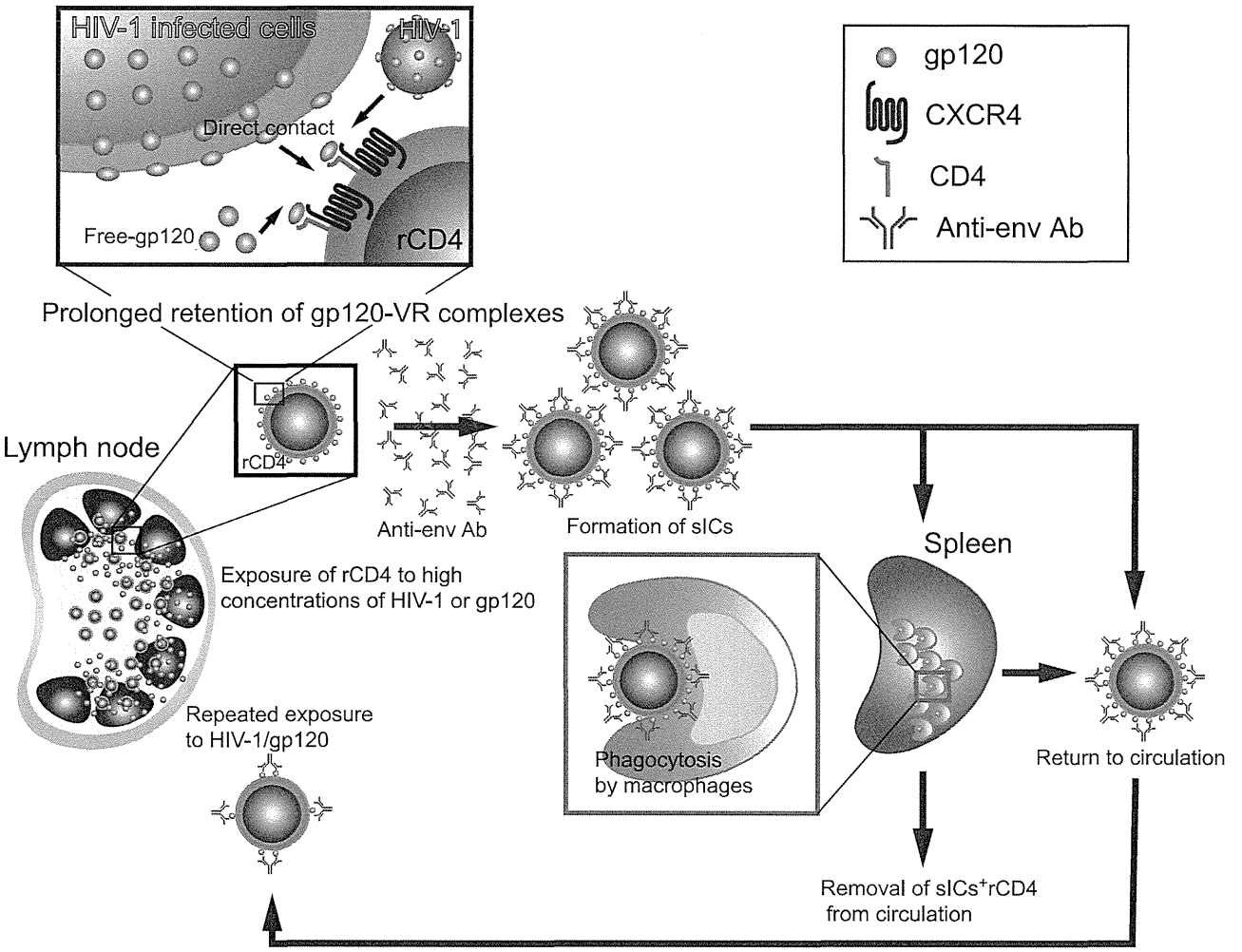

Figure 7. Schematic figure summarizes the causes and consequences of sIC$^+$ rCD4s. rCD4s continuously travel between the blood stream and LNs over a period of approximately 1 d. Because a large proportion of HIV-1 is produced in the LNs, the target T cells that migrate to the LNs are exposed to high concentrations of HIV-1, gp120, or ICs as well as anti-env Abs. Prolonged retention of gp120-VR complexes on rCD4s causes the retention of sICs in a manner that reflects the levels of HIV-1 exposure in the LNs. sIC$^+$ rCD4s are removed from circulation through ADCP or ADCC by macrophages or NK cells, respectively. The sIC$^+$ rCD4s that are not removed from circulation remigrate to the LNs to be exposed to a high concentration of gp120/HIV-1. The percentages and amounts of sICs on rCD4s in the blood reflect a balance of five factors, namely, the levels of virus production in lymphoid tissues, the levels of anti-env Abs, the turnover dynamics of sICs on rCD4s, the duration of repeated exposure by continuous migration to the lymphoid organs, and the levels of immunological elimination of sIC$^+$ rCD4s.
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H14-60) by the National Center for Global Health and Medicine Ethical Committee, and written informed consent was obtained from every subject.

Purification and Isolation of CD4$^+$ T, B, and NK Cells

The highly purified drCD4s from healthy donors, which were used as representative qCD4 T cells *in vivo*, were purified by negative selection with magnetic beads followed by T cell density gradient separation on a discontinuous Percoll gradient (Pharmacia Biotech, Uppsala, Sweden) as described previously [34]. The rCD4s were purified using the CD4$^+$ T Cell Isolation Kit II (Miltenyi Biotech, Auburn, CA) followed by magnetic depletion with anti-CD25 and anti-CD69 Abs. CD20$^+$ IgG^{dull} IgM^{dull} B cells were isolated using the B Cell Isolation Kit II (Miltenyi Biotech) followed by magnetic depletion with anti-IgG and anti-IgM Abs. NK cells and macrophages were isolated using the NK Cell Isolation Kit II and the Macrophage Isolation Kit II (Miltenyi Biotech), respectively.

Preparation of Activated qCD4s

Purified qCD4s were stimulated for 72 h with plate-bound anti-CD3 Abs (UCTH1:40 mg/ml) and anti-CD28 Abs (Lew-28:20 mg/ml) in RPMI 1640 containing fetal calf serum (FCS) at 37°C in humidified air containing 5% CO_2.

Kinetic Studies and Flow Cytometry

Kinetic studies of CXCR4 and CD4 expression were performed as previously described [30]. Briefly, A3.01 cells, qCD4s, and activated qCD4s were cultured in flat-bottom 96-well microtiter plates (Nalge Nunc, Penfield, NY) (in triplicate) with or without ActD (20 μg/ml), BFA (10 μg/ml), and/or cycloheximide (50 μg/ml). The concentrations of inhibitors used were as previously described [30]. The percent change in surface receptor expression

was calculated from the MFIs (**except for Fig. 1A and B**) or percentages of cells in predetermined gates (**Fig. 1A and B**).

For kinetic studies, qCD4s were incubated on ice with $gp120_{IIIB}$ (250 ng/ml), $gp120_{BaL}$ (250 ng/ml), or AT-2-inactivated HIV-1_{Lai}, HIV-1_{BaL}, or an HIV-1 clinical isolate (R5 strain as determine by biological assays) for 30 min in binding buffer (PBS with 10% FCS) and then washed with binding buffer. Aliquots of qCD4s exposed to either gp120 or HIV-1 were cultured with 10% FCS containing medium alone or medium containing 10% HI patient serum (pooled from five HIV-1-seropositive subjects). Cells that were cultured in medium without 10% FCS were stained with rabbit anti-gp120 antiserum (ABI) followed by anti-rabbit IgG-FITC (DAKO, Hamburg, Germany) and fixed with 1.0% paraformaldehyde. Cells that were cultured in patient serum-containing medium were stained with the same serum followed by anti-human IgG-FITC. The effects of T22 or Enf were studied by incubating cells with the drugs in binding buffer for 30 min on ice followed by exposure to gp120 or HIV-1 in binding buffer containing the corresponding drug. The exposed cells were washed thoroughly with binding buffer containing the corresponding drug, subsequently cultured in the absence (for T22 experiments) or presence (for Enf experiments) of drug for the indicated times. The percent change in cell-bound gp120 was calculated from MFIs.

Confocal Microscopy

Purified qCD4s were incubated with gp120 (250 ng/ml) or AT-2-inactivated HIV-1. Immunofluorescence was performed by serial staining with goat anti-CD4 polyclonal Abs (R&D Systems), Cy3-conjugated secondary Abs (Sigma), rabbit anti-gp120 antiserum (ABI), anti-rabbit IgG-FITC (Dako), biotinylated anti-CXCR4 monoclonal Abs (R&D Systems), and streptavidin-Qdot 605 (Life Technologies, Carlsbad, CA), in that order. The cells were then fixed with 4% paraformaldehyde. sICs were visualized by staining purified rCD4s with goat anti-CD4 polyclonal Abs, Cy3-conjugated secondary Abs, biotinylated $F(ab')^2$ anti-human Igs (Life Technologies), and streptavidin-Qdot 525, in that order. Multicolor confocal and DIC images with a 512×512 resolution were acquired using a Zeiss LSM510 system with a Plan-Apochromatic 63×1.4 NA oil immersion DIC objective (Carl Zeiss, Oberkochen, Germany) using multi-track scanning.

sIC Analysis

For phenotypic analysis, PBMCs purified using Ficoll-Paque were stained with anti-CD3-PerCP, anti-CD4-APC, anti-CD25-PE, anti-CD69-PE, and biotin-$F(ab')^2$ anti-human IgG (BioSource) or biotin-$F(ab')^2$ anti-human IgM (Life Technologies), in that order. After washing, the cells were stained with streptavidin-FITC. PBMCs from an HIV-1-seronegative donor were simultaneously stained as a negative control. For longitudinal analyses of sICs, purified PBMCs were cryopreserved at −80°C, and each sample set from the patient was labeled simultaneously.

Western Blotting, IgG Purification, and HIV-1 RNA in Situ Hybridization

Whole-cell lysates derived from gp120 (250 ng/ml)- or HIV-1-pulsed qCD4s or rCD4s purified from HIV-1-seropositive or healthy individuals were subjected to SDS-PAGE and were transferred to polyvinylidene difluoride membranes and blotted with antibodies against gp120 (Life Technologies), p24 (Life Technologies), or human-IgG (Dako) after blocking with TBST/5% milk. Proteins were visualized using the SuperSignal West Pico Chemiluminescent Kit (Thermo Fisher Scientific, Waltham, MA) and Biomax-MR film (Kodak, Rochester, NY). IgG purification and flow cytometry-based HIV-1 RNA *in situ* hybridization were performed using the Melon-Gel IgG Spin Purification kit (Thermo Fisher Scientific) and ViroTect (Invirion, Oak Brook IL), respectively.

Macrophage Ab-dependent Cellular Phagocytosis (ADCP), TUNEL, Fc-OxyBurst, and Chemotaxis Assays

Macrophages were cultured in X-VIVO 10 (Lonza, Zurich, Switzerland) containing 10% heat-inactivated human serum AB (Lonza). Macrophages were collected on day 5 of culture and labeled with Orange-CMTMR (5 nM) (Life Technologies). Target qCD4s were coated with gp120 (250 ng/ml unless otherwise specified) or AT-2-inactivated HIV-1 at 4°C for 1 h, washed thoroughly, and exposed to HI- or non-HI-patient serum for 1 h at 37°C. After labeling with CFSE, qCD4s were incubated with Orange-CMTMR-labeled macrophages (5:1) in X-VIVO 10 in glass bottom dishes (*Matsunami Glass*, Osaka, Japan). After fixation, the number of macrophages containing qCD4s was determined from three-dimensional reconstructions generated using an LSM 510 system. The percentage of macrophages that phagocytosed rCD4s was determined using approximately 500 macrophages per experiment. The TUNEL reaction was performed using the FragEL-DNA fragmentation detection kit (Oncogene, La Jolla State, CA). Fc-OxyBurst assays (Life Technologies) were performed according to the manufacturer's instructions. PBMCs from one HIV-1-seronegative individual were used as the standard for calculating the percent change and relative quantities of oxidative species generated by macrophages as follows: relative $O^{\bullet}$ production $= (F_{sample} - F_{min\text{-}sample})/(F_{ref} - F_{min\text{-}ref})$ where $F_{min\text{-}sample}$ and $F_{min\text{-}ref}$ are background MFIs in the patient sample and control, respectively. qCD4 chemotaxis activity was determined using 5-μm microchemotaxis plates (NeuroProbe, Gaithersburg, MD) as described [34].

Macrophage ADCP Time-lapse Microscopy

For time-lapse microscopy, two-color confocal and DIC images were collected every 30 s with a Zeiss LSM 510 system with a Plan-Neofluar 40×1.3 NA oil immersion DIC objective (Carl Zeiss).

NK Cell ADCC Assays

Purified qCD4s were coated with gp120 (250 ng/ml unless otherwise specified) or AT-2-treated HIV-1. Highly purified CFSE-labeled (3 nM) NK cells were incubated for 48 h with gp120/HIV-1-coated qCD4s in 10% serum from HIV-1-seropositive subjects, and cytotoxicity was determined by PI labeling (100 μg/ml). Cytokine effects were studied by culturing purified NK cells for 42 h with or without IL-2 (50 ng/ml) or IL-15 (10 ng/ml) in complete medium with 10% FCS. After the cells were labeled with CFSE, ADCC assays with gp120-coated qCD4s were performed.

Supporting Information

Figure S1 Rapid turnover of CXCR4 on A3.01 T lymphoma cells and activated qCD4s. (**a**) The schematic summarizes the inhibitory activities of the indicated compounds. (**b**) Effect of inhibitors on CXCR4 expression on A3.01 and activated qCD4s. (**c**) After T22 exposure, anti-CXCR4 mAb (12G5) binding to CXCR4 was assessed by FACS. Percent recovery was calculated using MFIs. Bars indicate SD. Data are representative of three independent experiments.
(EPS)

Figure S2 Turnover of cell-bound gp120 or HIV-1 on qCD4s or anti-CD3 Ab-exposed qCD4s. (**a**) Representative FACS data. (**b**) The quantity of cell-bound p24 on HIV-1_{Lai}-pulsed qCD4s was assessed by immunoblotting with anti-p24 Abs. qCD4s were pulsed with HIV-1_{Lai} in the presence or absence of Enf. Numbers indicate cell-bound p24 relative to cell-bound p24 at 0 h. Data are representative of three independent experiments.
(EPS)

Figure S3 HIV-1 patient serum contains sufficient levels of anti-gp120 Abs to form sICs on qCD4s. Summary of the percentages of Leu3a/CD4v4 (left) and the MFIs of sICs on qCD4s exposed to the indicated concentrations of the gp120 (right). $gp120_{IIIB}$ was incubated at various concentrations with qCD4s, which were then stained with Leu3a and CD4v4 or serum from HIV-1^+ patients.
(EPS)

Figure S4 cICs in the serum of viremic HIV-1^+ Pts are sufficient to form sICs on B cells but not on resting CD4^+ T cells. (**a, b**) Summary of the percentages (**a**) and representative FACS data (**b**) of IgM^+ or IgG^+ sICs or IgM^+ sIC formation on purified $CD20^+$ IgG^{dull} IgM^{dull} B cells after exposure to serum from a healthy control donor or HIV-1^+ Pts with various VLs. (**c, d**) Summary of the percentages (**d**) and representative FACS data (**c**) of fluorescence-based HIV-1 RNA *in situ* hybridization in B cells exposed to serum from a healthy control donor or HIV-1^+ Pts with various VLs. Plasma VLs are indicated next to the HIV-1^+ Pt numbers. (**e**) Summary of the percentages of sIg^+ rCD4s in gp120-pulsed or non-pulsed qCD4s that were exposed to serum (**gp120+serum or Serum**) or the percentages of sIg^+ rCD4s in non-pulsed qCD4s that were exposed to purified IgG (100 mg/ml) (**IgG**) from a healthy control or HIV-1^+ Pts with various VLs.
(EPS)

Figure S5 Time-lapse microscopy of phagocytosis of gp120-coated qCD4s and sIC^+ qCD4s by macrophages. (**a, b**) Representative time-lapse image sequence of phagocytosis of gp120-coated qCD4s (**a**) and sIC^+ qCD4s (**b**) by macrophages. The color overlay images show macrophages (Orange-CMTMR, red) and qCD4s (CFSE, green). Schematic figures and trajectories of qCD4s (various colors) and macrophages (red) are also shown.
(EPS)

Figure S6 Three-dimensional images of phagocytosis of sIC-coated qCD4s by macrophages. Data show 3D image reconstruction of deconvoluted stacks through X-Y-Z projections of fluorescence confocal micrographs of phagocytosis assays at 3 h. The color overlay images show macrophages (Orange-CMTMR, red) and qCD4s (CFSE, green).
(EPS)

Table S1 Percentage of expression of CR and FcγRII in B and CD4^+ T cells from patients and controls.
(DOCX)

Movie S1 Time-lapse microscopy of phagocytosis of gp120-coated qCD4s by macrophages. The color overlay images show macrophages (Orange-CMTMR, red) and qCD4 (CFSE, green).
(AVI)

Movie S2 Time-lapse microscopy of phagocytosis of sIC^+ qCD4s by macrophages. The color overlay images show macrophages (Orange-CMTMR, red) and qCD4 (CFSE, green).
(AVI)

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Author Contributions

Conceived and designed the experiments: YS. Performed the experiments: YS. Analyzed the data: YS. Contributed reagents/materials/analysis tools: YS HG NT SO. Wrote the paper: YS.

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Clinical significance of high anti-*Entamoeba histolytica* antibody titer in asymptomatic HIV-1-infected individuals

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ABSTRACT

Background. Anti-*Entamoeba histolytica* antibody (anti-Eh) is widely used in seroprevalence studies though its clinical significance has not been assessed previously.

Methods. Anti-Eh titer was measured at first visit to our clinic (baseline) in 1,303 HIV-1-infected patients. The time to diagnosis of invasive amebiasis was assessed by Kaplan-Meier method and risk factors for the development of invasive amebiasis were assessed by Cox proportional-hazards regression analysis. For patients who developed invasive amebiasis, anti-Eh titers at onset were compared with those at baseline and after treatment.

Results. The anti-Eh seroprevalence in the study population was 21.3% (277/1,303). Eighteen patients developed invasive amebiasis during the treatment-free period among 1,207 patients who had no history of previous treatment with nitroimidazole. Patients with high anti-Eh titer at baseline developed invasive amebiasis more frequently than those with low anti-Eh titer. Most cases of invasive amebiasis who had high anti-Eh titer at baseline developed within one year. High anti-Eh titer was the only independent predictor of future invasive amebiasis. Anti-Eh titer was elevated at the onset of invasive amebiasis in patients with low anti-Eh titer at baseline.

Conclusions. Asymptomatic HIV-1-infected individuals with high anti-Eh titer are at risk of invasive amebiasis probably due to exacerbation of subclinical amebiasis.

INTRODUCTION

Invasive amebiasis caused by *Entamoeba histolytica* is the second most common cause of parasite infection-related mortality worldwide, accounting for 40,000 to 100,000 deaths annually [1]. Recently, it was reported that invasive amebiasis is prevalent not only in developing countries where food or water is contaminated with stool, but also in East Asian developed countries (Korea, China, Taiwan and Japan) and Australia as a sexually transmitted infection (STI) [2-4]. On the other hand, the annual incidence of human immunodeficiency virus type 1 (HIV-1) infection is also on the rise among men who have sex with men (MSM) in these countries [5-8], with resultant growing concern regarding invasive amebiasis in HIV-1-infected MSM [9-14].

Serum anti-*E. histolytica* antibody (anti-Eh) is widely used as an index marker for the presence of amebiasis. It is used not only in developing countries [15-22] but also in developed countries where amebiasis is spreading as an STI [3, 9, 23-26]. Furthermore, the seroprevalence of anti-Eh antibody in HIV-1-infected individuals is generally higher than in HIV-1 negative ones [3, 9, 15, 24]. However, only limited information is available on the seroprevalence of amebiasis in Japan [25, 26] despite the increasing number of invasive amebiasis among HIV-1-infected individuals reported lately [27, 28].

Serum anti-Eh antibody is also widely used for the diagnosis of invasive amebiasis based on the high sensitivity and good differentiation ability from other amoeba species, such as *Entamoeba dispar* and *Entamoeba moshkovskii* [29]. However, the primary disadvantage of this method is that it cannot distinguish current

infection from past infection. Moreover, anti-Eh antibody titer can be elevated even in asymptomatic infected individuals, and seroconversion of anti-Eh was reported in the absence of any symptoms in longitudinal follow-up in endemic areas [14]. At present, the pathogenesis of amebiasis in asymptomatic anti-Eh-positive individuals remains poorly understood.

In the present study, we found high seroprevalence of anti-Eh antibody in HIV-1-infected adult Japanese. Retrospective analysis of these seropositive individuals indicated that those with high anti-Eh titer are prone to future invasive amebiasis. These findings highlight the clinical significance of anti-Eh positivity and enhance our understanding of the pathogenesis of invasive amebiasis.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the Human Research Ethics Committee of our hospital, the National Center for Global Health and Medicine, Tokyo. The study was conducted in accordance with the principles expressed in the Declaration of Helsinki.

Study design and population

The present study was a single-center retrospective cohort study. Our facility is one of the largest core hospitals for patients with HIV-1 infection in Japan, with >3000 registered patients. The study population was HIV-1-infected patients who were referred to our hospital for management of HIV-1 infection for the first time between January 2006 and April 2012.

Anti-*E. histolytica* antibody (anti-Eh) testing

Indirect fluorescent-antibody (IFA) assay was used for the detection of anti-Eh antibody in serum by using a slide pre-coated with fixed *E. histolytica*. This method can distinguish amebiasis caused by *E. histolytica* from that caused by other amoeba species, such as *E. dispar* and *E. moshkovskii.* The sensitivity and specificity of this method for the detection of *E. histolytica* infection are comparable with other methods, such as counterimmunoelectrophoresis and indirect hemagglutination amebic serology [29, 30]. The commercial kit, Amoeba-Spot IF (bioMerieux SA), is currently approved for the diagnosis of *E. histolytica* infection in Japan. Based on the instructions enclosed with the kit, the biological samples were initially diluted at 1:100 with phosphate-buffered saline (PBS) and then incubated for 30 minutes at room temperature on slides pre-coated with fixed *E. histolytica*. Then, the slides were washed with PBS twice, treated with the fluorescent-labeled anti-human antibodies, and incubated for another 30 minutes at room temperature. The slides were washed again, and cover slips with buffered glycerol were placed over the slides. Fluorescence in each slide was examined with fluorescence microscope and compared with negative control slides. Seropositivity was defined as positive response in serum sample diluted at 1:100, and anti-Eh titer was determined by the highest dilution for the positive response.

Development of invasive amebiasis in patients without history of nitroimidazole treatment

Newly-registered HIV-1-infected individuals who underwent anti-Eh testing at first visit were included in this analysis. Patients were excluded from the follow-up study 1) if they had been treated previously with nitroimidazole (metronidazole or tinidazole), or 2) if they were treated with nitroimidazole at first visit to the clinic. The clinical characteristics and results of serological tests for other STIs, such as syphilis, hepatitis B and C viruses (HBV and HCV), were collected from the medical records. The follow-up period spanned from the time of the first visit to May 2012, unless patients died from other causes during this period, dropped out, or were referred to other facilities.

The diagnosis of invasive amebiasis was based on the medical records of three different clinicians, and satisfied one of the following two criteria, as described previously [12-14] ; 1) identification of erythrophagocytic trophozoites in biological specimens (stool or biopsy sample) of HIV-1-infected patients with symptoms of invasive amebiasis, such as fever, tenesmus and diarrhea, 2) identification of liver abscess by imaging studies in seropositive (titer $\geq$ x100) patients with symptoms related to invasive amebiasis who showed clinical improvement after nitroimidazole monotherapy. For patients who developed invasive amebiasis during follow-up, we compared anti-Eh titer at the time of onset of invasive amebiasis with those at first visit (baseline) and after nitroimidazole therapy.

Statistical analysis

The patients' characteristics and results of serological tests on STIs were compared using chi-square test or Student t-test for qualitative or quantitative variables, respectively. The time to the diagnosis of invasive amebiasis was calculated from the date of the first visit of our hospital to the date of diagnosis of invasive amebiasis. Censored cases represented those who died, dropped out, or were referred to other facilities during the follow-up. The time from first visit to the diagnosis of invasive amebiasis was calculated by the Kaplan-Meier method followed by log-rank test to determine the statistical significance. The Cox proportional-hazards regression analysis was used to estimate the impact of anti-Eh titer at baseline on the incidence of invasive amebiasis. The impact of basic clinical characteristics, such as sexuality and serology status of other STIs, was estimated with univariate Cox proportional hazards regression. We also conducted multivariate Cox hazards regression analysis using variables identified in univariate analysis with P values of <0.20. In all analyses, statistical significance was defined as two-sided P value of <0.05. We used the hazard ratio (HR) and 95% confidence interval (95%CI) to estimate the impact of each variable on the development of invasive amebiasis. All statistical analyses were performed using The Statistical Package for Social Sciences (SPSS Inc., Chicago, IL).

RESULTS

Clinical characteristics of asymptomatic anti-Eh-positive HIV-1-infected patients

A total of 1,519 patients were referred to our hospital during the study period. Anti-Eh testing was conducted in 1,303 patients at first visit, including 73 with history of

invasive amebiasis, and anti-Eh was positive in 277 of these (21.3%). Among the anti-Eh-positive individuals, the rates of MSM (88.4%) and those with previous exposure to syphilis (TPHA test positive) (54.9%) and HBV (68.8%) were higher than those of anti-Eh-negatives individuals, indicating that sexually active MSM are prone to *E. histolytica* infection among HIV-1-infected individuals in Japan (Table 1). Eight patients were diagnosed with invasive amebiasis at first visit, including 7 cases of amebic colitis and 1 case of amebic liver abscess, and they were treated immediately with metronidazole.

Incidence of invasive amebiasis during follow-up of HIV-1 infected individuals

To assess the frequency of development of invasive amebiasis in patients free of symptomatic invasive amebiasis and who had not previously received nitroimidazole therapy, we excluded 96 patients from the analysis, including 73 patients because they had been treated previously for invasive amebiasis, and 23 patients (7 cases of amebic colitis, 1 case of amebic liver abscess, and 15 asymptomatic but anti-Eh-positive cases treated preemptively) because they were treated with nitroimidazole at first visit (Figure 1). The remaining 1,207 patients, including 195 anti-Eh-positive patients (16.2%), were followed-up for median period of 25.3 months (interquartile range: 7.0-47.2). During the follow-up period, 18 patients developed invasive amebiasis (median time to onset: 9.1 months), including amebic appendicitis in one patient (confirmed by identification of erythrophagocytic trophozoites in surgically removed specimen), amebic liver abscess in 6, and amebic colitis in 11 (confirmed by identification of erythrophagocytic trophozoites in stool samples). The median anti-Eh

titer at baseline was significantly higher among patients who developed invasive amebiasis than that among those who did not, but the other clinical and laboratory parameters were not different between the two groups (Table 2). Although no significant differences in the frequency of invasive amebiasis were evident in patients with x100 (P=0.77) and x200 (P=0.18) anti-Eh titers at baseline, compared with negative anti-Eh patients (< x100), the frequency was higher in patients with x400 (P<0.001), x800 (P=0.025), and ≥ x1600 (P<0.001) anti-Eh titers at baseline, compared with negative anti-Eh patients. Univariate and multivariate analyses also showed that future development of invasive amebiasis correlated only with high titer of anti-Eh antibody at baseline (≥ x400: Univariate, HR: 20.985, 95% CI: 8.085-54.467; multivariate, HR: 22.079, 95% CI: 7.964-61.215) (Table 3). Furthermore, the risk of development of invasive amebiasis was significantly higher in the high anti-Eh titer group (patients with anti-Eh titer ≥ x400 at baseline) than in the low anti-Eh titer group (patients with anti-Eh titer ≤ x200 at baseline) (log-rank test: χ^2 = 80.203, p<0.001, Kaplan-Meier estimate, Figure 2). Moreover, most patients of the high anti-Eh titer group developed invasive amebiasis during the first year of follow-up whereas those of the low anti-Eh titer group developed this complication more lately and new cases of invasive amebiasis were diagnosed throughout the follow-up period.

Transitional changes in anti-Eh titer among patients who developed amebiasis

The median anti-Eh titer was significantly higher at the onset of invasive amebiasis than that at first visit in patients with low baseline anti-Eh titer (≤ x200) (p=0.028,

Wilcoxon signed-rank test) (Figure 3). In contrast, the median anti-Eh titers at these two time points were not different in patients with high baseline anti-Eh titer (≥ x400) (p=0.18, Wilcoxon signed-rank test). Serum samples taken after nitroimidazole treatment (median time from the commencement of treatment 289 days [range 174-841]) were available in 10 patients. Anti-Eh titers were lower after the treatment in 7 of the 10 patients, compared with the baseline values. To define the natural decay of anti-Eh, we measured serum anti-Eh titers at 9 months after study enrollment in 37 patients with high anti-Eh titer at baseline but did not develop invasive amebiasis during the study period. The titers were lower, or similar to the baseline in 19 and 15 patients, respectively, while the remaining 3 patients showed 2-fold increase in the titer.

DISCUSSION

In the present study, the seroprevalence of anti-Eh antibody among HIV-1-infected patients was 21.3%, which was much higher than those reported in other developed countries where amebiasis is considered as an STI [3, 9, 23, 24]. In addition, our results showed that sexually active MSM tend to be seropositive for *E. histolytica* infection, in agreement with previous studies from our group [27, 28].

The pathogenesis of amebiasis, such as incubation period after cyst ingestion and the mechanism of spontaneous remission, remains unclear. Although previous study showed anti-Eh-positive children were more susceptible to *E. histolytica* infection than their seronegative counterparts [31], the clinical significance of anti-Eh seropositivity and its titer in asymptomatic individuals had not been fully assessed.

We measured serum anti-Eh IgM levels in 18 patients at the onset of invasive amebiasis [32], but the level was detectable only in 3 patients with amebic colitis and 1 patient with liver abscess. The present study demonstrated that patients with high anti-Eh titer ($\geq$ x400) at first visit developed invasive amebiasis much more frequently than those with low anti-Eh titer ($\leq$ x200). The cumulative risk for invasive amebiasis among patients with high anti-Eh titer at baseline rapidly increased during the first one year of follow-up but plateaued thereafter, suggesting that exacerbation of subclinical amebiasis occurs frequently within one year in these patients. On the other hand, the cumulative risk for invasive amebiasis among patients with low anti-Eh titer at baseline increased more slowly and developed at the same pace throughout the follow-up period, suggesting that the invasive amebiasis in these patients represented new infection rather than exacerbation of subclinical infection. The median anti-Eh titer at the onset of invasive amebiasis in patients of high anti-Eh titer group was not higher than that at first visit, whereas the titer increased at the onset compared with that at baseline in low anti-Eh titer group. In addition, uni- and multi-variate analyses identified high titer of anti-Eh antibody at baseline as the only significant risk factor for future development of invasive amebiasis; seropositivity to other STIs was not a significant factor. These results add support to the aforementioned hypothesis regarding the difference in the pathology of invasive amebiasis between the high and low anti-Eh groups. In this study, 15 asymptomatic but anti-Eh-positive patients were treated with metronidazole at first visit (excluded from the follow-up analysis study), and none of them developed invasive amebiasis (median follow-up period, 11.7

months), suggesting the potential effectiveness of preemptive therapy for asymptomatic individuals with high anti-Eh titer.

In conclusion, our results showed a relatively high prevalence of amebiasis in HIV-1-infected individuals in Japan, and that subclinical amebiasis is common among these individuals. The results emphasize the difficulty of disease control in not only individual patients with amebiasis but also in epidemiological control of this condition due to the long duration of subclinical infection of *E. histolytica*. Anti-Eh testing for high risk individuals could be helpful in early diagnosis of subclinical amebiasis, and early treatment of patients with such infection could prevent the development of invasive amebiasis and the transmission to others in the same community. Further studies to clarify the pathogenesis of invasive amebiasis are warranted.

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Declaration

The authors report no potential conflicts.

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