at that stage. Therefore, to avoid the emergence of drug resistance, there is a need for close monitoring of viral load and possible change of the treatment regimen before week 24 of NFV-containing therapy if viral load remains >400 copies/ml at week 12. In the present study, we did not address the influence of combination therapy on drug resistance because of the small number of patients. In this cohort, AZT plus 3TC or d4T plus 3TC were the main drugs used in combination with NFV (Table 1). In this regard, Squires et al. (2000) demonstrated that these two combinations do not affect the clinical efficacy.

NFV was well tolerated during the clinical trial. Only 4% of 696 patients discontinued the treatment by 24 weeks as a result of adverse events (Markowitz et al., 1998). In contrast, 10% ($n = 5$) of our patients discontinued treatment due to adverse events related to NFV. Among them, four stopped NFV due to drug eruptions, which appeared within 2 weeks of treatment. In the absence of such reaction soon after commencement of therapy, adherence to treatment with NFV-containing regimens was noted in such patients. Six patients changed their treatment due to virologic failure. However, new therapeutic regimens resulted in the suppression of viral load to undetectable in all cases according to their resistant profiles, indicating the importance of drug resistance testing in clinical practice (Gatanaga et al., 1999; Hirsch et al., 2000).

In conclusion, our results showed that NFV-containing regimens for PI-naïve patients are safe and effective when taken up to 108 weeks in daily clinical practice. There was no independent predictor for the emergence of drug resistance in clinical status, such as CD4 and viral load at baseline, prior AIDS diagnosis and prior use of RTI. Therefore, NFV can be used widely in PI-naïve patients. Virologic success at week 12 could predict continuation of treatment without the appearance of primary mutations thereafter.

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References

Aizawa S, Gatanaga H, Ida S, Sakai A, Tanaka M, Takahashi Y, Hirabayashi Y, Oka S. Clinical benefits of resistance assay for HIV-specific protease inhibitors: when to check and in whom? AIDS 1999;13:1278–9.

Albrecht MA, Bosch RJ, Hammer SM, Liou SH, Kessler H, Para MF, Eron J, Valdez H, Dehlinger M, Katzenstein DA. Nelfinavir, efavirenz, or both after the failure of nucleoside treatment of HIV infection. New Engl J Med 2001;345:398–407.

Division of AIDS. Division of AIDS table for grading severity of adult adverse experience. Rockville, MD: National Institute of Allergy and Infectious Diseases, 1996.

Easterbrook PJ, Newson R, Ives N, Pereira S, Moyle G, Gazzard BG. Comparison of virologic, immunologic, and clinical response of five different initial protease inhibitor-containing and nevirapine-containing regimens. J Aquir Immune Defic Syndr 2001;27:350–64.

Egger M, Hirschel B, Francioli P, Sudre P, Wirz M, Flepp M, Rickenbach M, Malinverni R, Vernazza P, Battegay M. Impact of new antiretroviral combination therapies in HIV infected patients in Switzerland: prospective multicenter study. Br Med J 1997;315:1194–9.

Fatkenheuer G, Theisen A, Rockstroh J, Grabow T, Wicke C, Becker K, Wieland U, Pfister H, Reiser M, Hegener P, Franzen C, Schwenk A, Salzberger B. Virological treatment failure of protease inhibitor therapy in an unselected cohort of HIV-infected patients. AIDS 1997;11:F113–6.

Gatanaga H, Aizawa S, Kikuchi Y, Tachikawa N, Genka I, Yoshizawa S, Yamamoto Y, Yasuoka A, Oka S. Anti-HIV effect of saquinavir combined with ritonavir is limited by previous long-term therapy with protease inhibitors. AIDS Res Hum Retrovir 1999;15:1493–8.

Gathe J, Chu A, Kass C, Paxton W. Three year experience with nelfinavir combination therapy. In: Program and Abstracts of the Thirteenth International AIDS Conference, Durban, July 9–14, 2000. Abstract 3236.

Grabar S, Le Moing V, Goujard C, Leport C, Kazatchkine MD, Costagliola D, Weiss L. Clinical outcome of patients with HIV-1 infection according to immunologic and virologic response after 6 months of highly active antiretroviral therapy. Ann Intern Med 2000a;133:401–10.

Grabar S, Pradier C, Le Corfec E, Lancar R, Allavena C, Bentata M, Berlureau P, Dupont C, Fabbro-Peray P,

Poizot-Martin I, Costagliola D. Factors associated with clinical and virological failure in patients receiving a triple therapy including a protease inhibitor. AIDS 2000b;14:141–9.

Gulick RM, Mellors JW, Havlir D, Eron JJ, Meibohm A, Condra JH, Valentine FT, McMahon D, Gonzalez C, Jonas L, Emini EA, Chodakewitz JA, Isaacs R, Richman DD. 3-Year suppression of HIV viremia with indinavir, zidovudine, and lamivudine. Ann Intern Med 2000;133:35–9.

Hammer SM, Squires KE, Hughes MD, Grimes JM, Demeter LM, Currier JS, Eron JJ, Jr, Feinberg JE, Balfour HH, Jr, Deyton LR, Chodakewitz JA, Fischl MA. A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. New Engl J Med 1997;337:725–33.

Hirsch MS, Brun-Vézinet F, D'Aquila RT, Hammer SM, Johnson VA, Kuritzkes DR, Loveday C, Mellors JW, Clotet B, Conway B, Demeter LM, Vella S, Jacobsen DM, Richman DD. Antiretroviral drug resistance testing in adult HIV-1 infection. J Am Med Assoc 2000;283:2417–26.

Lucas GM, Chaisson RE, Moore RD. Highly active antiretroviral therapy in a large urban clinic: risk factor for virologic failure and adverse drug reactions. Ann Intern Med 1999;131:81–7.

Markowitz M, Conant M, Hurley A, Schluger R, Duran M, Peterkin J, Chapman S, Patick A, Hendricks A, Yuen GJ, Hoskins W, Clendeninn N, Ho DD. A preliminary evaluation of nelfinavir mesylate, an inhibitor of human immunodeficiency virus (HIV)-1 protease, to treat HIV infection. J Infect Dis 1998;177:1533–40.

Mocroft A, Gill MJ, Davidson W, Phillips AN. Predictors of a viral response and subsequent virological treatment failure in patients with HIV starting a protease inhibitor. AIDS 1998;12:2161–7.

Mocroft A, Devereux H, Kinloch-de-Loes S, Wilson D, Madge S, Youle M, Tyrer M, Loveday C, Phillips AN, Johnson MA. Immunological, virological and clinical response to highly active antiretroviral therapy treatment regimens in a complete clinic population. AIDS 2000;14:1545–52.

Murphy EL, Collier AC, Kalish LA, Assmann SF, Para MF, Flanigan TP, Kumar PN, Mintz L, Wallach FR, Nemo GJ. Highly active antiretroviral therapy decreases mortality and morbidity in patients with advanced HIV disease. Ann Intern Med 2001;135:17–26.

Palella FJ, Jr, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ, Holmberg SD. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. New Engl J Med 1998;338:853–60.

Paredes R, Mocroft A, Kirk O, Lazzarin A, Barton SE, van Lunzen J, Katzenstein TL, Antunes F, Lundgren JD, Clotet B. Predictors of virological success and ensuring failure in HIV-positive patients starting highly active antiretroviral therapy in Europe. Results from the EuroSIDA study. Arch Intern Med 2000;160:1123–32.

Paris D, Ledergerber B, Weber R, Jost J, Flepp M, Opravil M, Ruef C, Zimmerli S. Incidence and predictors of virologic failure of antiretroviral triple-drug therapy in a community-based cohort. AIDS Res Hum Retrovir 1999;15:1631–8.

Paterson DL, Swindells S, Mohr J, Brester M, Vergis EN, Squier C, Wagener MM, Singh N. Adherence to protease inhibitor therapy and outcome in patients with HIV infection. Ann Intern Med 2000;133:21–30.

Patick AK, Duran M, Cao Y, Shugarts D, Keller MR, Mazabel E, Knowles M, Chapman S, Kuritzkes DR, Markowitz M. Genotypic and phenotypic characterization of human immunodeficiency virus type 1 variants isolated from patients treated with the protease inhibitor nelfinavir. Antimicrob Agents Chemother 1998;42:2637–44.

Podzamczer D, Ferrer E, Consiglio E, Gatell JM, Perez P, Perez JL, Luna E, Gonzalez A, Pedrol E, Lozano L, Azuaje C, Llibre JM, Casiro A, Aranda M, Barrufet P, Lacasa JM, Badia X, Casado A, Lupo S, Cahn P. Final 12-month results from the combine study: a randomized, open, multicenter trial comparing combivir (AZT/3TC) plus nelfinavir or nevirapine in HIV+ naïve patients. In: Program and Abstracts of the First IAS Conference on HIV Pathogenesis and Treatment, Buenos Aires, July 8–11, 2001. Abstract 7.

Rhone SA, Hogg RS, Yip B, Sherlock C, Conway B, Schechter MT, O'Shaughnessy MV, Montaner JS. The antiviral effect of ritonavir and saquinavir in combination amongst HIV-infected adults: results from a community-based study. AIDS 1998;12:619–24.

Roca B, Gomez CJ, Arnedo A. A randomized, comparative study of lamivudine plus stavudine, with indinavir or nelfinavir, in treatment-experienced HIV-infected patients. AIDS 2000;14:157–61.

Ruane P, Mendonca J, Timerman A, Cernohous P, Bauer E, Bernstein B, Sun E. Kaletra vs. nelfinavir in antiretroviral-naïve subjects: week 60 comparison in a phase III, blinded, randomized clinical trial. In: Program and Abstracts of the First IAS Conference on HIV Pathogenesis and Treatment, Buenos Aires, July 8–11, 2001. Abstract 6.

Seminari E, Maggiolo F, Villani P, Suter F, Pan A, Regazzi MB, Paolucci S, Baldanti F, Tinelli C, Maserati R. Efavirenz, nelfinavir, and stavudine rescue combination therapy in HIV-1-positive patients heavily pretreated with nucleoside analogues and protease inhibitors. J Aquir Immune Defic Syndr 1999;22:453–60.

Squires KE, Gulick R, Tebas P, Santana J, Mulanovich V, Clark R, Yangco B, Marlowe SI, Wright D, Cohen C, Cooley T, Mauney J, Uffelman K, Schoellkopf N, Grosso R, Stevens M. A comparison of stavudine plus lamivudine versus zidovudine plus lamivudine in combination with indinavir in antiretroviral naïve individuals with HIV infection: selection of thymidine analog regimen therapy (START 1). AIDS 2000;14:1591–600.

Staszewski S, Miller V, Sabin C, Carlebach A, Berger AM, Weidmann E, Helm EB, Hill A, Phillips A. Virological response to protease inhibitor therapy in an HIV clinic cohort. AIDS 1999;13:367–73.

Tebas P, Patick AK, Kane EM, Klebert MK, Simpson JH, Erice A, Powderly WG, Henry K. Virologic responses to a ritonavir-saquinavir-containing regimen in patients who had previously failed nelfinavir. AIDS 1999;13: F23–8.

Tsuchiya K, Matsuoka S, Hachiya A, Yasuoka A, Tachikawa N, Kikuchi Y, Genka I, Teruya K, Kimura S, Oka S. Accumulation of lopinavir resistance associated mutations over 3 years follow-up of patients on HAART; implication in salvage therapy. AIDS 2001;15:1183–4.

Serious Bradyarrhythmia That Was Possibly Induced by Lopinavir-Ritonavir in 2 Patients with Acquired Immunodeficiency Syndrome

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We describe 2 patients with acquired immunodeficiency syndrome who had potentially fatal bradyarrhythmia that occurred shortly after commencement of antiretroviral therapy. Lopinavir-ritonavir was the only drug that both patients were using.

Lopinavir-ritonavir is an effective option for the treatment of HIV type 1 (HIV-1)–infected individuals. However, we experienced 2 cases of serious bradyarrhythmia that were possibly induced by lopinavir-ritonavir.

Case report. A 22-year-old patient with hemophilia and HIV-1 infection was admitted to our hospital (International Medical Center of Japan, Tokyo) for salvage therapy for HIV-1 infection on 23 March 2001. The patient was also known to be infected with hepatitis B virus and hepatitis C virus (HCV). He was treated with trimethoprim-sulfamethoxazole, and coagulation factor VIII was administered as needed. At admission, the patient's CD4 cell count was 9 cells/μL and his HIV-1 load was 170,000 copies/mL. Since 1994, the patient had been treated aggressively with various antiretroviral drugs, including zidovudine, didanosine, stavudine, zalcitabine, saquinavir, nelfinavir, abacavir, efavirenz, and amprenavir.

On 27 March, the patient began receiving 3 mIU of IFN-α 3 times per week, and the dosage was subsequently increased to 6 mIU 3 times per week. Ribavirin (400 mg b.i.d.) was added during the second week of IFN-α therapy. The patient's clinical status improved in the subsequent 4 weeks. The patient began receiving treatment with lopinavir-ritonavir (400 mg/100 mg b.i.d.) and didanosine (400 mg q.d.) on 20 April. However, the patient complained of nausea and general malaise after receiving the first dose of lopinavir-ritonavir, and he felt chest discomfort 2 h after receiving the fourth dose of lopinavir-ritonavir (on day 2 of treatment). An electrocardiogram showed complete atrioventricular block (figure 1*A*). The serum electrolyte and creatine kinase levels were normal. All medications were discontinued except for trimethoprim-sulfamethoxazole. Complete atrioventricular block improved after the patient received an injection of atropine sulphate and a β-stimulant. The response to a challenge with lopinavir-ritonavir and didanosine was examined 50 days after the aforementioned episode of atrioventricular block. At that time, the patient was receiving only trimethoprim-sulfamethoxazole and the β-stimulant. Chest pain and palpitation occurred 10 h after the patient received the fifth dose of lopinavir-ritonavir. Electrocardiography performed at that time revealed Mobitz type I, second-degree atrioventricular block (figure 1*B*).

The second patient was a 60-year-old man with hemophilia and HIV-1 infection who was referred to our hospital for salvage therapy for HIV-1 infection on 11 September 2001. At admission, HCV infection, diabetes mellitus, duodenal ulcer, and idiopathic thrombocytopenia were diagnosed. At that time, the CD4 count was 36 cells/μL and the HIV-1 load was 3300 copies/mL. The patient had been treated aggressively with zidovudine, didanosine, stavudine, indinavir, and abacavir since 1995. After admission to the hospital, he was treated with sodium rabeprazole (a proton pump inhibitor), carbazochrome sodium sulfonate and tranexamic acid (for idiopathic thrombocytopenia), insulin replacement, and coagulation factor VIII, as required, in addition to zidovudine and abacavir.

On 12 September, antiretroviral therapy was switched to abacavir (300 mg b.i.d.), efavirenz (600 mg q.d.), and lopinavir-ritonavir (500 mg b.i.d.). The next morning, the patient was not able to take lopinavir-ritonavir because of dizziness induced by efavirenz that he had taken the preceding night. Subsequently, the patient felt sick, and bradycardia was documented 15 h after the patient received the most recent dose of lopinavir-ritonavir. An electrocardiogram showed sinus arrest with junctional escape rhythm (figure 1*C*). Serum electrolyte and creatine kinase levels were normal, and there were no signs of ischemic heart disease at this time. Consequently, all antiretroviral therapy was discontinued. Despite the administration of atropine

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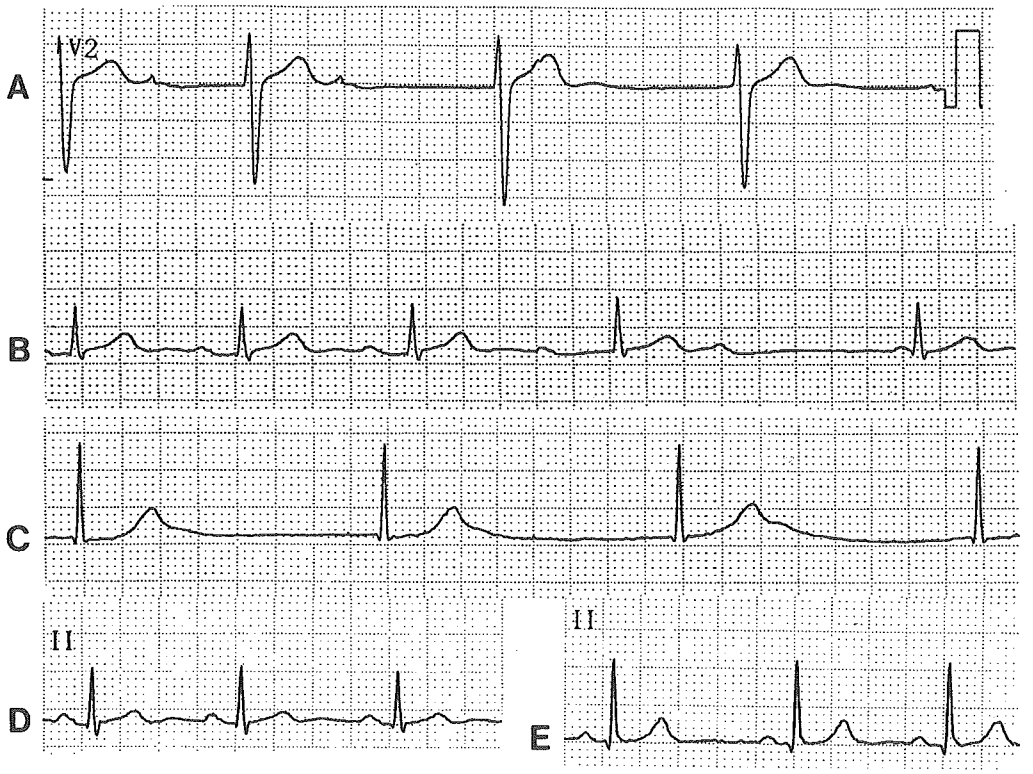

Figure 1. Electrocardiograms for 2 patients with arrhythmia. The electrocardiograms were recorded at 10 mm/mV on a paper speed of 25 mm/s. *A,* Complete atrioventricular block in the first patient (precordial lead V2). *B,* Mobitz type I, second-degree atrioventricular block recorded during the challenge test in the first patient (lead II). *C,* Sinus arrest with junctional escape rhythm in the second patient (lead II). *D,* Normal findings recorded on 23 March 2001 (before lopinavir-ritonavir was given) for the first patient (lead II). *E,* Normal findings recorded on 12 September 2001 (before lopinavir-ritonavir was given) for the second patient (lead II).

sulphate and β-stimulant, arrhythmia persisted for 20 h after the discontinuation of antiretroviral therapy.

Discussion. Both patients had no history of cardiac disease (including congenital abnormality), electrocardiograms obtained at admission showed normal sinus rhythm (figure 1*D*, 1*E*), and there was no family history of arrhythmia or sudden death. Direct infection of myocardial cells by HIV-1 [1] and cardiac muscle toxicity caused by reverse-transcriptase inhibitors [2] have been reported elsewhere. The common clinical features of the 2 cases were advanced-stage HIV-1 infection, HCV infection, and a long history of receiving antiretroviral therapy, especially with reverse-transcriptase inhibitors. How these clinical features were involved in the adverse events is unknown at present. Lopinavir-ritonavir is widely used in salvage therapy for HIV-1 infection [3] because of its strong antiretroviral activity and favorable pharmacokinetic properties [4]. In the first patient, we initially thought that the arrhythmia was caused by IFN-α, but the cause was determined to be either lopinavir-ritonavir or didanosine by a challenge test that involved the latter drugs. For the second patient, a challenge test was not performed. However, sinus arrest was documented shortly after the patient received combination therapy with lopinavir-ritonavir, abacavir, and efavirenz. The only common drug for the 2 patients was lopinavir-ritonavir.

Although the type of arrhythmia noted was different between these patients, to our knowledge, this is the first report of potentially fatal bradyarrhythmia that was possibly related to use of lopinavir-ritonavir. One can speculate that lopinavir-ritonavir had a toxic effect on cells in the sinus and/or atrioventricular node. The mechanism for this adverse event has yet to be elucidated in detail. As of July 2002, lopinavir-ritonavir has been prescribed to ~80 patients at our ambulatory clinic. We noted the bradyarrhythmia in these 2 patients because they were hospitalized at the commencement of therapy. Special attention should be directed toward signs of arrhythmia that appear shortly after patients start receiving therapy that contains lopinavir-ritonavir.

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References

1. Barbaro G, Lorenzo GD, Grisorio B, et al. Incidence of dilated cardiomyopathy and detection of HIV in myocardial cells of HIV-positive patients. N Engl J Med **1998**; 339:1093–9.
2. Brinkman K, Hofstede HJM, Burger DM, et al. Adverse effect of reverse transcriptase inhibitors: mitochondrial toxicity as common pathway. AIDS **1998**; 12:1735–44.
3. Tsuchiya K, Matsuoka S, Yasuoka A, et al. Accumulation of lopinavir resistance–associated mutations over 3 years follow-up of patients on HAART: implication in salvage therapy. AIDS **2001**; 15:1183–4.
4. Bertz R, Lam W, Brun S, et al. Multiple-dose pharmacokinetics (PK) of LPV/ritonavir (LPV/r) in HIV$^+$ subjects [abstract 0327]. In: Programs and abstracts of the 39th Interscience Conference on Antimicrobial Agents and Chemotherapy (San Francisco). Washington, DC: American Society for Microbiology, **1999**.

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

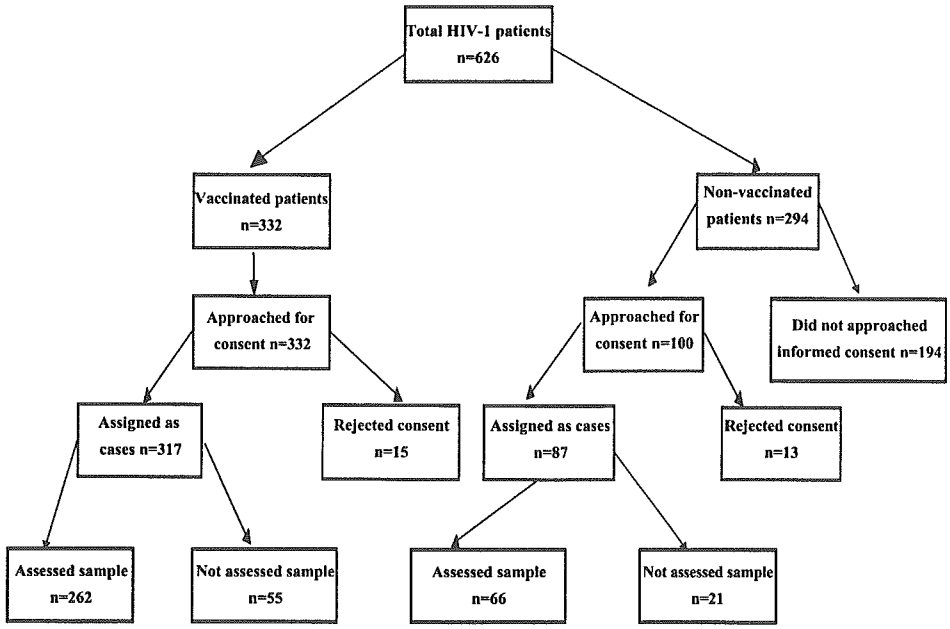

FIGURE 1. Profile of participants in this study.

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).

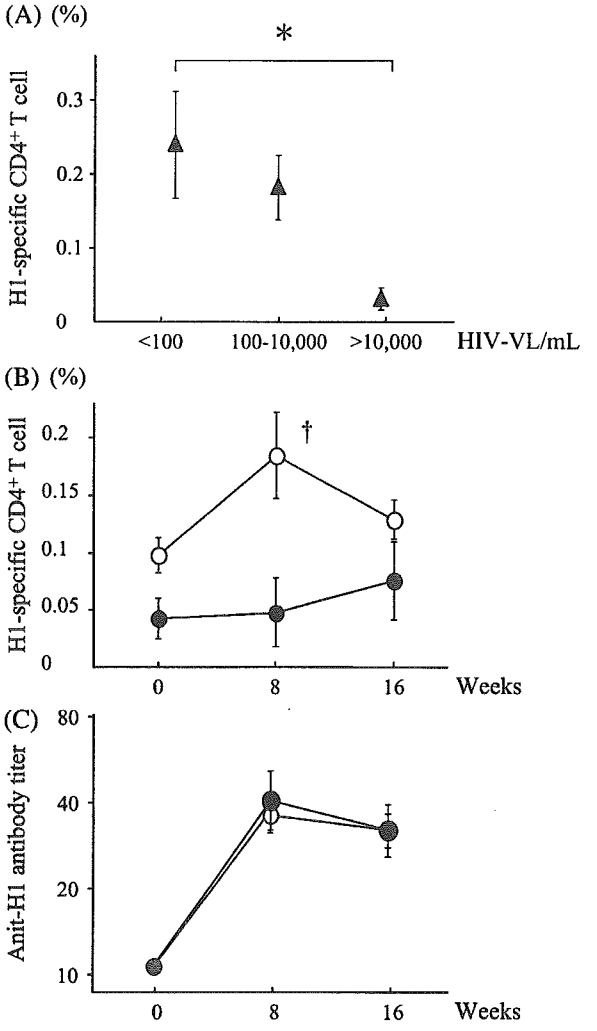

FIGURE 2. H1-specific CD4+ T-cell responses after influenza vaccine in baseline anti-H1 antibody–negative patients. A, Correlation of plasma HIV-1 viral load (HIV VL) and percentage of H1-specific CD4+ T cells. *H1-specific CD4+ T cells (▲) were significantly fewer in number in subjects with an HIV VL >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

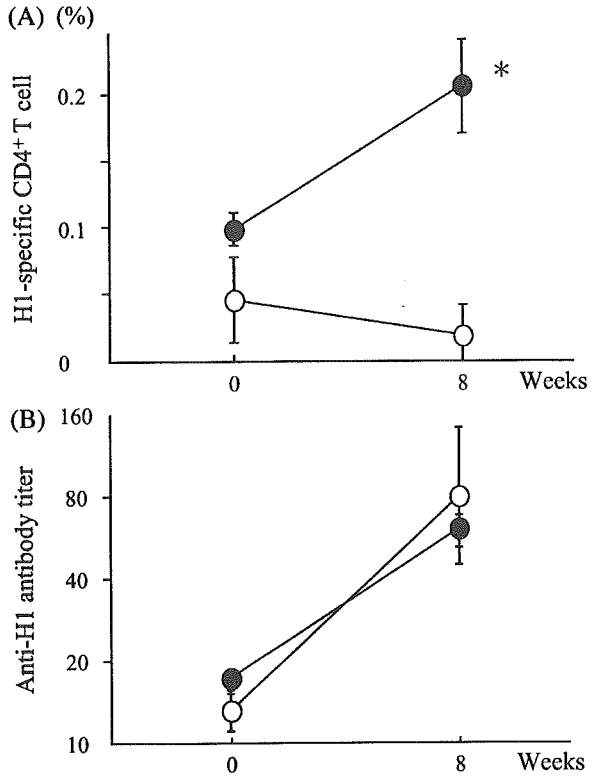

FIGURE 3. H1-specific $CD4^+$ T cells and anti-H1 antibody responses at week 8 after vaccination in influenza A/H1N1–infected patients. Five vaccinated individuals were infected with influenza A/H1N1. A, Percentage of H1-specific $CD4^+$ T-cell responses in infected (○; n = 4) and noninfected (●; n = 119) individuals. *H1-specific $CD4^+$ T cells responded better to influenza A/H1N1 in noninfected patients than in infected patients ($P < 0.05$). One sample of 5 influenza A/H1N1–infected individuals was not examined because the sample was not among the first 10 samples per day as stated in the text. B, Anti-H1 antibody titers in infected (○; n = 5) and noninfected (●; n = 249) individuals. The anti-H1 antibody response at week 8 was similar in both groups. Data are mean ± SEM.

baseline antibody-negative HIV-1–infected patients, the antibody responses to the single-shot vaccination were less effective than those in healthy patients. In contrast, however, in baseline antibody-positive HIV-1–infected patients, the antibody responses were similar or more effective than those in the healthy controls and the titers exceeded >40 U in most cases, irrespective of CD4 count. Previous studies demonstrated that an antibody titer >40 U could be used as an index of vaccine protection.[12,25] In our study, the antibody titer was <40 U in most patients who became infected with influenza. Considered together, these results suggest that the antibody response may support the clinical efficacy of influenza vaccination. Kroon et al[8] reported that postvaccination antibody titers were higher in previously vaccinated HIV-1–infected patients than in nonvaccinated patients, although the difference was not significant. In the present study, the antibody titers showed a better response in individuals positive at baseline for anti-HA antibody than in those negative for the antibody. Furthermore, the response was well sustained, irrespective of CD4 count. Thus, it is conceivable that annual vaccination is specifically important for all HIV-1–infected patients. Sustainability of the antibody titer raised by the vaccination is to be followed in a future study.

In the immunologic part of our study, we examined antibody responses and specific CD4 T cells. The antibody response was almost the same as that reported previously[8,9]; the response correlated with the CD4 count. In contrast, specific CD4 T cells were much more influenced by HIV VL than by CD4 count.[1,8–15] Therefore, the specific CD4 T cells were higher in patients treated with HAART than in those untreated. This result indicates that HAART improves HA-specific CD4 T cells like in other infections,[21] or, in other words, the heightened cellular response to the influenza vaccine suggests functional reconstitution of the immune system after HAART.

Our data indicate that the specific CD4 T-cell responses may be related to HIV VL. The specific CD4 T-cell response needs antigen presentation by dendritic cells.[26] HIV-1 infection impairs the function of antigen presentation of dendritic cells.[27] Therefore, specific CD4 T-cell responses may be profoundly decreased in patients with a high HIV VL.

It is interesting to note that the percentage of H1-specific CD4 T cells at week 8 was significantly lower in influenza A/H1N1–infected patients. It is conceivable that the response of HA-specific CD4 T cells at week 8 can predict the efficacy of influenza vaccine. Influenza-specific CD4 T cells provide help (as Th cells) to B cells for the production of antibody to influenza HA and neuraminidase[28,29] and also promote the generation of virus-specific $CD8^+$ cytotoxic T lymphocytes (CTLs).[26,30–33] Therefore, the specific CD4 T cell must have a protective role. This concept would be more reliable if we had analyzed H3-specific CD4 T cells rather than H1-specific CD4 T cells, because influenza A/H3N2 was the predominant subtype in this season. Further studies are necessary to elucidate this point.

Our study was designed as a prospective but nonrandomized study, because influenza vaccine has been already recommended for HIV-1–infected patients.[7] Practically, the number of nonvaccinated patients who did not participate in our study was higher than that of vaccinated patients (13% of nonvaccinated patients vs. 4.5% of vaccinated patients), and the violation rate of the study protocol was higher in nonvaccinated patients than in vaccinated patients (24.1% vs. 17.4%). Thus, 262 (78.9%) of 332 vaccinated patients and 66 (66%) of 100 nonvaccinated patients were analyzed in this study. Although a relatively high proportion of patients failed to complete the protocol, the main reason for the drop out may have been the lack of incentives and the need to visit our clinic on a fixed date for blood sampling. The vaccinated and nonvaccinated groups were well balanced in terms of baseline characteristics, however. Finally, we believe that the selection bias of participants, if any, is negligible.

In conclusion, our prospective study in a large population demonstrated that influenza vaccine provides protection of HIV-1–infected patients. In baseline antibody-negative patients, the 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. In contrast, in baseline antibody-positive patients, good antibody responses were observed, irrespective of CD4 counts. Annual vaccination of

HIV-1–infected patients is thus recommended. Therapy with HAART improves the cellular immune response to influenza HA.

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REFERENCES

1. Fine AD, Bridges CB, De Guzman AM, et al. Influenza A among patients with human immunodeficiency virus: an outbreak of infection at a residential facility in New York City. *Clin Infect Dis*. 2001;32:1784–1791.
2. Ho DD. HIV-1 viremia and influenza [letter]. *Lancet*. 1992;339:1549.
3. Stanley SK, Ostrowski MA, Justement JS, et al. Effect of immunization with a common recall antigen on viral expression in patients infected with human immunodeficiency virus type 1. *N Engl J Med*. 1996;334:1222–1230.
4. Couch RB. Editorial response: influenza, influenza virus vaccine, and human immunodeficiency virus infection. *Clin Infect Dis*. 1999;28:548–551.
5. Tasker SA, O'Brien WA, Treanor JJ, et al. Effects of influenza vaccination in HIV-infected adults: a double-blind, placebo-controlled trial. *Vaccine*. 1998;16:1039–1042.
6. Zanetti AR, Amendola A, Besana S, et al. Safety and immunogenicity of influenza vaccination in individuals infected with HIV. *Vaccine*. 2002;20 (Suppl 5):B29–B32.
7. Centers for Disease Control and Prevention. Prevention and control of influenza: recommendations of Advisory Committee on Immunization Practices (ACIP). *MMWR Morb Mortal Wkly Rep*. 2002;51(RR03):1–31.
8. Kroon FP, van Dissel JT, de Jong JC, et al. Antibody response to influenza, tetanus and pneumococcal vaccines in HIV-seropositive individuals in relation to the number of CD4+ lymphocytes. *AIDS*. 1994;8:469–476.
9. Fowke KR, D'Amico R, Chernoff DN, et al. Immunologic and virologic evaluation after influenza vaccination of HIV-1-infected patients. *AIDS*. 1997;11:1013–1021.
10. Kroon FP, Rimmelzwaan GF, Roos MTL, et al. Restored humoral immune response to influenza vaccination in HIV-infected adults treated with highly active antiretroviral therapy. *AIDS*. 1998;12(Suppl):F217–F223.
11. Fullar JD, Craven DE, Steger KA, et al. Influenza vaccination of human immunodeficiency virus (HIV)-infected adults: impact on plasma levels of HIV type 1 RNA and determinants of antibody response. *Clin Infect Dis*. 1999;28:541–547.
12. Tasker SA, Treanor JJ, Paxton WB, et al. Efficacy of influenza vaccination in HIV-infected persons. *Ann Intern Med*. 1999;131:430–433.
13. Skiest DJ, Machala T. Comparison of the effects of acute influenza infection and influenza vaccination on HIV viral load and CD4 cell counts. *J Clin Virol*. 2003;26:307–315.
14. Chadwick EG, Chang G, Decker MD, et al. Serologic response standard inactivated influenza vaccine in human immunodeficiency virus-infected children. *Pediatr Infect Dis J*. 1994;13:206–211.
15. Huang K-L, Ruben FL, Rinaldo CR, et al. Antibody responses after influenza and pneumococcal immunization in HIV-infected homosexual men. *JAMA*. 1987;257:2047–2050.
16. Brenchley JM, Hill BJ, Ambrozak DR, et al. T-cell subsets that harbor human immunodeficiency virus (HIV) in vivo: implications for HIV pathogenesis. *J Virol*. 2004;78:1160–1168.
17. Virelizier J-L, Allison AC, Shild GC. Antibody responses to antigenic determinants of influenza virus hemagglutinin. II. Original antigenic sin: a bone marrow-derived lymphocyte memory phenomenon modulated by thymus-derived lymphocytes. *J Exp Med*. 1974;140:1571–1578.
18. Burns WH, Billups LC, Notkins AL. Thymus dependence of viral antigens. *Nature*. 1975;256:654–656.
19. Lucas SJ, Barry DW, Kind P. Antibody production and protection against influenza virus in immunodeficient mice. *Infect Immun*. 1978;20:115–119.
20. Dowdle WN, Kendel AP, Noble GR. Influenza viruses. In: Lenette EH, Schmidt NJ, eds. *Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections*. 5th ed. Washington, DC: American Public Health Association; 1979:585–609.
21. Tanaka M, Yasuoka C, Genka I, et al. Sustained cytomegalovirus-specific CD4+ T cell response associated with prevention of recurrence of cytomegalovirus retinitis without secondary prophylaxis after highly active antiretroviral therapy in patients with AIDS. *AIDS Res Hum Retroviruses*. 2001;17:1749–1756.
22. Tanaka M, Hirabayashi Y, Gatanaga H, et al. Reduction in interleukin-2-producing cells but not Th1 to Th2 shift in moderate and advanced stages of human immunodeficiency virus type-1-infection: direct analysis of intracellular cytokine concentration in CD4+CD8− T cells. *Scand J Immunol*. 1999;50:550–554.
23. Hirabayashi Y, Tamura SI, Suzuki Y, et al. H-2-unrestricted adjuvant effect of cholera toxin B subunit on murine antibody responses to influenza virus hemagglutinin. *Immunology*. 1991;72:329–335.
24. Infectious Disease Surveillance Center, National Institute of Infectious Disease, Japan. Influenza 2002/2003 season. *Infectious Agents Surveillance Report*. 2003;24:281–282.
25. Treanor JJ. Influenza virus. In: Treanor J, Mandell J, Douglas RG Jr., et al, eds. *Principles and Practice of Infectious Diseases*. 4th ed. Philadelphia: Churchill Livingstone; 2000:1823–1849.
26. Maus MV, Kovacs B, Kwok WW, et al. Extensive replicative capacity of human central memory T cells. *J Immunol*. 2004;172:6675–6683.
27. Kawamura T, Gatanaga H, Borris DL, et al. Decreased stimulation of $CD4^+$ T cell proliferation and IL-2 production by highly enriched populations of HIV-infected dendritic cells. *J Immunol*. 2003;170:4260–4266.
28. Russell SM, Liew FY. T cells primed by influenza virion internal components can cooperate in the antibody response to hemagglutinin. *Nature*. 1979;280:147–148.
29. Lamb JR, Woody JN, Hartzman RJ, et al. *In vitro* influenza virus-specific antibody production in man: antigen-specific and HLA-restricted induction of helper activity mediated by cloned human T lymphocytes. *J Immunol*. 1982;129:1465–1470.
30. Biddison WE, Sharrow SO, Shearer GM. T cell subpopulations required for the human cytotoxic T lymphocyte response to influenza virus: Evidence for T cell help. *J Immunol*. 1981;127:487–491.
31. Reiss CS, Burakoff SJ. Specificity of helper T cell for the cytotoxic T lymphocyte response to influenza viruses. *J Exp Med*. 1981;154:541–546.
32. Kallas EG, Gibbons DC, Soucier H, et al. Detection of intracellular antigen-specific cytokines in human T cell populations. *J Infect Dis*. 1999; 179:1124–1131.
33. Hashimoto G, Wright PF, Karzon DT. Antibody-dependent cell-mediated cytotoxicity against influenza virus-infected cells. *J Infect Dis*. 1983;148: 785–794.

APPENDIX

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Homozygous *CYP2B6* *6 (Q172H and K262R) correlates with high plasma efavirenz concentrations in HIV-1 patients treated with standard efavirenz-containing regimens

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Abstract

Efavirenz (EFV) is metabolized by cytochrome P450 2B6 (*CYP2B6*) in the liver. We analyzed the genotypes of *CYP2B6* and their contribution to plasma EFV concentrations in 35 EFV-treated patients in International Medical Center of Japan. The mean plasma EFV concentration of patients with *CYP2B6* *6/*6 (Q172H and K262R) ($25.4 \pm 7.5\,\mu M$, $\pm$ SD, $n = 2$) was significantly higher than that of patients with genotypes *6 heterozygote ($9.9 \pm 3.3\,\mu M$, $n = 10$) or without alleles *6 ($8.0 \pm 2.6\,\mu M$, $n = 23$) ($p < 0.0001$). To confirm our result, we further analyzed nine patients (three with high EFV concentrations and arbitrarily selected six with normal EFV concentrations) treated in Osaka National Hospital, and it resulted that the only three patients with the high concentrations were the *6/*6 holder. EFV dose could be decreased in those patients harboring the genotype to reduce toxicity with compromising potency, representing the first step of the Tailor-Made therapy of HIV-1 infection.

Keywords: Cytochrome P450; Genetic polymorphism; HIV-1; Efavirenz; Plasma concentration

Efavirenz (EFV) is a non-nucleoside reverse transcriptase inhibitor that shows potent inhibitory activity against HIV-1 and is stated as a key drug of the first line regimens in the HIV-1 treatment Guideline [1]. However, a number of patients treated with EFV develop central nervous system symptoms including headache, dizziness, insomnia, and fatigue. These side effects are more frequent in patients with high plasma concentration of EFV [2,3] as well as worsen with long-term therapy, and are the main reason for poor adherence or interruption of therapy. EFV is reported to be metabolized by cytochrome P450 (CYP) 3A4 (*CYP3A4*) and 2B6 (*CYP2B6*) to hydroxylated metabolites in the liver [4]. The recent HIV-1 treatment Guideline stated that EFV is metabolized by *CYP3A* [1], whereas an in vitro study indicated that EFV is mainly metabolized by *CYP2B6* [5]. Furthermore, a pharmacogenetic study demonstrated the association of the homozygous variant of multidrug-resistance transporter (*MDR1*; gene product P-glycoprotein) C3435T and good immune recovery in patients treated with EFV-containing regimens [6]. In order to clarify the contribution of polymorphisms to plasma EFV concentration in vivo, we analyzed genotypes of *CYP2B6*, *CYP3A4*, and *MDR1*, and their correlation with plasma EFV concentrations.

Materials and methods

Patients. A total of 60 HIV-1 patients who were treated with EFV-containing regimens at the AIDS Clinical Center, International

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Medical Center of Japan (IMCJ), were examined for their allelic variants of *CYP2B6*, *CYP3A4*, and *MDR1*. Among them, 35 patients were on standard therapy of EFV-containing regimens (600 mg EFV once daily dosing with two nucleotide reverse transcriptase inhibitors) and fully adhered to the regimens based on self-reports. Their plasma EFV concentrations were measured and the correlation between variants and EFV concentrations was analyzed. We excluded those patients who were taking other agents that could potentially interact with plasma EFV concentration such as protease inhibitors and those taking EFV twice daily from the analysis of the correlation. The mean age and body weight of these 35 patients (34 males and 1 female) were 41.6 ± 11.5 years and 63.4 ± 10.9 kg, respectively. The median latency between commencement of treatment and analysis of EFV concentration was 76.9 weeks (range, 4–200). The means ± SD alanine aminotransferase level was 33.1 ± 18.4 U/L. Blood samples were taken between 10 and 14 h (mean, 12.0 h) after dosing. To confirm the results of patients treated at the IMCJ, we further analyzed the allelic variants of nine patients who were treated at the Osaka National Hospital (ONH) [three patients with high plasma EFV concentrations (one patient was taking only 200 mg EFV once daily due to severe side effects) and six patients with normal EFV concentrations]. The Ethics Committee for the Study of Human Genome in each hospital approved this study (IMCJ-H14-36, ONH-23) and all patients gave a written informed consent.

Genotyping. Genomic DNA was isolated from peripheral blood using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Genotyping of allelic variants of *CYP2B6* [7] [**1* (wild type), **2* (C64T), **3* (C777A), **4* (A785G), **5* (C1459T), **6* (G516T and A785G), **7* (G516T, A785G, and C1459T), and **8* (A415G)], CYP3A4 [**11* (C1088T; unstable form [8]), **12* (C1117T; has an altered testosterone hydroxylase activity [8]), **13* (C1247T; lack of expression [8]), **17* (T566C; exhibits lower turnover numbers for testosterone and chlorpyrifos [9]), and **18* (T878C; exhibits higher turnover numbers for testosterone and chlorpyrifos) [9] and *MDR1* C3435T was carried out using the allelic-specific fluorogenic 5′ nuclease chain reaction assay by the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). Each 25 μl PCR mixture contained 20 ng genomic DNA, 900 nM primers, 200 nM TaqMan minor groove binder (MGB) probes, and 12.5 μl TaqMan universal PCR master mix (Applied Biosystems). The primers and TaqMan MGB probes used in this study are summarized in Table 1. The thermal cycler program was set up at 50 °C for 2 min and 95 °C for 10 min, and then repeated 40 cycles with 95 °C for 15 s and 60 °C for 1 min.

Plasma efavirenz concentration. Plasma was isolated by centrifugation (10 min at 1800*g*) on the same day as blood sampling and stored at −80 °C until analysis. EFV concentration was measured by reverse-phase high performance liquid chromatography (HPLC) method [10] at BioMedical Laboratory (Saitama, Japan). HPLC was performed on an Inertsil ODS-3 column (5 μm, 250 × 4.6 mm; GL Sciences, Tokyo, Japan) at a flow rate of 1.2 ml/min with ultraviolet-detection at 247 nm. The mobile phase consisted of acetonitrile and water (65:35, v/v).

Statistical analysis. StatView version 5.0 software (SAS Institute, Cary, NC) was used for the comparison of different genotype groups. If one-way analysis of variance (ANOVA) was significant ($p < 0.05$), post hoc Scheffe's *F* test was applied.

Results and discussion

Frequency of genotypic variants of CYP2B6, CYP3A4, and MDR1

We first analyzed the frequency of genotypic variants of the 60 patients seen at IMCJ. The *CYP2B6* genotypes were **1/*1* in 28 patients, **1/*2* in 4, **1/*4* in 5, **1/*6* in

Table 1
Primers and TaqMan MGB probes used in this study

	Forward primer	Reverse primer	VIC probe (wild-type)	6-FAM probe (mutant)
CYP2B6				
C64T	CCTCACAGGACTCTTGCTACTC	AGGCGGTCATGGGTGTTAG	TGGTTCAGCGCCACC	CTGGTTCAGTGCCACC
A415G	CTGTGACCACTATGAGGGACTTC	CTGAGCCTCCTCCTGAATCC	ACACTCCGCTTTCCCAT	CACTCCGCTCTCCCAT
G516T	TCATGGACCCCACCTTCCT	GACGATGGAGCAGATGATGTTG	TTCCAGTCCATTACC	CTTCCATTCCATTACC
C777A	TGGAGAAGCACCGTGAAACC	GAGCAGGTAGGTGTCGATGAG	CCCAGCGCCCCCA	CCCCAGAGCCCCCA
A785G	TGGAGAAGCACCGTGAAACC	TGGAGCAGGTAGGTGTCGAT	CCCCCAAGGACCTC	CCCCAGGGACCTC
C1459T	CCCAGAAGACATCGATCTGACA	GAATGACCCTGGAATCCTTTGAC	AGATCCGCTTCCTG	AGATCTGCTTCCTGC
CYP3A4				
T566C	GGCCTACAGCATGGATGTGAT	TGGATTGTTGAGAGAGTCGATGTT	AGCACATCATTTGGA	AGCACATCATCTGGA
T878C	TCTTTCCTCTCCTTTCAGCTCTGT	GGTTTCATAGCCAGCAAAAATAAAG	CGATCTGGAGCTCG	CGATCCGGAGCTC
C1088T	TGTGCTACAGATGGAGTATCTTGACA	CATCCCATTGATCTCAACATCTTTT	TCTGAGCGTTTCATT	CTGAGCATTTCATTCA
C1117T	TCTTGACATGGTGGTGAATGAAA	CATCCCATTGATCTCAACATCTTTT	CCCTCTCAAGTCTC	CCTCTCAAATCTC
C1247T	AAAGTACTGGACAGAGCCTGAGAAG	GGAGGGCTCCCTTCCCA	TTCCTCCCTGAAAGG	CCTCCTTGAAAGGTA
MDR1				
C3435T	AACAGCCGGGTGGTGTCA	ATGTATGTTGGCCTCCTTTGCT	CTCACGATCTCTTC	CCTCACAATCTCTT

MGB, minor groove binder; VIC, vasoactive intestinal contractor; 6-FAM: 6-carboxyfluorescein.
Bold indicates the site of substitution.

Table 2
Frequency of CYP2B6 alleles and genotypes in 60 HIV-1 patients at IMCJ[a]

	Frequency (%)	95% CI
CYP2B6 allele		
**1*	78 (65)	56.5–73.5
**2*	9 (7.5)	3.8–14.2
**4*	10 (8.3)	4.4–15.3
**5*	2 (1.7)	0.3–6.0
**6*	21 (17.5)	10.7–24.3
CYP2B6 genotype		
**1/*1*	28 (46.7)	33.7–60.0
**1/*2*	4 (6.7)	1.8–16.2
**1/*4*	5 (8.3)	2.7–18.4
**1/*6*	13 (21.7)	12.1–34.2
**2/*4*	2 (3.3)	0.4–11.5
**2/*6*	3 (5.0)	1.0–13.9
**4/*4*	1 (1.7)	0.02–8.9
**4/*6*	1 (1.7)	0.02–8.9
**5/*5*	1 (1.7)	0.02–8.9
**6/*6*	2 (3.3)	0.4–11.5

95% CI, 95% confidence intervals.
[a] IMCJ, International Medical Center of Japan.

13, **2/*4* in 2, **2/*6* in 3, **4/*4* in 1, **4/*6* in 1, **5/*5* in 1, and **6/*6* in 2 (Table 2). The *CYP3A4* polymorphisms were only shown in T878C T/C heterozygote in three patients and other alleles were not found. *MDR1* C3435T polymorphisms were C/C in 19 patients, C/T in 31, and T/T in 10.

Correlation between the genotypic variants and EFV concentrations

Among the 35 patients who were on standard therapy of EFV-containing regimens, two had significantly higher plasma EFV concentrations (30.7 and 20.0 μM) than the other patients. *CYP2B6* genotype of the two patients was **6/*6* homozygote. The mean plasma EFV concentrations of patients with *CYP2B6* **6/*6* genotype (25.4 ± 7.5 μM, $n = 2$) were significantly higher than those of patients with **6* heterozygous genotypes (9.9 ± 3.3 μM, $n = 10$) and non-**6* alleles (8.0 ± 2.6 μM, $n = 23$) [one-way ANOVA ($p < 0.0001$) and post hoc Scheffe's F test showed statistically significant difference in plasma EFV concentration between **6/*6* genotype and **6* heterozygous genotypes ($p < 0.0001$), and non-**6* alleles ($p < 0.0001$)]. As shown in Table 3, the differences of patients' characteristics in each *CYP2B6* genotype were not significant, indicating that these characteristics did not influence the difference of EFV concentrations among the three genotypes. Then, we analyzed the additional nine samples (three with high EFV concentrations) obtained from the ONH and found that *CYP2B6* genotypes of the three patients with high EFV concentration were also **6/*6* genotype. Consequently, only five patients whose EFV concentrations were >20 μM had *CYP2B6* **6/*6* genotype (Fig. 1A). There was a significant correlation between *CYP2B6* **6/*6* genotype and high plasma EFV concentrations. In contrast, there was no correlation between *CYP2B6* **5*, *CYP3A4*, *MDR1* genotypes, and plasma EFV concentrations (Figs. 1B–D) in our small number of patients examined in this study.

Homozygous variant of *MDR1* C3435T has been shown to associate with responsiveness to EFV therapy [6]. However, no correlation was found between the C3435T polymorphisms and plasma EFV concentration in our study. Then, the plasma EFV concentration could not explain the favorable clinical result. EFV is a non-nucleoside reverse transcriptase inhibitor and, therefore, plays an anti-HIV-1 activity within HIV-1 infected cells but not in plasma. It remains to be elucidated whether or not the C3435T polymorphisms correlate with high intracellular EFV concentration.

Genetic polymorphism is known to be associated with variable level of *CYP2B6* expression in the liver. Especially, the expression levels of *CYP2B6* **6/*6* genotype are significantly lower than those of wild and other genotypes [7,11]. The high plasma EFV concentration may be explained by the low expression level of this genotype. Based on our new finding, extremely high plasma EFV concentration can be predicted by determining the genotype before commencement of EFV-containing therapy. In such patients, the EFV dose could be decreased to reduce the cost and more importantly the associated toxicity, without compromising its potency. In fact, one patient was treated with 200 mg EFV once daily due to severe side effects but had higher EFV concentrations than other patients with other genotypes. The frequency of the *CYP2B6* **6/*6* genotype in IMCJ patients was 3.3% (2 in 60 patients), whereas

Table 3
Patients' characteristics in each CYP2B6 genotype in 35 patients who were treated with standard EFV-containing therapy at IMCJ[a]

	Non-**6* genotypes	**6* heterozygote genotypes	**6/*6* genotype	*p*
n	23	10	2	
Male:female	23:0	9:1	2:0	n.s.
Age (years) (mean ± SD)	38.8 ± 8.2	45.3 ± 14.8	55.5 ± 19.1	n.s.
Weight (kg) (mean ± SD)	64.3 ± 11.5	58.6 ± 7.5	77.0 ± 5.1	n.s.
Alanine aminotransferase level (U/L) (mean ± SD)	31.0 ± 20.4	35.3 ± 14.0	46.5 ± 3.5	n.s.

n.s., not significant.
[a] IMCJ, International Medical Center of Japan.

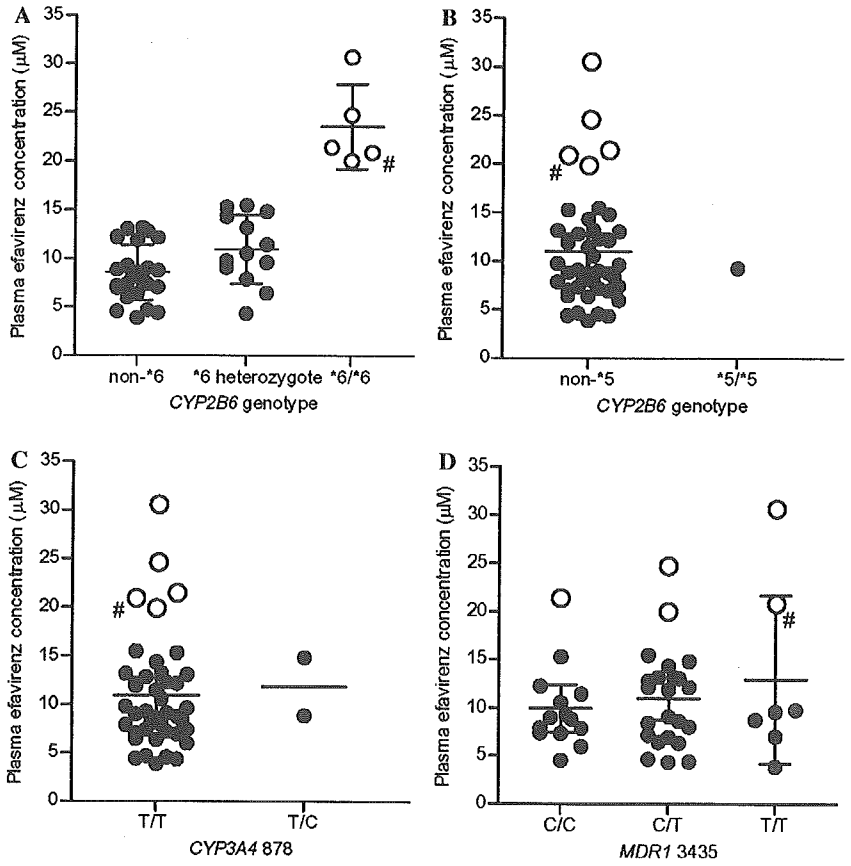

Fig. 1. Correlation between *CYP2B6 *6* genotypes (A), *CYP2B6 *5/*5* genotype (B), *CYP3A4* T878C genotype (C), *MDR1* 3435 genotypes (D), and plasma efavirenz concentrations. A total of 44 HIV-1 patients treated with standard EFV-containing regimens (35 from IMCJ and 9 from ONH) are depicted. Only homozygous genotypes of *CYP2B6* are represented in this figure [A (**6* genotypes) and B (**5/*5* genotype)]. Non-**6* genotypes ($n = 26$) include **1/*1* ($n = 18$), **1/*2* ($n = 2$), **1/*4* ($n = 3$), **2/*4* ($n = 2$), and **5/*5* ($n = 1$). **6* heterozygote genotypes ($n = 13$) include **1/*6* ($n = 9$), **2/*6* ($n = 3$), and **4/*6* ($n = 1$). Numbers of patients of *MDR1* 3435 C/C, C/T, and T/T genotypes are 14, 23, and 7 patients, respectively. Open circles: *CYP2B6 *6/*6* genotype holders, closed circles: other *CYP2B6* genotypes holders. Middle bar indicates mean, and upper and lower bars SD. (#) Patient on 200 mg EFV once daily.

the frequency was 6% in Caucasian population [7]. If these patients could be treated with low dose EFV based on genetic data of *CYP2B6 *6/*6* genotype, it could represent the first step of the Tailor-Made therapy of HIV-1 infection.

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References

[1] Department of Health and Human Services, Guidelines for the use of antiretroviral agents in HIV-infected adults and adolescents, March 23, 2004.

[2] C. Marzolini, A. Telenti, L.A. Decosterd, G. Greub, J. Biollaz, T. Buclin, Efavirenz plasma levels can predict treatment failure and central nervous system side effects in HIV-1-infected patients, AIDS 15 (2001) 71–75.

[3] C. Csajka, C. Marzolini, K. Fattinger, L.A. Decosterd, J. Fellay, A. Telenti, J. Biollaz, T. Buclin, Population pharmacokinetics and effects of efavirenz in patients with human immunodeficiency virus infection, Clin. Pharmacol. Ther. 73 (2003) 20–30.

[4] J.C. Adkins, S. Noble, Efavirenz, Drugs 56 (1998) 1055–1064.

[5] B.A. Ward, J.C. Gorski, D.R. Jones, S.D. Hall, D.A. Flockhart, Z. Desta, The cytochrome P450 2B6 (CYP2B6) is main catalyst of efavirenz primary and secondary metabolism: implication for HIV/AIDS therapy and utility of efavirenz as a substrate maker of CYP2B6 catalytic activity, J. Pharmacol. Exp. Ther. 306 (2003) 287–300.

[6] J. Fellay, C. Marzolini, E.R. Meaden, D.J. Back, T. Buclin, J.P. Chave, L.A. Decosterd, H. Furrer, M. Opravil, G. Pantaleo, D. Retelska, L. Ruiz, A.H. Schinkel, P. Vernazza, C.B. Eap, A. Telenti, Swiss HIV Cohort Study, Response to antiretroviral treatment in HIV-1-infected individuals with allelic variants of the multidrug resistance transporter 1: a pharmacogenetics study, Lancet 359 (2002) 30–36.

[7] T. Lang, K. Klein, J. Fischer, A.K. Nussler, P. Neuhaus, U. Hofmann, M. Eichelbaum, M. Schwab, U.M. Zanger, Extensive genetic polymorphism in the human CYP2B6 gene with impact on expression and function in human liver, Pharmacogenetics 11 (2001) 399–415.

[8] R. Eiselt, T.L. Domanski, A. Zibat, R. Mueller, E. Presecan-Siedel, E. Hustert, U.M. Zanger, J. Brockmoller, H.P. Klenk, U.A. Meyer, K.K. Khan, Y.A. He, J.R. Halpert, L. Wojnowski,

Identification and functional characterization of eight CYP3A4 protein variants, Pharmacogenetics 11 (2001) 447–458.
[9] D. Dai, J. Tang, R. Rose, E. Hodgson, R.J. Bienstock, H.W. Mohrenweiser, J.A. Goldstein, Identification of variants of CYP3A4 and characterization of their abilities to metabolize testosterone and chlorpyrifos, J. Pharmacol. Exp. Ther. 299 (2001) 825–831.
[10] P. Villani, M. Pregnolato, S. Banfo, M. Rettani, D. Burroni, E. Seminari, R. Maserati, M.B. Regazzi, High-performance liquid chromatography method for analyzing the antiretroviral agent efavirenz in human plasma, Ther. Drug Monit. 21 (1999) 346–350.
[11] H.J. Xie, U. Yasar, S. Lundgren, L. Griskevicius, Y. Terelius, M. Hassan, A. Rane, Role of polymorphic human CYP2B6 in cyclophosphamide bioactivation, Pharmacogenomics J. 3 (2003) 53–61.

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A Novel Subtype of GB Virus C/Hepatitis G Virus Genotype 1 Detected Uniquely in Patients With Hemophilia in Japan

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GB virus C (GBV-C) or hepatitis G virus (HGV) has been transmitted to Japanese patients with hemophilia through the frequent use of unheated blood products. Sequence analysis showed that most of the viruses isolated from these patients belonged to GBV-C/HGV genotype 1, which is usually found in persons from Africa. This may point to the origin of this virus in Japanese patients with hemophilia. The phylogeny of 11 GBV-C/HGV isolates from Japanese patients with hemophilia was investigated by a detailed analysis with a fragment spanning from the 5′ non-coding region to part of the E1 gene. Except for one that belonged to the genotype 3 cluster, all isolates were GBV-C/HGV type 1. Five main clades exist within the GBV-C/HGV genotype 1 sequences. These isolates are grouped in 2 defined clades. Three of the isolates are clustered in subtype 1c clade whereas the other 7 strains formed a new statistically well-supported monophyletic group (named subtype 1e). Our results suggest that GBV-C/HGV type 1 can at present be classified into at least 5 clades and in this group a majority of Japanese patients with hemophilia was infected with a GBV-C/HGV of a unique and newly described subtype within genotype 1. ***J. Med. Virol. 71:385–390, 2003.***

KEY WORDS: GBV-C; HGV; phylogenetics; genotyping

INTRODUCTION

GB virus C (GBV-C)/hepatitis G virus (HGV) were identified by two independent research groups [Simons et al., 1995; Linnen et al., 1996]. Sequence comparison showed that they are different isolates of the same virus. Although it was isolated initially from patients with hepatitis, and despite its similarity in genomic structure with the hepatitis C virus (HCV), it is unlikely that GBV-C/HGV is a cause of hepatitis [Mushahwar, 2000]. The virus has a worldwide distribution in all populations and is transmitted mainly by the parenteral route.

GBV-C/HGV is an enveloped positive-strand RNA virus with a genome size of about 9.3 kilobases (kb) belonging to the *Flaviviridae* family. In previous studies, only three genotypes have been found [Fukushi et al., 1996; Muerhoff et al., 1996]. Type 1 was found mainly in Africa and can be classified into four groups [Liu et al., 2000]. Type 2, consisting of two subtypes, is the most common in Europe, North America, and many other regions [Muerhoff et al., 1997; Smith et al., 1997]. Type 3 was found so far only in Asia. However, at least five major genotypes of GBV-C/HGV have been identified by sequence analysis of the 5′ non-coding region (5′NCR) or E2 gene [Handajani et al., 2000; Smith et al., 2000]. In addition to the three known genotypes, recent studies have identified two new genotypes from Myanmar, Vietnam, South Africa, and Indonesia [Naito et al., 1999; Tucker et al., 1999; Handajani et al., 2000]. It has been proposed that the Southeast Asian isolates be named type 4, and the South African isolates type 5 [Tucker and Smuts, 2000].

Japanese patients with hemophilia have been at high risk of infection with parentally transmitted viruses

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through contaminated blood products. It has been reported that the majority of Japanese patients with hemophilia have been infected with genotype 1 GBV-C/HGV strains [Toyoda et al., 1998]. However, the real phylogenetic relationships of these viruses with the other GBV-C/HGV genotype 1 isolates is unclear. In this study, we investigated the phylogeny of GBV-C/HGV isolates from Japanese with hemophiliac patients known by a more detailed analysis with a fragment spanning from the 5′ NCR to part of the E1 gene as compared to reported GBV-C/HGV sequences including many genotype 1 GBV-C/HGV strains.

METHODS

Serum Samples

Serum samples from 11 GBV-C/HGV RNA positive Japanese patients with hemophilia (all males, age at the collection of the sample; 31.9 years) had been collected at different times between 1997 and 2000. All patients were followed at Nagoya University Hospital regularly as outpatients, and GBV-C/HGV RNA was detected in a previous study [Toyoda et al., 1998]. For each sample, several aliquots were made immediately after sampling, and were stored at −80°C until processed.

The entire protocol has been approved by the ethics committees of Nagoya University Hospital, and was carried out in compliance with the Declaration of Helsinki. Written informed consent was obtained from all patients prior to the study.

RT-PCR and Sequencing

Viral RNA were extracted from the serum or plasma samples with the High Pure Viral RNA Kit (Roche Diagnostics, Mannheim, Germany) and cDNA was synthesized with random hexamer primers [Cornu et al., 1997]. A nested PCR was performed in the 5′NCR to part of the E1 gene. PCR products were purified from agarose gel and subjected to direct sequencing. PCR primers, reaction conditions, DNA purification, and sequencing method have been described elsewhere [Liu et al., 1998].

Phylogenetic Analysis

A BLAST search was carried out to obtain the other GBV-C/HGV sequences in the same amplified region available in the EMBL/GenBank database. Sequences were aligned with the GeneWorks software (version 2.5.1, Oxford Molecular, UK) followed by minimal manual editing. Phylogeny construction and evaluation were performed using the Phylip software package (version 3.573, University of Washington, Seattle, WA) [Felsenstein, 1989], with the neighbor-jointing method (NJ), the Fitch and Wagner parsimony method (pars), and the maximum likelihood method (ML). An empirical transition/transversion ratio of 2.5 was estimated by the PUZZEL software (version 4.0, Universitat Munchen, Munich, Germany) [Strimmer and von Haeseler, 1996], and was used with the Felsenstein model to calculate the evolutionary distances. The robustness of the NJ and pars trees were evaluated statistically by bootstrap analysis with 1,000 bootstrap samples [Felsenstein, 1985]. Since the ML method is already a statistical method (with a statistical evaluation of the branch length), no bootstrapping was done for it.

RESULTS

Eleven GBV-C/HGV RNA positive samples of the Japanese patients with hemophilia were tested by a nested PCR amplifying the 5′NCR to part of the E1 gene of the viral genome. The PCR fragments with the expected size of about 592 bp were amplified and sequenced directly from all 11 samples. The sequences were deposited in the EMBL database and assigned the accession numbers AJ496420 to AJ496430.

A total of 74 GBV-C/HGV sequences from different regions of the world were included in the phylogenetic analysis. Five major GBV-C/HGV genotypes were identified (Fig. 1). In general, types 1, 2, 3, 4, and 5 consist mainly of isolates from Africa, Europe and North America, Asia, Southeast Asia, South Africa, and Singapore, respectively. However, the type 5 isolates Zan13 and SG57 did not form a well-supported monophyletic group.

One out of the eleven isolates from Japanese patients' isolates with hemophilia is GBV-C/HGV genotype 3, whereas all the other isolates (10/11) are GBV-C/HGV genotype 1 in two different clades (Fig. 1). In the analysis of GBV-C/HGV genotype 1 sequences, 5 main clades were identified. Among the ten genotype 1 isolates of these patients, three clustered in the subtype 1c clade, whereas the other seven strains formed a new well-supported monophyletic group (clade 1e) with a bootstrap value of 100% in the NJ and pars methods (Fig. 1a and b). The branch length is also statistically significant ($P < 0.01$) in the ML tree (Fig. 1c).

The details of patients infected with GBV-C/HGV of the new group (clade e) and others are shown in Table I. All patients were co-infected with hepatitis C virus (HCV) and 6 patients were also co-infected with the human immunodeficiency virus (HIV). We could not find distinctive common characteristics of the patients with GBV-C/HGV with the new group as compared to others, except for the higher rate of co-infection with HCV subtype 1a and HIV.

DISCUSSION

Japanese patients with hemophilia were one of the high-risk groups for infection with parenterally transmitted viruses by the use of unheated blood products. It has been shown that about 20% of Japanese patients with hemophilia have been infected with GBV-C/HGV genotype 1 strains [Toyoda et al., 1998]. Their real phylogenetic relationships with other GBV-C/HGV genotype 1 isolates were not known due to the short length (208 bp) of the fragment analyzed and insufficient genotype 1 sequences for comparison. GBV-C/HGV genotype 1 isolates are usually detected in persons from