm)			
Subtype	At Entry	Year 1	Year 2	Year 3
E (n=49)	474±39	412±32	358±27	268±29
Non-E* (n=15)	553±57	497±67	358±40	305±47

* Non-E = B_{Thai} 3, B_{MN} = 6, Nontype = 6

Table XV : Summary of CD4 Declining Rate in different HIV-1 Subtypes in Asymptomatic or PGL patients with CD4 ≥ 200 cells/cu.mm. (Heterosexuals only, N=64)

	Change of CD4 (cells/cu.mm, mean ± S.E.)		
Subtype	Year 1	Year 2	Year 3
E (n=49)	63±38	54±29	90±29
Non-E (n=15)	56±51	139±40	53±43

Table XVI : Summary of Cumulative Disease Progression in different HIV-1 subtypes from Asymptomatic or PGL to ARC or AIDS (Heterosexuals only, N=64)

	Disease Progression		
Subtype	Yr0→Yr1	Yr1→Yr2	Yr2→Yr3*
E (n=49)	13/49 (27%)	24/49 (49%)	32/49 (65%)
Non-E (n=15)	2/15 (13.3%)	5/15 (33.3%)	7/15 (47%)

*$p < 0.05$ as compared to Non-E

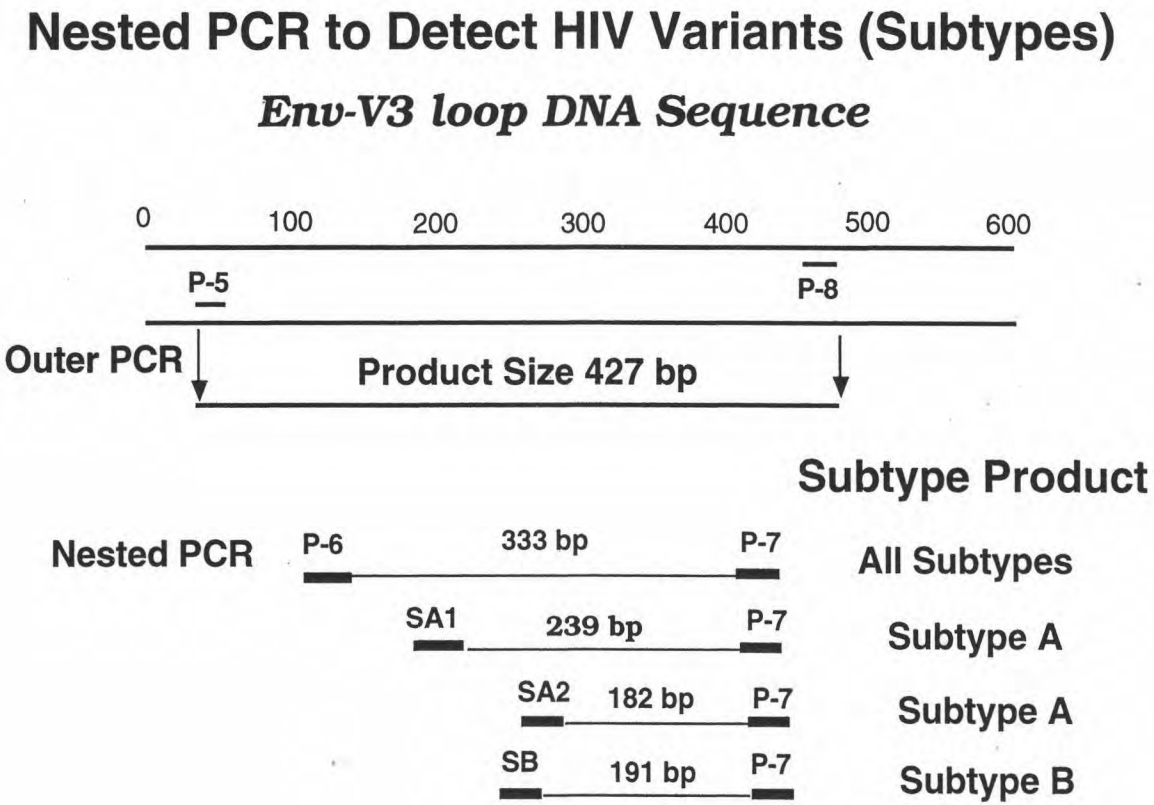

Figure 1. Principle of PCR-based Genotyping Analysis

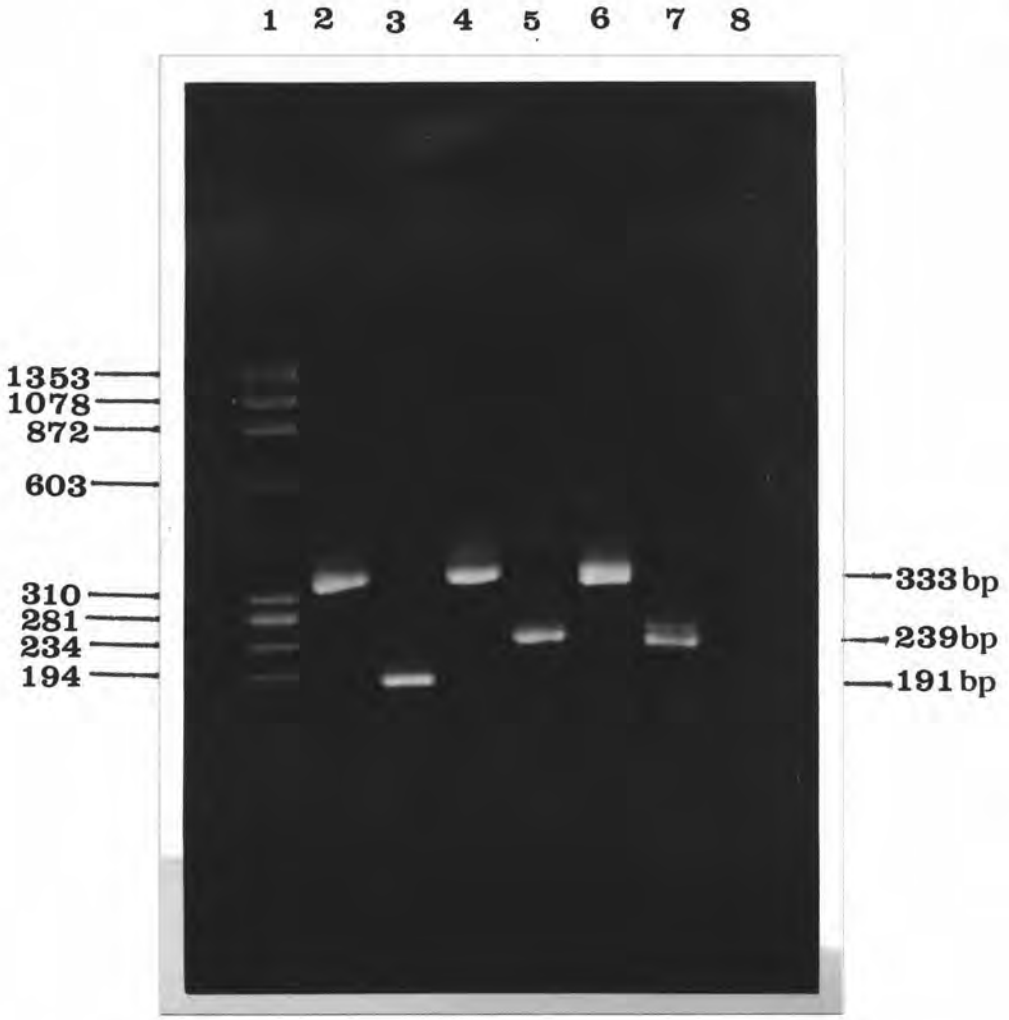

Figure 2. Example of Known Sequence Sample by Genotyping

lane 1 ; ϕX174/HaeIII fragment (markers)
lane 2 ; Sample with HIV-1 universal primers(6-7)[a]
lane 3 ; Sample with subtype selective primers(SA1-SB-7)[b]
lane 4 ; Sample with HIV-1 universal primers(6-7)[a]
lane 5 ; Sample with subtype selective primers(SA1-SB-7)[c]
lane 6 ; Sample with HIV-1 universal primers(6-7)[a]
lane 7 ; Sample with HIV-1 universal primers(SA1-SB-7)[c]
lane 8 ; Negative control

[a] Indicating the sample containing HIV-1 proviral DNA
[b] Indicating the sample containing HIV-1 subtype B_{Thai}
[c] Indicating the sample containing HIV-1 subtype E

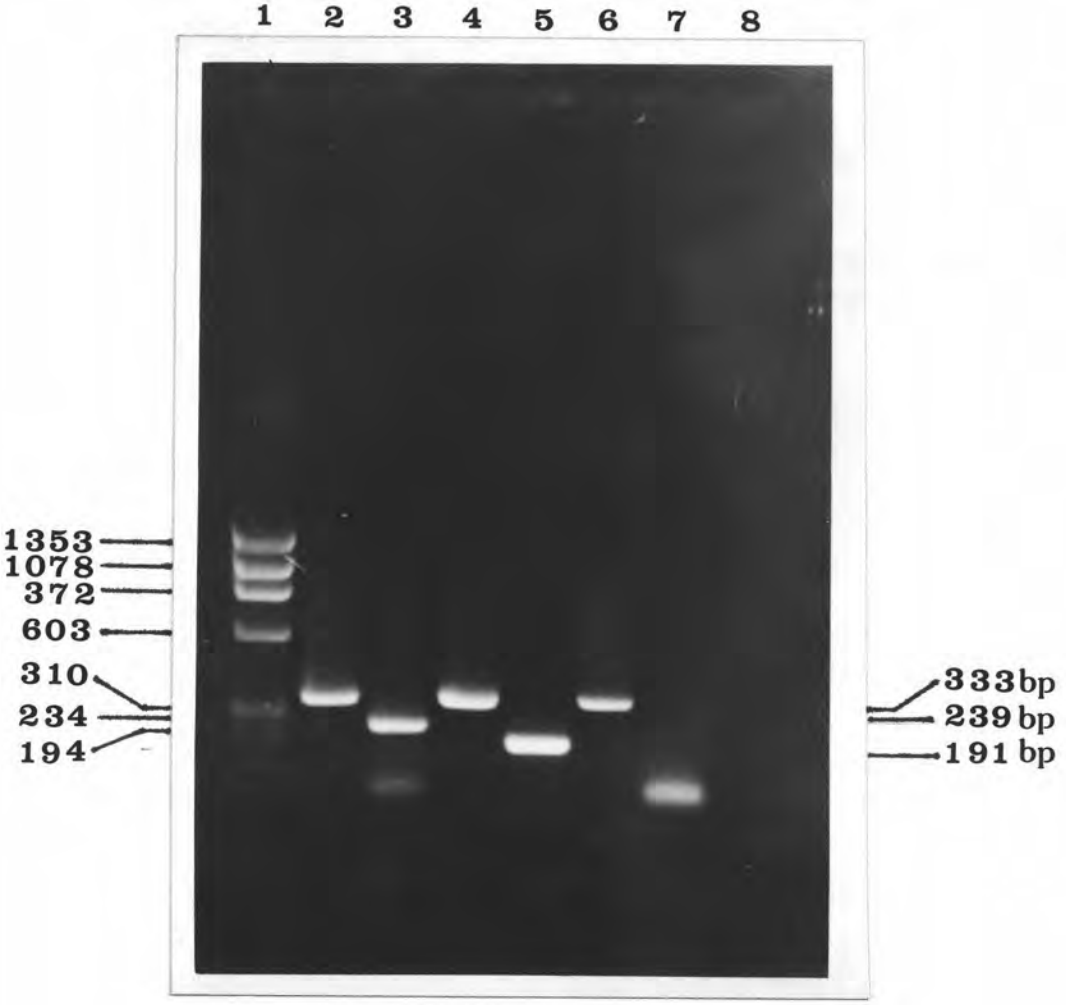

Figure 3. Example of Non-serotypable Sample by Genotyping

lane 1 ; ϕX174/HaeIII fragment (markers)
lane 2 ; Sample no.1 with HIV-1 universal primers.(6-7)
lane 3 ; " with primers SA1-SB-7 (indicating genotype E)
lane 4 ; Sample no.2 with HIV-1 universal primers.(6-7)
lane 5 ; " with primers SA1-SB-7 (indicating genotype B)
lane 6 ; Sample no.3 with HIV-1 universal primers.(6-7)
lane 7 ; " with primers SA1-SB-7 (indicating nontypable by genotype)
lane 8 ; Negative control

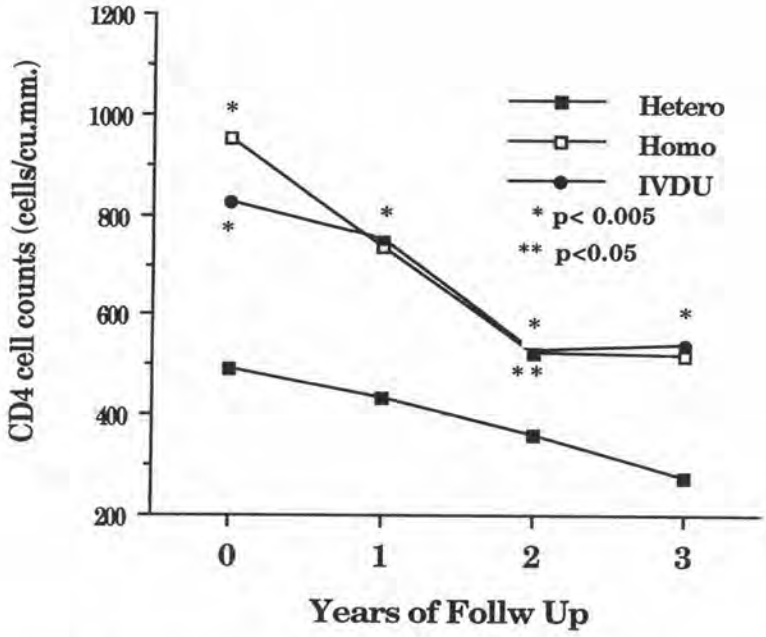

Figure 4. Means of CD4 Cell Counts of the Natural History Cohort
(Statisitcal analysis by the use of unpaired t-test)

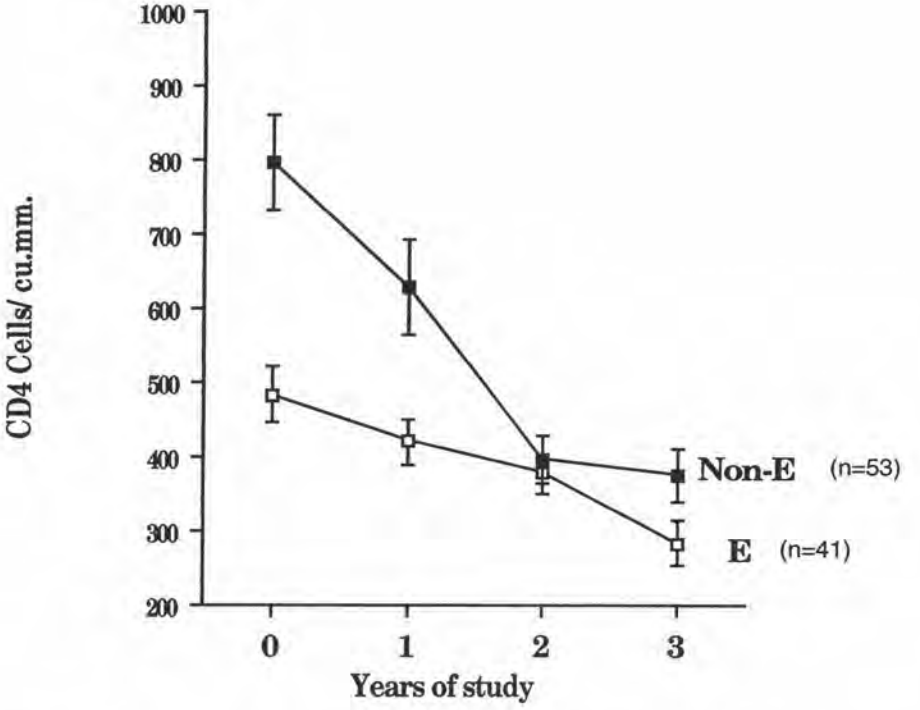

Figure 5. CD4 cell counts among the HIV-1 subtypes in the natural history cohort

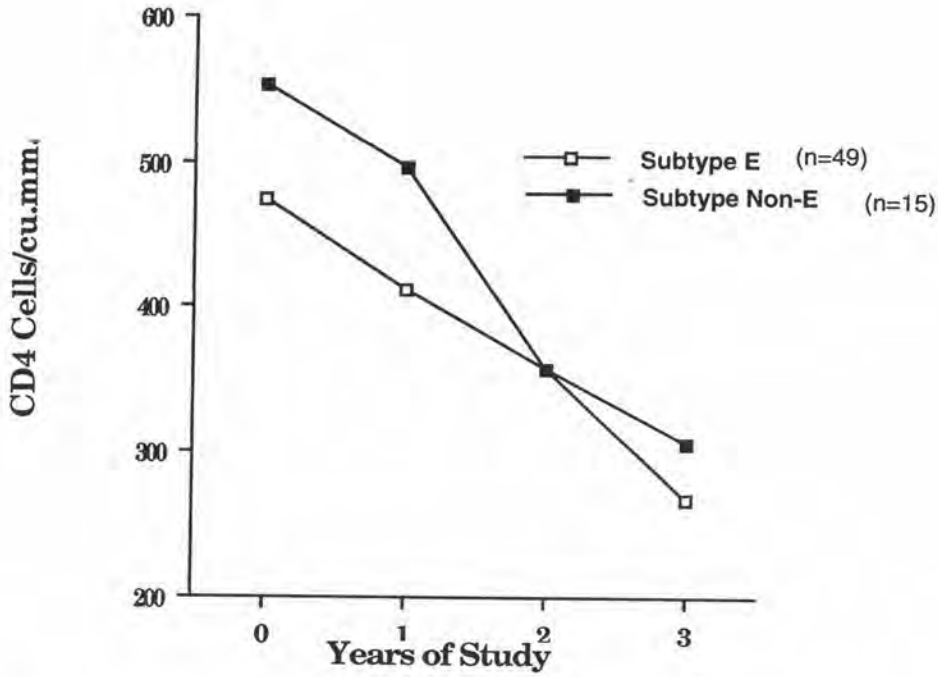

Figure 6. CD4 cell counts among the different subtypes in the heterosexual group (n=64)

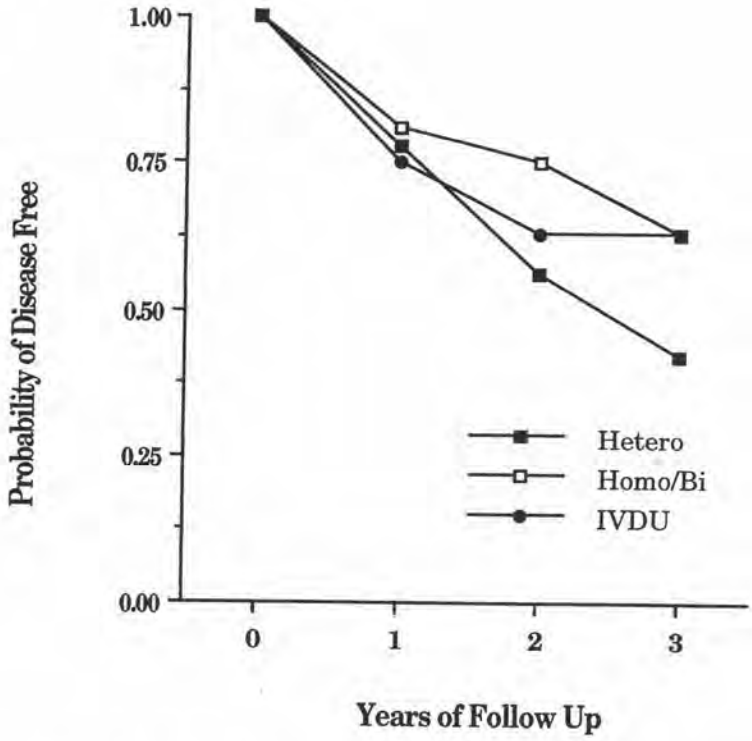

Figure 7. Analysis of disease progression among the three risk groups

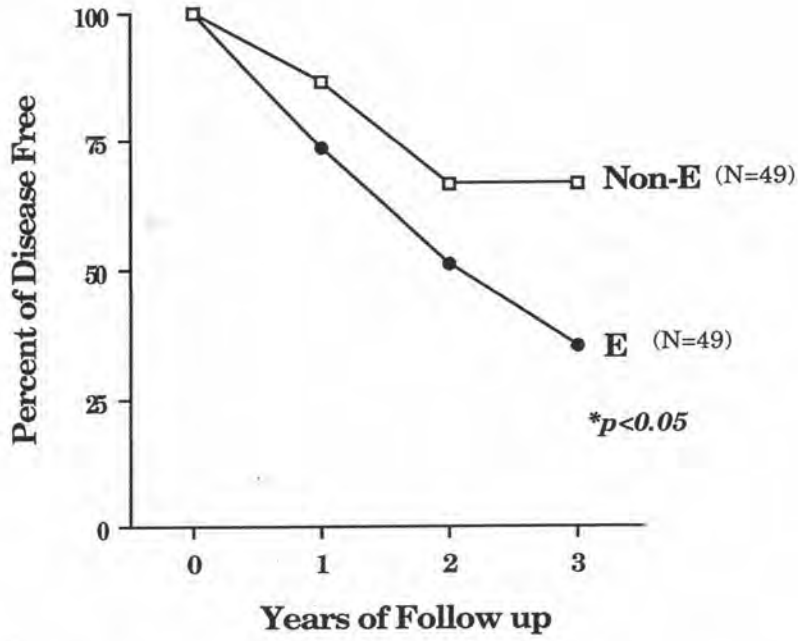

Figure 8. Natural Courses of HIV-1 Infected Heterosexual