Relationship between the level of HIV-1 proviral DNA and HIV-1- specific cytotoxic T lymphocyte response in HIV-1 infected Koreans

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Directed by Professor **June Myung Kim**

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Jun Yong Choi

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감사의 글

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능력이 많지 않은 저를 늘 애정과 관심으로 지켜봐 주시는 세열회 식구들의 따뜻한 성원에도 감사드립니다. 실험을 준비하고 진행해 나가는데 열과 성을 다하여 도움을 주신 김영화 선생님께 감사드립니다.

저를 사랑으로 보살펴 주신 어머님, 아버님과 처가 부모님께 감사드립니다. 마지막으로 늘 저를 믿고 따라주며 변함없는 사랑을 보내준 소중한 아내와 사랑스런 자녀 정우, 유진이와 함께 기쁨을 나누고 싶습니다.

저 자 씀

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Relationship between the level of HIV-1 proviral DNA and HIV-1- specific cytotoxic T lymphocyte response in HIV-1 infected Koreans

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Background: The use of highly active antiretroviral therapy (HAART) in the treatment of human immunodeficiency virus type 1 (HIV-1)-infected individuals has resulted in dramatic improvements in the clinical outcomes of many infected persons. However, HAART does not eliminate the persistently replicating, latent HIV-1 reservoir in patients, despite the viral suppression associated with the therapy. HIV-specific cytotoxic T lymphocytes (CTLs) play a central role in the immune response to HIV infection. The objective of this study is to ascertain the relationship between the level of HIV-1 proviral DNA and CTL response in HIV-infected Koreans.

Patients and Methods: Eighty-two HIV-1-infected Koreans who had been admitted to Severance Hospital, Yonsei University College of Medicine, were included in this study, after obtaining their informed consents. HLA class I genes were typed, using the sequencing-based typing method. CD4+ T cells were isolated from the peripheral blood mononuclear cells (PBMC) of HIV-1-infected individuals, using a human CD4 cell-separation kit. In order to determine the frequency of CD4+ T cells carrying HIV-1 provirus in infected individuals, real-time PCR was carried out. DNA (1 ng) from the CD4+ T cells was used as a template for the real-time PCR. An enzyme

linked immunospot (ELISPOT) assay, using HLA-I restricted peptides, was also performed to evaluate the responses of the HIV-1- specific cytotoxic T lymphocytes. Spearman's rank correlations or Pearson's correlation were used to correlate the frequency of HIV-1- specific CTLs with the frequency of CD4+ T cells harboring HIV-1 proviral DNA.

Results: No significant differences in the frequency of CD4+ T cells harboring HIV-1 proviral DNA were detected between patients receiving, and those who did not receive, HAART. There was, however, a statistically significant inverse correlation between the frequency of CD4+ T cells harboring HIV-1 proviral DNA and the CD4+ T cell counts in the peripheral blood of subjects (P=0.01, r=-0.307). Among the patients receiving HAART, the frequency of CD4+ T cells harboring HIV- 1 proviral DNA was lower in patients in whom viral load had been suppressed to levels below 25 copies/mL than in patients in whom viral load had not been suppressed (P=0.033). CTL response was also found to be higher in patients with unsuppressed viral loads than in patients whose viral loads had been suppressed to undetectable levels (P=0.02). In patients with the HLA-A genotype of HLA-A*0201, who also exhibited undetectable levels of plasma viral load due to suppression, the frequency of CD4+ T cells harboring HIV-1 proviral DNA did not correlate with the CTL response (P=0.604). Among the 28 patients studied with HLA-A genotype of HLA-A*0201, 18 (64.3%) exhibited sequence variations in the Gag $p17_{77-85}$ epitope. Among 26 patients studied with HLA-A genotype other than HLA-A02, 19 (73.1%) exhibited sequence variations in the Gag $p17_{77-85}$ epitope.

Conclusions: HAART does not eradicate the proviral DNA. This result is also consistent with the results of previous studies. CTL response was affected by plasma HIV RNA, but not by proviral DNA. There were immune escape mutants in these chronically HIV-1 infected Koreans. However, a comparison of viral sequences derived from the HLA-A*0201 positive individuals to sequences obtained from HLA-A02 negative individuals demonstrated some weak evidence for immune selective pressure, thus bringing into question the in vivo efficacy of immunodominant CTL responses during chronic HIV-1 infection. As neither HAART nor HIV-1 specific CTL eradicate proviral reservoirs, another strategy is clearly required in order to eradicate HIV infection.

Key Words: HIV, HIV infection, AIDS, proviral DNA, CTL response, escape mutant

Relationship between the level of HIV-1 proviral DNA and HIV-1- specific cytotoxic T lymphocyte response in HIV-1 infected Koreans

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I. Introduction

The use of highly active antiretroviral therapy (HAART) in the treatment of human immunodeficiency virus type 1 (HIV-1)-infected individuals has dramatically changed the clinical outcomes of many infected persons, and has contributed to current substantial declines in both the incidence of AIDS, and AIDS-related mortality[1]. However, replication-competent virus, HIV-1 proviral DNA (including 2 long terminal repeat circles), spliced and unspliced HIV-1 RNA in CD4+ T cells, and unidentified viral reservoirs have all been demonstrated in most infected individuals, even those in whom plasma viremia has been suppressed below detectable levels[2-8]. These sources of ongoing replication have emerged as the major obstacle to the eradication of HIV- 1. Continuous HAART treatment does not eliminate viral replication in the peripheral blood, or more specifically, in the memory (CD45RO) T lymphocytes[9-11]. HAART also does not prevent replication in cells harboring competent HIV-1 proviral DNA. These facts underline the impossibility of eradicating HIV-1 using only HAART[2,4,12-14].

The ability to generate high levels of broadly specific anti-HIV-1 cytotoxic T lymphocytes (CTL) is considered to be a critical component of the host immune response to HIV-1[15-21]. Studies of immune responses generated in HIV-infected individuals

indicate that CD8+ T cells play an important role in the control of viremia. The emergence of HIV-specific CD8+ CTL activity coincides with the clearance of viremia during primary HIV infection[15,18], and declines in CTL response tend to be associated with disease progression in infected individuals[17].

As with other chronic infectious diseases, it is possible that the long-term efficacy of combination antiretroviral therapy will require the augmentation of host immune responses, most notably, that of anti-HIV-1 CTL reactivity. Recent studies, however, have yielded contradictory evidence regarding the impact of combination antiretroviral therapy on anti-HIV-1 CTL reactivity. Several reports have asserted that the numbers of anti-HIV-1 CTL precursors, as measured *in vitro* in a 2-week, limiting dilution assay, increase due to suppressive antiretroviral therapy in acute and chronically infected patients[22-24]. Conversely, others have reported that the levels of circulating CD8+ CD38+ T cells, which bind HLA-A2 tetrameric HIV-1 Gag p17 and RT peptide complexes, actually decrease after the initiation of antiretroviral therapy in patients with advanced immunodeficiency[23,25-27]. Recently, another study has provided some evidence that triple-drug antiretroviral therapy fails to produce a sustained increases in anti-HIV-1 CD8+ T-cell function in HIV-1-infected patients with advanced immunodeficiency[28]. Two basic patterns of HIV-1-specific T-cell reactivity resultant from combination antiretroviral therapy have emerged. One involves an early rise, from very low pretreatment levels, in anti-HIV-1 CTL and IFN-γ producing CD8+ cells specific for HIV-1 Gag, Pol, and Env during triple-drug therapy. However, a decline to baseline levels was observed in most of these patients within 2 years. The second pattern of anti-HIV-1 CD8+ T-cell responses in the study was the total failure of CTL and IFN-γ reactivity to increase above the low baseline levels, throughout the entire 2 years of triple-drug therapy.

The mechanisms underlying the maintenance or disappearance of CTL after HAART remain unclear. The effects of CTL on persistent replicating reservoirs of HIV infection are also not yet well known.

I postulated that HIV-1 proviral DNA, reflecting the HIV-1 reservoir, might induce CTL responses in HIV-infected individuals.

The objective of this study was, then, to ascertain what relationship exists between the level of HIV-1 proviral DNA and CTL response in HIV-infected Koreans.

II. Patients and Methods

1. Study Patients

Eighty-two HIV-1-infected Koreans who had been admitted to Severance Hospital, Yonsei University College of Medicine, were enrolled in this study, after obtaining their informed consent. All subjects were chronically HIV-1 infected patients. Their HIV RNA levels were measured by nucleic acid sequence-based amplification, using the NucliSens®EasyQ Analyzer (Biomerieux, Boxtel, Netherlands). CD4+ or CD8+ T lymphocyte counts were calculated by multiplying the number of lymphocytes measured with an automatic cell counter, by the percentage of CD4+ or CD8+ antigen positive cells, using monoclonal antibody (Becton-Dickinson, New Jersey, USA).

2. HLA Genotyping

HLA class I genes were typed using the sequencing-based typing method (by Biosewoom Co, Seoul, Korea). Polymorphic regions (exon 1, intron 1, exon 2, intron 2, and exon 3) were amplified using a set of HLA class I locus-specific primers. Both strands of exons 2 and 3 were sequenced with an automated DNA sequencer, using nested sequencing primers, as has been previously described[29].

3. The frequency of CD4+ T cells harboring HIV-1 proviral DNA

A. Isolation of CD4+ T cells

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque density gradient centrifugation. CD4+ T cells were isolated from the PBMCs of HIV-1- infected individuals, using a human CD4 cell-separation kit (EasySepTM, StemCell Technologies, Vancouver, Canada).

B. Quantitative real-time HIV-1 DNA PCR

In order to determine the level of HIV-1 provirus-harboring CD4+ T cells in the

infected individuals, real-time polymerase chain reaction (PCR) was conducted, as described below. Genomic DNA was isolated from 1-2X106 purified CD4+ T cells, using a DNA isolation kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. DNA (1 ng) was then used as a template for real-time PCR, which was performed in an iCycler (Bio-Rad, CA, USA). The amplification reaction was done in triplicate, using 0.5 μM primers, a 0.2 μM fluorescent probe, 0.8 mM dNTPs, 5 mM MgCl2, and 2.5 U Platinum Taq Polymerase (Applied Biosystems, Foster City, CA, USA) in a total volume of 50 μL. Primers 5'-GGTCTCTCTGGTTAGACCAGAT-3' (5' primer) and 5'-CTGCTAGAGATTTTCCACACTG-3' (3' primer) were used, along with the fluorescent probe, 5'-6FAM-AGTAGTGTGTGCCCGTCTGTTTAMRA-3'. PCR conditions consisted of a denaturation step at 95℃ for 3 minutes, followed by 45 cycles of 15 seconds at 95℃, and 1 minute at 58℃. Serially diluted ACH-2 DNA (from the National Institute of Health, USA) was also subjected to PCR, as above, in order to obtain standard curves.

4. HIV-1 specific cytotoxic T lymphocyte response

An enzyme linked immunospot (ELISPOT) assay was performed in order to establish the number of IFN-γ producing cells, as described previously[28]. Nitrocellulose membranes in 96-microwell polyvinylidene difluoride-backed plates were coated overnight at 4℃ with 50 μL of anti-IFN-γ MAb per well. The antibody-coated plates were then washed four times with phosphate- buffered saline (PBS), and treated with 180 μL of RPMI medium containing 10% human serum per well, for 1 hour at 37℃. The responder cells for this assay were PBMCs or, when sufficient cells were available, CD8+ cells enriched by the negative selection of PBMC with antibody-coated magnetic beads (anti-CD4, anti-CD19, and anti-CD16 MAb) to remove CD4+ cells, B cells, and natural killer cells, respectively. A total of 10^5 to 10^6 of these PBMC or CD8+ cells were incubated overnight, at 37℃ in 5% CO_2, with HIV-1 peptides (10 μL/mL) in nitrocellulose membrane 96-well plates. The plates were washed four times with PBS containing 1% BSA, and 2 μL of the secondary antibody (biotin-conjugated anti-IFN-γ MAb 7-B6-1) per mL was added to each well; the plates were then incubated for 2 hours at 37℃ in CO_2. The plates were washed four

times with PBS containing 1% BSA, then treated with avidinbound, biotinylated horseradish peroxidase H for 1 hour at room temperature. The plates were then washed an additional three times with PBS containing 1% BSA and three times with PBS alone, followed by a 5-minute incubation with 100 μL of 3- amino-9-ethylcarbazole per well. The reaction was stopped with running tap water. Red-brown spots, representing single CD8+ T cells producing IFN-γ, were counted using a dissecting microscope. PBMC or CD8+ T cells stimulated with phytohemagglutinin were used as a positive control. The number of antigen-specific, CD8+ T-cell- producing IFN-γ cells was calculated by subtracting the number of spot-forming cells in the medium control from the peptide-stimulated cells.

5. Sequencing of HLA-A*0201 restricted epitopes

Genomic DNA from CD4+ T cells was used for a PCR template. The primers used in the Pol $RT_{476-484}$ PCR were 5'-GACAGCTGGACTGTCAATGAC-3' and 5'-GTGGCTTGCCAATACTCTGTC-3'. The primers used in the Gag $p17_{77-85}$ PCR were 5'-CCTGGCCTGTTAGAAACATCA-3' and 5'-TGCTCTTCCTCTATCTTGTCT-3'. PCR was carried out with 1 μL of template DNA, 20 pmol of each primer, and PreMix (Bioneer, Daejeon, Korea) containing 1 μg of Taq DNA polymerase in a total volume of 20 μL. Model 2400 PE Thermal Cycler (Applied Biosystems, San Jose, CA, USA) was used for DNA amplification, which was performed at 94℃ for 5 minutes, followed by 35 cycles at 94℃ for 30 seconds, 55℃ for 30 seconds, and 72℃ for 30 seconds. The PCR products were directly sequenced using the primers and the Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Warrington, UK).

6. Statistical analysis

Spearman's rank correlation or Pearson's correlation were used to measure correlations between ① the frequency of CD4+ T cells carrying HIV-1 proviral DNA and immunologic, virologic, and clinical parameters, and ② the frequency of HIV-1- specific CTLs and the numbers of CD4+ T cells carrying HIV-1 proviral DNA, immunologic, virologic, and clinical parameters.

III. Results

1. Study Patients

The characteristics of the enrolled patients are described in Table 1. Twenty-two of these were naive with respect to antiretroviral treatment at the moment of blood sampling, and 60 were receiving various HAART regimens. The mean CD4 count was 380±203 cells/ μL, and the mean plasma HIV RNA level was 69,998±387,302 copies/ mL.

Table 1. Baseline characteristics of patients included in the study

Characteristics	
Sex (no. of male/no of female)	71:11
Age (mean±standard deviation)	39.5±9.2(22-71)
No. of AIDS patients	34(41.5%)
Mean CD4 count (cells/μL)	380±203(2-890)
Mean CD8 count (cells/μL)	791±387(136-2355)
Mean plasma HIV RNA (copies/mL)	69,998±387,302(<25-3100000)
No. of patients on HAART	60(73.2%)

2. HLA-A genotyping

HLA-A genotypes were detected in 75 subjects. Allele frequencies at the HLA-A loci are shown in Table 2. For the HLA class I region, 9 antigens in the A locus were identified in 75 HIV-1 infected Koreans. A02 (28.0%), A24 (25.3%), A33 (14.0 %), A26 (12.7%), and A11 (10.7%) were the predominant antigens, and were all detected in frequencies exceeding 10%.

Table 2. Gene frequencies of HLA-A in 75 HIV-1 infected Koreans

Allele	No. of allele	Allele frequency(%)
A01	1	0.7
A02	42	28.0
A03	3	2.0
A11	16	10.7
A24	38	25.3
A26	19	12.7
A30	6	4.0
A31	4	2.7
A33	21	14.0
Heterozygote	60	80.0
Homozygote	15	20.0

3. The frequency of CD4+ T cells harboring HIV-1 proviral DNA and correlation between various immunologic and virologic parameters

The mean frequency, in all subjects, of HIV-1 proviral DNA-harboring CD4+ T cells was 48.4±92.1 pg/ng genomic DNA. No significant differences were detected in the frequencies of HIV-1 proviral DNA-harboring CD4+ T cells between the HAART-naive patients and the patients who were receiving HAART (48.9±81.0 pg/ng genomic DNA vs. 48.2±96.9 pg/ng genomic DNA, $P>0.05$). There was no correlation between duration of HAART and the frequency of CD4+ T cells carrying HIV-1 proviral DNA. There was, however, a statistically significant inverse correlation detected between the frequency of CD4+ T cells carrying HIV-1 proviral DNA, and the CD4+ T cell counts in the peripheral blood of subjects ($P=0.01$, $r=-0.307$, Figure 1). However, no correlation was discovered to exist between the frequency of CD4+ T cells carrying HIV-1 proviral DNA and CD8+ T cell counts ($P>0.05$, Figure 1). Among the patients receiving HAART, the frequency of CD4+ T cells harboring HIV-1 proviral DNA was lower in patients in whom viral loads had been suppressed to below 25 copies/ mL

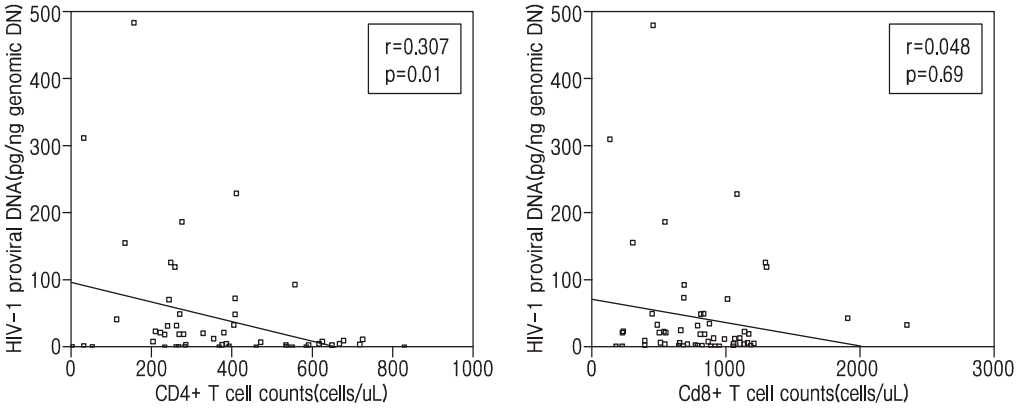

Figure 1. Relationship between the frequency of CD4+ T cells harboring human immunodeficiency virus type 1 (HIV-1) proviral DNA and immunologic parameters. Correlations were assessed between the frequency of cells harboring HIV-1 proviral DNA and the numbers of CD4+ and CD8+ T cells.

than in patients with unsuppressed viral loads (20.7±33.8 pg/ng of genomic DNA vs. 106.1±149.3 pg/ng of genomic DNA, P=0.033, Figure 2). There was no correlation between the duration of HAART and the frequency of CD4+ T cells harboring HIV-1 proviral DNA(P>0.05).

4. HIV-1 specific cytotoxic T lymphocyte response

We performed ELISPOT assays using HLA-A*0201 restricted peptide (Pol $RT_{476-484}$ ILKEPVHGV, and Gag $p17_{77-85}$ SLYNTVATL) in patients with HLA-A genotypes of A*0201. The mean CTL response was 532±679 SPCs/10^6 cells. CTL responses could not be correlated with either CD4+ T cell counts or CD8+ T cell counts (P>0.05, Figure 3). CTL response was higher in patients (n=13) with unsuppressed viral loads than in patients (n=13) in whom viral loads had been suppressed to undetectable levels (856 SPCs/10^6 cells vs. 196 SPCs/10^6 cells, P=0.02, Figure 4).

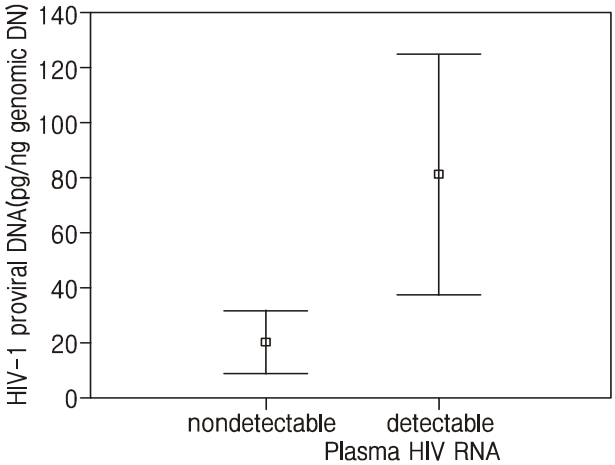

Figure 2. The frequency of HIV-1 proviral DNA-harboring CD4+ T cells in patients receiving HAART. The frequency of CD4+ T cells harboring HIV-1 proviral DNA was lower in patients in whom the viral load had been suppressed below 25 copies/ mL, than in patients with unsuppressed viral loads (20.7±33.8 pg/ng of genomic DNA vs. 106.1±149.3 pg/ng of genomic DNA, *P*=0.033).

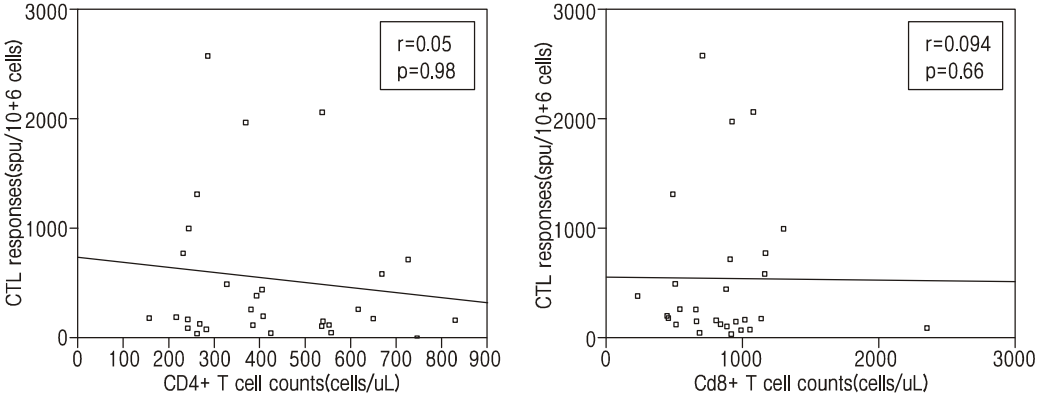

Figure 3. Relationship between cytotoxic T lymphocyte responses, as assessed by ELISPOT assays, and immunologic parameters in HIV-1 infected persons with HLA- A genotype of A*0201. Correlations were assessed between spot-forming units by ELISPOT assays, and CD4+ and CD8+ T cell counts

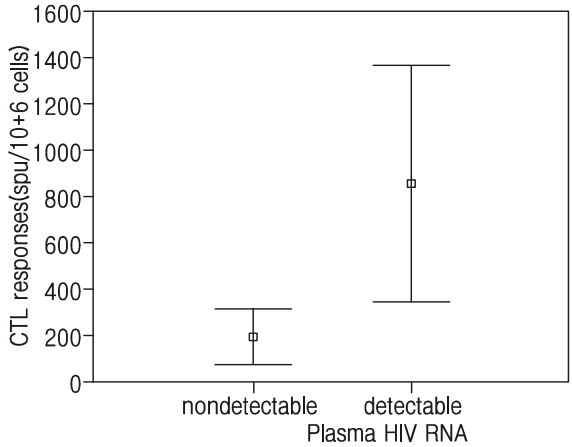

Figure 4. Cytotoxic T lymphocyte responses (CTL), as determined by ELISPOT assays, according to levels of plasma HIV RNA. CTL responses were higher in patients with unsuppressed viral loads than in patients whose viral loads had been suppressed to undetectable range (856 SPCs/10^6cells vs. 196 SPCs/10^6 cells, P=0.02).

5. Relationship between the level of HIV-1 proviral DNA and HIV-specific cytotoxic T lymphocyte response

In the patients with HLA-A genotype of HLA-A*0201, who also possessed undetectable plasma viral load levels, the frequency of HIV-1 proviral DNA-harboring CD4+ T cells did not correlate with CTL response(P=0.604, Figure 5).

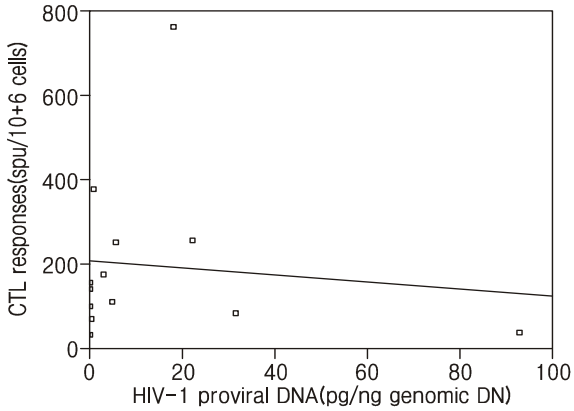

Figure 5. Relationship between the frequency of CD4+ T cells harboring human immunodeficiency virus type 1 (HIV-1) proviral DNA, and cytotoxic T lymphocyte responses,

as determined by ELISPOT assays, in HIV-1 infected persons with HLA-A genotypes of A*0201. Correlations were assessed between the frequency of CD4+ T cells harboring human immunodeficiency virus type 1 (HIV-1) proviral DNA, and spot- forming cells in ELISPOT assays.

6. Escape mutations within the HLA-A*0201-restricted CTL epitope

Among the 28 patients studied with HLA-A genotype of HLA-A*0201, 18 (64.3%) exhibited sequence variations in the Gag $p17_{77-85}$ epitope(Table 3).

Table 3. Gag $p17_{77-85}$ epitope sequence variation among HLA-A*0201-positive subjects

Subjects	Peptide									CD4+ T cell counts (cells/μL)	HIV RNA (copies/mL)	proviral DNA (pg/ng)	CTL responses (SPCs/10^6cells)
	I	L	K	E	P	V	H	G	V				
3	I	L	K	E	P	V	H	G	V	666	3,000	4.63	580
8	I	L	K	E	P	V	H	G	V	381	<25	22.27	255
10	I	L	K	**Q**	P	V	H	G	V	551	50		115
16	I	L	**R**	E	P	V	H	G	V	404	75,000	34.25	430
20	I	L	K	**V**	P	V	H	G	V	223	<25	21.76	
22	I	L	K	E	P	V	H	G	V	268	<25	0.46	
26	I	L	K	E	P	V	H	G	V	245	3,200	126.53	993
29	I	L	K	E	P	V	H	G	V	647	<25	2.81	175
30	I	L	K	E	P	V	H	G	V	243	67,000	71.33	158
33	I	L	K	E	P	V	H	G	V	259	<25	118.84	
34	I	L	K	**T**	P	V	H	G	V				
38	I	L	K	**H**	P	V	H	G	V	234	<25	18.12	763
40	I	L	K	E	P	V	H	G	V	262	<25		33
42	I	L	K	E	P	V	H	G	V	269	4,900	48.66	120
44	I	L	K	**Q**	P	V	H	G	V	827	<25		155
45	I	L	K	**Q**	P	V	H	G	V	407	24,000	48.83	195
48	I	L	K	E	P	V	H	G	V	286	3,200	2.77	2,570
49	I	L	K	E	P	V	H	G	V	157	22,000	482.82	175
51	I	L	K	**Q**	P	V	H	G	V	386	<25	4.86	110
61	I	L	K	E	P	V	H	G	V	535	2,300	2.58	2,053
64	I	L	K	**T**	P	V	H	G	V				
66	I	L	K	E	P	V	H	G	V	393	<25	0.84	378
69	I	L	K	E	P	V	H	G	V	327	21,000	20.57	483
72	I	L	K	**A**	P	V	H	G	V	555	<25	92.84	38
74	I	L	K	E	P	V	H	G	V	262	120	32.78	1298
75	I	L	**R**	E	P	V	H	G	V	725		11.15	708
78	I	L	K	E	P	V	H	G	V	616	<25	5.48	250
83	I	L	K	E	P	V	H	G	V	285	<25	0.15	70
85	I	L	K	E	P	V	H	G	V				
88	I	L	K	E	P	V	H	G	V	242	<25	31.42	83

There were no differences in CD4+ T cell counts, plasma HIV RNA, the frequency of HIV-1 proviral DNA-harboring CD4+ T cells, or CTL responses between the patients with and and those without escape mutants.

Among 26 patients studied with HLA-A genotypes other than HLA-A02, 19 (73.1 %) exhibited sequence variations in the Gag $p17_{77-85}$ epitope(Table 4).

Table 4. Gag $p17_{77-85}$ epitope sequence variation among HLA-A02-negative subjects

Subjects	Peptide									CD4+ T cell counts (cells/μL)	HIV RNA (copies/mL)	proviral DNA (pg/ng)
	S	L	Y	N	T	V	A	T	L			
4	S	L	Y	N	T	V	A	T	L	677	<25	10.04
5	S	L	**F**	N	T	V	A	T	L	411	620	228.74
7	S	L	Y	N	T	V	A	**V**	L	355	7,300	11.02
9	S	L	**F**	N	**A**	V	A	**V**	L	407	<25	72.94
15	S	L	Y	N	**A**	V	A	**V**	L	275	28,000	186.42
19	S	L	**F**	N	**L**	V	A	T	L	392	<25	2.29
23	S	L	**F**	N	**A**	V	A	**V**	L	591	<25	3.08
24	S	L	Y	N	T	V	A	T	L	31	1,500,000	311.13
25	S	L	**F**	N	T	V	A	T	L	584	7,500	0
27	S	L	Y	N	T	V	A	T	L	270	<25	19.53
32	S	L	Y	N	T	V	A	T	L	281	120,000	20.64
41	S	L	**F**	N	T	V	A	T	L	359	<25	42.04
43	S	L	**F**	N	T	V	A	T	L	268	3,600	181.79
46	S	L	Y	N	T	V	A	T	L	196	<25	94.43
47	S	L	Y	N	T	V	A	**V**	L	445	<25	1.02
53	S	L	**F**	N	T	**I**	A	T	L	111	2,800	394.69
55	S	L	**F**	N	T	V	A	T	L	393	710	22.60
56	S	L	**F**	N	**A**	V	A	**V**	L	70	<25	
57	S	L	**F**	N	T	V	A	T	L	890	<25	4.10
58	S	L	**F**	N	T	**I**	A	T	L	715	<25	0.22
65	S	L	Y	N	T	**I**	A	T	L	611	5,700	15.26
67	S	L	Y	N	T	V	A	T	L	275	<25	1.26
68	S	L	**F**	N	T	V	A	T	L	266	<25	2.49
73	S	L	**F**	N	T	V	A	T	L	226	81	248.89
77	S	L	**H**	N	T	V	A	**V**	L	461	<25	0.51
80	S	L	Y	N	T	V	A	T	L		3,600	77.02

Among 30 patients studied with HLA-A genotype of HLA-A*0201, 11 (36.7%) exhibited sequence variations in the Pol $RT_{476-484}$ epitope(Table 5).

Table 5. Pol $RT_{476-484}$ epitope sequence variation among HLA-A*0201-positive subjects

Subjects	Peptide									CD4+ T cell counts (cells/μL)	HIV RNA (copies/mL)	proviral DNA (pg/ng)	CTL responses (SPCs/10^6cells)
	S	L	Y	N	T	V	A	T	L				
3	S	L	Y	N	T	V	A	T	L	666	3,000	4.63	580
8	S	L	**F**	N	T	V	A	T	L	381	<25	22.27	255
10	S	L	**F**	N	T	V	A	T	L	551	50		115
16	S	L	**F**	N	T	V	A	**V**	L	404	75,000	34.25	430
20	S	L	**F**	N	T	V	A	T	L	223	<25	21.76	
22	S	L	**F**	N	**A**	**I**	A	**V**	L	268	<25	0.46	
26	S	L	**H**	N	T	V	A	**V**	L	245	3,200	126.53	993
29	S	L	Y	N	T	V	A	**A**	L	647	<25	2.81	175
30	S	L	Y	N	T	V	A	T	L	243	67,000	71.33	158
33	S	L	Y	N	T	V	A	T	L	259	<25	118.84	
37	S	L	**F**	N	**A**	V	A	**V**	L	113	9,600	41.56	
38	S	L	Y	N	T	V	A	T	L	234	<25	18.12	763
40	S	L	**H**	N	T	V	A	T	L	262	<25		33
42	S	L	Y	N	T	V	A	**A**	L	269	4,900	48.66	120
44	S	L	**F**	N	**A**	V	A	T	L	827	<25		155
45	S	L	**F**	N	T	**I**	A	T	L	407	24,000	48.83	195
48	S	L	Y	N	T	V	A	T	L	286	3,200	2.77	2,570
49	S	L	Y	N	T	V	A	T	L	157	22,000	482.82	175
51	S	L	**F**	N	T	V	A	T	L	386	<25	4.86	110
61	S	L	**F**	N	T	V	A	T	L	535	2,300	2.58	2,053
66	S	**V**	**F**	N	**L**	V	A	**V**	L	393	<25	0.84	378
69	S	L	**H**	N	T	V	A	T	L	327	21,000	20.57	483
72	S	L	**F**	N	T	V	A	T	L	555	<25	92.84	38
74	S	L	Y	N	T	V	A	T	L	262	120	32.78	1298
75	S	L	Y	N	T	V	A	T	L	725		11.15	708
78	S	L	Y	N	T	V	A	T	L	616	<25	5.48	250
83	S	L	Y	N	T	V	A	T	L	285	<25	0.15	70
88	S	L	Y	N	**L**	V	A	T	L	242	<25	31.42	83

Among 32 patients studied with HLA-A genotypes other than HLA-A02, 10(31.3 %) exhibited sequence variations in the Pol $RT_{476-484}$ epitope(Table 6).

Table 6. Pol $RT_{476-484}$ epitope sequence variation among HLA-A02-negative subjects

Subjects	Peptide									CD4+ T cell counts (cells/μL)	HIV RNA (copies/mL)	proviral DNA (pg/ng)
	I	L	K	E	P	V	H	G	V			
4	I	L	K	E	P	V	H	G	V	677	<25	10.04
5	I	L	K	**T**	P	V	H	G	V	411	620	228.74
7	I	L	K	E	P	V	H	G	V	355	7,300	11.02
9	I	L	K	E	P	V	H	G	V	407	<25	72.94
15	I	L	K	E	P	V	H	G	V	275	28,000	186.42
19	I	L	K	E	P	V	H	G	V	392	<25	2.29
23	I	L	**R**	**T**	P	V	H	G	V	591	<25	3.08
24	I	L	K	E	P	V	H	G	V	31	1,500,000	311.13
25	I	L	K	E	P	V	H	G	V	584	7,500	
27	I	L	K	**A**	P	V	H	G	V	270	<25	19.53
32	I	L	K	E	P	V	H	G	V	281	120,000	20.64
35	I	L	K	**R**	P	V	H	G	V	381	1,200	22.16
36	I	L	K	E	P	V	H	G	V	472	6,300	6.18
39	I	L	K	E	P	V	H	G	V			
41	I	L	K	**H**	P	V	H	G	V	359	<25	42.04
46	I	L	K	E	P	V	H	G	V	196	<25	94.43
47	I	L	K	E	P	V	H	G	V	445	<25	1.02
53	I	L	K	**T**	P	V	H	G	V	111	2,800	394.69
55	I	L	K	E	P	V	H	G	V	393	710	22.60
57	I	L	K	E	P	V	H	G	V	890	<25	4.10
58	I	L	K	E	P	V	H	G	V	715	<25	0.22
65	I	L	K	E	P	V	H	G	V	611	5,700	15.26
67	I	L	K	E	P	V	H	G	V	275	<25	1.26
68	I	L	K	E	P	V	H	G	V	266	<25	2.49
71	I	L	K	E	P	V	H	G	V	603	<25	100.12
73	I	L	K	**V**	P	V	H	G	V	226	81	248.89
76	I	L	K	**T**	P	V	H	G	V	54	<25	
77	I	L	K	E	P	V	H	G	V	461	<25	0.51
80	I	L	K	E	P	V	H	G	V		3,600	77.02
82	I	L	K	**D**	P	V	H	G	V	2	94,000	
84	I	L	**N**	E	P	V	H	G	V	625	<25	8.50
87	I	L	K	E	P	V	H	G	V	284	99, 000	

IV. Discussion

The use of HAART in the treatment of HIV-1 infected individuals has contributed to sizable declines in both the incidence of AIDS, and AIDS-related mortality 1. However, even continuous HAART treatment does not result in the complete elimination of viral replication in the peripheral blood[9-11], and HAART is also unable to halt replication in cells containing competent HIV-1 proviral DNA. These facts underscore the impossibility of eradicating HIV-1 using only HAART[2,4,12-14].

Three phases of viral decay following HAART have been described[30]. The decreased plasma viral load in the first phase reflects two processes of intrinsic decay: the clearance of free virions from the plasma with a half-life (t1/2) of <6 h, and the decay of short-lived infected CD4+ T lymphocytes, with a t1/2 of 1-2 days[31]. The second phase represents the clearance of the viral reservoir from infected macrophages and mononuclear cells in the lymphoid tissues, with a t1/2 of 1-4 weeks[32]. The observation that memory CD4+ lymphocytes have a mean inter-mitotic period of 22 weeks is consistent with the t1/2 of proviral DNA in the PBMCs of patients receiving HAART[31,33]. It also suggests that CD4+ T cells may serve as latent reservoirs for the third phase.

HAART also does not result in the elimination of HIV-1 reservoirs, as reflected by the frequency of HIV-1 proviral DNA-harboring CD4+ T cells. This finding is consistent with the reports of other researchers. Modeling studies built on projections of decay curves have estimated that in a setting of prolonged suppression of plasma viremia by antiretroviral therapy, to <50 copies of HIV RNA per milliliter, it would require from 7 to 70 years for the pool of latently infected cells to be completely eliminated[34]. Furthermore, the reservoir of latently infected cells is replenished during minor rebounds of virus replication. These rebound episodes may occur intermittently, even in patients who for the most part are treated successfully, and major viremic rebounds will certainly occur in patients whose therapy is interrupted for a period of weeks or longer[34]. Differences in HIV-1 proviral levels according to the degree of suppression of plasma HIV RNA in patients receiving HAART might be caused by the dynamic differences between the three phases of viral decay following HAART.

The inverse correlation existing between CD4+ T cell counts and HIV-1 provirus

levels is also consistent with the findings of another study[32]. In that study, a significant inverse correlation was demonstrated to exist between the frequency of HIV-1 proviral DNA-bearing CD4+ T cells, and CD4+ T cell counts. A similar pattern was discovered with regard to the CD4+:CD8+ T cell ratio in HIV-1 infected individuals receiving HAART, and in whom plasma viremia had been suppressed below the limit of detection for prolonged periods of time.

The ability to generate high levels of broadly specific anti-HIV-1 CTL is considered to be a critical component of the host immune response to HIV-1[15-21]. CTL recognize peptides derived from endogenously synthesized viral proteins, which are processed intracellularly and presented as a complex with MHC class I molecules and beta 2-microglobulin at the surface of infected cells[35]. The specific interaction of the T cell receptor heterodimer and the peptide/MHC complex results in the activation of CTL response, and the subsequent control of viral replication via both cytolytic and noncytolytic mechanisms[36], thereby providing an overall antiviral effect, which is likely to be important in both the acute and chronic phases of infection[37,38].

In this study, we examined CTL responses directed against HIV-1-derived epitopes restricted by HLA-A*0201, the most common class I allele in most ethnic populations. The magnitude of CTL response was not correlated with proviral burden in these chronically infected persons. Augmentation of CTL responses requires sufficient levels of antigen, functional T-helper-cell responses, and intact antigen presentation networks[39-42]. HIV-1 infection appears to disturb both the antigen presentation networks and the T-helper-cell responses[43-46], and these disturbances may be greater in subjects treated during chronic infection than in those treated during early infection[39,43]. These disturbances would also affect the ability of CD8+ T cells to respond to antigen. Minimal expression of viral antigens has also been observed in the resting state of T cells. Therefore, the augmentation of CTL responses could not, by itself, affect latency reservoirs.

Another study had suggested that CD8+ T cells exhibit potent suppressive activity against HIV replication in the latent viral reservoir, via direct cellular contact, in patients who are naturally long-term nonprogressors, or in those treated with HAART[47]. This study suggested that other antiviral activities of CD8+ T cells, other than CTLs, were responsible for the suppression of HIV replication in the resting CD4+ T cell

reservoirs.

Although the selection pressure associated with antiviral drug therapies has been well documented, less is known about the immune selection pressure exerted by CTL[48]. At least one study of primary infection has demonstrated the emergence of viral sequence variants not recognized by the initial CTL response[49], but this has not been a universal finding[50]. Other studies have generated conflicting data regarding immune selection pressure on the population of viral variants in chronic HIV infection[38,51-54]. Other studies have suggested that CTL may be present, but ineffective *in vivo*. Such impairment may be related to a lack of sufficient helper cell function[55,56].

The emergence of the immune escape mutant is considered to be one of the main obstacles to immune-based therapy, most notably in the development of therapeutic vaccines. In this study, there were a large proportion of CTL escape variants in patients. However, a comparison of viral sequences derived from HLA-A*0201 positive individuals to sequences obtained from HLA-A02 negative individuals demonstrated only weak evidence suggesting immune selective pressure, thus bringing into question the in vivo efficacy of immunodominant CTL responses present during chronic HIV-1 infection.

This study has some limitations. It features cross-sectional findings, and the sequence variations of epitopes were not compared with the sequences of Korean reference strains. The serial follow-up of viral sequence variation and comparison of sequences to those of Korean reference strains would facilitate a more precise characterization of the CTL escape mutant. The heterogeneity of the study population constitutes another limitation of this study.

Despite some limitations, this study delineates the limitations inherent in HAART and other immune-based strategies, as have previous studies. It is clear that another strategy will be necessary with regard to eradication of HIV infection.

V. Conclusion

HAART did not eradicate proviral DNA, as had been established in previous studies. CTL responses were affected by plasma HIV RNA, but not by proviral DNA. Immune escape mutants were detected in these chronically HIV-1 infected Koreans, and the immune escape mutant is considered to be one of the main obstacles to immune-based therapy. However, a comparison of viral sequences derived from HLA-A*0201 positive individuals to sequences obtained from HLA-A02 negative individuals demonstrated some weak evidence for immune selective pressure, thus bringing into question the *in vivo* efficacy of immunodominant CTL responses during chronic HIV-1 infection. As HAART and HIV-1 specific CTL are insufficient to eradicate proviral reservoirs, another strategy must be devised in order to eradicate HIV infection.

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Abstract (In Korean)

한국인 HIV 감염자에서 HIV-1 특이 세포독성 반응과 CD4 양성 세포 내 provirus의 상관관계

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연구배경: HIV 감염자에서 강력한 항레트로바이러스 치료(highly active antiretroviral therapy, HAART)는 획기적으로 그 임상 경과를 호전시키는 것이 증명되었으며, AIDS 관련 사망률을 현저히 감소시켰다. 하지만 HAART로 HIV 감염을 완치시킬 수 없는데, 이는 HAART를 시행해도 proviral DNA 형태의 latent reservoir가 존재하기 때문이다. 최근 HIV 감염을 치료하는데 있어서 숙주 면역 기능의 역할이 강조되고 있으며, HIV 특이 세포독성 T림프구 반응(cytotoxic T lymphocyte response, CTL 반응)은 HIV에 대한 숙주 면역기능 중에서 가장 중요한 역할을 한다. 연구자는 HAART를 시행해도 없어지지 않는 HIV의 reservoir를 반영한다고 알려져 있는 proviral DNA의 level이 CTL 반응에 영향을 미칠 것이라고 가정하였다. 본 연구의 목적은 한국인 HIV 감염자에서 HIV-1 proviral DNA level과 CTL 반응 간의 상관관계를 규명하는 것이다.

대상 및 방법: 연세대학교 의과대학 세브란스병원에 내원한 82명의 한국인 HIV-1 감염자를 대상으로 하였다. Sequencing based typing 방법으로 HLA I 유전형을 결정하였다. 말초혈액 단핵구로부터 human CD4 세포분리 kit를 사용하여 CD4 양성 T림프구를 분리하였다. 전체 CD4 양성 T세포 중에서 HIV-1 provirus가 있는 CD4 양성 세포의 비율을 결정하기 위하여 CD4 양성 T세포의 genomic DNA를 주형으로 real time PCR을 수행하였다. CTL response 정도를 측정하기 위하여 HLA-I 제한 peptide를 사용하여 Enzyme linked immonospot (ELISPOT) assay를 수

행하였다. HIV-1 특이 CTL 반응과 HIV-1 provirus가 있는 CD4 양성 세포의 비율 간의 상관 관계는 Spearman's rank correlation이나 Pearson's correlation을 사용하여 검증하였다.

결과: HIV-1 provirus가 있는 CD4 양성 세포의 비율은 HAART를 시행받은 환자와 시행받지 않은 환자 간에 유의한 차이가 없었다. HIV-1 provirus가 있는 CD4 양성 세포의 비율은 CD4 양성 T세포수와 유의한 역의 상관관계를 나타냈다(P=0.01, r=-0.307). HAART를 시행 중인 환자들에서 혈장 바이러스 농도가 검출한계 미만으로 억제되어있는 환자들에서의 HIV-1 provirus가 있는 CD4 양성 세포의 비율이 바이러스 농도가 억제되지 않은 환자보다 낮았다(P=0.033). CTL 반응은 혈장 바이러스 농도가 억제되지 않은 환자들이 혈장 바이러스 농도가 억제된 환자들보다 높았다(P=0.02). 혈장내 바이러스 농도가 억제되어 있는 HLA-A02 유전형을 지닌 환자에서 HIV-1 provirus가 있는 CD4 양성 세포의 비율과 CTL 반응은 상관 관계가 없었다(P=0.604). HLA-A*0201 유전형을 지닌 환자들 중 64.3%에서 Gag $p17_{77\text{-}85}$ epitope 부위의 escape variation이 나타났다. HLA 유전형이 A02가 아닌 환자들 중 73.1%에서 Gag $p17_{77\text{-}85}$ epitope 부위의 염기서열 변이가 나타났다.

결론: HAART는 기존의 연구 결과와 마찬가지로 HIV proviral DNA를 없애지 못하는 것으로 나타났다. CTL 반응은 proviral DNA에 의해서는 영향받지 않고 혈장내 바이러스억제 여부에 의해서만 영향 받는 것으로 나타났다. 대상 한국인 HIV 만성 감염자에서 면역 escape mutant가 있었다. 그러나, HLA-A*0201 유전형인 군과 HLV-A02 유전형이 아닌 군 사이의 HLA-A*0201 제한 epitope의 아미노산 염기서열을 비교해 본 결과는 면역 선택압력이 만성 HIV 감염자의 생체내에서 큰 영향을 미치지 못할 가능성이 있음을 나타냈다. HAART와 CTL 이 provirus reservoir를 없앨 수 없으므로, HIV 완치를 위한 다른 전략이 필요하다고 사료된다.

핵심되는 말: HIV, HIV 감염, 후천성 면역결핍증, proviral DNA, 세포독성림프구반응, 탈출 변이