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Sustained High Levels of Serum Interferon-g During HIV-1 Infection: A Specific Trend Different from Other Cytokines

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Abstract

The expression levels of various cytokines increase with the progression of HIV-1 infection. However, the effects of antiretroviral therapy (ART) on serum cytokine levels have not been fully determined. In this study we measured serum cytokine levels of 35 HIV-1-infected Japanese adults. We first performed a cross-sectional study and observed that TNF- α , IL-6, IL-10, IL-18, and IL-7 levels all showed significant increases in those with advanced disease, and that this had a significant negative correlation with the CD4 cell count. However, IFN-g levels did not show this relationship. A longitudinal study in 18 HIV-1-infected patients with a CD4 cell count <350/mL revealed that the introduction of ART reduced cytokine levels. Significant reductions of IL-7, IL-10, IFN-g, and IL-18 levels were observed on days 30, 60, 90, and 90 after the initiation of ART, respectively. These results indicate a discrepancy between cross-sectional and longitudinal studies of serum levels of IFN-g. To clarify this, we investigated serum IFN-g levels in each patient. In 5 of the 15 patients IFN-g levels did not decrease, even after ART initiation, and remained at 5 pg/mL or higher on day 120 after ART initiation. Higher IFN-g levels (>5 pg/mL) were also observed in 2 of 7 asymptomatic patients, and 2 of 11 patients who underwent ART for 1 year or longer. These data demonstrate that IFN-g levels in some patients increased and remained high even after the initiation of ART, which was a specific observation different from those of the other cytokines.

Introduction

CYTOKINES ARE INTERCELLULAR SIGNALING MOLECULES that regulate the differentiation, proliferation, and activation of immune cells (11,12). They are primarily secreted by immune cells, and they exert their biological effects by binding to specific receptors on these (autocrine) or other target cells. Cytokines serve as the immune response molecules against infective microorganisms, and also have various physiological functions in inflammation, allergy, development, and hematopoiesis. The cytokines include interferon (IFN), tumor necrosis factor (TNF), and colony-forming factor, as well as the interleukins (ILs), which all show different functions *in vivo*. Common characteristics shared by these cytokines include effectiveness, even in trace amounts, transient secretion, and a short half-life. Thus serum cytokine levels are generally low and are often undetectable in healthy individuals. Another common feature of cytokines is the presence of complicated networks or cascades, which regulate their mutual effects additively, synergistically, or antagonis-

tically. The Th-1/Th-2 balance represents one of these networks (6). This balance determines the differentiation of naive T cells into Th-1 cells for cellular immunity, or Th-2 cells for humoral immunity.

Human immunodeficiency virus (HIV)-1 infects CD4⁺ cells to destroy the immune system, leading to the development of acquired immunodeficiency syndrome (AIDS). After HIV-1 infection, 5–10 y will pass without symptoms in most patients. This period is called the asymptomatic carrier (AC) period. Although apparently asymptomatic in this period, HIV-1 gradually destroys the immune system and decreases the number of CD4⁺ T lymphocytes (the CD4 cell count). When the CD4 count drops below 200/mL, various opportunistic infections, including AIDS indicator diseases, will develop. Antiretroviral therapy (ART) with multiple agents was developed in the second half of the 1990s, markedly improving the prognosis of HIV-1-infected patients. This therapy is called highly-active antiretroviral therapy (HAART), which continuously suppresses viral replication and restores the function of the immune system in HIV-1-infected patients.

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Many studies have been conducted on the importance of cytokines in the pathogenesis of HIV-1 infection because it affects the immune system (11). In the pre-HAART era, the expression levels of various cytokines reportedly increased, along with the progression of immunodeficiency (1,3). Since in previous studies researchers have reported that a Th-1/Th-2 imbalance is strongly involved in HIV pathogenesis (5,7), the cytokines responsible for the Th-1/Th-2 balance, such as IL-2 (18), IL-6 (4), IL-10 (2), and IFN- γ , have been studied in detail (1). On the other hand, in the post-HAART era, the increased cytokine expression was decreased by ART (14,16,22,23,25). However, only a few cytokines examined in the pre-HAART era that exhibit abnormal expression have been studied. It has not yet been determined whether this abnormal cytokine expression is caused by biological responses to viral proliferation, or whether it is induced secondary to other infections

caused by the immunodeficiency. In fact, the abnormal cytokine expression may be caused by latent opportunistic infections due to a decreased CD4 cell count. In this study we investigated the cytokines with expression levels that differed from those of the other cytokines, by measuring their serum levels primarily in patients who underwent ART, in order to identify the cytokines directly involved in HIV-1 infection.

Materials and Methods

Patients

We first obtained written informed consent from 35 HIV-1-infected adults who regularly visited our hospital, and collected sera from these patients. The median age of the patients (34 male and 1 female) was 39 y (range 28–73 y). All of the patients were Japanese. The putative infection routes

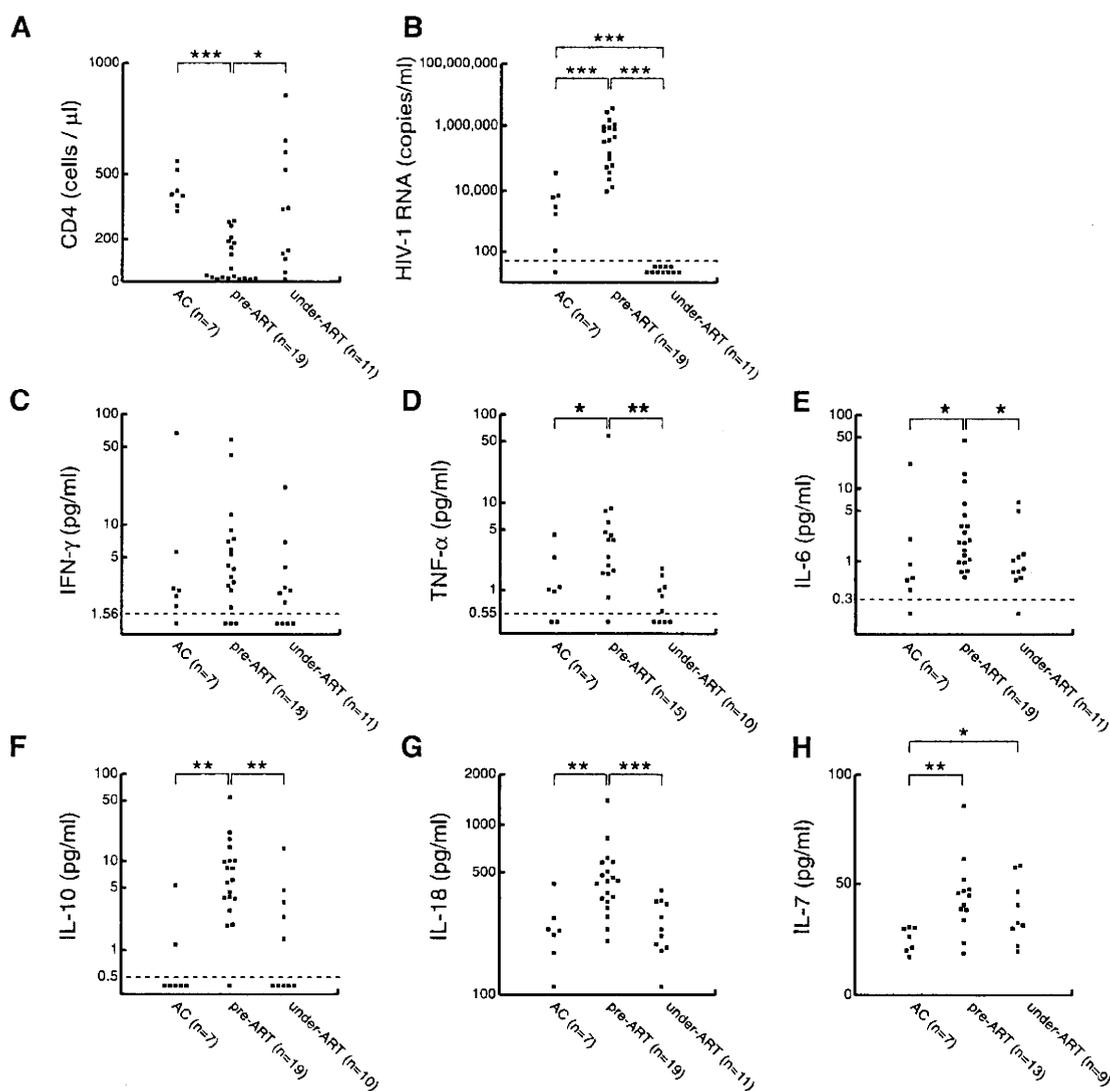


FIG. 1. Serum cytokine levels in asymptomatic carrier (AC), pre-antiretroviral therapy (pre-ART), and under-ART groups. (A and B) Shown are the CD4 cell counts and HIV-1 RNA levels of the HIV-1-infected subjects in the three study groups. (C–H) These plots show the values of the indicated cytokines in the three study groups. Statistical comparisons were made using the Kruskal-Wallis test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

of these patients included homosexual contact (29 patients), heterosexual contact (5 patients), and transfusion for hemophilia (1 patient). We then measured the CD4 cell counts by flow cytometry using the whole-blood lysis method. Plasma HIV-1 RNA levels were measured using reverse-transcriptase PCR (Amplicor HIV-1 monitor test; Roche Molecular Diagnostics Corp., Indianapolis, IN). This study was reviewed and approved by the Institutional Review Board, National Hospital Organization, Osaka National Hospital (approval number 0542).

Cross-sectional and longitudinal studies

The patients were divided into the following three groups: AC, pre-ART, and under-ART. They were then subjected to a cross-sectional study to compare serum cytokine levels. The AC group included asymptomatic patients with CD4 cell counts of 350/mL or higher. The pre-ART group included untreated patients (ART naive) with CD4 cell counts less than 350/mL, from whom samples were collected after treatment for opportunistic infections. The under-ART group included patients who underwent ART for 1 year or longer, and whose plasma HIV-1 RNA levels were below the detection limit. Patients in the pre-ART group were subjected to a longitudinal study, and samples were collected from them periodically after ART introduction for comparison with the baseline.

Measurement of serum cytokines

Serum cytokine levels were measured with a sandwich enzyme-linked immunosorbent assay (ELISA). The following reagents were used: IFN- γ (human IFN- γ ELISA; Bender MedSystems, Vienna, Austria), IL-6 (Quanti Glo human IL-6 chemiluminescent immunoassay; R&D Systems, Inc., Minneapolis, MN), IL-10 (human IL-10 ultra-sensitive ELISA kit; Invitrogen, Carlsbad, CA), IL-18 (human IL-18 ELISA; Medical & Biological Laboratories Co., Ltd., Nagoya, Japan), IL-1 (Quantikine HS human IL-1 immunoassay; R&D Systems), IL-2 (Quantikine human IL-2 immunoassay; R&D Systems), IL-4 (human IL-4 ultra-sensitive immunoassay; R&D Systems), IL-12 (Quantikine HS human IL-12 immunoassay; R&D Systems), IL-7 (Quantikine HS human IL-7 immunoassay; R&D Systems), TNF- α (Quanti Glo human TNF- α chemiluminescent immunoassay; R&D Systems), IL-17 (Quantikine human IL-17 immunoassay; R&D Systems), and IL-23 (Quantikine human IL-23 immunoassay; R&D Systems). The measurements were carried out according to the manufacturers' instructions.

Statistical analysis

Multiple comparisons were carried out using the Kruskal-Wallis non-parametric analysis of variance (ANOVA) test. The Spearman rank test was used for correlation, and the Wilcoxon signed-rank test was used for paired data. The significance level was set at $p < 0.05$.

Results

We measured the serum cytokine levels in 35 HIV-1-infected patients. IL-1b, IL-2, IL-4, IL-12, IL-17, and IL-23 were not considered in the analysis because they were below the detection limits in the majority of the samples (60% or above) (data not shown). To examine the relationship between the

serum cytokine levels and HIV-1 infection, the patients were classified into three groups on the basis of their CD4 counts and the introduction of ART: AC, pre-ART, and under-ART groups, and their serum cytokine levels were compared (cross-sectional study). The CD4 counts and plasma HIV-1 RNA levels of the groups are shown in Fig. 1A and B. As presented in Fig. 1D-G, serum TNF- α , IL-6, IL-10, and IL-18 levels in the pre-ART group showed significant increases compared with the corresponding values in the AC and under-ART groups. The IL-7 levels were significantly higher in the pre-ART group than in the AC group, but comparable

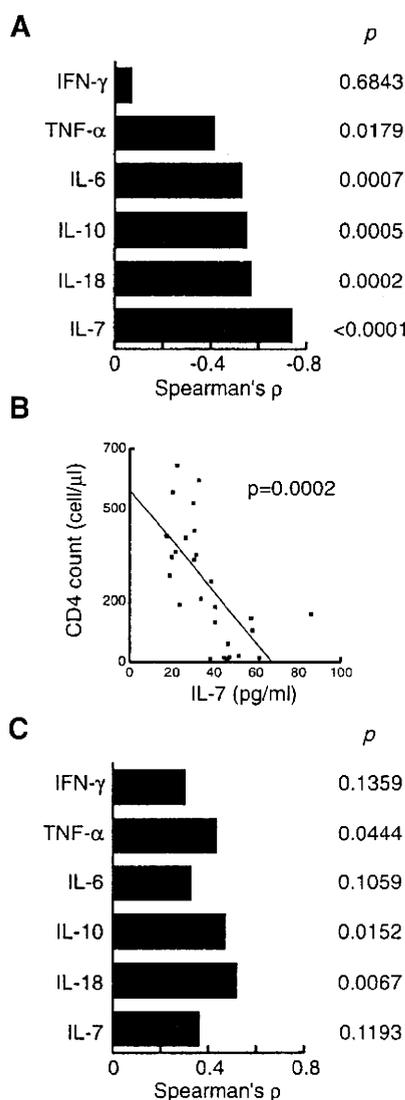


FIG. 2. Association of serum cytokine levels in HIV-1-infected patients with CD4 cell counts and plasma HIV-1 RNA levels. (A) The bars indicate Spearman's rank correlation coefficients (r). (B) Linear regression analysis was used to investigate the relationship of IL-7 levels with CD4 cell counts (slope = -8.3 , $r^2 = 0.42$). (C) Correlation with plasma HIV-1 RNA levels. The bars indicate Spearman's rank correlation coefficients (r).

with those in the under-ART group (Fig. 1H). On the other hand, IFN- γ levels did not differ significantly among the three groups (Fig. 1C). Thus, except for IFN- γ , 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- γ , 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- α /IL-18 and plasma HIV-1 RNA levels (Fig. 2C). Thus except for IFN- γ , 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

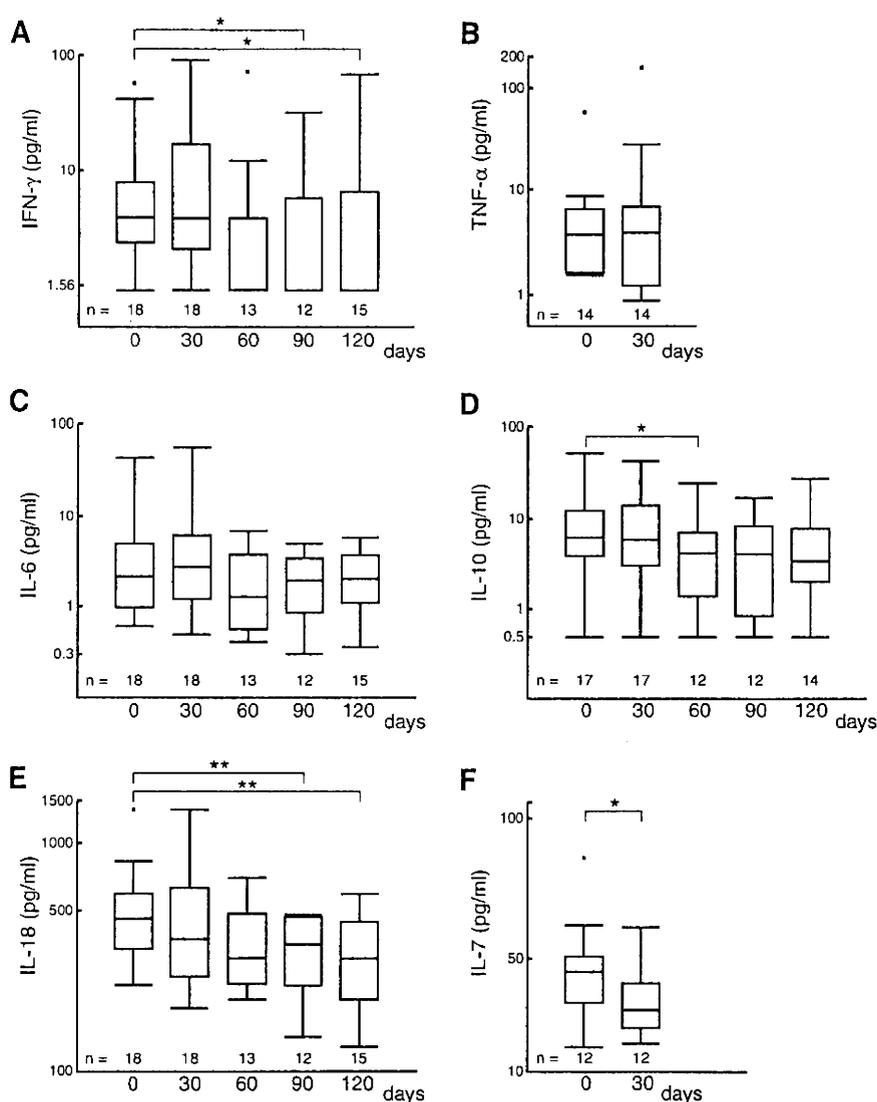


FIG. 3. Serum cytokine levels in HIV-1-infected patients before initiation of and during ART (A-F). The values shown of the indicated cytokines before, and at 30, 60, 90, and 120 d from the start of ART, are shown using box-and-whisker plots, representing the minimum, 25th percentile, median, 75th percentile, 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- γ , IL-6, and TNF- α) showed upward trends. Four cytokines (IFN- γ , 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- γ 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- γ (Fig. 1C). However, our longitudinal observations demonstrated a significant decrease in IFN- γ 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- γ levels were gradually suppressed by ART. However, in 5 of the 15 patients, IFN- γ 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- γ 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- γ levels (>5 pg/mL). Thus, during the AC period or later, the IFN- γ 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

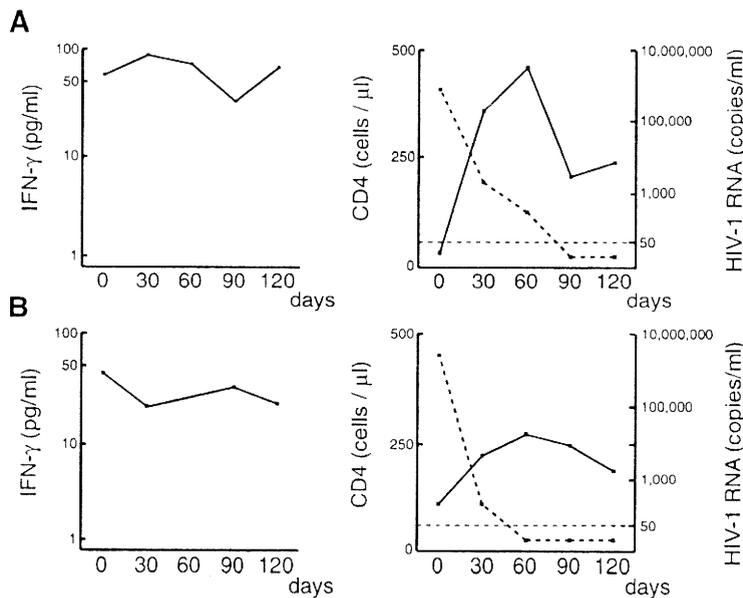


FIG. 4. Sustained elevation of serum IFN- γ levels during ART in HIV-1-infected individuals (A and B). Shown are values of serum IFN- γ (left), CD4 cell counts (right, solid lines), and levels of plasma HIV-1 RNA (right, dashed lines), of two typical patients at the indicated time points after the initiation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

cytes 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.

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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

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

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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.

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/ μ l, 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.

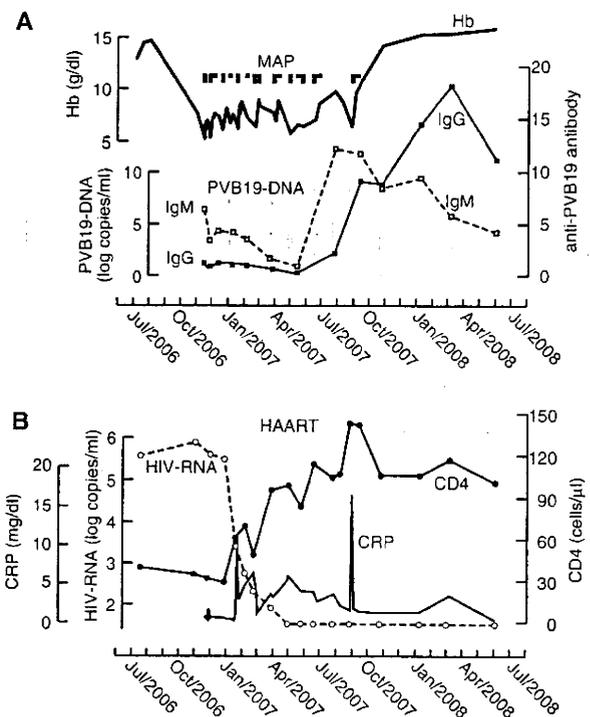


Fig. 1 The patient's clinical course with the changes in the hemoglobin levels and immunological status at the primary and persistent PVB19 infection and at the resolution of PRCA. **a** The upper solid line shows the time course of changes in the hemoglobin (Hb) level. The closed boxes indicate transfusion 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

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, Denka 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/ μ l, 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/ μ l), 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 10¹⁰ to 10⁶ 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 10⁶ 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 10¹⁰ 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 10³–10⁵ 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.

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

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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; Ndembu 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 μ L of plasma was diluted

with 500 μ L 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

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	<i>n</i>	(%)
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.

(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.

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

more frequently to treatment-naïve patients. Regarding the drug-resistant mutations shown in Table 3, 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)

^aNumbers of cases and the proportions in parentheses are listed.

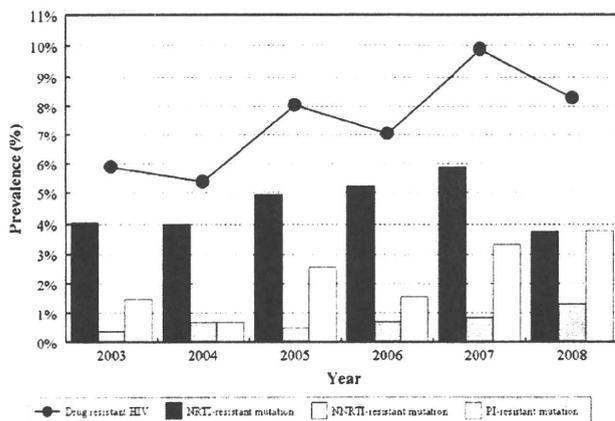


Fig. 1. Annual overall prevalence of drug-resistant HIV-1 (solid circles) in Japan increased in treatment-naïve patients in Japan from 2003 to 2008. The most prevalent mutation in each year was associated with resistance to nucleoside reverse transcriptase inhibitor (NRTI) treatment. Annual prevalence 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.

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.

^{**} $p < 0.01$.

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/ μ L 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;

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.

* $p < 0.05$.

** $p < 0.01$.

$p < 0.01$) (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

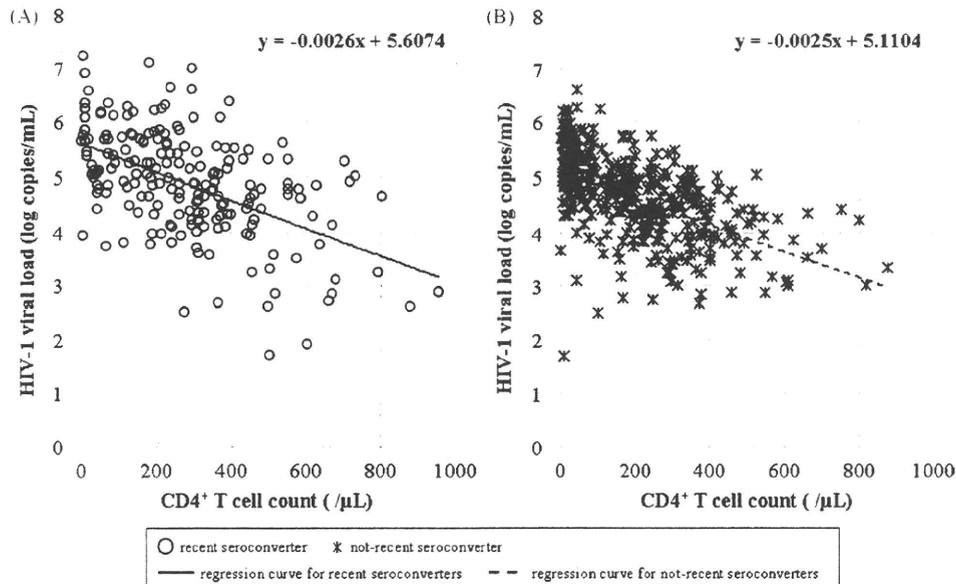


Fig. 2. Scatter plots of viral load and CD4+ T cell counts for (A) recently seroconverted patients (○), and (B) not recently seroconverted patients (*) determined by BED assay. Regression curves and their equations are shown for each group.

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; Mintsá-Ndong et al., 2009; Ndembí 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 com-

munity, 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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2010.07.008.

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