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Brain Magnetic Resonance Imaging Screening Is Not Useful for HIV-1-Infected Patients Without Neurological Symptoms

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Abstract

We investigated the diagnostic usefulness of brain magnetic resonance imaging (MRI) screening in HIV-1-infected patients without neurological symptoms in detecting intracranial diseases at early stages. In this retrospective analysis, the study patients were HIV-1-infected patients who underwent brain MRI scan in clinical practice between 2001 and 2013. We excluded patients with MRI for (1) follow-up examination for prediagnosed intracranial diseases, (2) cancer staging, (3) screening mycobacterium/bacteria/fungi disease proliferation in the brain, and (4) evaluation for meningitis/encephalitis. The study patients ($n=485$) were classified into two groups: those who underwent brain MRI scan without any neurological symptoms/signs (asymptomatic patients, $n=158$) and those who underwent MRI due to such symptoms (symptomatic patients, $n=327$). Asymptomatic patients had lower CD4 counts than symptomatic patients (median 78 versus 241/μl). Intracranial diseases were detected in three (2%) of the asymptomatic patients [two toxoplasmosis and one progressive multifocal leukoencephalopathy (PML)] compared to 58 (19%) of the symptomatic patients (the χ^2 test, $p<0.01$). The latter included toxoplasmosis ($n=10$), PML ($n=7$), cytomegalovirus encephalitis ($n=3$), primary central nervous system lymphoma ($n=3$), cryptococcoma/meningitis ($n=3$), and HIV-associated dementia ($n=17$). Among symptomatic patients, intracranial diseases were common in those with slurred speech (3/6, 50%), seizure (4/10, 40%), eyesight/vision abnormality (5/16, 31%), altered mental status (8/31, 26%), and hemiplegia/numbness (13/50, 26%). For patients with CD4 count $<200/\mu$l, intracranial diseases were detected in only 3 (3%) of 144 asymptomatic patients, compared with 46 (32%) of 113 symptomatic patients ($p<0.01$). Brain MRI screening for HIV-1-infected patients without neurological symptoms is of little value.

Introduction

Patients with advanced HIV-1 infection are prone to develop intracranial opportunistic diseases, such as toxoplasma encephalitis, primary central nervous system lymphoma (PCNSL), progressive multifocal leukoencephalopathy (PML), and cytomegalovirus (CMV) encephalitis.[1] Although the introduction of antiretroviral therapy (ART) substantially decreased the incidence of neurological opportunistic infections,[2,3] such diseases have high associated mortality even with appropriate treatment, and recurrences and residual neurological deficits can occur.[4,5] Because delayed diagnosis of these intracranial diseases has a detrimental effect on patients with HIV-1 infection,[5,6] early diagnosis, not to mention prevention, of such diseases is of importance.

Brain magnetic resonance imaging (MRI) is often preferred to computed tomography (CT) in establishing the diagnosis of many of these diseases due to its superior sensitivity to subtle white matter and meningeal disease.[7–10] However, there is no information on the utility of brain MRI screening for HIV-1-infected patients without neurological symptoms/signs in detecting intracranial opportunistic diseases at early stages. This observational study was designed to assess the usefulness of brain MRI screening of such patients with HIV-1 infection.

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Materials and Methods

Study design, setting, and participants

We conducted an observational single-center study to investigate the usefulness of brain MRI screening in HIV-1-infected patients without neurological symptoms who warrant investigation for intracranial diseases. The study was conducted at the AIDS Clinical Center, National Center for Global Health and Medicine (NCGM), Tokyo, the largest referral center for HIV care in Japan.[11] The study patients were those who fulfilled the following inclusion criteria: HIV-1-infected patients who underwent brain MRI scan in clinical practice between June 2001 and August 2013. In addition, the following exclusion criteria were applied: patients who underwent brain MRI for (1) follow-up examination during the study period because of intracranial diseases such as opportunistic infections, stroke, or malignancy, which were diagnosed prior to the referral to our clinic, (2) staging of malignant tumors, (3) screening mycobacterium/bacteria/fungi disease proliferation in the brain in patients who were already diagnosed with mycobacterial diseases or bacteremia or fungemia, and (4) evaluation of meningitis/encephalitis.

The study patients ($n=485$) were classified into those who underwent brain MRI scan without any neurological symptoms, such as seizure, altered mental status, hemiplegia/numbness, headache, or fever (asymptomatic patients, $n=158$), and those who underwent MRI due to the abovementioned symptoms, which can suggest a focal brain lesion[5] (symptomatic patients, $n=327$). Asymptomatic patients included those who underwent MRI due to positive antitoxoplasma IgG antibody ($n=38$) and positive serum cryptococcal antigen ($n=1$). At our clinic, patients with a low CD4 cell count (typically less than 200/μl) often underwent brain MRI even though they had no neurological symptoms/signs that would warrant a brain imaging examination to rule out intracranial opportunistic infections or malignancy at early stages.

The study was approved by the Human Research Ethics Committee of NCGM. All patients included in this study provided written informed consent for their clinical and laboratory data to be used and published for research purposes. The study was conducted according to the principles expressed in the Declaration of Helsinki.

Measurements

At our hospital, brain MRI was routinely read by one experienced radiologist and the findings were confirmed by another radiologist. Furthermore, the MRI diagnosis was confirmed by reviewing the medical records and follow-up brain imaging when available. The diagnostic criteria for cryptococcal meningitis, cytomegalovirus encephalitis, and toxoplasmic encephalitis were those adopted by the AIDS Clinical Trials Group (ACTG)-A5164.[12] HIV-associated dementia in this study was diagnosed based on the MRI findings, which included generalized atrophy and prominent white matter changes plus cognitive impairment based on the chart review, and not necessarily required neurocognitive function tests.[8] The reasons for conducting an MRI were also extracted from the medical records. Baseline characteristics and HIV-1-related variables at the time of brain MRI were also extracted from the medical records. They included age, sex, ethnicity, history of AIDS, route of HIV-1 transmission, treatment status for HIV-1 infection (either treatment naive or experienced), CD4 cell count, and HIV viral load. For CD4 count and HIV load, we used data collected closest to and preceding by up to 3 months the day of the brain MRI. In Japan, because the prescription period under the health care system is limited to 3 months, patients need to visit the HIV Clinic at least once every 3 months for prescriptions as well as monitoring CD4 cell count and HIV-1 load.[11]

Statistical analysis

Baseline characteristics were compared between asymptomatic and symptomatic patients using the Student's t-test and χ^2 test (Fisher's exact test) for continuous and categorical variables, respectively. Prevalence of intracranial diseases was calculated among asymptomatic patients and compared to that of symptomatic patients with the χ^2 test. The logistic regression model was used to estimate the associations of lack of neurological symptoms/signs over the presence of such symptoms/signs with the MRI findings of intracranial diseases. The model was adjusted for age, sex, CD4 count, HIV treatment status, and history of AIDS. Subgroup analysis included the prevalence of intracranial diseases in patients with a CD4 count $<200/\mu$l. Statistical significance was defined as two-sided p values <0.05. We used odds ratios (ORs) with 95% confidence intervals (95% CIs). All statistical analyses were performed with The Statistical Package for Social Sciences ver. 21.0 (SPSS, Chicago, IL).

Results

The study included 485 patients who underwent a brain MRI scan in clinical practice, of whom 158 had no neurological symptoms (asymptomatic) and 327 did have such symptoms (symptomatic). Of the total patients, 475 (98%) were Asians, 446 (92%) were males, and 365 (75%) were infected with HIV-1 through homosexual contact (Table 1). The median age of the study patients was 41 [interquartile range (IQR) 34–51]. Asymptomatic patients had a lower CD4 count [median 78/μl, interquartile range (IQR) 21–237, symptomatic: 241/μl, 60–470 ($p<0.01$)] and higher HIV-1 viral load [4.84 $\log_{10}$/ml, IQR 2.97–5.62, symptomatic: 2.95 $\log_{10}$/ml, 1.70–5.11 ($p<0.01$)] than symptomatic patients. Asymptomatic patients were more likely to be treatment naive (68% versus 41%, $p<0.01$) and have a history of AIDS (62% versus 47%, $p<0.01$). There was no significant difference in other baseline characteristics between the two groups (Table 1).

Among the 158 asymptomatic patients, brain MRI screening detected toxoplasmosis ($n=2$) and PML ($n=1$, with CD4 43/μl), i.e., a prevalence of intracranial diseases of 2%. The two patients with toxoplasmosis underwent brain MRI due to positive antitoxoplasma IgG antibody with a titer of 20,480 (CD4 168/μl) and 1,280 (CD4 16/μl) IU/ml. In asymptomatic patients who underwent brain MRI due to positive antitoxoplasma IgG antibody, intracranial diseases were detected in 3 (8%) out of 38 patients (Table 2). On the other hand, brain MRI for symptomatic patients detected 58 intracranial diseases with a prevalence of 19%. The cases included toxoplasmic encephalitis ($n=10$), PML ($n=7$), CMV encephalitis ($n=3$), PCNSL ($n=3$), cryptococcosis/meningitis ($n=3$), herpes simplex virus encephalitis ($n=1$), HIV-associated dementia ($n=17$), acute cerebral infarction ($n=8$), gummatous

TABLE 1. CLINICAL CHARACTERISTICS OF THE STUDY PATIENTS ACCORDING TO NEUROLOGICAL SYMPTOMS

	All patients (n=*485*)	*Patients without neurological symptoms* (n=*158*)	*Patients with neurological symptoms* (n=*327*)	p *value*
Male sex, *n* (%)	446 (92)	146 (92)	300 (92)	0.86
Age[†]	41 (34–51)	42 (33–52)	41 (35–49)	0.95
Asian, *n* (%)	475 (98)	154 (98)	321 (98)	0.74
CD4 cell count (/μl)[a]	178 (41–420)	78 (21–237)	241 (60–470)	<0.01
HIV-1 load ($\log_{10}$/ml)[a]	4.20 (1.70–5.26)	4.84 (2.97–5.61)	2.95 (1.70–5.11)[b]	<0.01
Homosexual contact, *n* (%)	364 (75)	117 (74)	247 (76)	0.74
Treatment naive, *n* (%)	240 (50)	107 (68)	133 (41)	<0.01
History of AIDS, *n* (%)	250 (52)	98 (62)	152 (47)	<0.01

[a]Median (interquartile range).
[b]Data on HIV-1 load are not available for two patients.

syphilis ($n=1$), tuberculoma ($n=1$), metastatic cancer ($n=1$), chronic subdural hematoma ($n=1$), schwannoma ($n=1$), and progressive supranuclear palsy ($n=1$) (Table 2). In asymptomatic patients, intracranial diseases were less likely to be detected by brain MRI, compared to symptomatic patients [by univariate and multivariate analysis (OR=0.1; 95% CI, 0.03–0.29; $p<0.01$) (adjusted OR=0.1; 95% CI, 0.02–0.17; $p<0.01$)]. Patients with higher CD4 counts were also less likely to have intracranial diseases (per 100/μl increment, adjusted OR=0.7; 95% CI, 0.55–0.83; $p<0.01$). Among the symptomatic patients, those who presented with slurred speech, seizure, eyesight/vision abnormality, altered mental status, and hemiplegia/numbness were highly likely to have intracranial diseases, with a prevalence of 50%, 40%, 31%, 26%, and 26%, respectively (Table 3).

Subgroup analysis limited to data of patients with CD4 count of <200/μl showed that the abovementioned three intracranial diseases were detected in 144 asymptomatic patients with a prevalence of 3%, compared to 46 (32%) of 113 symptomatic patients (asymptomatic over symptomatic, OR=0.1; 95% CI, 0.02–0.19; $p<0.01$) (Table 2). Only a few intracranial opportunistic diseases were diagnosed in patients with a CD4 count of ≥200/μl; PCNSL ($n=1$), HIV-associated dementia ($n=4$), acute cerebral infarction ($n=6$), metastatic cancer ($n=1$), and progressive supranuclear palsy ($n=1$).

Discussion

In this observational study of patients who underwent brain MRI screening in clinical practice, only 2% of patients without neurological symptoms/signs that warranted investigation of intracranial diseases were found to have intracranial diseases, whereas a significantly higher prevalence (19%) of intracranial diseases was detected in patients who underwent brain MRI due to such symptoms. Among patients with a CD4 count of <200/μl, who are reported to be at high risk for intracranial diseases,[5,10] the result was similar; 3% and 32% of asymptomatic and symptomatic patients, respectively, were found to have intracranial diseases. On the other hand, high detection rates of intracranial diseases by brain MRI were observed in patients who presented with slurred speech (50%), seizure (40%), eyesight/vision abnormality (31%), altered mental status (26%), and hemiplegia/

TABLE 2. PREVALENCE OF INTRACRANIAL DISEASES DETECTED BY BRAIN MAGNETIC RESONANCE IMAGING ACCORDING TO NEUROLOGICAL SYMPTOMS

Intracranial diseases	*Patients without neurological symptoms* (n=*158*)	*Patients without neurological symptoms with CD4 <200/µl* (n=*144*)	*Patients with neurological symptoms* (n=*327*)	*Patients with neurological symptoms with CD4 <200/µl* (n=*113*)	*Positive toxoplasma Ab and without neurological symptoms* (n=*38*)
Toxoplasmosis	2 (1)	2 (2)	10 (3)	10 (7)	2 (1)
PML	1 (1)	1 (1)	7 (2)	7 (5)	1 (1)
HIV-associated dementia			17 (6)	13 (9)	
Malignant lymphoma			4 (1)	3 (2)	
CMV encephalopathy			3 (1)	3 (2)	
Cryptococcoma/meningitis			3 (1)	3 (1)	
HSV encephalopathy			1	1	
Gummatous syphilis			1	1	
Tuberculoma			1	1	
Metastatic cancer			1		
Cerebral infarction			8 (3)	2 (1)	
Others			3 (1)	2 (1)	
Total	3 (2)	3 (3)	59 (19)	46 (32)	3 (8)

Data are numbers (percentages) of patients.
Ab, antibody; PML, progressive multifocal leukoencephalopathy; CMV, cytomegalovirus; HSV, herpes simplex virus.

Table 3. Prevalence of Intracranial Diseases Detected by Brain Magnetic Resonance Imaging According to Neurological Symptom Categories

	Intracranial diseases	*Prevalence of intracranial diseases*
Slurred speech ($n=6$)	Cerebral infarction $n=2$ PML $n=1$	50%
Seizure ($n=10$)	Toxoplasmosis $n=2$ PML $n=1$ HSV encephalitis $n=1$	40%
Eyesight/vision abnormality ($n=16$)	Malignant lymphoma $n=2$ HIV-associated dementia $n=2$ Metastatic cancer $n=1$	31%
Altered mental status ($n=31$)	Toxoplasmosis $n=2$ HIV-associated dementia $n=2$ Cryptococcoma/meningitis $n=2$ PML $n=1$ Tuberculoma $n=1$	26%
Hemiplegia/numbness ($n=50$)	Cerebral infarction $n=5$ Toxoplasmosis $n=3$ PML $n=3$ HIV-associated dementia $n=1$ Other $n=1$	26%
Neurocognitive impairment ($n=62$)	HIV-associated dementia $n=9$ Cerebral infarction $n=1$ CMV encephalitis $n=2$	19%
Fever work-up ($n=12$)	Malignant lymphoma $n=1$ HIV-associated dementia $n=1$	17%
Dizziness/vertigo/tinnitus ($n=45$)	Toxoplasmosis $n=1$ PML $n=1$ Malignant lymphoma $n=1$ HIV-associated dementia $n=1$ CMV encephalitis $n=1$	11%
Abnormal ophthalmologic examination ($n=11$)	HIV-associated dementia $n=1$	9%
Headache ($n=49$)	Toxoplasmosis $n=2$	4%
Syncope ($n=16$)		0%

PML, progressive multifocal leukoencephalopathy; HSV, herpes simplex virus; CMV, cytomegalovirus.

numbness (26%). The present study indicates that brain MRI screening for HIV-1-infected patients without neurological symptoms/signs, even those with a low CD4 count (< 200/μl), is of little value. In contrast, MRI screening is useful for patients with particular neurological symptoms/signs. These findings can help reduce unnecessary brain MRI examinations and can be helpful in clinical decision making.

Interestingly, in both of the two asymptomatic toxoplasmic encephalitis patients who underwent brain MRI screening because of positive antitoxoplasma IgG antibody, the antibody titer was very high (20,480 IU/ml and 1,280). Together with the fact that the prevalence of intracranial diseases in asymptomatic patients with positive antitoxoplasma IgG antibody was higher (8%) than the 2% in the entire group of asymptomatic patients, brain MRI screening for patients without neurological symptoms/signs who presented with high antitoxoplasma antibody may be of value and clinically justifiable.

Our study has certain limitations. First, because brain MRI was performed at the discretion of the treating physician, patient selection bias, especially among those without neurological symptoms/signs, cannot be ruled out. However, we had a large number of study patients, and considering the availability and cost of an MRI scan, the results of the present study are of value and are useful in clinical decision making. Second, because endemic opportunistic infections vary depending on the region[13,14] and the majority of our patients were Asian, the results of the present study might not be applicable to patients in other regions. Third, in this study the diagnosis of HIV-associated dementia was based on the MRI findings plus cognitive impairment based on a chart review, and the patients did not necessarily undergo neurocognitive function tests.[8] This is because the present study included patients from 2001, long before the diagnostic Frascati criteria for an HIV-associated neurocognitive disorder that required neurocognitive function tests were established.[15]

In conclusion, although our results suggest that brain MRI screening is of little value in HIV-1-infected patients without neurological symptoms/signs that warrant investigation on intracranial diseases, it should be performed in HIV-1-infected patients who present with particular neurological symptoms, such as slurred speech and seizure.

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Author Disclosure Statement

No competing financial interests exist.

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Short Report: Asymptomatic Intestinal Amebiasis in Japanese HIV-1–Infected Individuals

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Abstract. Seventy-one asymptomatic human immunodeficiency virus-1 (HIV-1) -infected individuals who underwent colonoscopy for detection of diseases other than amebiasis were included in this study. Ulcerative lesions caused by *Entamoeba histolytica* were identified by colonoscopy and biopsy in 11.3% (8 of 71) of individuals. Stool microscopic examination hardly identified *Entamoeba*, whereas serum antibody against *E. histolytica* was often elevated in patients with subclinical intestinal amebiasis. Human leukocyte antigen (HLA) class II allele against *E. histolytica* infection (DQB1*06:01) was frequently identified in these patients. This study emphasizes the endemic nature of *E. histolytica* infection in our cohort and the difficulties in epidemiological control.

INTRODUCTION

Invasive amebiasis caused by *Entamoeba histolytica* is the second most common cause of parasite infection-related mortality worldwide, accounting for 40,600 to 73,800 deaths annually.[1] Recent studies indicated that invasive amebiasis is prevalent in not only developing countries, where food or water is contaminated with stool, but also, East Asian developed countries, including Japan, as a sexually transmitted infection.[2–5] We reported previously high seropositivity for *E. histolytica* among asymptomatic human immunodeficiency virus-1 (HIV-1)-infected individuals in Japan and showed relatively high incidence of invasive amebiasis in that population, probably because of exacerbation of subclinical infection.[6] Other groups also reported that serum antibody against *E. histolytica* can be elevated, even in asymptomatic-infected individuals, and that seroconversion was seen in the absence of any symptoms in longitudinal follow-up in endemic areas.[7] These results indicate that subclinical infection of *E. histolytica* is frequent in high-risk populations, making it difficult to control *E. histolytica* endemicity.

Evidence suggests that human leukocyte antigen (HLA) type plays a role in amebiasis. For example, Duggal and others[8] reported previously that HLA DQB1*0601 seemed to provide protection against *E. histolytica* infection in Bangladeshi children.

This cross-sectional study was designed to determine the prevalence of ulcerative lesions associated with *E. histolytica* infection in asymptomatic HIV-1–infected individuals in Japan. We also examined the pathogenesis of subclinical intestinal amebiasis and the role of HLA genotypes.

MATERIALS AND METHODS

Ethics statement. The study was approved by the Human Research Ethics Committee of our hospital, the National Center for Global Health and Medicine in Tokyo. The study was conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all participants. No children were included in the study.

Study design and participants. This cross-sectional study included HIV-infected patients who underwent colonoscopy between June of 2010 and June of 2013. One week before colonoscopy, each patient filled out a questionnaire about lower gastrointestinal symptoms based on the Gastrointestinal Symptom Rating Scale (GSRS) rating on a seven-graded Likert scale.[9] Asymptomate for lower gastrointestinal diseases was defined as GSRS scores of one or two for three questions on the diarrhea syndrome domain (diarrhea, loose stools, and urgent need to defecate) and one question on bloody stool.[10] Serum antibody testing against *E. histolytica* was performed in all participants on the day of colonoscopy. Serum antibody was tested by indirect fluorescent antibody assay using whole *E. histolytica* antigen according to the protocol described in the instruction sheet of the approved kit (bioMerieux, SA). Seropositivity was defined as positive response in a serum sample diluted at 1:100 ($\times 100$), and anti-Eh titer was determined by the highest dilution for the positive response. HLA type was determined by standard sequence-based genotyping (HLA Laboratory, Kyoto, Japan). The diagnosis of subclinical intestinal infection of *E. histolytica* was established on confirmation of one or two of the following two criteria: (1) identification of amebic trophozoites in biopsy specimens from gross ulcerative lesions obtained during colonoscopy and/or (2) no pathogens identified in biopsy specimens of gross ulcerative lesion, which were compatible with amebic ulcer,[11] but ulcerative lesion resolved completely after metronidazole monotherapy as confirmed by colonoscopy.

Statistical analysis. The patients' characteristics and serum positivities for anti-*E. histolytica* antibody were compared using χ^2 or Mann–Whitney *U* test for qualitative or quantitative variables, respectively. Statistical significance was defined as two-sided P value < 0.05. All statistical analyses were performed using The Statistical Package for Social Sciences (SPSS Inc., Chicago, IL).

RESULTS

Study population. In total, 380 HIV-1–infected individuals were enrolled during the study period, and 71 patients met the criteria of no symptoms for lower gastrointestinal diseases according to the GSRS. The most common reason for colonoscopy was colorectal cancer screening (N = 48), whereas

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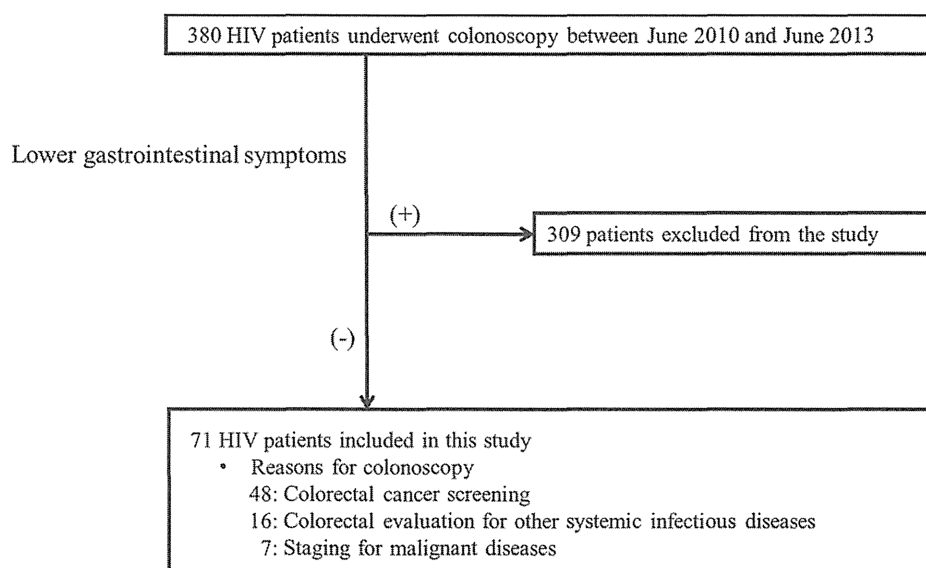

FIGURE 1. Flow diagram of the patient recruitment process. Lower abdominal symptoms were collected based on the GSRS rating on a seven-graded Likert scale at 1 week before colonoscopy.

the other 23 patients underwent colonoscopy for evaluation of progression of malignancies or infections (e.g., malignant lymphoma, Kaposi's sarcoma, tuberculosis, and cytomegalovirus) (Figure 1).

Frequency of intestinal amebic infection among asymptomatic HIV-1–infected individuals. Amebic colitis was confirmed in eight (11.3%) cases. Gross ulcerative lesions were identified by colonoscopy in all eight cases. Amebic trophozoite was identified in the biopsy specimens of five cases (Figure 2). Although amebic trophozoites were not identified in the biopsy specimens of the other three cases, their sera were positive for antibody against *E. histolytica*. In all patients, the ulcerative lesions resolved completely after metronidazole monotherapy.

Clinical features and presentation of patients with and without intestinal amebic infection. As shown in Table 1, patients with amebic intestinal ulcerative lesions tended to be younger, be male homosexuals, have low CD4 counts, and have high HIV-RNA levels, although these differences were not statistically significant. Multiple ulcerative lesions were found in four cases (50%), and the most frequently involved location was the cecum (five cases; 62.5%). Serum antibody against *E. histolytica* was positive in 7 of 8 (87.5%) patients with amebic intestinal ulcerative lesions compared with positivity in only 11 of 63 (17.5%) patients without amebic ulcerative lesions (Table 2).

From the limited data on fecal occult blood testing (FOB) and stool microscopic examination before treatment in cases with amebic ulcerative lesions, FOB was positive in two of three cases (66.7%), and the cyst form, not trophozoite form, *Entoamoeba* was found in only one of four cases (25%).

HLA class II allele frequencies in patients with and without subclinical intestinal amebiasis. HLA data were available for 57 patients (7 of 8 patients with amebiasis and 50 of 63 patients without amebiasis) in our study. We investigated the relation between HLA alleles identified in more than five patients (frequency > 10%) and subclinical intestinal amebiasis. HLA DQB1*06:01 allele was significantly more frequent in patients with subclinical intestinal amebiasis than those without it (Table 3). All the HLA DQB1*06:01 holders were heterozygotes. The frequency of the HLA DRB1*15:02 allele was also significantly higher in patients with subclinical intestinal amebiasis ($P = 0.05$); 7 of 10 patients with HLA DQB1*06:01 also held HLA DRB1*15:02. No colonic amebic ulceration was detected in DQB1*06:01 (−)/DRB1*15:02 (+) patients. Thus, DQB1*06:01 seemed to be the primary HLA allele associated with subclinical intestinal amebiasis in the study population.

DISCUSSION

The pathogenesis of amebiasis remains unclear, including the incubation period after cyst ingestion and the mechanism of spontaneous remission. We reported previously high seroprevalence of *E. histolytica* (21.3%) in HIV-1–infected individuals and that the majority of these patients (78.3%) had no history of invasive amebiasis. In that study, the patients were considered to be at high risk for developing symptomatic amebic infection in longitudinal follow-up (about 20% within the first 1 year of the follow-up period).[6] Based on those results, we speculated the presence of subclinical intestinal amebiasis in patients positive for antibody against *E. histolytica* in the serum resulting in high frequency of symptomatic amebic diseases thereafter, although we did not identify the lesions of *E. histolytica* in these individuals in that study. However, Okamoto and others[12] reported that intestinal ulcerative lesions of *E. histolytica* were rare based on colonoscopic examination in the general population in Japan with positive FOB (0.1%; 4 of 5,193). Our group reported previously that patients with cecal amebic ulcers were sometimes asymptomatic.[11] In this regard, however, the clinical significance of *E. histolytica* infection in asymptomatic individuals had not been fully assessed. In this study, we identified gross amebic ulcers by colonoscopy in 11.2% of asymptomatic HIV-1–infected individuals.

Detection of intestinal amebiasis in asymptomatic individuals is important for not only treatment but also, epidemiological control, especially in endemic areas, because individuals

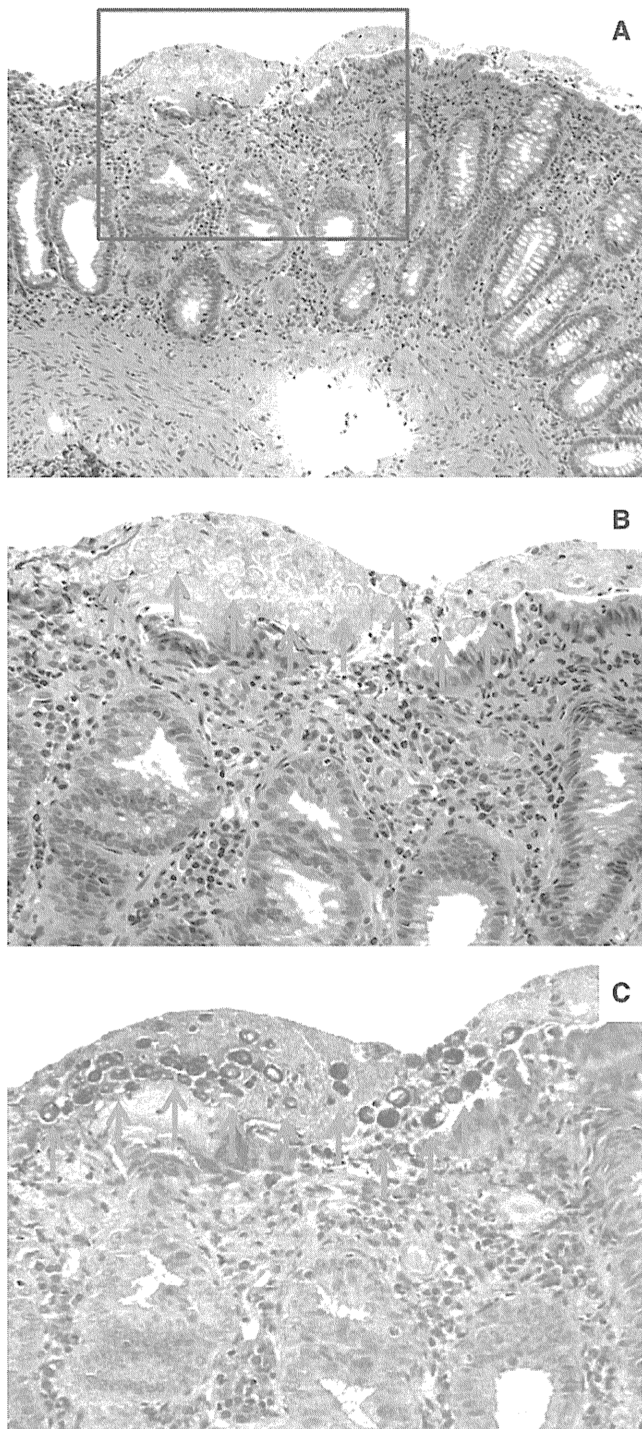

FIGURE 2. Histopathological findings in subclinical intestinal amebiasis. Colonic tissue section was obtained during colonoscopy from a representative asymptomatic patient. *E. histolytica* on the surface of large-intestinal mucosa was clearly stained with periodic acid-Schiff (PAS) staining (green arrows). (**A**) Hematoxylin-eosin staining, ×100. (**B**) Higher magnification of the boxed area in **A**. Hematoxylin-eosin staining, ×400. (**C**) PAS staining, ×400.

with intestinal amebic ulcers can act as a reservoir for *E. histolytica*. However, it is sometimes difficult to identify amebiasis in these individuals, because they lack typical abdominal symptoms related to amebiasis, such as tenesmus, diarrhea, and dysentery. Moreover, our results showed that stool microscopic examination hardly identified amebiasis in these individuals. FOB is more sensitive than stool microscopic examination. However, FOB was positive in 72.7% (16 of 22) of patients free of amebic ulceration. Serum antibody against *E. histolytica* might be a sensitive marker of amebic ulcer in asymptomatic individuals. However, low titers of serum antibody were frequently found in individuals without amebic ulcer. The optimal cutoff value of antibody titer for amebic ulcer is still unclear (for cutoff titer of ×100, sensitivity is 87.5%, and specificity is 82.5%, whereas for cutoff titer ×400, sensitivity is 75.0%, and specificity is 95.2%) (Table 2).

Interestingly, our analysis showed high frequency of HLA DQB1*06:01 heterozygote in patients with subclinical intestinal amebiasis. This allele was reported previously to provide protection against *E. histolytica* infection in Bangladeshi patients.[8] One possible explanation is that ulcerative lesions could occur asymptomatically in patients with HLA DQB1*06:01 and that their immune system could prevent the development of invasive disease from *E. histolytica*, resulting in the high frequency of subclinical intestinal amebiasis observed in our cross-sectional analysis. Genetic differences between Bangladeshi and Japanese patients should also be considered. HLA DQB1*06:01 and DRB1*15:01 were the most common haplotypes in Bangladesh, although they were not identified in our patients.

TABLE 1

Characteristics of patients with and without subclinical intestinal amebiasis

	Amebiasis	No amebiasis	*P* value
n	8	63	
Age (years), median (range)	39 (27–62)	51 (26–81)	0.07
Male sex (%)	8/8 (100%)	56/63 (88.9%)	1.00
Men who have sex with men (%)	8/8 (100%)	44/63 (69.8%)	0.10
Past history of amebiasis (%)	0/8 (0%)	7/63 (11.1%)	1.00
CD4/μL, median (range)	301 (70–584)	436 (21–1,697)	0.28
HIV-RNA (LC/mL), median (range)	4.02 (UD–5.41)	UD (UD–5.85)	0.09

LC/mL = log 10 copies per milliliter; UD = undetectable.

TABLE 2

Clinical presentation of patients with and without subclinical intestinal amebiasis

	Amebiasis	No amebiasis	*P* value
n	8	63	
Serum positivity for anti-*E. histolytica* antibody (%)	7/8 (87.5%)	11/63 (17.5%)	< 0.001
< ×100	1	52	
×100	1	5	
×200	0	3	
×400	3	2	
×800	1	1	
×1,600	2	0	
Site of intestinal amebiasis			
Cecum	5		
Ascending	3		
Transverse	1		
Descending	0		
Sigmoid	1		
Rectum	4		

TABLE 3
Frequencies of HLA class II alleles in patients with and without amebiasis

	Patients with amebiasis ($N = 7$)	Patients without amebiasis ($N = 50$)	P value
DRB1			
*04:03	1 (14.3%)	5 (10.0%)	0.56
*04:05	3 (42.9%)	16 (32.0%)	0.68
*04:06	1 (14.3%)	5 (10.0%)	0.56
*09:01	1 (14.3%)	17 (34.0%)	0.41
*11:01	0 (0.0%)	6 (12.0%)	1.00
*13:02	0 (0.0%)	7 (14.0%)	0.58
*15:01	1 (14.3%)	7 (14.0%)	1.00
*15:02	3 (42.9%)	5 (10.0%)	0.050
DQB1			
*03:01	1 (14.3%)	11 (22.0%)	1.00
*03:02	2 (28.6%)	12 (24.0%)	1.00
*03:03	1 (14.3%)	20 (40.0%)	0.24
*04:01	3 (42.9%)	16 (32.0%)	0.68
*05:02	1 (14.3%)	3 (6.0%)	0.42
*05:03	0 (0.0%)	6 (12.0%)	1.00
*06:01	5 (71.4%)	5 (10.0%)	0.001
*06:02	1 (14.3%)	7 (14.0%)	1.00
*06:04	0 (0.0%)	7 (14.0%)	0.58

Data are numbers and frequencies of patients harboring each HLA allele. HLA data were available in 57 patients. HLA alleles identified in more than five patients (> 10%) were considered.

Additional studies are needed to examine the effects of host genetic factors on *E. histolytica* infection and the development of invasive disease. Interestingly, not only HLA but also, mutation of the leptin receptor were reported to be associated with amebic infection.[13]

In conclusion, intestinal amebic ulcerative lesions were frequently found in asymptomatic HIV-1–infected Japanese individuals who could otherwise act as reservoirs for new infection in other high-risk populations. Additional studies of subclinical infection are needed to control the *E. histolytica* endemicity.

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Acute Hepatitis C in HIV-1 Infected Japanese Cohort: Single Center Retrospective Cohort Study

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Abstract

Objectives: HCV co-infection is a poor prognostic factor in HIV-1-infected patients. Although the number of newly reported patients who show seroconversion is increasing, the clinical features are still unclear, especially in Asian countries.

Design: A single-center retrospective cohort study of patients diagnosed between 2001–2012.

Methods: Acute hepatitis C (AHC) was diagnosed upon detection of high serum ALT (>100 IU) followed by anti-HCV seroconversion. Clinical characteristics, HIV-1-related immunological status and IL-28B genotypes (rs12979860, rs8099917) were collected. We compared these variables between patients with and without spontaneous clearance of HCV and between responders and non-responders to treatment with pegylated interferon (PEG-IFN) plus ribavirin.

Results: Thirty-five patients were diagnosed with AHC during the study period. The majority (96.9%) were MSM. Three were lost to follow-up. Seventy-five percent of patients with AHC (24/32) were asymptomatic and found incidentally to have high serum ALT. Compared to those who did not show spontaneous clearance, patients with spontaneous HCV viral clearance showed more symptoms and more severe abnormalities related to acute hepatitis. Spontaneous clearance was seen in 4 out of 28 patients with CC+TT genotype, but not in 6 patients with IL-28B CT+TG genotype. PEG-IFN plus ribavirin treatment was initiated in 12 out of 28 cases without spontaneous clearance. The sustained virological response rate was high (81.8%, 9/11), even in cases with CT+TG genotype infected with HCV genotype 1b (SVR 2/2).

Conclusions: Careful attention to AHC is needed in HIV-1-infected MSM. Early diagnosis and PEG-IFN plus ribavirin treatment should be considered for AHC cases.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All data supporting our conclusions are included within the manuscript. Original data of our retrospective analyses are available in medical records of our hospital.

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Introduction

The estimated worldwide prevalence of hepatitis C virus (HCV) infection is 2–3% [1]. HCV co-infection increases morbidity rate in HIV infected individuals, and previous meta-analysis reported mortality among patients co-infected with HCV was 1.35 times higher than that among patients with HIV-infection alone even in the highly active antiretroviral therapy (HAART) era [2]. In HIV-1/HCV co-infected patients, progression to liver cirrhosis and hepatocellular carcinoma (HCC) is faster than that in patients without HIV-1 infection [3]. Furthermore, the response to treatment with pegylated interferon (PEG-IFN) plus ribavirin (RBV) in HIV-positive patients with chronic HCV infection is poor (sustained virological response: SVR 19–40%), compared with patients infected with HCV alone (SVR 54–61%) [4–9].

The risk of HCV acquisition via heterosexual intercourse is estimated to be very low [10]. Recently, however, a high incidence of HCV seroconversion has been reported in HIV-1 infected men who have sex with men (MSM) [11–13]. These results suggest that new HCV infection can be a potential future problem in the clinical management of HIV-1 infected patients. On the other hand, a favorable response to treatment with PEG-IFN plus RBV for acute hepatitis C (AHC) relative to that for chronic one has been reported in HCV-infected (SVR 85–98%) [14,15] and HIV/HCV co-infected patients (SVR 60–80%) [16,17]. In this regard, the recent guidelines recommend PEG-IFN plus RBV treatment

for AHC in HIV-1 co-infected patients [18–20]. However, data of AHC among HIV-1 infected patients is still limited, especially from Asian countries.

The response to treatment with PEG-IFN plus RBV is closely associated with the interleukin-28B (IL-28B) genotype, which encodes interferon-λ3 (IFN-λ3), in chronic HCV hepatitis, even in HIV-1 co-infected cases [21–23]. Furthermore, HCV mono-infected individuals with favorable IL-28B genotype (CC at rs12979860, TT at rs8099917) seem to achieve spontaneous clearance of HCV compared to those with non-favorable genotypes [21,22,24,25]. To our knowledge, there are no studies on the effect of IL-28B genotype on the natural course and response to treatment of AHC in HIV-1 infected individuals in Asian population [24,26,27].

In the last 12 years, 35 patients with HIV-1 infection were diagnosed with AHC in our hospital. In the present retrospective study, we report the results of analysis of data of 32 of these cases, and discuss the factors associated with spontaneous HCV clearance and response to treatment with PEG-IFN plus RBV (Fig. 1).

Methods

Study Design

This single-center retrospective cohort study was conducted in accordance with the ethical principles of the Declaration of Helsinki and of Good Clinical Practice. The ethics committee of National Center for Global Health and Medicine approved the study. All patients provided written informed consent.

Study Participants

The medical records of HIV-1 infected patients in our institution, the largest HIV clinic in Japan, admitted and treated between January 2001 and December 2012, were retrospectively reviewed. AHC was defined according to the following criteria; elevation of alanine transferase (ALT) >100 IU/L accompanied by seroconversion of anti-HCV antibody, and exclusion of other causes (e.g., acute hepatitis B and drug-induced hepatitis). Patients who were lost to follow-up within 1 year from the diagnosis of AHC were excluded from the analysis since they could not be assessed for clinical presentation including spontaneous clearance. Spontaneous clearance was defined as a decrease in HCV RNA to undetectable level without treatment within one year from the diagnosis and remaining as such thereafter. For patients receiving PEG-IFN plus RBV treatment, we assessed the SVR rate. SVR was defined as continued undetectable HCV RNA at 24 weeks

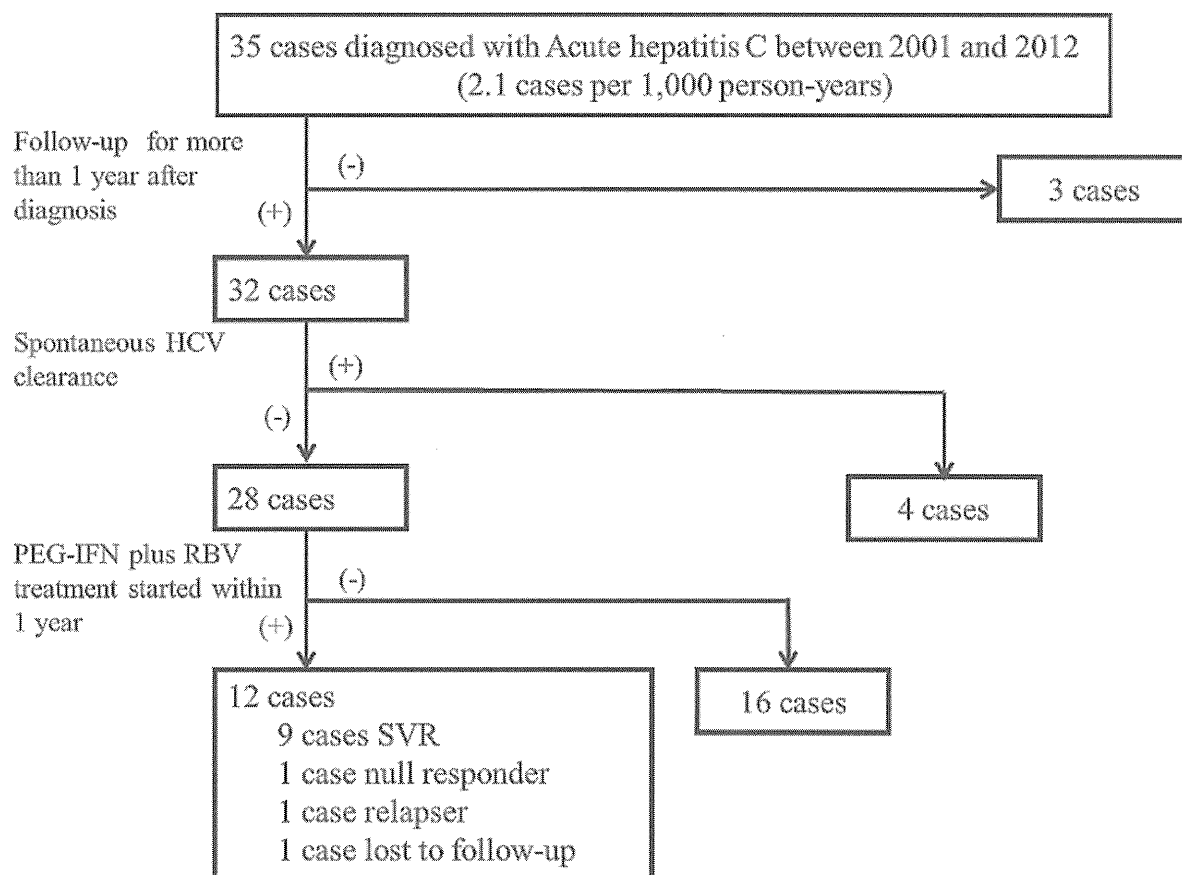

Figure 1. Patient enrollment process. Acute hepatitis C (AHC) was defined as elevation of alanine transaminase (ALT) >100 IU/L accompanied by seroconversion of anti-hepatitis C virus (HCV) antibody. Three patients could not be followed up for 1 year after diagnosis and were excluded from further analysis. HCV cleared spontaneously in 4 cases. PEG-IFN plus RBV treatment was initiated within 1 year of diagnosis of AHC in 12 out of 28 patients who did not show spontaneous clearance. One patient with missing treatment data following transfer to another clinic about two weeks after initiation of IFN plus RBV, was excluded from analysis related to the effect of PEG-IFN plus RBV. PEG-IFN: pegylated interferon, RBV: ribavirin.
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Table 1. Characteristics of AHC patients (n = 32).

	All patients (n = 32)	Spontaneous clearance (n = 4)	Non-spontaneous clearance (n = 28)	P-value
Age (years)	40 [30–58]	44 [37–56]	40 [30–58]	0.361
Male sex	32 (100)	4 (100)	28 (100)	-
Men who have sex with men	31(96.9)	4 (100)	27 (96.4)	1.000
IL-28B genotypes (rs12979860+rs8099917)				
CC+TT genotype	26 (81.2)	4 (100)	22 (78.6)	0.416
CT+TG genotype	6 (18.8)	none	6 (21.4)	-
TT+GG genotype	none	none	none	-
Injecting drug users	4 (12.5)	none	4 (14.3)	1.000
Received ART at diagnosis	29 (90.6)	4 (100)	25 (89.3)	1.000
CD4 count at diagnosis (cells/µL)	420 [167–824]	317 [184–616]	424 [167–824]	0.424
HIV-RNA at diagnosis (copies/mL)	UD [UD -9.4×10^4]	50 [UD-50]	42.5 [UD-9.4×10^4]	0.737

Data are number (%) of patients or median [range].
ART, antiretroviral therapy; UD, undetectable.
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after completion of therapy. Baseline characteristics, status of HIV-1 infection, history of injecting drug usage (IDU), symptoms related to AHC (fatigue and jaundice), laboratory data abnormalities from AHC (ALT, T-bil), treatment of HCV infection and histological findings of liver biopsy, where available, and were collected from the medical records. We compared these variables between patients with and without spontaneous clearance of HCV and between responders and non-responders to treatment with PEG-IFN plus RBV.

HCV Analysis

For each patient, titers of anti-HCV antibody were measured by a third-generation Latex aggregation assay (Ortho HCV Ab LPIA test III, Ortho Clinical Diagnostics, NJ) at first visit to our hospital and at diagnosis of AHC. Serum HCV RNA at each time point was extracted automatically (Cobas Ampliprep, Roche InVitro Diagnostics, Switzerland). Thereafter, cDNA was prepared and its titer was measured by quantitative polymerase chain reaction (Cobas TaqMan 48, Roche In Vitro Diagnostics). Direct sequencing was performed using DNA probe assay by ABI PRISM 3100 (Applied Biosystems, Foster City, CA). Finally, the

Table 2. Clinical presentation of AHC patients (n = 32).

	All patients (n = 32)	Spontaneous clearance (n = 4)	Non-spontaneous clearance (n = 28)	P-value
No symptoms	24 (75)	1 (25)	23 (82.1)	-
Symptoms	8 (25)	3 (75)	5 (17.9)	0.039
Fatigue	8 (25)	3 (75)	5 (17.9)	-
Jaundice	2 (6.25)	1 (25)	1(3.6)	-
Peak Alanine transaminase level (IU/L)	661 [117–2194]	707 [1237–2126]	614 [117–2194]	0.072
Peak total bilirubin level (mg/dL)	1.9 [0.7–17.0]	9.8 [4.2–17.0]	1.6 [0.7–6.8]	0.002
HCV genotype				
1a	1/27 (3.7)	None	1/2 (4.3)	-
1b	19/27 (70.4)	3/4 (75)	16/23 (69.6)	-
2a	4/27 (14.8)	1/4 (25)	3/23 (13)	-
2b	3/27 (11.1)	None	3/23 (13)	-
Not available	5	None	5	
HCV-RNA at diagnosis (Log IU/mL)	6.6 [1.9–7.8][¶]	6.6 [4.9–6.8][†]	6.6 [1.9–7.8][‡]	0.594
Latency to HCV clearance (wks)*	-	11 [7–31]	-	-

Data are number (%) of patients or median [range] values.
*Time between AHC diagnosis and HCV clearance (weeks).
[¶]Data of 6 patients not available for analysis.
[†]Data of 5 patients not available for analysis.
[‡]Data of 1 patient not available for analysis.
doi:10.1371/journal.pone.0100517.t002

Table 3. Comparison of patients of the SVR and non-SVR groups.

	All patients (n = 11)	SVR (n = 9)	Non-SVR (n = 2)
Age (years)	38 [30–58]	38 [30–48]	52 [47–58]
Male sex	11 (100)	9 (100)	2 (100)
Men who have sex with men	11 (100)	9 (100)	2 (100)
IL-28B genotype			
CC+TT genotype	9 (81.8)	7 (77.8)	2 (100)
CT+TG genotype	2 (18.2)	2 (22.2)	None
Injecting drug users	1 (9.1)	None	1 (50)
Received ART before treatment	10 (90.9)	8 (88.9)	2 (100)
CD4 count before treatment (cells/μL)	382 [230–655]	440 [272–655]	238 [254–278]
HIV-RNA before treatment (copies/mL)	UD [UD-3.3×10^4]	UD [UD-3.3×10^4]	UD [305–610]
HCV genotype			
1b	10 (90.9)	8 (88.9)	2 (100)
2a	1 (9.1)	1 (11.1)	None
HCV-RNA before treatment (Log IU/mL)	6.3 [3.3–7.8]	6.3 [5–7.8]	5.7 [3.6–8.0]
Latency to AHC diagnosis (months)*	3.2 [0.9–6.9]	3.2 [0.9–6.9]	4.4 [3.7–5.1]
Duration of PEG-IFN+RBV therapy (wks)	43 [11–72]	43 [11–72]	36 [11–60]
Latency to HCV clearance (wks)¶	-	8 [3–16]	-
RVR	3 (27.2)	3 (33.3)	None
EVR	7 (63.6)	6 (66.7)	1 (50)
Histopathology positive for liver fibrosis			
F0	3/6	3	0
F1	2/6	1	1
F2	1/6	0	1
F3	0	0	0

Data are number (%) of patients or median [range] values.
*Time between AHC diagnosis and initiation of therapy (months).
¶Time between initiation of therapy and HCV clearance (weeks).
ART, antiretroviral therapy; UD, undetectable; PEG-IFN+RBV, pegylated interferon plus ribavirin; SVR, sustained viral response; EVR, early viral response; RVR, rapid viral response.
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genotype was determined from the amino acid sequences of 5 – untranslated region [28].

Genotyping of IL-28b Alleles

Genomic DNA was isolated from peripheral blood mononuclear cells, using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). SNPs, rs12979860, and rs 8099917 were genotyped, using the TaqMan Drug Metabolism Assays by ABI PRISM 7900 HT sequence detection system (Applied Biosystems) according to the instructions provided by the manufacturer. The researchers responsible for genotyping were blinded to clinical data of the patients.

Statistical Analysis

The patients' characteristics and results of differences in viral clearance and virological response were compared using chi-square test (for qualitative variables) or Mann-Whitney U-test (for quantitative variables). Statistical significance was defined at two-sided p value of <0.05. All statistical analyses were performed with The Statistical Package for Social Sciences Version 21 (SPSS Inc, Chicago, IL).

Results

Patient Enrollment

A total of 35 patients were diagnosed with AHC during the study period. The incidence of AHC was 2.1 cases per 1,000 person-years. Three patients who were lost to follow-up within 1 year after diagnosis of AHC were excluded from the analysis. No deaths or fulminant hepatitis were recorded during the study period. Spontaneous HCV clearance was achieved by 4 patients, including 2 patients in whom HCV clearance was achieved within 3 months of diagnosis of AHC. The median time between diagnosis of AHC and HCV clearance was 11 weeks (range, 7–31 weeks). Among the 28 patients who did not show spontaneous HCV clearance, treatment with PEG-IFN plus RBV was initiated within 6 months of diagnosis in 9 patients and between 6 and 12 months of diagnosis of AHC in 3 patients (6.1, 6.4 and 6.9 months, respectively), whereas treatment was not initiated in the remaining 16 patients due to cost (n = 7) or other comorbidity (depression, history of epilepsy) (**Fig. 1**).

Patients' Characteristics and Clinical Presentations of AHC

The characteristics and clinical presentation of AHC patients are listed in **Tables 1** and **2**, respectively. All patients were

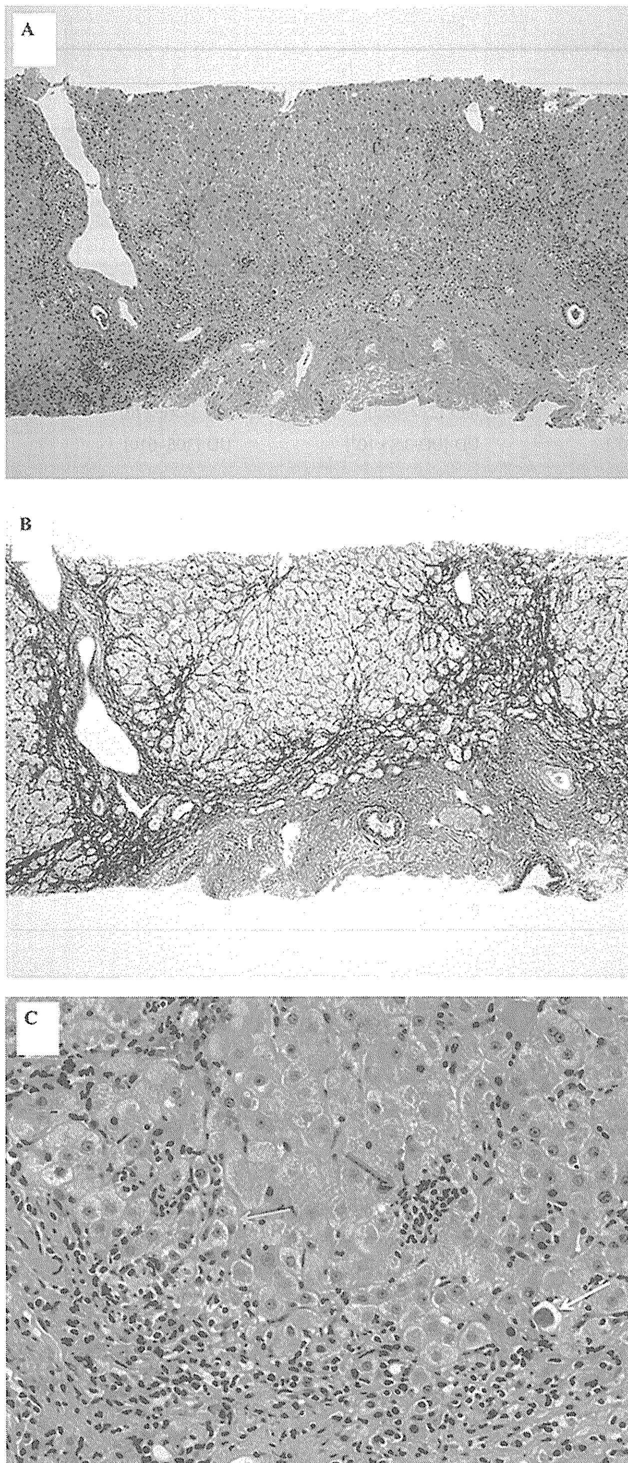

Figure 2. Histological findings in needle liver biopsy specimen from the patient who showed null-response (Table 3). The pretreatment biopsy specimen obtained at 13 weeks after AHC diagnosis showed stage 2 fibrosis (F2) according to the classification of chronic hepatitis C (New Inuyama Classification). (A and B) Formation of bridging fibrosis by fibrous and cellular expansion in the portal tract. (C) Magnified view showing centrilobular piece-meal necrosis (green arrow), acid folic body (yellow arrow) and spotty necrosis (red arrow). (A) Hematoxylin-eosin stain, x100, (B) Silver impregnation stain, x100, (C) Hematoxylin-eosin stain, x400. PEG-IFN: pegylated interferon, RBV: ribavirin, AHC: acute C hepatitis.
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Japanese men, including 31 (96.9%) MSM. Twenty nine patients (90.6%) received antiretroviral therapy (ART) and HIV-RNA was well suppressed in these patients. Four patients (12.5%) had a history of IDU, whereas none had a history of occupational exposure to HCV or blood transfusion. Although there was no significant difference between patients with and without spontaneous HCV clearance, the IL-28B CC+TT genotype was rs12979860 and rs8099917 in all 4 patients who showed spontaneous clearance and none of the patients with IL-28B CT+TG genotype showed spontaneous clearance (**Table 1**).

The majority of patients with AHC (24/32, 75%) were asymptomatic at the onset of AHC. High ALT was identified incidentally at routine visit for HIV-1 infection, with subsequent tests confirming the diagnosis of AHC. Compared to patients who did not show spontaneous clearance, patients with spontaneous clearance showed more severe clinical presentation of hepatitis (symptomatic, with higher serum total bilirubin and ALT value at diagnosis). The most frequent HCV genotype was 1b (70.4%). At diagnosis, HCV RNA was higher than 5.0 LC/mL in 24 out of 26 patients (**Table 2**).

Response to Treatment with PEG-IFN Plus RBV

Treatment with PEG-IFN plus RBV was initiated in 12 patients within 1 year of diagnosis of AHC (median interval from AHC diagnosis, 3.2 months). We assessed the response to treatment in only 11 patients; the other patient was lost to follow-up within two weeks of treatment initiation (**Fig. 1**, **Table 3**). SVR was achieved in 9 of 11 patients (81.8%) despite the high incidence of HCV genotype 1b and high viral load.

Two patients did not achieve SVR. Both patients were infected by genotype 1b with high viral load, and treatment was initiated within 6 months of diagnosis. One achieved viral clearance within 12 weeks (early virological response: EVR) but showed viral rebound at 15 weeks after completion of the treatment (relapser), whereas viral clearance was not achieved during treatment in the other patient (null-responder). Both patients were relatively older and their CD4 counts were lower, compared to those with SVR, although statistical analysis was not performed due to the small number of cases. In patients with SVR, the median time between initiation of therapy and clearance of HCV was 8 weeks (range, 3–16 weeks). Surprisingly, both patients with IL-28B CT+TG alleles achieved SVR despite genotype 1b and high viral load, although we could not compare the SVR rate among different genotypes since only one patient was infected with genotype 2a in this study.

Histological Findings of AHC in HIV-1 Co-infected Patients

HBs antigen was negative and ALT was within the normal range in the year preceding AHC in all 6 patients, whereas HBs Ab and/or HBc Ab was positive in 5 patients. No pre-existing factors of liver fibrosis other than HIV infection were evident before AHC. Liver biopsy was performed in 6 patients before treatment with PEG-IFN plus RBV. The median interval between diagnosis of AHC and biopsy was 4.3 months (range, 3.3–6.1 months). Fibrotic changes were confirmed in 3 cases by hematoxylin-eosin staining and silver impregnation staining (**Fig. 2**, **Table 3**). These lesions were paler-staining by Victoria Blue stain, indicating that the fibrotic areas did not reflect chronic changes.

Discussion

In the present study, we identified 35 cases of AHC during the study period and nearly all such patients (34/35) were MSM, and

the most frequent HCV genotype was 1b (19/27). These findings are consistent with previous reports from other countries [11–13]. In this regard, a high incidence of HCV seroconversion in HIV-1 infected MSM was reported recently by two separate groups [11–13]. The same studies also reported that genotype 1b was the major genotype among their patients [11–13], and that HCV infection was frequently not detected during the acute phase and diagnosed only at the chronic stage mainly due to the lack of symptoms.

Similar to the previous reports on AHC, 75% of our cases were asymptomatic, and only 6.3% of the study population showed mild elevation of serum ALT (100 IU/L < ALT <150 IU/L). In this regard, ALT elevation during acute HCV infection is often relatively transient, and therefore could be easily missed during routine clinical care. The need of regular screening for anti-HCV antibody in HIV-1 infected MSM is controversial, and the recommendations are different in guidelines from different developed countries [29,30]. Our results emphasize the importance of regular ALT monitoring and HCV re-screening at the time of mild ALT elevation during follow-up, especially in high-risk populations such as sexually active MSM.

There are few reports on the relationship between IL-28B CC+TT genotype and spontaneous clearance of HCV [21,31]. In the present study, spontaneous HCV clearance was seen in 4 out of 26 patients with IL-28B CC+TT genotype, whereas no spontaneous HCV clearance was seen in all 6 patients with IL-28B CT+TG genotype. Although this difference could not be confirmed to be statistically significant due to the small number of patients (4 patients), this is, to our knowledge, the first report on the relation between IL-28B and spontaneous HCV clearance during AHC in HIV-1 co-infected patients in Asian population. Our study also showed that the severity of clinical symptoms was an important factor related to spontaneous HCV clearance. Further investigation is needed for a better understanding of the pathogenesis of AHC, especially factors involved in spontaneous clearance.

The use of PEG-IFN plus RBV treatment for AHC within 6 months of diagnosis is now recommended for HIV-1 co-infected cases [17–19] although data on the response of HIV-1 infected individuals with AHC to the PEG-IFN plus RBV remain limited. One study reported spontaneous clearance of HCV between 6 and 12 months of diagnosis [32]. In this regard, it is sometimes difficult in the clinical setting to start PEG-IFN plus RBV treatment within 6 months of diagnosis because some patients have comorbidities and complications other than HIV and HCV. In our analysis, 9 of 11 patients (81.8%), including 2 patients whose treatment was initiated between 6 and 12 months of diagnosis, achieved SVR despite high rate of genotype 1b infection (SVR 90.0% among those with genotype 1b virus). Furthermore, HCV genotype 1b-infected patients carrying the IL-28B CT+TG genotype (n = 2), which is a predictor of poor response to the treatment of chronic HCV infection, achieved SVR. These results emphasize the advantage of the PEG-IFN plus RBV treatment for AHC.

Little is known about the progression of AHC to liver fibrosis in patients with HIV/HCV co-infection [33], although rapid progression of liver fibrosis during the chronic phase is well recognized [3]. Fierer et al. [34] reported that the development of fibrosis occurs even in the acute phase of HCV infection in HIV-Infected men. In the present study with limited cross-sectional analysis of liver biopsies after AHC, fibrosis was detected in 3 out of 6 cases, which is consistent with the above report of Fierer et al. [34]. Moreover, SVR was not achieved in 2 out of 3 patients who showed liver fibrosis, whereas the other 3 patients without fibrosis achieved SVR (**Table 3**). These results emphasize the clinical importance of early diagnosis and early treatment for AHC in HIV-1 infected individuals.

In conclusion, the potential of AHC should always be considered in HIV-1 infected MSM, even in asymptomatic case, who present with mild ALT elevation. Favorable response can be expected if anti-HCV treatment is initiated during the early phase. Further investigation is needed to determine the predictor(s) of spontaneous HCV clearance, appropriate timing of treatment initiation, and duration of treatment.

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Author Contributions

Conceived and designed the experiments: MI KW TK YK SO HG. Performed the experiments: MI KW TK YN MY TI NM. Analyzed the data: MI KW TK HG. Contributed reagents/materials/analysis tools: MI KW TK YN MY TI NM YK SO HG. Contributed to the writing of the manuscript: MI KW KT HG.

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Superimposed Epitopes Restricted by the Same HLA Molecule Drive Distinct HIV-Specific CD8+ T Cell Repertoires

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Superimposed epitopes, in which a shorter epitope is embedded within a longer one, can be presented by the same HLA class I molecule. CD8+ CTL responses against such epitopes and the contribution of this phenomenon to immune control are poorly characterized. In this study, we examined HLA-A*24:02–restricted CTLs specific for the superimposed HIV Nef epitopes RYPLTFGWCF (RF10) and RYPLTFGW (RW8). Unexpectedly, RF10-specific and RW8-specific CTLs from HIV-1–infected HLA-A*24:02+ individuals had no overlapping Ag reactivity or clonotypic compositions. Single-cell TCR sequence analyses demonstrated that RF10-specific T cells had a more diverse TCR repertoire than did RW8-specific T cells. Furthermore, RF10-specific CTLs presented a higher Ag sensitivity and HIV suppressive capacity compared with RW8-specific CTLs. Crystallographic analyses revealed important structural differences between RF10– and RW8–HLA-A*24:02 complexes as well, with featured and featureless conformations, respectively, providing an explanation for the induction of distinct T cell responses against these epitopes. The present study shows that a single viral sequence containing superimposed epitopes restricted by the same HLA molecule could elicit distinct CD8+ T cell responses, therefore enhancing the control of HIV replication. This study also showed that a featured epitope (e.g., RF10) could drive the induction of T cells with high TCR diversity and affinity. ***The Journal of Immunology*****, 2014, 193: 77–84.**

Cytotoxic CD8+ T lymphocytes recognize target cells through the recognition of peptides 8–11 aa long that are presented by MHC class I (MHCI) molecules (1–3). Of note, two epitopes in which a shorter one is embedded within a longer one are defined as superimposed epitopes, and they have been shown to be presented by the same MHCI molecule (4–6). A number of studies have reported CTL responses against such superimposed peptides in the context of an HIV-1 infection (7–10). For instance, these responses include CTLs specific for HLA-B57–restricted p24 Gag-derived peptides, for example, KI8 (residues 162–169, KAFSPEVI) and KF11 (residues 162–172, KAFSPEVIPMF), as well as for HLA-B*35:01–restricted Nef-derived peptides, for example, VY8 (residues 74–81, VPLRPMTY) and RY11 (residues 71–81, RPQVPLRPMTY). However, the biological relevance of this phenomenon remains unclear. Indeed, although it is speculated that CTLs can show cross-reactivity toward superimposed epitopes and work together effectively against HIV-infected targets, the functional synergism of these cells has not been studied in detail.

HLA-A*24:02 is the most frequent HLA class I allele in Japan, being found in ~70% of Japanese individuals (11) and of those infected with HIV-1 (12, 13). This allele also occurs in the range from ~25–64% in other Asian countries and in 18% in white populations (14, 15). Therefore, the study of immune responses to epitopes restricted by this allele is important for our understanding of HIV pathogenesis and vaccine development. We previously reported that Nef138-10 (RYPLTFGWCF, RF10) is an immunodominant CTL epitope in HLA-A*24:02+ Japanese individuals chronically infected with HIV-1 (16, 17). Of interest, Nef138-8 (RYPLTFGW, RW8) is also defined as an optimal epitope presented by HLA-A*24:02 in white individuals (18). Although these superimposed epitopes elicit effective specific CTL responses important for the control of HIV-1 replication in HLA-A*24:02+ individuals, the overlap in terms of reactivity and antiviral ability between RF10- and RW8-specific CTLs remains unknown.

In the present study, we performed a comprehensive analysis of CTL responses specific for RW8- and RF10-superimposed epitopes by using multiple approaches. We used RF10 and RW8 tetramers to identify and isolate cells from chronically HIV-1–infected HLA-A*24:02+ Japanese individuals, α- and β-chain TCR repertoire analyses at the single-cell level to assess the degree of overlap between responses, and crystallographic approaches to reveal the structural basis of RF10– and RW8–HLA-A*24:02 interactions. We report unanticipated differences between RF10- and RW8-specific CTLs, that is, the elicitation of totally distinct CTL responses against superimposed HIV-1 epitopes restricted by the same HLA molecule, as well as distinct TCR repertoires between the featured (RF10) and featureless (RW8) epitope-specific CTLs.

Materials and Methods

Patient samples

The study was approved by the Ethics Committees of Kumamoto University and the National Center for Global Health and Medicine. Informed consent

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Abbreviations used in this article: MFI, mean fluorescence intensity; MHCI, MHC class I; tet, tetramer.

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was obtained from all individuals according to the Declaration of Helsinki. Twenty-three HLA-A*24:02^{+} treatment-naive individuals chronically infected with HIV-1 and eight HLA-A*24:02^{+} HIV-1 seronegative individuals were recruited (Supplemental Table I). Their plasma and PBMCs were separated from whole blood. HLA types were determined by standard sequence-based genotyping.

HIV-1–specific CTL clones

Ag-specific CTL clones were generated as previously described (17). Briefly, RW8- and RF10-specific CTL cell lines were first obtained by stimulating PBMCs from patient KI-158 with cognate peptides. Peptide-specific CTL clones were then generated from the cell lines by limiting dilution in 96-well U-shaped plates cocultured with 1×10^6 irradiated feeder PBMCs from healthy donors and 1×10^5 irradiated C1R-A*2402 cells prepulsed with RW8 or RF10 peptide at 1 μM concentration. All CTL clones were cultured in 200 μl cloning medium (RPMI 1640 containing 10% FBS, 200 U/ml rIL-2, and 2.5% PHA soup) and stimulated weekly with irradiated C1R-A*2402 cells prepulsed with RW8 or RF10 peptide.

Tetramer staining

HLA-A*24:02 tetrameric complexes were synthesized as previously described (19). For tetramer-binding assays, CTL clones were stained with PE-conjugated RW8 or RF10 tetramers at various concentrations (0–1000 nM) at 37°C for 30 min before staining with FITC-conjugated anti-CD8 mAb at 4°C for 30 min. The cells were analyzed by using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star). For TCR avidity measurements, the tetramer concentration that yielded the EC_{50} mean fluorescence intensity (MFI) was calculated by probit analysis.

Cell line

C1R-A*2402 and RMA-S-A*2402 cells were previously generated by transfecting *HLA-A*24:02* genes into C1R cells and RMA-S cells, respectively (5, 20, 21). The C1R cell line is a human B cell lymphoblastoid line lacking surface expression of HLA-A and partially HLA-B molecules. It was derived from a normal B cell line, Hmy2, through three rounds of mutagenesis and selection with anti-HLA mAb (22). RMA-S cells are a TAP2 deficiency cell line derived from RMA cells. They express high levels of empty MHC molecules (i.e., not carrying endogenous peptides on the cell surface) when cultured at 26°C and very low levels when cultured at 37°C (23). RMA-S-A*2402 and C1R-A*2402 cells were cultured in RPMI 1640 medium containing 10% FCS and 0.2 mg/ml hygromycin B.

Peptide-binding assay

The binding of peptides to HLA-A*24:02 molecules was tested as previously described (24). Briefly, RMA-S-A*2402 cells were precultured at 26°C for 14–18 h and then incubated at the same temperature for 1 h with either RW8 or RF10 peptide at various concentrations (0–100 nM). Thereafter, they were incubated at 37°C for 3 h. After incubation, the peptide-pulsed cells were stained with anti-HLA class I α3 domain mAb TP25.99 (19) and subsequently with FITC-conjugated sheep IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). The MFI was measured by flow cytometry (FACSCanto II).

Replication suppression assay

Two HIV-1 virus laboratory strains, NL-432-10F and NL-M20A-10F, were used in these assays. They were generated from NL-43 or NL-M20A by site-directed mutagenesis to carry the RYPLTFGWCF sequence (17). The ability of HIV-1–specific CTLs to suppress HIV-1 replication was examined as previously described (17). Briefly, primary CD4^{+} T cells were infected with NL-432-10F and NL-M20A-10F, respectively, for 6 h before being washed with R10 medium. The cells were then cocultured with HIV-1–specific CTL clones at various E:T ratios. Ten microliters of culture supernatant was collected at day 6, and the concentration of p24 Ag in it was determined by performing p24 ELISA (ZeptMetrix, Buffalo, NY).

^{51}Cr-release assay

The cytotoxic potential of CTL clones against C1R-A*2402 prepulsed with appropriate peptide at various concentrations (0–100 nM) was determined as previously described (25). Briefly, C1R-A*2402 cells were labeled with 100 μl ^{51}Cr for 1 h before washing and then pulsed for 1 h with peptides. Effector cells were cocultured for 4 h at 37°C with target cells (2×10^3/well) at an E:T ratio of 2:1. After centrifugation, 100 μl supernatant was collected and analyzed with a gamma counter. The specific lysis was calculated as [(cpm experiment − cpm supernatant)/(cpm maximum − cpm supernatant)] × 100.

Ex vivo single-cell TCR repertoire analysis and assessment of TCR diversity

Cryopreserved PBMC samples from patients were thawed, divided, and immediately stained with RW8 or RF10 tetramers, followed by staining with anti-CD3 mAb (Pacific Blue), anti-CD8 mAb (FITC), and 7-aminoactinomycin D. RW8 and RF10 tetramer^{+}CD3^{+}CD8^{+}7-aminoactinomycin D^{-} cells were sorted into a 96-well plates (Bio-Rad) by using a FACSAria I (BD Biosciences). Unbiased identification of TCR α- and β-chain usage was assessed as previously described (26). An Illustra ExoStar (GE Healthcare, Little Chalfont, U.K.), which contains alkaline phosphatase and exonuclease 1, was used to remove unincorporated primers and nucleotides from amplification reaction for the subsequent tailing reaction. The names of all identified TCR genes were given based on the international ImMunoGeneTics information system nomenclature (27). The diversity of TCR clonotypes was calculated by using both the number of different clonotypes and Simpson's diversity index for both α- and β-chains and the formula $D_s = 1 - \Sigma\{[n_i(n_i - 1)]/[N(N - 1)]\}$, where n_i is the TCR clone size of the ith clonotype and N is the total number of TCR sequences sampled. This index uses the relative frequency of each clonotype to calculate a diversity index ranging between 0 and 1, with 0 being minimal and 1 being maximal diversity (28).

Crystallization, data collection, and processing

Soluble peptide–HLA-A*24:02 complexes were prepared as previously described (29). HLA-A*24:02 molecules were purified by Superdex 200 10/300 GL gel-filtration chromatography (GE Healthcare). All crystallization attempts were performed by the hanging drop vapor diffusion method at 18°C with a protein/reservoir drop ratio of 1:1. Crystals were seen after 3–5 d in 0.1 M MES (pH 6.5) and 12% (w/v) polyethylene glycol at 20,000 g/mol. The crystals were briefly soaked in reservoir solution containing 17% (v/v) glycerol, mounted on an x-ray machine with a nylon loop, and then flash-cooled in a stream of gaseous nitrogen. Diffraction data were collected by using beamline NE3A in the KEK Synchrotron Facility (Tsukuba, Japan) and an ADSC Q270 imaging-plate detector at a wavelength of 1.0 Å. Data were indexed, integrated, and scaled by using HKL2000. The data collection statistics are shown in Table I. Data were analyzed by molecular replacement by use of Phaser in CCP4. We used the A24VYG molecule as the search model (Protein Data Bank accession no. 2BCK, http://www.rcsb.org/pdb/home/home.do). All of the structures were further refined by several rounds of refinement made by using the PHENIX program. The refinement statistics are given in Table I.

Results

*Effective induction of RW8- or RF10-specific CTL responses in HIV-1–infected HLA-A*24:02^{+} patients*

To assess the degree of overlap between CTL responses specific for two superimposed Nef epitopes (RW8 and RF10), we first generated HLA-A*24:02 tetramers with RW8 or RF10 peptides (RW8-tet and RF10-tet, respectively) and compared ex vivo frequencies of 8-mer– or 10-mer–specific CD8^{+} T cells in 23 treatment-naive HLA-A*24:02^{+} individuals with chronic HIV-1 infection. In eight HIV-1 seronegative HLA-A*2402^{+} donors, the frequencies of RW8 and RF10 tetramer^{+} CD8^{+} T cells were 0.080 ± 0.009 and 0.045 ± 0.022% (mean ± SD), respectively (Fig. 1A). We evaluated the mean ± 3 SD as positive staining and therefore considered 0.10 and 0.11% of tetramer^{+} CD8+ cells as positive values for RW8-specific and RF10-specific T cells, respectively (Fig. 1B, dashed line). Among the 23 HIV-1–infected individuals studied, 14 and 19 were positive for RW8-specific CTLs and RF10-specific CTLs, respectively (Fig. 1A). Thirteen of the 23 individuals analyzed (56.5%) presented both RW8- and RF10-specific T cells (Fig. 1B). The magnitude of RW8-specific and RF10-specific T cells correlated with one another across individuals (Fig. 1B). However, this correlation was modest, indicating that these populations did not overlap entirely. In fact, the frequency of RF10 tetramer^{+} CD8^{+} cells was significantly higher than that of RW8 tetramer^{+} CD8^{+} cells (Fig. 1A). Taken together, these results indicate that both RW8-specific and RF10-specific CTLs could be effectively elicited in HLA-A*24:02^{+} individuals with a chronic HIV-1 infection; however,

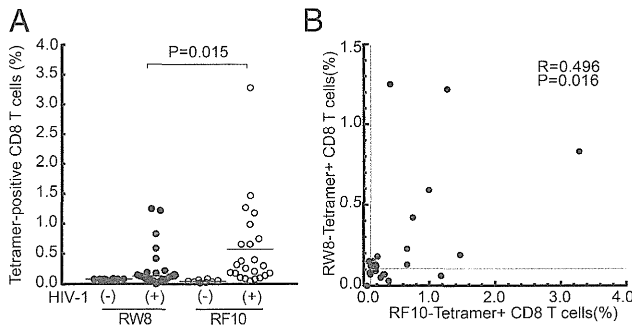

FIGURE 1. Frequencies of RW8- and RF10-specific $CD8^+$ T cells in individuals with chronic HIV-1 infection. (**A**) Frequencies of total RW8 and RF10 $tetramer^+$ $CD8^+$ T cells in 23 chronically HIV-1–infected treatment-naive HLA-A*24:02^+ individuals and in 8 HLA-A*24:02^+ uninfected controls. Statistical analyses were conducted by using the nonparametric Mann–Whitney *U* test. (**B**) Correlations between RW8 and RF10 $tetramer^+$ $CD8^+$ T cell frequencies in HIV-1–infected HLA-A*24:02^+ individuals. Of $tetramer^+$ $CD8^+$ cells, 0.10 and 0.11% were considered as positive values for RW8-specific and RF10-specific T cells, respectively (the dashed line indicates the threshold), as described in the text. The correlation was determined by using the Spearman rank test.

they do not appear to be equivalent, which begs the question of their cross-reactive potential.

Distinct reactivity of RW8- and RF10-specific CTLs

To investigate whether RW8- or RF10-specific CTLs could cross-recognize the superimposed epitopes, we first performed concurrent RW8 and RF10 tetramer (RW8-tet and RF10-tet, respectively) staining of PBMCs from HIV-1–infected donors. In patients presenting both RF10- and RW8-specific $CD8^+$ T cells, these cells did not seem to be RW8 and RF10 cross-reactive, as they failed to stain for both tetramers simultaneously. A representative case (patient KI-158) is shown in Fig. 2A. To analyze further the fine reactivity toward these epitopes, we next established CTL clones from patient KI-158 presenting both RF10- and RW8-specific $CD8^+$ T cells upon initial selection and stimulation with RW8 or RF10 peptides. RF10- and RW8-specific clones were clearly discriminated by using both tetramers together at the same concentration (Fig. 2B). We performed staining using different concentrations of the specific tetramers to measure the TCR avidity of representative RF10- or RW8-specific clones. CTL52 clone (RW8-specific) exhibited a strong affinity for RW8-tet but not for RF10-tet, whereas the CTL173 (RF10-specific) clone exhibited a strong affinity for RF10-tet but not RW8-tet (Fig. 2C), indicating that CTL52 and CTL173 clones had TCRs with high affinity for RW8 peptide–HLA-A*24:02 and RF10 peptide–HLA-A*24:02 complexes, respectively.

Next, we tested the functional avidity of RF10- and RW8-specific clones. RF10-specific clones (CTL170 and CTL173) effectively killed RF10 peptide–pulsed cells but failed to kill RW8 peptide–pulsed targets even at a high concentration of RW8 peptide (Fig. 2D), indicating that these RF10-specific clones did not cross-recognize the 8-mer peptide. RW8-specific clones (CTL52 and CTL72) recognized both RW8 peptide– and RF10 peptide–pulsed targets, but the cytotoxic activity of these clones against RW8 peptide–pulsed target cells was 10- to 50-fold higher than that against the RF10 peptide–pulsed ones (Fig. 2E). Although RW8 clones presented some cross-reactivity toward RF10, they recognized the RW8 peptide with greater efficiency than RF10. Altogether, these results indicate that RW8-specific and RF10-specific CTLs displayed no or poor cross-reactivity for their respective superimposed epitopes.

Different TCR usage between RW8- and RF10-specific $CD8^+$ T cells

The lack of cross-reactivity between RW8-specific and RF10-specific $CD8^+$ T cells implies that distinct clonotypes should

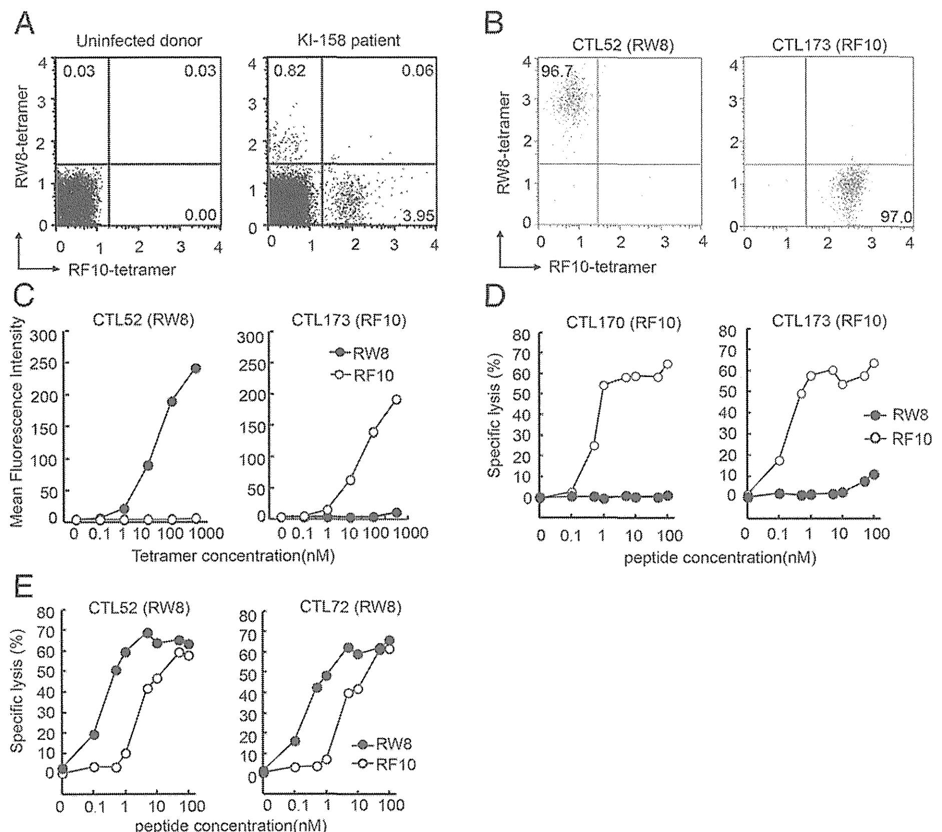

FIGURE 2. Recognition of RW8 and RF10 peptides by RW8- or RF10-specific CTL clones. (**A**) Simultaneous RW8 and RF10 tetramer staining of PBMCs from a representative HIV-1–infected donor and an HIV-1–uninfected healthy individual. The percentage of $tetramer^+$ cells among $CD8^+$ cells is shown. (**B**) Simultaneous RW8 and RF10 tetramer staining of CTL52 and CTL173 clones. The percentage of $tetramer^+$ cells among $CD8^+$ cells is shown. (**C**) RW8 and RF10 tetramer staining of CTL52 and CTL173 clones at various concentrations. Tetramer binding to CTL52 and CTL173 clones was analyzed by flow cytometry, and the data are shown as MFI. (**D** and **E**) Cytolytic activities of HLA-A*24:02–restricted RF10 (CTL170/CTL173)-specific (D) and RW8 (CTL52/CTL72)-specific (E) CTL clones toward HLA-A*24:02^+ target cells (C1R-A*2402) prepulsed with the indicated peptide at various concentrations.