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Characteristic of HIV-1 in V3 Loop Region Based on Seroreactivity and Amino Acid Sequences in Thailand

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SUMMARY **The third variable (V3) domain of the envelop (env) protein has been used for determining genetic subtype and phenotypic characteristics of human immunodeficiency virus type 1 (HIV-1) isolates. Based on the seroreactivity of the HIV-1 subtype by V3 peptide binding enzyme immunoassay (EIA) of 351 samples obtained in 1998 from HIV-1 infected individuals and AIDS patients, we found that 283 (80.6%) were subtype E, 20 (5.7%) were subtype B, 28 (8.0%) were cross-reactive between both types and 20 (5.7%) were non-typeable. The degree of seroreactivity of HIV-1 subtype E decreased significantly when the amino acid at the crown of the V3 loop was substituted from a GPGQ motif to GPGR motif. Interestingly, AIDS patients who had V3 sequences of subtype E as GPGR motif had a stronger immunoreactivity to GPGQ motif peptides than to GPGR motif peptides, in contradiction for their proviral sequences. The results suggested that mutations in the V3 loop may lead to a changed immunoreactivity that makes HIV-1 mutants unrecognizable or allow escape from the primary immune response by means of neutralizing sensitivity. In connection with vaccine development, it should be pointed out that the combination of V3 sequencing and peptide EIA could provide a novel approach to obtain a primarily infected virus sequence as a target for a preventive AIDS vaccine.**

The interests in the human immunodeficiency virus type 1 (HIV-1), the etiologic agent of AIDS, are concentrated around its genetic, antigenic and biological variations, particularly with regard to the relevance of this variability to the HIV vaccine development. The variability is relatively high in the external envelope protein gp120. Within the V3 loop of this protein resides the principal neutralizing determinant.[1] As a consequence, this determinant becomes an important target for vaccine development. Type specificity has been observed in both antibody and T cell responses as a result of variations in V3 sequences.[2,3] To determine the trend of HIV-1 genetic subtypes in the population, the V3 region is chosen to elucidate reactivity to peptides from A through H HIV-1 subtypes.[4]

Several studies have shown that the V3 region has additional functions in virus replication and that determinants of virus tropism lie within this region. Changes in amino acids flanking the tip of the V3 loop sequence are sufficient to change the cell tropism of the virus.[5] At least three positions including several amino acid residues in V3 are involved in determining macrophage-tropism.[6,7] Further, a high net charge of V3 due to additional basic amino acids is related to a syncytium inducing (SI) and fast replicating virus phenotype.[5] Fast replicating SI variants emerge in 50% of HIV-1 seropositive individuals preceding progression to AIDS. Slow replicating, non-syncytium inducing (NSI) variants are predominant in the asymptomatic stage and persist throughout all stages of HIV-1 infection.[8,9] In general, the NSI isolates such as those from brain tissues or cerebrospinal fluid had the characteristics

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of monocyte-tropism with no cytopathic appearance in T cells. This is probably important for persistence, possibly by forming a major viral reservoir in the body.[10] Because of the persistence of the NSI in monocyte-tropic variants,[8] these variants were suggested to be another target for vaccine development.

By using V3 loop peptide binding enzyme immunoassay (peptide EIA) and V3 loop sequence analysis, we identified virus subtypes for HIV-1 positive specimens in 1998 and investigated whether the variations in length and amino acids of the V3 loop correlate with the change of viral biological characteristics in this study. Serum and DNA samples were used for peptide EIA and V3 loop sequence analysis, respectively.

MATERIALS AND METHODS

From July to December 1998, a total of 351 serum samples were obtained from 251 asymptomatic HIV-1 infected individuals (heterosexuals, n = 160; intravenous drug users, n = 91) and 100 AIDS patients. Among the AIDS patients, peripheral blood mononuclear cells (PBMC) were collected in 20 cases for HIV-1 DNA preparations. The samples were collected from the asymptomatic HIV-1 infected individuals and the AIDS patients with their consent forms at Siriraj Hospital in Bangkok and Bamrasnaradura Hospital in Nonthaburi Province, respectively. Their ages ranged from 27 to 54 years old with the average of 37 years and the ratio of male:female was 4:1.

Seroreactivity of HIV-1 env subtypes by peptide EIA

The procedure for EIA used in this study was described previously.[11] Based on consensus sequence of the V3 loop in each subtype, synthetic peptides were designed with a variation in amino acids and length. Each peptide was added with a non virally coded aspartic acid (D) to the N terminal end to improve binding to the plates. All peptides were synthesized by BioSynthesis Inc., U.S.A. The panel of peptides used were as follows: subtype B (B), DKSIHLGPGQAWYI (consensus B); subtype E (E/Q), DTSITIGPGQVFYR (consensus E); (E/R), DTSITIGPGRVFYR and (E/M), DTSITIGPGQVFYRTGDI.

Peptide EIA was performed on microplates (NUNC-Maxisorb Loose, Denmark) that were coated with 100 µl/well of the peptides, solubilized in carbonate buffer pH 9.6, at a final concentration of 0.55 µg/well. The coated plates were stored overnight at 4°C. On the next day, antigen was aspirated and plates were blocked with 200 µl per well of skim milk buffer at 37°C for 1.5 hours (5% non-fat dry milk and 0.3% Tween 20 in PBS, pH 7.2), followed by three washings with 0.05% Tween 20 in PBS, pH 7.2 (PBS-T). One hundred microliters of diluted sera (1:500) in skim milk buffer were added to the antigen-coated plates and incubated for 1 hour at 37°C. Bound antibodies were detected with peroxidase conjugated goat anti-human immunoglobulin (Ig) diluted in skim milk buffer (1:4,000) and tetramethylbenzidine/H_2O_2 substrate after washing five times with PBS-T between each step. Absorbance at 450 nm against 630 nm was measured. A cutoff of 0.3 was used throughout the study, with dual reaction (subtype B/E) further clarified as monoreactive (subtype B or E) if one peptide's optical density (OD) was three times greater than that of the other peptides (OD).

DNA sequencing of the V3 loop region

HIV-1 DNA sequences corresponding to the V3 loop were amplified from 20 DNA samples of the AIDS group by polymerase chain reaction (PCR).[12] The following V3 loop region-specific primers were used: JA9 (5'CACAGTACAATGTACACATC-3') and JA12 (5'ACAGTAGAAAAATTCCCCTC-3') for outer primers and JA10 (5'AAATGGCAGTCTAGCAGAAG-3') and JA11 (5'-ACAATTTCTGGGTCCCCTCC-3') for inner primers. The PCR products of the V3 loop region were expected at 341 base pairs when detected by a 2% gel electrophoresis with the ethidium bromide staining. The positive PCR products were then purified by using high pure PCR products purification kit (Boeringer Mannheim). The purified products were processed for PCR cycle sequencing with a big dye terminator reaction mixture (DNA sequencing kit, PE Applied Biosystems) and thermal cycles for 25 cycles. Each round of thermal cycle was set at 96°C, 30 seconds for denaturation, 56°C, 15 seconds for annealing and 60°C, 4 minutes for extension. After PCR reactions, the products were purified to get rid of the excess dye by using the centrisep kit (Perkin Elmer) before DNA sequencing with the automated DNA sequencer ABI PRISM377. The results from DNA sequencing were then analyzed for amino acid sequences by DNASIS program.

RESULTS

The seroreactivity of HIV-1 subtypes by peptide EIA on the 351 serum samples showed 283 (80.6%) as subtype E, 20 (5.7%) as subtype B, 28 (8.0%) as cross-reactive between both subtypes and 20 (5.7%) as non-typeable. The proportion of subtype E was highest

in heterosexuals while subtype B was more predominant in injecting drug users (IDU). The cross-reactivity between subtypes B and E was found mostly in IDU and the non-typeable specimens were higher in the AIDS group than in any other group (Table 1).

Based on the sequence of consensus HIV-1 subtype E,[11] three sequences of the V3 loop peptide with a variation in length of 13 and 17 amino acids (E/Q and E/M peptides, respectively) and the substitutional amino acid from the GPGQ motif to the GPGR motif at the crown of the V3 loop (E/R peptide) were chosen to study the changes in seroreactivity of HIV-1 subtype E sera. As shown in Fig. 1, the percentage of positive seroreactivity was similar when different lengths of E/Q peptide (98.9%) and E/M peptide (97.9%) were used. In contrast, the rate of positive seroreactivity was low (13.8%) when E/R peptide was used.

When the phenotypes of 20 AIDS patients were considered regarding their V3 sequences, it was found that all of their sera showed high reactivity with E/Q and E/M peptides but low reactivity with E/R and B peptides (Table 2). Interestingly, 12 out of 20 V3 loop sequences from the AIDS group contained different loop apices, namely, GPGR (8 cases), GPGL (2 cases), GPGH (1 case) and GPGK (1 case). Even in these cases, their sera were highly reactive with the E/Q and E/M peptides (GPGQ) but not with the E/R peptide (GPGR). Among the eight cases with a GPGR loop, the results clearly showed a high seroreactivity with GPGQ in contradiction to their DNA sequences.

DISCUSSION

At present, HIV-1 subtype E has emerged as the main variant in the Southeast Asian epidemic.[13] In Thailand, HIV-1 subtype E accounts for approximately 95% of HIV positive cases in heterosexuals and is found increasingly in IDUs. Among IDUs, the proportion of incident infection due to subtype E increased from 2.6% in 1988 to 43.8% in 1993.[14] In our study, when peptides representing consensus B and E were used on 351 serum samples collected in 1998, subtype E (80.6%) appeared to be the predominant one. Focussing on the IDU group, the proportion of subtype E was still increasing from 43.8% in 1993[14] to 64.8% (59/91 cases) in 1998 in this study (Table 1). Recently, Subbarao *et al.*[15] reported that 79.7% of HIV-1 seroconverters in IDU cohort in Bangkok from 1995 to 1998 were identified as subtype E and 20.3% were as subtype B infections in IDU group.

Although specific V3 peptides predictive of HIV-1 subtypes have been found there is still significant cross-reactivity.[4] From this study, 28 (8%) and 20 (5.7%) specimens were cross-reactive and non-typeable, respectively. These results suggest that it may be necessary to use or include alternative methods such as heteroduplex mobility assay,[14] peptide competition assay[16] or DNA sequencing assay[14] for more definitive subtype determination. In this study, patients bb 32 and bb 53 were found to have subtype E by direct sequencing but were non- typeable by peptide EIA (Table 2). These non-typeable cases in the AIDS group demonstrated low seroactivity to any used peptides as shown in Table 2, indicating that these patients may be at the final stage of HIV-1 infection associated with a low level of anti-V3 loop antibodies due to the progression of immunodeficiency. In contrast, the cases of cross-reactivity in peptide EIA were main-ly observed in asymptomatic heterosexual and IDU groups. It is likely that these individuals could still keep producing antibodies against the new variants which possessed mutations in the V3 loop and es-

Table 1 HIV-1 subtype in HIV-1 infected groups by peptide EIA

Group	Total	Subtype			
		E[a](%)	B[a](%)	B/E[b](%)	Non- typeable[c] (%)
HIV infected groups					
-Heterosexual	160	141 (40.2)	4 (1.1)	10 (2.8)	5 (1.4)
-IDU	91	59 (16.8)	13 (14.3)	15 (4.3)	4 (1.1)
-AIDS	100	83 (23.4)	3 (0.9)	3 (0.9)	11 (3.2)
Total	351	283 (80.6)	20 (5.7)	28 (8.0)	20 (5.7)

[a]Monoreactive; [b]Cross-reactive; [c]Non-reactive.

Table 2 Immunoreactive and amino acid sequences of HIV-1 subtype E in 20 cases of AIDS patients

No. of AIDS group	OD in various peptides by peptide EIA				V3 amino acid sequences															
	B	E/Q	E/R	E/M	T	S	I	T	I	G	P	G	Q	V	F	Y	R	T	G	D	I
bb1	0.4	>3.0	0.3	>3.0	-	-	L	-	V	-	-	-	R	-	-	-	-	-	-	A	-
bb24	0.3	1.2	0.1	0.8	A	-	T	R	-	-	-	-	R	-	-	-	-	-	-	-	-
bb25	0.3	1.4	0.1	1.2	-	R	-	-	-	-	-	-	R	-	-	-	-	-	-	E	-
bb26	0.4	1.2	0.1	0.9	-	-	-	-	A	-	-	-	R	-	-	-	-	-	-	E	-
bb36	0.2	>3.0	0.5	>3.0	-	R	-	-	-	-	-	-	R	-	-	-	-	-	-	E	-
bb38	0.1	2.4	0.2	2.5	-	-	V	P	-	-	-	-	R	-	-	-	-	-	-	-	-
bb50	0.1	2.1	0	2.4	-	-	V	-	-	-	-	-	R	-	-	-	-	-	-	-	-
bb13	0.4	2.1	0.1	2.8	-	-	V	-	-	-	-	-	-	-	W	-	-	-	-	A	-
bb18	0.4	>3.0	0.3	>3.0	-	-	M	-	-	-	-	-	-	-	I	-	-	-	-	-	-
bb19	1.1	>3.0	0.5	>3.0	-	-	V	H	-	-	-	-	-	-	-	-	-	-	-	-	-
bb21	0.4	>3.0	0.2	>3.0	-	-	-	H	-	-	-	-	-	-	-	Q	-	-	-	V	
bb22	0.1	>3.0	0	2.6	M	-	T	R	-	-	-	-	-	-	-	-	-	-	-	-	-
bb31	0.1	1.9	0.1	1.7	-	G	-	-	-	-	-	-	L	-	-	-	-	-	-	E	-
bb33	0.2	>3.0	0.1	>3.0	-	R	-	-	-	-	-	-	L	-	-	-	-	-	-	E	-
bb35	0.1	2.6	0.1	2.3	-	T	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-
bb37	0.3	2.4	0.1	1.9	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-
bb40	0.3	0.6	0.2	1.0	I	-	M	-	R	-	-	-	H	-	Y	-	-	-	-	-	-
bb52	0.2	0.7	0.1	0.7	-	-	M	-	-	-	-	-	-	-	-	S	-	-	-	-	-
bb32[a]	0.2	0.1	0.1	0.1	-	R	-	-	-	-	-	-	R	-	-	-	-	-	-	-	-
bb53[a]	0.2	0.2	0	0.2	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-

Note: The immunoreaction was determined by the optical density (OD) in peptide EIA

[a]non-typeable by peptide EIA

caped former immune responses. Consequently, these subjects might demonstrate such cross reactivity.

Regarding the V3 principal neutralizing determinant, Schreiber *et al.*[17] reported that the type-specific antibodies are mainly directed to discontinuous epitopes consisting in their native form of an entire V3 loop, while group-specific antibodies are preferentially directed to linear epitopes on the tip of the loop. In the latter case, at least eight amino acids from the GPG apical V3 loop were found to elicit neutralizing antibodies.[18] In the present study, we have tested seroreactivity against linear V3 epitope only and found that the degree of seroreactivity was decreased when comparing an amino acid substitution at the crown of the V3 loop with the different lengths of peptides. As shown in Fig. 1, both E/Q (13 amino acids) and E/M peptides (17 amino acids) that had the same GPGQ motif demonstrated a high seroreactivity while E/R peptides (13 amino acids) with a GPGR motif exhibited low reactivity. This means that the amino acid substitution from the GPGQ to the GPGR motif might lead to a drastic change of antigenicity that resulted in a weak immunoreactivity in the sera. Quite interestingly, the AIDS patients who have genetic sequences with a GPGR motif and a strong seroreactivity to the E/Q peptide but a weak response to the E/R peptide might indicate that the GPGQ-type virus which caused the initial infection changed to new variants in order to escape the primary host immune response and that the V3 epitope of the GPGR-type variant might not have a strong antigenicity in HIV-infected individuals. This is consistent with the report by Subbarao *et al.*[15] that most of the cases primarily infected with subtype E HIV in an IDU cohort in Bangkok displayed GPGQ-genotype. Our data could provide a novel insight for the strategies to design an AIDS vaccine in the sense that the target

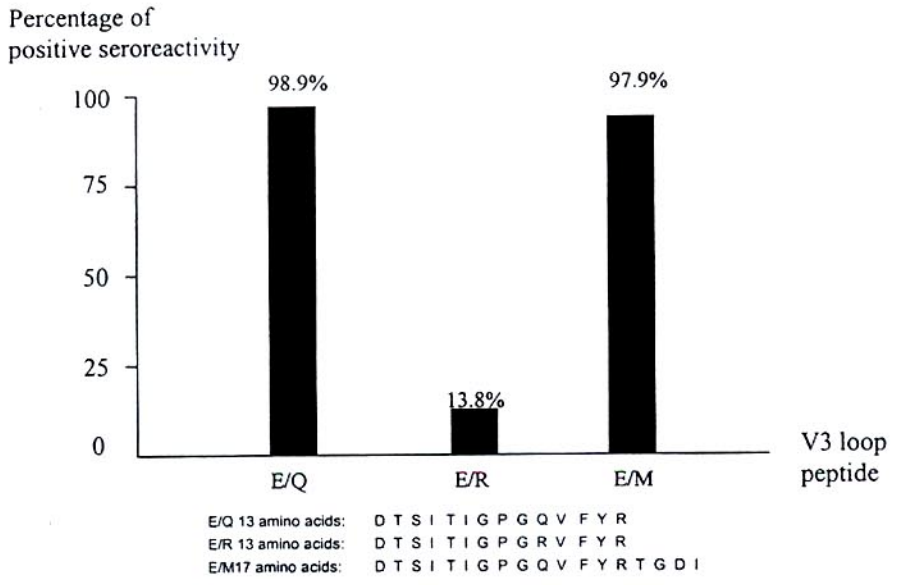

Fig. 1 The percentage of positive seroreactivity in 283 HIV-1 subtype E sera by using three V3 loop peptides (E/Q, E/R and E/M peptides) in the peptide EIA.

virus isolate should be determined by a combination of V3 sequencing and peptide EIA as we demonstrated in this study and that the GPGQ-type subtype E virus should be high priority target for developing a preventive AIDS vaccine.

Scarlatti *et al.*[19] clearly demonstrated that *in vivo* evolution of HIV-1 co-receptor usage from CCR5 to other chemokine receptors was correlated with disease progression. Only one amino acid substitution at position 311 (serine or glycine to arginine) within the V3 loop or an increase of the net positive charge of the entire V3 loop were associated with a change of co-receptor usage. In our case, 4 out of 20 AIDS patients possessed an arginine residue at position 311 as a major provirus V3 sequence. These variants may exhibit a co-receptor usage change which might lead to the onset of AIDS. Further clarification of genetic and antigenic characteristics of the V3 loop in HIV-1 subtype E as demonstrated in this study is epidemiologically meaningful and has implications for the HIV vaccine development.

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REFERENCES

1. Javaherian K, Langlois AJ, La Rosa GJ, *et al.* Broadly neutralizing antibodies elicited by the hypervariable neutralizing determinant of HIV-1. Science 1990; 250: 1590-3.
2. Palker TJ, Clark ME, Langlois AJ, *et al.* Type-specific neutralization of the human immunodeficency virus with antibodies to env-encoded synthetic peptides. Proc Natl Acad Sci USA 1988; 85: 1932-6.
3. Takahashi H, Merli S, Putney S, *et al.* A single amino acid interchange yield reciprocal CTL specificities for HIV-1 gp160. Science 1989; 246: 118-21.
4. Devito C, Levi M, Hinkula J, *et al.* Seroreactivity to HIV-1 V3 subtypes A to H peptides of Argentinian HIV-positive sera. J Acquir Immune Defic Syndr 1998; 17: 156-9.
5. De Jong JJ, Goudsmit J, Keulen W, *et al.* Human immunodeficiency virus type 1. Clones chimeric for the envelope V3 domain differ in syncytium formation and replication capacity. J Virol 1992; 66: 757-65.
6. Shioda T, Levy JA, Cheng-Mayer C. Small amino acid changes in the V3 hypervariable region of gp120 can affect the T cell-line and macrophage tropism of human immunodeficiency virus type 1. Proc Natl Acad Sci USA 1992; 89: 9434-8.
7. Xiao L, Goldman SM, Lai AA, *et al.* CCR-5 coreceptor usage of non-syncytium-inducing primary HIV-1 is independent of phylogenetically distinct global HIV-1 isolates: delineation of consensus motif in the V3 domain that predicts CCR-5 usage. Virology 1998; 240: 83-92.
8. Schuitemaker H, Kootstra NA, De Goede REY, *et al.* Monocytotropic human immunodeficiency virus type 1 (HIV-1) variants detectable in all stages of HIV infection lack T cell line tropism and syncytium inducing ability in primary T cell culture. J Virol 1991; 65: 356-63.
9. Termette M, Gruters RA, De Wolf F, *et al.* Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of AIDS obtained from studies on a panel of sequential HIV isolates. J Virol 1989;

63: 2118-25.

10. Schuitemaker H, Koot M, Kootstra NA, *et al.* Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T cell tropic virus population. J Virol 1992; 66: 1354-60.
11. Pau CP, Lee-Thomas S, Auwanit W, *et al.* High specific V3 peptide enzyme immunoassay for serotyping HIV-1 specimens from Thailand. AIDS 1993; 7: 337-40.
12. Albert J, Fenyo EM. Simple sensitive and specific detection of HIV-1 in clinical specimens by polymerase chain reaction with nested primers. J Clin Microbiol 1990; 28: 1560-4.
13. Mastro TD, Zhang KL, Panda S, *et al.* HIV infection and AIDS in Asia. In: Pizzo PA, Wilfert CM, eds. Pediatric AIDS: The challenge of HIV infection in infants, children and aldolescents. 3rd edition. Williams and Wilkins, Baltimore: 1998; 47-63.
14. Wasi C, Herring B, Raktham S, *et al.* Determinantion of HIV-1 subtypes in injecting drug users in Bangkok, Thailand, using peptide-binding enzyme immunoassay and heteroduplex mobility assay: evidence of increasing infection with HIV-1 subtype E. AIDS 1995; 9: 843-9.
15. Subbarao S, Vanichseni S, Hu DJ, *et al.* Genetic characterization of incident HIV type 1 subtype E and B strains from a prospective cohort of injecting drug users in Bangkok, Thailand. AIDS Res Hum Retroviruses 2000; 16: 699-707.
16. Jansson M, Wahren B, Albert J, *et al.* Peptide serology for analysis of the inter and intra individual variation in the HIV-1 V3 doman. AIDS 1994; 8: 413-21.
17. Schreiber M, Wachsmuth C, Muller H, *et al.* The V3-directed immune response in natural human immunodeficiency virus type 1 infection is predominantly directed against a variable, discontinuous epitope presented by the gp120 V3 domain. J Virol 1997; 71: 9198- 205.
18. Javaherian K, Langlois AJ, McDanal C, *et al.* Principal neutralizing domain of the human immunodeficiency virus type 1 envelope protein. Proc Natl Acad Sci USA 1989; 86: 6768-72.
19. Scarlatti G, Tresoldi E, Bjorndal A, *et al. In vivo* evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression. Nat Med 1997; 3: 1259-65.