factors for Shiga toxin–producing *Escherichia coli* infection in Australia. Clin Infect Dis 2009; 49:249–256.
2. Watanabe Y, Ozasa K, Mermin JH, et al. Factory outbreak of *Escherichia coli* O157:H7 infection in Japan. Emerg Infect Dis 1999; 5: 424–428.
3. Centers for Disease Control and Prevention. CDC health alert: multiple states investigating a large outbreak of *E. coli* O157:H7 infections. Atlanta: US Department of Health and Human Services, 2006.
4. Balagué C, Khan AA, Fernandez L, et al. Occurrence of non-O157 shiga toxin-producing *Escherichia coli* in ready-to-eat food from supermarkets in Argentina. Food Microbiol 2006; 23:307–313.
5. Estrada-Garcia T, Cerna JF, Thompson MR, Lopez-Saucedo C. Faecal contamination and enterotoxigenic. *Escherichia coli* in street-vended chili sauces in Mexico and its public health relevance. Epidemiol Infect 2002; 129: 223–226.
6. Ericsson CD, Pickering LK, Sullivan P, DuPont HL. The role of location of food consumption in the prevention of travelers' diarrhea in Mexico. Gastroenterology 1980; 79:812–816.
7. Lopez-Saucedo C, Cerna JF, Villegas-Sepulveda N, et al. Single multiplex polymerase chain reaction to detect diverse loci associated with diarrheagenic *Escherichia coli*. Emerg Infect Dis 2003; 9:127–131.
8. Estrada-García T, Cerna JF, Paheco-Gil L, et al. Drug-resistant diarrheogenic *Escherichia coli*, Mexico. Emerg Infect Dis 2005; 11:1306–1308.
9. Paniagua GL, Monroy E, García-González O, Alonso J, Negrete E, Vaca S. Two or more enteropathogens are associated with diarrhoea in Mexican children. Ann Clin Microbiol Antimicrob 2007; 6:17.
10. Estrada-Garcia T, Lopez-Saucedo C, Thompson-Bonilla R, et al. Association of diarrheagenic *Escherichia coli* Pathotypes with infection and diarrhea among Mexican children and association of atypical Enteropathogenic *E. coli* with acute diarrhea. J Clin Microbiol 2009; 47:93–98.
11. Mead PS, Griffin PM. *Escherichia coli* O157: H7. Lancet 1998; 10;352:120712.

Reprints or correspondence: Dr Teresa Estrada-Garcia, Dept of Molecular Biomedicine, CINVESTAV-IPN, Av. IPN No. 2508, Col. Zacatenco, Mexico DF, CP 07360, Mexico (testrada @cinvestav.mx).

Clinical Infectious Diseases 2010; 50:450–1

1058-4838/2010/5003-0023$15.00
DOI: 10.1086/649888

Serum (1→3) β-D-Glucan as a Noninvasive Adjunct Marker for the Diagnosis and Follow-Up of *Pneumocystis jiroveci* Pneumonia in Patients with HIV Infection

To the Editor—We read with great interest the Brief Report by Watanabe et al [1] about the value of the (1→3) β-D-glucan (BG) assay as an adjunct for the diagnosis of *Pneumocystis jiroveci* pneumonia (PJP) in patients with AIDS. We congratulate the authors because their report has a large study population (111 patients with PJP and a control group with 425 patients). However, we would appreciate your taking into account the following observations.

First, we wonder whether the report is of a prospective study or a retrospective analysis of test performance in those patients with confirmed PJP.

Second, when using a control group, it is important to define accurately the risk factors of the matched control group, because the possible development of PJP is dependant on the host risk(s) for disease. From our point of view, in this setting, the control group should include human immunodeficiency virus (HIV)–positive patients with $CD4^+$ cell counts ≤200 cells/mm^3 or a $CD4^+$ cell percentage ≤14% with a clinical respiratory infection. These characteristics are not described by the authors in their control group.

Third, we would like to remark that the accuracy of a diagnostic test is defined by calculating the cutoff value, the sensitivity, the specificity, and the positive and negative predictive values. In their study, Watanabe et al [1] only report the sensitivity and specificity. In the clinical settings in which BG is used, negative predictive value is high, and it is consequently important to rule out the diagnosis of PJP and other invasive fungal diseases.

Fourth, bacterial pneumonia is a common respiratory infection in this subpopulation, with a 20% rate of positive blood culture results. Both gram-positive and gram-negative bacteremias have been reported to be the source of false-positive BG results [2]. The administration of some antibiotics may also be a cause of BG reactivity [3]. We miss these pertinent data in the cohort assessed by Watanabe et al [1], because both are possible confounding factors.

Fifth, Watanabe et al [1] state that serum BG levels are not suitable for monitoring the response to treatment and that they do not always return to normal levels during treatment. We agree that BG does not return to normal levels during the course of treatment, because 3 weeks is not enough time to achieve a serological cure, which usually requires several weeks after the end of treatment [4]. Our group [4] and others [5, 6] have reported that the kinetics of measured BG (Fungitell; Associates of Cape Cod) suggest that decreasing levels of BG correspond to a favorable response to treatment (Figure 1), whereas increasing levels are associated with treatment failure [4].

Therefore, we believe that prospective

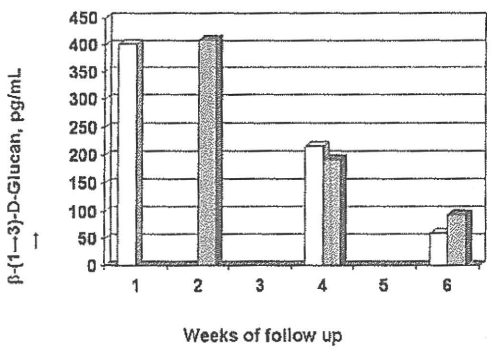

Figure 1. Kinetics of (1→3) β-D-glucan levels in a patient with *Pneumocystis jiroveci* pneumonia and human immunodeficiency virus infection *(white bars)* and in a renal transplant recipient with *P. jiroveci* pneumonia *(gray bars)*. Both patients responded to the treatment and survived the infection.

studies are needed to further evaluate the accuracy of serum BG assay for the diagnosis and follow-up of PJP in HIV-positive patients.

Acknowledgments

We thank Dr. Malcolm A. Finkelman for his critical reading of this letter.

Financial support. Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, PI070107 (to A.d.P), PI07134 (to M.S.C.), and PI070376 (to J.P.); Department of Education, Universities and Research Basque Government, IT-264–07 (to J.P.); Río Hortega Research Educational Contract from the Instituto de Salud Carlos III (to J.L.G); and Pfizer Spain (educational grant to A.d.P.).

Potential conflicts of interest. All authors: no conflicts.

Amalia del Palacio,[1] Jara Llenas-García,[2] María Soledad Cuétara,[3] Federico Pulido,[2] Rafael Rubio,[2] José Pontón,[4] and Ángel del Palacio-Pérez-Medel[5]

[1]Servicios de Microbiología and [2]Unidad VIH, Departamento de Medicina Interna del Hospital Universitario 12 de Octubre, and [3]Servicio de Microbiología del Hospital Severo Ochoa, Leganés, Madrid, and [4]Departamento de Inmunología, Microbiología y Parasitología, Facultad de Medicina y Odontología, Universidad del País Vasco, Bilbao, Vizcaya, Spain

References

1. Watanabe T, Yasuoka A, Tanuma J, et al. Serum (1→3) β-D-glucan as a noninvasive adjunct marker for the diagnosis of *Pneumocystis* pneumonia in patients with AIDS. Clin Infect Dis **2009**; 49:1128–1131.
2. Mennik-Kersten MASH, Ruegenbrink D, Verweig PE. *Pseudomonas aeruginosa* as a cause of 1,3-β-D-glucan assay reactivity. Clin Infect Dis **2008**; 46:1930–1931.
3. Mennik-Kersten MASH, Verweig PE. Non-culture based diagnostics of opportunistic fungi. Infect Dis Clin North Am **2006**; 20:711–717.
4. Cuétara MS, Alhambra A, Chaves F, Moragues MD, Pontón J, del Palacio A. Use of a serum (1→3) β-D-glucan assay for diagnosis and follow-up of *Pneumocystis jiroveci* pneumonia. Clin Infect Dis **2008**; 47:1364–1366.
5. Marty FM, Koo S, Bryar J, Baden LR. (1→3) β-D-glucan assay positivity in patients with *Pneumocystis (carinii) jiroveci* pneumonia. Ann Intern Med **2007**; 147:70–72.
6. Marty FM, Koo S, Bryar J, Baden LR. (1→3) β-D-glucan assay positivity in oncologic patients with *Pneumocystis jiroveci* pneumonia (PCP). [abstract M-1606]. In: Program and abstracts of the 46th Interscience Conference on Antimicrobial Agents and Chemotherapy (San Francisco). Washington, DC: American Society for Microbiology, 2006:425.

Reprints or correspondence: Dr Amalia del Palacio, Dept of Microbiology, Hospital Universitario 12 de Octubre, Madrid, Spain (apalacioh.hdoc@salud.madrid.org).

Clinical Infectious Diseases 2010; 50:451–2

1058-4838/2010/5003-0024$15.00
DOI: 10.1086/649889

Reply to del Palacio et al

To the Editor—We thank Palacio et al [1] for their interest in our study [2]. We would like to reply to their 5 comments.

First, our study [2] was performed retrospectively and analyzed >100 confirmed cases of *Pneumocystis* pneumonia (PCP).

Second, patients who had human immunodeficiency virus infection but did not have PCP were used as a control group, regardless of their $CD4^+$ cell counts in our original study. Among the 425 control patients, 273 had $CD4^+$ cell counts that were <200 cells/μL. If we analyzed data for these 273 patients, the median serum β-D-glucan level was 8.6 pg/mL (range, 1.0–283.0 pg/mL), which was almost same as the median value for all 425 control patients. This data indicates that serum β-D-glucan level is not influenced by $CD4^+$ cell counts.

Third, using the cutoff value of 23.2 pg/mL, positive predictive value and negative predictive value were 67.3% and 98.9%, respectively.

Fourth, the diagnosis of PCP was established by identification of *Pneumocystis jirovecii* in bronchoalveolar lavage (BAL) fluid with use of Diff-Quik (Dade Behring) staining method. We also examined the same sample by Gram stain and Ziehl-Neelsen stain for the detection of bacteria and mycobacteria, respectively, in BAL fluid. In addition, there were no patients who had comorbidity due to bacterial pneumonia or pulmonary tuberculosis. Therefore, it is unlikely that our data were biased by confounding factors, such as bacterial pneumonia or administration of antibiotics.

Fifth, as we mentioned in our report [2], β-D-glucan levels generally decrease soon after treatment in patients who experience a good clinical course, as Palacio et al [1] have presented, and levels are normalized several months or years after treatment in all patients. However, β-D-glucan levels are elevated in ~20% of patients during the early phase of treatment, and β-D-glucan levels seldom return to the normal level within a 21-day treatment period. In accordance with the Centers for Disease Control and Prevention guidelines [3], treatment of PCP is usually completed by 21 days in our hospital, regardless of the β-D-glucan levels, and there were no patients who experienced relapse caused by the cessation of treatment after 21 days. Therefore, it is apparent that increase of β-D-glucan levels soon after treatment does not always indicate treatment failure. Consequently, we concluded that serum β-D-glucan is a useful adjunct marker for diagnosis of PCP but is not suitable for monitoring of the disease.

Acknowledgments

We thank all staff of the AIDS Clinical Center for the care of patients.

Financial support. The Ministry of Health, Labour, and Welfare of Japan (AIDS-H18–008).

Potential conflicts of interests. All authors: no conflicts.

Tamayo Watanabe[1,2] and Shinichi Oka[1,2]

[1]AIDS Clinical Center, International Medical Center of Japan, Tokyo, and [2]Division of Infectious Diseases, Center for AIDS Research, Kumamoto University, Kumamoto, Japan

References

1. del Palacio A, Llenas-García J, Soledad Cuétara M, et al. Serum (1→3) β-D-glucan as a noninvasive adjunct marker for the diagnosis and follow-up of *Pneumocystis jiroveci* pneumonia in patients with HIV infection. Clin Infect Dis **2010**; 50:451–2 (in this issue).
2. Watanabe T, Yasuoka A, Tanuma J, et al. Serum (1→3) β-D-glucan as a noninvasive adjunct marker for the diagnosis of Pneumocystis pneumonia in patients with AIDS. Clin Infect Dis **2009**; 49:1128–1131.
3. Kaplan JE, Benson C, Holmes KH, Brooks JT, Pau A, Masur H. Guidelines for prevention and treatment of opportunistic infections in HIV-infected adults and adolescents: recommendations from CDC, the National Institutes of Health, and the HIV Medicine Association of the Infectious Diseases Society of America. MMWR Recomm Rep **2009**; 58(RR-4):1–207.

Reprints or correspondence: Dr Shinichi Oka, AIDS Clinical Center, International Medical Center of Japan, 1-21-1, Toyama, Shinjuku-ku, Tokyo 162-8655, Japan (oka@imcj.hosp.go.jp).

Clinical Infectious Diseases 2010; 50:452–3

1058-4838/2010/5003-0025$15.00
DOI: 10.1086/649890

Zoonotic Sporotrichosis in Rio de Janeiro, Brazil: A Protracted Epidemic yet to Be Curbed

To the Editor—The zoonotic transmission of sporotrichosis seems to be rare worldwide. However, since 1998, an increasing number of cases have been reported in humans in Rio de Janeiro, Brazil, the vast majority of them associated with contact with cats affected by the same condition [1, 2]. From 1998 through 2004, there were 759 humans and 1503 cats diagnosed with this mycosis by *Sporothrix schenckii* isolation in biological specimens at the Evandro Chagas Clinical Research Institute, Oswaldo Cruz Foundation (IPEC/Fiocruz). This represents an enormous increment vis-à-vis previous findings, because during the previous 12 years, there were only 13 cases in the same reference center [2].

From 2005 through 2008, 804 human patients were diagnosed with sporotrichosis, corresponding to an annual increase of 85%. The most affected population remains unchanged, with a predominance of women aged 40–49 years who are engaged in domestic duties and are from deprived social strata. Close contact with cats, either with clinically evident disease or with no symptoms, was reported in 91% of the human cases. Bites and/or scratches were reported by 68% of these patients, suggesting such lesions as the putative mean of transmission of the fungus.

The clinical picture [3] comprised 66% of presentations belonging to the lymphocutaneous form, 25% of the fixed form, and 9% of patients with disseminated lesions (ie, disseminated cutaneous forms, with or without extracutaneous lesions). Also worthy of notice is the apparent overlapping with other infectious conditions, such as human immunodeficiency virus infection (14 patients), tuberculosis (3), leprosy (2), and human T-lymphotropic virus infection (2). Among those patients infected by human immunodeficiency virus, 36% presented the disseminated severe form of the disease.

The drug of choice to treat these patients has been oral itraconazole (n = 514; 64% of patients), and terbinafine was used in 184 (23%) patients. Amphotericin B was very seldom used (6 patients). Almost 2% of clinically cured patients had clinical relapses (reemergence of their lesions), whereas 11% (n = 90) of the patients did not need to be treated, because of spontaneous cure. Patients were followed from 3–6 months after the end of therapy. Nine percent of the patients were lost to follow-up. Six patients were hospitalized, with 2 deaths. Irrespective of the drug regimens, 89% of the cases were cured.

It is still not certain how the infectious agent has been disseminated throughout the Rio de Janeiro municipality and its outskirts, but it is beyond reasonable doubt that the close interaction with cats represents a key form of transmission of the fungus. Felines have very close contact with contaminated soil and organic matter and constitute a reservoir of this agent [4, 5]. An improper destination given to ill or dead cats was mentioned by 71% of their current/former owners (most cats were just abandoned or died without receiving a proper burial or cremation). Such nonhygienic practices most likely foster the sustained dissemination of the mycosis, contributing to its current epidemic (en route to endemization?) status, which has yet to be curbed in Rio de Janeiro's metropolitan area.

Acknowledgments

Potential conflicts of interest. All authors: no conflicts.

Dayvison Francis Saraiva Freitas,[1] Antonio Carlos F. do Valle,[1] Rodrigo de Almeida Paes,[2] Francisco I. Bastos,[3] and Maria Clara G. Galhardo[1]

[1]Laboratório de Pesquisa Clínica em Dermatologia Infecciosa and [2]Laboratório de Micologia, Instituto de Pesquisa Clínica Evandro Chagas, and [3]Instituto de Comunicação e Informação Científica e Tecnológica, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil

References

1. Barros MBL, Schubach AO, Valle ACF, et al. Cat-transmitted sporotrichosis epidemic in Rio de Janeiro, Brazil: description of a series of cases. Clin Infect Dis 2004; 38:529–535.
2. Schubach A, Barros MB, Wanke B. Epidemic sporotrichosis. Curr Opin Infect Dis 2008; 21(2):129–133.
3. Rippon JW. Medical mycology: the pathogenic fungi and the pathogenic actinomycetes. 3rd ed. Philadelphia: WB Saunders, 1988:325–352.
4. Dustan RW, Reiman KA, Langhan RF. Feline sporothricosis. J Am Vet Med Assoc 1986; 189: 880–883.
5. Schubach TM, Schubach AO, Okamoto T, et al. Evaluation of an epidemic of sporotrichosis in cats: 347 cases (1998–2001). J Am Vet Med Assoc 2004; 224:1623–1629.

Reprints or correspondence: Dayvison Francis Saraiva Freitas, Rua Barata Ribeiro, 185-ap.905, Copacabana, Rio de Janeiro, RJ, Brasil. CEP 22011-001 (dayvison.freitas@ipec.fiocruz.br).

Clinical Infectious Diseases 2010; 50:453

1058-4838/2010/5003-0026$15.00
DOI: 10.1086/649891

Genetic Determinants of Antibiotic Resistance in Diarrheagenic *Klebsiella pneumoniae* Subspecies *ozaenae*: An Emerging Enteropathogen in Senegal

To the Editor—Diarrheal diseases are common in developing countries. They are usually caused by bacteria like *Salmonella, Shigella, Escherichia coli, Campylobacter,* and *Vibrio cholerae.* Members of the genus *Klebsiella* are found as normal flora in the human intestinal tract; some strains are considered to be enteropathogenic and are able to cause diarrhea both in immunocompetent and immunocompromised individuals [1, 2]. Although this pathogen is responsible for diarrhea, the molecular mechanism of its pathogenesis remains unclear. Virulence factors includ-

INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Clinical Symptoms and Courses of Primary HIV-1 Infection in Recent Years in Japan

Hideta Nakamura, Katsuji Teruya, Misao Takano, Kunihisa Tsukada, Junko Tanuma, Hirohisa Yazaki, Haruhito Honda, Miwako Honda, Hiroyuki Gatanaga, Yoshimi Kikuchi and Shinichi Oka

Abstract

Background The natural course of HIV-1 infection includes 10 years of an asymptomatic period before the development of AIDS. However, in Japan, the disease progression process seems faster in recent years.

Methods The study subjects were 108 new patients with primary HIV-1 infection during the period from 1997 through 2007. We evaluated their clinical symptoms and laboratory data, and then analyzed disease progression in 82 eligible patients. Disease progression was defined as a fall in CD4 count below 350/μL and/or initiation of antiretroviral therapy.

Results Ninety percent of the patients were infected via homosexual intercourse. All patients had at least one clinical symptom (mean; 4.75±1.99) related to primary HIV-1 infection, with a mean duration of 23.2 days (±14.8) and 53.3% of them had to be hospitalized due to severe symptoms. The mean CD4 count and viral load at first visit were 390/μL (±220.1) and 4.81 log10/mL (±0.78), respectively. None developed AIDS during the study period. Estimates of risk of disease progression were 61.0% at 48 weeks and 82.2% at 144 weeks. In patients who required antiretroviral therapy, the median CD4 count was 215/μL (range, 52-858) at initiation of such therapy. Among the patients with a CD4 count of <350/μL at first visit, 53% never showed recovery of CD4 count (>350/μL) without antiretroviral therapy.

Conclusion Despite possible bias in patient population, disease progression seemed faster in symptomatic Japanese patients with recently acquired primary HIV-1 infection than the previously defined natural course of the disease.

Key words: HIV-1, primary infection, disease progression

(Intern Med 50: 95-101, 2011)
(DOI: 10.2169/internalmedicine.50.4137)

Introduction

The natural course of HIV-1 infection has been well described in large cohorts from the United States and Europe before the introduction of highly active antiretroviral therapy (HAART); primary HIV-1 infection (PHI) is followed by a clinical latency, usually lasting around 10 years, which precedes the eventual collapse of the immune system (1, 2). However, there is a common feeling among clinicians at present that the natural disease progression of recently infected patients is faster than in previous years (3, 4). Disease progression depends on various factors such as HLA type (5), concomitant infections (6, 7), and available medical resources (8). In addition to these factors, events occurring during PHI could also determine the natural course of the disease. Initial studies suggested that patients with more symptoms related to primary PHI and longer duration of illness exhibit faster rates of progression to AIDS (9-13). Plasma viral load at a set point is also an independent predictor of disease progression (14, 15). However, to determine the viral set point is sometimes difficult. Therefore, for clinicians, the severity of clinical symptoms is the only predictor of subsequent disease progression. The latency be-

AIDS Clinical Center, National Center for Global Health and Medicine, Japan
Received for publication June 28, 2010; Accepted for publication September 29, 2010
Correspondence to Dr. Shinichi Oka, oka@acc.ncgm.go.jp

tween the development of PHI and commencement of HAART is also important in the present HAART era.

The main aim of this study was to evaluate the natural disease progression of recently infected Japanese patients. To determine whether or not the disease progression of recently infected patients is accelerated, their CD4 decline was compared with that of hemophiliacs infected before 1985 as the first HIV-1 infection in Japanese.

Furthermore, we also evaluated the correlation between initial CD4 count, viral load, and clinical events and subsequent changes in CD4 and/or time to start HAART in symptomatic Japanese patients with PHI.

Patients and Methods

Study site and patients with PHI

This study was conducted at the AIDS Clinical Center (ACC), National Center for Global Health and Medicine (NCGM: formerly International Medical Center of Japan). The NCGM (925 beds) is a tertiary general hospital located in central Tokyo and the ACC is the main referral clinic for treatment of HIV infected patients in Japan. As part of the follow-up service, HIV-1 infected patients usually visit the ACC on a monthly basis and CD4 count and viral load are measured at each visit. In the present retrospective study, we reviewed the medical records of 108 patients with PHI who were newly diagnosed with PHI between 1997 through 2007 at the ACC. We had conducted a clinical trial of structured treatment interruptions in patients with PHI from November 2000 through December 2002 and 26 patients were enrolled in that trial (16, 17). In terms of the data of these 26 patients, only the initial clinical and laboratory data were included in the present analysis, while all other data, such as time to events, were excluded from this study. To compare the natural CD4 decline of previously and recently infected patients, CD4 counts of 42 Japanese hemophiliacs recorded in the database in 1988 were analyzed as a previous control. Japanese hemophiliacs were infected with HIV-1 through contaminated blood products before 1985 (the estimated mean year of infection was 1983). Therefore, CD4 counts at the end of 1988 were the data at least 3 years after infection. In this comparison, the number of eligible recently infected patients was 59 patients; untreated and CD4 count at 3 years after infection was available.

Definition of PHI

PHI was diagnosed based on the presence of the following three criteria: 1) negative or incomplete western blot finding at the first visit with subsequent change to positive, 2) negative or weakly reactive enzyme-linked immunosorbent assay (ELISA) result for plasma HIV-1 RNA, and 3) confirmed HIV-1 infection on the first visit with documentation of negative ELISA result within 6 months. Symptomatic PHI was defined as PHI accompanied by at least one symptom related to acute retroviral syndrome, such as fever, lymphadenopathy, or skin rash.

Definition of disease progression

Disease progression was defined as fall in CD4 count below 350/μL and/or initiation of antiretroviral therapy. Specifically, patients with an AIDS-defined illness [listed under Centers for Disease Control and Prevention (CDC) category C], patients with AIDS requiring initiation of HAART, and those with severe symptomatic PHI on HAART were defined to have disease progression. The selection of a cutoff value of 350/μL for CD4 count was based on the fact that treatment is generally indicated during the chronic phase of infection when CD4 count falls below 350/μL (18). Patients were considered to be in immunologic progression at the first visit when the initial CD4 count was <350/μL and never subsequently reached 350/μL. For patients who showed a spontaneous increase in subsequent CD4 counts to ≥350/μL (such recovery occurred within 3 months from the first visit in all such patients), disease progression was set to have started at the time when such change in CD4 count occurred.

Statistical analysis

Continuous variables are presented as mean value ± SD. Categorical variables were presented as absolute numbers and proportions. Time to events was analyzed by the Kaplan-Meier survival curves, and compared using log-rank test. For patients who did not experience the events described above, data were censored at their last visit. To evaluate the differences between patients groups, the Student t test and χ^2 test were used when appropriate. The relationships between variables were analyzed by the Spearman rank-over correlation test. Statistical significance was defined as $p<0.05$. Data were analyzed using SPSS for Windows (version 15, SPSS, Inc., Chicago, IL).

Results

Table 1 lists the demographics of the enrolled patients with PHI. All patients had at least one documented symptom consistent with PHI (median 5; range 1-11). Fever, cervical lymphadenopathy, pharyngitis, and rash were found in more than 50% of patients (Table 2). The mean duration of symptoms was 23.2 days (SD ±14.8). Fifty-eight (53.7%) patients had to be hospitalized due to severe clinical symptoms. The initial viral loads in hospitalized patients were significantly higher than those of non-hospitalized patients. A longer duration of symptoms was associated with higher initial viral load (R=0.31, p=0.002) (Fig. 1A), and lower CD 4 count (R=-0.22, p=0.03) (Fig. 1B). Consequently, a higher viral load slightly was correlated with a lower CD4 count at the first visit (R=-0.22, p=0.033) (Fig. 1C).

Disease progression was analyzed in 82 patients. None of the patients had AIDS-defining events. Estimates of the risk of disease progression were 50.6% at 24 weeks, 61.0% at 48 weeks, 67.0% at 96 weeks, and 82.2% at 144 weeks

Table 1. Baseline Characteristics of 108 Patients with Primary HIV-1 Infection in this Study

Characteristics	Total number or mean (±SD) or %	Hospitalized patients (n = 58)	Non-hospitalized patients (n = 50)	p
Age (year)	31.8±8.48	32±9.07	31±7.82	NS
Sex				
Male	102	56	46	NS
Female	6	2	4	NS
Predisposing factor				
MSM	97	53	44	NS
Heterosexual	8	3	5	NS
IDU	1	0	1	NS
Unknown	2	2	0	NS
PMH of STD	75 (69.7)	44 (40.4)	31 (29.3)	NS
Syphilis	49 (45.5)	27 (25.3)	21 (20.2)	NS
Acute hepatitis A	11 (10.1)	6 (6.1)	5 (4.0)	NS
Acute hepatitis B	36 (33.3)	22 (20.2)	14 (13.1)	NS
Amebiasis	10 (9.1)	9 (8.0)	1 (1.1)	0.035
Others	7 (6.1)	2 (2.0)	5 (4.1)	NS
No. of symptoms	4.75±1.99	4.98±1.94	4.48±2.04	NS
Duration of symptoms (days)	23.2±14.8	27.8±13.1	18.0±15.1	0.001
Laboratory findings				
CD4 count/μL	390.0±220.1	356.1±204.1	443.7±236.0	0.06
HIV RNA $\log_{10}$/mL	4.81±0.78	5.03±0.68	4.48±0.81	0.001
STI trial*	26	12	14	NS

*Patients enrolled in a clinical trial of structured treatment interruptions in recently HIV-1-infected patients. Abbreviations; MSM: men who have sex with men, PMH of STD: past medical history of sexual transmitted diseases, STI: structured treatment interpretations, IDU: intravenous drug user, Others: genital herpes infection, chlamydial urethral infection condyloma acuminata, NS: not significant

Data are presented as mean ± SD or percentage (%) unless otherwise indicated

Table 2. Symptoms and Physical Findings Observed in the Patients with >10% Frequencies (n=108)

Symptoms and physical findings	frequency (%)
Fever	91
Lymphadenopathy	63
Pharyngitis	53
Rash	50
Diarrhea	37
Fatigue	32
Headache	26
Myalgia	20
Weight loss	19
Nausea	16
Appetite loss	14
Neurological sign	13
Hepatomegaly	13
Thrush	12

(Fig. 2). Eighteen of 34 (53.3%) patients with an initial CD 4 cell count below 350 cells/μL had immunologic progression at the first visit. Their CD4 counts never increased above 350/μL until initiation of HAART. Forty-eight (58.5%) required initiation of HAART in this study. The reasons for the initiation of HAART were severe clinical symptoms related to PHI in 16 patients and immunologic progression in 32 patients. The median CD4 count of those patients at initiation of HAART was 215/μL (range, 52-858).

We analyzed the clinical course in 66 patients (excluding 26 patients who enrolled in a clinical trial of structured treatment interruptions in PHI and 16 patients who received HAART for PHI) to determine the factors associated with disease progression. Half of these patients (33 patients) required hospitalization. As shown in Fig. 3A, the mean time to disease progression of the hospitalized patients [57.4 weeks, 95% confidence interval (95%CI); 34.9-79.8 weeks] was shorter than that of the non-hospitalized (33 patients, 94.4 weeks, 95%CI; 71-117 weeks, p=0.002). Among the 32 patients with CD4 count >350/μL at first visit, 24% had documented disease progression within 1 year, whereas among 34 patients with CD4 count <350/μL at first visit, 76.4% showed disease progression (Fig. 3B). The mean times to disease progression for the two groups were 111.9 weeks (95%CI; 92.8-131) and 39.5 weeks (95%CI; 18.6-60.5), respectively (p<0.001). Disease progression in 39 patients with high viral load (≥5.0 $\log_{10}$/mL) was not significantly different (p=0.41) from that in 27 patients with low viral load (<5.0 $\log_{10}$/mL) (Fig. 3C). The number of symptoms was not significantly different in each group (Fig. 3D). The mean time to disease progression was 69.8 weeks (95% CI; 47.2-92.5) in patients with a high viral load and 80.4 weeks (95%CI; 54.9-105.8) in those with a low viral load.

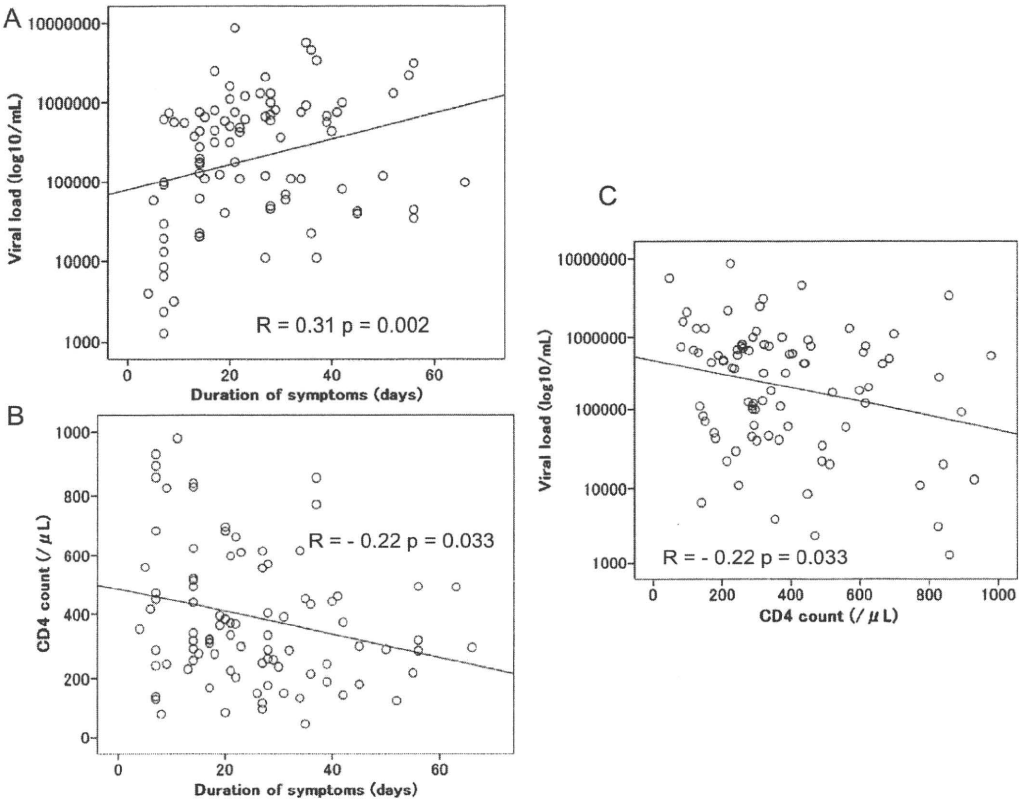

Figure 1. **Correlations among plasma viral load, CD4 count, and clinical symptoms. A; Plasma viral load correlated with duration of symptoms (R=0.31, p=0.002). B; CD4 count correlated inversely with duration of symptoms (R=-0.22, p=0.033). C; plasma viral load correlated inversely with CD4 count (R=-0.22, p=0.033).**

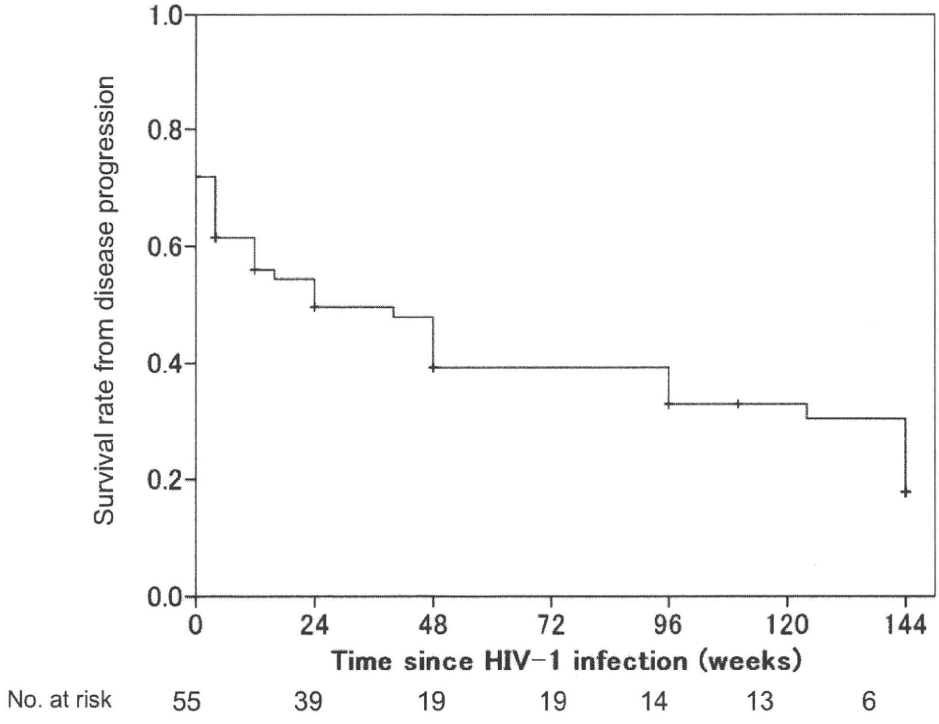

Figure 2. **Progression-free survival in 82 patients. Progression was defined as CD4 count <350/μL or initiation of HAART. No. at risk: the number of CD4 count >350/μL or HAART naïve patients**

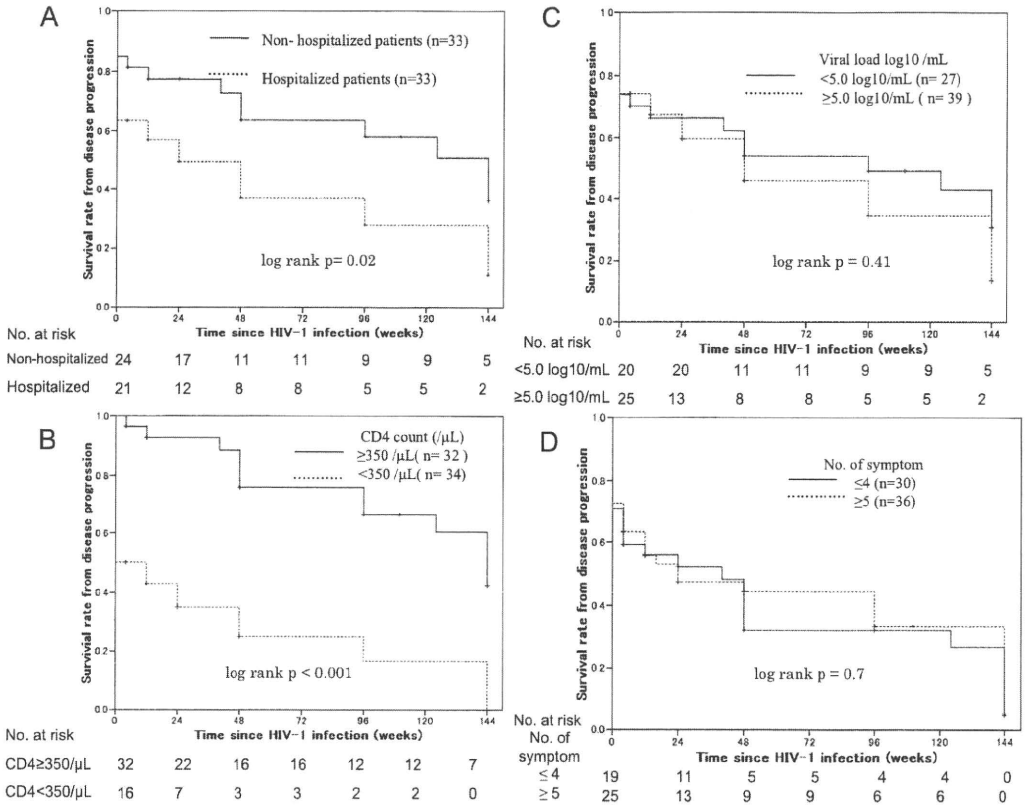

Figure 3. Progression-free survival among 66 patients according to rate of hospitalization, baseline CD4 count, and viral load. No. at risk: the number of CD4 count >350/μL or HAART naïve patients. A; Solid line: patients who required hospitalization due to PHI, dashed line: patients who did not require hospitalization (p=0.02, by log-rank test). B; Solid line: patients with CD4 count >350/μL at first visit, dashed line: patients with CD4 count <350/μL (p<0.001). C; Solid line: patients with viral load <5.0 log_{10}/mL, dashed line: patients with viral load ≥5.0 log_{10}/mL (p=0.41). Disease progression was defined as CD4 count <350/μL or initiation of HAART. D; Solid line: patients with the number of PHI symptoms ≤4, dashed line: patients with the number of PHI symptoms ≥5 (p=0.7, by log-rank test).

Comparison of percentage of recently infected patients with CD4 counts >350/μL at 3 years after infection and that of hemophiliacs as the first HIV-1 infected population in Japanese is shown in Fig. 4. The percentage (13.5%) of recently infected patients was significantly lower than that (47.6%) of Japanese hemophiliacs (p<0.001), clearly indicating the rapid decline of CD4 count in recently infected patients.

Discussion

In this study, we demonstrated rapid disease progression of symptomatic PHI Japanese patients in this decade. However, when we divided our study subjects into two groups according to the first half (1997-2002) and the latter half (2003-2007), disease progression of each group was not different (data not shown). In contrast, disease progression surrogated with natural CD4 decline of recently infected patients was significantly accelerated compared with Japanese hemophiliacs infected with HIV-1 before 1985. However, there are two quite different backgrounds; one is the route of infection and the other is the year of infection. Almost all hemophiliac patients are also co-infected with hepatitis C but do not have other sexually transmitted diseases (STDs). In contrast, most patients in the present study were infected via homosexual intercourse with many other STDs that may facilitate acceleration of the disease progression (7). In the present study, 69.7% patients had a past medical history of STDs, and the mean number of STDs was 1.08/patient (0: 31.3%, 1: 37.4%, 2: 23.2%, 3: 8.1%). In this regard, most published data on disease progression were obtained from men who have sex with men (MSM) cohorts (1, 2). Therefore, it is unlikely that the recent rapid disease progression is due to Japanese MSM. Whether or not the rapid disease progression in the recently HIV-1-infected Japanese can be generalized is to be elucidated in future studies.

Some HLA types are protective against disease progression such as HLA-B57 (19) and HLA-B51 (20) because HLA-restricted cytotoxic T lymphocytes (CTLs) play an important role on viral control. On the other hand, virus can easily escape from CTLs (17, 21). In some prevalent HLA types, escape virus can transmit and accumulate in the population (21). In this situation, some HLA types are no more

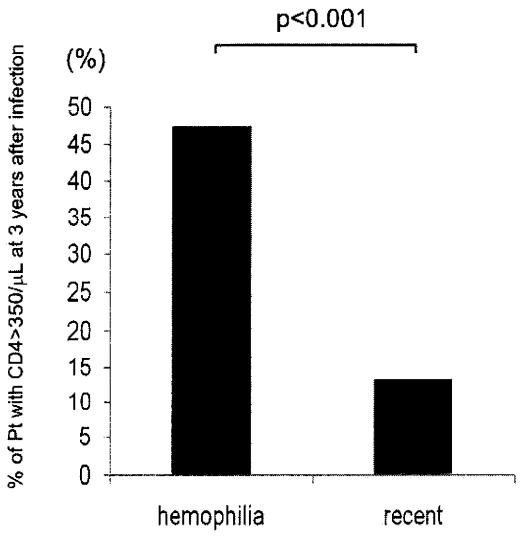

Figure 4. **Comparison of percentage of previously and recently infected patients with CD4 counts >350/μL at 3 years after infection. In this analysis, Japanese hemophiliacs (designated "hemophilia" in the figure) were regarded as a previously infected patient, because they were infected with HIV-1 before 1985. The number of hemophiliacs was 42 patients. The eligible number of recently infected patients (designated "recent" in the figure) was 59 patients; infected with HIV-1 after 1997, untreated, and CD4 count at 3 years after infection.**

protective. The HLA distribution is different in Americans compared to Japanese. Another possible hypothesis for the different disease progression is that Japanese hemophiliacs were exposed to HIV-1 through contaminated blood products imported from US as the first Japanese population infected with the virus around 1983. However, in recent years, most HIV-1 infection in Japanese is transmitted from Japanese patients. It can be postulated that current HIV-1 in Japan has adapted to the Japanese population, indicating acquisition and accumulation of escape virus from immune pressure of the otherwise protective HLA in Japanese population (21). From a negative point of view, the situation is similar to the epidemic of drug-resistance virus in treatment of naïve patients (22). The clinical relevance of the prevalence of immune escape virus in Japanese is a potentially serious matter in terms of the natural course of HIV-1 infection.

In the present study, all patients have had at least one symptom associated with PHI. During the follow-up period, no patient developed AIDS, whereas around 70% of the patients experienced immunologic progression as defined by a CD4 count <350/μL. It is noteworthy that the majority of these patients exhibited immunologic progression within 3 years and, surprisingly, >60% of them were documented within the first year. HAART was initiated in nearly 60% of patients during this period, including initiation for PHI-related severe symptoms in 20% of these patients. Previous studies on PHI have suggested that the number, duration, and/or severity of symptoms can predict faster disease progression to AIDS (23, 24). Our findings are compatible with these previous studies. Considered together, these results suggest that the duration of illness rather than the number of symptoms is more likely to be a major determinant of immunological progression. The estimated risks of disease progression were more than 50% by week 24 and 80% by week 144. Comparison with those observed elsewhere during the natural course of HIV-1 infection (24), these disease progression rates are surprisingly high. Among the patients with CD4 counts >350/μL at first visit, a quarter of them showed disease progression within 1 year. In contrast, in patients with CD4 count <350/μL, three quarters of them showed disease progression within the same period. Goujard et al (25) suggested possible recovery of CD4 count after the primary infection phase even in patients with very low count because it fluctuates during that period. In contrast, our results suggest that patients with a CD4 count of <350/μL during primary infection should be monitored carefully because spontaneous recovery of CD4 cell count during primary infection was rare. This cautionary remark could also apply to patients with a CD4 count of >350/μL because they exhibited nearly 60% risk of disease progression within 3 years. These observations may allow more targeted clinical monitoring and timely initiation of HAART. The impact of a short-term HAART during symptomatic primary infection on the subsequent disease progression needs to be elucidated in future study.

Although we included all recent seroconverters during the study period, it could be argued that this study carries some institution bias (i.e., a high proportion of cases with severe disease). However, the present finding of a surprisingly rapid disease progression in our patient population is new. Whether or not the natural course of disease progression has recently become accelerated in other countries or other cohorts is a matter of great interest.

The authors state that they have no Conflict of Interest (COI).

Acknowledgement

The authors thank all clinical staffs of the AIDS Clinical Center. This study was supported in part by a Grant-in-Aid for AIDS Research from the Ministry of Health, Labour, and Welfare of Japan.

References

1. Collaborative Group on AIDS Incubation and HIV Survival. Time from HIV-1 seroconversion to AIDS and death before widespread use of highly-active antiretroviral therapy: a collaborative reanalysis. Lancet **355**: 1131-1137, 2000.
2. Lyles RH, Munoz A, Yamashita TE, et al. Natural history of human immunodeficiency virus type 1 viremia after seroconversion and proximal to AIDS in a large cohort of homosexual men. Multicenter AIDS cohort study. J Infect Dis **181**: 872-880, 2000.
3. Crum-Cianflone N, Eberly L, Zhang Y, et al. Is HIV becoming more virulent? Initial CD4 counts among HIV seroconverters during the course of the HIV epidemic: 1985-2007. Clin Infect Dis **48**: 1285-1292, 2009.

4. Muller V, Maggiolo F, Suter F, et al. Increasing clinical virulence in two decades of the Italian HIV epidemic. PLoS Pathog **5**: e1000454, 2009.
5. Carrington M, O'Brien SJ. The influence of HLA genotype on AIDS. Annu Rev Med **54**: 535-551, 2003.
6. McShane H. Co-infection with HIV and TB: double trouble. Int J STD AIDS **16**: 95-100, 2005.
7. Palacios R, Jimenez-Onate F, Aguilar M, et al. Impact syphilis infection on HIV viral load and CD4 cell counts in HIV-infected patients. J Acquir Immun Defic Syndr **44**: 356-359, 2007.
8. Duncombe C, Kerr SJ, Ruxrungtham K, et al. HIV disease progression in a patient cohort treated via a clinical research network in a resource limited setting. AIDS **28**: 169-178, 2005.
9. Pedersen C, Katzenstein T, Nielsen C, et al. Prognostic value of serum HIV-RNA levels at virologic steady state after seroconversion: relation to CD4 cell count and clinical course of primary infection. J Acquir Immune Defic Syndr Hum Retrovirol **16**: 93-99, 1997.
10. Vanhems P, Hirschel B, Phillips AN, et al. Incubation time of acute human immunodeficiency virus (HIV) infection and duration of acute HIV infection are independent prognostic factors of progression to AIDS. J Infect Dis **182**: 334-337, 2000.
11. Vanhems P, Lanbert J, Cooper DA, et al. Severity and prognosis of acute human immunodeficiency virus type 1 illness: a dose-response relationship. Clin Infect Dis **26**: 323-329, 1998.
12. Sterling T, Vlahov D, Astemborski J, et al. Initial plasma HIV-1 RNA levels and progression to AIDS in women and men. N Engl J Med **344**: 720-725, 2001.
13. Henrard DR, Daar E, Farzadegan H, et al. Virologic and immunologic characterization of symptomatic and asymptomatic primary HIV-1 infection. J Acquir Immune Defic Syndr Hum Retrovirol **9**: 305-310, 1995.
14. Schacker TW, Hughes JP, Shea T, et al. Biological and virologic characteristics of primary HIV infection. Ann Intern Med **128**: 613-620, 1998.
15. Lefrere JJ, Roudot-Thoraval F, Mariotti M, et al. The risk of disease progression is determined during the first year of human immunodeficiency virus type 1 infection. J Infect Dis **177**: 1541-1548, 1998.
16. Fujiwara M, Tanuma J, Koizumi H, et al. Different abilities of escape mutant-specific cytotoxic T cells to suppress replication of escape mutant and wild-type human immunodeficiency virus type 1 in new hosts. J Virol **82**: 138-147, 2008.
17. Tanuma J, Fujiwara M, Teruya K, et al. HLA-A*2402-restricted HIV-1 specific T lymphocytes and escape mutation after ART with structured treatment interruptions. Microbes Infect **10**: 689-698, 2008.
18. Thompson MA, Aberg JA, Cahn P, et al. Antiretroviral treatment of adult HIV infection; 2010 recommendations of the international AIDS Society-USA Panel. JAMA **304**: 321-333, 2010.
19. Goulder PJ, Bunce M, Krausa P, et al. Novel, cross-restricted, conserved, and immunodominant cytotoxic T lymphocyte epitopes in slow progressors in HIV type 1 infection. AIDS Res Hum Retroviruses **10**: 1691-1698, 1996.
20. Kawashima Y, Kuse N, Gatanaga H, et al. Long-term control of HIV-1 in hemophiliacs carrying slow-progressing allele HLA-B* 5101. J Virol **84**: 7151-7160, 2010.
21. Kawashima Y, Pfafferott K, Frater J, et al. Adaptation of HIV-1 to human leukocyte antigen class I. Nature **458**: 641-645, 2009.
22. Little SJ, Holte S, Routy JP, et al. Antiretroviral-drug resistance among patients recently infected with HIV. N Engl J Med **347**: 385-394, 2002.
23. Vanhems P, Lambert J, Cooper DA, et al. Severity and prognosis of acute immunodeficiency virus type 1 illness: a dose-response relationship. Clin Infect Dis **26**: 323-329, 1998.
24. Lavreys L, Baeten JM, Chohan V, et al. Higher set point plasma viral load and more-severe acute HIV type 1 (HIV-1) illness predict mortality among high-risk HIV-1-infected African women. Clin Infect Dis **42**: 1333-1339, 2006.
25. Goujard C, Bonarek M, Meyer L, et al. CD4 cell count and HIV DNA level are independent predictors of disease progression after primary HIV type 1 infection in untreated patients. Clin Infect Dis **42**: 709-715, 2006.

High Performance Liquid Chromatography Using UV Detection for the Simultaneous Quantification of the New Non-nucleoside Reverse Transcriptase Inhibitor Etravirine (TMC-125), and 4 Protease Inhibitors in Human Plasma

Atsushi HIRANO,[a,b,c] Masaaki TAKAHASHI,*[a,b] Eri KINOSHITA,[a] Masaaki SHIBATA,[a] Toshiharu NOMURA,[a] Yoshiyuki YOKOMAKU,[b] Motohiro HAMAGUCHI,[b] and Wataru SUGIURA[b]

[a] *Department of Pharmacy, National Hospital Organization Nagoya Medical Center;* [b] *Clinical Research Center, National Hospital Organization Nagoya Medical Center; 4-1-1 Sannomaru, Naka-ku, Nagoya, Aichi 460-0001, Japan; and* [c] *Department of Medicinal Informatics, Graduate School of Medicine, Kanazawa University; 13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan.*

Received March 4, 2010; accepted May 10, 2010; published online May 20, 2010

Etravirine (TMC-125, ETV) is a second-generation non-nucleoside reverse transcriptase inhibitor (NNRTI) that demonstrates potent activity against NNRTI-resistant strains of human immunodeficiency virus type-1 (HIV-1). Thus, ETV has been used in combination with ritonavir-boosted protease inhibitor (PI) and integrase inhibitor for therapy-experienced HIV-1-infected patients. On the other hand, as ETV is a substrate and inducer of cytochrome P450 3A4 (CYP3A4), ETV may induce metabolism of PI and alter the concentrations of co-administered PIs. In order to ensure optimal drug efficacy and prevention of resistance, it is essential to monitor plasma concentrations of ETV and PIs. Here we describe the application of HPLC with UV detection for the simulatancous assay of ETV and 4 PIs, darunavir (DRV), atazanavir (ATV), ritonavir (RTV) and lopinavir (LPV). In this study, the calibration curve of each drug was linear with the average accuracy ranging from 93.6 to 110.9%. Both intra- and interday coefficients of variation for each drug were less than 11.6%. The mean recovery of all drugs ranged from 88.0 to 97.5%. The limit of quantification was 0.04, 0.04, 0.04, 0.05 and 0.07 μg/ml for ETV, DRV, ATV, RTV and LPV, respectively. These results demonstrate that our HPLC-UV method can be used for routine determination of plasma concentrations of ETV and 4 PIs in clinical settings.

Key words etravirine; HPLC; protease inhibitor; therapeutic drug monitoring

Etravirine (TMC-125, ETV) is a second-generation non-nucleoside reverse transcriptase inhibitor (NNRTI) that demonstrates potent activity against NNRTI-resistant strains of human immunodeficiency virus type-1 (HIV-1). According to the DUET studies (randomized, double-blind, placebo-controlled trials), overall, ETV was well tolerated in treatment-experienced patients infected with HIV-1, with its safety and tolerability profile generally comparable to placebo at week 24.[1,2] Additionally, 48-week data pooled from these studies showed greater virologic and immunologic responses compared with placebo.[3]

In the latest HIV treatment, ETV has been used in combination with ritonavir-boosted protease inhibitor (PI) and integrase inhibitor for therapy-experienced HIV-1-infected patients. On the other hand, as ETV is a substrate and inducer of cytochrome P450 3A4 (CYP3A4), ETV may induce metabolism of PI and alter the concentrations of co-administered PIs.[1] In order to ensure optimal drug efficacy and prevention of resistance, it is essential to monitor plasma concentrations of ETV and PIs.

Fayet *et al.*[5] and Quaranta *et al.*[6] succeeded in determining plasma concentrations of ETV and other drugs through the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS). Rezk *et al.*[7] have also developed a method to measure plasma concentrations of ETV and PIs by LC-MS. LC-MS or LC-MS/MS assay is very sensitive and accurate. However, MS equipment is very expensive and unavailable in conventional hospital laboratories. Therefore, development of alternate methods is necessary.

Recently, D'Avolio *et al.*[8] reported a new HPLC method that employs a photo diode array (HPLC-PDA) for quantification of ETV and other antiretroviral drugs. This method is simple, reliable, and sensitive, using cost-effective instrumentation when compared with others.[5–7] However, this method requires a solid phase extraction. Furthermore, in general hospitals, a UV detector coupled with HPLC is more popular than a PDA detector. The HPLC-UV method is a user-friendly assay that is readily adaptable to standard laboratory equipment for routine therapeutic drug monitoring (TDM).

In this study, we propose the simultaneous quantitative assay of ETV and 4PIs, darunavir (DRV), atazanavir (ATV), ritonavir (RTV) and lopinavir (LPV) in a simple procedure that is derived from a previously established HPLC-UV method.[9] This method can be applied to pharmacokinetic studies of PIs and ETV, the newest NNRTI, and it is useful when evaluating the clinical significance of TDM for these drugs.

MATERIALS AND METHODS

Chemicals ETV and DRV were supplied by Tibotec Pharmaceuticals Ltd. (Eastgate Village, Eastgate, Little Island, Co., Cork, Ireland). LPV and RTV were generously provided by Abbott Laboratories (Abbott Park, IL, U.S.A). ATV was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (New Brunswick, NJ, U.S.A.). The internal standard (IS), 6,7-dimethyl-2,3-di(2-pyridyl)quinoxaline, was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Acetonitrile, methanol, ethyl acetate and *n*-hexane (Kanto

* To whom correspondence should be addressed. e-mail: masaakit@nnh.hosp.go.jp

Chemical, Tokyo, Japan) were HPLC grade. Sodium carbonate was purchased from Katayama Chemical (Osaka, Japan). Water was deionized and osmosed using a Milli-Q® system (Millipore, Bedford, MA, U.S.A.). All other chemicals were of analytical grade and have been described in our previous report.[9]

Standard Solutions Stock solutions of tested drugs and IS were prepared by accurately dissolving weighed amounts of each reference compound in water/ethanol (50 : 50, v/v) to yield concentrations of 191.0 μg/ml for ETV, 85.2 μg/ml for DRV, 502.0 μg/ml for ATV, 425.0 μg/ml for RTV, 95.1 μg/ml for LPV, and 588.0 μg/ml for IS. These stock solutions were stored at −80 °C until the day of analysis. Each stock solution was diluted in drug-free plasma to yield concentrations of 0.08, 0.14, 0.42, 1.04 and 4.17 μg/ml for ETV, 0.07, 0.12, 0.37, 0.93 and 3.72 μg/ml for DRV, 0.09, 0.15, 0.44, 1.10 and 4.38 μg/ml for ATV, 0.07, 0.12, 0.37, 0.93 and 3.71 μg/ml for RTV, 0.08, 0.14, 0.42, 1.04 and 4.15 μg/ml for LPV.

Chromatography The HPLC system consisted of a Waters pump (model 515), a 717 plus autosampler, and a 2487 dual λ absorbance detector coupled to the Empower™ software (Waters, Milford, MA, U.S.A.). The analytical column was a Radial-Pak Nova-Pak C_{18} column (4 μm, 8×100 mm, Waters) protected by Guard-Pak Inserts Nova-Pak C_{18} precolumn. Absorbance was measured at 205 nm, with separations performed at 30 °C. The mobile phase consisted of 39% 50 mM phosphate buffer (pH 6.2), 22% methanol and 39% acetonitrile. The assay run time was 30 min with a flow rate of 1.8 ml/min. Drugs were quantified by measuring the peak areas under the chromatograms.

Sample Preparation A total of 2 ml of ethyl acetate/*n*-hexane (50 : 50, v/v) containing the IS (1.18 μg/ml) and 1 ml of 0.5 M sodium carbonate were added to a 500 μl plasma sample. The mixture was vortexed and then centrifuged at 3500×***g*** for 5 min. The organic layer was separated and evaporated to dryness. The dried material was then dissolved in 100 μl of a mobile phase solution and centrifuged at 13000×***g*** for 5 min. Lastly, 25 μl of the upper solution was injected into the HPLC column.

The institutional review board of the National Hospital Organization Nagoya Medical Center approved this study. Plasma samples were prepared from patients after obtaining written informed consent.

Validation Intra- and interday precision values using this method were estimated by assaying control plasma containing five different concentrations of each drug five times on the same day and on three separate days to obtain the coefficient of variation (CV). Accuracy was determined as the percentage of the nominal concentration. Drug recovery from plasma was evaluated by analyzing triplicate samples with or without extraction. Plasma samples spiked with known amounts of both drugs and IS were extracted as usual. Blank plasma samples that contained only the IS were extracted and subsequently spiked with the same amount of analytes to give the 100% reference. The recovery was assessed by comparing the peak area ratio (analytes/IS) of extracts. The limit of quantification was defined as the lowest concentration for which both the CV% and the percent of deviation from the nominal concentration were less than 20%.

RESULTS

Plasma Sample Chromatograms Figure 1A is a chromatogram of a spiked plasma sample containing 3.77 μg/ml of DRV, 1.18 μg/ml of IS, 4.44 μg/ml of ATV, 3.76 μg/ml of RTV, 1.69 μg/ml of ETV and 4.21 μg/ml of LPV. Under the described chromatographic conditions, retention times were 3.4, 4.4, 8.3, 10.5, 11.7, 13.0 min for DRV, IS, ATV, RTV, ETV and LPV, respectively. At a detection wavelength of 205 nm, assays performed on drug-free human plasma demonstrated that there were no interfering peaks during the intervals of interest for the retention times (Fig. 1B).

Figure 2A is a chromatogram of a plasma sample from an HIV-1-infected patient treated with raltegravir, ETV, ATV, RTV and lamivudine. The patient was a Japanese male aged 37 years with a body weight of 72.6 kg. His $CD4^+$ T cell count was 302/μl with a viral load of 7200 copies/ml. ETV and other antiretroviral agents were administered for 7 d. The plasma concentration at trough was 0.30, 0.48 and 0.24 μg/ml for ETV, ATV and RTV, respectively.

Figure 2B shows a chromatogram of a plasma sample from an HIV-1-infected patient treated with raltegravir, ETV, DRV, RTV and lamivudine. The patient was a Brazilian male aged 49 years with a body weight of 83.0 kg. His $CD4^+$ T cell count was 157/μl with a viral load of 44 copies/ml. ETV

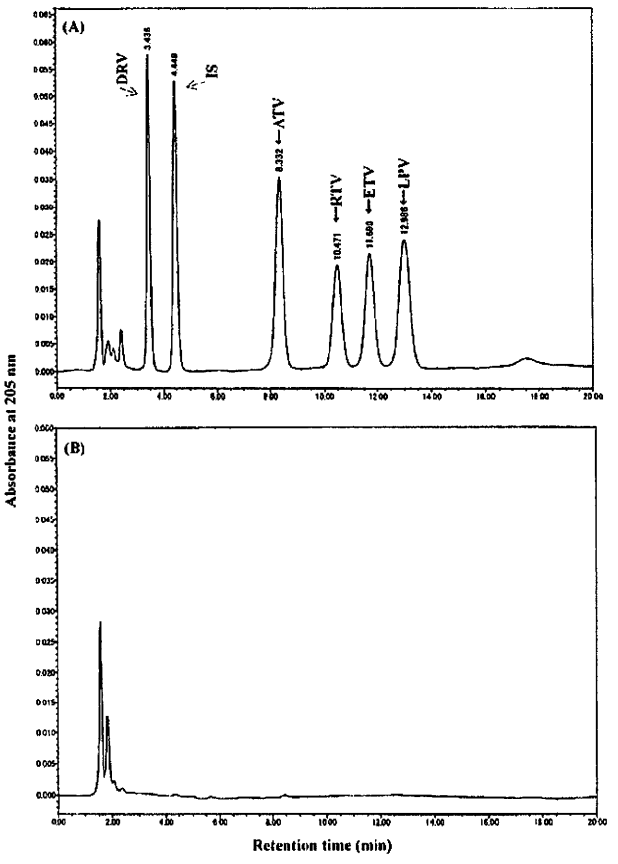

Fig. 1. Chromatograms Obtained after Extraction of (A) Spiked Plasma Sample Containing 3.77 μg/ml of DRV, 1.18 μg/ml of IS, 4.44 μg/ml of ATV, 3.76 μg/ml of RTV, 1.69 μg/ml of ETV and 4.21 μg/ml of LPV, and (B) Drug-Free Human Plasma Sample from a Healthy Volunteer

DRV, darunavir; IS, internal standard; ATV, atazanavir; RTV, ritonavir; ETV, etravirine; LPV, lopinavir.

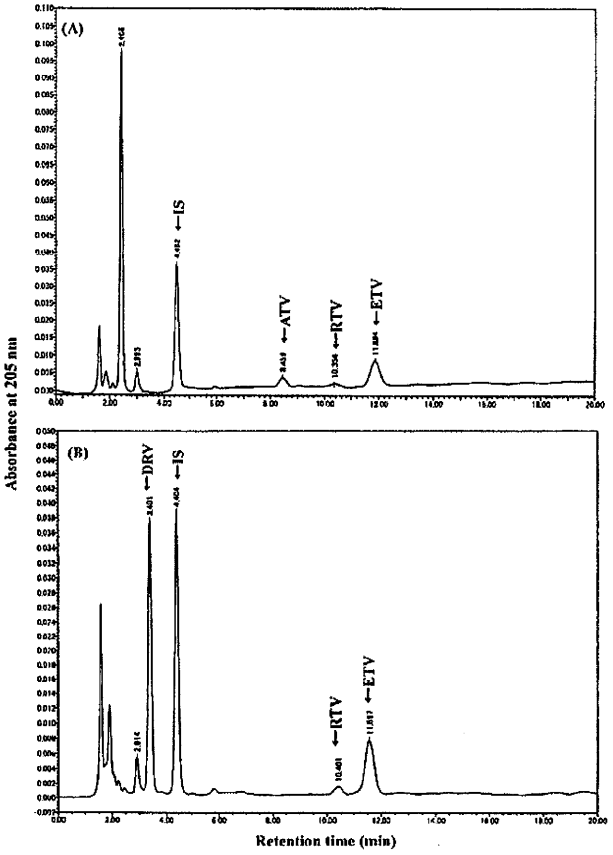

Fig. 2. Chromatograms Obtained after Extraction of a Plasma Sample from an HIV-1-Infected Patient (A) Treated with Raltegravir, ETV, ATV, RTV and Lamivudine, and (B) Treated with Raltegravir, DRV, ETV, RTV and Lamivudine

DRV, darunavir; IS, internal standard; ATV, atazanavir; RTV, ritonavir; ETV, etravirine.

and other antiretroviral agents were administered for 3 weeks. The plasma concentration at trough was 0.33, 3.30, and 0.29 μg/ml for ETV, DRV and RTV, respectively.

In chromatograms of Figs. 2A and B, the peaks of raltegravir and lamivudine were not detected.

Precision, Accuracy, Recovery, Linearity and Limit of Quantification The precision and accuracy for all tested drugs are shown in Table 1. The analyses show satisfactory precision with intra- and interassay coefficients of variation less than 11.6%. Accuracies ranged from 93.6 to 110.9%. The mean recovery of all drugs ranged from 88.0 to 97.5%. The regression coefficients of determination (R^2) values of the calibration curves for each drug were 0.99 or greater. The limit of quantification was 0.04, 0.04, 0.04, 0.05 and 0.07 μg/ml for ETV, DRV, ATV, RTV and LPV, respectively.

DISCUSSION

ETV has activity *in vitro* against viral strains with mutations that confer resistance to efavirenz and nevirapine.[11] The EC_{50} of ETV was < 100 nM (43.5 ng/ml) against clinically derived recombinant viruses resistant to at least one of the currently marketed NNRTIs. However, clinical investigations have yet to determine the therapeutic range of ETV concentrations that are associated with the desired therapeutic response. In addition, there is the potential problem for complex drug interactions due to the fact that ETV is a substrate and inducer of CYP3A4, as well as a substrate and inhibitor of 2C9 and 2C19.[1] In clinical treatment, ETV is co-administered with other antiretroviral agents including RTV-boosted PI. Therefore, too-low or too-high plasma concentrations of these drugs may decrease treatment efficacy or increase the risk of adverse effects. To solve these problems, a simple drug monitoring system for these agents is needed. Here we describe the application of HPLC with UV detection for simultaneously assaying ETV and 4 PIs. HPLC-UV equipment is frequently used in conventional hospital laboratories.

Table 1. Intra- and Interday Precision and Accuracy for ETV, ATV, RTV, LPV and DRV

	Expected (μg/ml)	Intraday (n = 5)		Interday (n = 15)		Accuracy (%)
		Measured (μg/ml)	CV (%)	Measured (μg/ml)	CV (%)	
ETV	0.08	0.09 ± 0.00	3.0	0.09 ± 0.00	4.9	110.9 ± 2.8
	0.14	0.14 ± 0.01	3.9	0.14 ± 0.01	3.9	99.4 ± 3.9
	0.42	0.42 ± 0.01	1.2	0.42 ± 0.01	3.0	99.8 ± 3.0
	1.04	1.03 ± 0.01	0.5	1.03 ± 0.01	1.4	98.9 ± 1.4
	4.17	4.17 ± 0.03	0.8	4.14 ± 0.05	1.3	99.2 ± 1.2
ATV	0.09	0.09 ± 0.00	2.2	0.09 ± 0.01	6.5	103.1 ± 6.7
	0.15	0.16 ± 0.00	2.6	0.16 ± 0.01	3.3	103.6 ± 3.4
	0.44	0.45 ± 0.01	1.4	0.44 ± 0.01	2.4	100.8 ± 2.5
	1.10	1.10 ± 0.00	0.4	1.12 ± 0.02	2.1	101.4 ± 2.1
	4.38	4.37 ± 0.01	0.3	4.38 ± 0.04	0.9	99.9 ± 0.9
RTV	0.07	0.07 ± 0.01	8.1	0.07 ± 0.01	8.0	93.6 ± 7.5
	0.12	0.12 ± 0.00	3.3	0.12 ± 0.01	4.4	100.3 ± 4.4
	0.37	0.37 ± 0.01	2.3	0.37 ± 0.01	3.9	99.6 ± 3.9
	0.93	0.93 ± 0.01	1.4	0.94 ± 0.02	2.2	101.1 ± 2.2
	3.71	3.72 ± 0.07	1.9	3.69 ± 0.06	1.7	99.6 ± 1.6
LPV	0.08	0.08 ± 0.01	11.6	0.09 ± 0.01	9.3	107.3 ± 10.0
	0.14	0.14 ± 0.00	2.7	0.14 ± 0.01	3.7	99.2 ± 3.7
	0.42	0.42 ± 0.01	2.7	0.42 ± 0.01	3.4	101.1 ± 3.9
	1.04	1.04 ± 0.01	0.8	1.05 ± 0.02	2.1	100.5 ± 2.1
	4.15	4.14 ± 0.02	0.4	4.16 ± 0.07	1.6	100.1 ± 1.6
DRV	0.07	0.07 ± 0.01	7.6	0.08 ± 0.01	9.9	108.4 ± 10.7
	0.12	0.12 ± 0.01	4.7	0.12 ± 0.00	3.9	99.7 ± 3.9
	0.37	0.37 ± 0.01	1.9	0.37 ± 0.01	2.8	100.8 ± 2.8
	0.93	0.93 ± 0.01	0.9	0.93 ± 0.02	1.9	100.5 ± 1.9
	3.72	3.70 ± 0.04	1.0	3.72 ± 0.03	0.9	100.1 ± 0.9

ETV, etravirine; ATV, atazanavir; RTV, ritonavir; LPV, lopinavir; DRV, darunavir; CV, coefficient of variation.

In this study, the calibration curve of each drug was linear with the average accuracy ranging from 93.6 to 110.9%. Both intra- and interday coefficients of variation for each drug were less than 11.6%. These results demonstrate that our HPLC-UV method has advantages in both reproducibility and accuracy in measuring plasma concentration of ETV and 4 PIs in a single run.

In our clinical cases, the ETV plasma concentrations, measured at trough, were 0.30 or 0.33 μg/ml for the HIV-1-infected patients. These values were similar to the previously reported findings in DUET studies.[1,2] In each case, the trough concentration of DRV or ATV was more than the suggested minimum target trough value in the guideline.[1] The viral load has been decreasing in these patients and treatment success is expected in the future. We, thus, proposed maintaining the current daily dose of these drugs. Conversely, the peaks of co-administered raltegravir and lamivudine were not

detected because these drugs were not extracted from plasma by our liquid liquid extraction technique. As these drugs are not metabolized by cytochrome P450, there are no drug interactions with ETV.

In conclusion, we have successfully constructed a protocol for the simultaneous quantification of ETV and 4 PIs by HPLC-UV. We believe our method enables accurate monitoring of ETV and co-administered PIs and may guide optimized administration of these drugs and prevent potential drug interactions and toxicity in treatment.

Acknowledgments This study was supported in part by a Grant-in-Aid for Clinical Research from the National Hospital Organization to MT. Janssen Pharmaceutical K.K. also provided funding for this study.

REFERENCES AND NOTES

1) Madruga J. V., Cahn P., Grinsztejn B., Haubrich R., Lalezari J., Mills A., Pialoux G., Wilkin T., Peeters M., Vingerhoets J., de Smedt G., Leopold L., Trefiglio R., Woodfall B., *Lancet*, **370**, 29 38 (2007).
2) Lazzarin A., Campbell T., Clotet B., Johnson M., Katlama C., Moll A., Towner W., Trottier B., Peeters M., Vingerhoets J., de Smedt G., Baeten B., Beets G., Sinha R., Woodfall B., *Lancet*, **370**, 39 48 (2007).
3) Katlama C., Haubrich R., Lalezari J., Lazzarin A., Madruga J. V., Molina J. M., Schechter M., Peeters M., Picchio G., Vingerhoets J., Woodfall B., De Smedt G., *AIDS*, **23**, 2289 2300 (2009).
4) The Panel on Clinical Practices for Treatment of HIV Infection Convened by the Department of Health and Human Services (DHHS). Published at http://AIDSinfo.nih.gov, 1 December 2009.
5) Fayet A., Beguin A., Zanolari B., Cruchon S., Guignard N., Telenti A., Cavassini M., Gunthard H. F., Buclin T., Biollaz J., Rochat B., Decosterd L. A., *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, **877**, 1057 1069 (2009).
6) Quaranta S., Woloch C., Paccou A., Giocanti M., Solas C., Lacarelle B., *Ther. Drug Monit.*, **31**, 695 702 (2009).
7) Rezk N. L., White N. R., Jennings S. H., Kashuba A. D., *Talanta*, **79**, 1372 1378 (2009).
8) D'Avolio A., Baietto L., Siccardi M., Sciandra M., Simiele M., Oddone V., Bonora S., Di Perri G., *Ther. Drug Monit.*, **30**, 662 669 (2008).
9) Takahashi M., Yoshida M., Oki T., Okumura N., Suzuki T., Kaneda T., *Biol. Pharm. Bull.*, **28**, 1286 1290 (2005).
10) Product Information, INTELENCE (etravirine tablet). Tibotec Therapeutics, Bridgewater, NJ, U.S.A.: http://www.intelence-info.com/intelence/
11) Andries K., Azijn H., Thielemans T., Ludovici D., Kukla M., Heeres J., Janssen P., De Corte B., Vingerhoets J., Pauwels R., de Bethune M. P., *Antimicrob. Agents Chemother.*, **48**, 4680 4686 (2004).

HIV-2 CRF01_AB: First Circulating Recombinant Form of HIV-2

Shiro Ibe, PhD, Yoshiyuki Yokomaku, MD, PhD,* Teiichiro Shiino, PhD,† Rie Tanaka, PhD,‡ Junko Hattori, PhD,* Seiichiro Fujisaki, PhD,* Yasumasa Iwatani, PhD,*§ Naoto Mamiya, MD, PhD,* Makoto Utsumi, MD, PhD,* Shingo Kato, PhD,‡ Motohiro Hamaguchi, MD,* and Wataru Sugiura, MD, PhD*†§*

Background: Five HIV-2–seropositive cases were recently identified in Japan, outside the HIV-2 endemic area of West Africa. To clarify the molecular epidemiology of HIV-2 in Japan, we analyzed sequences of these cases in detail.

Methods: HIV-2 genetic groups were determined by *gag* and *env* sequences. For suspected recombinant isolates, the genetic structure was determined by full-length genomic analyses. To understand the history and evolution of HIV-2 recombinant isolates, we estimated the time of most recent common ancestor by Bayesian Markov chain Monte Carlo method.

Results: Three isolates were determined as recombinants of groups A and B, and their mosaic genome structures were identical with that of 7312A, a recombinant isolate reported in 1990 from Côte d'Ivoire. Our 3 isolates and 7312A fulfilled the criteria for determining a circulating recombinant form (CRF). These isolates were verified by the Los Alamos HIV sequence database as the first CRF of HIV-2, HIV-2 CRF01_AB. The mean time of most recent common ancestor of CRF01_AB was estimated as between 1964 and 1973, several decades after the estimated emergence of HIV-2.

Conclusions: We recently identified HIV-2 CRF01_AB cases in Japan. This ectopic observation of the virus outside its original endemic area suggests an ongoing global spread of HIV-2 CRF01_AB.

Key Words: circulating recombinant form, CRF01_AB, HIV-2, molecular epidemiology

(*J Acquir Immune Defic Syndr* 2010;54:241–247)

INTRODUCTION

One million people worldwide are infected with HIV-2. The distribution of HIV-2, unlike the global epidemic of HIV-1, is still mainly restricted to West Africa and several European and Asian countries.[1–4] HIV-2 has been characterized as less pathogenic than HIV-1,[5–11] with more than 75% of HIV-2–infected cases remaining asymptomatic throughout their clinical course.[4] HIV-2 can be genetically classified into 8 groups, A to H, which have equivalent genetic distances to those of HIV-1 groups but not subtypes, with groups A and B circulating in the human population.[12–16] In addition, 2 different AB recombinants (7312A and 510-03) have been identified in West Africa,[12,13,17–19] but their circulation has not been identified to date.

In Japan, only 2 HIV-2–infected cases have been reported, but both were infected abroad.[20,21] Inside the country, there has been no evidence of HIV-2 transmission and circulation. Here we report 5 HIV-2–infected cases recently identified in Japan. Of these 5 cases, 3 were shown by full-length genomic analysis to be infected with the same type of recombinant virus determined to be the first circulating recombinant form (CRF) of HIV-2.

METHODS

HIV-2 Samples and Quantification of HIV Plasma Viral Loads

Among 843 HIV/AIDS cases registered at the Nagoya Medical Center (NMC), Japan from 1994 to 2008 (for demographic characteristics, see **Table, Supplemental Digital Content 1**, http://links.lww.com/QAI/A49), 5 cases (3 males and 2 females) were diagnosed serologically as HIV-2 infected. To better understand the molecular epidemiology of HIV-2 infection in Japan, we analyzed the HIV-2 genetic groups of the 5 cases.

Plasma HIV-1 viral loads were measured by the Cobas Amplicor HIV-1 monitor test v1.5 (Roche Diagnostics, Tokyo, Japan) or the Cobas TaqMan HIV-1 test (Roche Diagnostics),

Received for publication November 21, 2009; accepted March 4, 2010.

From the *Department of Infection and Immunology, Clinical Research Center, National Hospital Organization Nagoya Medical Center, Nagoya, Japan; †AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan; ‡Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo, Japan; and §Department of AIDS Research, Nagoya University Graduate School of Medicine, Nagoya, Japan.

Supported by a Grant-in-Aid for AIDS research from the Ministry of Health, Labor, and Welfare of Japan (H19-AIDS-007).

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions this article on the journal's Web site (www.jaids.com).

Correspondence to: Wataru Sugiura, MD, PhD, Clinical Research Center, National Hospital Organization Nagoya Medical Center, 4-1-1 Sannomaru, Naka-ku, Nagoya, Aichi 460-0001, Japan (e-mail: wsugiura@nih.go.jp).

whereas plasma HIV-2 viral loads were measured by an in-house quantification assay, the Poisson quantification method described elsewhere.[22,23] In brief, total RNA was extracted from 500 μL of plasma sample using the QIAamp UltraSens Virus Kit (QIAGEN, Tokyo, Japan). Reverse transcription (RT) and nested polymerase chain reaction (PCR) (RT-nested PCR) were performed using serially diluted RNA samples, and HIV-2 viral loads were statistically calculated using results from samples diluted to near the endpoint. (For details of RT-nested PCR reaction mixtures and thermal programs, see **Table, Supplemental Digital Content 2**, http://links.lww.com/QAI/A50).

Genomic DNA Sequencing

HIV-2 proviral DNAs were purified from peripheral blood mononuclear cells using the DNA blood mini kit (QIAGEN). To determine HIV-2 genetic groups, *gag* (777 bps: 1163 to 1939 according to SIVmac239) and *env* (454 bps: 7300 to 7753) gene fragments were amplified by nested PCR using LA Taq polymerase (Takara Bio, Shiga, Japan) and previously reported[13,24] primers: gagA, gagB, gagC, and gagF for *gag*, and PFD1, LTR9574, EB2, and EB5 for *env*. To determine full-length genomic sequences, 4 DNA fragments containing (1) 5′ long terminal repeat (LTR) (915 bps: 31 to 945), (2) *gag* to *nef* genes (9122 bps: 899 to 10020), (3) 3′ LTR (791 bps: 9463 to 10252), and (4) the joining point of the circular 2 LTR form (597 bps: 10085 to 10279 and 1 to 402) were amplified by nested PCR using 8 primer pairs (see **Table, Supplemental Digital Content 3**, http://links.lww.com/QAI/A51). The following PCR program was used: denaturation (2 minutes at 94°C) followed by 40 cycles of PCR (94°C: 15 seconds, 60°C: 30 seconds, and 70°C: 1 minute/1000 bps). Sequencing was performed using a 3730 DNA Analyzer (Applied Biosystems, Tokyo, Japan).

Phylogenetic Tree Analysis and Determination of Recombinant Genome Structures

Multiple sequence alignment was performed using CLUSTAL W, and genetic distances were calculated based on the maximum composite likelihood model using MEGA software v4.[25] Phylogenetic trees were constructed using the neighbor-joining method.

Complete full-length genomic sequences of 4 HIV-2 group A strains (ALI, BEN, CAM2CG, and UC2), 3 HIV-2 group B strains (D205, EHO, and UC1), and SIVmac239, (a rhesus macaque-adapted simian immunodeficiency viral isolate) were used as reference sequences. After realigning the sequence set, recombinant breakpoints were determined by similarity plotting, bootscanning, and informative site analysis using SimPlot software, v3.5.1.[26]

Estimated Times of the Most Recent Common Ancestors

Evolutionary rates, chronological phylogenies, and other evolutionary parameters were estimated from 17 full-length or near full-length HIV-2/SIV genomic sequences (see **Table, Supplemental Digital Content 4**, http://links.lww.com/QAI/A52) using the Bayesian Markov chain Monte Carlo (MCMC) method implemented in BEAST v1.4.8.[27] The alignment data for the full-genome sequences were processed into 2 subsets consisting of sequences corresponding to the group A or B region of HIV-2 AB-recombinant virus. Bayesian MCMC analyses were performed using a relaxed molecular clock model.[28] The nucleotide substitution model was evaluated by the hierarchical likelihood ratio test using PAUP v4.0 beta[29] with MrModeltest (Nylander JAA. 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University), and the general time-reversible model[30] was adopted with both invariant sites and gamma-distributed site heterogeneity for 4 rate categories. The coalescent model used in the analyses was a logistically growing population because the population size of HIV-2 seemed constant in the early phase followed by exponential growth in the recent period.[31] Each Bayesian MCMC analysis was run for 40 million states and sampled every 10,000 states. Posterior probabilities were calculated with a burn-in of 4 million states and checked for convergence using Tracer v1.4. The posterior distribution of the substitution rate obtained from the heterochronous sequences was subsequently incorporated as a prior distribution for the evolutionary rate of HIV-2 genome regions A and B, thereby adding a timescale to the phylogenetic histories of the HIV-2 strains and enabling the times of most recent common ancestor (tMRCAs) to be estimated.[32]

Accession Numbers

Nucleotide sequences have been registered as #AB499685 to AB499695 in the DNA databank of Japan.

TABLE 1. Demographic and Clinical Characteristics of Patients Diagnosed as HIV-2 Infected

Patient #	Year	Sex	Age (Yrs)	Nationality	Risk Factor for Infection	$CD4^+$ Cell Count (Cells/μL)	HIV-1 Viral Load (Copies/mL)*	HIV-2 Viral Load (Copies/mL)	Western Blot†		Opportunistic Infections
									HIV-1	HIV-2	
NMC307	2004	M	28	Nigerian	Hetero	241	<50	350,000	I	P	Tuberculosis
NMC678	2007	F	28	Japanese	Hetero	883	<50	ND	I	P	—
NMC716	2007	M	36	Nigerian	Hetero	4	<50	680,000	I	P	Candidiasis
NMC786	2008	M	38	Ghanaian	Hetero	1	<40	60,000	N	I	Candidiasis, CMV infection
NMC842	2008	F	34	Japanese	Hetero	110	<40	25,000	N	P	—

*Detection limits of Cobas Amplicor HIV-1 monitor v1.5 and Cobas TaqMan HIV-1 tests were 50 and 40 copies/ml, respectively.
†New LAV Blot I and II kits (Bio-Rad Laboratories, Tokyo, Japan) were used.
CMV, cytomegalovirus; F, female; Hetero, heterosexual contact; I, intermediate; M, male; ND, not detected; N, negative; P, positive.

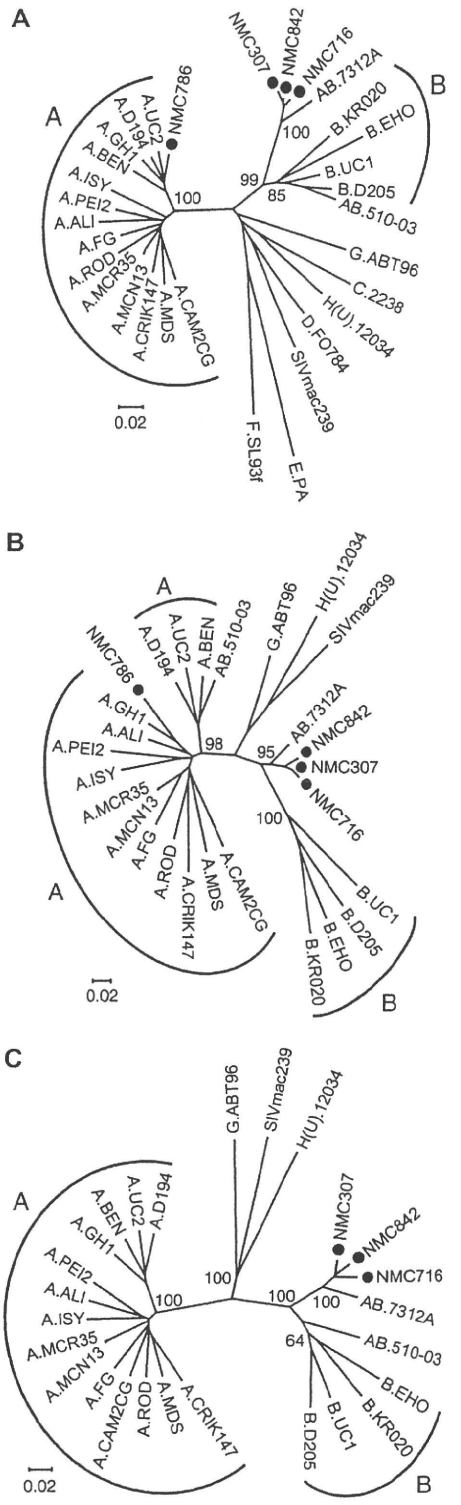

FIGURE 1. Phylogenetic tree analyses of HIV-2 isolates identified in this study. Phylogenetic tree analyses are shown using the following: A, HIV-2 *gag* gene sequences (bps: 1163 to 1939 in the reference SIVmac239 sequence); B, *env* gene sequences (bps: 7300 to 7753); and C, full-length or near full-length genomic sequences. Phylogenetic trees were constructed by the neighbor-joining method. Bootstrap values were calculated by 1000 analyses and are shown at the major tree nodes. Scale bar represents 0.02 nucleotide substitutions per site. Each reference HIV-2 strain is represented by its genetic group and name. HIV-2 isolates identified in this study (NMC307, NMC716, NMC786, and NMC842) are shown by filled circles.

RESULTS

HIV-2 Infection Confirmed by Nucleotide Amplification in Four AIDS Cases

Profiles of 5 HIV-2–seropositive cases are summarized in Table 1. The 3 males were from West African countries, a major endemic area for HIV-2, and suspected as seropositive before arriving in Japan. However, 2 females, both Japanese, were suspected to be recently infected within Japan based on their interviews. All their risk factors were heterosexual contacts, and no personal connection was confirmed among any of these cases. Thus, these 5 cases were independently infected with HIV-2 on different occasions. Notably, 4 cases (NMC307, NMC716, NMC786, and NMC842) were found at advanced stage AIDS with low $CD4^+$ cell counts and high HIV-2 viral loads, accompanied by opportunistic infections (Table 1). One case (NMC678) was found at an asymptomatic stage with high $CD4^+$ cell count and undetectable viremia. HIV-1 RNAs were undetectable in all 5 cases, indicating that they were infected by HIV-2 alone.

The First Circulating Recombinant Form Discovered in HIV-2: HIV-2 CRF01_AB

HIV-2 genetic groups were determined by both *gag* and *env* sequences. We were successful in analyzing 4 AIDS cases, however, we failed to amplify these 2 genes and analyze in asymptomatic case NMC678. One isolate (NMC786) was clearly classified into group A in phylogenetic tree analysis (Fig. 1A, B). On the other hand, isolates NMC307, NMC716, and NMC842 formed an independent cluster with a reference AB recombinant isolate 7312A (Fig. 1A, B). To better understand the detailed genomic structures of the 3 suspected AB recombinants, full-length genomic sequences of the 3 cases were analyzed. In the phylogenetic tree with full-length or near full-length reference sequences (Fig. 1C), NMC307, NMC716, NMC842, and 7312A formed an independent cluster with a high bootstrap value of 100%, suggesting these 4 isolates are the same type of AB-recombinant virus.

We next compared their genomic structures. As shown in Fig. 2A, similarity plotting and bootscanning analyses revealed that the recombinant breakpoints of our 3 isolates perfectly matched those of 7312A. This finding was supported by subregion phylogenetic analyses (Fig. 2B). In conclusion, NMC307, NMC716, and NMC842 are AB-recombinant forms with a mosaic genome structure identical to that of 7312A, demonstrating that they are the same type of HIV-2 AB-recombinant form.

The minimum requirement for declaring a new CRF, as proposed by the Los Alamos HIV sequence database in 1999, is at least 3 cases with no direct linkage, accompanied with near full-length sequences.[33,34] These CRF nomenclature

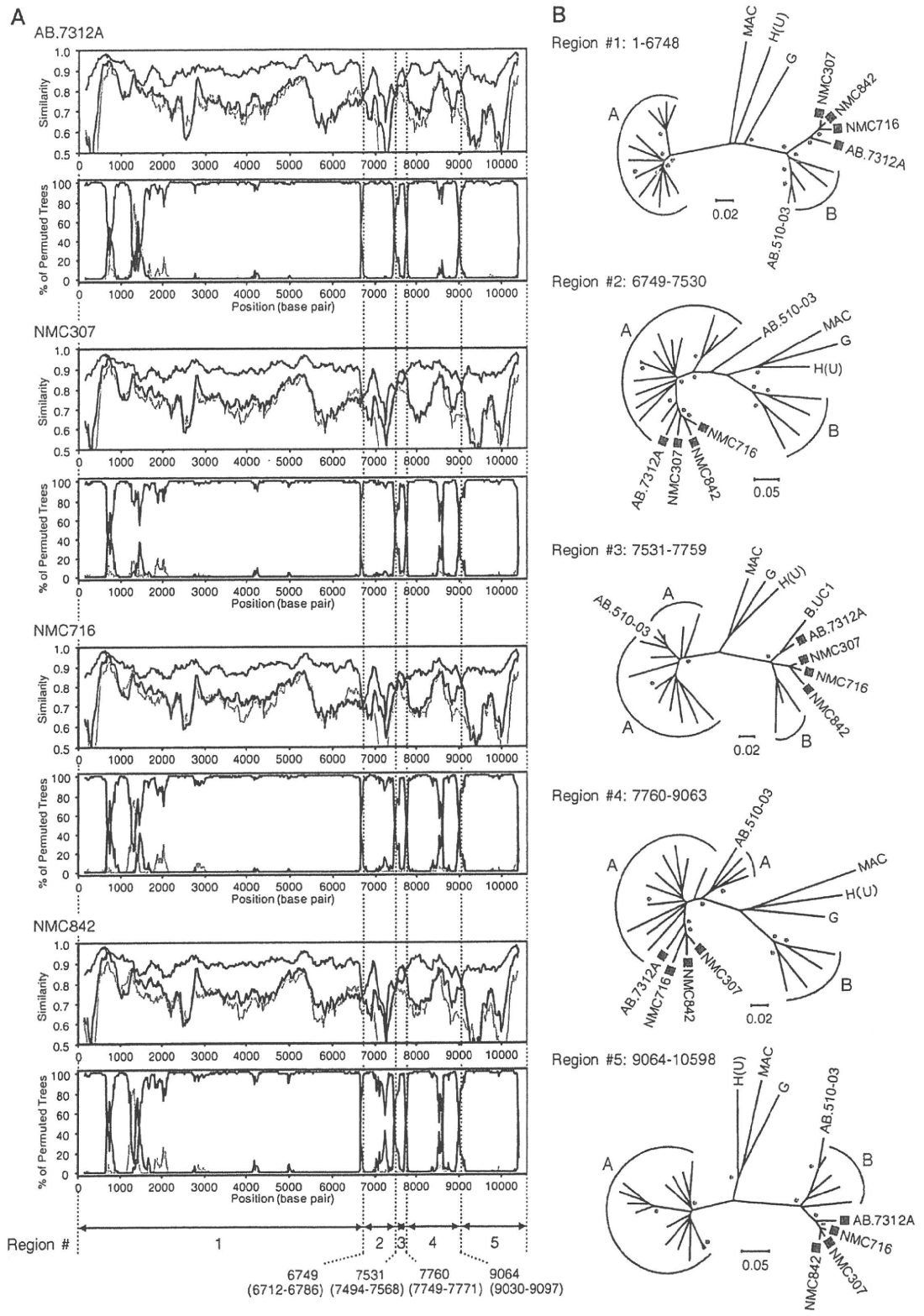

FIGURE 2. Determination of mosaic genome structures of HIV-2 AB recombinants. A, Similarity plotting (top) and bootscanning (bottom) data for each case of AB.7312A, NMC307, NMC716, and NMC842. Plots for consensus group A, consensus group B, and SIVmac239 are shown in red, blue, and gray, respectively. Both similarity plotting and bootscanning were performed with window and step sizes of 300 and 20 nucleotides, respectively. Bootscanning was performed using the neighbor-joining algorithm with 500 replicates. Each position of the 4 recombinant breakpoints is represented in the aligned sequence data set as the midpoint and

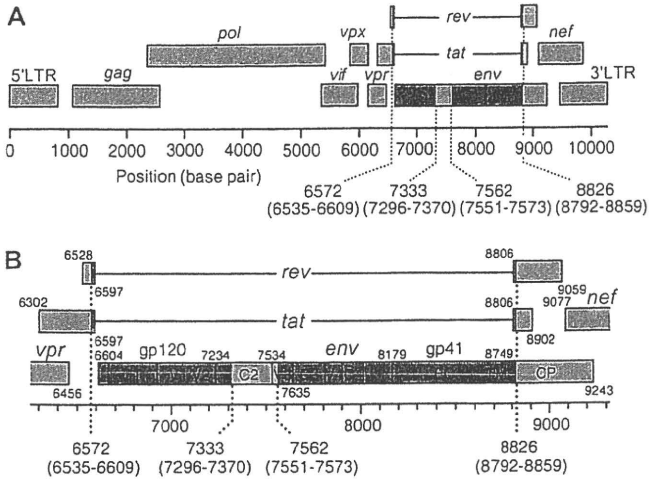

FIGURE 3. Schematic drawings for the genomic structure of HIV-2 CRF01_AB. A, Whole genomic structure; and B, Details around the *env* gene are represented. Regions belonging to group A and B are shown in red and blue, respectively. Numbering positions were adjusted to the reference SIVmac239 sequence.[35,36] Each position of 4 recombinant breakpoints is represented as the midpoint and range. C, constant region; CP, cytoplasmic domain; EC, extracellular domain; gp, glycoprotein; TM, transmembrane domain; V, variable region.

requirements are perfectly fulfilled with full-length genomic sequence information for 4 cases independently infected on different occasions with the AB recombinant identified by us and others.[12,13,19] Our data were carefully reviewed by editors of the Los Alamos HIV sequence database and confirmed as the first CRF discovered in HIV-2. They decided that the least confusing and most consistent way to name this new strain was to call it HIV-2 CRF01_AB.

The genomic structure of CRF01_AB is shown in Fig. 3. Interestingly, all 4 recombinant breakpoints of the CRF were located near or within the *env* gene (Fig. 3A). Further detailed analysis revealed that CRF01_AB possessed a chimeric gp120 containing a backbone of group A and a partial C2V3 fragment of group B and a chimeric gp41 containing extracellular and transmembrane domains of group A and a cytoplasmic domain of group B (Fig. 3B).

CRF01_AB Emerged Approximately in the Mid 20th Century

To estimate the time of CRF01_AB emergence, the time of the most recent common ancestor (tMRCA) of the recombinant was calculated by the Bayesian MCMC method. The mean substitution rates per year for the group A and B regions were estimated as 2.22×10^{-3} and 1.64×10^{-3}, respectively (Table 2), and the mean tMRCAs for groups A and B were estimated from 1921 to 1929, and from 1909 to 1948, respectively (Table 3). Similar results[31,37] validate our estimations. Finally, the mean tMRCA of CRF01_AB was estimated from 1964 to 1973. As the emergent times for groups A and B were estimated in the early 20th century, several decades seem to have been required for CRF01_AB to emerge. Concerning the geographical origin of the recombinant form, 3 of 4 isolates (7312A, NMC307, and NMC716) were identified in West Africans from Côte d'Ivoire and Nigeria. As these 2 countries were reported as sites of an epidemic in HIV-2 group A and B strains,[38,39] the most likely geographical origin of CRF01_AB is the south coastal area of West Africa.

DISCUSSION

In this study, we identified 3 HIV-2 AB recombinants with the same recombination pattern as 7312A, an isolate reported in Côte d'Ivoire in 1990.[12,13,19] These 4 isolates are determined as the first CRF of HIV-2, named CRF01_AB. It is noteworthy that all 3 of our cases infected with CRF01_AB were found at the AIDS stage. Considering that more than 75% of HIV-2–infected cases have a prognosis of remaining asymptomatic throughout their lifetimes[4] and that few HIV-2–seropositive cases were reported in Japan in the last 2 decades, 3 HIV-2 cases in the AIDS stage infected with the same CRF and identified in the past 5 years is highly unusual. Regarding the incubation periods for AIDS development in the 3 cases, not much information was available except for NMC842. This case was found to be seronegative for HIV-1/2 when tested in 2000. Thus, this case seems to have developed AIDS at most within 8 years, same as the median incubation period for AIDS development in HIV-1 infections (7.7–12.3 years).[40–45] As for the other 2 cases (NMC307 and NMC716), they developed AIDS at 28 and 36 years old (Table 1), which is significantly younger than age 65, reported as the peak of death by HIV-2 infections.[46,47] Though the number of cases identified is still small, we are concerned that the CRF01_AB might have acquired higher pathogenicity through recombination and adaptation to humans. As shown in Figure 3B, CRF01_AB has a recombination in the C2V3 region, the site of the major determinant for anti-envelope host immune responses and a functional domain for the chemokine receptor–binding site. The chimeric structure in the C2V3 region may confer advantages in host immune escape and viral replication capacity.

According to tMRCA analysis of the 4 isolates, CRF01_AB is estimated to have emerged sometime between 1964 and 1973. Interestingly, the mean tMRCA of the 3 isolates collected at NMC was estimated from 1982 to 1995 (Table 3), a later estimate than that of the 4 isolates, suggesting ongoing selection and evolution of CRF01_AB through transmission which has been taking place from the era of the 7312A isolate to the NMC isolates.

In conclusion, we report here the first CRF of HIV-2, CRF01_AB. Although national borders worldwide have

range (bottom). B, Subregion phylogenetic tree analyses. Phylogenetic trees were individually constructed by the neighbor-joining method using 5 subregion sequences. The HIV-2 isolates identified in this study (NMC307, NMC716, and NMC842) and AB.7312A are shown by green filled squares. Bootstrap values were calculated from 1000 analyses, and values greater than 95% are shown as orange dots at tree nodes. Scale bar represents 0.02 or 0.05 nucleotide substitutions per site. MAC, SIVmac239.

TABLE 2. Parameters in Bayesian MCMC Analysis for HIV-2/SIV Phylogenetic Inferences

	Substitution Rate Per Year		Coefficient of Variation		Population Size	
Data Set	Mean	95% HPD	Mean	95% HPD	Mean	95% HPD
Group A Region	2.22×10^{-3}	6.86×10^{-4}–3.68×10^{-3}	0.173	0.076–0.293	405.2	98.3–830.2
Group B Region	1.64×10^{-3}	5.99×10^{-4}–2.87×10^{-3}	0.269	0.170–0.395	341.2	93.3–668.9
Combined*	1.87×10^{-3}	6.39×10^{-4}–3.32×10^{-3}	0.235	0.088–0.382	357.9	93.3–709.2

*Combined data were produced from the 2 subsets, "group A region" and "group B region," using a LogCombiner program.
HPD, highest posterior density.

TABLE 3. Estimated TMRCAs of Monophyletic Clades in the HIV-2/SIV Lineage

	Group A region		Group B region		Combined	
Data set	Mean	95% HPD	Mean	95% HPD	Mean	95% HPD
Clade						
NMC isolates*	1982	1960–1996	1995	1987–2002	1990	1974–2002
CRF01_AB†	1964	1933–1985	1973	1956–1986	1971	1949–1986
Group A	1921	1864–1963	1929	1882–1964	1927	1879–1964
Group B	1909	1837–1962	1948	1915–1973	1934	1879–1973
HIV-2/SIV	1818	1670–1923	1821	1697–1930	1822	1693–1926

*This clade consisted of our 3 CRF01_AB isolates: NMC307, NMC716, and NMC842.
†This clade consisted of all 4 CRF01_AB isolates: 7312A, NMC307, NMC716, and NMC842.
HPD, highest posterior density;
SIV, simian immunodeficiency virus.

become more porous than ever, it is still surprising that the same recombinant strain was harvested in Japan, an island nation remote from the original endemic area, West Africa. This ectopic observation of the virus outside its endemic area suggests an ongoing global spread of HIV-2 CRF01_AB.

ACKNOWLEDGMENTS

We thank Dr. Thomas Leitner and editors of the Los Alamos HIV sequence database for discussing our data and naming the new HIV-2 circulating recombinant form. We thank Dr. Koya Ariyoshi for critical reading of our article and Claire Baldwin for her help in preparing the article.

REFERENCES

1. Marlink R. Lessons from the second AIDS virus, HIV-2. *AIDS*. 1996;10: 689–699.
2. Schim van der Loeff MF, Aaby P. Towards a better understanding of the epidemiology of HIV-2. *AIDS*. 1999;13:S69–S84.
3. Bock PJ, Markovitz DM. Infection with HIV-2. *AIDS*. 2001;15:S35–S45.
4. de Silva TI, Cotten M, Rowland-Jones SL. HIV-2: the forgotten AIDS virus. *Trends Microbiol*. 2008;16:588–595.
5. Whittle H, Morris J, Todd J, et al. HIV-2-infected patients survive longer than HIV-1-infected patients. *AIDS*. 1994;8:1617–1620.
6. Marlink R, Kanki P, Thior I, et al. Reduced rate of disease development after HIV-2 infection as compared to HIV-1. *Science*. 1994;265: 1587–1590.
7. Kanki PJ, Travers KU, MBoup S, et al. Slower heterosexual spread of HIV-2 than HIV-1. *Lancet*. 1994;343:943–946.
8. Adjorlolo-Johnson G, De Cock KM, Ekpini E, et al. Prospective comparison of mother-to-child transmission of HIV-1 and HIV-2 in Abidjan, Ivory Coast. *JAMA*. 1994;272:462–466.
9. Ota MO, O'Donovan D, Alabi AS, et al. Maternal HIV-1 and HIV-2 infection and child survival in The Gambia. *AIDS*. 2000;14:435–439.
10. O'Donovan D, Ariyoshi K, Milligan P, et al. Maternal plasma viral RNA levels determine marked differences in mother-to-child transmission rates of HIV-1 and HIV-2 in The Gambia. MRC/Gambia Government/ University College London Medical School working group on mother-child transmission of HIV. *AIDS*. 2000;14:441–448.
11. Schim van der Loeff MF, Jaffar S, Aveika AA, et al. Mortality of HIV-1, HIV-2 and HIV-1/HIV-2 dually infected patients in a clinic-based cohort in The Gambia. *AIDS*. 2002;16:1775–1783.
12. Gao F, Yue L, White AT, et al. Human infection by genetically diverse SIV_{SM}-related HIV-2 in West Africa. *Nature*. 1992;358:495–499.
13. Gao F, Yue L, Robertson DL, et al. Genetic diversity of human immunodeficiency virus type 2: evidence for distinct sequence subtypes with differences in virus biology. *J Virol*. 1994;68:7433–7447.
14. Chen Z, Luckay A, Sodora DL, et al. Human immunodeficiency virus type 2 (HIV-2) seroprevalence and characterization of a distinct HIV-2 genetic subtype from the natural range of simian immunodeficiency virus-infected sooty mangabeys. *J Virol*. 1997;71:3953–3960.
15. Yamaguchi J, Devare SG, Brennan CA. Identification of a new HIV-2 subtype based on phylogenetic analysis of full-length genomic sequence. *AIDS Res Hum Retroviruses*. 2000;16:925–930.
16. Damond F, Worobey M, Campa P, et al. Identification of a highly divergent HIV type 2 and proposal for a change in HIV type 2 classification. *AIDS Res Hum Retroviruses*. 2004;20:666–672.
17. Ndembi N, Abraha A, Pilch H, et al. Molecular characterization of human immunodeficiency virus type 1 (HIV-1) and HIV-2 in Yaoundé, Cameroon: evidence of major drug resistance mutations in newly diagnosed patients infected with subtypes other than subtype B. *J Clin Microbiol*. 2008;46:177–184.
18. Yamaguchi J, Vallari A, Ndembi N, et al. HIV type 2 intergroup recombinant identified in Cameroon. *AIDS Res Hum Retroviruses*. 2008; 24:86–91.
19. Robertson DL, Hahn BH, Sharp PM. Recombination in AIDS viruses. *J Mol Evol*. 1995;40:249–259.
20. Kusagawa S, Imamura Y, Yasuoka A, et al. Identification of HIV type 2 subtype B transmission in East Asia. *AIDS Res Hum Retroviruses*. 2003; 19:1045–1049.
21. Utsumi T, Nagakawa H, Uenishi R, et al. An HIV-2-infected Japanese man who was a long-term nonprogressor for 36 years. *AIDS*. 2007;21: 1834–1835.
22. Kato S, Hanabusa H, Kaneko S, et al. Complete removal of HIV-1 RNA and proviral DNA from semen by the swim-up method: assisted