P. S., Chang, Y., and Peiper, S. C. (2001) *J. Mol. Biol.* **313,** 1181–1193
34. Blanpain, C., Lee, B., Vakili, J., Doranz, B. J., Govaerts, C., Migeotte, I., Sharron, M., Dupriez, V., Vassart, G., Doms, R. W., and Parmentier, M. (1999) *J. Biol. Chem.* **274,** 18902–18908
35. Govaerts, C., Bondue, A., Springael, J. Y., Olivella, M., Deupi, X., Le Poul, E., Wodak, S. J., Parmentier, M., Pardo, L., and Blanpain, C. (2003) *J. Biol. Chem.* **278,** 1892–1903
36. Sarafianos, S. G., Das, K., Hughes, S. H., and Arnold, E. (2004) *Curr. Opin. Struct. Biol.* **14,** 716–730
37. Sparks, S., *et al.* (2005) *12th Conference on Retroviruses and Opportunistic Infections,* Abstr. 77 AQ: P
38. Lalezari, J., *et al.* (2004) *44th Interscience Conference on Antimicrobial Agents and Chemotherapy,* Abstr. H-1137b AQ: Q

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Design and synthesis of novel HIV-1 protease inhibitors incorporating oxyindoles as the P'_2-ligands

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Abstract—A series of novel oxyindole-derived HIV-1 protease inhibitors were designed and synthesized based upon our X-ray crystal structure of inhibitor **2** (TMC-114) bound to HIV-1 protease. The effects of substituents, spirocyclic rings, and ring sizes have been investigated. A number of inhibitors exhibited low nanomolar inhibitory potencies against HIV protease.

The AIDS epidemic has grown into one of the most pressing medical concerns of our time.[1] The advent of highly active antiretroviral therapy (HAART) with HIV protease inhibitors and reverse transcriptase inhibitors has resulted in an improved quality of life, enhanced HIV management, and halted the progression of AIDS.[2] However, drug side effects and the emergence of drug-resistance are making these therapies ineffective.[3] In our continuing effort to develop new inhibitors that maintain their potencies against mutant strains of HIV, we have recently reported the design and synthesis of a novel inhibitor (**2**, now known as TMC-114 or Darunavir, Fig. 1) which is currently undergoing phase III clinical trials.[4,5] This inhibitor is exceedingly potent against wild-type ($K_i = 15 \pm 1$ pM, $n = 4$ and $ID_{50} = 1.4 \pm 0.25$ nM, $n = 5$) as well as resistant viruses.[4]

Subsequently, to gain molecular insight into the ligand-binding site interaction, we determined a high resolution X-ray crystal structure of this inhibitor bound to HIV-1 protease.[6] An intriguing feature of this structure is the presence of a tetracoordinated critical water molecule that donates its hydrogen bonds to the urethane carbonyl and one of the sulfonamide oxygens of the inhibitor and accepts two hydrogen bonds from the N–H of Ile 50 and Ile 50' amides of the HIV protease. This tight bound water molecule is also present in saquinavir-bound HIV-1 protease as well as other protein–ligand complexes.[7] Based on this key interaction, we postulated that an oxyindole derivative could be designed to interact with this critical water molecule as well as to fill the S'_2 region of the enzyme active site effectively. Such inhibitor with a basic amine

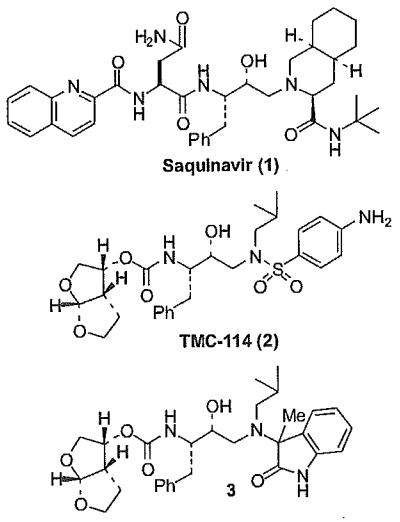

Figure 1. HIV protease inhibitors.

Keywords: HIV proptease; Inhibitors; Oxyindole; TMC-114; Design; Synthesis.
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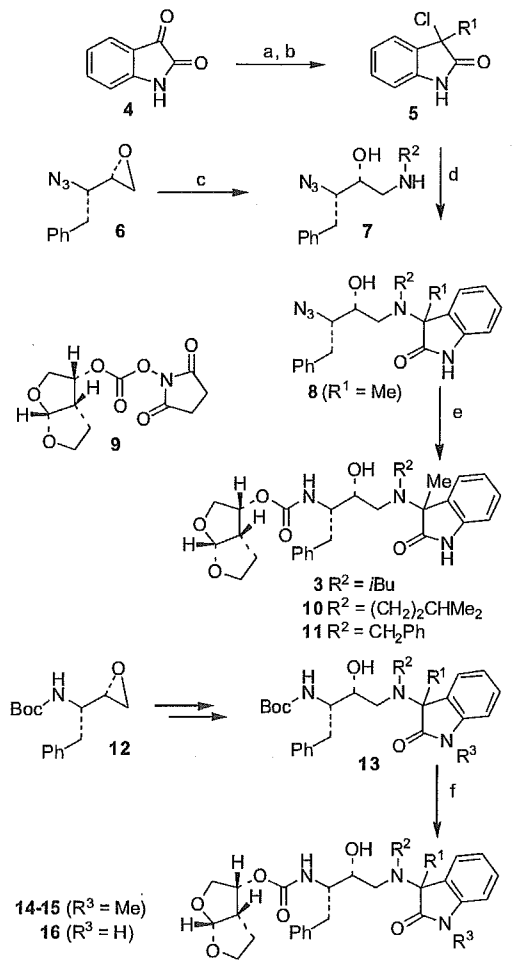
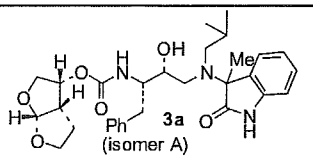
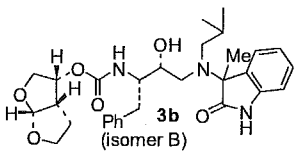
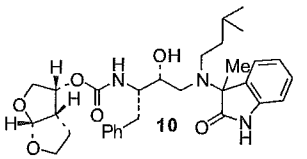
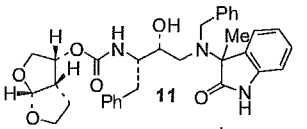
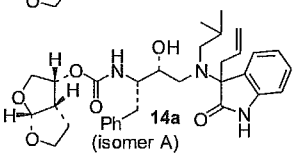
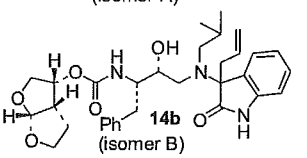
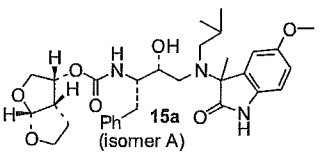
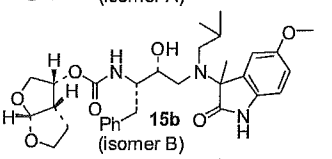
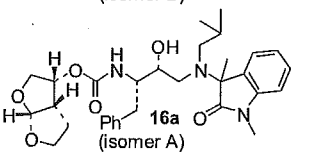
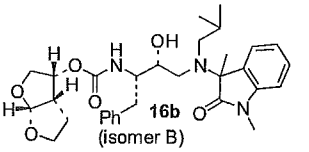
functionality may improve absorption profiles. Oxyindoles have been previously utilized in several FDA-approved drugs.[8] Herein, we report our preliminary results of these investigations in which an oxyindole ring has been incorporated in the P_2' position of inhibitor **2**. This has resulted in a series of inhibitors with subnanomolar enzyme inhibitory potencies. We have also examined the feasibility of spirocyclic oxyindole derivatives as P_2'-ligands. However, acyclic inhibitors were more potent than their cyclic counterparts.

The general synthesis of various oxyindole-derived inhibitors is outlined in Scheme 1. As shown, commercially available isatin was reacted with 2.2 equiv of the appropriate alkyl Grignard reagent at 0 °C to provide the corresponding tertiary alcohol in 57–72% yield.[9] Chlorination of the resulting alcohol using thionyl chloride and triethylamine in CH_2Cl_2 produced chloride **5** in good overall yield (57–76%).[10] Reaction of optically active azido epoxide **6**[11] with the appropriate amine in isopropanol at reflux gave the corresponding secondary amine **7** in essentially quantitative yield. Reaction of the respective amine **7** with chloride **5** (R^1=Me) and triethylamine in acetonitrile smoothly provided oxyindole derivative **8** as a mixture (1:1 ratio by ^1H NMR analysis) of diastereomers in excellent yields (81–94%). Both diastereomers for inhibitor **3** were separated by silica gel chromatography. Catalytic hydrogenation of various azides **8** with optically active bis-tetrahydrofuranyl



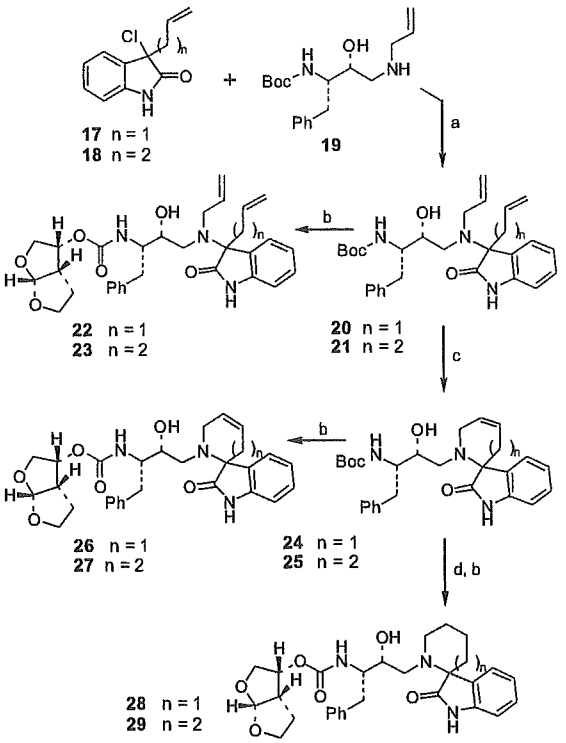
Scheme 1. Reagents and condition: (a) R^1MgBr, THF, 0 °C; (b) $SOCl_2$, TEA, CH_2Cl_2; (c) R^2NH_2, *i*-PrOH; (d) CH_3CN, TEA; (e) **9**, H_2, Pd/C, THF; (f) i—TFA, CH_2Cl_2; ii—**9**, TEA, CH_2Cl_2.

Table 1. Inhibitory activity of oxyindole derivatives

Entry	Compound	K_i (nM)
1	 **3a** (isomer A)	6 ± 0.6
2	 **3b** (isomer B)	3 ± 0.3
3	 **10**	7 ± 0.05
4	 **11**	26 ± 2.5
5	 **14a** (isomer A)	2 ± 0.3
6	 **14b** (isomer B)	7 ± 0.7
7	 **15a** (isomer A)	102 ± 4.9
8	 **15b** (isomer B)	130 ± 12.5
9	 **16a** (isomer A)	42 ± 3.2
10	 **16b** (isomer B)	60 ± 8

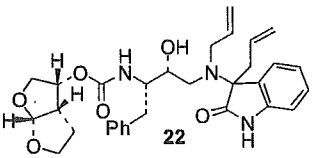
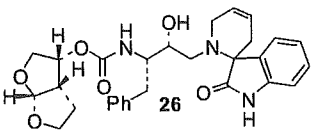
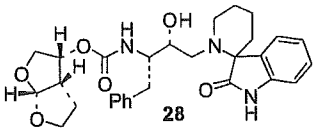
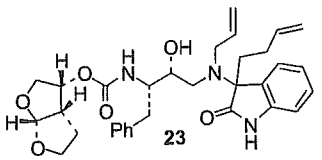
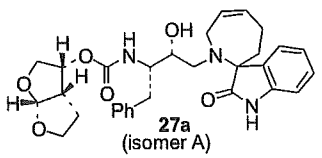
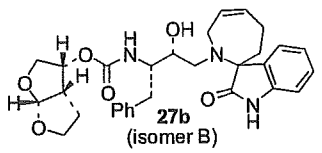
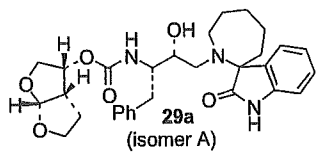
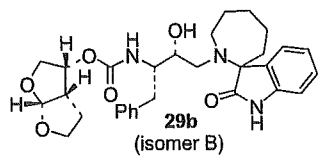
carbonate **9** in THF in the presence of triethylamine afforded optically pure inhibitors **3a** and **3b** as well as diastereomeric mixture of **10** and **11** in good yields (60–75%). Preparation of inhibitors **14–16** was carried out with commercially available Boc-protected epoxide **12** as starting material. Epoxide opening followed by reaction with chloride **5** provided the corresponding Boc derivatives **13**. Diastereomers were separated by silica gel chromatography using 25% ethyl acetate in hexane as the eluent. Removal of the Boc group by exposure to TFA followed by the reaction of the resulting amine with mixed carbonate **9**[12] in the presence of triethylamine in CH_2Cl_2 furnished the final inhibitors **14–16** in good yield (47–65%). Thus, the corresponding oxyindole diastereomers for **14–16** were prepared in an optically active form. Stereochemical identity of the oxyindole ring chiral center was not determined for our preliminary studies.

The inhibitory potencies of various oxyindole-derived inhibitors are shown in Table 1. The assay protocol of Toth and Marshall[13] was utilized and the values denote mean values from two determinations. As can be seen, both oxyindole diastereomers of the *N*-isobutyl analogs **3a** and **3b** have shown potencies of 6 and 3 nM, respectively. It appears that S_2'-enzyme active site has only a slight preference for one diastereomer over the other. Either *R* or *S* absolute configuration of the oxyindole chiral center in **3** seems to bind into HIV protease S_2'-active site effectively. Nevertheless, our attempts to assign stereochemical identity of the oxyindole ring were unsuccessful. Diastereomeric mixtures of *N*-isoamyl (**10**) and *N*-benzyl (**11**) derivatives also showed good activity, with the larger benzyl analog being less potent (26 vs 7 nM). Introduction of an allyl group at the oxyindole C-3 center (**14a** and **14b**) resulted in a separable mixture of diastereomers which showed comparable potency (2 and 7 nM, respectively) with that of the methyl analogs (**3a** and **3b**). In an effort to interact with the residues in the active site, we have incorporated a 5-methoxy substituent on the oxyindole aromatic ring. Thus, 5-methoxyisatin was converted to inhibitors **15a** and **15b** as a diastereomeric mixture (1:1 ratio by ^1H NMR analysis)



Scheme 2. Reagents and conditions: (a) CH_3CN, TEA, reflux; (b) i—TFA, CH_2Cl_2; ii—**9**, TEA, CH_2Cl_2; (c) Grubbs' 1st generation catalyst, CH_2Cl_2, 42 °C; (d) H_2, 10% Pd/C, MeOH.

Table 2. Inhibitory activity of spirocyclic derivatives

Entry	Compound	K_i (nM)
1	 **22**	5 ± 0.5
2	 **26**	126 ± 0.5
3	 **28**	>1000
4	 **23**	47 ± 1.1
5	 **27a** (isomer A)	>1000
6	 **27b** (isomer B)	>1000
7	 **29a** (isomer A)	>1000
8	 **29b** (isomer B)	>1000

and the mixture was separated. However, these inhibitors have shown significantly lower inhibitory activity compared to unsubstituted inhibitors **3**. The *N*-methyl oxyindole derivative was also synthesized and the individual diastereomers (**16a** and **16b**) displayed K_i values of 42 and 60 nM, respectively. The fact that the potency displayed an approximately 10-fold decrease (as compared to compounds **3a** and **3b**) suggests that the oxyindole N–H may be participating in hydrogen bonding with the enzyme active site.

We have also examined the feasibility of spirocyclic oxyindole derivatives as the P_2'-ligand. It has been shown by us and others that constrained rings in the HIV protease active site significantly improved enzyme inhibitory activity.[14,15] Our preliminary molecular modeling suggested that such spirocycles can make effective interaction in the active site. Scheme 2 shows the synthesis of six- to seven-membered spirocyclic oxyindole-derived inhibitors. Opening epoxide **12** with allylamine in *i*-PrOH provided quantitative yield of secondary amine **19**. The olefinic chlorooxyindoles (**17**, 48%) and (**18**, 52%) were prepared following the same 2-step sequence as described in Scheme 1. Reactions of these chlorooxyindoles with amine **19** afforded tertiary amines **20** and **21** as 1:1 mixtures of diastereomers (by ^{1}H NMR) in 68–82% yield. These diastereomers could be separated and the mixture was utilized in subsequent reactions. The dienes were then subjected to ring closing metathesis using Grubbs' first generation[16] catalyst in refluxing CH_2Cl_2 to provide six and seven-membered spirocycles **24** and **25**, respectively, in excellent yield (80–85%). The seven-membered ring diastereomers were separated at this point by silica gel chromatography, while the six-membered ring was used as a 1:1 mixture of diastereomers. The unsaturated rings were converted into urethane derivatives **26** and **27** containing P_2-bis-tetrahydrofuranyl ligand following the standard protocol described in Scheme 1. Saturated inhibitors **28** and **29** were prepared by removal of Boc from **24** and **25** and reaction of the resulting amines with carbonate **9** in the presence of triethylamine in CH_2Cl_2 followed by catalytic hydrogenation of the resulting olefins (60–65% yield).

The spirocyclic oxyindole derivatives were assayed and their potencies are displayed in Table 2. Acyclic compounds **22** and **23** showed good activity (5 and 47 nM, respectively) and were generally consistent with those observed for similar compounds shown in Table 1. Interestingly, there is a significant reduction in inhibitory potency for the corresponding six and seven-membered unsaturated and saturated spirocyclic inhibitors. As shown, inhibitor with a cyclohexene ring has shown a K_i value of 126 nM. However, saturation of the double bond provided compound **28** with very little activity (K_i value > 1 μM). Also, all spirocyclic derivatives with a seven-membered ring have displayed no significant enzyme inhibitory activity. A closer inspection of the preliminary model structure reveals that the oxyindole carbonyls of the spirocyclic derivatives do not overlap with the sulfone oxygen of **2** that effectively interacts with the tight-bound water molecule in the active site. Further structural modifications of the oxyindole derivatives are necessary for effective binding in the active site.

We have determined the antiviral activity of **3a** and **3b** against HIV-1_{IIIb} in MT-2 cells. The results are summarized in Table 3. The IC_{50} values shown were determined based on the inhibition of HIV-induced cytopathogenicity in MT-2 cells. All assays were conducted in duplicate, and the values with standard deviation denote mean values from two or three. As can be seen, the antiviral activity of these compounds was substantially limited compared to saquinavir.[17] To improve antiviral potency, further modifications of functionalities are in progress. To gain molecular insight, an energy minimized model of 3(*R'*)-configuration of oxyindole derivative **3** was created (Fig. 2). The structure was built based on our published crystal structure of **2**-complexed with

Table 3. Antiviral activity of **3a** and **3b**

Inhibitor	IC_{50} (μM)	CC_{50} (μM)
3a	0.30 ± 0.071	>10
3b	0.48 ± 0.38	>10
Saquinavir	0.005 ± 0.002	>10

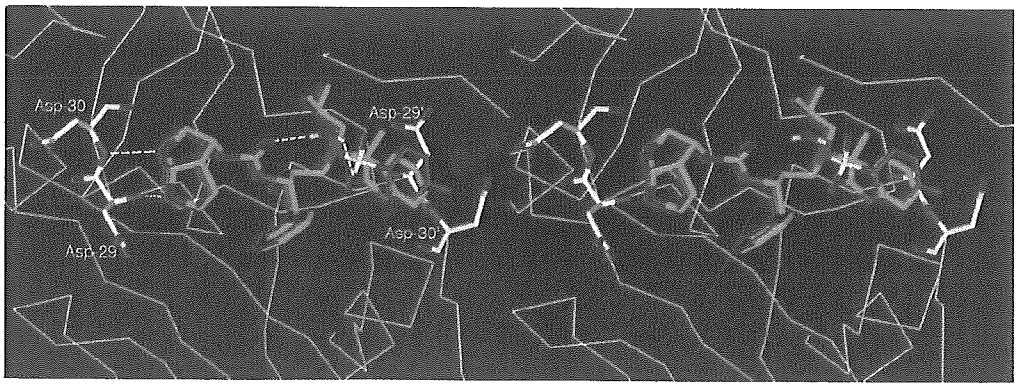


Figure 2. (3'*R*)-Oxyindole isomer of compound **3** modeled into the active site of HIV-1 protease. The inhibitor (green), superimposed upon the crystal structure of TMC-114 (magenta).

HIV-1 protease.[6] The conformation of **3** was optimized using the MMFF94x force field.[18]

In summary, a series of novel HIV protease inhibitors incorporating oxyindole-derived P_2'-ligand has been designed, synthesized, and evaluated. The oxyindole derivatives can be readily prepared from isatin. The oxyindole derivatives incorporate a basic amine functionality. Various 3-alkyl substitutents on the oxyindole rings resulted in inhibitors with low nanomolar potency. In general, acyclic inhibitors are considerably more potent than their cyclic counterparts. Preliminary structure–activity studies have shown that the lactam N–H is critical to enhanced potency. We have also investigated the feasibility of spiro oxyindoles as the P2′-ligands. However, spirocyclic inhibitors have shown significantly reduced potencies compared to their acyclic counterparts. Further design and optimization of these inhibitors are currently underway.

Acknowledgment

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References and notes

1. United Nations. 2004 Report on the Global HIV/AIDS Epidemic: 4th Global Report. New York, USA, 2004.
2. (a) Flexner, C. *N. Engl. J. Med.* **1998**, *338*, 1281; (b) Cihlar, T.; Bischofberger, N. *Annu. Rep. Med. Chem.* **2000**, *35*, 177.
3. Tamalet, C.; Pasquier, C.; Yahi, N.; Colson, P.; Poizot-Martin, I.; Lepeu, G. *J. Med. Virol.* **2000**, *61*, 181.
4. (a) Ghosh, A. K.; Shin, D. W.; Swanson, L.; Krishnan, K.; Cho, H.; Hussain, K. A.; Walters, D. E.; Holland, L.; Buthod, J. *Il Farmaco* **2001**, *56*, 29; (b) Ghosh, A. K.; Kincaid, J. F.; Cho, W.; Walters, D. E.; Krishnan, K.; Hussain, K. A.; Koo, Y.; Cho, H.; Rudall, C.; Holland, L.; Buthod, J. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 687; (c) Koh, Y.; Nakata, H.; Maeda, K.; Ogata, H.; Bilcer, G.; Devasamudram, T.; Kincaid, J. F.; Boross, P.; Wang, Y.-F.; Tie, Y.; Volarath, P.; Gaddis, L.; Harrison, R. W.Weber, I. T.; Ghosh, A. K.; Mitsuya, H. *Antimicrob. Agents Chemother.* **2003**, *47*, 3123; (d) Yoshimura, K.; Kato, R.; Kavlick, M. F.; Nguyen, A.; Maroun, V.; Maeda, K.; Hussain, K. A.; Ghosh, A. K.; Gulnik, S. V.; Erickson, J. W.; Mitsuya, H. *J. Virol.* **2002**, *76*, 1349.
5. (a) De Meyer, S.; Azijn, H.; Surleraux, D.; Jochmans, D.; Tahri, A.; Pauwels, R.; Wigerinck, P.; de Bethune, M.-P. *Antimicrob. Agents Chemother.* **2005**, *49*, 2314; (b) De Meyers, S.; Peeters, M. Conference on Retroviruses and opportunistic Infections (11th CROI), February 8–11, 2004, San Francisco, CA. Abstracts 533 and 620.
6. Tie, Y.; Boross, P. I.; Wang, Y.-F.; Gaddis, L.; Hussain, A. K.; Leshchenko, S.; Ghosh, A. K.; Louis, J. M.; Harrison, R. W.; Weber, I. T. *J. Mol. Biol.* **2004**, *338*, 341.
7. Swain, A. L.; Miller, M. M.; Green, J.; Rich, D. H.; Schneider, J.; Kent, S. B. H.; Wlodawer, A. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 8805.
8. (a) Gunasekara, N. S.; Spencer, C. M.; Keating, G. M. *Drugs* **2002**, *62*, 1217; (b) Gallagher, G., Jr.; Lavanchy, P. G.; Wilson, J. W.; Hieble, J. P.; DeMarinis, R. M. *J. Med. Chem.* **1985**, *28*, 1533; (c) Haynes, J.; Obiako, B.; Babal, P.; Stevens, T. *Am. J. Physiol. Heart Circ. Physiol.* **1999**, *276*, H1877; (d) Liu, Y.; Liu, D.; Printzenhoff, D.; Coghlan, M. J.; Harris, R.; Kraft, D. S. *Eur. J. Pharmacol.* **2002**, *435*, 153.
9. (a) Alvarez, R. G.; Hunter, I. S.; Suckling, C. J.; Thomas, M.; Vitinius, U. *Tetrahedron* **2001**, *57*, 8581; (b) Sharma, V. M.; Prasanna, P.; Adi Seshu, K. V.; Renuka, B.; Laxman Rao, C. V.; Sunil Kumar, G.; Prasad Narasimhulu, P.; Aravind Babu, P.; Puranik, R. C.; Subramanyam, D. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2303.
10. Powers, J. C. *J. Org. Chem.* **1966**, *31*, 2627.
11. Ghosh, A. K.; Thompson, W. J.; Holloway, M. K.; McKee, S. P.; Duong, T. T.; Lee, H. Y.; Munson, P. M.; Smith, A. M.; Wai, J. M.; Darke, P. L.; Zugay, J. A.; Emini, E. A.; Schleif, W. A.; Huff, J. R.; Anderson, P. S. *J. Med. Chem.* **1993**, *36*, 2300.
12. (a) Ghosh, A. K.; Thompson, W. J.; Fitzgerald, P. M. D.; Culberson, J. C.; Axel, M. G.; McKee, S. P.; Huff, J. R.; Anderson, P. S. *J. Med. Chem.* **1994**, *37*, 2506; (b) Ghosh, A. K.; Leshchenko, S.; Noetzel, M. *J. Org. Chem.* **2004**, *69*, 7822; (c) Ghosh, A. K.; Chen, Y. *Tetrahedron Lett.* **1995**, *36*, 505.
13. Toth, M. V.; Marshall, G. R. *Int. J. Pept. Protein Res.* **1990**, *36*, 544.
14. (a) Ghosh, A. K.; Swanson, L.; Liu, C.; Cho, H.; Hussain, A.; Walters, D. E.; Holland, L.; Buthod, J. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1993; (b) Ghosh, A. K.; Swanson, L. M.; Cho, H.; Leshchenko, S.; Hussain, K. A.; Kay, S.; Walters, D. E.; Koh, Y.; Mitsuya, H. *J. Med. Chem.* **2005**, *48*, 3576.
15. (a) Fairlie, D. P.; Tyndall, J. D. A.; Reid, R. C.; Wong, A. K.; Abbenante, G.; Scanlon, M. J.; March, D. R.; Bergman, D. A.; Chai, C. L. L.; Burkett, B. A. *J. Am. Chem. Soc.* **1996**, *118*, 8511; (b) Abbenante, G.; Bergman, D. A.; Brinkworth, R. I.; March, D. R.; Reid, R. C.; Hunt, P. A.; James, I. W.; Dancer, R. J.; Garnham, B.; Stoermer, M. L.; Fairlie, D. P. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2531; (c) Reid, R. C.; Pattenden, L. K.; Tyndall, J. D. A.; Martin, J. L.; Walsh, T.; Fairlie, D. P. *J. Med. Chem.* **2004**, *47*, 1641; (d) Glenn, M. P.; Pattenden, L. K.; Reid, R. C.; Tyssen, D. P.; Tyndall, J. D. A.; Birch, C. J.; Fairlie, D. P. *J. Med. Chem.* **2002**, *45*, 371.
16. Grubbs, R. H.; Chang, S. *Tetrahedron* **1998**, *54*, 4413.
17. Roberts, N. A.; Martin, J. A.; Kinchington, D.; Broadhurst, A. V.; Craig, J. C.; Duncan, I. B.; Galpin, S. A.; Handa, B. K.; Kay, J.; Krohn, A.; Lambert, R. W.; Merrett, J. H.; Mills, J. S.; Parkes, K. E. B.; Redshaw, S.; Ritchie, A. J.; Taylor, D. L.; Thomas, G. J.; Machin, P. *Science* **1990**, *248*, 358.
18. Halgren, T. A. *J. Comput. Chem.* **1999**, *20*, 720.

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Frequent Detection of Epstein-Barr Virus and Cytomegalovirus but Not JC Virus DNA in Cerebrospinal Fluid Samples from Human Immunodeficiency Virus-Infected Patients in Northern Thailand

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Applying nested-PCRs, we frequently detected DNA of Epstein-Barr virus and cytomegalovirus but not JC virus in cerebrospinal fluid samples from 140 human immunodeficiency virus-infected patients with central nervous system symptoms in northern Thailand. Despite the low incidence of primary central nervous system lymphoma or cytomegalovirus encephalitis among Thai AIDS patients, Epstein-Barr virus and cytomegalovirus infections in the central nervous system are common.

According to reports from the Thai Ministry of Public Health, opportunistic infections are common in the central nervous system (CNS) of Thai AIDS patients and have caused a significant portion of mortality. Cryptococcal meningitis was noted as 20.3% of the first AIDS defining illness in northern Thailand; toxoplasma encephalitis was 5.3%; tuberculous meningitis was also seen, though the exact prevalence in the total number of AIDS patients is unknown (2). Virus infections in the CNS such as Epstein-Barr virus (EBV), cytomegalovirus (CMV), and JC virus (JCV) can result in life-threatening consequences as they cause primary CNS lymphoma, cytomegalovirus encephalitis, and progressive multifocal leukoencephalopathy, respectively. In developed countries, PCR tests to detect EBV, CMV and JCV DNA in the cerebrospinal fluid (CSF) have been used as a supplemental diagnostic test (5). However, in developing countries, such a test is not available and very limited data have been reported about the prevalence of virus infections in the CNS. The objective of this study is to investigate the significance of EBV, CMV, and JCV infections in the CNS of human immunodeficiency virus (HIV)-infected Thais in northern Thailand.

From March 2001 to June 2003, CSF samples of 140 HIV-1-infected patients at the day care center clinic or the HIV/AIDS ward in Lampang Hospital, which is a Thai government referral hospital for Lampang province in northern Thailand, were examined as they were clinically suspected of having opportunistic infections in the CNS and did not have any contraindication for lumbar puncture. Consequently, 163 CSF samples including follow-up CSF samples were taken. All CSF samples were initially examined for routine laboratory tests such as cell count, protein concentration, sugar level, bacterial and fungal culture, Indian ink stain, Gram stain, acid-fast bacilli stain, and a latex agglutination test for cryptococcal antigen (PASTOREX, Bio-Rad, France). After the routine laboratory tests, residual CSF samples were stored at −80°C until DNA extraction.

All study patients gave informed consent when they participated in the Lampang HIV cohort study, which was approved by the Thai government ethics committee. DNA was extracted from 200 μl of CSF (QIAGEN blood mini DNA extraction kit, QIAGEN, California), eluted with 50 μl of distilled water, and 10 ul were used as the target for PCR. PCR amplifications were performed using ExTaq DNA polymerase (TaKaRa Biomedical, Osaka, Japan) and nested primer sets targeting specific sequences of virus genes as previously published: the EBNA-1 gene for EBV (PCR product, 209 bp) (3), immediately early protein gene for CMV (146 bp) (1), and regulatory regions for JCV (approximately 396 bp) (7, 10).

Diagnosis of EBV and CMV infection was made on the basis of the size of amplicons, but for the diagnosis of JCV, we further sequenced PCR products. The positive control for EBV PCR was DNA extracted from Namalwa cells as previously described (11). DNA extract from culture supernatant of CMV-producing fibroblast cells was used as a positive control for CMV PCR. DNA extract from the urine of a healthy JCV carrier was used as a positive control for JCV PCR. The detection limit of nested PCR for EBV and CJV was evaluated as previously described (10, 11). The detection limit of CMV PCR was approximately 100 copies/ml of CSF, which was estimated by a limiting dilution method using a DNA sample, of which the number of CMV copies was determined by a quantitative real-time PCR (Mitsubishi-Kagaku BCL, Tokyo, Japan).

The median (interquartile range; range) of age among 140 patients was 33 years (30 to 37 years; 20 to 63 years); 93 patients (66.4%) were male. $CD4^+$ T-cell count data were

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TABLE 1. Clinical characteristics of study patients[a]

Parameter	No. of patients (%)
Symptoms	
Altered mental status	37 (27.0)
Focal sign	18 (13.1)
Chronic headache	121 (88.3)
Fever	104 (75.9)
Diagnosis of CNS infection	
Cryptococcal meningitis[b]	98 (70.0)
Toxoplasmic encephalitis[b]	10 (7.1)
Tuberculous meningitis	6 (4.3)
Aseptic meningitis	3 (2.1)
No diagnosis	24 (17.1)
Antiretroviral drug therapy	
None	131 (93.6)
Two drugs	3 (2.1)
Three drugs	4 (2.9)
Unknown	2 (1.4)
Status at discharge	
Improved	99 (70.7)
Dead	29 (20.7)
Referred to another hospital	7 (5.0)
Unknown	5 (3.6)

[a] Medical records were available for 137 patients.
[b] Includes one case with both cryptococcal meningitis and toxoplasmic encephalitis.

available in 48 patients; the median (IQR; range) was 16 (7 to 42/μl; 0 to 605/μl). Clinical pictures of the patients are summarized in Table 1. Cryptococcal meningitis was by far the most common opportunistic infection in the CNS. There was no case of primary CNS lymphoma, CMV encephalitis, or progressive multifocal leukoencephalopathy. However, one patient developed clinical symptoms of progressive multifocal leukoencephalopathy during the follow-up period.

Thirty-one of 140 patients (22.1%) were positive for EBV PCR and 16 of 140 (11.4%) patients were positive for CMV PCR. Six patients were positive for both EBV and CMV PCR. More than one CSF sample was collected from 20 patients. The results of CMV PCR were concordant in all pairs of samples, but the results of EBV PCR were discordant in five pairs. None of the 140 first CSF samples was positive for JCV PCR. However, JCV was detected in the second CSF sample of one cryptococcal meningitis case. We found that patients with EBV DNA in the CSF tended to be older than the other patients and had a significantly higher protein concentration and a higher number of cells in the CSF (Table 2). We did not find any factor significantly associated with CMV DNA detection in CSF.

We found that EBV infection in the CNS is common in advanced HIV-infected patients in northern Thailand. This frequency was higher than the result of a similar study in Italy (4). The majority of our study patients were suffering from cryptococcal meningitis, but the detection rate of EBV DNA did not significantly differ according to the clinical diagnosis of cryptococcal meningitis. A significant association of EBV detection with a CSF cell count raised the concern that we may have detected EBV in the lymphocytes circulating in the peripheral blood, which invaded the CSF, rather than EBV of the CNS involvement. However, EBV was also often detected in patients without a CSF cell: EBV DNA was detected in CSF from 9 (19.6%) of 46 patients with a CSF cell count of 0.

Several studies from Western countries have shown a high sensitivity and specificity of EBV PCR in CSF for diagnosing primary CNS lymphoma (5). However, we have not seen any primary CNS lymphoma cases in our experience of having seen over 2,400 HIV-1-infected patients at the day care center clinic from its establishment on October 1995 to July 2004. Furthermore, the government report of adult AIDS patients from 1994 to 1998 showed that there were 98 primary CNS lymphoma cases, which represented only 0.1% of all reported first AIDS-defining illness in Thailand (2). According to the Thai national

TABLE 2. Factors associated with EBV or CMV DNA detection in the CSF[a]

Parameter	Median no. of patients (IQR)					
	EBV			CMV		
	PCR positive (n = 31)	PCR negative (n = 109)	P	PCR positive (n = 16)	PCR negative (n = 124)	P
Age (yr)	35 (31–42)	33 (30–36)	0.069	32 (29–38)	33 (30–37)	0.7
No. female	11 (35.5%)	36 (33.1%)	0.79	6 (37.5%)	41 (33.1%)	0.72
CSF cell count (/μl)	8 (0–66)	4 (0–10)	0.045	6 (0–18)	4 (0–16)	0.97
CSF protein concn (mg/dl)	80 (55–160)	53 (34–90)	0.003	75 (30–88)	57.5 (40–100)	0.96
Clinical diagnosis[b]						
Cryptococcal meningitis	21 (70.0%)	76 (69.7%)		12 (75.0%)	85 (69.1%)	
Toxoplasmisc encephalitis	4 (13.3%)	5 (4.6%)	0.40	1 (6.3%)	8 (6.5%)	0.65
Tubercular meningitis	1 (3.3%)	5 (4.6%)		0 (0.0%)	6 (4.9%)	
Aseptic meningitis	0 (0.0%)	3 (2.8)		1 (6.3%)	2 (1.6%)	
No apparent CNS infection	4 (13.3%)	20 (18.3%)		2 (12.5%)	22 (17.9%)	
Symptoms[c]						
Altered mental status	12 (40.0%)	25 (23.4%)	0.07	4 (25.0%)	33 (27.3%)	0.85
Headache	27 (90.0%)	94 (87.9%)	0.75	15 (93.8%)	106 (87.6%)	0.47
Focal sign	4 (13.3%)	14 (13.1%)	0.97	1 (6.3%)	17 (14.0%)	0.34
Fever	22 (73.3%)	82 (76.6%)	0.71	11 (68.8%)	93 (76.9%)	0.48
Death at discharge[d]	5 (17.2%)	24 (24.2%)	0.43	4 (25.0%)	25 (22.3%)	0.81

[a] Data are median (interquartile range) or number of patients (%).
[b] One case with cryptococcal meningitis and toxoplasmic encephalitis was excluded from the analysis.
[c] Medical records were available for 137 patients.
[d] Survival status at discharge was known for 128 patients.

guideline for clinical management of HIV/AIDS patients (8), if patients with a focal sign have poor response to the toxoplasma encephalitis therapy, further investigation with computed tomography scan is recommended to exclude other space-occupying lesions such as primary CNS lymphoma, and the computed tomography scan is available at most government referral hospitals in Thailand. However, this clinical practice may underdiagnose a minimal primary CNS lymphoma, which does not cause CNS symptoms.

Because of a high mortality rate of symptomatic Thai patients (9), patients with a small primary CNS lymphoma might have died due to other opportunistic infections before the primary CNS lymphoma lesion became large and caused CNS symptoms. Recently the Thai government pharmaceutical organization has started mass production of generic antiretroviral drugs. If many insidious primary CNS lymphoma cases exist in Thailand, we expect to see more patients with apparent primary CNS lymphoma lesions as the antiretroviral drug-treated patients survive longer. Alternatively, it is plausible that Thai patients are less susceptible to the development of primary CNS lymphoma and that EBV DNA detection in CSF from AIDS patients does not supplement the diagnosis of primary CNS lymphoma in Thailand.

In our experiences at Lampang Hospital, CMV retinitis is common among our advanced HIV-infected patients, but we have not seen any case with CMV encephalitis. This rarity of CMV encephalitis may be due to the difficulty of making a firm diagnosis in Thailand, since it requires magnetic resonance imaging or biopsy, which is not widely available, and the disease does not induce characteristic clinical symptoms. Our data on CMV PCR warn that we may be overlooking patients with CMV encephalitis.

Progressive multifocal leukoencephalopathy cases have been reported but are not common in Thailand (2, 6). At Lampang Hospital, we had one male patient who presented with hemiparesis and was diagnosed with progressive multifocal leukoencephalopathy on the basis of computed tomography scan findings and clinical course. His CSF was negative for JCV PCR, but this result does not exclude progressive multifocal leukoencephalopathy as the sensitivity of JCV PCR is not high (5). We found one case in which JCV virus was detected in the CSF of the second lumbar puncture. This patient did not have any other CNS symptoms besides headache, but he died shortly after the diagnosis of cryptococcal meningitis. We think that a low prevalence of JCV DNA detection is compatible with our clinical impression, that is, progressive multifocal leukoencephalopathy cases are there but not common, though more patients would be detected if brain magnetic resonance imaging were available.

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REFERENCES

1. **Brytting, M., V. A. Sundqvist, P. Stalhandske, A. Linde, and B. Wahren.** 1991. Cytomegalovirus DNA detection of an immediate early protein gene with nested primer oligonucleotides. J. Virol. Methods **32:**127–138.
2. **Chariyalertsak, S., T. Sirisanthana, O., Saengwonloey, and K. E. Nelson.** 2001. Clinical presentation and risk behaviors of patients with acquired immunodeficiency syndrome in Thailand, 1994–1998: regional variation and temporal trends. Clin. Infect. Dis. **32:**955–962.
3. **Cinque, P., M. Brytting, L. Vago, A. Castagna, C. Parravicini, N. Zanchetta, A. D. Monforte, B. Wahren, A. Lazzarin, and A. Linde.** 1993. Epstein-Barr virus DNA in cerebrospinal fluid from patients with AIDS-related primary lymphoma of the central nervous system. Lancet **342:**398–401.
4. **Cinque, P., L. Vago, H. Dahl, M. Brytting, M. R. Terreni, C. Fornara, S. Racca, A. Castagna, A. D. Monforte, B. Wahren, A. Lazzarin, and A. Linde.** 1996. Polymerase chain reaction on cerebrospinal fluid for diagnosis of virus-associated opportunistic diseases of the central nervous system in HIV-infected patients. AIDS **10:**951–958.
5. **Cinque, P., P. Scarpellini, L. Vago, A. Linde, and A. Lazzarin.** 1997. Diagnosis of central nervous system complications in HIV-infected patients: cerebrospinal fluid analysis by the polymerase chain reaction. AIDS. **11:**1–17.
6. **Dujneungkunakorn, T., S. Sungkanuparph, A. Vibhagool, W. Pairoj, W. Chantratita, C. Srichunrusami, J. Laothamatas, and T. Pulkes.** 2002. Detection of JC virus infection in progressive multifocal leukoencephalopathy: the first documented case in Thailand. J. Med. Assoc. Thai. **85:**1139–1144.
7. **Kato, A., T. Kitamura, T. Takasaka, T. Tominaga, A. Ishikawa, H-Y Zheng, and Y. Yogo.** 2004. Detection of the archetypal regulatory region of JC virus from the tonsil tissue of patients with tonsillitis and tonsilar hypertrophy. J. Neurovirol. **10:**244–249.
8. **Ministry of Public Health of Thailand.** 2000. National guidelines for the clinical management of HIV infection in children and adults, 6th edition. Ministry of Public Health, Bangkok, Thailand.
9. **Pathipvanich, P., K. Ariyoshi, A. Rojanawiwat. S. Wongchoosie, P. Yingseree, K. Yoshiike, P. Warachit, and P. Sawanpanyalert.** 2003. Survival benefit from non-highly active antiretroviral therapy in a resource-constrained setting. J. Acquir. Immune Defic. Syndr. **32:**157–160.
10. **Sugimoto, C., D. Ito, K. Tanaka, H. Matsuda, H. Saito, H. Sakai, K. Fujihara, Y. Itoyama, T. Yamada, J. Kira, R. Matsumoto, M. Mori, K. Nagashima, and Y. Yogo.** 1998. Amplification of JC virus regulatory DNA sequences from cerebrospinal fluid: diagnostic value for progressive multifocal leukoencephalopathy. Arch. Virol. **143:**249–262.
11. **Tachikawa, N., M. Goto, Y. Hoshino, H. Gatanaga, A. Yasuoka, T. Wakabayashi, H. Katano, S. Kimura, S. Oka, A. Iwamoto, et al.** 1999. Detection of Toxoplasma gondii, Epstein-Barr virus, and JC virus DNAs in the cerebrospinal fluid in acquired immunodeficiency syndrome patients with focal central nervous system complications. Intern. Med. **38:**556–562.

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Unrelated cord blood transplantation for a human immunodeficiency virus-1-seropositive patient with acute lymphoblastic leukemia

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The concurrent use of highly active antiretroviral therapy (HAART) improves results of high-dose chemotherapy with autologous stem cell transplantation (SCT) for human immunodeficiency virus-1 (HIV)-associated lymphomas.[1] Recently, successful allogeneic SCT from HLA-matched sibling donors was reported in HIV-infected patients.[2–4] Here, we describe the first case of an HIV-infected patient with acute lymphoblastic leukemia (ALL) who underwent umbilical cord blood transplantation (CBT).

In July 1996, a 23-year-old Japanese woman presented with fever and genital herpes. She was confirmed as seropositive for HIV, probably transmitted from her boyfriend. In March 2001, a real-time quantitative polymerase chain reaction (PCR) analysis showed that the HIV-RNA level was elevated to 25 000 copies/ml (lower limit of detection, 50). The CD4 count decreased to 28/μl. Therefore, HAART consisting of 60 mg stavudine, 300 mg lamivudine, and 600 mg efavirenz was initiated. In July 2001, the HIV-RNA level decreased to 220 copies/ml, and the CD4 count increased to 129/μl. In May 2003, her complete blood count tests showed a white blood cell count (WBC) of 3990/μl with 29% lymphoblasts. Bone marrow (BM) examination showed hypercellularity with 96% lymphoblasts, which were positive for CD4, CD10, CD13, CD19, CD33, CD34, and HLA-DR. Cytogenetic analysis disclosed the presence of t(9;22)(q34;q11) in 12 of 20 metaphases. The $p190^{BCR\text{-}ABL}$ transcript was shown by a reverse transcriptase (RT)-PCR analysis. She was diagnosed as Philadelphia chromosome-positive ALL. She achieved hematological complete remission after two courses of chemotherapy. She has been taking HAART during and after the chemotherapy and her HIV-RNA level continued to be below detectable levels. She was negative for hepatitis B virus surface antigen and anti-hepatitis C virus antibody, and positive for anti-cytomegalovirus antibody. As she had no HLA-matched related or unrelated BM donors, the patient underwent CBT from an unrelated donor with mismatches at two loci (HLA-B and DR) in September 2003 (Figure 1). The numbers of total nucleated cells and CD34-positive cells in the cord

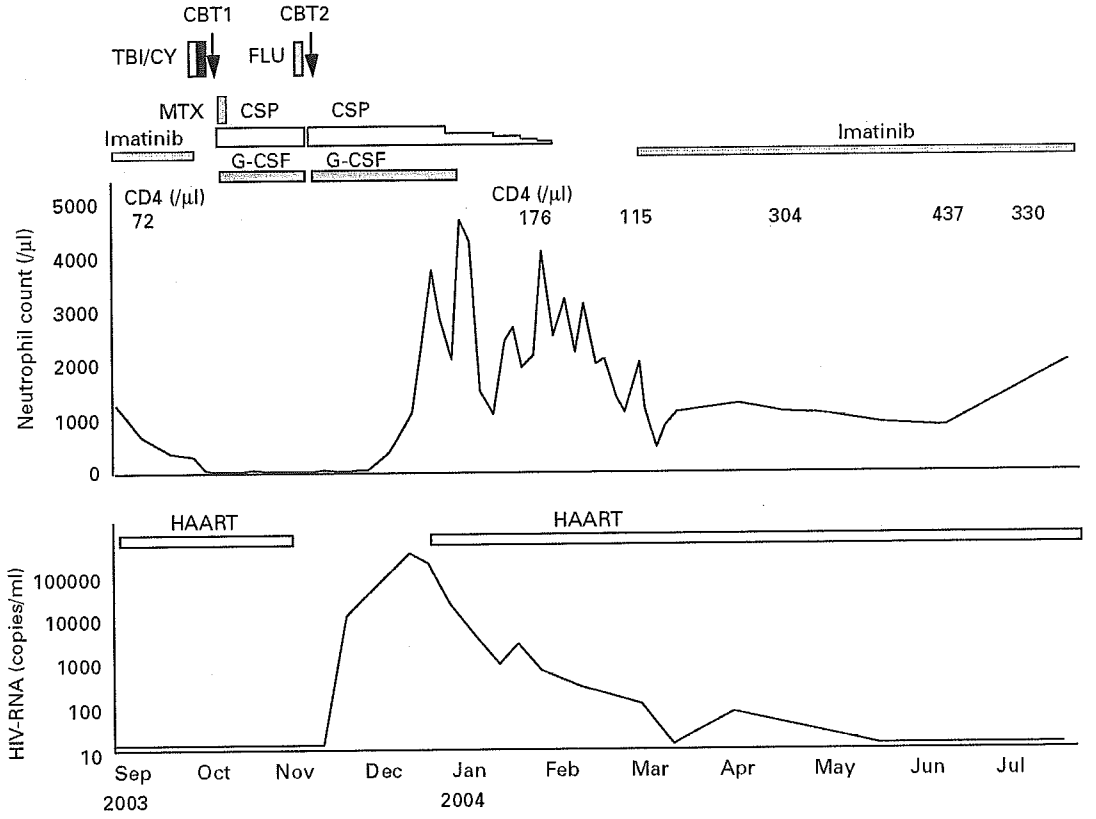

Figure 1 Clinical course of the patient.

blood (CB) unit were 2.9×10^7/kg and 0.76×10^5/kg, respectively. The conditioning regimen included 12 Gy total body irradiation and 120 mg/kg cyclophosphamide. Graft-versus-host disease (GVHD) prophylaxis consisted of cyclosporine and methotrexate. The patient tolerated the procedure well with minimal regimen-related toxicity. Owing to possible myelosuppression, HAART was discontinued on day +28. On day +33, her WBC remained below 100/μl and all of the BM cells were shown to be derived from the recipient. At 40 days after the first CBT, second CBT was performed from an unrelated donor with a one-locus mismatch at HLA-DR. The numbers of total nucleated cells and CD34-positive cells in the CB unit were 2.1×10^7/kg and 0.46×10^5/kg, respectively. The conditioning regimen included 40 mg/m^2 fludarabine for 3 days. Cyclosporine was administered for GVHD prophylaxis. A neutrophil count consistently greater than 500/μl was achieved on day +27. Full donor chimerism of BM cells was shown on day +28. The HIV-RNA level increased to 3×10^6 copies/ml on day +31. After the administration of HAART from day +38, the HIV-RNA levels returned to below detectable levels from day +195, and the CD4 count increased to above 300/μl from day +170. No bacterial or fungal infections were documented during the first and second CBT processes and cytomegalovirus reactivation was successfully treated with ganciclovir and foscarnet. Grade I acute GVHD occurred, but resolved without any additional immunosuppressants. No chronic GVHD was observed. An RT-PCR analysis showed continuous negative test results for the p190$^{BCR\text{-}ABL}$ transcript until the last follow-up evaluation at 15 months post-CBT.

CBT for adults has been associated with a high rate of early transplantation-related mortality (TRM).[5,6] However, our single-institution experience showed a 1-year TRM of 9% and 2-year disease-free survival of 74% in 68 adults after CBT.[7] Both CB donors and the patient in the present study were Japanese. The lesser genetic diversity in a single ethnic population in our studies might be associated with the favorable outcomes of CBT, such as the lower rates of severe acute GVHD. Although our results suggest that CBT is feasible for HIV-infected patients on HAART, the safety and efficacy should be further examined by prospective studies.

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References

1 Krishnan A, Zaia J, Forman SJ. Should HIV-positive patients with lymphoma be offered stem cell transplants? *Bone Marrow Transplant* 2003; **32**: 741–748.
2 Schlegel P, Beatty P, Halvorsen R, McCune J. Successful allogeneic bone marrow transplant in an HIV-1-positive man with chronic myelogenous leukemia. *J Acquir Immune Defic Syndr* 2000; **24**: 289–290.
3 Sora F, Antinori A, Piccirillo N *et al.* Highly active antiretroviral therapy and allogeneic CD34(+) peripheral blood progenitor cells transplantation in an HIV/HCV coinfected patient with acute myeloid leukemia. *Exp Hematol* 2002; **30**: 279–284.
4 Kang EM, de Witte M, Malech H *et al.* Nonmyeloablative conditioning followed by transplantation of genetically modified HLA-matched peripheral blood progenitor cells for hematologic malignancies in patients with acquired immunodeficiency syndrome. *Blood* 2002; **99**: 698–701.
5 Laughlin MJ, Eapen M, Rubinstein P *et al.* Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. *N Engl J Med* 2004; **351**: 2265–2275.
6 Rocha V, Labopin M, Sanz G *et al.* Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. *N Engl J Med* 2004; **351**: 2276–2285.
7 Takahashi S, Iseki T, Ooi J *et al.* Single institute comparative analysis of unrelated bone marrow transplantation and cord blood transplantation for adult patients with hematological malignancies. *Blood* 2004; **104**: 3813–3820.

Modified Dynabeads Method for Enumerating CD4+ T-Lymphocyte Count for Widespread Use in Resource-Limited Situations

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Summary: The Dynabeads method showed the potential for enumerating CD4+ T lymphocytes (CD4 count) in HIV-1–infected individuals. The large volume of Dynabeads required for 1 sample and complex procedure made the method expensive and not easy for use, however. To decrease the cost and simplify the procedure, we reduced the volume of the Dynabeads, added wash times, and skipped over the staining step so as to count the CD4 cells directly under an optical microscope. The CD4 count of 246 blood samples using our modified Dynabeads method (DynabeadsCD4) showed a significant correlation with that obtained by flow cytometry (FlowcytoCD4) ($r = 0.91$ [$P < 0.0001$]; slope = 1.03, intercept = −16). The sensitivity and specificity for a CD4 count less than 200 cells/μL were 79% and 94%, and for a CD4 count less than 350 cells/μL, the sensitivity and specificity were 95% and 88%, respectively. The positive and negative predictive values for a CD4 count less than 350 cells/μL were 97% and 83%, respectively. The systematic error was 8 cells/μL (95% confidence interval [CI]: 0.4–16). The cost of Dynabeads for 1 sample was less than $1.00; thus, the estimated cost per DynabeadsCD4 test is less than $3.00, including the cost of other disposable materials. Our modified method is simple, economic, and accurate enough to monitor antiretroviral therapy in resource-limited situations.

Key Words: CD4, monitoring, Dynabeads, resource-limited situations

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The CD4+ T-lymphocyte count (CD4 count) is an important surrogate marker for the clinical course of HIV infection, such as initiation of prophylactic treatment of opportunistic infections, initiation of antiretroviral therapy (ART), and monitoring the response to ART.[1–4] In developed countries, the CD4 count is usually measured by flow cytometry, which is considered to be the standard reference method.[3,4] In resource-limited areas, however, flow cytometry is available only in limited settings such as tertiary medical centers because it requires expensive reagents and well-trained technicians. Furthermore, equipment maintenance is another difficult issue, because a technical support system is needed in areas afflicted with frequent electrical power failures, which could potentially cause machine-related problems.

In recent years, lower cost and less technically demanding methods for enumerating CD4 cells have been tried but have not been used widely even in resource-limited settings for various reasons.[4,5] In the World Health Organization (WHO) guidelines for treatment of HIV-infected individuals in resource-limited environments, a total lymphocyte count (TLC) of 1200 cells/μL is recommended to represent a CD4 count threshold of 200 cells/μL in making a decision regarding therapy when the CD4 count is unavailable.[1] In addition, various research groups have recommended the use of a TLC,[5] absolute lymphocyte count or TLC,[6] and TLC combined with hemoglobin measurement[7] as surrogate markers for monitoring ART. These studies suggested that the lymphocyte count might have some value in monitoring ART. The lymphocyte count is readily available and inexpensive, but it is not sufficiently adequate to predict the absolute CD4 count in many settings.[4]

Among several low-cost and less technically demanding methods,[8–15] the Dynabeads assay, which uses magnetic particles coated with a monoclonal antibody to CD4 to capture CD4+ cells, seems to be a good candidate as an alternative to flow cytometry based on its good correlation with the results of flow cytometry.[8,10,11,13] According to the protocol recommended by the manufacturer, however, CD4 and CD8 cells are enumerated at the same time using a large volume of Dynabeads. The large volume of Dynabeads used in each assay is also relatively expensive (approximately $5), particularly for poor settings. In addition, division of the samples into 2 aliquots during the procedure might jeopardize the accuracy of the results. Moreover, in this assay, the cells are lysed and nuclei are stained to count them, which makes the operation complex.

For monitoring ART in HIV infections, only the CD4 component is necessary and only the CD4 count (not CD8 count) is mentioned in ART guidelines.[1,2] For this reason,

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further modifications are needed for the expanded use of the CD4 count in resource-limited areas. In the present study, we modified the protocol to make it simple and inexpensive so that it could be applied widely in resource-limited facilities.

MATERIALS AND METHODS

Study Population and CD4 Enumeration

This study included 242 adult patients infected with HIV-1 who regularly consulted the AIDS Clinical Center of the International Medical Center of Japan between June and October 2003. The inclusion criteria were a CD4 count less than 1000 cells/μL and consent granted to participate in the study. Patient age ranged from 20 to 78 years (mean ± SD: 40 ± 11.5). A total of 315 blood samples were collected using EDTA-containing tubes and tested for CD4 count within 4 hours by flow cytometry (FlowcytoCD4; Coulter-EPICS XL-MCL, Beckman-Coulter, Fullerton, CA) with CD45–fluorescein isothiocyanate (FITC)/CD4-phycoerythrin (RD1)/CD8-pheycoerythrin-Texas Red (ECD)/CD3-pheycoerythrin-cyanin 5.1 (PC5) (Beckman-Coulter). The CD4 cell count in the rest of the blood sample was enumerated using Dynabeads (Dynabeads CD4; Dynal Biotech ASA, Oslo, Norway) within 24 hours. When different protocols such as 25-μL and 5-μL volumes of CD4 Dynabeads or 10 and 30 minutes of incubation time were compared, the same sample was used in each experiment.

Modified Protocol (Original Protocol) of the Dynabeads Method

A well-mixed whole-blood sample (125 μL) was placed into a 1.5-mL microtube containing 375 μL (350 μL) of buffer (0.1% bovine serum albumin in phosphate-buffered saline [PBS]). CD14 Dynabeads were suspended with buffer (1:1 diluted buffer); 5 μL (25 μL) of CD14 Dynabeads was then added to the microtube containing the blood sample and the tube was inverted several times and then incubated in Dynal MX-1 for 10 minutes. The tube was spun down in a microcentrifuge and then placed in magnetic particle concentration for microcentrifuge tubes (Dynal MPC-S) (6 tubes per batch) for 2 minutes, followed by transfer of the entire volume (division into 2 200-μL aliquots) of monocyte-depleted blood into a new microtube. In the next step, 5 μL (25 μL) of CD4 Dynabeads was added to the tube and incubated in Dynal MX-1 for 30 minutes (10 minutes). The cells were washed with 500 μL of buffer, vortexed gently, and spun down; the tube was then placed in Dynal MPC-S for 2 minutes, the wash buffer was discarded, and the tube was removed from the Dynal MPC-S. The cells were washed 3 more times (once), resuspended by adding 125 μL of buffer, and kept at 4°C until counting (50 μL of lysing solution was added, followed by thorough vortexing for resuspension, and the cells were allowed to stand for 5 minutes, after which 50 μL of acridine orange staining solution was added and the sample was kept in darkness until counting). Finally, the sample was vortexed well, 10 μL of cells was applied to a hemocytometer, and the mononuclear cells with attached Dynabeads were counted as $CD4^+$ cells under a light microscope (the number of nuclei was determined under a fluorescence microscope). After reducing the volume of CD4 Dynabeads, it was not difficult to count the CD4 cells under an optical microscope, even without staining. All procedures were performed at room temperature at approximately 23°C. All Dynabeads-related equipments and reagents were products of Dynal Biotech ASA.

Statistical Analysis

All data are expressed as mean ± SD. StatView v5.0 software was used to analyze the correlation and single linear regression between DynabeadsCD4 and FlowcytoCD4. *P* values were calculated by 2-sided test and considered as significant if at a level less than 5%. All confidence intervals were 2-sided, with a significant level of 5%.

RESULTS

First, we examined the influence of a reduced volume of CD14 (from 12.5 μL to 5 μL) Dynabeads on monocyte depletion. The percentage of monocytes in 5 blood samples was analyzed by flow cytometry before and after treatment with 5 μL of CD14 Dynabeads. The result showed that 5 μL of CD14 Dynabeads deleted 92.4% to 97.5% (average = 95.6%) of monocytes from 125 μL of whole blood. The remaining experiments were performed using 5 μL of CD14 Dynabeads. Next, we examined the influence of a reduced volume of CD4 Dynabeads on the CD4 count in 23 samples. The volume of CD4 Dynabeads was reduced from 25 μL to 5 μL, but the incubation time was still 10 minutes (like that of original protocol), which we called modified protocol 1. CD4 counts by the original protocol and modified protocol 1 correlated significantly with those determined by flow cytometry: DynabeadsCD4 by the original protocol ($r = 0.90$ [$P < 0.0001$]; slope = 1.05, intercept = −32) and DynabeadsCD4 by modified protocol 1 ($r = 0.92$ [$P < 0.0001$]; slope = 1.05, intercept = 26). These results indicated that DynabeadsCD4 obtained by using the reduced volume of CD4 Dynabeads with a 10-minute CD4 separation correlated well with FlowcytoCD4. When the number of samples was increased to 56, however, the mean DynabeadsCD4 of 56 samples by modified protocol 1 was 269 ± 140 cells/μL compared with a mean FlowcytoCD4 of 336 ± 178 cells/μL (Table 1). The difference was −67 cells/μL ($P < 0.0001$). This result suggested that the 10-minute CD4 separation time was too short. We then examined the effect using a reduced volume of Dynabeads and a different incubation time.

Next, with 5 μL of CD4 Dynabeads, we lengthened the CD4 separation time from 10 minutes to 30 minutes in 34 samples. The correlations between DynabeadsCD4 and FlowcytoCD4 were $r = 0.91$ ($P < 0.0001$) and $r = 0.94$ ($P < 0.0001$), with slopes of 1.05 and 1.0 and intercepts of 22 and 8, for 10 and 30 minutes of incubation time, respectively. The mean difference with flowcytoCD4 was −32 cells/μL ($P = 0.008$) and −8 cells/μL ($P = 0.42$), respectively. According to these data, the 30-minute incubation time for CD4 separation yielded a better result than that of the 10-minute incubation time. We then fixed the protocol as 5 μL of CD14 Dynabeads with 10 minutes of incubation time and 5 μL of CD4

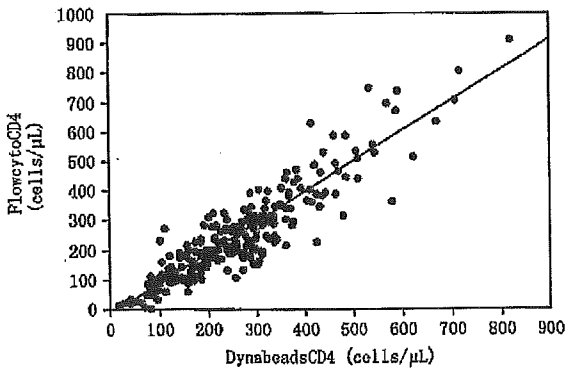
TABLE 1. DynabeadsCD4 Determined by Different Protocols Compared with FlowcytoCD4

Protocol	Mean ± SD (Cells/μL)		Mean Difference		Regression Line		
	DynabeadsCD4	FlowcytoCD4	Cells/μL (95% CI)	*P*	Intercept	Slope	r^2
Original (n = 59)	364 ± 166	372 ± 193	−8 (−32 to 16)	0.521	−2	1.026	0.775
Modified 1 (n = 56)*	269 ± 140	336 ± 178	−67 (−93 to −41)	<0.0001	50	1.061	0.698
Modified 2 (n = 246)†	262 ± 136	254 ± 154	8 (0.4 to 16)	0.0396	−16	1.031	0.829

†Modified 2 (the final one): modified protocol with 30 minutes of CD4 separation.
*Modified 1: modified protocol with 10 minutes of CD4 separation.
$P > 0.05$ for all intercepts, $P < 0.0001$ for all slopes and r^2.

Dynabeads with 30 minutes incubation time (which we called modified protocol 2) and tested 246 samples. DynabeadsCD4 showed a significant correlation with FlowcytoCD4 ($r = 0.91$ [$P < 0.0001$]; slope = 1.03, intercept = −16; Fig. 1). At less than 200 cells/μL, the sensitivity and specificity of DynabeadsCD4 compared with FlowcytoCD4 were 79% and 94%, respectively, and at less than 350 cells/μL, the sensitivity and specificity were 95% and 88%, respectively. The mean DynabeadsCD4 was 262 ± 135 cells/μL and that of FlowcytoCD4 was 254 ± 154 cells/μL (see Table 1). The difference in the mean values was 8 cells/μL (95% confidence interval [CI]: 0.4–16; $P = 0.04$), with a random error of 64 cells/μL. The positive and negative predictive values of DynabeadsCD4 and FlowcytoCD4 for less than 200 cells/μL and less than 350 cells/μL were 90% and 87% and 97% and 83%, respectively. Other factors (eg, on therapy vs. off therapy, male vs. female) had no influence on DynabeadsCD4 (data not shown).

Table 2 shows the results of a comparison between the original protocol and our modified protocol. In our modified protocol, volumes of CD14 and CD4 Dynabeads were reduced from 12.5 μL and 25 μL, respectively, to 5 μL each against 125 μL of whole blood. Accordingly, the cost of the Dynabeads test decreased from $2.84 to $0.89. The incubation time for CD4 separation was prolonged to 30 minutes to obtain a better yield. In our protocol, after monocyte depletion, we transferred all treated blood to a new microtube for CD4 cell separation because we did not consider the CD8 count. We also skipped over lysis and nuclear staining steps so as to simplify the procedure.



FlowcytoCD4 = −16.484 + 1.031 × DynabeadsCD4; $r^2 = 0.829$

FIGURE 1. Correlation analysis of CD4+ T-lymphocyte count (CD4 count) using Dynabeads method and CD4 count using flow cytometry.

DISCUSSION

To attain the "3 by 5" goal of effective ART promoted by the WHO, precise monitoring of ART is indispensable. Low cost, in addition to good accuracy, is thus an important issue. In this regard, maintenance of a "high-tech" machine for long-term monitoring may be impossible. The Dynabeads method is currently used as an alternative method to flow cytometry for CD4 count in a number of countries. In this study, we successfully modified the protocol of the Dynabeads method to make it more suitable in resource-limited areas with 2 goals in mind: reasonable cost and sufficient accuracy.

TABLE 2. Comparison Between the Original Protocol and Modified Protocol 2 for Enumeration of CD4 Count

Step	Original Protocol	Modified Protocol 2
Buffer (μL)	350	375
Blood (μL)	125	125
CD14 Dynabeads (μL)	25 (1:1 dilution)	5
Incubation temperature (°C)	RT	RT
Incubation duration (min)	10	10
Monocyte-depleted supernatant (μL)	200*	505†
CD4 Dynabeads (μL)	25	5
Incubation temperature (°C)	RT	RT
Incubation duration (min)	10	30
Repeat of washing (total min)	2 (10 min)	4 (20 min)
Staining time (min)	5 min	—
Resuspension volume (μL)	—	125
Time of total experiment per sample (min)	50	75
Samples comfortably analyzed per operator	12–18	12–18
Cost of CD14 Dynabeads ($)	1.63	0.65
Cost of CD4 Dynabeads ($)	1.21	0.24
Total cost of Dynabeads ($)	2.84	0.89

*Transferring 200 μL to a new tube.
†Transferring the entire volume to a new tube.
Values are for 1 test.
RT indicates room temperature (approximately 23°C).

In the present study, DynabeadsCD4 obtained by using the original protocol also showed a good result (see Table 1). During the operation, we found 2 problems with the original protocol, however. One was the transfer of 200 μL of blood from 500 μL of blood to a new tube after monocyte depletion. This step might lead to inaccurate results because we could not mix the blood well while the tube was on the Dynal MPC-S. The other was that too many free Dynabeads (which did not attach to CD4 cells) and red blood cells were identified when the cells were counted under a light microscope. This might be the reason for recommending lysis of the cells, staining the nuclei, and using a fluorescent microscope in the last step of the original protocol. In our modified protocol, the entire sample was transferred to a new tube after monocyte depletion. The number of free Dynabeads decreased after the volume of CD4 Dynabeads was reduced. Furthermore, we washed the sample 4 times after CD4 cell separation in spite of the original protocol recommending washing only twice. The red blood cells could be almost completely removed by 4 washes, especially when the washing buffer had been discarded completely at each wash. These modifications made a direct count under a light microscope possible.

After reduction of the volume of Dynabeads used in the assay, the cost of reagents used for analysis of 1 sample decreased to less than $1.00. Thus, the total cost of 1 CD4 count, including other disposable materials such as syringes, tubes, and tips, could be less than $3.00.

In conclusion, the present study demonstrated that our final modified protocol of Dynabeads assay could be used as a good alternative to flow cytometry with sufficient accuracy, reliability, and simplicity at a reasonable cost. Therefore, the assay could be suitable for monitoring ART in resource-limited settings.

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REFERENCES

1. World Health Organization. Scaling up antiretroviral therapy in resource limited settings: guidelines for a public health approach. Available at: http://www.who.int/hiv/. Accessed April 2002.
2. Department of Health and Human Services. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Available at: http://AIDsinfo.nih.gov. Accessed July 14, 2003.
3. Brando B, Barnett D, Janossy G, et al. Cytofluorimetric methods for assessing absolute numbers of cell subsets in blood. *Cytometry.* 2000; 42:327–346.
4. Crowe S, Turnbull S, Oelrichs R, et al. Monitoring of human immunodeficiency virus infection in resource-constrained countries. *Clin Infect Dis.* 2003;37(Suppl 1):S25–S35.
5. Badri M, Wood R. Usefulness of total lymphocyte count in monitoring highly active antiretroviral therapy in resource-limited settings. *AIDS.* 2003;17:541–545.
6. Jacobson AM, Liu L, Khayam-Bashi H, et al. Absolute or total lymphocyte count as a marker for the CD4 T lymphocyte criterion for initiating antiretroviral therapy. *AIDS.* 2003;17:917–919.
7. Spacek AL, Griswold M, Quinn CT, et al. Total lymphocyte count and hemoglobin combined in an algorithm to initiate the use of highly active antiretroviral therapy in resource-limited settings. *AIDS.* 2003;17:1311–1317.
8. Diagbouga S, Chazallon C, Kazatchkine MD, et al. Successful implementation of a low-cost method for enumerating CD4+ T lymphocytes in resource-limited settings: the ANRS 12-26 study. *AIDS.* 2003;17:2201–2208.
9. Brinchmann JE, Leivestad T, Vartdal F. Quantification of lymphocyte subsets based on cells directly from blood. *J Immunogenet.* 1989;16:177–183.
10. Didier J, Kazatchkine M, Demouchy C, et al. Comparative assessment of five alternative methods for CD4 T-lymphocyte enumeration for implementation in developing countries. *J Acquir Immune Defic Syndr.* 2001; 26:193–195.
11. Lyamuya EF, Kagoma C, Mbena EC, et al. Evaluation of the FACScount, TRAx CD4 and Dynabeads methods for CD4 lymphocyte determination. *J Immunol Methods.* 1996;195:103–112.
12. Nicholson JK, Velleca W, Jubert S, et al. Evaluation of alternative CD4 technologies for the enumeration of CD4 lymphocyte. *J Immunol Methods.* 1994;177:43–54.
13. Lyamuya EF, Schechter M, Echeverria de Perez G, et al. Multicentre evaluation of alternative methods for CD4 lymphocyte determination [abstract Mo.A.513]. Presented at the International AIDS Conference, Vancouver, July 1996.
14. Carella AV, Moss MW, Provost V, et al. A manual bead assay for the determination of absolute CD4+ and CD8+ lymphocyte counts in human immunodeficiency virus-infected individuals. *Clin Diagn Lab Immunol.* 1995;2:623–625.
15. Gaudernack G, Lundin KE. Rapid immunomagnetic phenotyping of cells. *J Immunogenet.* 1989;16:169–175.

Efficacy and Immunologic Responses to Influenza Vaccine in HIV-1–Infected Patients

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Summary: Influenza vaccine is recommended for HIV-1–infected patients. The present prospective study was conducted to evaluate the clinical efficacy and immunologic responses to the vaccine. From November 1 to December 27, 2002, 262 HIV-1–infected patients received a trivalent influenza subunit vaccine, whereas 66 did not. Influenza illness occurred in 16 vaccinated and 14 nonvaccinated patients (incidence = 6.1% [95% confidence interval (CI): 4%–10%] in vaccinated vs. 21.2% [CI: 13%–35%] in nonvaccinated persons, $P < 0.001$; relative risk = 0.29 [CI: 0.14–0.55]). Influenza vaccine provided clinically effective protection against influenza illness in HIV-1–infected patients. In baseline antibody-negative patients, anti-H1 and anti-H3 antibody responses to the vaccination were significant in those patients with a CD4 count >200 cells/μL compared with those with a CD4 count <200 cells/μL ($P < 0.05$). In contrast, in baseline antibody-positive patients, good antibody responses were observed irrespective of CD4 counts, like the healthy controls. Based on these results, annual vaccination is recommended. Specific CD4 responses correlated with HIV-1 viral load (VL), especially in patients treated with highly active antiretroviral therapy (HAART) compared with those without HAART ($P < 0.01$), although the clinical efficacy did not correlate with HIV-1 VL. HAART may enhance the immunologic efficacy of influenza vaccine.

Key Words: HIV-1, influenza, vaccination, antibody response, specific CD4

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After the recent approval of various anti-influenza drugs and rapid diagnosis kits for influenza infection by the Ministry of Health, Labor, and Welfare of Japan, it has become easier to diagnose this infection. Along with the developments in diagnostic methods and treatment of the infection, influenza vaccination programs have been actively applied in HIV-1–infected individuals. Influenza virus infection may be more prolonged in individuals with immunodeficiency[1] and can cause a transient increase in plasma HIV-1 viral load (VL)[2] that might become relevant to the clinical course of HIV-1 infection.[2,3] Therefore, influenza vaccine has been generally recommended for HIV-1–infected patients,[4–6] as is already stated in the guidelines of the Advisory Committee on Immunization Practices.[7] Few studies have reported the protective effect of such vaccination in patients with HIV-1 infection, however. Previous studies demonstrated that the number of CD4 T cells (CD4 count) could predict the efficacy of and/or antibody response to the vaccine but did not clearly demonstrate the correlation between the vaccine efficacy and HIV VL.[1,8–15]

Activated memory $CD4^+$ T cells are the predominant target of HIV-1,[16] and the antibody response to hemagglutinin (HA) is T-cell dependent.[17–19] Therefore, highly active antiretroviral therapy (HAART) may reconstitute the immune function of not only the antibody responses but T helper (Th)–cell responses. In this large prospective clinical study, we investigated the clinical efficacy of influenza vaccine in HIV-1–infected patients and correlated it with the immune response to the vaccine as determined by increased antibody titer and/or HA-specific CD4 T cells.

MATERIALS AND METHODS

Study Design and Participants

A 0.5-mL dose of single-shot trivalent influenza subunit vaccine, which contains 15 μg of influenza virus strains A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), and B/Shanton/7/87, was prepared for adults in the 2002 through 2003 winter season in Japan. All HIV-1–infected patients who consulted the outpatient clinic of the AIDS Clinical Center at the International Medical Center of Japan from November 1 to December 27, 2002 were advised to receive the vaccine, although the final decision was left to the individual. In previous seasons, nearly half of HIV-1–infected patients received influenza vaccine in our clinic. This study was designed to be prospective in nature but nonrandomized. Only individuals, vaccinated and nonvaccinated, who understood the purpose of the study were enrolled, without any incentives. To keep selective bias to a minimum, all vaccinated and consecutive first-come 100 nonvaccinated patients were asked to participate in this study. All study participants gave

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informed consent, and the institutional ethical committee approved this study (protocol IMCJ-141). Twenty-six hospital staff members who were vaccinated with the same vaccine batch were enrolled as healthy immunized controls after consenting to participate in this study. Among them, 4 had no anti-influenza antibodies before vaccination. All participants were asked to visit to our clinic at least at week 0, 8, and/or 16 after enrollment to allow the withdrawal of 17 mL of blood at each visit for analysis of immunologic responses and routine examinations, including CD4 count and HIV VL.

Definition and Diagnosis of Influenza Virus Infection

In this study, influenza infection (illness) was defined if the patient had flulike symptoms associated with at least 1 adjunct diagnosis such as a serologic or virologic diagnosis. Flulike symptoms were defined as a fever of ≥38.0°C combined with 2 of the following 5 clinical symptoms: cough, rhinitis, myalgia, sore throat, and headache. All participants were asked to visit the clinic if they developed flulike symptoms. To avoid a bias in the clinical diagnosis, a history of influenza vaccination was written out on a separate colored sheet, which was removed from medical records before the outpatient clinic physician attended and examined the patient. The serologic diagnosis was defined as a >4-fold rise in anti-influenza antibody titer compared with before and 4 weeks after the symptoms. In addition, a change of the antibody titer from <10 to 40 U was defined as a 4-fold rise. Patients who had only the antibody rise but no flulike symptoms were not considered to have influenza-related illness. The virologic diagnosis was made by means of viral culture and/or a Rapidvue influenza test kit (Quidel, San Diego, CA) using a nasal or throat swab.

Laboratory Investigations

At each visit, CD4 T cells were enumerated by standard flow cytometry and HIV VL was measured using the Roche Amplicor assay kit, version 1.5 (Roche Diagnostic Systems, Branchburg, NJ). Antibody responses to each of the 3 individual vaccine components were examined by the standard hemagglutinin inhibition (HAI) assay.[20] Titers ≥40 U were defined as protective, and a >4-fold rise in the antibody titer was considered an adequate response in previously antibody-negative patients.

For assessment of HA-specific CD4 T-cell responses, intracellular γ-interferon (IFN) production was examined by flow cytometry using the method described previously.[21,22] Because of the limited availability of peripheral blood mononuclear cells (PBMCs), we analyzed the H1-specific CD4 T cells only. Because fresh PBMCs must be used for this assay, as a result of a labor limitation, only the first 10 participants per day were examined on any particular day. Briefly, HA was purified from influenza virus strain, A/New Caledonia/20/99 (H1N1), as described previously.[23] PBMCs were isolated from the fresh heparinized blood and cultured (2×10^6 cells/mL) with diluted H1 plus anti-CD28 antibody (1 μg/mL) or medium alone for 16 hours at 37°C. Brefeldin A (10 μg/mL) was added to each sample in the final 5 hours of incubation. After 16 hours of stimulation, the cells were collected and stained with anti-CD4 allophycocyanin antibody (Beckman Coulter, Fullerton, CA) and anti-CD69–fluorescent isothiocyanate antibody (Becton Dickinson). Subsequently, the cells were fixed and permeabilized to examine for the intracellular production of γ-IFN as described previously.[21,22] The flow cytometry analysis was performed by means of the FACSCalibur fluorescence-activated cell sorter with CellQuest software (BD Biosciences, San Jose, CA), and 10,000 CD4 T cells were collected for each analysis.

Statistical Analysis

The data on HA-specific CD4 T cells are presented as the arithmetic mean ± SEM. The data on anti-HA antibody titer are presented as the geometric mean. Statistical analyses were performed using StatView 5.0 software (Abacus Concepts, Berkeley, CA). Differences in the proportion of influenza virus infection between vaccinated and nonvaccinated groups were analyzed by the χ^2 test. Multiple logistic regression analysis was used to identify factors that contributed to protection against influenza illness. For the analyses of immune responses, participants were stratified by their CD4 count or HIV VL. Changes in antibody titer and HA-specific CD4 T cells were analyzed using the Kruskal-Wallis test or the Mann-Whitney U test. In all tests, a P value <0.05 was considered significant.

RESULTS

Subjects

During the period of vaccination, 626 HIV-1–infected patients visited our clinic, and 332 of these received the vaccine, whereas 294 did not. Among them, 317 of those vaccinated and 87 of 100 approached to participate as nonvaccinated patients agreed to participate in the present study. Consequently, 76 patients dropped out of the study (55 of 317 vaccinated patients and 21 of 87 nonvaccinated patients). There were no characteristic differences at baseline between the analyzed and drop-out patients (data not shown). None of the patients dropped out from the study because of HIV-1 disease progression, and none received anticancer or immunosuppressive agents during this study. The final composition of the study group based on compliance with the study protocol, including visits on the fixed dates, was 262 vaccinated (82.6%) and 66 nonvaccinated (75.9%) patients (Fig. 1). Table 1 summarizes the baseline characteristics of the participants.

Efficacy of Influenza Vaccine

The peak of the influenza epidemic of the 2002 through 2003 winter season in Japan was documented during the fourth week of January 2003 and was predominantly caused by influenza A/H3N2. The prevalence of influenza infection in this season was the third highest in the last decade.[24] In this study, 30 participants were diagnosed as having definitive influenza illness (5 patients with A/H1N1 strain, 16 with A/H3N2 strain, and 9 with B strain). Six patients were confirmed to have an influenza illness by flulike symptoms, positive viral cultures, positive influenza test kit results, and a >4-fold rise in antibody titer (1 with H1N1 strain, 1 with H3N2 strain, and

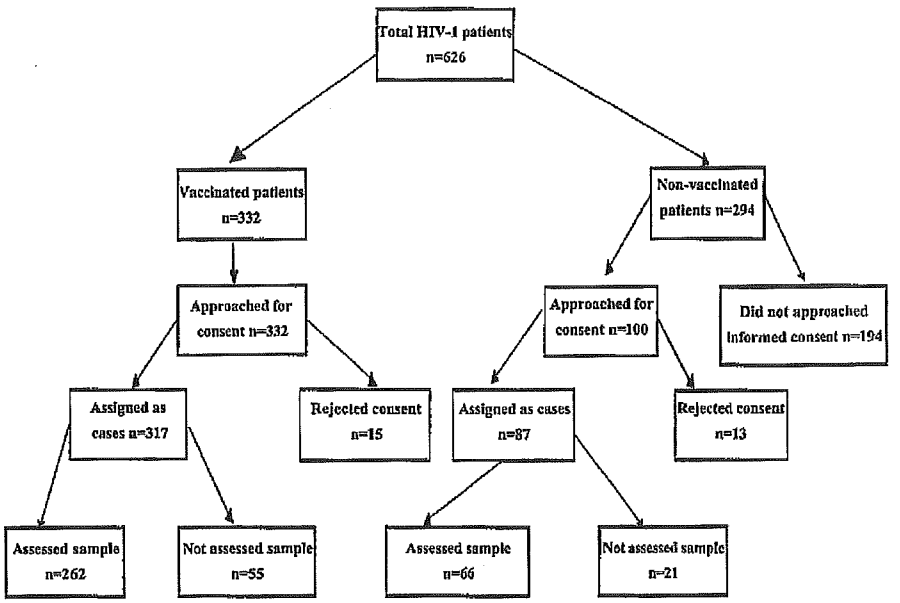

FIGURE 1. Profile of participants in this study.

4 with B strain); 3 by the symptoms, positive viral cultures, and antibody rise (2 with H1N1 strain and 1 with H3N2 strain); 5 by the symptoms, influenza test kit results, and antibody rise
(1 with H1N1 strain, 2 with H3N2 strain, and 2 with B strain); and 16 by the antibody rise between the symptoms (1 with H1N1 strain, 12 with H3N2 strain, and 3 with B strain). In total, 16 of 262 vaccinated patents had influenza illness (6.1%, confidence interval [CI]: 0.04–0.1) and 14 of 66 nonvaccinated patients had the illness (21.2%, CI: 0.13–0.35). The difference in the incidence between the 2 groups was significant ($P < 0.001$). The relative risk (RR) of influenza illness in vaccinated patients was 0.29 (CI: 0.14–0.55; $P < 0.001$) compared with nonvaccinated patients (Table 2). Eight patients who had a >4-fold rise in anti-H3 antibody titers between week 8 and week 16 without any clinical symptoms were not regarded as having influenza illness.

In patients with a CD4 count >200 cells/μL, the incidence of influenza illness in vaccinated patients (6.2%) was significantly lower than in nonvaccinated patients (21.0%) ($P < 0.001$). Conversely, in patients with a CD4 count <200 cells/μL, the same comparison showed no significant difference. Nevertheless, the incidences of influenza illness in vaccinated (5.9%) and nonvaccinated (22.2%) patients were the same as the incidence in patients with a CD4 count >200 cells/μL. Therefore, this analysis had lack of power because of the small number of nonvaccinated patients in this stratum. In vaccinated and nonvaccinated patients, the differences in the incidence were significant in patients with HAART ($P < 0.002$) and without HAART ($P < 0.05$) (see Table 2). When CD4 count was entered as a continuous variable, multivariate analysis using the logistic regression model identified vaccination ($P < 0.001$) and CD4 count ($P < 0.05$) but not HIV VL as independent predictors of influenza illness in HIV-1–infected patients.

In patients with influenza illness, 4 of 16 vaccinated patients and 4 of 14 nonvaccinated patients received an anti-influenza drug. None of the patients with influenza illness developed pneumonia that required treatment or hospitalization during the study period. Vaccination did not significantly change the HIV VL or CD4 count at weeks 8 and 16.

TABLE 1. Baseline Clinical and Immunologic Characteristics of Participants*

	Vaccinated	Nonvaccinated	*P*
No. participants (n)	262	66	—
Male/female ratio	7:1	15:1	n.s.
Median age, y (range)	41 (20–78)	40 (20–61)	n.s.
Received HAART (%)	75.2%	72.3%	n.s.
Median CD4 count at vaccination, μL (range)	380 (40–1137)	374 (66–1025)	n.s.
Median CD8 count at vaccination, μL (range)	778 (54–2649)	751 (163–1929)	n.s.
Median HIV VL at vaccination, $\log_{10}$/mL (range)	2.5 (1.5–6.2)	2.5 (1.5–6.4)	n.s.
Prior anti-H1 antibody-positive (%)	29.4%	26.4%	n.s.
Prior anti-H3 antibody-positive (%)	32.3%	30.3%	n.s.

*All participants were Japanese.
n.s. indicates not significant.

Anti-Hemagglutinin Antibody Responses Before and After Vaccination

HAI antibody titers against HA antigens (H1 and H3) were tested before and 8 and 16 weeks after vaccination (Table 3). To evaluate the effect of the single-shot influenza vaccine, subjects were divided into 2 groups based on the HAI titer before vaccination: the baseline HAI antibody-negative and antibody-positive groups. Furthermore, we excluded from this

TABLE 2. Incidence of Influenza Illness

	Vaccinated		Nonvaccinated		
	Illness/Patients	Rate (95% CI)	Illness/Patients	Rate (95% CI)	χ^2 Test
All patients	16/262	6.1% (0.04–0.1)	14/66	21.2% (0.13–0.35)	$P < 0.001$
CD4 count					
<200 cells/μL	3/51	5.9% (0.02–0.15)	2/9	22.2% (0.06–0.55)	n.s.
≥200 cells/μL	13/211	6.2% (0.03–0.1)	12/57	21.0% (0.12–0.33)	$P < 0.001$
HAART					
+	12/197	6.1% (0.04–0.1)	10/48	20.8% (0.11–0.34)	$P < 0.002$
−	4/65	6.2% (0.02–0.14)	4/18	22.2% (0.09–0.45)	$P < 0.05$

Incidence of influenza illness in healthy immunized controls was 3.8% (1 of 26, 95% CI: 0.01–0.19).
n.s. indicates not significant.

analysis the 13 patients who received the vaccination but had influenza illness (5 with H1N1 strain and 8 with H3N2 strain) during the study period so as to evaluate the antibody responses by the vaccination. The 8 patients who showed a >4-fold rise in anti-H3 antibody titers between week 8 and week 16 without any clinical symptoms were also excluded from this analysis, because the antibody rise in these cases was thought to be caused by influenza virus but not by vaccination. In the baseline HAI-negative group, the antibody responses to both antigens were significantly different compared with those in stratified HIV-1–infected patients by CD4 count (<200 cells/μL and ≥200 cells/μL; $P < 0.05$) at week 8 and week 16. These titers were low compared with those of the healthy immunized controls in both strata, however. In those with a CD4 count <200 cells/μL, 12 (27.9%) of 43 patients and 12 (32.4%) of 37 patients showed more than a 4-fold rise in the antibody responses against anti-H1 and anti-H3, respectively. In contrast, in those patients with a CD4 count >200 cells/μL, 62 (44.6%) of 139 patients and 61 (46.9%) of 130 patients showed a >4-fold rise in the antibody responses against anti-H1 and anti-H3, respectively. Although differences in the percentages of patients who showed both anti-H1 ($P = 0.05$) and anti-H3 ($P = 0.12$) antibody responses of the different CD4 strata were only marginal, there was a tendency for the single-shot vaccination to be more effective in terms of antibody responses in patients with a CD4 count >200 cells/μL. The antibody responses in both groups were not influenced by HIV VL (<100 copies/mL and ≥100 copies/mL; data not shown).

In the baseline HAI antibody-positive group, HAI titers to both antigens remained high and the sustainability of the antibody titers in HIV-1–infected patients was similar to those of the healthy controls, irrespective of CD4 counts (see Table 3). In terms of the antibody rise, in those with a CD4 count <200 cells/μL, 5 of 8 patients and 1 of 6 patients showed more than a 4-fold rise in the antibody response against anti-H1 and

TABLE 3. Anti-HA Antibody Responses After Vaccination in Baseline Anti-HA Antibody-Negative and Positive Individuals

	Anti-HA Antibody Responses* After Vaccination in HIV-1 Patients†						Healthy Immunized Controls	
	Stratum 1 (CD4 count <200 cells/μL)			Stratum 2 (CD4 count ≥200 cells/μL)				
	Week 0	Week 8	Week 16	Week 0	Week 8	Week 16	Week 0	Week 8
Baseline anti-H1 Ab-negative	n = 43			n = 139			n = 4	
Anti-H1 Ab responses	<10	26‡ (10–1280)	23‡ (10–1280)	<10	42 (10–1280)	36 (10–1280)	<10	135 (40–320)
Baseline anti-H3 Ab-negative	n = 37			n = 130			n = 4	
Anti-H3 Ab responses	<10	25‡ (10–640)	23‡ (10–1280)	<10	34 (10–1280)	32 (10–640)	<10	135 (40–320)
Baseline anti-H1 Ab-positive	n = 8			n = 67			n = 22	
Anti-H1 Ab responses	44 (20–320)	353 (40–1280)	208 (80–160)	54 (20–1280)	158 (20–1280)	143 (20–1280)	80 (20–640)	86 (20–640)
Baseline anti-H3 Ab-positive	n = 6			n = 73			n = 22	
Anti-H3 Ab responses	32 (20–80)	46 (20–160)	71 (20–640)	41 (20–1280)	105 (20–1280)	87 (10–1280)	59 (20–320)	66 (20–320)

*The data presented here are the geometric mean of anti-HA antibody titer. Range of the absolute titer is shown in parentheses.
†To analyze antibody responses to vaccination, patients with influenza infection were excluded from this analysis.
‡$P < 0.05$ compared with the respective value of stratum 2.
Ab indicates antibody. Change of the antibody titer from <10 to 40 U was considered a 4-fold rise.

anti-H3. Conversely, in those with a CD4 count >200 cells/μL, 16 of 67 patients and 19 of 73 patients showed more than a 4-fold rise.

Anti-H1 and Anti-H3 Antibody Responses in Patients With Influenza Illness Despite Vaccination

A total of 16 patients (5 with H1N1 strain, 8 with H3N2 strain, and 3 with B strain) had influenza illness among the vaccinated group during this study period. In the 5 patients with H1N1 illness, 3 were baseline anti-H1 antibody-negative and 2 had the antibody. Among the 3 baseline anti-H1 antibody-negative patients, 2 were infected before week 8 and 1 was infected after week 8. In the patient infected after week 8, no anti-H1 antibody was detected at week 8. In each of the 2 baseline anti-H1 antibody-positive patients, the titer was 20 U. Both patients were infected before week 8. In the 8 patients with H3N2 illness, 6 were baseline anti-H3 antibody-negative and 2 were positive for the antibody. In the 6 baseline anti-H3 antibody-negative patients, all were infected after week 8. Among these 6 patients, 4 were negative for anti-H3 antibody at week 8, whereas 2 had a 4-fold rise in the antibody before infection. In each of the 2 baseline anti-H3 antibody-positive patients, the titer was 20 U. Both patients were infected after week 8. Anti-H3 antibody at week 8 was increased to 40 U (a 2-fold rise) only in 1 patient. Overall, among the 9 infected patients (1 with H1N2 strain and 8 with H3N2 strain) in whom the antibody responses at week 8 could be evaluated, only 2 had a >4-fold rise of the antibody response before infection.

H1-Specific CD4 T-Cell Response Before and After Vaccination in Baseline Anti-H1 Antibody-Negative Subjects

H1-specific CD4 T-cell responses at week 8 were HIV VL dependent ($P < 0.005$) but not CD4 count dependent (Fig. 2A). Therefore, H1-specific CD4 T-cell responses were significantly increased by vaccination in HAART-treated patients ($P = 0.001$), because HIV VL was decreased by HAART (see Fig. 2B). In contrast, responses of HAI antibody titer were not different between HAART-treated and antiretroviral-naive patients (see Fig. 2C).

Comparison of Immune Responses to H1 Antigen at Week 8 Between Influenza A/H1N1–Infected and –Uninfected Patients

Five individuals were infected with influenza A/H1N1 during this season. HAI antibody titers at 8 weeks after the vaccination were not different between the infected and uninfected individuals. In contrast, H1-specific CD4 T-cell responses at week 8 were significantly low in the infected persons compared with those in the uninfected persons ($P < 0.05$; Fig. 3).

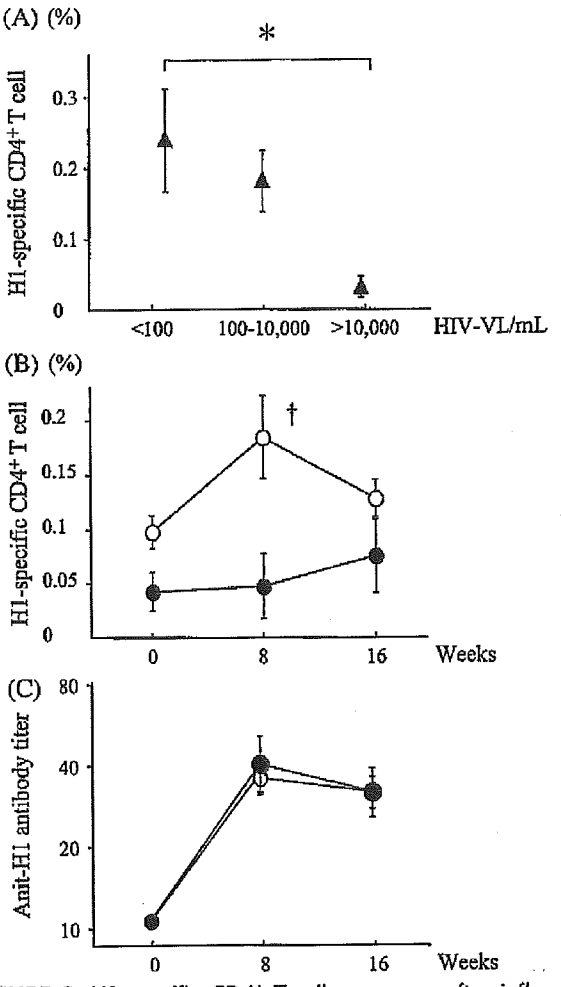

FIGURE 2. H1-specific CD4+ T-cell responses after influenza vaccine in baseline anti-H1 antibody–negative patients. A, Correlation of plasma HIV-1 viral load (HIV VL) and percentage of H1-specific CD4+ T cells. *H1-specific CD4+ T cells (▲) were significantly fewer in number in subjects with an HIV VL >10,000 copies/mL ($P < 0.005$). The number of samples with an HIV VL <100 copies/mL was 53, there were 19 samples with 100 to 10,000 copies/mL, and there were 11 samples with >10,000 copies/mL, because H1-specific CD4+ T cells were only examined in the first 10 samples per day as stated in the text. B, Changes in the percentage of H1-specific CD4+ Tcells in highly active antiretroviral therapy (HAART)–treated; (○; n = 63) and antiretroviral-naive patients (●; n = 12). †HAART-treated patients had significantly greater numbers of H1-specific CD4+ T cells at week 8 ($P < 0.01$) than antiretroviral-naive patients. C, Changes in anti-H1 antibody titer in HAART-treated (○; n = 131) and antiretroviral-naive patients (●; n = 35). Anti-H1 antibody responses were similar in both groups. Data are mean ± SEM.

DISCUSSION

Our prospective study confirmed many conclusions of previously reported small studies. First, we confirmed the protective effect of influenza vaccine in HIV-1–infected patients.[8–15] Second, anti-H1–specific and anti-H3–specific antibody responses were examined in HIV-1–infected patients after vaccination, and the responses were confirmed to be dependent on CD4 counts.[8–11]

To clarify the efficacy of a single-shot vaccination, we divided the participants by the positivity of anti-H1– and anti-H3–specific antibodies before vaccination and found that in