Table 1. Cellular genes differentially expressed in hepatitis C virus (HCV) core transgenic mouse liver

	Upregulated	Downregulated
Lipid metabolism	NPC1 Catalase Very long chain acyl-CoA dehydrogenase Carboxylesterase selenoprotein P Carbonic anhydrase Adipose differentiation-related protein Bilirubin/phenol family UDP glucuronosyltransferase	Stearoyl-CoA desaturase Sterol-carrier protein X Alpha-enolase carnitine acetyltransferase Gal beta 1,4(3) GlcNAc alpha 2,3-sialyltransferase Very long chain acyl-CoA synthetase Liver transferrin 4-Hydroxyphenylpyruvate dioxygenase LAF1 transketolase *s*-Adenosylmethionine synthetase Apolipoprotein A-II
Transcription and cell proliferation	Int-6 GCN5L1 *H. sapiens* 8.2k-Da differentiation factor USF1 Initiation factor eIF-4AI Human elongation factor-1-delta Sui1	Human guanine nucleotide regulatory protein Alpha-fetoprotein Retinol binding protein
Inflammation	Alpha-1 protease inhibitor 3 Hemopexin	Alpha-2-macroglobulin LMW prekininogen Complement component C3 AHSG(alpha 2 HS-glycoprotein) homologue
Others	Microvascular endothelial differentiation gene 1 Diazepam-binding inhibitor Argininosuccinate synthetase Skeletal muscle alpha-tropomyosin Ampd3 gene DNA-binding protein	Vitronectin Epithelin 1 and 2 Murinoglobulin

microsomal triglyceride transfer protein (MTP) by HCV core protein[26]; this inhibits the secretion of very low density protein (VLDL) from the liver, yielding an increase of triglycerides in the liver. The last pathway involves sterol regulatory element-binding protein (SREBP)-1c, which regulates the production of triglycerides and phospholipids. In HCV core gene transgenic mice, SREBP-1c is activated, whereas neither SREBP-2 nor SREBP-1a is upregulated.[27]

In relation to lipid metabolism, the core protein has also been found to interact with retinoid X receptor (RXR)-α.[28] RXR-α is one of the nuclear receptors, which forms a homodimer or heterodimers with other nuclear receptors, including PPAR (peroxisome proliferator-activated receptor)-α, and plays a pivotal role in the regulation of the expression of genes relating to lipid metabolism, cell differentiation, and proliferation. In fact, the core protein of HCV activates genes that have an RXR-α-responsive element as well as those with a PPAR-α-responsive element, both in mice and in cultured cells.[28] Based on these results, we, then, examined the expression and function of PPAR-α in the liver of core gene transgenic mice.

PPAR-α activation in HCV-associated hepatocarcinogenesis

PPAR-α, one of the PPAR genes, plays a central role as a heterodimer with RXR-α in regulating fatty acid transport and catabolism. It is also known as a molecular target for lipid-lowering fibrate drugs.[29] On the other hand, prolonged administration of PPAR-α agonists causes HCC in rodents. Currently, there is little evidence that the low-affinity fibrate ligands are associated with human cancers, but it is possible that chronic activation of high-affinity ligands could be carcinogenic in humans.[29]

The level of PPAR-α protein was increased in the liver of core gene transgenic mice as early as 9 months of age. PPAR-α protein is accumulated with age in the nuclei of hepatocytes together with cyclin D1 protein. However, the level of PPAR-α mRNA was not increased at any age. By pulse-chase experiment, the stability of nuclear PPAR-α was increased in the presence of the core protein. In line with the increase of PPAR-α protein, target genes of PPAR-α were activated in the liver of core gene transgenic mice; these genes include

cyclin D1, cyclin-dependent kinase (CDK)-4, acy-CoA oxidase, and peroxisome thiolase.[30] However, in general, the activation of PPAR-α leads to improvement but not aggravation of steatosis. Then, what is the function of PPAR-α activation that is observed in the core gene transgenic mice?

To clarify the role of PPAR-α activation in pathogenesis of steatosis and HCC, we mated a core gene transgenic mouse with a PPAR-α knockout (KO) mouse and studied the phenotype. PPAR-α KO mice have reduced expression of target genes of PPAR-α, and have mild steatosis in the liver, as expected.[31] It was unanticipated, however, that steatosis was absent in PPAR-α-null or -heterozygous core gene transgenic mice but present in PPAR-α-intact core gene transgenic mice at the age of 9 or 24 months.[30] 8-Hydroxy deoxyguanosine (8-OHdG) and peroxylipids, both of which are markers for oxidative stress, were decreased in PPAR-α KO core gene transgenic mice. Mitochondrial dysfunction in the core gene transgenic mice, which contributes to overproduction of oxidative stress,[19] was also improved in PPAR-α KO core gene transgenic mice.

Finally, PPAR-α KO core gene transgenic mice did not develop HCC at the age of 24 months, whereas about one-third of PPAR-α-intact core gene transgenic mice did. It should be noted that core gene transgenic mice that are heterozygous for the PPAR-α gene also did not develop HCC.[32] When clofibrate, a peroxisome proliferator, was administered for 24 months to PPAR-α-heterozygous mice, either with or without the core gene, HCC developed in a higher rate in the core gene (+) mice with greater PPAR-α activation. It should be noted that steatosis was present only in core gene (+) PPAR-α-heterozygous mice. In summary, steatosis and HCC developed in PPAR-α-intact but not in PPAR-α-heterozygous or PPAR-α-null core gene transgenic mice, indicating that not the presence but the persistent activation of PPAR-α would be important in hepatocarcinogenesis by HCV core protein. In general, PPAR-α acts to ameliorate steatosis, but with the presence of mitochondrial dysfunction, which is also provoked by the core protein, the core-activated PPAR-α may exacerbate steatosis. Persistent activation of PPAR-α with "strong" ligands such as the core protein of HCV could be carcinogenic in humans, although the low-affinity fibrate ligands are not likely associated with human cancers.

HCV core protein causes "fatty acid spiral"

Figure 2 illustrates our current hypothesis for the role of lipid metabolism in HCV-associated hepatocarcinogenesis. Immune-mediated inflammation should also play a pivotal role in hepatocarcinogenesis in HCV infection. However, in HCV infection, the core protein induces steatosis through the aforementioned pathways, leading to "fatty acid spiral" in the presence of the mitochondrial complex 1 dysfunction and PPAR-α activation, both of which are caused by the core protein. These intracellular alterations would contribute to hepatocarcinogenesis by inducing oxidative stress overproduction and cell-growth signal activation. In such a sense, the core protein of HCV is not a classical-type oncoprotein, but rather seems to contribute to hepatocarcinogenesis by modulating intracellular metabolism and signaling.

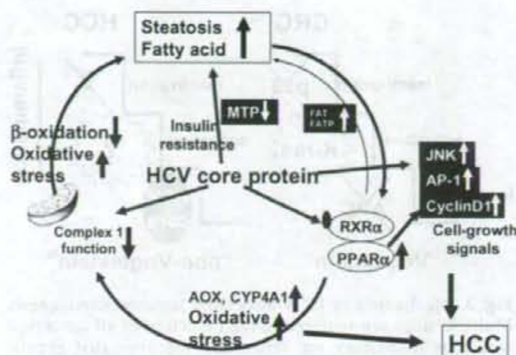

Fig. 2. "Fatty acid spiral" by HCV core protein. In HCV infection, the core protein induces steatosis via several pathways, leading to "fatty acid spiral" in the presence of the mitochondrial complex 1 dysfunction and PPAR-α activation, both of which are also caused by the core protein. These intracellular alterations would contribute to hepatocarcinogenesis by inducing oxidative stress overproduction and cell-growth signal activation. In such a sense, the core protein of HCV is not a classical type oncoprotein, but rather seems to contribute to hepatocarcinogenesis by modulating intracellular metabolism and signaling. *HCV*, hepatitis C virus; *HCC*, hepatocellular carcinoma; *ROS*, reactive oxygen species; *JNK*, c-Jun N-terminal kinase; *ERK*, extracellular signal-regulated kinase; *AP-1*, activating protein-1; *RXR-α*, retinoid X receptor-α; *PPAR-α*, peroxisome proliferator activated receptor-α; *AOX*, acyl-CoA oxidase; *CYP*, cytochrome P450; *MTP*, microsomal triglyceride transfer protein; *FAT*, fatty acid translocase; fatty acid transport protein

The HCV protein may allow some steps in multistep hepatocarcinogenesis to be skipped

The results of our studies on transgenic mice have indicated a carcinogenic potential of the HCV core protein in vivo; thus, HCV would be directly involved in hepatocarcinogenesis. In research studies of carcinogenesis, the theory outlined by Kinzler and Vogelstein[33] has gained wide popularity. They have proposed that the

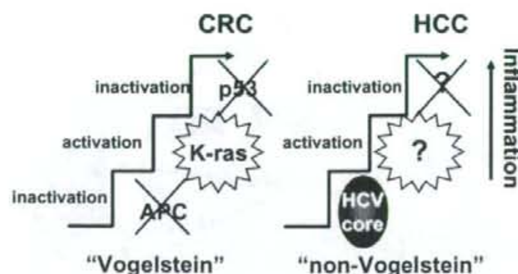

Fig. 3. Mechanism of HCV-associated hepatocarcinogenesis. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps may be skipped in the development of HCC in the presence of the core protein. The overall effects achieved by the expression of the core protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations required for carcinogenesis. By considering such a "non-Vogelstein-type" process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers

development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations. They have deduced that mutations in the APC gene for inactivation, those in K-*ras* for activation, and those in the p53 gene for inactivation accumulate, which cooperate toward the development of colorectal cancer.[33] Their theory has been extended to the carcinogenesis of other cancers as well, called "Vogelstein-type" carcinogenesis (Fig. 3).

On the basis of the results we obtained for the induction of HCC by the HCV core protein, we would like to introduce a different mechanism for hepatocarcinogenesis in HCV infection. We do allow multistages in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, may be skipped in the development of HCC in HCV infection to which the core protein would contribute (see Fig. 3). The overall effect achieved by the expression of the viral protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations required for carcinogenesis.

By considering such a "non-Vogelstein-type" process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers.[34] Now it does not seem so difficult as before to determine why HCC develops in persistent HCV infection at an outstandingly high incidence. Our theory may also give an account of the nonmetastatic and multicentric de novo occurrence characteristics of HCC, which would be the result of persistent HCV infection.

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Short Communication

Prevalence of hepatitis B virus infection in Japanese patients with HIV

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Patients with HIV infection are frequently infected with hepatitis viruses, which are presently the major cause of mortality in HIV-infected patients after the widespread use of highly active antiretrovirus therapy. We previously reported that approximately 20% of HIV-positive Japanese patients were also infected with hepatitis C virus (HCV). Hepatitis B virus (HBV) infection may also be an impediment to a good course of treatment for HIV-infected patients, because of recurrent liver injuries and a common effectiveness of some anti-HIV drugs on HBV replication. However, the status of co-infection with HIV and HBV in Japan is unclear. We conducted a nationwide survey to determine the prevalence of HIV–HBV co-infection by distributing a questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan. Among the 5998 patients reported to be HIV positive, 377 (6.4%) were positive for the hepatitis B surface antigen. Homosexual men accounted for two-thirds (70.8%) of the HIV–HBV co-infected patients, distinct from HIV–HCV co-infection in Japan in which most of the HIV–HCV co-infected patients were recipients of blood products. One-third of HIV–HBV co-infected patients had elevated serum alanine aminotransferase levels at least once during the 1-year observation period. In conclusion, some HIV-infected Japanese patients also have HBV infection and liver disease. A detailed analysis of the progression and activity of liver disease in co-infected patients is needed.

Key words: co-infection, hepatitis B, HIV, liver disease.

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a major public health problem worldwide, along with hepatitis C virus (HCV) and HIV infections. In the USA, the estimated prevalence of HBV is less than 1%, but approximately 1 million people are persistently infected.[1] The prevalence of HIV in the USA is also <1%, and the virus is estimated to have infected approximately 800 000 people.[2] Because of the common transmission routes, that is, parenteral transmission routes, many people with HIV infection are also infected with HBV. Among the HIV-positive people in the USA, the prevalence of HBV co-infection is 6–14%.[1,2] Before the introduction of highly active antiretroviral therapy (HAART) in 1996, most patients with HIV infection died of HIV-associated opportunistic infections, such as *Pneumocystis jiroveci* pneumonia and cytomegaloviral infection. Since the widespread use of HAART, the mortality associated with HIV infection has declined. However, the reduction in mortality due to opportunistic infection, has left patients co-infected with HIV and hepatitis viruses faced with the menace of progressive liver diseases due to HBV infection,[3,4] in addition to HCV infection.[5]

HBV co-infection or superinfection of HIV-infected patients leads to several problematic situations. First, HBV infection tends to develop into persistent infection in HIV-infected patients,[1,6,7] which is a rare event in healthy adults, although it substantially depends on the genotype of HBV.[8] It results in the acceleration of the development of cirrhosis and eventually hepatocellular carcinoma. Second, some nucleoside reverse transcriptase inhibitors (NRTI) used in HAART also have

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inhibitory effects on the replication of HBV.[9–12] A careless administration or discontinuation of NRTI on HIV–HBV co-infected patients may cause reactivation and/or aggravation of hepatitis B. In addition, the administration of anti-HBV drugs in HIV–HBV co-infection may lead to the development of drug resistance.[11,12] Third, liver injury occurs more frequently in patients on HAART who are co-infected with HIV and HBV than those infected with HIV only.[9,10]

Importantly, co-infection with HIV and HCV increases the morbidity and mortality of HIV-infected patients in Japan,[13] where the prevalence of HIV infection is increasing linearly, and is exceptionally high among developed countries.[14] There are more than 14 000 HIV-positive people in Japan as of 2006, according to the AIDS National Survey in Japan,[14] and approximately 0.8 million chronic HBV carriers.[15] However, the prevalence of co-infection with HIV and HBV in Japan has not been clarified to date. Therefore, we conducted a nationwide study by distributing a postal mail-based questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan.

PATIENTS AND METHODS

IN THE QUESTIONNAIRE, the following information was obtained from the hospitals regarding the number of patients who visited the hospitals at least once between January and December in 2006: (i) the number of HIV-positive patients; (ii) the number of hepatitis B surface antigen (HBsAg)-positive patients among (i); (iii) the number of patients among (ii) who were determined at least once to have a serum alanine aminotransferase (ALT) level higher than 100 IU/L; (iv) the number of HIV-positive patients that contracted HIV from blood products; (v) the number of HBsAg-positive patients among (iv), (vi) the number of patients among (v) who were determined at least once to have a serum ALT level higher than 100 IU/L; (vii) the number of HIV-positive patients among homosexual men, (viii) the number of HBsAg-positive patients among (vii), (ix) the number of patients among (viii) who were determined at least once to have a serum ALT level higher than 100 IU/L; (x) the number of HIV-positive patients that contracted HIV through intravenous drug use (xi) the number of HBsAg-positive patients among (x), (xii) the number of patients among (xi) who had at least one determination of a serum ALT level more than 100 IU/L; (xiii) the number of HIV-positive patients whose transmission routes were classified as "others"; (xiv) the number of HBsAg-positive patients among (xiii); and (xv) the number of patients among (xiv) who were determined at least once to have a serum ALT level higher than 100 IU/L.

The questionnaire was sent to the 372 hospitals belonging to the HIV/AIDS Network of Japan by mail. Answers were mostly returned by mail and in some cases by fax. The list of the hospitals in the HIV/AIDS Network of Japan can be viewed at http://www.acc.go.jp/mLhw/mLhw_frame.htm.

RESULTS

THE QUESTIONNAIRE WAS sent to all 372 hospitals that were on the list of the hospitals in the HIV/AIDS Network of Japan in January 2006. Two hundred and seven hospitals (55.6%) responded within the indicated period. In total, 5998 patients were reported to be HIV positive. The collection rate of 55.6% was higher than that (47.8%) for a questionnaire HIV–HCV co-infection study carried out in 2003.[15] It may appear rather low, particularly considering the number of reported HIV-positive people in 2006, which was approximately 14 000, according to the AIDS National Survey in Japan.[14] However, not all of the HIV-positive people were going to hospitals, and the answers to the questionnaire were obtained from most of the major hospitals in the HIV/AIDS Network in big cities around Japan. This suggests that not all, but a majority of HIV-positive Japanese patients were enrolled in the study.

Among the 5998 patients reported to be HIV positive, 377 (6.3%) patients were positive for HBsAg (Table 1). Of these 377 patients, 122 (32.4%) had elevated serum ALT levels at least one time during the 1-year observation period.

The HBV prevalence rates, when fractionated by the routes of transmission, were as follows: among the 508 HIV-positive patients who contracted HIV from blood products, such as unheated concentrated coagulation factors, only 30 (5.9%) were HBsAg positive, which shows a marked contrast to the prevalence of HCV in this cohort (Fig. 1).[16] Among the 23 intravenous drug users, three (13.0%) were HBsAg positive. Among the 3213 HIV-positive patients who were homosexual men, 267 (8.3%) were HBsAg positive. In the remaining 2254 patients who were HIV-positive and whose route of HIV transmission was classified as "others", most contracted HIV heterosexually. This number (2254) showed a substantial increase from the 1316 obtained in the questionnaire for the HIV–HCV co-infection study in 2003, while the total number of HIV-positive patients increased from 4877 to 5998.[16] Among these, 77 (3.4%)

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Table 1 Prevalence rates of hepatitis B virus infection among HIV-positive patients

Routes of transmission	No. patients	HBsAg positive (% in HIV positive according to route)	ALT >100 IU/L (% in HBsAg positive according to route)
Blood products	508 (5.9%)	30 (40.0%)	12
Homosexual men	3213 (8.3%)	267 (32.2%)	86
Drug addicts	23 (13.0%)	3 (66.7%)	2
Others (heterosexual etc.)	2254 (3.4%)	77 (28.6%)	22
Total	5998	377 (6.3%)	122 (32.4%)

ALT, serum alanine aminotransferase; HBsAg, hepatitis B surface antigen.

were HBsAg positive. In terms of the route of HIV infection, 267 (70.8%) of the 377 patients were homosexual men among the HIV–HBV co-infected patients. This shows a contrast to the status of HIV–HCV co-infection, in which the majority of HIV–HCV co-infected Japanese patients contracted both viruses from blood products.[16]

There were one or more HIV-positive patients in 154 (74.4%) of the 207 hospitals in the HIV/AIDS Network of Japan (Table 2). Twenty four (11.6%) of 207 hospitals had 20–49 HIV-positive patients, and 16 (7.7%) hospitals had 50 or more HIV-positive patients. There were one or more patients who were co-infected with HIV and HBV in 64 (30.9%) of the 207 hospitals. There were 10 or more HIV–HBV co-infected patients in nine (4.3%) hospitals, all of which had 50 or more HIV-positive patients (Table 2). HIV–HBV co-infected patients were concentrated in specific hospitals in big cities around Japan. In particular, in the Kanto area, HIV–HBV co-infected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area.

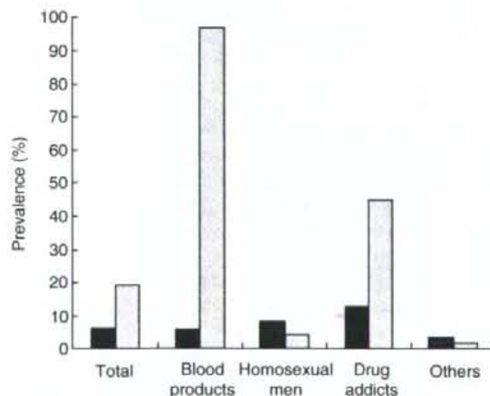

Figure 1 Prevalence rates of persistent hepatitis B virus and hepatitis C virus infections in the HIV-positive population sorted by the HIV risk group. (■), HBsAg, hepatitis B surface antigen; (□), anti-HCV, antibody to hepatitis C virus. *Prevalence rates of anti-HCV are obtained from Koike K *et al.*[16]

DISCUSSION

ALONG WITH THE increase in the number of HIV-infected patients in Japan, co-infection with HIV and hepatitis viruses has become a major medical issue. HBV infection of HIV-positive patients raises several difficult problems: HBV infection tends to develop into persistent infection, even in adults; some NRTI used in HAART also have inhibitory effects on the replication of HBV, the improper administration, or discontinuation of which may lead to drug resistance; and HIV–HBV co-infected patients on HAART have liver injuries more frequently than HIV-monoinfected patients. It is important to determine the status of HBV infection in HIV-positive patients.

According to the statistics of the Ministry of Health, Labor, and Welfare of Japan, the number of reported HIV-positive people was slightly over 14 000 in 2006.[14] In the present study, 6.4% of HIV-positive patients were positive for HBsAg, the most reliable marker for ongoing HBV infection. It might have been advantageous if

Table 2 Number of hospitals categorized according to the number of patients infected with HIV and those co-infected with HIV and hepatitis B virus (HBV)

No. HIV (+)/ HBV (+)	No. HIV(+)				
	0	1–19	20–49	50+	Total
0	53	76	13	1	143
1–9	0	38	11	6	55
10+	0	0	0	9	9
Total	53	114	24	16	207

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serum HBV–DNA levels were determined, but unfortunately, HBV–DNA level determination was not a routine laboratory test in most hospitals. In addition, considering that the antibody to the hepatitis B core antigen might be the only marker of ongoing HBV infection in some immuno-compromised patients, it would also be advantageous if this viral marker were available. These issues should be investigated in future studies. Comments from hospitals to the questionnaire included one indicating that not all HIV-positive patients underwent a test for serum HBsAg, suggesting the actual prevalence of HBsAg in HIV-infected patients might be higher than 6.4%.

In a previous questionnaire study of HIV–HCV co-infection, the prevalence of HCV infection among HIV-infected patients was 19.2%;[16] the prevalence of HBV infection (6.4%), is one-third of it. The lower positivity for HBsAg than for the anti-HCV antibody among those who contracted HIV through blood products accounts for this difference: almost all (96.9%) of the patients who contracted HIV through blood products were also anti-HCV antibody positive.[16] It should be noted that among the homosexual male patients who were HIV positive, 8.3% were HBsAg positive, which is twice as high as that of the anti-HCV antibody in these populations. A higher prevalence of HBV infection as a sexually transmitted infection than that of HCV[17] may explain the high prevalence of HBV infection in HIV-positive homosexual men. Similarly, a HBV prevalence of 3.4% in heterosexually transmitted HIV-positive patients is higher than that of the general Japanese population of the same age.[15]

Of the 377 patients who were HBsAg positive, 122 (32.4%) had elevated serum ALT levels at least once in the 1-year observation period. In this type of study using a questionnaire, it is difficult to obtain the details of patients' data, including age, body weight, and the degrees of liver injuries and fibrosis. If detailed items were included in the questionnaire, then the collection rate would be low. This time, to obtain a high collection rate, we asked whether the patients with HBsAg showed an elevated ALT level higher than 100 IU/L at least once during the 1-year observation period. We thereby do not have details on liver disease in HIV–HBV co-infected patients in the current study. Nonetheless, one-third of HIV–HBV co-infected patients have moderate liver injuries, either chronic hepatitis B or adverse effects of drugs, and are waiting for an aid for the amelioration of liver disease. A detailed analysis of the progression and activity of liver disease in HIV–HBV co-infected patients is expected.

The collection rate of the present questionnaire from the hospitals belonging to the HIV/AIDS Network was 55.6% (207 of 372). This was higher than that (47.8%) in the HIV–HCV co-infection questionnaire study carried out in 2003. The reason for this increase is not clear, but presumably the questionnaire conducted in 2003 has raised awareness among hospital staff regarding the relevance of hepatitis virus and HIV co-infection in clinical practice.

In the current study, both Japanese patients and those of other nationalities/ethnicities were included in the study. Although the ratio of newly diagnosed HIV-positive foreign people has been declining to approximately 10% in 2006, the one in total HIV positive still accounts for approximately 25% in Japan. Because the rates of the HBV carrier are different among countries, it is ideal to analyze the HBV prevalence separately according to the nationalities/ethnicities. However, in the current survey to the hospitals in HIV/AIDS Network of Japan, nationality/ethnicity was not itemized in order to make the questionnaire simple. If we would attempt to obtain such data under the approval of the ethical committee in each hospital, the response rate to questionnaire would be extremely lowered.

To establish measures that decrease the morbidity and mortality of HIV–HBV co-infected patients, it is essential to determine the current status of co-infection. In the present study, the number and transmission routes of HIV–HBV co-infected patients in Japan were determined for the first time, although detailed information on the severity and progression of liver disease in HIV–HBV co-infected patients has not been obtained yet. Undoubtedly, this will be the first step towards improving the prognosis and quality of life of Japanese patients co-infected with HIV and HBV.

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Original Article

Prevalence of coinfection with human immunodeficiency virus and hepatitis C virus in Japan

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People with human immunodeficiency virus (HIV) infection are frequently infected with hepatitis C virus (HCV), because of the common transmission routes. Since the dissemination of hyperactive antiretrovirus therapy (HAART), the morbidity and mortality associated with HIV infection have declined. However, the reduction in mortality due to opportunistic infection has made HCV-associated liver diseases the leading cause of mortality in Western countries. A similar situation is assumed in Japan, but the status of coinfection with HIV and HCV is unclear. We conducted a nationwide survey to determine the prevalence of coinfection with HIV and HCV by distributing a questionnaire to the hospitals in the HIV/AIDS Network of Japan. Among 4877 patients reported to be HIV-positive, 935 (19.2%) were also positive for the anti-HCV antibody. Most (84.1%) of the patients coinfected with HIV and HCV were recipients of blood products. These data, for the first time, show the current status of coinfection with HIV and HCV in Japan. A detailed analysis of the progression and severity of liver diseases in the coinfected patients is expected.

Key words: coinfection, hepatitis C, HIV, liver disease

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection and human immunodeficiency virus (HIV) infection are major public health problems worldwide. In the USA, the estimated prevalence of the anti-HCV antibody is 1.8%, with 2.7 million people having HCV-RNA detected in their blood, indicative of ongoing HCV infection.[1] The prevalence of HIV is <1%, and the virus is estimated to have infected approximately 800 000 people.[2] Because of the common transmission routes, that is, parenteral ones, many people with HIV infection are also infected with HCV.[3] Before the introduction of hyperactive antiretroviral treatment (HAART) in 1996, most people with HIV infection died of HIV-associated opportunistic infections such as *Pneumocystis carinii* (currently called *P. jiroveci*) pneumonia and cytomegaloviral infection. Since the dissemination of HAART, the morbidity and mortality associated with HIV infection have declined. However, the reduction in mortality due to opportunistic infection has made patients coinfected with HIV and HCV faced with the menace of progressive liver diseases due to HCV infection in the United States and Europe.[4,5]

Coinfection with HIV has been shown to increase the HCV load in HCV infection,[6] being a negative prognostic factor for clearance of HCV in anti-HCV therapy using interferon.[7,8] It also accelerates the development of cirrhosis and, eventually, hepatocellular carcinoma. Although still controversial, coinfection with HIV and HCV yields a more rapid progression to acquired immunodeficiency syndrome (AIDS) in some cases.[9,10] Importantly, coinfection with HIV and HCV will increase the morbidity and mortality of HIV-infected patients also in Japan, where the prevalence of HIV infection is increasing in a linear fashion, exceptionally among developed countries.[11] There are more than 10 000 HIV-positive people in Japan as of the end of 2004, according to the AIDS National Survey in Japan,[12] and approximately 1.8 million chronic HCV carriers, according to the estimation by the Ministry of Health, Labor and Welfare (MHLW) of Japan. However, unfortunately, the prevalence of coinfection with HIV and HCV in Japan has been unclarified to date. Therefore, we conducted a nationwide study by distributing an

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email-based questionnaire to the hospitals in the HIV/AIDS Network of Japan.

METHODS

IN THE QUESTIONNAIRE, the following information was obtained from hospitals regarding the number of patients who visited the hospitals at least once between January and December 2003: (1) the number of HIV-positive patients; (2) the number of anti-HCV-positive patients among (1); (3) the number of HCV-RNA-positive patients among (2); (4) the number of HIV-positive patients who contracted HIV from blood products; (5) the number of anti-HCV-positive patients among (4); (6) the number of HCV-RNA-positive patients among (5); (7) the number of HIV-positive patients among men who have sex with men (MSM); (8) the number of anti-HCV-positive patients among (7); (9) the number of HCV-RNA-positive patients among (8); (10) the number of HIV-positive patients who contracted HIV through intravenous drug use; (11) the number of anti-HCV-positive patients among (10); (12) the number of HCV-RNA-positive patients among (11); (13) the number of HIV-positive patients whose transmission routes were classified as 'others'; (14) the number of anti-HCV-positive patients among (13); and (15) the number of HCV-RNA-positive patients among (14).

The questionnaire was sent to the 366 hospitals in the HIV/AIDS Network of Japan by email. When emails were returned with a failure of delivery, the questionnaire was forwarded by post. Answers were mostly returned by email, and in some cases by fax. The list of the hospitals in the HIV/AIDS Network of Japan can be browsed at: http://www.acc.go.jp/mLhw/mLhw_frame.htm.

RESULTS

THE QUESTIONNAIRE WAS sent to all 366 hospitals that were on the list of hospitals in the HIV/AIDS Network of Japan in January 2004. One hundred and seventy-six hospitals (48.1%) responded within the indicated period. A collection rate of 47.8% may appear rather low, particularly considering the number of reported HIV-positive people, 10 000, in 2004 according to the statistics of the MHLW of Japan.[12] However, not all the HIV-positive cases are visiting hospitals, and answers to the questionnaire were obtained from most of the major hospitals in the HIV/AIDS Network in big cities around Japan. These factors suggest that not all but a majority of HIV-positive patients in Japan were enrolled in the study.

Table 1 Number of hospitals categorized by the number of patients infected with HIV and those coinfected with HIV and HCV

No. of HIV(+)/HCV(+)	No. of HIV(+)				
	0	1–19	20–49	50+	Total
0	43	52	5	1	101
1–9	0	45	9	3	57
10+	0	2	4	12	18
Total	43	99	18	16	176

There were one or more HIV-positive patients in 133 of 176 (75.6%) hospitals; there were no HIV-positive patients in the remaining 43 hospitals (Table 1). Eighteen of 176 (10.2%) hospitals had 20–49 HIV-positive patients, and 16 (9.1%) hospitals had 50 or more HIV-positive patients. On the other hand, there were one or more patients who were coinfected with HIV and HCV in 75 (42.6%) of 176 hospitals, and there were 10 or more HIV/HCV coinfected patients in 18 (10.2%) hospitals. HIV/HCV coinfected patients were concentrated in specific hospitals in big cities around Japan. In particular, in the Kanto area, HIV/HCV coinfected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area (Fig. 1). Of the 16 hospitals with 50 or more HIV-positive patients and of the 18 hospitals with 10 or more HIV/HCV coinfected patients, 12 were the same hospitals (Table 1). Hospitals with 10 or more HIV/HCV coinfected patients, but with less than 50 HIV-positive patients had the characteristic that most HIV-positive patients contracted HIV from blood products.

In total, 4877 patients were reported to be HIV-positive. Among these, 935 (19.2%) were positive for anti-HCV (Table 2). Of these 935 patients, 780 were HCV-RNA-positive, although it should be noted that not all the patients underwent HCV-RNA testing.

HCV prevalence when fractionated by routes of transmission was as follows. Among 811 HIV-positive patients who contracted HIV from blood products such as unheated concentrated coagulation factors, 786 (96.9%) were anti-HCV-antibody-positive. Of 20 intravenous drug users, nine (45.0%) were anti-HCV-antibody-positive. Among 2730 HIV-positive patients who were MSM (men who have sex with men), 114 (4.2%) were anti-HCV positive. In the remaining 1316 HIV-positive patients whose routes of HIV transmission

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Figure 1 Nationwide distribution of hospitals in the HIV/AIDS Network of Japan that a number of HIV-positive or HIV/HCV coinfected patients are visiting regularly. Note that in the Kanto area, HIV/HCV coinfected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area. (△) hospitals with 1–19 HIV-positive patients; (□) hospitals with 20–49 HIV-positive patients; (○) hospitals with 50+ HIV-positive patients. Hatched figures: hospitals with 10 or more HIV/HCV coinfected patients. Closed figures: hospitals with less than 10 HIV/HCV coinfected patients. For easier visual comprehension, hospitals with 19 or less HIV-positive patients and 9 or less HIV/HCV coinfected patients are omitted from the figure.

were classified as "others", most of whom contracted HIV heterosexually, 26 (2.0%) were anti-HCV-antibody-positive. On the other hand, in HIV/HCV coinfected patients, 786 (84.1%) of 935 patients were recipients of blood products. Thus, the majority of HIV/HCV coinfected patients in Japan are those who contracted HIV, and most likely also HCV, from blood products.

DISCUSSION

ACCORDING TO THE statistics of the MHLW of Japan, the number of reported HIV-positive people was just over 10 000 in 2004.[12] The total number of HIV-positive patients in the current study is approximately half of that. By a simple calculation, there would be about 1900 HIV/HCV coinfected patients in Japan. However, because HIV-positive patients who contracted HIV from blood products are almost all registered in Japan and most of them should have been enrolled in this survey, the number of HIV/HCV coinfected patients is likely smaller than 1900. It is regrettable that not all the patients underwent HCV-RNA testing, but it is unavoidable in this type of questionnaire-based study. In some cases, the existence of a positive anti-HCV antibody indicates a memory of a remote HCV infection.

Almost all of the patients who contracted HIV through blood products were also anti-HCV-antibody-positive, suggesting that both viruses were transmitted through the same route. In MSM patients who were HIV-positive, approximately 4% were anti-HCV-antibody-positive, which is about threefold higher than the prevalence of HCV in Japan.[13] In people aging from 40 to 50 years old in the general Japanese population, whose ages are similar to those of the MSM patients in the current study, the prevalence of HCV is less than 0.5%.[13] Therefore, an HCV prevalence of 4% in MSM

Table 2 Prevalence of HCV infection in HIV-positive patients

Routes of transmission	No. of patients	Anti-HCV-positive	HCV-RNA-positive†
Blood products	811	786 (96.9%)	667
MSM‡	2730	114 (4.2%)	98
Drug addicts	20	9 (45.0%)	8
Others (heterosexual etc.)	1316	26 (2.0%)	7
Total	4877	935 (19.2%)	780

†Not all patients were subjected to HCV-RNA test. ‡MSM, men who have sex with men.

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HIV-positive patients is quite high, suggesting the same route of the transmission of HIV and HCV, and a more intensive exposure to HCV or more susceptibility to HCV in these HIV-positive patients. Similarly, an HCV prevalence of 1.4% in heterosexually transmitted HIV-positive patients is higher than that of the general Japanese population of the same age.

To establish measures that decrease the morbidity and mortality of HIV/HCV coinfected patients, it is essential to recognize the current status of the coinfection. In the present study, the number and transmission routes of HIV/HCV coinfected patients in Japan were first described, although detailed information on the progression of HCV-associated liver diseases in HIV/HCV coinfected patients has not yet been obtained. Undoubtedly, this will be the first step for improving the prognosis and quality of life of patients coinfected with HIV and HCV in Japan. A detailed analysis of the progression and severity of HCV-associated liver diseases is expected.

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Short Communication

Reversal Periods and Patterns from Drug-Resistant to Wild-Type HIV Type 1 after Cessation of Anti-HIV Therapy

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ABSTRACT

Anti-HIV drug-resistant virus reverts to wild type following discontinuation of antiretroviral therapy (ART). This study aimed to determine the reversal period. ART was discontinued in 16 patients harboring drug-resistant viruses. Resistant mutations of reverse transcriptase (RT) and protease (PR) genes of plasma- and peripheral blood mononuclear cells (PBMC)-derived viruses were examined by direct sequencing monthly until the disappearance of mutants (median follow-up period: 8.9 months). Only wild-type virus was detected in 50% of patients at 6.3 months (quartiles, 3.2–20.7 months) and at 9.2 months (quartiles, 5.7–13.8 months) in plasma- and PBMC-derived viruses, respectively, after ART interruption. Among the 133 resistance-associated mutations identified at ART interruption, half the RT and PR mutations shifted to wild type in 3.2 months in plasma, 6.7 months of RT, and 5.7 months of PR in PBMC, respectively. In plasma- and PBMC-derived viruses, the PR mutations reverted earlier than the RT mutations. These results could be relevant as to when to perform drug-resistance testing.

THE EMERGENCE OF DRUG-RESISTANCE-ASSOCIATED MUTATIONS leads to treatment failure and may limit future treatment options. Therefore, inclusion of drug-resistance testing is recommended in anti-HIV-1 treatment guidelines, especially after failure of standard regimens.[1,2] A number of studies showed that drug-resistance testing improved the benefits of antiretroviral therapy (ART).[3–8] For drug resistance testing, plasma and peripheral blood mononuclear cell (PBMC) can be used as clinical specimens.[9] Using direct sequencing, we reported previously the earlier detection of resistant mutations in plasma than in PBMC.[10] Accordingly, we recommended the use of plasma for early detection of drug resistance during therapy in those patients who fail to respond to antiretroviral treatment. Clinically, even when patients develop virologic failure [rebound of plasma HIV-1 viral load (VL)], the CD4 count remains sufficiently high for treatment interruption, at least in some patients. In such cases, the timing of genotypic drug resistance testing is of practical importance. Discontinuation of treatment causes the reversion of resistance mutations to wild-type viruses.[11–18] Previous studies indicated that resistance mutations of plasma viruses could rapidly become undetectable either partially or entirely from 14 days to 4 months after ART cessation.[12–18] The reversion of mutations to wild type is considered to be due to the low replication fitness of mutant variants and outgrowth of wild type viruses when the drug-selective pressure is withdrawn.[17,21–22] However, the time course and pattern of this reversion have not been studied in detail in heavily treated patients. Clarification of this issue will help determine the most appropriate time and sample for performing genotypic-resistance testing after ART cessation.

The study subjects were 16 HIV-1-infected patients who had been known to have drug-resistance virus beforehand and discontinued antiretroviral therapy from August 1998 through December 2002 for a variety of reasons. All patients regularly consulted the AIDS Clinical Center at the International Medical Center of Japan, Tokyo, and gave written informed consent. Their demographic data and clinical characteristics at the time of quitting ART are listed in Table 1. Their blood samples were

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TABLE 1. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF PATIENTS[a]

Pt	Sex	Age (years)	Risk factor	CD4 cells/μl	log_{10} VL month 0	log_{10} VL month 1	Duration of ART (months)	Drugs ever used	Reasons of ART stop
1	M	40	Bisexual	404	5.2	5.3	70.4	AZT, 3TC, d4T, ddI, NFV, RTV, SQH	Virological failure
2	M	23	Hemophilia	103	5	5	46.6	ddC, d4T, 3TC, AZT, ddI, SQH, NFV, IDV	Virological failure
3	M	36	MSM	209	3.5	3.5	91.8	AZT, 3TC, d4T, ddI, NFV	Virological failure
4	M	22	Hemophilia	116	5.1	4.3	71.9	ddC, d4T, 3TC, ABC, ddI, AZT, EFV, SQH, NFV, APV	Virological failure
5	M	26	Hemophilia	30	5.7	5.2	50.2	d4T, 3TC, ddI, AZT, NFV, IDV, RTV	Virological failure
6	M	28	Hemophilia	93	3.6	3.7	129.7	d4T, ddI, AZT, 3TC, ABC, ddC, EFV, RTV, SQH, IDV, NFV, APV	Virological failure
7	M	29	Hemophilia	698	1.7	5	108.6	AZT, 3TC, d4T, ddI, ABC, EFV, RTV, SQH	Side effects
8	M	24	Hemophilia	35	5.1	5.1	67	AZT, 3TC, ddC, SQH, RTV	Virological failure
9	F	42	Heterosexual	690	1.8	4.4	90	AZT, ddI, d4T, 3TC, ABC, EFV, SQH, APV, NFV	Poor adherence
10	M	19	Hemophilia	586	1.8	4.2	107.6	AZT, ddC, d4T, 3TC, ddI, ABC EFV, IDV, RTV, NFV, SQH, LPV/r	Poor adherence
11	M	24	Hemophilia	644	1.7	4.5	117.6	AZT, ddI, 3TC, IDV, RTV	Poor adherence
12	M	22	Hemophilia	138	4.5	4.5	53.4	AZT, ddC, d4T, 3TC, ddI, NVP, SQH, RTV, IDV	Virological failure
13	M	34	Bisexual	276	1.7	3.6	47.6	d4T, 3TC, ddI, ABC, AZT, NFV, IDV, RTV, LPV/r	Poor adherence
14	M	42	MSM	420	4.3	5.3	49.8	AZT, 3TC, d4T, ddI, IDV, RTV, SQH	Virological failure
15	M	39	Hemophilia	544	1.7	4.5	140.8	AZT, d4T, 3TC, NVP, NFV	Side effects
16	M	37	Bisexual	525	4.4	4.6	69.9	AZT, ddC	Virological failure
Mean		30.4		344	3.5	4.5	82		

[a]M, male; F, female; MSM, men having sex with men; VL, HIV-1 viral load in plasma; month 0, time when ART was stopped; month 1, 1 month after ART was stopped. AZT, zidovudine; 3TC, lamivudine; d4T, stavudine; ddI, didanosine; ddC, zalcitabine; ABC, abacavir; EFV, efavirenz; NVP, nevirapine; NFV, nelfinavir; RTV, ritonavir; SQH, saquinavir hard gel capsule; DOV, indinavir; APV, amprenavir; LPV/r, lopinavir + ritonavir.

collected monthly. Measurements of VL (Amplicor HIV-Monitor, Roche Molecular Systems, Inc., NJ) and CD4 and CD8 lymphocyte counts (monoclonal antibodies and flow cytometry) were performed at each blood sampling.

PBMC were separated by centrifugation from 7 ml EDTA-treated blood. PBMC and plasma were stored at −80°C until sequence analysis. The method of sequence analysis was reported previously.[10] Briefly, total RNA was extracted from 100 μl plasma and DNA was extracted from 1×10^6 PBMCs (SMITEST Ex R&D Kit, Japan). The RNA sample was subjected to reverse transcription (RT) followed by nested polymerase chain reaction (PCR) using primers targeting the RT gene and protease (PR) gene, respectively. A DNA sample was also subjected to nested PCR using the same primers for the same targets. The primers covered 1–100 base pairs of PR and 40–240 base pairs of RT. Sequences of primer sets were published elsewhere.[10] Direct sequencing was performed on a 3730 DNA Analyzer (Applied Biosystems). A heterozygous base sequence was identified when the electrogram showed a minor peak at >50% of the major peak. The amino acid sequence was deduced with the GENETYX-WIN version 4.1 (Software Development, Tokyo) and the amino acid substitutions related to drug resistance were estimated from published data.[2] The clade of HIV-1 was determined by the sequences of RT and PR genes.

The reversal period was defined as the time interval between the date of ART interruption and the date of the disappearance of mutations confirmed by direct sequencing. When mutations (all minor mutations, in some patients) did not revert, the reversal period was defined as the date ART stopped to the date most mutations shifted to the wild-type amino acid sequence (for example, see Table 2; protease residues of plasma virus at month 5.9 of patient 2). As all HIV-1s amplified in this study were HIV-1 clade B, we regarded L63P as the polymorphism. The major mutant residues included M41L, A62V, D67N, K70R, L74V, M184V, G190S, L210W, T215F/Y, and K219E/Q of RT mutations and D30N, L33F, M46I, G48V, V82A/F, I84V, and L90M of PR mutations.[2] The follow-up period was the time interval from when ART was interrupted to when the resistance mutations disappeared.

A Kaplan–Meier survival curve was used to estimate the continuous periods of resistance mutations. The Mann–Whitney *U* test was used for group comparisons, the Wilcoxon signed rank test was used for paired comparison of the reversal period, the paired *t*-test was used for changes in CD4 count and HIV-1 viral load, and correlation analysis was used for the relationship between the reversal period and baseline CD4 count or baseline viral load, respectively. StatView version 5 was used for analysis and a *p* value less than 5% was considered statistically significant.

As shown in Table 1, most patients enrolled in this study had been treated over a long period of time [mean ART period: 82 months (SD, 31.6; range, 46.6–140.8 months)]. The reasons for discontinuation of ART were virologic failure in 10 cases, poor adherence in 4 cases, and side effects in 2 cases. The median follow-up period was 8.9 months (range: 2–25 months) and all patients provided blood samples for testing. None of the patients received any ART during the follow-up period. CD4 counts of 10 patients were more than 200/μl at the time of ART discontinuation. After withdrawal of ART, the CD4 count decreased a mean value of 66/μl 1 month later and continued to decrease until the disappearance of resistant mutations. The VL of 6 patients (patients 7, 9, 10, 11, 13, and 15) who discontinued ART because of side effects or poor adherence ranged from <50 to 650 copies/ml at the time of ART cessation. The VL of these patients rebounded to a mean of 4.2 $\log_{10}$ copies/ml 1 month later (designated as rebounded virus) but showed a plateau level thereafter. The VL of the other 10 patients who discontinued ART for virologic failure was stable after ART cessation.

In all 16 patients, a total of 133 resistance mutation residues with 59 RT and 74 PR were found in plasma and PBMC. The concordance of mutant residues between plasma and PBMC was 96.2% (RT mutations 93.2%, PR mutations 98.6%). All 16 patients possessed RT resistance mutations but 4 of them had no PR mutations (Table 2). In PR, both plasma and PBMC-derived viruses had 26 major resistance and 48 minor resistance residues. In contrast in RT, 52 and 50 major RT residues and 7 and 9 minor RT residues were detected in plasma and PBMC, respectively. The results showed that the resistance mutations could shift to wild type after 1 month or could persist for as long as 22 months after treatment stopped. Interestingly, in 6 patients with viral load rebound, the rebounded viruses in 5 patients (patients 7, 10, 11, 13, and 15) had the same resistant mutations as their predecessor viruses 1 month after ART cessation and then reverted to wild type thereafter. In patient 9, the rebounded virus was a wild-type virus.

As shown in Fig. 1A, after ART interruption, only wild-type virus was detected in 50% of patients at 6.3 months (quartiles, 3.2–20.7 months) and at 9.2 months (quartiles, 5.7–13.8 months) in plasma- and PBMC-derived viruses, respectively. In Fig. 1B, the reversion of 133 resistance mutations is shown by a Kaplan–Meier survival curve. Fifty percent of both PR and RT resistance mutations shifted to wild type in 3.2 months in plasma (quartiles, 1.5–3.7 months for PR, 2–10 months for RT). However, in PBMC, 50% of PR and RT mutations disappeared in 5.7 (quartile, 3.2–6.7 months) and 6.7 (quartile, 3.5–12 months) months, respectively. The reversal period of PR and RT mutations in plasma was 2.5 and 3.5 months, respectively, less than that in PBMC (both $p < 0.05$). Furthermore, the PR mutations shifted to wild type much more rapidly than RT mutations in both plasma and PBMC, although the half life of both mutation residues were the same in plasma (Wilcoxon test $p < 0.05$). In terms of the reversal period of major and minor mutations, there were no difference between them both in the PR and RT regions of plasma- or PBMC-derived viruses. There were no relationships found between the reversal periods of RT and PR mutations and the baseline CD4 cell count, baseline VL, and changes in these two surrogate markers 1 month later (data not shown).

Figure 2 shows how the mutation residues disappeared after ART cessation. We roughly divided the reversal process into two patterns. The first pattern was that resistant mutations persisted for some time and then disappeared abruptly (Fig. 2A). Most PR mutations of plasma viruses, 50% of PR mutations of provirus, and 50% of RT mutations in both types of specimens showed this pattern. The second pattern was that of a gradual decrease of mutations followed by their disappearance or persistence (Fig. 2B). One-third of RT mutations showed this pattern. Overall, all major mutations of RT and PR genes disappeared in all patients after withdrawal of ART. In contrast, the minor mutations did not disappear in some patients.

Table 2. Resistance Mutations and Reversal Period in Plasma and PBMC after ART Cessation

Pt	Sample	Months after ART cessation	Reverse transcriptase residues	Months after ART cessation	Protease residues
1	Plasma	0	41L, 69D, 118I, 210W, 215Y	0	10I, 30N, 33F, 71T, 84I, 88D, 90M
		3.2	—[a]	3.2	—
	PBMC	0	41L, 69D, 118I, 210W, 215Y	0	10I/L, 30N/D, 33F/L, 71T/A, 84I/V, 88D/N, 90M
		3.2	—	3.2	—
2	Plasma	0	41L, 67N, 69D, 118I, 210W, 215Y	0	10I, 20M, 36I, 48V, 54V, 82A
		15.2[b]	41L, 210W/R	5.9	10F, 36I
	PBMC	0	41L, 67N, 69D, 118I, 210W, 215Y	0	10I, 20M, 36I, 48V, 54V, 82A
		15.2[b]	41L, 118I, 215Y	9	10F, 36I
3	Plasma	0	41L, 44D, 184V, 215Y	0	30N, 71V, 77I, 88D
		7	—	4.6	—
	PBMC	0	41L, 44D, 184V, 215Y	0	30N, 71V, 77I, 88D
		8.6	—	4.6	—
4	Plasma	0	41L, 74V, 184V, 215Y	0	10I, 20I/M, 71V, 73S, 84V, 90M
		2.8	—	4.8	—
	PBMC	0	41L, 184V, 215Y	0	10I/L, 20I, 71V/A, 73S/G, 84V/I, 90M
		4.8	—	4.8	—
5	Plasma	0	41L, 44D, 67N, 210W, 215Y	0	10I, 46I, 71T, 73S, 77I, 82F, 90M
		7.9	—	3.3	10I, 77I
	PBMC	0	41L, 44D, 67N, 184V, 210W, 215Y	0	10I, 46I, 71V, 73S, 77I, 82F, 90M
		12.5	—	6.7	10I, 77I
6	Plasma	0	41L, 74V, 184V, 215Y	0	10I, 46I, 54L, 71V, 77I, 84V, 90M
		6.3	—	1.4	77I
	PBMC	0	41L, 74V/L, V118I, 184V/M, 190G/S, 210W, 215Y	0	10I, 20M/K, 46I, 54L/I, 71V, 77I, 84V, 90M
		11.3	—	3.2	77I
7	Plasma	0	67N, 70R, 184V, 219Q	0	N[c]
		1	67N/D, 70R, 184V/M, 219Q/K		
		3.2	—		
	PBMC	0	67N, 70R, 184V, 219Q	0	N
		9.2	—		
8	Plasma	0	41L, 184V, 215F	0	20R, 36I, 54V, 71V, 82A, 90M
		5.7	—	3.7	—
	PBMC	0	41L, 184V, 215F	0	20R, 36I, 54V, 71V, 82A, 90M
		5.7	—	5.7	20R,
9	Plasma	0	41L, 67N, 70R, 215F, 219E	0	10L/I, 36I, 73S, 77I, 90M
		1	—	1	—
	PBMC	0	67N, 184M/N, 210W, 219E	0	10L/I, 71T/I, 73S, 77I, 90M
		2	—	1	—

10	Plasma	0	41L, 67N, 215F, 219Q	0	10I, 36I, 46I, 53L, 71V, 84V, 90M
		1	41L, 67N, 215F, 219Q		
		8.5[d]	219Q/K	3.7	10I, 36I
	PBMC	0	41L/M, 67N, 70R, 118V/I, 184V/M, 215F, 219Q	0	10I, 36I, 46I, 53L/F, 71V, 84V, 90M
		8.5[d]	67N, 70R, 219Q	7.9	10I, 36I
11	Plasma	0	—	0	N
		1.5	67N, 70R, 219Q		
		24	67N, 69N/D, 219Q		
	PBMC	0	N	0	N
12	Plasma	0	184V, 62V	0	10I, 48V, 71T, 77I, 82A, 90M
		2	—	2.2	71T, 77I
	PBMC	0	184V, 62V	0	10I, 48V, 71T, 77I, 82A, 90M
		2.2	—	5	71T, 77I
13	Plasma	0	67N, 184V	0	10I, 46I, 71V, 77I, 88S
		1	67N, 184V	1	10I, 36M/I, 71V/T
		3.5	—	2.3	10I, 71V/T
	PBMC	0	67N/D, 184V/M	0	10I, 46M/I, 71V/T, 77I
		13.8	—	7.4	10I, 36I/M, 71V/A
14	Plasma	0	184V	0	10I, 20R, 24I, 36I, 53L, 54V, 71T, 82A
		1	—	1	—
	PBMC	0	N	0	10I/L, 20R/K, 24I/L, 36I, 53L/F, 54V, 71V/A, 82V/A
				6.2	—
15	Plasma	0	67N, 70R, 219Q	0	N
		1	67N, 70R		
		20.7	—		
	PBMC	0	67N, 70R, 219Q	0	N
		22.5	67N, 219Q		
16	Plasma	0	67N/D/G, 69A/D, 70R, 219Q	0	N
		19.5	219Q		
	PBMC	0	69A/D, 70R, 219Q	0	N
		19.5	69A/D, 219Q		

[a]—, wild type.
[b]This patient died at this time point with RT mutations detected.
[c]N, no resistance mutations.
[d]New ART was introduced at the time.

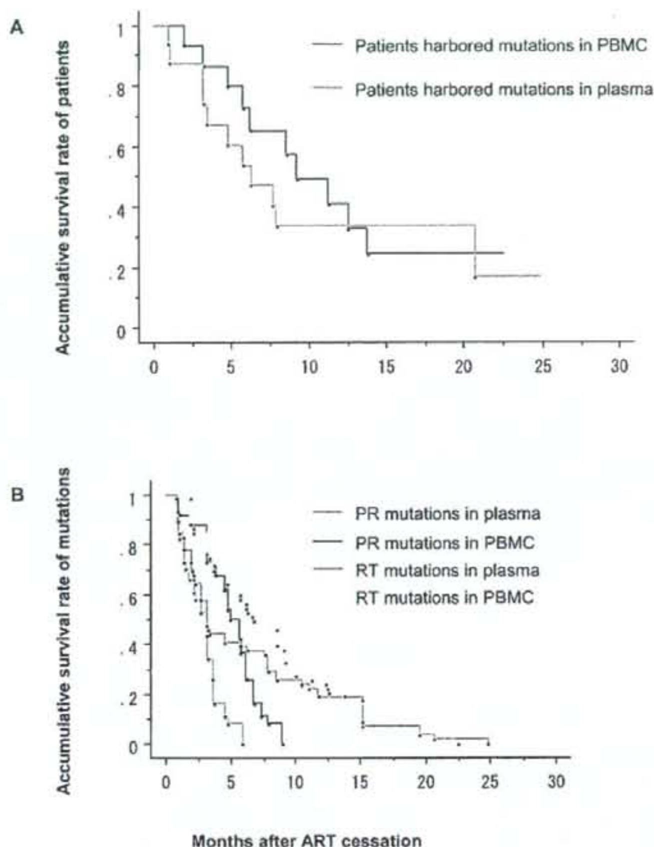

FIG. 1. (**A**) Kaplan–Meier curves showing percent of 16 patients with drug resistance mutations in plasma or PBMC. (**B**) Kaplan–Meier curves showing percent of 133 drug resistance mutations (59 RT and 74 PR) in plasma or PBMC. PR, in plasma vs. in PBMC ($p < 0.05$); RT, in plasma vs. in PBMC ($p < 0.05$); PR vs. RT ($p < 0.05$).

We designed the present study with the main objectives of determining the duration of the reversal period from the presence of resistant viruses to wild-type viruses and of elucidating the reversal patterns of plasma- and PBMC-derived viruses after discontinuation of ART. To determine the duration of the reversal period (i.e., from resistant mutations of RT and PR genes of plasma viruses and proviruses to wild type), sequential specimens of plasma and PBMC from patients with resistance mutations were sequenced after ART was interrupted. We found that the PR and RT resistance mutations shifted to wild type much more rapidly in plasma than in PBMC after ART cessation. In 3.2 months after ART stopped, 50% of the resistance mutations in plasma-derived viruses shifted to wild type and 50% of the major mutations of both RT and PR regions were undetected by direct sequencing. This period was similar to that reported by other investigators.[13,14,16–20] However, 50% of the mutations of RT and PR were detected by 6.7 and 5.7 months, respectively, when PBMC samples were used. Accordingly, when the patient develops virologic failure and drug resistance testing is performed using plasma sample after 3.2 months of ART cessation, the results of the test should be interpreted with caution, especially when deciding subsequent ART regimens, because 50% of mutation residues were undetectable by testing. When a resistant virus is not detected by drug-resistant testing, therapy using the same antiretroviral drugs or the same class of agents that reveal cross resistance is usually associated with early drug failure by previously acquired resistant viruses.[23,24] Therefore, like other recommendations,[1,2] drug-resistance testing should be performed soon after ART cessation. However, according to our data, the testing period could be postponed for 2.5 months (from 3.2 to 5.7 months) after ART withdrawal if PBMCs are used instead for plasma. In this regard, PBMC is a suitable candidate specimen for drug-resistance testing during off therapy.

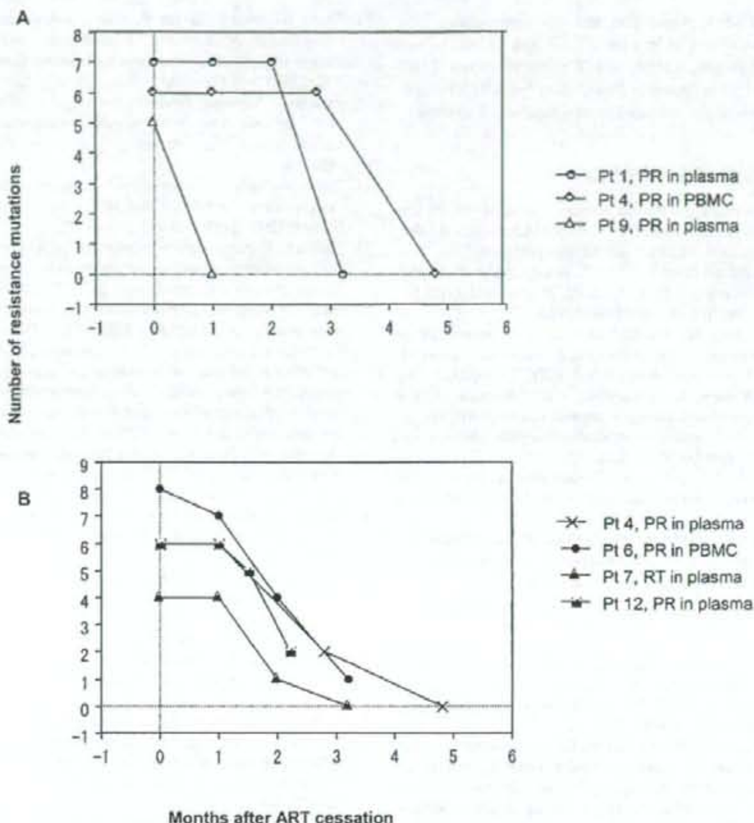

FIG. 2. Two Representative patterns of resistance mutations reverted to wild type after ART cessation. (A) Steep disappearance pattern of resistance mutations; (B) gradual reversal pattern of resistance mutations. RT, reverse transcriptase; PR, protease.

Drug-resistance testing is not advised for patients with VL <1000 copies/ml since amplification of the virus is unreliable.[1,2] However, if ART has to be discontinued because of ART-related toxicities and VL was undetectable at the time of discontinuation, the timing of the test is a practical question. Others report[17] a sharp reduction in the number of mutations at the time of viral load increase in patients during structured treatment interruption. Our results showed that at 1 month after ART cessation, VL dramatically increased from <1000 copies/ml to >4 $\log_{10}$ copies/ml in 6 patients who stopped treatment due to causes other than ART failure. However, the rebounded viruses in 5 of these 6 patients were still resistance mutant but not the wild-type virus. We previously reported that drug resistance mutations emerged gradually when therapy failed.[10] In contrast, the results here showed that nearly 50% of the mutations disappeared abruptly when ART completely stopped. Thus, waiting for several months after ART withdrawal until stabilization of the VL may potentially result in missing important information for selecting the subsequent therapeutic regimen. Therefore, in such situations, drug-resistance testing should be performed after 1 month to obtain a reliable result after ART withdrawal.

We previously studied the emergence of drug resistance during therapy and reported that the appearance of drug resistance in plasma viruses precedes that in proviruses by more than 1 year and recommended the use of plasma samples for drug-resistance testing during therapy.[10] Considering the high concordance of resistance mutations between plasma and PBMC, and the long persistence period of mutations in PBMC, we conclude that when ART stopped, if PBMC could be used as the sample for resistance assay, the test period may be postponed for 3 months.

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