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Antibody Reactivity to HIV-1 Vpu in HIV-1/AIDS Patients on Highly Active Antiretroviral Therapy

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Key Words
Anti-Vpu antibody · IgG subclasses · *vpu* gene · Viral load · Total IgG · Disease progression · Highly active antiretroviral therapy · AIDS

Abstract
Human immunodeficiency virus type 1 (HIV-1) Vpu protein promotes both extracellular release of viral particles and degradation of CD4 in the endoplasmic reticulum. The correlation of anti-Vpu antibody (Ab) reactivity to Vpu and AIDS disease progression was studied in 162 HIV-1/AIDS patients after they had received highly active antiretroviral therapy (HAART) for 1 year. Anti-Vpu Ab reactivity was analyzed by Western blot using a recombinant Vpu protein. Results showed that at baseline (prior to initiation of HAART), 31.5% of patients (51/162) had anti-Vpu Ab. The proportion of anti-Vpu Ab in patients with CD4 counts ≥500, 200–500 and <200/mm^3 were 40.6, 34.7 and 14.3%, respectively (χ^2 test, $p < 0.05$). In addition, decreasing levels of anti-Vpu Ab reactivity were significantly correlated with increasing levels of HIV-1 viral load. After receiving HAART for 1 year, 7 of 111 anti-Vpu Ab-negative patients (6.3%) seroconverted (– → + group) and 8 of 51 anti-Vpu Ab-positive (15.7%) patients became negative (+ → – group). Among 104 anti-Vpu Ab-negative patients, 40 were selected for analysis of the *vpu* gene. All of them had an intact *vpu* gene. Patients were further divided into four groups according to their anti-Vpu Ab serostatus and anti-HIV-1 Ab was measured. The results showed that only the anti-Vpu Ab seroconverted group (– → +) had increased serum levels of anti-HIV-1 Abs after 1 year of HAART, while the other three groups (+ → +, – → – and + → –) had decreased serum levels of anti-HIV-1 Abs after 1 year of HAART ($p < 0.05$). In conclusion, the presence of anti-Vpu Ab is associated with improved prognosis following HIV-1 infection, and seroconversion of anti-Vpu Ab in patients on HAART indicates significant recovery of immunity.

Introduction

There are six regulatory genes, *tat, rev, vif, vpu, vpr* and *nef,* encoded by the human immunodeficiency virus type 1 (HIV-1) [12]. Among these genes, the *vpu* gene only presents in HIV-1, not in HIV-2 [8]. The *vpu* gene product Vpu is an integral membrane phosphoprotein with two well-described functions: enhancement of viral particle release and induction of the proteolysis of several important membrane proteins, including CD4 and major histocompatibility complex class I molecules [2, 15, 21].

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It has been reported that antibodies (Abs) to HIV-1 regulatory proteins are less readily detected in AIDS patients, especially those in the most advanced stages of disease [7, 23]. Regarding the Ab reactivity to Vpu, discrepant results were obtained from the following three studies. Using Western blot (WB) assays with a recombinant protein containing 49 amino acids from the C-terminal region of Vpu, Matsuda et al. [19] reported a bimodal distribution of anti-Vpu Ab seropositive rates in HIV-1-infected patients with Walter Reed (WR) stages 2–6. Reiss et al. [22], using enzyme immunoassays (EIAs) with a recombinant Vpu fusion protein, demonstrated that there was no association between the Ab reactivity pattern to Vpu and the patients' clinical outcome. Finally, Schneider et al. [24] used synthetic peptides in EIA to show that there was a correlation between anti-Vpu Ab and the WR stages, while the Ab rates were not in a bimodal distribution.

To resolve the discrepant results mentioned above and to understand the change in Ab reactivity to Vpu in patients on highly active antiretroviral therapy (HAART), WB assays with a full-length recombinant Vpu protein were developed in this study. Besides CD4 counts, we used HIV-1 viral load (VL) as a disease progression marker for the correlation study, since it has been shown that HIV-1 VL is the best predictor of disease progression to AIDS and death [20]. Furthermore, total serum immunoglobulin (Ig) G and anti-HIV-1-specific Abs were evaluated before therapy and after the patients had been on HAART for 1 year.

Methods

The HIV-1/AIDS Patient Cohort

Between March and April 1997, 162 patients who attended the outpatient clinics of the Taipei Venereal Disease Control Institute were enrolled in this study. Informed consent was obtained from all the patients. Blood samples had been collected before the patients started to receive HAART with two nucleoside analogue HIV-1-reverse transcriptase inhibitors and one protease inhibitor. During the treatment, serum and plasma samples were collected every 4 months and stored at −80°C for further analysis. In addition, a questionnaire was used to record the patients' demographic data, symptoms and signs, CD4/CD8 cell counts and medications. The protocol was approved by the institute's review board (No. VGHIRB-90-05-06A).

Quantitative HIV-1 RNA Analysis

Plasma samples were analyzed using the Quantiplex bDNA signal amplification system, second generation (Chiron Corp., Emeryville, Calif., USA), for the measurement of HIV-1 viral RNA. Each sample was tested in duplicate. The detection sensitivity of the assay is 500 copies/ml. Detailed procedures of the assay have been described previously by the manufacturer [11].

Construction of an HIV-1 Vpu Expression Plasmid

The HIV-1 *vpu* gene was amplified from HIV-1 proviral DNA of HXB2 strain [25] using PCR with primers vpu-1 (5′-GCGGAATTCAAATGCAACCTATACAAATA) and vpu-2 (5′-GCGGTCGACCTACAGATCATCAACATC). The upstream primer (vpu-1) contained an *Eco*RI restriction enzyme site and the downstream primer (vpu-2) contained a *Sal*I restriction enzyme site. The PCR conditions were as recommended by the manufacturer (Perkin-Elmer Cetus, Norwalk, Conn., USA), except that the concentration of $MgCl_2$ was 2 m*M* and that of the primers was 150 n*M*. Thirty cycles of amplification were performed in a DNA thermal Cycler (Perkin-Elmer Cetus) using the manufacturer's Amplitaq *Taq* DNA polymerase. Each PCR cycle employed a primer annealing step at 50°C for 30 s and an extension step at 72°C for 1 min. Immediately after amplification, SDS and EDTA were added to the PCR reaction (0.1% and 5 m*M*, respectively) and the DNA was precipitated with 2.5 *M* ammonium acetate and 70% ethanol. After digestion with *Eco*RI and *Sal*I, the DNA fragment was isolated by elution from agarose gel electrophoresis. The fragment, about 290 bp in size, was ligated to a vector, pGEX-KG [13], which had previously been digested with *Eco*RI and *Sal*I. The resultant plasmid, designated pGEX-VPU, was further confirmed by automated DNA sequencing (Model 373A, Version 1.0.2, Applied Biosystems, Foster City, Calif., USA) [26]. The plasmid pGEX-KG was also used for the induction and purification of the glutathione S-transferase (GST) protein, which served as a control in the WB assay.

Induction of the Recombinant Vpu Protein

For protein induction, a single ampicillin-resistant colony of BL21 (DE3) [13] harboring pGEX-VPU was picked and grown at 37°C in Luria-Bertani broth supplemented with ampicillin. When the density of the culture reached 0.5–0.7 at OD (optical density) 550, isopropyl-β-thiogalactopyranoside (IPTG; Boehringer Mannheim Biochemicals, Indianapolis, Ind., USA) was added to a final concentration of 0.4 m*M*. After induction for 3.5 h, bacteria were collected for protein purification. Detailed procedures of protein purification have been described previously [13].

WB Assay and IgG Subclass Determination

Recombinant proteins purified from glutathione agarose beads were used as antigens in the WB assay. The quantity of each recombinant protein used for an SDS-12.5% polyacrylamide minigel (Hoefer SE260) was as follows: 2 mg of GST-Vpu and 3 mg of GST. Serum samples at a dilution of 1:100 were analyzed for their anti-Vpu Ab reactivity using WB assay. Detailed procedures of the WB assay have been described previously [6]. A panel of positive control serum samples was used in the WB assay to identify the GST-Vpu recombinant protein: a rabbit anti-Vpu antiserum [18], an anti-GST monoclonal Ab [27], a rabbit anti-GST fusion protein antiserum-R4 [5] and a serum sample from an HIV-1-infected patient (No. 472).

The intensity of the Ab reactivity was measured using densitometry (1D Main program, American Applied Biotechnology, Fullerton, Calif., USA). The magnitude of the anti-Vpu Ab reactivity was determined by comparing the intensity of the reactive band of each patient in the WB with that of the serum No. 472 (1+ = Ab reactivity equal to or less than the Ab reactivity of serum No. 472; 2+ = Ab reactivity more than that of serum No. 472).

In addition, WB assay was used to determine the IgG subclass of anti-Vpu Ab in the serial serum samples of the anti-Vpu Ab seroconverters. Patient serum at a dilution of 1:50 was added to WB strips containing the recombinant Vpu protein. After incubation at 37°C for 90 min, the strips were washed 5 times and mouse monoclonal Abs anti-human IgG1-Fc, IgG2-Fc, IgG3-hinge and IgG4-Fc (Chemicon International Inc., Temecula, Calif., USA) at a dilution of 1:200 were added to different strips from the same patient. After incubation at 37°C for 40 min, the strips were washed 5 times and incubated with sheep anti-mouse Ig-horseradish peroxidase-linked whole Ab (Amersham Co., Arlington Heights, Ill., USA) at a dilution of 1:200 at 37°C for 40 min. After being washed 5 times, the strips were reacted with the substrate solution [0.05% 3,3′-diaminobenzidine (Sigma) and 0.05% H_2O_2 in PBS] at room temperature for 10 min.

DNA Sequencing of the vpu Gene

Peripheral blood mononuclear cells from 40 patients who did not have anti-Vpu Ab were collected for DNA sequencing of their HIV-1 *vpu* gene. Genomic DNA was obtained by phenol-chloroform extraction and ethanol precipitation. PCR was used to amplify the *vpu* gene with nested primers. One microgram of genomic DNA was used as the template for the first round of PCR. The reaction mixture contained 20 m*M* Tris-HCl (pH 8.0), 100 m*M* KCl, 0.1 m*M* EDTA, 1 m*M* DTT, 0.5% Tween 20, 0.5% Nonidet P-40, 50% glycerol, 2 m*M* $MgCl_2$, 200 μ*M* each of the deoxynucleoside triphosphates, 15 pmol of each of the primers and 0.5 μl of TaKaRa Ex Taq™ DNA polymerase (HT Biotechnology, Cambridge, UK). The primer pairs used in this study were ED3/ED14 (for the first PCR) [10] and 154.1/KPN (for the nested PCR and also for the automated DNA sequencing) [17]. The amplification conditions in the first-round PCR were 94°C for 15 s, 58°C for 45 s and 72°C for 1 min, for 35 cycles. A 1-μl aliquot of the first-round PCR product was used as the template for the nested PCR. The conditions for the nested PCR were 94°C for 15 s, 60°C for 45 s and 72°C for 1 min, for 35 cycles.

To remove the excess nucleotides and primers, the nested PCR products were treated with exonuclease I (0.3 U/μl of reaction) and shrimp alkaline phosphatase (0.08 U/μl of reaction) (Amersham Biosciences) at 37°C for 40 min, followed by inactivation at 80°C for 20 min. The DNA sequencing reaction was carried out in a half reaction volume (10 μl) using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). After removing the unincorporated free dyes by using ethanol-sodium acetate precipitation, the samples were run on the ABI PRISM™ 3700 DNA Analyzer (Applied Biosystems).

Nucleotide Sequence Accession Numbers

The nucleotide sequences of *vpu* gene from different patients have been deposited into the GenBank, and their accession numbers are AY137613 to AY137652 for the following patients: B-VPU24, B-VPU61, B-VPU609, B-VPU605, B-VPU590, B-VPU59, B-VPU103, B-VPU57, B-VPU239, B-VPU104, B-VPU63, B-VPU82, B-VPU256, B-VPU735, B-VPU301, B-VPU451, B-VPU1204, B-VPU139, B-VPU113, B-VPU133, B-VPU938, B-VPU126, B-VPU36, B-VPU377, B-VPU142, B-VPU563, B-VPU851, B-VPU532, B-VPU60, B-VPU44, B-VPU102, B-VPU380, B-VPU389, B-VPU556, CRF01_AE-VPU141, CRF01_AE-VPU529, CRF01_AE-VPU396, CRF01_AE-VPU432, CRF01_AE-VPU125 and CRF01_AE-VPU579. For phylogenetic tree analysis, several well-characterized reference sequences were selected to represent subtypes A–H. The subtypes and GenBank accession numbers of these strains were U455 (A, M62320), UG273 (A, L22957), UG275 (A, L22951), 92UG037 (A, U51190), SF2 (B, K02007), MN (B, M17449), JRFL (B, U63632), ETH2220 (C, U46016), 92BR025 (C, U52953), NDK (D, M27323), Z2Z6 (D, M22639), CM240X (AE, U54771), BZ126A (F, L22082), BZ163A (F, L22085), HH8793 (G, AF061641), SE6165 (G, AF061642), 92NG083 (AG, U88826), IBNG (AG, L39106), 90CR056 (H, AF005496) and SIVCPZUS (AF103818).

Phylogenetic Tree Analysis

The nucleotide sequences of *vpu* gene from 40 patients were edited using the BioEdit program [14], and the resultant 246-bp DNA fragments (nucleotide residues 6062–6307 of HXB2) were subjected to phylogenetic tree analysis. The neighbor-joining method and the Kimura 2-parameter distance matrix listed in the molecular evolutionary genetic analysis were used [16]. The full-length *vpu* genes were aligned with sequences of reference strains representing various HIV-1 subtypes (Los Alamos HIV database).

Quantification of Total Serum IgG and HIV-1-Specific Ab

Total serum IgG was measured using the N Antisera to Human Immunoglobulins Kit (Behringwerke AG, Marburg, Germany), and HIV-1 specific Ab was measured using the Abbott HIV-1/HIV-2 3rd Generation plus EIA (Abbott Co., North Chicago, Ill., USA). For the measurement of the IgG, an optimal amount of the patient's serum was diluted at 1:400 and reacted with rabbit anti-human IgG antiserum before it was analyzed using the Behring Nephelometer. The coefficients of variation of the nephelometry assay were less than 3% [1]. The Abbott HIV-1/HIV-2 EIA can detect both IgG and IgM to HIV-1 and HIV-2 antigens (HIV-1 recombinant gp41, p24 and HIV-2 recombinant gp36) simultaneously. For the measurement of the HIV-1-specific Ab, a 1:1,000 dilution of patient's serum was incubated with a polystyrene bead coated with recombinant HIV-1 and HIV-2 proteins in an EIA plate. Each patient's serum was tested in duplicate. The procedures were as recommended by the manufacturer.

Statistical Analysis

Microsoft Excel was used to construct a database and the SAS program was used for statistical analysis. The patients were divided into three groups according to their CD4 count and four groups according to their VL: CD4 $\geq 500/mm^3$ (group A), $200 \leq$ CD4 $< 500/mm^3$ (group B) and CD4 $< 200/mm^3$ (group C); VL undetectable or <500 copies/ml (group 1), $500 <$ VL $< 4{,}180$ copies/ml (group 2), $4{,}180 <$ VL $< 19{,}770$ copies/ml (group 3) and VL $> 19{,}770$ copies/ml (group 4). Fisher's exact test, Mantel-Haenszel χ^2 test or ANOVA was used to compare the rates and magnitudes of anti-Vpu Ab reactivity among the different groups mentioned above, as appropriate.

Results

Generation of a Recombinant Vpu Protein for the WB Assay

The 0.29-kb DNA fragment obtained from PCR was subcloned into the expression vector pGEX-KG in frame with GST. The resultant plasmid, designated pGEX-VPU, contains the full-length HIV-1 *vpu* gene, which was

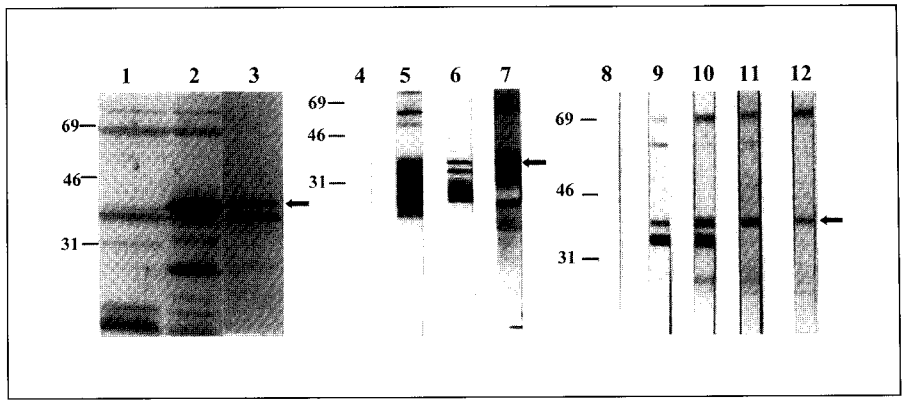


Fig. 1. Induction and purification of a recombinant Vpu protein for WB assay. Lanes 1–3: Commassie blue staining; lanes 4–12: WB assay. Antigens: lane 1: bacterial lysates before IPTG induction; lane 2: bacterial lysates after IPTG induction; lanes 3–12: purified products from the glutathione agarose beads which had reacted with bacterial lysates from lane 2. Abs: lanes 4 and 8: normal human serum (1:100 dilution); lane 5: a rabbit anti-GST fusion protein antiserum (1:100 dilution); lane 6: anti-GST monoclonal Ab from supernatant of a hybridoma culture (1:1 dilution); lane 7: a rabbit anti-Vpu antiserum (1:200 dilution); lanes 9–12: serum samples from HIV-1/AIDS patients (1:100 dilution). Molecular weight markers in kD are shown at the left margins of the panels. Arrows = GST-Vpu recombinant protein.

confirmed by DNA sequencing (data not shown). The plasmid pGEX-VPU was used to transform *Escherichia coli* and the GST-Vpu recombinant protein was induced by IPTG (fig. 1, lane 2). A GST-Vpu fusion protein 39 kD in size was identified in the glutathione agarose bead fraction by a rabbit anti-Vpu antiserum in a WB assay (fig. 1, lane 7). The GST-Vpu recombinant protein was further confirmed by using a rabbit anti-GST fusion protein antiserum or a monoclonal Ab against GST in a WB assay (fig. 1, lanes 5 and 6).

Correlation between the Anti-Vpu Ab Reactivity and AIDS Disease Progression

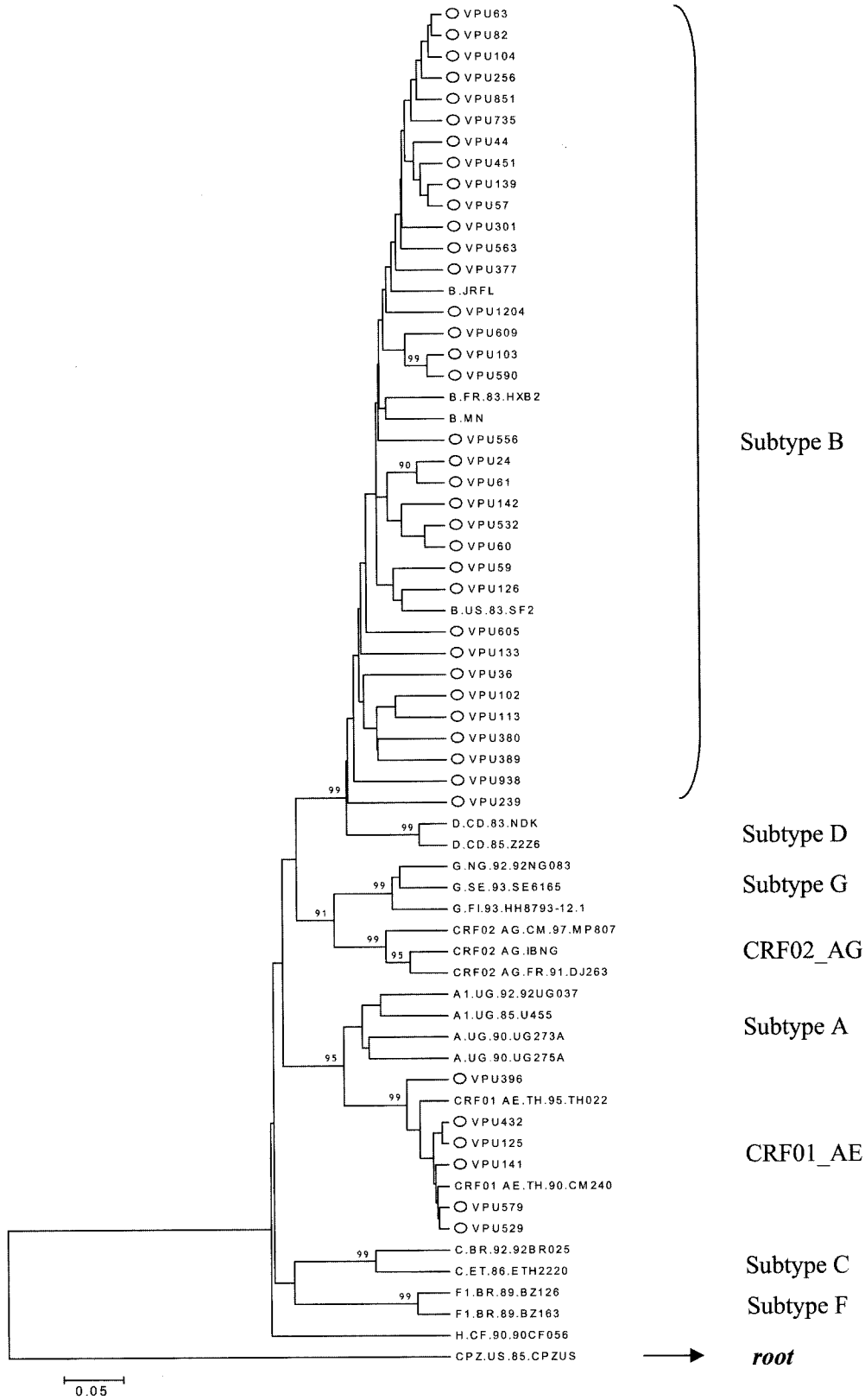
A total of 162 HIV-1-infected patients participated in this study. Before HAART, HIV-1 VL and CD4 counts of all the patients were measured and the correlation of both markers was statistically significant (Spearman's $r = -0.41$, $p < 0.0001$). The cohort was divided into groups A, B and C according to their CD4 counts and groups 1, 2, 3 and 4 according to their VL, and their Ab reactivity to Vpu was compared using a WB assay (fig. 2, right panel).

As shown in table 1, before HAART, 51 of 162 HIV-1/AIDS patients (31.5%) had anti-Vpu Ab. The anti-Vpu Ab-positive rates among the three groups of patients with CD4 counts ≥ 500, between 200 and 500 and <200 were 40.6, 35.6 and 15.0%, respectively, and the differences between the rates were statistically significant (χ^2 test, $p = 0.019$). The Ab reactivity was further quantified by densitometry and scored as either 1+ or 2+ by comparing the intensity of the reactive bands with a positive control. The results showed that significant associations were found between the levels of anti-Vpu Ab reactivity and CD4 counts (Mantel-Haenszel χ^2 test, $p = 0.012$, $\gamma = -0.35$).

In terms of the association of the anti-Vpu Ab reactivity and VL, 41% of patients with VLs <500 copies/ml had anti-Vpu Ab, and the seropositive rates decreased from 43.6 to 27.5 and 10.8% in different groups of patients with increasing levels of VL. In addition, the mean optic densities (OD) of the anti-Vpu Ab reactivity, measured by densitometry, were significantly different among the four groups of patients with different VLs (ANOVA, $p = 0.014$) and there was a significant association between different levels of anti-Vpu Ab reactivity and VLs (table 1, Mantel-Haenszel χ^2 test, $p = 0.0006$, $\gamma = -0.39$).

Changes in Anti-Vpu Ab Reactivity and CD4 Counts after HAART for 1 Year

As shown in table 2, after receiving HAART for 1 year, 51 patients exhibited substantial changes in their CD4 counts and had changes in their disease stages; 88.2% (45/51) moved upward to a healthier status, while only 11.8% moved downward. After HAART, the number of patients in the healthy carrier stage (CD4 $\geq 500/mm^3$) almost doubled, from 31 to 59, and the number of patients in the AIDS stage (CD4 $<200/mm^3$) decreased from 37 to 25.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VPU82
VPU104
VPU256
VPU851
VPU735
VPU44
VPU451
VPU139
VPU57
VPU301
VPU563
VPU377
B.JRFL
VPU1204
VPU609
VPU103
VPU590
B.FR.83.HXB2
B.MN
VPU556
VPU24
VPU61
VPU142
VPU532
VPU60
VPU59
VPU126
B.US.83.SF2
VPU605
VPU133
VPU36
VPU102
VPU113
VPU380
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D.CD.85.Z2Z6
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VPU141
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C.ET.86.ETH2220
F1.BR.89.BZ126
F1.BR.89.BZ163
H.CF.90.90CF056
CPZ.US.85.CPZUS
0.05
Subtype B
Subtype D
Subtype G
CRF02_AG
Subtype A
CRF01_AE
Subtype C
Subtype F
root

2

Table 1. Anti-Vpu Ab reactivities in groups of HIV-1-infected patients with different CD4 cell counts or HIV-1 VLs

	Positive Ab reactivity to Vpu			Negative Ab reactivity to Vpu
	all	1+	2+	
Total patients (n = 162)	51 (31.5)	38 (23.5)	13 (8.0)	111 (68.5)
CD4 cell count				
Group A: ≥ 500/mm^3 (n = 32)	13 (40.6)	7 (21.9)	6 (18.8)	19 (59.4)
Group B: 200 < CD4 < 500/mm^3 (n = 95)	33 (34.7)	27 (28.4)	6 (6.3)	62 (65.3)
Group C: <200/mm^3 (n = 35)	5 (14.3)	4 (11.4)	1 (2.9)	30 (85.1)
VL				
Group 1: ≤ 500 copies/ml (n = 46)	19 (41.3)	14 (30.4)	5 (10.9)	27 (58.7)
Group 2: 500 < copies ≤ 4,180/ml (n = 39)	17 (43.6)	12 (30.8)	5 (12.8)	22 (56.4)
Group 3: 4,180 < copies ≤ 19,770/ml (n = 40)	11 (27.5)	8 (20.0)	3 (7.5)	29 (72.5)
Group 4: > 19,770 copies/ml (n = 37)	4 (10.8)	4 (10.8)	0 (0.0)	33 (89.2)

Figures in parentheses represent percentages, except where otherwise indicated. Statistical analysis of CD4 cell counts: Fisher's exact test (2-tailed), p = 0.03; Mantel-Haenszel χ^2 test, p = 0.006, $\gamma = -0.368$. Statistical analysis of VLs: Fisher's exact test (2-tailed), p = 0.025; Mantel-Haenszel χ^2 test, p = 0.001, $\gamma = -0.375$.

The patients' anti-Vpu Ab reactivity was evaluated again after they had received HAART for 1 year. Seven of 111 anti-Vpu Ab-negative patients (6.3%) seroconverted (– → + group), 8 of 51 anti-Vpu Ab-positive patients (15.7%) became seronegative (+ → – group) and 147 patients had no change in their anti-Vpu Ab status. In addition, among patients in group B (AIDS-related complex) and group C (AIDS), the anti-Vpu Ab-positive rate of patients whose CD4 cell counts moved upward to better stages was significantly higher than that of the patients whose CD4 cell counts stayed the same or moved downward to worse stages [the sums of the numbers in the two boxed areas in table 2: 19/45 (42.2%) vs. 18/80 (22.5%); χ^2 test, $p < 0.05$].

Total Serum IgG and Anti-HIV-1 Abs in Patients with Changed or Unchanged Anti-Vpu Ab Status

The patients were divided into four groups (+ → +, + → –, – → + and – → –) according to their status of anti-Vpu Ab reactivity before and after HAART. Total serum IgG and HIV-1-specific Abs of the patients before and after HAART were analyzed. As shown in table 3, the means of the total IgG were significantly different among the four groups before HAART ($p < 0.05$). After receiving HAART for 1 year, only the seroconverted (– → +) group had increased levels of total IgG and anti-HIV-1 Abs, while the other three groups had decreased levels of both markers ($p < 0.01$ and $p < 0.05$, respectively).

IgG Subclasses of the Vpu-Specific Abs in the Seroconverters

Mouse monoclonal Abs against different human IgG subclasses (IgG1, IgG2, IgG3 and IgG4) were used in the WB to distinguish the subclass of the anti-Vpu Ab in the patients belonging to the seroconverted (– → +) group. Sequential serum samples at 4-month intervals from 5 seroconverters were used for the analysis. The results showed that 3 patients generated both IgG1 and IgG2 anti-Vpu Abs, 1 patient (patient No. 963) generated IgG1, IgG2 and IgG3 anti-Vpu Abs and 1 patient (patient No. 916) generated both IgG2 and IgG4 anti-Vpu Abs.

Analysis of the vpu Gene

Since a number of HIV-1 isolates were naturally *vpu*(–) viruses due to Vpu deletions or inactivation of the initiation codon, we decided to analyze the *vpu* gene of the HIV-1 isolates from patients who lacked an immunological response to Vpu in this study. Forty of 104 anti-Vpu Ab-seronegative cases were analyzed and none of them

Fig. 2. Phylogenetic tree analysis of 40 HIV-1 isolates (circles) according to their *vpu* gene. The consensus neighbor-joining trees were obtained from 1,000 bootstrap samples of aligned *vpu* sequences corresponding to the nucleotide sequence 6070–6302 of HXB2 from different HIV-1 isolates. Sequences from different subtypes or circulating recombinant forms (CRFs) were also included for analysis.

Table 2. Rates of patients with anti-Vpu Ab who remained in the same or changed the CD4 T cell counts category after receiving HAART for 1 year

Before HAART			After receiving HAART for 1 year			
CD4 counts group			CD4 ≥ 500	200 < CD4 < 500	CD4 ≤ 200	Total
A CD4 ≥ 500	31 patients	→	27	4	0	
Anti-Vpu Ab+	13 (41.9%)		10 (37.0%)	2 (50.0%)		
B 200 < CD4 < 500	88 patients	→	31	55	2	
Anti-Vpu Ab+	31 (35.2%)		14 (45.2%)	14 (25.5%)	1 (50.0%)	
C CD4 ≤ 200	37 patients	→	1	13	23	
Anti-Vpu Ab+	6 (16.2%)		1 (100%)	4 (30.8%)	3 (13.0%)	
Total	156 patients	→	59	72	25	156
Anti-Vpu Ab+	50 (32.1%)		25 (42.4%)	20 (27.8%)	4 (16.0%)	49 (31.4%)

Table 3. Total IgG and anti-HIV-1 Abs in HIV-1/AIDS patients organized into four groups based on the status of anti-Vpu Ab reactivity before and after HAART for 1 year

Anti-Vpu Ab[1]	Total IgG, mg/dl			Anti-HIV-1 Ab, OD ratio		
	n	before HAART	change after HAART[2]	n	before HAART	change after HAART[2]
+ → +	43	1,704.6 ± 595.2	−210.0 ± 519.2	38	2.64 ± 0.65	−0.14 ± 0.52
+ → −	8	1,771.0 ± 377.3	−240.0 ± 393.1	8	2.99 ± 0.79	−0.69 ± 0.51
− → +	7	1,567.3 ± 517.7	220.4 ± 415.7	7	1.87 ± 1.17	0.59 ± 0.78
− → −	104	1,694.2 ± 481.4	−111.6 ± 395.6	95	2.64 ± 0.80	−0.28 ± 0.52
ANOVA		F = 2.7921	F = 8.2405		F = 1.475	F = 3.335
		p < 0.01	p < 0.001		p = 0.2238	p < 0.05

Total IgG and anti-HIV-1 Ab values are shown as mean ± standard deviation.

[1] Groups of patients with different patterns of anti-Vpu Ab reactivity before (left of the arrow) and after (right of the arrow) receiving HAART for 1 year.

[2] The levels of total IgG or anti-HIV-1 Ab after 1 year of HAART minus the levels of IgG or anti-HIV-1 Ab before the treatment.

had a *vpu*-deleted mutant. Phylogenetic tree analysis showed that 34 were infected with HIV-1 subtype B and 6 were infected with HIV-1 CRF01_AE (fig. 2).

Discussion

In this study, we used a recombinant GST-Vpu fusion protein in WB assay to analyze anti-Vpu Ab reactivity in an HIV-1/AIDS patient cohort receiving HAART. The GST-Vpu fusion protein, about 39 kD in size, has been identified by several antisera, including a rabbit anti-Vpu antiserum [18], a monoclonal Ab against GST [27] and a rabbit polyclonal anti-GST fusion protein antiserum [5]. Since the size of the GST portion of the fusion protein was about 26 kD, the Vpu portion of the fusion protein was about 13 kD, which is smaller than the expected size of full-length Vpu (16 kD). It is likely that the hydrophobic region of the transmembrane domain misfolds in the cytoplasm of *E. coli*. This may lead to proteolytic cleavage of the fusion protein and generation of smaller Vpu products. As shown in lane 6 of figure 1, the large band migrating below the GST-Vpu (arrow) may represent the smaller Vpu products.

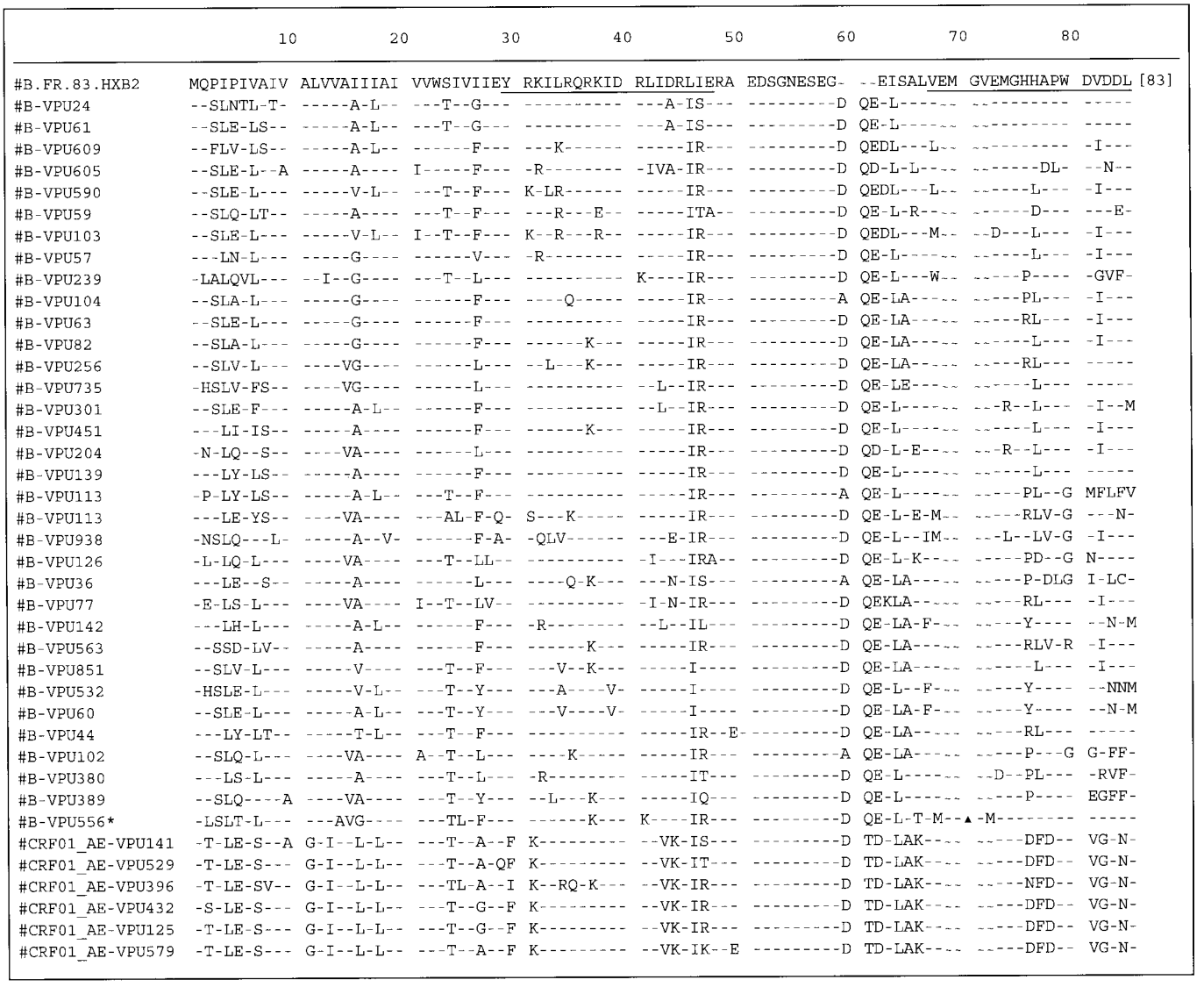

Fig. 3. Alignment of the predicted amino acid sequences of the Vpu protein from 40 isolates in the present study. The amino acid sequence of Vpu from HXB2 is shown at the top. Dashes indicate amino acids identical to those of HXB2, and ▲ indicates insertion of six amino acids – HHAPAP. The sequences underlined are peptides used in an EIA for the detection of anti-Vpu Ab by Schneider et al. [24] and ~ indicates deletion.

In this study, 31.5% of HIV-1/AIDS patients were found to have anti-Vpu Ab, and decreasing levels of anti-Vpu Ab reactivity correlated with AIDS disease progression. The seropositive rate of anti-Vpu Ab in this study was higher than the rate (26%) reported by Schneider et al. [24]. In both our study and that of Schneider et al. [24], it was found that anti-Vpu Ab is associated with disease progression. The discrepancy in the rates of anti-Vpu Ab may be due to the cohorts used in the studies and the methods employed to detect the anti-Vpu Ab. Instead of using a recombinant protein in WB assay, Schneider et al. [24] used synthetic peptides containing 18 carboxyl-terminal amino acids of the Vpu protein in EIA to detect the anti-Vpu Ab. As shown in figure 3, the synthetic peptide that Schneider et al. [24] used (underlined sequence at the carboxyl-terminal region of HXB2) in the EIA contains fewer conserved epitopes than the central region of the Vpu protein.

Furthermore, to rule out the possibility that the 104 anti-Vpu Ab-negative patients were infected with an HIV-1 *vpu* gene-deleted/mutated variant, we selected 40 cases from among them for DNA sequencing. The results showed that all of the major HIV-1 that they had contained intact *vpu* genes. The predicted amino acid sequences of the Vpu proteins are presented in figure 3. In comparison with the Vpu sequence of HXB2, most of the Taiwanese isolates had the L46I mutation, 60–62DQE insertion and 69–72EMGV deletion. In addition, there were signature patterns of the Vpu proteins either from clade B or from CRF01_AE.

Previously, Schneider et al. [24] reported that HIV-1 patients at WR stages 1 and 2 had significantly higher rates (36 and 42%) of anti-Vpu Ab compared with patients classified into WR stages 3–6 (11%). Their results are consistent with the data that we have presented in this study (table 1). In addition, neither study demonstrated an increase in anti-Vpu Ab-seropositive rates in the final stage of AIDS disease progression, a phenomenon observed previously by Matsuda et al. [19].

Previously, using the same WB assay format, we analyzed Ab reactivity to different HIV-1 antigens in the same AIDS patient cohort and found that the Ab-positive rates for HIV-1 integrase, Gag-p24, Nef and Tat proteins were 93.3, 81.0, 77.1 and 50.3%, respectively [3, 4]. Therefore, Vpu is the least immunogenic protein among the above-mentioned HIV-1 antigens. The Vpu protein not only has a low seropositive rate in HIV-1-infected people, but also stimulates less Ab reactivity than other HIV-1 antigens. For instance, in previous studies, the magnitudes of Ab reactivity of anti-integrase, anti-Gag and anti-Nef Abs were graded from 1+ to 4+, where 1+ was defined as Ab reactivity equal to or less than that of a positive serum sample at a dilution of 1:10,000 [3, 4]. In this study, however, the anti-Vpu Ab reactivity was graded from 1+ to 2+, with 1+ being defined as Ab reactivity equal to or less than that of a positive serum at a dilution of 1:100.

Before HAART, the mean of the IgG levels in patients with anti-Vpu Ab was 1,723.1 ± 338.8 mg/dl, which was slightly higher than that in patients without anti-Vpu Ab (1,667.3 ± 466.5 mg/dl). In addition, patients with 2+ anti-Vpu Ab reactivity had a higher level of total IgG than patients with 1+ anti-Vpu Ab reactivity (1,851.9 ± 499.1 vs. 1,668.2 ± 583.3 mg/dl).

In this study, we followed up the patients' anti-Vpu Ab reactivity after they had received HAART for 1 year and found that 7 of 111 anti-Vpu Ab-negative patients (6.3%) seroconverted and 8 of 43 anti-Vpu Ab-positive persons (15.7%) lost the Ab reactivity. Previously, in the same cohort, we found that 8 of 38 anti-Nef Ab-negative patients (21.1%) seroconverted, while only 9 of 125 anti-Nef Ab-positive patients (7.2%) lost the Ab reactivity [4]. The difference in the anti-Vpu and anti-Nef Ab seroconversion rates also reflects the degree of immunogenicity of these viral antigens.

In terms of IgG subclasses, most of the anti-Vpu Ab seroconverters generated both IgG1 and IgG2 anti-Vpu Abs after HAART, while only 1 seroconverter generated IgG2 and IgG4 anti-Vpu Abs. It has been postulated that T helper 1 cells induce the production of opsonizing Abs belonging to certain IgG subclasses, e.g. IgG1 and IgG3 in humans, whereas T helper 2 cells initiate the humoral response by activating naïve B cells to secrete IgM and induce other Ab isotypes, including IgG2, IgG4, IgA and IgE [9]. Therefore, the seroconversion of anti-Vpu Ab in patients receiving HAART may reflect the restoration of T helper cell immunity.

When both the total IgG and HIV-1 specific Abs were compared before and after HAART, the 'anti-Vpu Ab seroconverters' were the only group of patients who had increased levels of total IgG and anti-HIV-1 Abs. Furthermore, the changes in HIV-1-specific Abs were significantly different among those four groups (table 3). Therefore, seroconversion of anti-Vpu Ab during HAART may indicate significant recovery of the patient's immune system. Further studies are needed to elucidate whether the specific Abs increased in the anti-Vpu Ab seroconverters are neutralizing Abs, and whether seroconversion of anti-Vpu Ab following HAART is associated with survival in AIDS patients.

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