with those in the under-ART group (Fig. 1H). On the other hand, IFN-g levels did not differ significantly among the three groups (Fig. 1C). Thus, except for IFN-g, all of the other cytokines were found to be increased in the immunocompromised patients.

Subsequently, the correlation between the serum cytokine levels and CD4 counts was analyzed using Spearman's rank test. Except for IFN-g, all of the other cytokines exhibited a significant negative correlation with the CD4 cell counts (Fig. 2A). Among the cytokines we examined, IL-7 levels showed the strongest negative correlation (Spearman's $r = -0.74$); a similarly negative correlation was noted in the regression analysis (Fig. 2B). Serum cytokine and plasma HIV-1 RNA levels were also examined. Since the plasma HIV-1 RNA levels were below the detection limit in all of the patients in the under-ART group, and this might have led to bias-based errors, the under-ART group was excluded from this assay. A significant but weak correlation was noted between TNF-a/IL-18 and plasma HIV-1 RNA levels (Fig. 2C). Thus except for IFN-g, the levels of the cytokines analyzed in this study increased with disease progression, and correlated with clinical indicators such as decreased CD4 cell counts and increased plasma HIV-1 RNA levels.

Finally, a longitudinal study was conducted on the pre-ART group to examine the effects of ART on serum cytokine levels. ART was introduced for all 19 patients in the pre-ART group. One patient in this group did not show an optimal virological response 24 wk after the introduction of ART, so this patient was excluded from the analysis. Fig. 3 shows the serum cytokine levels before the introduction of ART, and on

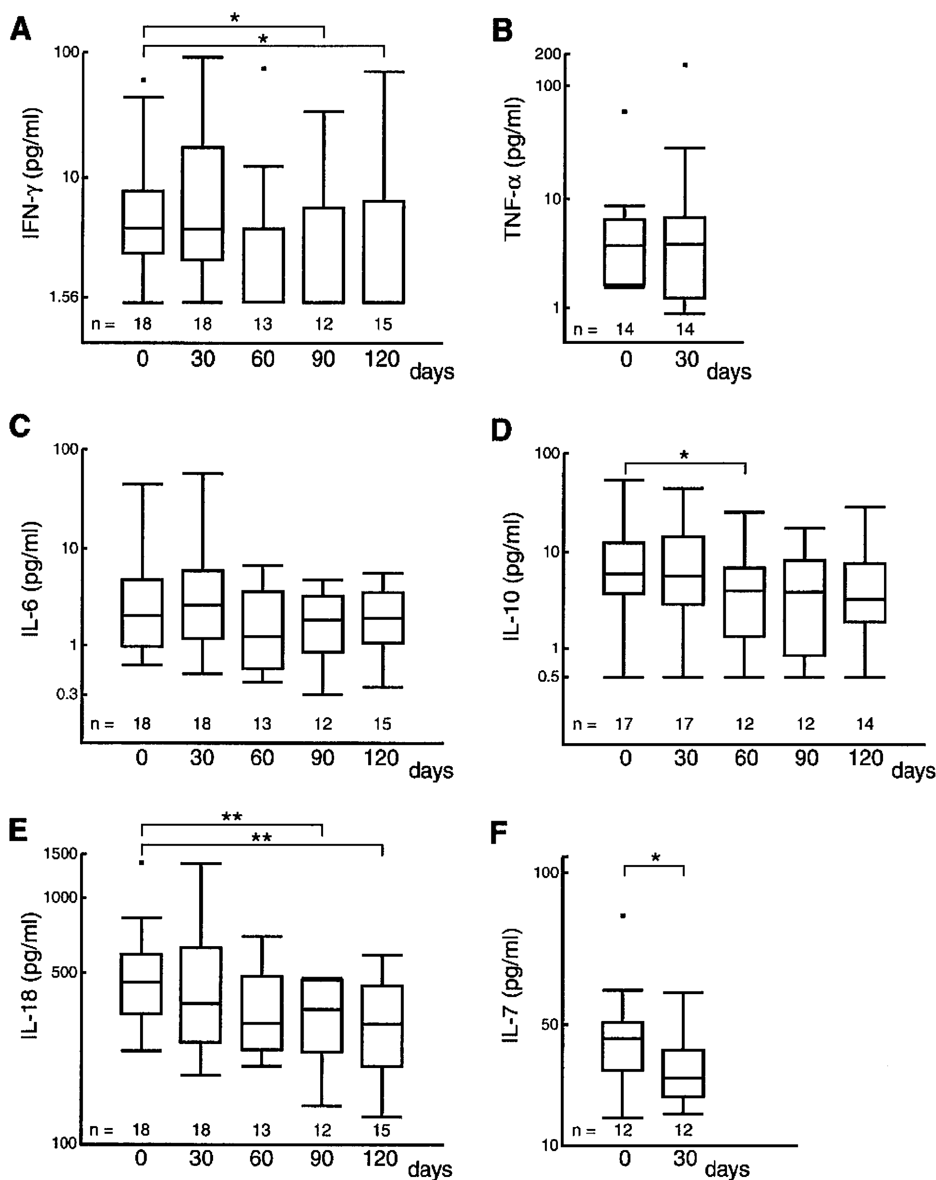

FIG. 3. Serum cytokine levels in HIV-1-infected patients before initiation of and during ART (**A–F**). The values shown of the indicated cytokines before, and at 30, 60, 90, and 120 d from the start of ART, are shown using box-and-whisker plots, representing the minimum, 25th percentile, median, 75th percentile, maximum, and outlying values. Statistical analyses versus baseline levels were carried out using Wilcoxon's matched-pair signed-rank test (*$p < 0.05$, **$p < 0.01$).

days 30, 60, 90, and 120 after its introduction. On day 30, only IL-7 levels showed significant decreases compared with baseline levels. Some cytokines (IL-10 and IL-18) showed no change, while others (IFN-g, IL-6, and TNF-a) showed upward trends. Four cytokines (IFN-g, IL-6, IL-10, and IL-18), when measured over time, revealed downward trends on day 60 and beyond; IL-10 showed a significant decrease on day 60, and IFN-g and IL-18 showed significant decreases on days 90 and 120. Thus, the IL-7 level rapidly declined after the initiation of ART, while the expression of the other cytokines decreased slightly later.

There was no correlation between the pre-ART and under-ART groups for IFN-g (Fig. 1C). However, our longitudinal observations demonstrated a significant decrease in IFN-g levels upon initiation of ART (Fig. 3A). To resolve this discrepancy, we examined the patients in the pre-ART group in more detail. In most of the patients, the IFN-g levels were gradually suppressed by ART. However, in 5 of the 15 patients, IFN-g levels did not decrease, even after the initiation of ART, and remained at 5 pg/mL or higher at day 120 after ART initiation, regardless of the ART-induced virological response. The data of two representative patients are shown in Fig. 4. In the patients shown in Fig. 4A, whose plasma HIV-1 RNA levels were maintained below the detection limit, the IFN-g levels were above 30 pg/mL, even at 3 y after the initiation of ART. In addition, 2 of 7 patients in the AC group, and 2 of 11 patients in the under-ART group, had higher IFN-g levels (>5 pg/mL). Thus, during the AC period or later, the IFN-g levels in some patients increased, and remained high even after the initiation of ART.

Discussion

Here we demonstrated changes in the serum cytokine levels in HIV-1-infected patients. The serum levels of many cytokines increased with disease progression, and were decreased by the initiation of ART. The abnormal cytokine expression patterns may be explained by two possible mechanisms: the direct effect of immune destruction by HIV-1, and the effects of opportunistic infection. In this study, 14 (74%) patients in the pre-ART group developed AIDS. However, since the samples were collected from all of the patients after the treatment of opportunistic infections, the effects of these opportunistic infections on abnormal cytokine expression patterns may be limited. On the other hand, it is important to note that there were changes in the cytokine levels after the initiation of ART. On day 30 after ART introduction in the pre-ART group, all cytokines except for IL-7 remained unchanged or increased. At this point, the HIV-1 RNA levels in the blood were decreased in all 18 patients, and the CD4 cell counts were increased in 16 (89%) patients. This indicates that the cytokine levels increased despite virological suppression and immune restoration. Immune reconstitution inflammatory syndrome (IRIS), a seemingly paradoxical pathological condition, has been extensively described (20). This is a condition in which the existing opportunistic infection is exacerbated, and/or a new opportunistic infection develops after the introduction of ART, presumably due to the restoration of immune responses against a pathogen that existed prior to ART. Of the 18 patients in the pre-ART group, only 2 developed clinically apparent IRIS. One patient experienced a relapse of an existing CMV infection, and the other patient newly developed an atypical mycobacterial infection, an AIDS-indicator disease, after the introduction of ART. In addition to these 2 patients, several other patients developed IRIS, but required no specific treatment, at 2–4 weeks after initiation of ART. In these patients, the increased cytokine levels observed on day 30 after ART introduction were associated with immune restoration, suggesting that these immune responses may have been mounted against a potential

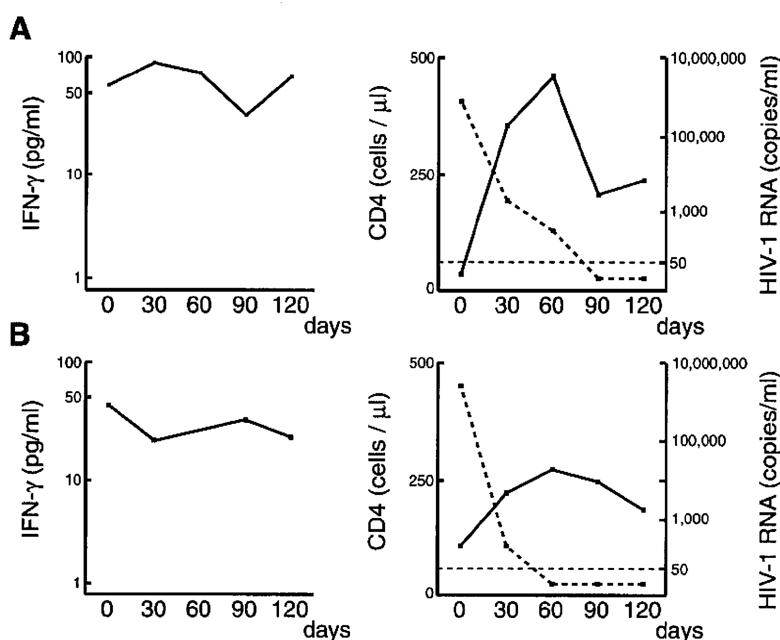

FIG. 4. Sustained elevation of serum IFN-g levels during ART in HIV-1-infected individuals (**A** and **B**). Shown are values of serum IFN-g (left), CD4 cell counts (right, solid lines), and levels of plasma HIV-1 RNA (right, dashed lines), of two typical patients at the indicated time points after the initiation of ART.

infectious agent. The direct effects of HIV-1 infection cannot be completely overlooked. However, the abnormal cytokine levels seen in immunocompromised patients may be at least partially associated with opportunistic infections.

After ART initiation, many cytokines showed no change or showed upward trends; on the other hand, IL-7 rapidly decreased. This appeared to be associated with the physiological actions of IL-7. Other cytokines are associated with immune responses and inflammatory reactions against microorganisms; however, the main function of IL-7 is for hematopoiesis (24). IL-7 mainly acts on hematopoietic stem cells, and induces their differentiation into T lymphocytes. The IL-7 levels were higher in the under-ART group, including those patients with poor recovery of CD4 cell counts, compared to the AC group (Fig. 1H). In addition, the IL-7 levels and CD4 counts show a marked negative correlation (Fig. 2B). These results are consistent with findings previously reported (14,15), suggesting that IL-7 is physiologically induced by decreased CD4 cell counts. Currently, IL-7 is receiving attention as a cytokine that increases the CD4 cell count (17), and is the focus of many clinical studies investigating whether IL-7 administration may induce $CD4^+$ lymphocyte expansion in HIV-1-infected patients (13,19).

In this study we demonstrated characteristic increases in IFN-g levels due to HIV-1 infection. ART has been reported to decrease the serum levels of many of the cytokines that are elevated in patients with HIV-1 infection (16,22,23,25), and increases the levels of IL-21, which are reduced in patients with HIV-1 infection (9,10). To the best of our knowledge, ours is the first study to report that cytokine levels remain unchanged by ART, despite their abnormally high levels. IFN-g levels were high in some patients not only during the AC period, but also in patients with sustained suppression of viral replication and immune restoration by ART. This suggests the potential induction of IFN-g expression by HIV-1. This may be the first report on the above-mentioned phenomenon, probably because these high levels are not necessarily sustained in all cases, and thus changes occurring following the initiation of ART should be studied in more detail. Although the sustained high IFN-g levels observed in some patients is thought to be due to individual differences in immune responses against HIV-1, or the genetic characteristics of HIV-1 (8), or both, we could find no clinical data associated with increased serum IFN-g levels to support this. Unlike the total CD4 cell counts and viral loads presented here, the total CD8 cell counts and their kinetics after the initiation of ART were not associated with changes in IFN-g levels (data not shown). However, the IFN-g levels were increased in 9 of 33 patients (27%), and these patients account for a significant proportion, thus yielding important findings. IFN-g is a cytokine used as an immunocompetence indicator in HIV-1 vaccine studies (21). It has been reported that Th-2 cell numbers tend to increase with the progression of HIV-1 infection, and that IFN-g is one of the key cytokines for differentiation into Th-1 cells (6). Thus IFN-g may play an essential role, different from those of other cytokines, in the pathogenesis of HIV-1 infection. One possible mechanism behind the sustained high serum IFN-g levels seen despite ART's introduction may be that IFN-g production by HIV-1-specific $CD8^+$ T lymphocytes is driven by HIV-1 viremia, and could even be induced by ongoing viral replication during ART. Only a small population of $CD8^+$ T lymphocytes may be involved in IFN-g production, because there was no association between total CD8 cell counts and serum IFN-g levels. In future studies, we intend to investigate the role of this cytokine with a focus on acute HIV-1 infection, in which HIV-1-specific $CD8^+$ T lymphocytes are preferentially expanded to control viral replication.

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Author Disclosure Statement

No competing financial interests exist.

References

1. Alonso K, Pontiggia P, Medenica R, and Rizzo S: Cytokine patterns in adults with AIDS. Immunol Invest 1997;26:341–350.
2. Ameglio, F., Cordiali Fei P, Solmone M, *et al.*: Serum IL-10 levels in HIV-positive subjects: correlation with CDC stages. J Biol Regul Homeost Agents 1994;8:48–52.
3. Aziz N, Nishanian P, and Fahey JL: Levels of cytokines and immune activation markers in plasma in human immunodeficiency virus infection: quality control procedures. Clin Diagn Lab Immunol 1998;5:755–761.
4. Breen EC, Rezai AR, *et al.*: Infection with HIV is associated with elevated IL-6 levels and production. J Immunol 1990; 144:480–484.
5. Clerici M, Hakim FT, Venzon DJ, Blatt S, Hendrix CW, Wynn TA, and Shearer GM: Changes in interleukin-2 and interleukin-4 production in asymptomatic, human immunodeficiency virus-seropositive individuals. J Clin Invest 1993;91:759–765.
6. Clerici M, and Shearer GM: The Th1-Th2 hypothesis of HIV infection: new insights. Immunol Today 1994;15:575–581.
7. Clerici M, Stocks NI, Zajac RA, Boswell RN, Lucey DR, Via CS, and Shearer GM: Detection of three distinct patterns of T helper cell dysfunction in asymptomatic, human immunodeficiency virus-seropositive patients. Independence of CD4+ cell numbers and clinical staging. J Clin Invest 1989; 84:1892–1899.
8. Greco G, Fujimura SH, Mourich DV, and Levy JA: Differential effects of human immunodeficiency virus isolates on beta-chemokine and gamma interferon production and on cell proliferation. J Virol 1999;73:1528–1534.
9. Iannello A, Boulassel MR, Samarani S, *et al.*: Dynamics and consequences of IL-21 production in HIV-infected individuals: a longitudinal and cross-sectional study. J Immunol 2010;184:114–126.
10. Iannello A, Tremblay C, Routy JP, Boulassel MR, Toma E, and Ahmad A: Decreased levels of circulating IL-21 in HIV-infected AIDS patients: correlation with CD4+ T-cell counts. Viral Immunol 2008;21:385–388.

11. Kedzierska K, and Crowe SM: Cytokines and HIV-1: interactions and clinical implications. Antivir Chem Chemother 2001;12:133–150.
12. Kishimoto T, Akira S, and Taga T: Interleukin-6 and its receptor: a paradigm for cytokines. Science 1992;258:593–597.
13. Levy Y, Lacabaratz C, Weiss L, *et al.*: Enhanced T cell recovery in HIV-1-infected adults through IL-7 treatment. J Clin Invest 2009;119:997–1007.
14. Llano A, Barretina J, Gutierrez A, Blanco J, Cabrera C, Clotet B, and Este JA: Interleukin-7 in plasma correlates with CD4 T-cell depletion and may be associated with emergence of syncytium-inducing variants in human immunodeficiency virus type 1-positive individuals. J Virol 2001;75:10319–10325.
15. Napolitano LA, Grant RM, Deeks SG, *et al.*: Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis. Nat Med 2001; 7:73–79.
16. Orsilles MA, Pieri E, Cooke P, and Caula C: IL-2 and IL-10 serum levels in HIV-1-infected patients with or without active antiretroviral therapy. APMIS 2006;114:55–60.
17. Rosenberg SA, Sportes C, Ahmadzadeh M, *et al.*: IL-7 administration to humans leads to expansion of CD8+ and CD4+ cells but a relative decrease of CD4+ T-regulatory cells. J Immunother 2006;29:313–319.
18. Scott-Algara D, Vuillier F, Marasescu M, de Saint Martin J, and Dighiero G: Serum levels of IL-2, IL-1 alpha, TNF-alpha, and soluble receptor of IL-2 in HIV-1-infected patients. AIDS Res Hum Retroviruses 1991;7:381–386.
19. Sereti I, Dunham RM, Spritzler J, *et al.*: IL-7 administration drives T cell-cycle entry and expansion in HIV-1 infection. Blood 2009;113:6304–6314.
20. Shelburne SA, Montes M, and Hamill RJ: Immune reconstitution inflammatory syndrome: more answers, more questions. J Antimicrob Chemother 2006;57:167–170.
21. Streeck H, Frahm N, and Walker BD: The role of IFN-gamma Elispot assay in HIV vaccine research. Nat Protoc 2009;4:461–469.
22. Stylianou E, Aukrust P, Kvale D, Muller F, and Froland SS: IL-10 in HIV infection: increasing serum IL-10 levels with disease progression—down-regulatory effect of potent antiretroviral therapy. Clin Exp Immunol 1999;116:115–120.
23. Stylianou E, Bjerkeli V, Yndestad A, *et al.*: Raised serum levels of interleukin-18 is associated with disease progression and may contribute to virological treatment failure in HIV-1-infected patients. Clin Exp Immunol 2003132:462–466.
24. Surh CD, and Sprent J: Homeostasis of naive and memory T cells. Immunity 2008;29:848–862.
25. Torre D, Speranza F, Martegani R, Pugliese A, Castelli F, Basilico C, and Biondi G: Circulating levels of IL-18 in adult and paediatric patients with HIV-1 infection. AIDS 2000; 14:2211–2212.

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CASE REPORT

Immune reconstitution to parvovirus B19 and resolution of anemia in a patient treated with highly active antiretroviral therapy

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Abstract Immune reconstitution inflammatory syndrome (IRIS) is an unsolved problem in the treatment of human immunodeficiency virus (HIV)-1 infection. Despite the high seroprevalence of parvovirus B19 (PVB19) among HIV-1-positive patients, reports on PVB19-induced anemia, especially that associated with PVB19-related IRIS, in these patients are limited. We present the case of a man with acquired immunodeficiency syndrome who developed severe transfusion-dependent anemia and was seropositive and borderline positive for immunoglobulin-M and IgG antibodies against PVB19, respectively. PVB19-DNA was also detected in his serum. The patient was diagnosed with pure red cell anemia (PRCA) caused by a primary PVB19 infection and was treated with periodical blood transfusions. However, he subsequently tested negative for IgG antibodies and developed chronic severe anemia with high levels of PVB19 viremia. This indicated a transition from primary to persistent infection. After initiation of highly active antiretroviral therapy, the patient showed an inflammatory reaction with rapid deterioration of anemia and seroconversion of the IgG antibody to PVB19. Subsequently, PRCA was completely resolved, but the patient's serum still contained low levels of PVB19-DNA. Thus, this was a case of IRIS associated with PVB19 infection. Our report highlights the significance of seroconversion to PVB19 in the diagnosis of IRIS and re-emphasizes the finding that persistently high levels of PVB19 viremia after primary infection are probably because of the lack of protective antibodies.

Keywords HIV-1 infection · Parvovirus B19 · Pure red cell anemia · Immune reconstitution inflammatory syndrome

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Introduction

Human immunodeficiency virus (HIV)-1 is known to infect $CD4^+$ T lymphocytes and cause acquired immunodeficiency syndrome (AIDS) by decreasing the number of $CD4^+$ cells. In the mid-1990s, a new and specific treatment, namely, highly active anti-retroviral therapy (HAART), was developed to treat HIV-1 infection; HAART is a combination therapy comprising administration of two or three classes of antiretroviral drugs. This therapy induces long-term suppression of viral proliferation and immunological reconstitution in HIV-1-infected patients and thus increases their survival rate. Although HAART cures opportunistic infections by restoring the immune system, it can also induce an inflammatory reaction that is characterized by the aggravation of a preexisting opportunistic infection and the emergence of other infectious diseases that were not observed before the initiation of HAART. This phenomenon, termed as immune reconstitution inflammatory syndrome (IRIS), is thought to be caused by an immunological reaction to a pathogen that was present in the host before the antiviral therapy [1]. This paradoxical syndrome poses a major problem in the patients who undergo HAART.

Human parvovirus B19 (PVB19) belongs to the genus *Erythrovirus*. PVB19 is the predominant pathogenic erythrovirus in humans and is the prototype strain for

genotype 1 [2]. PVB19 has been shown to cause erythema infectiosum in children as well as acute red cell aplasia in patients who have conditions causing hematopoietic stress, such as hemolytic anemia; this virus has also been implicated in the pathogenesis of rheumatic arthritis, myocarditis, nephritis, fulminant liver disease, and many other conditions [3]. In HIV-1-positive patients, PVB19 may persistently infect erythroid precursor cells, evade elimination by the immune system, and cause transfusion-dependent pure red cell anemia (PRCA) [4]. PVB19-related anemia can be resolved by treatment with intravenous immunoglobulin (Ig) [5]. However, this treatment often has a transient beneficial effect, and AIDS patients might experience a relapse of anemia. Therefore, AIDS patients may require periodic administration of intravenous Ig or blood transfusions. In recent years, some reports have shown that complete remission of PVB19-associated PRCA can be achieved by treating patients with HAART [6–8]. Although patients with HIV-1 infection show high seroprevalence of PVB19 [9], few reports have been published on primary or persistent PVB19 infection, particularly PVB19-related IRIS, in HIV-1-infected patients. In this report, we describe the case of a man with AIDS who presented with chronic PVB19-induced PRCA and IRIS after undergoing HAART. We focus on the relationship between the clinical presentation and immunological status in this condition.

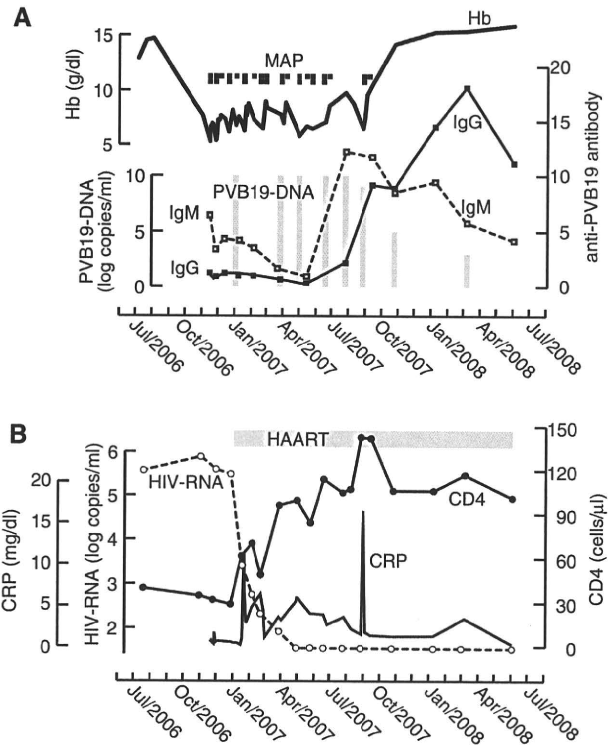

Fig. 1 The patient's clinical course with the changes in the hemoglobin levels and immunological status at the primary and persistent PVB19 infection and at the resolution of PRCA. **a** The *upper solid line* shows the time course of changes in the hemoglobin (Hb) level. The *closed boxes* indicate transfusion of 2 U of packed red blood cells. The *lower solid line* and the *broken line* represent the EIA indices of anti-PVB19 IgG and IgM antibodies, respectively. The *gray bars* show the concentration of serum PVB19-DNA. **b** Time course of changes in the CD4 cell count, plasma HIV-1-RNA levels, and CRP concentrations as well as the duration of HAART are shown

Case report

A 54-year-old HIV-1-positive man visited our hospital in May 2006. He had been diagnosed with *Pneumocystis jiroveci* pneumonia and treated with sulfamethoxazole/trimethoprim in February 2006. His initial CD4 cell count was 35 cells/μl, and the plasma HIV-1 RNA level was 250,000 copies/ml. The results of other laboratory analyses were normal, except for the presence of slight anemia (hemoglobin level 11.5 g/dl). He reported that he had traveled abroad to Southeast Asia for personal reasons.

In November 2006, he re-visited our hospital, and his hemoglobin level had decreased to 7.7 g/dl. He did not show any other symptoms, such as fever, rash, or arthralgia, or any signs of cardiac, renal, or hepatic disorders. He did not report any direct contact with patients having erythema infectiosum. Two weeks later, he experienced dyspnea and was hospitalized immediately. Severe anemia was detected (hemoglobin 5.3 g/dl), and blood transfusions were performed (Fig. 1; Table 1). Gastrointestinal bleeding and hemolytic anemia were ruled out. PVB19 infection was suspected, and an immunoassay [Parvo B19 IgM-enzyme immunoassay (EIA); "SEIKEN," Denka Seiken, Tokyo, Japan] revealed anti-PVB19 IgM antibodies in the serum.

A qualitative polymerase chain reaction (PCR) analysis performed at BML Inc. (Tokyo, Japan) revealed the presence of erythrovirus DNA in the serum. The commercial assays for PVB19-DNA can detect erythrovirus DNA, including the DNA of the newly described erythrovirus variants (genotype 2 and 3) [2]. Quantitative assessment, i.e., real-time PCR analysis, was not performed at this point. The anti-PVB19 IgG antibody index assessed using Parvo B19 IgG-EIA (Seiken, Denak Seiken) was borderline positive (0.92). Examination of a bone marrow aspirate revealed an aplastic marrow (myeloid/erythroid ratio 63:1). Neither parasites nor hemophagocytic cells were found in the aspirate. Although typical giant proerythroblasts were not observed, acute PRCA caused by primary PVB19 infection was diagnosed. The patient was transfused with 6–8 U of blood per month. The anti-PVB19 IgG antibody index gradually reduced and changed from borderline-positive to negative, and the anemia did not improve; these findings indicated a transition from primary PVB19 infection to chronic and persistent infection. Intravenous Ig

Table 1 Summarizing conditions of PVB19 infection, anemia, and immunological findings

Date (year/month)	PVB19 infection	Anemia	Decision of EIA		PVB19-DNA (log copies/ml)	CD4 cell count	HAART
			PVB19-IgG	PVB19-IgM			
2006/11	Primary infection	Acute anemia	+−	+	ND	35	−
2007/1	Persistent infection with high level viremia	Chronic anemia	−	+	10	29	−
2007/9	IRIS	Deterioration	+	+	9	142	+
2007/11	Low level viremia	Remission of PRCA	+	+	3–5	106	+

ND not determined

therapy was not administered because it is an expensive procedure.

From January 2007, HAART with tenofovir, emtricitabine, and efavirenz were initiated. The patient's CD4 cell count gradually increased, and his HIV-1 viral load became undetectable after May 2007. At the beginning of July 2007, the CD4 cell count had increased to 105 cells/μl, and seroconversion of IgG antibody was observed. Although the serum PVB19-DNA level was unchanged, the hemoglobin level increased to 9.8 g/dl, and the periodical blood transfusions were discontinued.

Two months after the last transfusion, the patient experienced episodes of dizziness and visited our hospital. His hemoglobin level rapidly deteriorated to 6.5 g/dl, and blood transfusion was repeated. The serum PVB19 load had reduced tenfold (from 10^{10} to 10^9 copies/ml). After 3 days, he developed fever and neutropenia (1,100 cells/μl), and circulating atypical lymphocytes were detected. Serum biochemical assessments showed elevated concentrations of lactate dehydrogenase (LDH) (395 IU/l) and C-reactive protein (CRP) (16.47 mg/dl). No other symptoms such as rash or arthralgia and no signs of cardiac, renal, or hepatic disorders were observed. The patient's symptoms disappeared, the abnormal test results reverted to normal within a few days, and the anemia rapidly improved. No further red blood cell transfusions were required. In October 2007, the patient's hemoglobin level was within the normal range, and the PVB19-DNA load decreased to 10^5 copies/ml. Although reexamination of bone marrow aspirate was not performed, PVB19-induced PRCA was completely resolved, and PVB19 IgG antibody was persistently detected; PVB19-DNA (10^3 copies/ml), however, was still detected.

Discussion

IRIS is a serious condition that can occur after the initiation of HAART. This syndrome is usually self-limited, but it may worsen and necessitate intervention. In our case, the patient presented with transient inflammatory responses, such as fever, shortly before the remission of PRCA. The laboratory results revealed leucopenia, atypical lymphocytes, and elevation of the serum LDH level; these findings were similar to those of a nonspecific response to viral infections. Additionally, his anemia rapidly worsened despite showing some improvement shortly before this episode. His immunological state was improving: the CD4 cell count rose, seroconversion to anti-PVB19 IgG antibody was observed, and the serum PVB19-DNA level showed a slight but significant decrease. On the basis of these paradoxical findings, we thought that this was an episode of IRIS. The recent literature contains only two reports of severe IRIS. In one of the cases, the patient presented with acute encephalitis [10]. In that case, the patient had persistent PVB19 infection, and the complication of chronic PRCA was treated with intravenous Ig therapy. Four weeks after initiating HAART, anemia developed rapidly with acute onset of ataxia and aphasia. Such a progression was unexpected because encephalitis is a rare complication in PVB19 infection. In the other case, acute and transient anemia developed after the initiation of HAART, although no anemia and PVB19 infection were detected before HAART [11]. Serum antibodies to PVB19 had not been fully confirmed in either of these cases. In all three cases, rapid deterioration of anemia was observed after HAART; this finding seems to be a typical presentation in IRIS associated with HAART for PVB19 infection. With the exception of anemia, the symptoms and pathogenic conditions observed in our case are different from those observed in the two above-mentioned cases. Our case seems to be the most typical presentation of IRIS because (1) the patient was proven to have a chronic PVB19 infection before HAART, (2) the immunological parameters, such as the CD4 cell count and IgG antibody production, showed an improvement during the course of IRIS, and (3) the patient developed symptoms resembling those of acute viral infection. The diagnosis of PVB19-associated IRIS with atypical features may be difficult because of the lack of diagnostic criteria. However, the

findings in our case suggested that seroconversion to antibody against PVB19 and the presence of anemia are helpful in diagnosing PVB19-related IRIS.

The production of neutralizing antibodies plays a pivotal role in the immune control of PVB19 infection [3]. Specific IgM and IgG antibodies are produced 2 and 3 weeks, respectively, after primary PVB19 infection, and these antibodies are responsible for the elimination of PVB19. The EIA kits used in this case could be used only for qualitative assessments. However, the EIA indices of anti-PVB19 IgG and IgM antibodies can indicate the titer of antibodies because these EIA kits include strong positive controls with EIA indices that are at least higher than 1.5, and the EIA indices of the clinical samples were up to 15 and showed good reproducibility. When evaluated on the basis of EIA indices, the anti-PVB19 IgM antibody level showed moderate elevation during primary PVB19 infection. In contrast, the samples were weakly negative for the anti-PVB19 IgG antibodies. These observations suggested that the class-switch recombination of B lymphocytes was markedly disturbed during the infection, and this was probably because of the dysfunction of $CD4^+$ T lymphocytes. Lack of these protective antibodies may lead to the transition from primary to persistent infection and permit high-level PVB19 viremia [12].

In the pre-HAART era, some cases of chronic PRCA with persistent PVB19 infection were treated with intravenous Ig therapy [5], in which the patients were administered neutralizing antibodies to PVB19. This treatment results in a rapid decrease in the copy number of blood PVB19-DNA from 10^{10} to 10^6 copies/ml and improvement of anemia [13]. However, this treatment has a transient effect, and many patients show recurrence of PRCA after the treatment. It should be noted that this therapy cannot clear the PVB19-DNA in the blood, and the DNA persists at levels of about 10^6 copies/ml. These observations suggest that administration of neutralizing antibody alone is insufficient for eliminating PVB19. In our case, the patient developed chronic PRCA, and the serum PVB19-DNA level was 10^{10} copies/ml. Even after seroconversion to anti-PVB19-DNA IgG antibody and the resolution of PRCA, the viremia persisted, and the patient had viral loads of 10^3–10^5 copies/ml. We cannot exclude the possibility that this might be caused by the production of an incomplete neutralizing antibody [7]. PVB19 was, however, not eliminated in our patient, as was also the case in previous reports of treatment with intravenous Ig. This indicates the importance of immune mechanisms other than humoral immune responses [14], such as those involving cytotoxic T lymphocytes.

In our case, the relationship among clinical observations, immunoserological findings and the serum viral load could be evaluated because intravenous Ig was not administered and immune recovery was prolonged. Physicians must note that PVB19 can cause severe anemia in HIV-1-infected patients [14], and detection of PVB19-DNA must be performed in immunosuppressed patients because of the lack of specific antibodies [12]. In addition, detailed investigations on immune reactions to PVB19 will facilitate a better understanding of the mechanism underlying immune reconstitution by HAART.

Written informed consent was obtained from the patient for publication of this case report. A copy of the written consent is available for review from the journal's Editor-in-Chief.

Acknowledgments We are thankful to the patient who provided written consent for the publication of this case report.

Conflict of interest The authors have no conflicts of interest to declare.

References

1. Shelburne SA, Montes M, Hamill RJ. Immune reconstitution inflammatory syndrome: more answers, more questions. J Antimicrob Chemother. 2006;57:167–70.
2. Servant A, Laperche S, Lallemand F, Marinho V, De Saint Maur G, Meritet JF, et al. Genetic diversity within human erythroviruses: identification of three genotypes. J Virol. 2002;76:9124–34.
3. Young NS, Brown KE. Parvovirus B19. N Engl J Med. 2004;350:586–97.
4. Koduri PR. Parvovirus B19-related anemia in HIV-infected patients. AIDS Patient Care STDS. 2000;14:7–11.
5. Koduri PR, Kumapley R, Valladares J, Teter C. Chronic pure red cell aplasia caused by parvovirus B19 in AIDS: use of intravenous immunoglobulin—a report of eight patients. Am J Hematol. 1999;61:16–20.
6. Mylonakis E, Dickinson BP, Mileno MD, Flanigan T, Schiffman FJ, Mega A, et al. Persistent parvovirus B19 related anemia of seven years' duration in an HIV-infected patient: complete remission associated with highly active antiretroviral therapy. Am J Hematol. 1999;60:164–6.
7. Chen MY, Hung CC, Fang CT, Hsieh SM. Reconstituted immunity against persistent parvovirus B19 infection in a patient with acquired immunodeficiency syndrome after highly active antiretroviral therapy. Clin Infect Dis. 2001;32:1361–5.
8. Ware AJ, Moore T. Resolution of chronic parvovirus b19-induced anemia, by use of highly active antiretroviral therapy, in a patient with acquired immunodeficiency syndrome. Clin Infect Dis. 2001;32:E122–3.
9. Azevedo KM, Setubal S, Camacho LA, Velarde LG, Oliveira SA. Seroepidemiological study of human parvovirus B19 among human immunodeficiency virus-infected patients in a medium-sized city in Rio de Janeiro, Brazil. Mem Inst Oswaldo Cruz. 2009;104:901–4.
10. Nolan RC, Chidlow G, French MA. Parvovirus B19 encephalitis presenting as immune restoration disease after highly active antiretroviral therapy for human immunodeficiency virus infection. Clin Infect Dis. 2003;36:1191–4.
11. Intalapaporn P, Poovorawan Y, Suankratay C. Immune reconstitution syndrome associated with parvovirus B19-induced pure

red cell aplasia during highly active antiretroviral therapy. J Infect. 2006;53:79–82.
12. Kurtzman GJ, Cohen BJ, Field AM, Oseas R, Blaese RM, Young NS. Immune response to B19 parvovirus and an antibody defect in persistent viral infection. J Clin Investig. 1989;84:1114–23.
13. Morelli P, Bestetti G, Longhi E, Parravicini C, Corbellino M, Meroni L. Persistent parvovirus B19-induced anemia in an HIV-infected patient under HAART. Case report and review of literature. Eur J Clin Microbiol Infect Dis. 2007;26:833–7.
14. Taguchi H, Takahashi T, Goto M, Nakamura T, Iwamoto A. Acute parvovirus B19 infection during anti-retroviral therapy. J Infect Chemother. 2001;7:110–2.

INTERNAL MEDICINE

□ CASE REPORT □

Three Cases of Fungemia in HIV-Infected Patients Diagnosed Through the Use of Mycobacterial Blood Culture Bottles

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Abstract

We treated three cases of fungemia in HIV-infected patients. These cases were caused by *Candida albicans*, *Cryptococcus neoformans*, and *Penicillium marneffei*, respectively, and all were diagnosed through the use of mycobacterial blood culture bottles. Although the detection of the etiologic agents of fungal infection is difficult, it has been shown that blood culture media for mycobacteria are more effective for the detection of fungemia than media for aerobes and anaerobes. Some reports have shown that Bactec Myco/F lytic bottles were useful for the diagnosis of fungemia in clinical samples. Here, we report the successful use of BacT MB bottles.

Key words: mycobacterial blood culture bottle, BacT MB bottle, fungemia, HIV

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Introduction

The incidence of fungemia, and especially of that caused by *Candida* spp., has recently been increasing. Because diagnosis and treatment with antifungal agents tend to be delayed in such cases, the mortality rate is high (1, 2). Part of the reason for this is that the estimated sensitivity of candidemia detection methods using standard aerobic and anaerobic blood culture bottles is only about 50% (3-6). Blood culture media for mycobacteria, however, are more suitable for detecting fungi *in vitro* than these traditional media are (7). In addition, a few reports have shown that, in clinical situations, Bactec Myco/F Lytic bottles manufactured by Becton Dickinson (Franklin Lakes, NJ, USA) yield better accuracy in detecting fungemia (8, 9). In the cases reported here, we used BacT MB bottles manufactured by bioMérieux (Marcy l'Etoile, France), and showed that they, too, were useful in the diagnosis of three different fungemia cases in human immunodeficiency virus (HIV)-positive patients.

Case Report

Case 1

A 53-year-old Japanese HIV-infected man with a 1-month history of dizziness was referred to us. Four months previously, he had started trimethoprim-sulfamethoxazole for *Pneumocystis* pneumonia and anti-retroviral therapy (lopinavir/ritonavir+ tenofovir/emtricitabine). On admission, his CD4 lymphocyte count was 12/μL and his viral load was under 50 copies/mL. Brain MRI showed a 3-cm ring-enhanced tumor in his cerebellum. After open biopsy, he was diagnosed with malignant lymphoma (diffuse large B cell type). Whole-brain radiation therapy was started. One month later, he was treated with meropenem against extended-spectrum beta-lactamase (ESBL)-producing *Kleb-*

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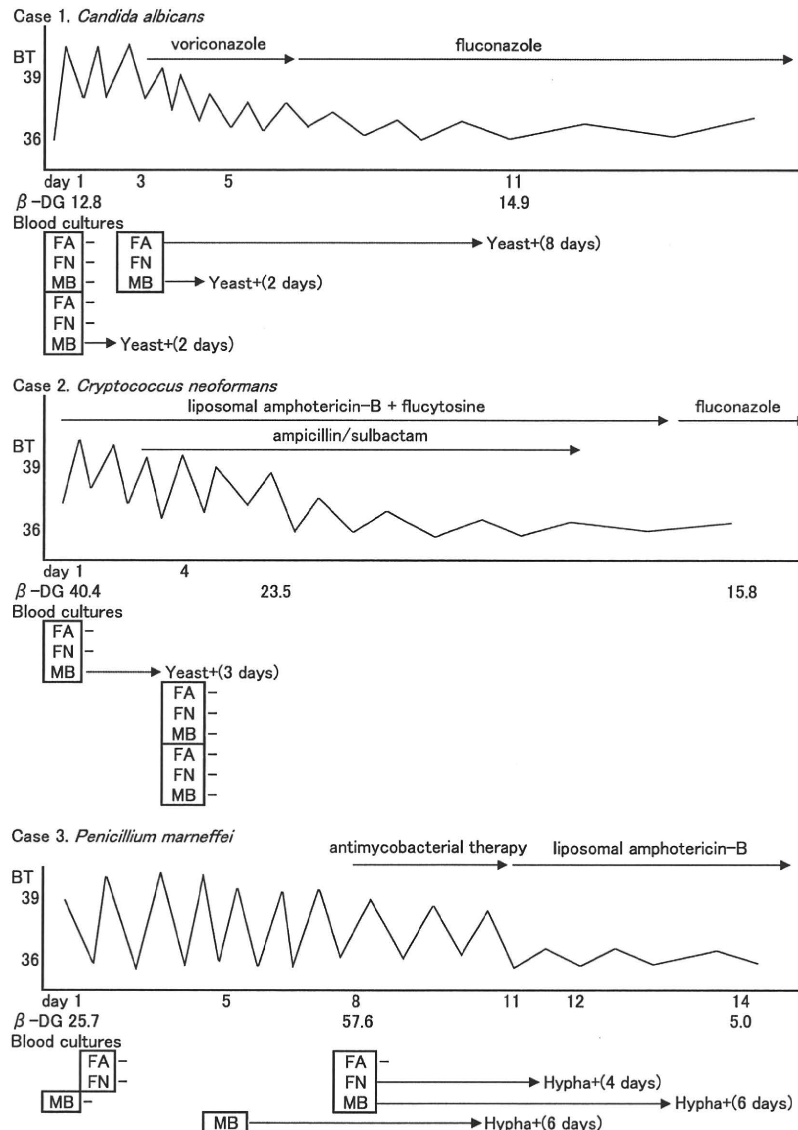

Figure 1. The timing of blood cultures and detection for the three cases. BT: body temperature (℃), β-DG: serum 1 → 3-β-D-glucan (pg/mL), FA, FN, and MB, BacT FA (aerobic bottle), BacT FN (anaerobic bottle), and BacT MB (mycobacterial bottle), respectively. - represents negative in culture. ( ) indicates the number of incubation days.

siella pneumoniae sepsis, and with vancomycin against methicillin-resistant *Staphylococcus epidermidis* (MRSE) sepsis. Initially his fever abated, but after antibiotic treatment he experienced another spike fever (defined hereafter as day 1). We took blood cultures in six bottles: two bottles for aerobes (BacT FA), two for anaerobes (BacT FN), and two for mycobacteria (BacT MB). Two days later (day 3), yeast was growing in one mycobacterial bottle (Fig. 1). We drew another set of blood cultures on day 3 to determine whether the yeast growth indicated a true fungemia or a contamination. Because the patient was in severe distress, we started voriconazole empirically, though it is known to interact with lopinavir/ritonavir. Yeast was found on day 5 in the mycobacterial bottle cultured on day 3, and on day 11 in the aerobic bottle cultured on day 3. All three yeasts were identified as *Candida albicans*. Since the same organism was detected in each of three bottles which had been taken on different days, we considered this case to be a true fungemia. They were sensitive to fluconazole, so we switched the patient from voriconazole to fluconazole. Serum 1→3-β-D glucan was not elevated, measuring 14.9 pg/mL at most. An ophthalmologist confirmed no endophthalmitis. The patient had no central venous catheter, and the entry point of candidemia was unknown. After treatment for candidemia, he was found to have a brain abscess, cellulitis, and a skin abscess at the site of bone marrow examination. He recovered from these serious infections and was discharged home.

Case 2

A 44-year-old Japanese HIV-infected man with a 3-week history of fever and headache was referred to us. He had chronic hepatitis B virus (HBV) infection. His CD4 lymphocyte count was 40/μL, and his viral load was 40,000 copies/

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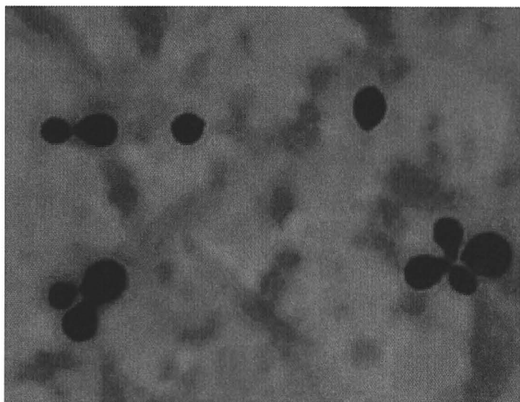

Figure 2. Gram-stained yeasts (*C. neoformans*) from a mycobacterial bottle in Case 2 (×1000).

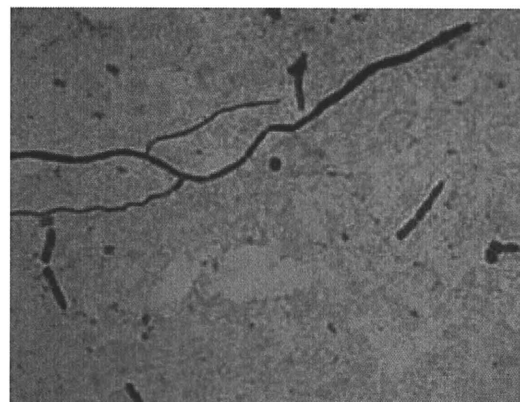

Figure 3. Gram-stained hyphae (*P. marneffei*) from a mycobacterial bottle in Case 3 (×1000).

mL. On admission (defined hereafter as day 1), we took one set of blood cultures including one bottle each for aerobes (BacT FA), anaerobes (BacT FN), and mycobacteria (BacT MB) (Fig. 1). Encapsulated yeasts were detected from the cerebrospinal fluid. We suspected *Cryptococcus* meningitis and accordingly started both liposomal amphotericin-B and flucytosine. We also used ampicillin/sulbactam for aspiration pneumonia. The patient's 1→3-β-D glucan was slightly elevated at 40.4 pg/mL. On day 4, yeasts were found growing in the only mycobacterial bottle (Fig. 2). We took two additional sets of blood cultures, which were all negative, probably because antifungal therapy had already been started. These yeasts were identified as *Cryptococcus neoformans*. After treatment for *Cryptococcus* meningitis, we started antiretroviral therapy (atazanavir+ritonavir+ tenofovir/emtricitabine), and the patient was discharged home.

Case 3

A 30-year-old Japanese HIV-infected man with a 10-day history of fever, cervical and subclavian lymphadenopathy was referred to us. He had traveled to Thailand several months previously. His CD4 lymphocyte count was 10/μL, and his viral load was 140,000 copies/mL. On the day after admission (defined hereafter as day 1), we took a single blood culture in a mycobacterial bottle (BacT MB) to test for *Mycobacterium avium* complex (Fig. 1). On day 2, we also drew blood cultures into aerobic (BacT FA) and anaerobic (BacT FN) bottles. These three bottles were all negative. Another mycobacterial bottle was taken on day 5, and aerobic, anaerobic, and mycobacterial bottles were taken on day 8. At this point we started antimycobacterial therapy empirically. Hyphae were observed on day 11 growing from the mycobacterial bottle taken on day 5 (Fig. 3), on day 12 from the anaerobic bottle taken on day 8, and on day 14 from the mycobacterial bottle taken on day 8. All of those hyphae were identified as *Penicillium marneffei*. The culture from a subclavian lymph node biopsy tested positive for the same organism. The patient's serum 1→3-β-D glucan was elevated at 57.6 pg/mL. We started liposomal amphotericin-B and he became afebrile. After anti-retroviral therapy (fosamprenavir+tenofovir/emtricitabine), he was discharged home.

Discussion

The incidence of fungemia, especially that caused by *Candida* spp., has recently been increasing (1, 2). The diagnosis of candidemia is frequently difficult, however, because the efficacy of fungemia detection using traditional aerobic and anaerobic bottles is estimated at only 50% (3-6). In general, serum 1→3-β-D-glucan is not sufficiently sensitive or specific to serve as a diagnostic marker for fungemia (4). Delayed diagnosis leads to poor prognosis: the mortality rate is over 40 percent (1, 2, 5). *Cryptococcus* meningitis is somewhat easier to diagnose, because in most cases it can be detected in cerebrospinal fluid. One report, however, has described a case of *Cryptococcus* meningitis that was not detected in cerebrospinal fluid but only through blood culture (8). *Penicillium marneffei* infection is rare in Japan but common in Southeast Asia. In cases of delayed diagnosis, the mortality rate is about 75% (9).

We have described the detection of three different fungal species, *Candida albicans*, *Cryptococcus neoformans*, and *Penicillium marneffei*, through the use of BacT MB bottles. The positivity rates of *C. albicans* detection were 33% (1/3 bottles) using aerobic bottles (BacT FA), 0% (0/3 bottles) using anaerobic bottles (BacT FN), and 67% (2/3 bottles) using mycobacterial bottles (BacT MB) (Table 1). The aerobic bottles (BacT FA) required 8 days of incubation before yielding results, while the mycobacterial bottles (BacT MB) required only 2 days. Mycobacterial bottles therefore exhibited the highest sensitivity and the shortest incubation period. The positivity rates of *C. neoformans* detection were 0% (0/3 bottles) using BacT FA bottles, 0% (0/3 bottles) using BacT FN bottles, and 33% (1/3 bottles) using BacT MB bottles (Table 1); in other words, *C. neoformans* was detected only when a mycobacterial bottle was used. The positivity rates of *P. marneffei* detection were 0% (0/2 bottles) using BacT FA bottles, 50% (1/2 bottles) using BacT FN bottles, and 67% (2/3 bottles) using BacT MB bottles (Ta-

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Table 1. Positivity Rates and Detection Times for Aerobic, Anaerobic, and Mycobacterial Bottles

	Case 1 *Candida albicans*	Case 2 *Cryptococcus neoformans*	Case 3 *Penicillium marneffei*
Positivity rate of aerobic bottle(BacT FA)	0.33 (1/3bottles)	0 (0/3bottles)	0 (0/2bottles)
anaerobic bottle(BacT FN)	0 (0/3bottles)	0 (0/3bottles)	0.5 (1/2bottles)
mycobacterial bottle(BacT MB)	0.67 (2/3bottles)	0.33 (1/3bottles)	0.67 (2/3bottles)
Detection time of aerobic bottle(BacT FA)	8 days	–	–
anaerobic bottle(BacT FN)	–	–	4 days
mycobacterial bottle(BacT MB)	2 days	3 days	6 days

ble 1). The mean number of days required for incubation was 4 days for BacT FN and 6 days for BacT MB. For *P. marneffei*, therefore, the mycobacterial bottle again exhibited the highest sensitivity, while the anaerobic bottle required the shortest incubation period. All three of these cases were completely cured through treatment with appropriate antifungal therapies. No other organisms were found in any other blood culture bottles.

About 200 HIV-positive patients are admitted to our hospital each year. When these HIV patients are febrile, we routinely take six bottles of blood culture, two each for the detection of aerobes, anaerobes, and mycobacteria. The required amounts of blood are 10 mL for each aerobic or anaerobic bottle and 5 mL for each mycobacterial bottle. Our laboratory uses the BacT/ALERT 3D automated blood culture system.

Between 2000 and 2005, we took 552 sets of aerobic and anaerobic blood cultures and 390 sets of mycobacterial blood cultures from 684 HIV-positive patients. The positivity rate among aerobic and anaerobic cultures was 3.81% (21/552 sets); three of the 21 positive results were considered to have been contaminations. The organisms involved in the true-positive cases were *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Cryptococcus neoformans*, and *Candida guilliermondii*. The positivity rate among mycobacterial cultures was 1.74% (7/390 sets); all of these involved only *Mycobacterium avium*.

The main reason for culturing blood in mycobacterial bottles is to detect miliary tuberculosis or *Mycobacterium avium* complex. In the three cases described here, however, the routine use of mycobacterial bottles for these other purposes led to early diagnosis of fungemia, not mycobacteremia. In Case 1, the first *Candida* culture grew in only one mycobacterial bottle, but because we had suspected opportunistic infection, we did not assume that it represented a contamination. Because continuous fungemia was demonstrated in another set of blood cultures, *Candida* was determined to be the etiologic agent. Having noticed that the *Candida* grew well in the mycobacterial bottle, we were not surprised when *Cryptococcus* and *Penicillium* were also detected through the use of mycobacterial bottles in Cases 2 and 3. Before witnessing these three cases, when fungi or bacteria other than mycobacteria grew in a mycobacterial bottle, we had suspected that the bottle had been contaminated.

Two kinds of blood culture bottles for mycobacteria are available: the Bactec Myco/F Lytic bottle manufactured by Becton Dickinson, and the BacT MB bottle manufactured by bioMérieux. Bactec Myco/F Lytic bottles are designed to detect both mycobacteria and fungi, but BacT MB bottles are designed to detect mycobacteria only, and not fungi. Nevertheless, both mycobacterial bottles were superior to aerobic and anaerobic bottles for fungal detection *in vitro* (7). One report has stated that, in a clinical situation, Bactec Myco/F Lytic bottles exhibited higher sensitivity and shorter incubation periods in the detection of *Candida albicans* and *Candida glabrata* than aerobic bottles (Bactec Plus Aerobic/F) did (5). Another report has shown that the routine use of Bactec Plus Aerobic/F, Plus Anaerobic/F and Myco/F Lytic bottles for immunocompromised hosts, such as patients in the ICU, permitted highly efficient *Candida albicans* detection (6). That study examined 1,253 blood culture sets (3,759 bottles) in two years. From these sets, 62 yeasts were isolated. The positivity rates were 7.33% among Plus Aerobic/F bottles (44/600 bottles), 1.13% among Plus Anaerobic/F bottles (5/441 bottles), and 25.4% among Mycocis IC/F bottles (48/189 bottles).

Because the present report includes only three fungemia cases, it may not be appropriate to compare these results with those of their reports, but our data correspond well with those from the larger studies in showing that mycobacterial blood cultures can detect fungi with a higher sensitivity than aerobic or anaerobic cultures offer.

Nevertheless, it is very difficult to estimate the true positivity rate of fungemia detection through the use of these blood cultures. Especially among HIV-positive patients, even if serum 1→3-β-D-glucan is elevated, this is frequently caused by *Pneumocystis* pneumonia, not by fungemia. Thus, the direct detection of fungi from blood cultures is particularly important in this area.

Some antibiotics are included inside Bactec Myco/F Lytic bottles to inhibit the growth of bacteria other than mycobacteria or fungi. No antibiotics are included inside BacT MB bottles. The reason why fungi grow so well in mycobacterial bottles is unclear. Both fungi and mycobacteria grow well in aerobic environments, and fungi grow faster than mycobacteria. Accordingly it is possible that, in cases of co-infection with fungi and mycobacteria, the mycobacteremia will be

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overlooked.

Each mycobacterial bottle requires an extra 5 mL of blood from the patient, as well as laboratory space for its storage. The mycobacterial bottles also cost three times as much as typical aerobic or anaerobic bottles (7, 10). The efficacy of aerobic and anaerobic detection is so low that the regular use of mycobacterial bottles is not recommended in the case of community-acquired infection. Mycobacterial bottles are suitable for patients with high risk of fungemia, including immunocompromised hosts and patients with central venous catheters in place. The prognosis of fungemia is still not very good, but early diagnosis leads to early antifungal treatment which is more likely to result in a complete cure.

We have encountered three cases of fungemia in HIV-infected patients, caused by *Candida albicans*, *Cryptococcus neoformans*, and *Penicillium marneffei*, respectively, all of which were diagnosed through blood culture in BacT MB bottles. Blood culture in aerobic and anaerobic bottles alone would not have been sufficient in these cases. We have found that BacT MB bottles are also useful for the isolation of fungi in clinical situations. More data are required to confirm the usefulness of these mycobacterial bottles for the detection of fungemia in immunocompromised hosts.

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References

1. Morrell M, Fraser VJ, Kollef MH. Delaying the empiric treatment of candida bloodstream infection until positive blood culture results are obtained: a potential risk factor for hospital mortality. Antimicrob Agents Chemother **49**: 3640-3645, 2005.
2. Edmond MB, Wallace SE, McClish DK, Pfaller MA, Jones RN, Wenzel RP. Nosocomial bloodstream infections in United States hospitals: a three-year analysis. Clin Infect Dis **29**: 239-244, 1999.
3. Jones JM. Laboratory diagnosis of invasive candidiasis. Clin Microbiol Rev **3**: 32-45, 1990.
4. Odabasi Z, Mattiuzzi G, Estey E, et al. Beta-D-glucan as a diagnostic adjunct for invasive fungal infections: validation, cutoff development, and performance in patients with acute myelogenous leukemia and myelodysplastic syndrome. Clin Infect Dis **39**: 199-205, 2004.
5. Meyer MH, Letscher-Bru V, Jaulhac B, Waller J, Candolfi E. Comparison of Mycosis IC/F and plus Aerobic/F media for diagnosis of fungemia by the bactec 9240 system. J Clin Microbiol **42**: 773-777, 2004.
6. Chiarini A, Palmeri A, Amato T, Immordino R, Distefano S, Giammanco A. Detection of bacterial and yeast species with the Bactec 9120 automated system with routine use of aerobic, anaerobic, and fungal media. J Clin Microbiol **46**: 4029-4033, 2008.
7. Horvath LL, George BJ, Murray CK, Harrison LS, Hospenthal DR. Direct comparison of the BACTEC 9240 and BacT/ALERT 3 D automated blood culture systems for candida growth detection. J Clin Microbiol **42**: 115-118, 2004.
8. Sivasangeetha K, Harish BN, Sujatha S, Parija SC, Dutta TK. Cryptococcal meningoencephalitis diagnosed by blood culture. Indian J Med Microbiol **25**: 282-284, 2007.
9. Wu TC, Chan JW, Ng CK, Tsang DN, Lee MP, Li PC. Clinical presentations and outcomes of *Penicillium marneffei* infections: a series from 1994 to 2004. Hong Kong Med J **14**: 103-109, 2008.
10. Fricker-Hidalgo H, Lebeau B, Pelloux H, Grillot R. Use of the BACTEC 9240 System with Mycosis-IC/F blood culture bottles for detection of fungemia. J Clin Microbiol **42**: 1855-1856, 2004.

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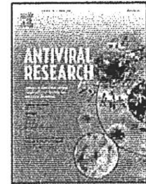

Trends in transmitted drug-resistant HIV-1 and demographic characteristics of newly diagnosed patients: Nationwide surveillance from 2003 to 2008 in Japan

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ABSTRACT

The emergence and transmission of drug-resistant human immunodeficiency virus-1 (HIV-1) compromises antiretroviral treatment for HIV-1. Thus, testing for drug resistance is recommended at diagnosis and before initiating highly active antiretroviral treatment. We conducted an epidemiological study enrolling newly diagnosed patients between 2003 and 2008 in our nationwide surveillance network. In the 6-year study period, the prevalence of drug-resistant HIV-1 among 2573 patients, consisting mainly of Japanese men in their late-30s and infected through male-to-male sexual contacts, followed an increasing trend from 5.9% (16/273) in 2003 to 8.3% (50/605) in 2008. Nucleoside reverse transcriptase inhibitor-associated mutations predominated in each year, with T215 revertants being the most abundant. The predictive factor for drug-resistant HIV-1 transmission was subtype B (OR = 2.36; p = 0.004), and those for recent HIV-1 infection were male gender (OR = 3.79; p = 0.009), MSM behavior (OR = 1.67; p = 0.01), Japanese nationality (OR = 2.31; p = 0.008), and subtype B (OR = 5.64; p < 0.05). Continued activities are needed to raise awareness of the risks of HIV-1 infection and complications of drug-resistant strains. Continued surveillance is also needed to understand trends in the HIV-1 epidemic.

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Abbreviations: HIV-1, human immunodeficiency virus type 1; HAART, highly active antiretroviral therapy; PI, protease inhibitor; HBV, hepatitis B virus; HCV, hepatitis C virus; PR, protease; RT, reverse transcriptase; RT-PCR, reverse transcription polymerase chain reaction; CRF, circulating recombinant form; NRTI, nucleoside RT inhibitor; NNRTI, non-nucleoside RT inhibitor; OR, odds ratio; CI, confidence interval; MSM, men who have sex with men; IDU, intravenous drug user.

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1. Introduction

The emergence of drug-resistant human immunodeficiency virus type 1 (HIV-1) among patients under highly active antiretroviral therapy (HAART) limits the successful suppression of HIV-1 replication. Several years after the introduction of HAART, drug-resistant strains are being detected among newly diagnosed HAART-naïve patients, suggesting the transmission of drug-resistant HIV-1 from the treatment-exposed population. Thus, treatment-naïve patients have been recommended by the US Department of Health and Human Services, International AIDS Society-USA, and other drug-resistance testing guidelines to undergo drug resistance testing at diagnosis and before initiation of HAART (DHHS, 2009; Hirsch et al., 2000, 2008). Indeed, choosing effective antiretrovirals according to the results obtained from this testing has led to successful control of HIV-1 infection. Furthermore, the drug resistance testing at diagnosis helps to understand transmission of drug-resistant HIV-1 in HAART-naïve individuals which in turn may help prevent transmission events.

The prevalence of drug-resistant HIV-1 among treatment-naïve patients has been closely monitored and reported from many countries. Before and early in the HAART era, when only mono or dual therapy was available, the prevalence was as high as 10–20% (Boden et al., 1999; Gómez-Cano et al., 1998; Tambussi et al., 1998). However, after the introduction of antiretrovirals with better pharmacokinetics, such as ritonavir-boosted protease inhibitor (PI), the emergence of drug-resistant viruses seemed to decrease (Gallego et al., 2001; Maia Teixeira et al., 2006).

Furthermore, despite the great number of HIV-1-infected patients, the prevalence tended to be low in developing countries where patients had limited or no access to antiretroviral drugs, e.g., 0–4.2% in Africa (Bártolo et al., 2009; Mintsa-Ndong et al., 2009; Ndembi et al., 2008; Pillay et al., 2008), 1.5% in Cambodia (Nouhin et al., 2009), and 2.6% in Vietnam (Ishizaki et al., 2009). In contrast, in countries where antiretroviral drugs are more accessible, the prevalence has been higher, e.g., 5.2% in Thailand (Apisarnthanarak et al., 2008), 9.4% in Taiwan (Chang et al., 2008), 10.0% in India (Lall et al., 2008), 7.8% in Portugal (Palma et al., 2007), 9.0% in Germany (Sagir et al., 2007), 9.5% in Belgium (Vercauteren et al., 2008), 10.9% in France (Chaix et al., 2009), and 15.9% in the US (Eshleman et al., 2007).

In Japan, since the first HIV-1-infected case was identified in 1985, the annual number of reported cases has been increasing every year, reaching 15 451 by the end of 2008. With more people getting infected, larger numbers of patients are starting anti-HIV-1 treatment and the risk of emerging drug-resistant HIV-1 is increasing. To understand the trends in drug-resistant HIV-1 in Japan, a nationwide surveillance project has been in effect since 2003. In our previous report of surveillance results from 2003 to 2004, the prevalence of drug-resistant HIV-1 in newly diagnosed patients was 4.0% (Gatanaga et al., 2007). We have continued collecting and analyzing data from newly diagnosed HIV-1-infected patients at participating clinical and research facilities in Japan. We report here the prevalence of drug-resistant HIV-1 among newly diagnosed therapy-naïve patients between 2003 and 2008.

2. Materials and methods

2.1. Sample

The study population included all the HIV-1-infected patients newly diagnosed between January 2003 and December 2008 at any of the participating HIV/AIDS clinics. Drug resistance genotypic tests were performed at 12 laboratories including 8 clinical laboratories at HIV/AIDS clinics, 3 public health laboratories, and the National Institute of Infectious Diseases. After patients agreed to participate in our surveillance project and gave informed consent, peripheral blood was drawn with EDTA added, and their demographic and clinical information were collected. Demographic information included age, gender, nationality, and risk behavior. Clinical data included HIV-1 viral loads, CD4$^+$ T cell counts, status of hepatitis B and C virus (HBV, HCV) co-infection, baseline sequence data, and drug-resistant amino acid mutations.

This study was conducted according to the principles in the Declaration of Helsinki, and was approved by the ethical committee of the National Institute of Infectious Diseases, Japan. By Japanese law, HIV-1-infected patients must be reported to the Japanese Ministry of Health, Labour, and Welfare upon diagnosis. The numbers reported to the Ministry are considered the "official numbers" of newly diagnosed HIV/AIDS cases, and were used as comparison controls to evaluate our study population.

2.2. Drug resistance genotypic testing

Drug resistance genotypic testing was performed using in-house protocols. Briefly, viral RNA was extracted from patient plasma samples. HIV-1 protease (PR, 1–99 amino acids) and the N-terminal region of reverse transcriptase (RT, 1–240 amino acids) were amplified in reverse transcription polymerase chain reaction (RT-PCR) followed by nested PCR using in-house primer sets. Subsequently, the amplified PCR products were purified and their sequences were analyzed by direct sequencing method using an automated sequencer. The resulting electropherograms were analyzed using commercially available software. The quality of testing methods used at each participating facility was assessed and confirmed for detection of drug-resistant mutations (Fujisaki et al., 2007). Thus, detection of drug-resistant mutations was consistent among facilities.

2.3. Determination of HIV-1 subtypes and drug-resistant HIV-1

HIV-1 subtypes were determined using the sequences of HIV-1 PR and RT genes obtained in the drug resistance genotypic testing explained above. Each sequence was aligned with the reference sequences of HIV-1 subtypes A through K, and circulating recombinant forms (CRFs), all of which were obtained from the Los Alamos HIV Databases (Los Alamos, 2010), using ClustalW, and phylogenetic trees were constructed using the neighbor-joining method with bootstrap value of 1000.

The resulting sequences were compared to that of HXB2 to judge the presence of amino acid mutations. The drug-resistant mutations were determined according to criteria of the HIV Drug Resistance Database of Stanford University (Bennett et al., 2009). Thus, a sample was considered to harbor drug-resistant HIV-1 if it possessed any of the following mutations: in the PR gene, L23I, L24I, D30N, V32I, M46I/L, I47V/A, G48V/M, I50V/L, F53L/Y, I54V/L/M/A/T/S, G73S/T/C/A, L76V, V82A/T/F/S/C/M/L, N83D, I84V/A/C, I85V, N88D/S, and L90M (indicating PI resistance); in the RT gene, M41L, K65R, D67N/G/E, T69D/insertion, K70R/E, L74V/I, V75M/T/A/S, F77L, Y115F, F116Y, Q151M, M184V/I, L210W, T215Y/F/I/S/C/D/V/E, K219Q/E/N/R (indicating nucleoside RT inhibitor [NRTI] resistance), and L100I, K101E/P, K103N/S, V106M/A, V179F, Y181C/I/V, Y188L/H/C, G190A/S/E, P225H, M230L (indicating non-nucleoside RT inhibitor [NNRTI] resistance).

2.4. BED assay

The time of HIV-1 seroconversion was estimated in randomly selected samples as recent (within 155 days) or not recent using the BED assay (Calypte HIV-1 BED Incidence EIA, BioRad) according to the Manufacturer's instruction. Briefly, 5 μL of plasma was diluted

with 500 μL of sample diluent in the kit, and the proportion of anti-HIV-1 IgG to a total IgG in the sample was measured by optical density.

2.5. Statistical analysis

Statistical analyses were performed using R software (SAS Institute). Chi-square or Fisher's exact probability tests were used to determine associations among patients' demographic characteristics, nationality, BED assay results, and transmission of drug resistance. The odds ratio (OR) and 95% confidence intervals (CI) were calculated for all the variables. Recent and not-recent seroconversion groups were examined for differences in HIV-1 viral loads by analysis of covariance (ANCOVA), with $CD4^{+}$ T cell count as the covariate.

3. Results

3.1. Majority of treatment-naïve patients are Japanese men who have sex with men (MSM) in mid-30s

The demographics of the 2573 newly diagnosed HIV-1-infected patients enrolled between 2003 and 2008 are summarized in Table 1. Male (n = 2397, 93.2%), Japanese (90.1%), and those infected through male-to-male sexual contact (68.9%) predominated, and the median age was 35. For the female cases (n = 170), high-risk heterosexual contact was the major risk factor (n = 152, 89.4%), and approximately half were non-Japanese (n = 63, 41.4%). Further analysis showed a significant association between the transmission route and nationality, i.e., most Japanese patients were infected through male-to-male sexual contact, while non-Japanese patients were infected by other routes (OR = 5.60; 95% CI 4.14–7.63; $p < 0.01$) (Table 2). It should be noted that sexual contacts (92.1%) are the major risk factor for HIV-1 infection in Japan. On the other hand, injecting drug usage, one of the high risk factors in other countries, accounts for only 0.4%.

HBV and/or HCV co-infection, an important clinical factor affecting prognosis and treatment of HIV infection (Ockenga et al., 1997; Piroth et al., 2000), was found to have a prevalence of 8.4% of 2101 patients, and 4.7% of 2071, respectively (Table 1). These prevalence rates did not change significantly throughout the study period (supplementary Table 1). HBV co-infection was found to be significantly associated with subtype B (OR = 2.04; $p < 0.05$) or infection through male-to-male sexual contact (OR = 1.66; $p < 0.05$).

3.2. Subtype B HIV-1 predominates in Japan

Of 2573 plasma samples collected during the study period, the sequences of PR and RT genes were successfully amplified and analyzed in 2536 (98.6%) and 2534 (98.5%) samples, respectively. Of these, we examined sequences of the PR-RT region from 2496 cases by phylogenetic tree analysis to determine the distribution of HIV-1 subtypes in Japan. Subtype B HIV-1 was found to predominate among the study population (n = 2194, 87.9%). The remaining non-B subtypes included 210 (8.4%) CRF01_AE, 30 (1.2%) C, 19 (0.8%) CRF02_AG, 18 (0.7%) A, 9 (0.4%) G, 7 (0.3%) F, 5 (0.2%) D, and 1 (0.04%) CRF08_BC (Table 1). In addition, 1 recombinant case of K/C, A/K, and D/B was detected in 2005, 2006, and 2007, respectively. These non-B subtype viruses were found mostly among the heterosexually infected population (223/302, 73.8%). In contrast, subtype B HIV-1 was found in the vast majority of MSM (1700/1773, 95.9%). In terms of nationality, Japanese patients, most of whom were MSM, were infected with subtype B HIV-1. On the other hand, only about a half of non-Japanese patients harbored subtype B HIV-1, and the remaining half were infected with non-B HIV-1, such as CRF01_AE (OR = 8.85; 95% CI 6.46–12.1; $p < 0.01$) (Table 2). This result is reasonable considering that the predominant HIV-1 subtype differs by country, and our study population included many Thais and Malaysians. In addition, this result suggests that subtype B HIV-1 is transmitted in a closed community of MSM, while non-B subtype strains are spread in wider areas among those infected through high-risk heterosexual contacts.

Table 1
Demographic characteristics of newly diagnosed HIV/AIDS patients.

	6-Year total (2573)	
Age		
Average	37.4	
Median	35	
Mode	35	
Quartile (Q1, Q3)	29, 43	
Nationality	n	(%)
Japanese	2319	(90.1)
Non-Japanese	225	(8.7)
Asian	83	(3.2)
Oceanian	4	(0.2)
North American	17	(0.7)
South American	58	(2.3)
European	10	(0.4)
African	26	(1.0)
Unspecified[a]	27	(1.0)
Unknown	29	(1.1)
Transmission category		
Male	2397	(93.2)
Male-to-male sexual contact	1773	(68.9)
High-risk heterosexual contact	369	(14.3)
Sexual contact	75	(2.9)
IDU	8	(0.3)
Other[b]	26	(1.0)
Unidentified	146	(5.7)
Female	170	(6.6)
High-risk heterosexual contact	152	(5.9)
IDU	3	(0.1)
Other[b]	5	(0.2)
Unidentified	11	(0.4)
Unknown	6	(0.2)
Unidentified	6	(0.2)
Hepatitis co-infection[c]		
HBV		
(+)	176	(8.4)
(−)	1925	(91.6)
Unknown	472	
HCV		
(+)	98	(4.7)
(−)	1973	(95.3)
Unknown	502	
HIV-1 subtype[c]		
B	2194	(87.9)
non-B	302	(12.1)
AE	210	(8.4)
C	30	(1.2)
AG	19	(0.8)
A	18	(0.7)
G	9	(0.4)
F	7	(0.3)
D	5	(0.2)
Other	4	(0.2)
Unidentified	77	

[a] Unspecified individuals in the nationality category were identified only as of non-Japanese origin.
[b] Other transmission categories include mother-to-child, blood products, transfusion, and needle stick.
[c] Prevalence of subtypes, HBV, and HCV was calculated after omitting the unidentified or unknown data. DU, intravenous drug user; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1.

3.3. Prevalence of drug-resistant HIV-1 is increasing in Japan

A total of 194 cases (7.7%) in the 6-year study period were found to harbor HIV-1 strains with at least one major drug-resistant muta-

Table 2
Characteristics of newly diagnosed Japanese and non-Japanese HIV/AIDS patients.

	Nationality (n)			Odds ratio
	Japanese	Non-Japanese	Unknown	
Gender				
Male	2224	151	22	11.45*
Female	95	74	1	
Unknown[b]			6	
Transmission category				
Male-to-male sexual contact	1691	73	9	5.60[a,*]
High-risk heterosexual contact	399	114	7	
Sexual contact	72	4	0	
Other	29	10	2	
Unidentified[b]	128	24	11	
Subtype				
B	2051	118	25	8.85*
Non-B	198	101	3	
Unidentified[b]	70	6	1	
BED assay (n = 640)				
Recent	220	13	0	2.31*
Not recent	351	48	8	
Drug-resistant HIV-1				
Detected	173	16	5	1.05
Not detected	2146	209	24	

[a] Odds ratios for the transmission category were calculated between male-to-male sexual contact and other categories which include high-risk heterosexual contact, sexual contact, and other.
[b] Unknown and Unidentified cases were omitted in calculation of odds ratio.
* $p < 0.01$.

tion conferred by PIs, NRTIs, or NNRTIs. The annual prevalence of drug-resistant mutations shown in Fig. 1 had an overall tendency to increase from 5.9% (16/273) in 2003 to 8.3% (50/605) in 2008. The most prevalent mutation in each year was NRTI-associated resistance, with 11 (4.0%), 12 (4.0%), 21 (5.0%), 23 (5.2%), 28 (5.9%), and 23 (3.7%) cases, followed by PI- and NNRTI-associated mutations. PI-resistant major mutations were detected in 63 cases (2.5%), and NNRTI-associated mutations were detected only in 20 cases (0.8%). These data reflect the type of antiretrovirals being prescribed in treated population. In other words, NRTIs have a long history of being prescribed including the period of mono and dual therapy; thus, NRTIs have been more frequently used. As a consequence, NRTI-resistant HIV-1 has emerged and been transmitted

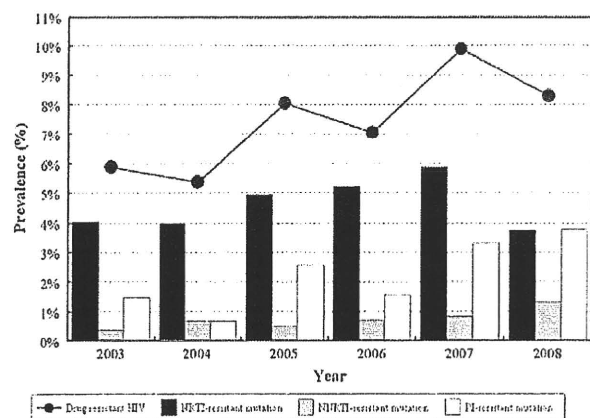

Fig. 1. Annual overall prevalence of drug-resistant HIV-1 (solid circles) in Japan increased in treatment-naïve patients in Japan from 2003 to 2008. The most prevalent mutation in each year was associated with resistance to nucleoside reverse transcriptase inhibitor (NRTI) treatment. Annual prevalence of drug-resistance mutations was categorized by antiretroviral drug class (NRTIs, solid black bars; non-nucleoside reverse transcriptase inhibitors [NNRTIs], horizontally striped bars; protease inhibitors [PIs], solid white bars). Drug-resistant HIV-1 was counted once even when the strain contained multiple drug-resistant mutations. Each drug-resistant mutation was counted even when multiple mutations were detected in one patient.

more frequently to treatment-naïve patients. Regarding the drug-resistant mutations shown in Table 3, T215 revertants (T215X) (3.2%), M184I/V (0.5%), K103N (0.6%), and M46I/L (1.7%) accounted for the majority of detected mutations in contrast to other muta-

Table 3
Drug-resistant mutations in newly diagnosed HIV/AIDS patients, by class of antiretroviral drugs.

	6-Year total (2573)	
	n	(%)
NRTI[a]		
M41L	11	(0.4)
K65R	1	(0.0)
D67N/G/E	7	(0.3)
T69D	8	(0.3)
69INS	1	(0.0)
K70R/E	2	(0.1)
L74V/I	3	(0.1)
V75A/M	2	(0.1)
Y115F	3	(0.1)
M184V/I	12	(0.5)
L210W	5	(0.2)
T215X	81	(3.2)
K219Q/E/N/R	4	(0.2)
NNRTI[a]		
L100I	1	(0.0)
K101E	2	(0.1)
K103N	14	(0.6)
V106A/M	1	(0.0)
Y181C/I/V	3	(0.1)
P225H	1	(0.0)
P236L	1	(0.0)
PI[a]		
L24I	1	(0.0)
D30N	5	(0.2)
V32I	3	(0.1)
M46I/L	44	(1.7)
I47V/A	2	(0.1)
V82A/L	2	(0.1)
I85V	5	(0.2)
N88D/S	7	(0.3)
L90M	4	(0.2)

[a] Numbers of cases and the proportions in parentheses are listed.

Table 4
Predictive factors for transmission of drug-resistant HIV-1.

	Drug-resistant HIV-1 (n)		Odds ratio
	(+)	(−)	
Gender			
Male	183	2214	1.92
Female	7	163	
Nationality			
Japanese	173	2146	1.05
Non-Japanese	16	209	
Transmission category			
Male-to-male sexual contact	130	1643	0.91
High-risk heterosexual contact	37	484	
Sexual contact	15	60	
Other	1	40	
Unidentified[a]	11	152	
Subtype			
B	180	2014	2.36**
Non-B	11	291	
Unidentified	3	77	

[a] For calculation of odds ratio, unidentified cases were omitted.
** $p<0.01$.

Table 5
Predictive factors for recent or not-recent seroconversion determined by BED assay, $n = 640$.

	Seroconversion (n)		Odds ratio
	Recent (n = 233)	Not recent (n = 407)	
Gender			
Male	229	377	3.79**
Female	4	25	
Unknown[b]	0	5	
Nationality			
Japanese	220	351	2.31**
Non-Japanese	13	48	
Unknown[b]	0	8	
Transmission category			
Male-to-male sexual contact	189	293	1.67[a,*]
High-risk heterosexual contact	24	70	
Sexual contact	7	11	
Other	0	4	
Unidentified[b]	13	29	
Subtype			
B	224	350	5.64**
Non-B	6	53	
Unidentified[b]	3	4	
Drug-resistant HIV			
Detected	14	37	0.64
Not detected	219	370	

[a] Odds ratio for the transmission category was calculated between male-to-male sexual contact and other categories which include high-risk heterosexual contact, sexual contact, and other.
[b] Unknown or unidentified cases were omitted in calculation of odds ratio.
* $p<0.05$.
** $p<0.01$.

tions that were detected only sporadically throughout the study period (supplementary Table 2).

Analysis of possible predictive factors for transmission of drug-resistant HIV-1 showed that individuals infected with subtype B HIV-1 had a significantly higher tendency to harbor drug-resistant HIV-1 than non-B subtypes (OR = 2.36; 95% CI = 1.27–4.88; $p < 0.01$) (Table 4). Other possible predictive factors, including male gender (OR = 1.92; 95% CI = 0.89–4.93; $p = 0.1$), Japanese nationality (OR = 1.05; 95% CI = 0.62–1.92; $p = 1$), and MSM behavior (OR = 0.91; 95% CI 0.66–1.26; $p = 0.57$), were not significant predictive factors in our study population. These results indicate that the chance of getting infected with drug-resistant HIV-1 was the same for anyone regardless of gender, nationality, or risk behavior.

3.4. *MSM are diagnosed earlier than heterosexually infected individuals*

To examine awareness of HIV infection, especially of risk behavior, and to characterize HIV-testing patterns among the HIV-infected population, we estimated the time of seroconversion by quantifying the amount of anti-HIV antibody in plasma samples. Of 640 randomly selected samples in 2007 and 2008, 233 (36.4%) were classified by BED assay with a cut-off value of 0.8 as recently infected (<155-day seroconversion), while the remaining 407 (63.4%) were classified as not recently infected (Table 5). For the recently and not recently infected groups, the average $CD4^+$ T cell count and HIV-1 viral load were 285 and 215 cells/μL and 5.1×10^5 and 1.4×10^5 copies/mL, respectively. Recently infected individuals were shown by ANCOVA with $CD4^+$ T cell counts as the covariate, to have significantly higher HIV-1 viral loads than not recently infected cases (Fig. 2). These data support that the BED assay had precisely determined early infected cases.

With respect to risk behavior, the highest rate of recent infection was in MSM (39.2%), followed by either homo- or heterosexual contacts (38.9%), and heterosexual contacts (25.0%). No patients infected through a risk behavior other than sexual contacts were categorized as recently infected. Whereas 37.8% of male patients were determined to be recently infected, only 13.8% of female patients were categorized as recently infected. These findings were reinforced by statistical analysis. Recent HIV-1 infection was significantly predicted by male gender (OR = 3.79; 95% CI 1.29–15.17; $p < 0.01$), MSM behavior (OR = 1.67; 95% CI = 1.11–2.54; $p = 0.01$), Japanese nationality (OR = 2.31; 95% CI 1.20–4.76; $p < 0.01$), and infection with subtype B HIV-1 (OR = 5.64; 95% CI = 2.37–16.33; $p < 0.01$) (Table 5). In other words, Japanese males, especially those who were MSM, were more aware of being at high risk of HIV-1 infection and got tested more often than non-Japanese. In contrast, females, individuals of non-Japanese origin, heterosexuals, and non-subtype-B-infected persons, had low awareness of the risks of HIV-1 infection.

Regarding associations between the time of diagnosis and drug-resistant HIV transmission event, time of diagnosis did not differ significantly between those harboring and those not harboring drug-resistant HIV-1 (OR = 0.64; 95% CI = 0.31–1.24; $p = 0.18$) (Table 5), suggesting that transmission of drug-resistant HIV-1 is not a recent trend, but has been ongoing since the first antiretroviral, AZT, was introduced in 1986.

4. Discussion

Our study results show that the proportion of drug-resistant HIV-1 among newly diagnosed cases in Japan increased slightly (by 2.4%) from 2003 to 2008, with fluctuations from year to year. Drug-resistant HIV-1 in HAART-naïve patients are transmitted from HAART-experienced patients with inadequate adherence or from other treatment-naïve individuals with drug-resistant strains, but not yet diagnosed or tested for drug-resistant HIV-1 (de Mendoza et al., 2005). Hence, drug-resistant mutations detected in the naïve population should be tightly related to trends in antiretroviral use in the treated population. Antiretrovirals available in the early days of the HAART era, especially, had short half-lives and low genetic barriers for drug resistance acquisition, making the viruses easily resistance prone. On the other hand, new antiretroviral drugs, such as lopinavir, atazanavir, amprenavir and darunavir, have been developed so that they have improved pharmacokinetics and higher genetic barriers, thus the viruses have less chance of developing drug resistance (Dunn et al., 2008; Lima et al., 2008; Zajdenverg et al., 2009). In the present study, we found that drug-resistant mutations detected among treatment-naïve patients were

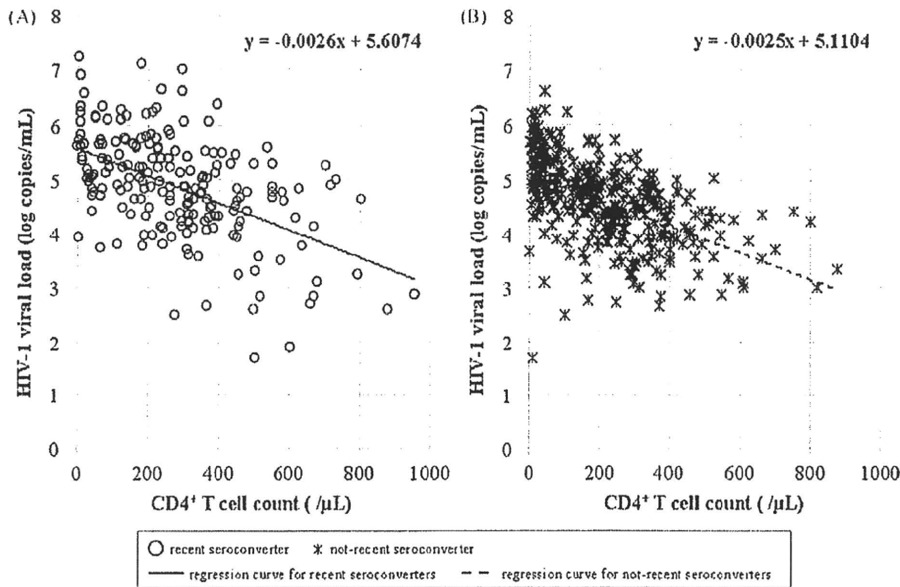

Fig. 2. Scatter plots of viral load and CD4+ T cell counts for (A) recently seroconverted patients (○), and (B) not recently seroconverted patients (*) determined by BED assay. Regression curves and their equations are shown for each group.

associated especially with antiretrovirals used prior to and early in the HAART era. It should be noted that contrary to the reports from the United States and many of European countries (Audelin et al., 2009; Vercauteren et al., 2009; Wheeler et al., 2010), the prevalence of NNRTI-resistant variants have been determined to be low in Japan, less than 1% in the study period 2003–2007 and 1.3% in 2008 being the highest. This difference is due to the situation in Japan that delavirdine had never been used and even nevirapine is only rarely prescribed. Nonetheless, strains with T215X, M46I/L, K103N, and M184V/I mutations were detected every year, suggesting that these strains are stably maintained in individuals and in high-risk populations even under antiretroviral drug-free environments. This finding is supported by the insignificant difference in prevalence of drug-resistant HIV-1 between recently and not recently infected groups. These results raise the concern that such drug-resistant strains may have become some epidemic strains actively transmitted among newly diagnosed HIV/AIDS patients. Furthermore, considering the presence of low frequent variants, the prevalence of drug-resistant mutations in this report may be higher if more sensitive techniques, such as allele-specific PCR and ultra-deep sequencing, are applied to test the samples (Halvas et al., 2010; Varghese et al., 2009). Further studies employing such techniques are needed to understand the detailed epidemic in Japan.

In investigating predictive factors for transmission of drug-resistant strains, we found that the only predictive factor was subtype B HIV-1 (OR = 2.36, $p < 0.01$). The lower transmission risk of drug-resistant strains in non-B HIV-1 can be explained by patients' countries of origin. We observed a significant relationship between non-B subtype HIV-1 and non-Japanese patients, most of whom were from developing countries with limited access to antiretrovirals. Thus, our finding agrees with reports of low prevalence drug-resistant HIV-1 transmission in developing countries (Bártolo et al., 2009; Ishizaki et al., 2009; Mintsa-Ndong et al., 2009; Ndembi et al., 2008; Nouhin et al., 2009; Pillay et al., 2008).

Interestingly, a high proportion of Japanese MSM was diagnosed as recently infected compared to patients of non-Japanese origin, and females determined by BED assay. This result may be due to successful prevention programs targeting the MSM community, so that they have become more aware of their risks of HIV-1 infection. On the other hand, many of non-Japanese patients are seen at hospitals long after HIV infection is established. In addition, women tend to be ignorant of the risks of HIV infection, thus they are often diagnosed upon a prenatal HIV screening test.

Although MSM was not a predictive factor for transmission, this group included 130 cases with drug-resistant HIV-1, the highest prevalence among all the transmission categories. Therefore, those who are involved in prevention programs should take one step further to remind the MSM community about drug-resistant HIV-1 and the limited choice of effective antiretrovirals. HIV-1 transmission has been reported to be prevented in models that assessed the effect of HIV-1 testing for wider populations and immediate initiation of antiretroviral therapy (Granich et al., 2009). Although this model seems very appealing, our results suggest the importance of not forgetting the emergence and transmission of drug-resistant HIV-1 and the limited selection of antiretroviral drugs. It is important to continue surveying newly diagnosed HIV/AIDS patients to keep track of trends in drug-resistant HIV-1 transmission, to reveal high-risk populations with low awareness of HIV infection, to propose effective programs to prevent transmission of drug-resistant HIV-1, and to develop antiretroviral drugs with improved pharmacokinetics/pharmacodynamics. All these efforts may bring us one step closer to eradicating HIV-1.

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