Clinical Significance of High Anti-*Entamoeba histolytica* Antibody Titer in Asymptomatic HIV-1-infected Individuals

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

Methods. Anti-*E. histolytica* titer was measured at first visit to our clinic (baseline) in 1303 patients infected with human immunodeficiency virus type 1 (HIV-1). 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-*E. histolytica* titers at onset were compared with those at baseline and after treatment.

Results. The anti-*E. histolytica* seroprevalence in the study population was 21.3% (277/1303). Eighteen patients developed invasive amebiasis during the treatment-free period among 1207 patients who had no history of previous treatment with nitroimidazole. Patients with high anti-*E. histolytica* titer at baseline developed invasive amebiasis more frequently than those with low anti-*E. histolytica* titer. Most cases of invasive amebiasis who had high anti-*E. histolytica* titer at baseline developed within 1 year. High anti-*E. histolytica* titer was the only independent predictor of future invasive amebiasis. Anti-*E. histolytica* titer was elevated at the onset of invasive amebiasis in patients with low anti-*E. histolytica* titer at baseline.

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

Keywords. seroprevalence; *Entamoeba histolytica*; HIV-1; anti-*E. histolytica* antibody; amebiasis.

Invasive amebiasis caused by *Entamoeba histolytica* is the second most common cause of parasite infection-related mortality worldwide, accounting for 40 000–100 000 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-*E. Histolytica*) 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-*E. histolytica* 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].

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Serum anti-*E. histolytica* 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-*E. histolytica* antibody titer can be elevated even in asymptomatic infected individuals, and seroconversion of anti-*E. histolytica* 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-*E. histolytica* -positive individuals remains poorly understood.

In the present study, we found high seroprevalence of anti-*E. histolytica* antibody in HIV-1-infected adult Japanese. Retrospective analysis of these seropositive individuals indicated that those with high anti-*E. histolytica* titer are prone to future invasive amebiasis. These findings highlight the clinical significance of anti-*E. histolytica* 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 Testing

Indirect fluorescent-antibody (IFA) assay was used for the detection of anti-*E. histolytica* antibody in serum by using a slide precoated 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 precoated 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-*E. histolytica* 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-*E. histolytica* 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 and 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 3 different clinicians and satisfied one of the following 2 criteria, as described elsewhere [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 $\geq \times 100$) 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-*E. histolytica* 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 χ^2 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-*E. histolytica* titer at baseline on the incidence of invasive amebiasis. The impact of basic clinical characteristics,

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Table 1. Characteristics of All Patients Who Underwent Anti-*E. histolytica* Testing (n = 1303)

	Anti-*E. histolytica* Negatives (n = 1026)	Anti-*E. histolytica* Positives (n = 277)	*P* Value
Age, years (range)	36 (18–77)	37 (19–74)	.06
Japanese nationality, no. (%)	921 (89.8%)	250 (90.3%)	.81
Male sex, no. (%)	960 (93.6%)	272 (98.2%)	.003
MSM, no. (%)	789 (76.9%)	245 (88.4%)	<.001
TPHA test positive, no. (%)	366/1012 (36.2%)	151/275 (54.9%)	<.001
HBV exposure,[a] no. (%)	524/1017 (51.5%)	187/272 (68.8%)	<.001
HCV Ab positive, no. (%)	40/1011 (4.0%)	5/273 (1.8%)	.09
Past history of IA, no. (%)	13 (1.3%)	60 (21.7%)	<.001
Diagnosis of IA at first visit, no. (%)	1 (0.1%)	7 (2.5%)	<.001

Abbreviations: Ab, antibody; Anti-*E. histolytica*, anti *Entamoeba histolytica* antibody; HBV, hepatitis B virus; HCV, hepatitis C virus; IA, invasive amebiasis; MSM, men who have sex with men; TPHA, *Treponema pallidum* hemagglutination.

[a] HBV exposure: HBsAg-positive or HBsAb-positive, and/or HBc-Ab positive.

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 < .20. In all analyses, statistical significance was defined as 2-sided *P* value of < .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-*E. histolytica*-positive HIV-1-infected Patients

A total of 1519 patients were referred to our hospital during the study period. Anti-*E. histolytica* testing was conducted in 1303 patients at first visit, including 73 with history of invasive amebiasis, and anti-*E. histolytica* was positive in 277 of these (21.3%). Among the anti-*E. histolytica*-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-*E. histolytica*-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-*E. histolytica*-positive cases treated preemptively) because they were treated with nitroimidazole at first visit (Figure 1). The remaining 1207 patients, including 195 anti-*E. histolytica*-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 1 patient

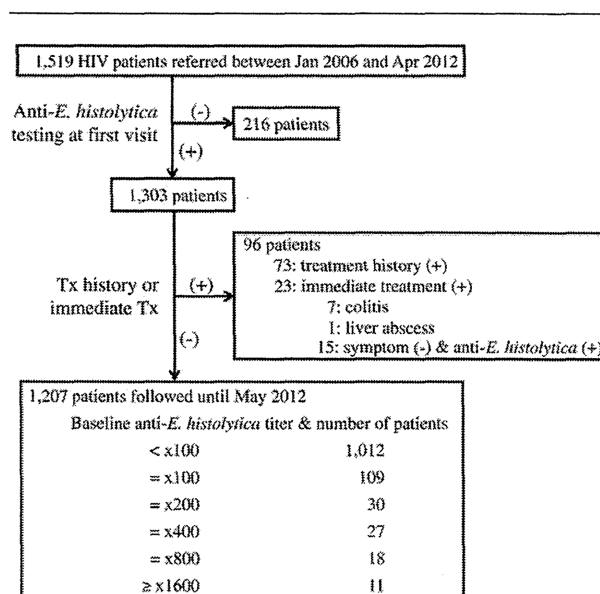

Figure 1. Flow diagram of patient recruitment process. Abbreviations: Anti-*E. histolytica*, anti-*Entamoeba histolytica* antibody; IA, invasive amebiasis; Tx, treatment.

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Table 2. Comparison of Clinical Characteristics of Patients With and Without Invasive Amebiasis

	Amebic Colitis (n = 11)	Extraintestinal IA[a] (n = 7)	Non-IA (n = 1189)	*P* Value IA vs Non-IA
Age (years), average (SD)	35.9 (12.3)	38.2 (11.0)	37.5 (10.8)	.81
Japanese nationality, no. (%)	10 (90.9)	6 (85.7)	1068 (89.8)	.71
Male sex, no. (%)	11 (100)	7 (100)	1119 (94.1)	.62
MSM, no. (%)	11 (100)	6 (85.7)	929 (78.1)	.15
TPHA test-positive, no. (%)	5 (45.5)	2 (28.6)	451/1175 (38.4)	.91
HBV exposure,[a] no. (%)	6 (54.5)	5 (71.4)	630/1178 (53.5)	.15
HCVAb-positive, no. (%)	0/11 (0)	0/7 (0)	42/1172 (3.6)	1.00
Anti-*E. histolytica* at baseline, median (IQR)	×100 (<×100–×800)	×400 (×100–×400)	<×100 (<×100–<×100)	<.001
Anti-*E. histolytica* at the onset of IA, median (IQR)	×800 (×200–×800)	×400 (×100–×800)	. . .	
Follow-up period, median months (IQR)	7.8 (3.3–25.1)	10.5 (4.9–17.9)	25.5 (7.0–47.3)	

Data were compared using χ^2 test, Student *t* test, or Mann–Whitney *U* test for qualitative or quantitative variables, respectively.

Abbreviations: Ab, antibody; Anti-*E. histolytica*, anti *Entamoeba histolytica* antibody; HBV, hepatitis B virus; HCV, hepatitis C virus; IA, invasive amebiasis; IA, invasive amebiasis; IQR, interquartile range; MSM, men who have sex with men; SD, standard deviation; TPHA, *Treponema pallidum* hemagglutination.[a]Extraintestinal cases include one case of appendicitis and 6 cases of liver abscess.

(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-*E. histolytica* 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 2 groups (Table 2). Although no significant differences in the frequency of invasive amebiasis were evident in patients with ×100 ($P = .77$) and ×200 ($P = .18$) anti-*E. histolytica* titers at baseline, compared with negative anti-*E. histolytica* patients (<×100), the frequency was higher in patients with ×400 ($P < .001$), ×800 ($P = .025$), and ≥×1600 ($P < .001$) anti-*E. histolytica* titers at baseline, compared with negative anti-*E. histolytica* patients. Univariate and multivariate analyses also showed that future development of invasive amebiasis correlated only with high titer of anti-*E. histolytica* antibody at baseline (≥×400: Univariate, HR: 20.985, 95% confidence interval [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-*E. histolytica* titer group (patients with anti-*E. histolytica* titer ≥×400 at baseline) than in the low anti-*E. histolytica* titer group (patients with anti-*E. histolytica* titer ≤×200 at baseline; log-rank test: $\chi^2 = 80.203$, $P < .001$, Kaplan-Meier estimate, Figure 2). Moreover, most patients of the high anti-*E. histolytica*

Table 3. Risk Analysis for Development of Invasive Amebiasis by Cox Proportional Hazard Regression Model

	Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
older age (by 1 y)	0.989 (.947–1.033)	.624		
Japanese nationality	1.334 (.305–5.840)	.702		
Male sex	21.884 (.002–241297.39)	.516		
MSM	4.318 (.573–32.518)	.156	4.048 (.488–33.584)	.195
TPHA test-positive	0.901 (.348–2.335)	.831		
HBV exposure-positive	2.183 (.778–6.124)	.138	1.839 (.644–5.249)	.255
HCVAb-positive	0.047 (.000–2697.344)	.584		
Anti-*E. histolytica* titer ≥×400	20.985 (8.085–54.467)	<.001	22.079 (7.964–61.215)	<.001

The Cox proportional-hazards regression analysis was used to estimate the impact of anti-*E. histolytica* 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. Multivariate Cox hazards regression analysis using variables identified in univariate analysis with *P* values of <.20. In all analyses, statistical significance was defined as *P* value of <.05.

Abbreviations: Ab, antibody; Anti-*E. histolytica*, anti *Entamoeba histolytica* antibody; CI, confidence interval; HBV, hepatitis B virus; HCV, hepatitis C virus; IA, invasive amebiasis; IA, invasive amebiasis; IQR, interquartile range; MSM, men who have sex with men; TPHA, *Treponema pallidum* hemagglutination.

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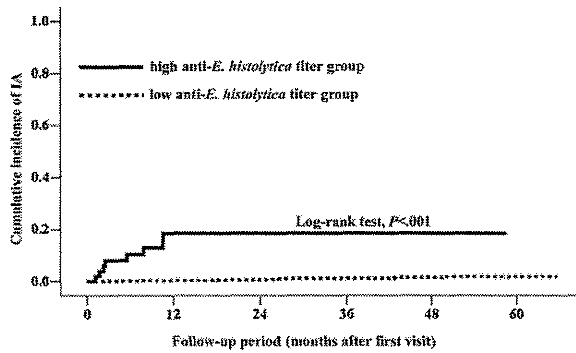

Figure 2. Incidence of invasive amebiasis in low and high anti-*E. histolytica* titer groups. Differences in the time from first visit to the diagnosis of invasive amebiasis (IA) between the low anti-*E. histolytica* titer group (≤×200 at baseline) and high anti-*E. histolytica* titer group (≥×400 at baseline) were analyzed by Kaplan-Meier method. Log-rank test was used to determine the statistical significance. Abbreviations: Anti-*E. histolytica*, anti-*Entamoeba histolytica* antibody; IA, invasive amebiasis.

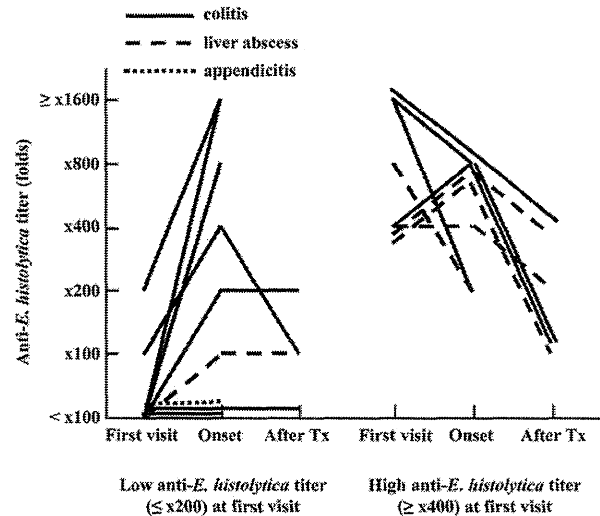

Figure 3. Anti-*E. histolytica* titer before and after diagnosis of invasive amebiasis. Anti-*E. histolytica* titer at the onset of IA was compared to that at baseline (first visit to the clinic) by Wilcoxon signed-rank test. Anti-*E. histolytica* titers after treatment were measured at 219 days [range: 174–252] and 367 days [272–841] after the completion of treatment of patients with low and high anti-*E. histolytica* titer at first visit, respectively. Abbreviations: Anti-*E. histolytica*, anti-*Entamoeba histolytica* antibody; IA, invasive amebiasis.

titer group developed invasive amebiasis during the first year of follow-up, whereas those of the low anti-E. histolytica titer group developed this complication more lately and new cases of invasive amebiasis were diagnosed throughout the follow-up period.

Transitional Changes in Anti-*E. histolytica* Titer Among Patients Who Developed Amebiasis

The median anti-*E. histolytica* titer was significantly higher at the onset of invasive amebiasis than that at first visit in patients with low baseline anti-*E. histolytica* titer (≤×200; $P = .028$, Wilcoxon signed-rank test) (Figure 3). In contrast, the median anti-*E. histolytica* titers at these 2 time points were not different in patients with high baseline anti-*E. histolytica* titer (≥×400; $P = .18$, 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-*E. histolytica* titers were lower after the treatment in 7 of the 10 patients, compared with the baseline values. To define the natural decay of anti-*E. histolytica*, we measured serum anti-*E. histolytica* titers at 9 months after study enrollment in 37 patients with high anti-*E. histolytica* 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, whereas the remaining 3 patients showed 2-fold increase in the titer.

DISCUSSION

In the present study, the seroprevalence of anti-*E. histolytica* 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-*E. histolytica*-positive children were more susceptible to *E. histolytica* infection than their seronegative counterparts [31], the clinical significance of anti-*E. histolytica* seropositivity and its titer in asymptomatic individuals had not been fully assessed. We measured serum anti-*E. histolytica* immunoglobulin M (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-*E. histolytica* titer (≥×400) at first visit developed invasive amebiasis much more frequently than those with low anti-*E. histolytica* titer (≤×200). The cumulative risk for invasive amebiasis among patients with high anti-*E. histolytica* 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-*E. histolytica* titer at baseline increased more slowly and

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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-*E. histolytica* titer at the onset of invasive amebiasis in patients of high anti-*E. histolytica* 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-*E. histolytica* titer group. In addition, uni- and multivariate analyses identified high titer of anti-*E. histolytica* 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-*E. histolytica* groups. In this study, 15 asymptomatic but anti-*E. histolytica*-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-*E. histolytica* 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-*E. histolytica* 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.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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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 (× 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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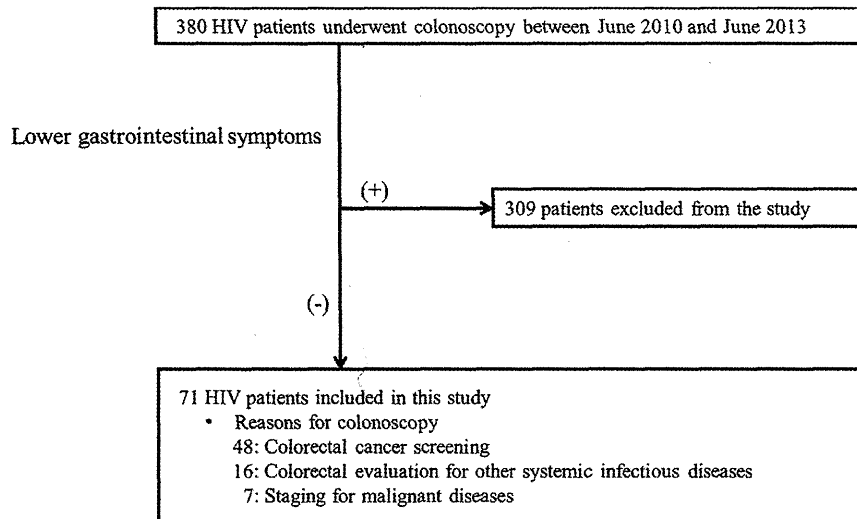

FIGURE 1. Flow diagram of the patient recruitment process. Lower abdominal symptoms were collected based on the GSRS rating on a seven-graded Likert scale at 1 week before colonoscopy.

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

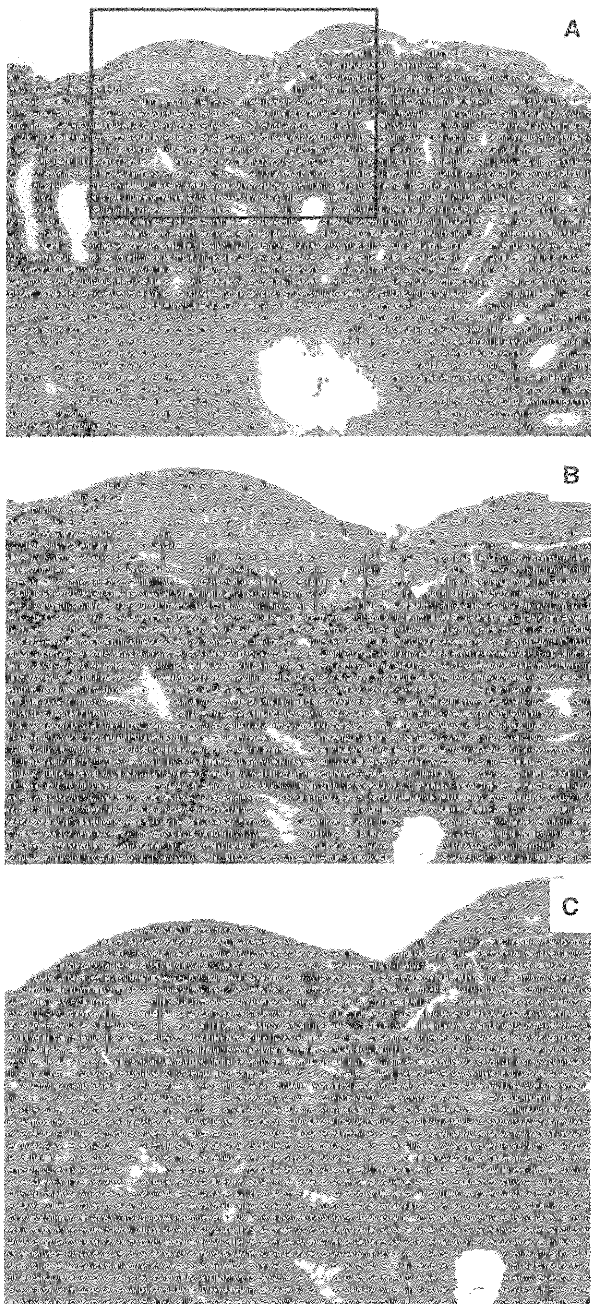

FIGURE 2. Histopathological findings in subclinical intestinal amebiasis. Colonic tissue section was obtained during colonoscopy from a representative asymptomatic patient. *E. histolytica* on the surface of large-intestinal mucosa was clearly stained with periodic acid-Schiff (PAS) staining (green arrows). (**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

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.

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 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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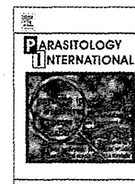

Direct evidence for the atovaquone action on the *Plasmodium* cytochrome bc_1 complex

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ABSTRACT

Atovaquone, a coenzyme Q analogue has been indicated to specifically target the cytochrome bc_1 complex of the mitochondrial respiratory chain in the malarial parasite and other protozoan. Various mutations in the quinone binding site of the cytochrome *b* gene of *Plasmodium* spp. such as M133I, L144S, L271V, K272R, Y268C, Y268S, Y268N, and V284F are suggesting to associate with resistance to atovaquone. There is no direct evidence of relation between the mutations and resistance to atovaquone in *Plasmodium* parasite that has been available. Technical difficulties in isolating active assayable mitochondria in the malarial parasite hinder us to obtain direct biochemical evidence to support the relation between the mutations and drug resistance.

The establishment of a mitochondrial isolation method for the malaria parasite has allowed us to test the degree of resistance of *Plasmodium berghei* isolates to atovaquone directly. We have tested the activity of dihydroorotate (DHO)-cytochrome *c* reductase in various *P. berghei* atovaquone resistant clones in the presence of a wide concentration range of atovaquone. Our results show the IC_{50} of *P. berghei* atovaquone resistant clones is much higher (1.5 up to 40 nM) in comparison to the atovaquone sensitive clones (0.132–0.465 nM). The highest IC_{50} was revealed in clones carrying Y268C and Y268N mutations (which play an important role in atovaquone resistance in *Plasmodium falciparum*), with an approximately 100-fold increase. The findings indicate the importance of the mutation in the quinone binding site of the cytochrome *b* gene and that provide a direct evidence for the atovaquone inhibitory mechanism in the cytochrome bc_1 complex of the parasite.

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1. Introduction

The expansion of drug-resistant strains of *Plasmodium falciparum* has caused serious global health problems in malaria treatment. Several new drugs have been introduced including new derivatives of quinoline-based compounds, but resistance of the parasite to those drugs developed very rapidly [1]. Different biochemical pathways in the malaria parasite are needed as the pharmacological target for the new affordable drugs.

Ubiquinone is a coenzyme involved in the mitochondrial electron transport, and serves as a point of contact between pyrimidine metabolism and energy metabolism. Atovaquone (a hydroxy-1,4-naphthoquinone derivative), that shares structural similarity with ubiquinone, has been suggested to be a potent and specific inhibitor of the parasite cytochrome bc_1 complex, and its molecular target is known to be the ubiquinol oxidation pocket (Q_o site) of the cytochrome bc_1 complex [2–4]. The drug has a broad spectrum activity against various apicomplexan parasites and mutations in the *cyt b* gene were shown to associate with resistance to atovaquone in various organisms, such as *Plasmodium* species, *Toxoplasma*, *Pneumocystis jirovecii* and yeast [5–11]. However, no direct evidence of relation between the mutations and resistance has been available by using intact mitochondria isolated from the malarial parasite, although biochemical analysis of the mutant has been reported using cell free extract [12].

Our group has reported various mutations in the quinone binding site of the *cyt b* gene of *Plasmodium berghei* such as M133I, L144S, L271V, K272R, Y268C, Y268S, Y268N, and V284F by using the mouse model with continuous atovaquone pressure [5,13] and all of those mutations have been proven to associate with resistance to atovaquone

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of other *Plasmodium*. However, the technical difficulties in isolating active mitochondria in the malarial parasite hinder us to obtain direct biochemical evidence to support the aforementioned results.

Following the establishment of the mitochondrial isolation method in the malaria parasite [14], we further tested the activity of dihydroorotate (DHO)-cytochrome *c* reductase in various *P. berghei* atovaquone resistant and sensitive clones in the presence of a wide concentration range of atovaquone for the sensitivity of the enzymes to the drug which might be altered by the cytochrome *b* mutations. We found that mutations in the quinone binding site of the cytochrome *b* gene resulted in a various sensitivity to atovaquone and provide a direct evidence for the atovaquone inhibitory mechanism in the parasite cytochrome bc_1 complex.

2. Material and method

2.1. Malaria parasites

P. berghei ANKA strains from Leiden through the gift of Dr. Andy Waters, Leiden University Medical Center, Netherland were used throughout this study.

Atovaquone-resistant mutant isolates were derived from *P. berghei* ANKA strain in the previous study [5,13] with different mutations in the cytochrome *b* (*PbLSJ2* with Y268C; *PbLSJ3* with L271V, K272R; *PbLSJ1* with Y268N; *PbSK2A1T* with M133I, V284F; *PbSK2A1Tb* with M133I, L271V; *PbSK1A2* with V284F; and *PbSR1* with L144S, V284F).

The parasites were maintained by serial blood passage and inoculated intraperitoneally into 10- to 12-week-old BALB/c mice at approximately 10^6 parasitized red blood cells/mouse. The parasitemia from several random mice were examined every two days until giving raise to 40–50% parasitemia.

2.2. Antimalarial drugs

Atovaquone was kindly provided by Dr. Mary Pudney of the Wellcome Research Laboratories, UK. A 10^{-2} M stock solution was dissolved in dimethyl sulfoxide and stored at −20 °C.

2.3. Preparation of mitochondrial fraction

A protocol to prepare active mitochondria from mouce blood infected with *P. berghei* was established as described below with a slight modification from the protocol for *P. falciparum*[14].

2.3.1. Isolation and purification of erythrocytes

Mouse blood infected with *P. berghei* parasites (7–8 days post infection with 40–50% parasitemia) was collected by cardiac puncture using a sterile disposable syringe and transferred into heparinized tube. Subsequently, platelet rich plasma and white blood cells were removed by the addition of 1/10 volume of 3.8% sodium citrate and 0.027 M NaCl to the tube, and then the solution was mixed gently and centrifuged at 800 *g* for 10 min [TOMY LX-120 (TOMY SEIKO Co Ltd, Japan)]. The supernatant including buffy coat (whitish layer: about 1/3 portion from the top of pellet) was discarded by Pasteur pipette. Ten volumes of 0.85% NaCl containing 4% dextran were added to the pellet, mixed gently and followed by centrifugation at 20 *g* for 10 min to remove the remaining white blood cells. The suspension follows a filtration through a leucofilter (Sepacell RN-10 Asahi Medical Co Ltd, Japan) that was pre-equilibrated with phosphate-buffered saline (PBS; 0.14 M NaCl, 0.01 M Na_2HPO_4; pH 7.2). Filtrate was centrifuged at 1750 *g* for 10 min to collect pure erythrocytes.

Animal care and experimental procedures were performed according to the Guidelines for Animal Experiments, The University of Tokyo.

2.3.2. Isolation of P. berghei mitochondria from infected erythrocytes

The pure parasite-infected erythrocytes were harvested and treated with 0.15% (w/v) saponin in PBS. After washing with PBS, the cells were disrupted with N_2 cavitation method using Cell Disruption Bomb (Parr Instrument Company, USA) at 1200 psi for 15 min at 4 °C in 5 volumes of H-medium (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 5 mM $MgCl_2$, 4 mM HEPES, 5 mM KH_2PO_4; pH 7.2) supplemented with 0.1% BSA, 1 mM PMSF and protease inhibitor cocktail (component of protease inhibitor cocktail: 2 mM AEBSF, 1 mM EDTA, 130 μM Bestatin, 14 μM E-64, 1 μM Leupeptin and 0.3 μM Aprotonin). Unbroken cells and cell debris were removed by centrifugation at 700 *g* for 8 min at 4 °C. The mitochondrial fraction was then recovered as a precipitate by centrifugation at 10,000 *g* for 10 min at 4 °C.

2.4. Enzyme assay

Since the activity of the *Plasmodium* bc_1 complex was too low for kinetic study of the enzyme, we measured the activity of DHO-cytochrome *c* reductase, which is more reliable than the ubiquinol-cytochrome *c* assay. DHO-cytochrome *c* activity consists of two enzymes, dihydroorotate dehydrogenase (DHODH) and bc_1 complex (Fig. 1). DHODH and DHO-cytochrome *c* reductase activities using dichlorophenol indophenol (DCIP) and cytochrome *c* as an electron acceptor, respectively, from various atovaquone-resistant and -sensitive *P. berghei* clones were measured in the presence of a wide concentration range of atovaquone.

2.4.1. DHODH activity

Spectrophotometric enzyme assay was performed using ubiquinone-2 and DCIP as electron acceptors [15]. DHODH activity was measured by recording the absorbance change of DCIP at 600 nm (UV-3000 spectrophotometer; Shimazu, Japan), following the addition of 500 μM DHO to 30 mM Tris–HCl (pH 8.0), 74 μg/ml DCIP, 100 μM ubiquinone-2 and 2 mM KCN at 25 °C.

2.4.2. DHO-cytochrome c reductase activity

DHO-cytochrome *c* reductase activity was measured by recording the change in absorbance of cytochrome *c* at 550 nm following the addition of 500 μM DHO to 30 mM Tris–HCl (pH 8.0), 20 μM cytochrome *c* and 2 mM KCN. In this system, electrons from DHO are transferred to ubiquinone by DHODH, and then finally transferred to cytochrome *c* by bc_1 complex.

2.5. Molecular modeling

Since atovaquone is smaller in molecular size than stigmatellin but relatively similar to 5-*n*-heptyl-6-hydroxy-4,7-dioxobenzothiazole (HHDBT), we have referred to the crystal structure of yeast cytochrome bc_1 complexed with HHDBT as shown in Fig. 2. Molecular modeling was carried out on NEC workstations, starting with the X-ray structure of yeast cytochrome bc_1 complexed with HHDBT (pdbID: 1P84). Structural parameters of atovaquone were calculated with the Dundee PRODRG2 server (http://davapc1.bioch.dundee.ac.uk/prodrg/). Atovaquone molecule was manually aligned in the same manner of HHDBT with the graphic program O [16]. The molecular dynamics and continuous energy minimization were calculated with the CNS software [17].

3. Result and discussions

In the present study, we directly showed that *P. berghei* resistant to antimalarial drug atovaquone was attributed to the mutations of mitochondria *cyt b* gene at the Q_o site. Mutations M133I and L271V, and mutation Y268N show specific activity about half of the wild type isolate, indicating that there may be a little defect in the respiratory function. Other mutant isolates show similar activities to the wild type. The findings may be associated with the fact that there are some

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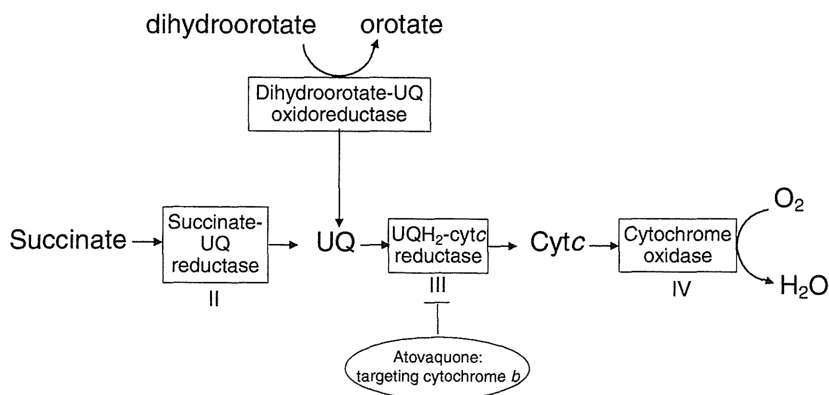

Fig. 1. Role of mitochondrial respiratory chain in malaria parasite. Respiratory chain of *Plasmodium* is composed from active complex II (succinate-ubiquinone reductase), III (quinol-cytochrome *c* reductase), IV (cytochrome *c* oxidase) and dihydroorotate dehydrogenase (DHODH). Ubiquinone (UQ) or commonly called coenzyme Q plays an important role in the respiratory chain of *Plasmodium* mitochondria. It is a point of contact between pyrimidine metabolism and energy metabolism. Many antimalarial agents contain a quinone structure. The enzymes mediating electron transfer between the enzymes and ubiquinone are considered to be good targets.

significant structural differences in the atovaquone binding pocket in *Plasmodium* enzyme.

Direct measurement of ubiquinol-cytochrome *c* reductase activity in *Plasmodium* cytochrome bc_1 complex is difficult because the activity of *Plasmodium* enzyme is too low to estimate and significant non-enzymatic redox reaction between ubiquinol and cytochrome *c* could not be eliminated. Therefore, we measured the activity of DHO-cytochrome *c* reductase (composed of DHODH, endogenous ubiquinone and cytochrome bc_1 complex; Fig. 1), which allowed us to obtain reproducible data by showing constant cytochrome *c* reduction during the measurement with negligible non-enzymatic reaction.

To ascertain that the atovaquone specifically inhibited the bc_1 complex, the DHODH activity in the presence of atovaquone was independently measured first. The DHODH activity was not affected by atovaquone at doses up to 100 μM indicating that atovaquone sensitivity of bc_1 complex can be examined by measuring DHO-cytochrome *c* reductase activity of the mitochondria. Specific activities of DHO-cytochrome *c* reductase from the atovaquone mutant isolates and the wild type are shown in Table 1. The data obtained in this study highlighted variations in specific activities of enzyme and IC_{50} to atovaquone, which explain the difference in atovaquone sensitivity.

The mutation sites acquiring resistance to atovaquone were all localized to the Q_o site and classified into three groups on the basis of acquired IC_{50} values. All of the mutant mitochondria showed higher IC_{50} values (1.45–43.5 nM) than that of wild type (0.327 nM). The highest IC_{50} was found in clones carrying the Y268C or Y268N mutations with an approximately 100 fold increase, 42.5 and 43.5 nM, respectively. The L271V/K272R, and M133I/L271V mutations had a slightly moderate effect, with IC_{50} values around 20 nM. The M133I/V284F, L144S/V284F and V284F mutations exhibited the lowest but significant effect, with IC_{50} values between 1.45 and 7.2 nM.

Although there are many X-ray structures of cytochrome bc_1 complexes from different organisms available [18–22], the mechanism of quinol oxidation has been discussed based on the inhibitor bound structures due to the lack of the complex structure with ubiquinol at Q_o site. Among the cytochrome bc_1 complex 3-D structures available in the database, the yeast complex has the highest similarity to those of apicomplexan parasites, especially around the ubiquinol oxidation pocket at the Q_o site (61% identical in amino acid sequence). Two high-resolution crystal structures complexed with Q_o site inhibitor, stigmatellin and HHDBT have been reported [21,23]. Reaction mechanism of ubiquinol oxidation in bifurcated manner transferring two electrons to the [2Fe–2S] cluster and heme b_L has been discussed

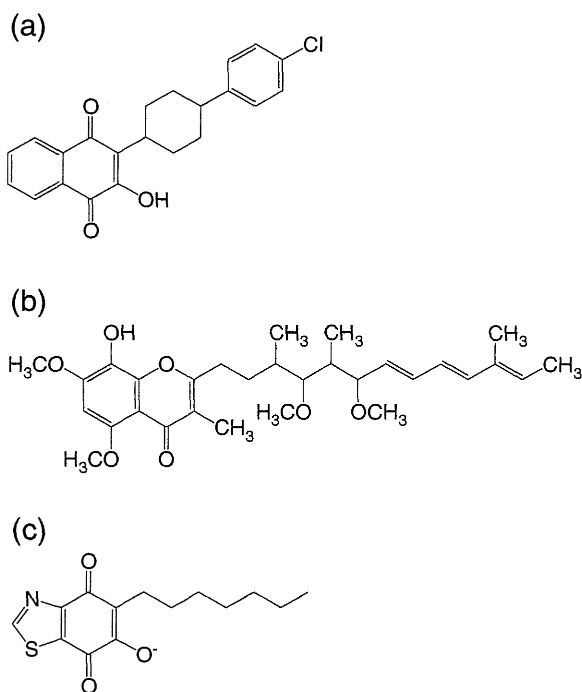

Fig. 2. Chemical Structures of Q_o site inhibitors. (a) Atovaquone, (b) stigmatellin, and (c) 5-*n*-heptyl-6-hydroxy-4,7-dioxobenzothiazole (HHDBT).

Table 1
Inhibitory effect of atovaquone against DHO-cytc activity.

Isolates	Atovaquone*	Mutation	Specific activity (nmol/mg/min)	IC_{50} (nM)
P. falciparum	S	–	2.56	0.052
P. berghei L (PbL)	S	–	22.7	0.327 ± 0.141
PbLSJ2.1	R	Y268C	22.4	42.5 ± 5.9
PbLSJ1.1	R	Y268N	13.0	43.5 ± 3.9
PbLSJ3.1	R	L271V, K272R	20.4	19.5
PbSK2A1Tb	R	M133I, L271V	13.0	20.0
PbSK2A1T	R	M133I, V284F		6.17 ± 0.7
PbSK1A2**	R	V284F	26.5	1.45
PbSR-1**	R	L144S, V284F	18.5	7.2

* S represents for sensitive parasite while R for resistant parasite.
** These isolates were reported previously by Syafruddin et al.[5].

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extensively and a single occupancy model from the structural analysis of HHDBT and stigmatellin bindings at the Q_o site based on the yeast X-ray structures has been proposed [21].

It was desirable to have the crystal structure of cytochrome bc_1 from the malaria parasites, but is obviously difficult to get the crystals of them. Yeast bc_1 variants, to which atovaquone-resistance mutations found in the malarial parasites are introduced, revealed that their relative increase in IC_{50} correlated with that in calculated atovaquone-binding energy [10]. Molecular basis for atovaquone resistance in *P. jirovecii* has also been proposed based on the homology model in the yeast cytochrome bc_1 complex [24,25]. In our case, based on the directly measured IC_{50} values of *Plasmodium* enzymes and the energy minimized docking model, the binding mode of atovaquone was revealed to be similar to that of HHDBT to the yeast cytochrome bc_1. In the yeast crystal structure, the oxygen atom of O6 from HHDBT forms hydrogen bond to His[181] of the Rieske iron–sulfur protein (ISP) and Tyr[279] of the cyt *b* at the Q_o site [23]. Since atovaquone has similar oxygen atom of O6 at the hydroxynaphthoquinone head group, it may form the hydrogen bond to His[181] of malaria ISP in the same manner. It was reasonable to presume that the head group of atovaquone bound to the back of Q_o site analogously to that of HHDBT and the chlorophenyl ring extends to the portal of Q_o site. The same hydrogen bond of atovaquone to His[181] has been observed by electron paramagnetic resonance (EPR) spectrum in the yeast bc_1 complex [24].

We have investigated atovaquone binding mode at the Q_o site of malaria cytochrome *b* based on the IC_{50} values of atovaquone-resistant variants and the available homologous 3D structures. It is clear that Tyr[268] is very important for atovaquone binding at the Q_o site as evidenced by the highest IC_{50} value. In the yeast crystal structures, the corresponding Tyr[279] is involved in the binding of stigmatellin and HHDBT, and provides a week CH–O hydrogen bond to inhibitors (Fig. 3a, b) [23]. Atovaquone might also need Tyr[268] for a CH–O hydrogen bonding to stabilize the head group in the Q_o site. It should be noted that IC_{50} of Y268C was 42.5 nM for *P. berghei*, while the equivalent yeast variant, Y279C, was between 1 and 2 μM [10].

The Leu[282] and Arg[283] in the yeast cytochrome *b*, which correspond to Leu[271] and Lys[272] in the malaria cyt *b*, localized to the vicinity of Tyr[279] residue in the yeast 3-D structure (Fig. 3c). The Leu[271] and Lys[272] mutations conferred a 50 fold increase in resistance to atovaquone based on the IC_{50} value, but mutation in the corresponding residues in yeast cytochrome bc_1 apparently did not affect the HHDBT binding [23]. The resistance caused by Arg[272] is also observed by mutational analysis in *Rhodobacter capsulatus*. Sensitivity to atovaquone increased 3 fold by I304M and R306K double mutations in *R. capsulatus*, which corresponds to the sequence conversion from *Saccharomyces cerevisiae* to *P. berghei* at Met[270] and Lys[272][26]. It therefore suggests that these two residues might be responsible for the specific binding of atovaquone to the malarial cytochrome bc_1. The positions of these residues are at the opposite to the presumed interacting region of atovaquone head group within Q_o site (Fig. 3b, c). Obvious difference in the chemical structures of atovaquone and HHDBT lies between the relatively inflexible cyclohexyl–chlorophenyl side-chain and the simple alkyl chain of HHDBT (Fig. 2). The cyclohexyl–chlorophenyl side chain might specifically interact with the residues from ef- to F-helices including Leu[271] and Lys[272], and consequently increased the binding affinity of atovaquone to the cytochrome *b* (Fig. 4). Thus, ef-helix, which contains Tyr[268] and Leu[271], is the key domain for atovaquone resistance.

The M133I mutation had certainly affected the resistance to atovaquone. The corresponding Met[139] in the yeast structure has van der Waals interactions with the tip of HHDBT at the back side of Q_o site (Fig. 3b, c). Atovaquone is expected to bind in the same manner as discussed above. The mutation of Met[133] to Ile with branched side chain at this point might only affect the inhibitor binding. The Leu[144] and Val[284] mutations conferred a feeble resistance to atovaquone. The mutations were localized to the portal of Q_o site and might hinder atovaquone from smoothly entering the Q_o site, because the head

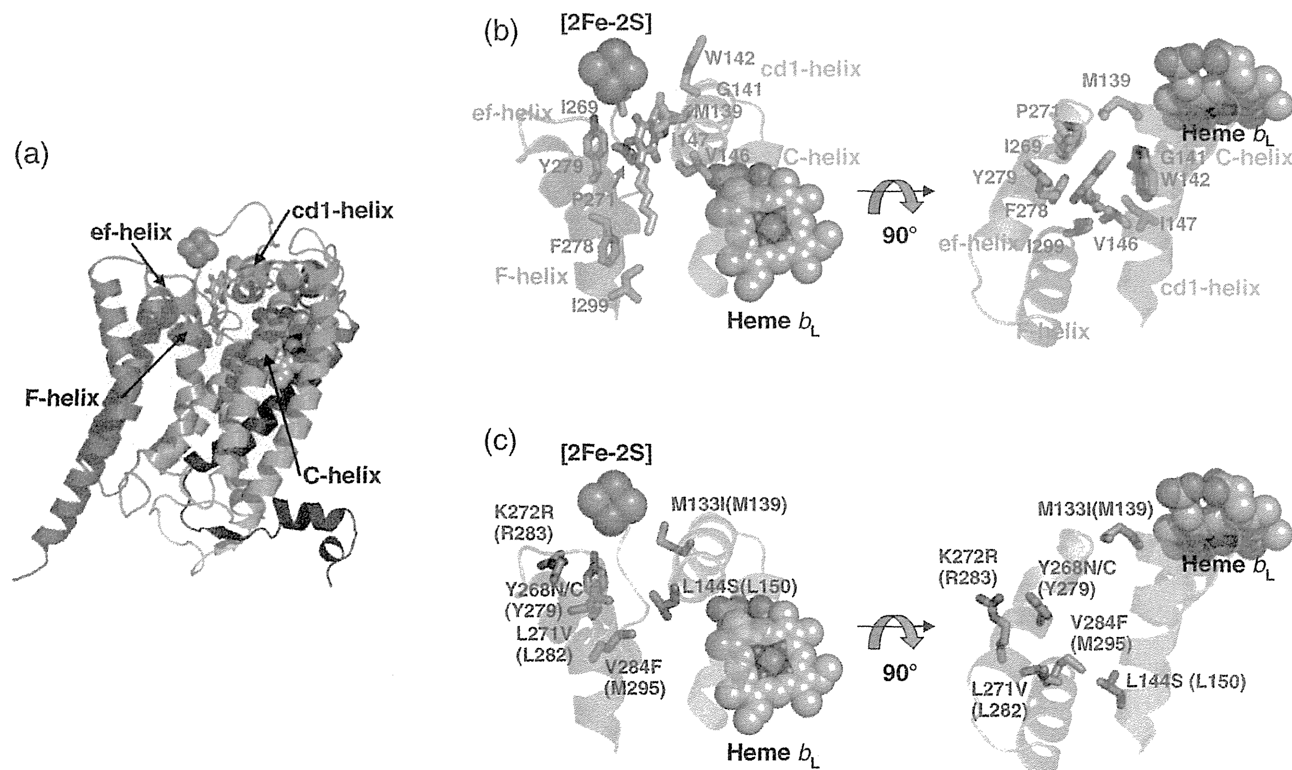

Fig. 3. Active site structure of the *S. cerevisiae* cytochrome *b* complexed with HHDBT (pdb:1P84). (a) Schematic representation of the cytochrome *b* extracted from the whole complex. (b) Q_o site of the yeast cytochrome *b* showing the residues interacting with HHDBT. (c) Q_o site showing the distribution of atovaquone resistant mutational sites.

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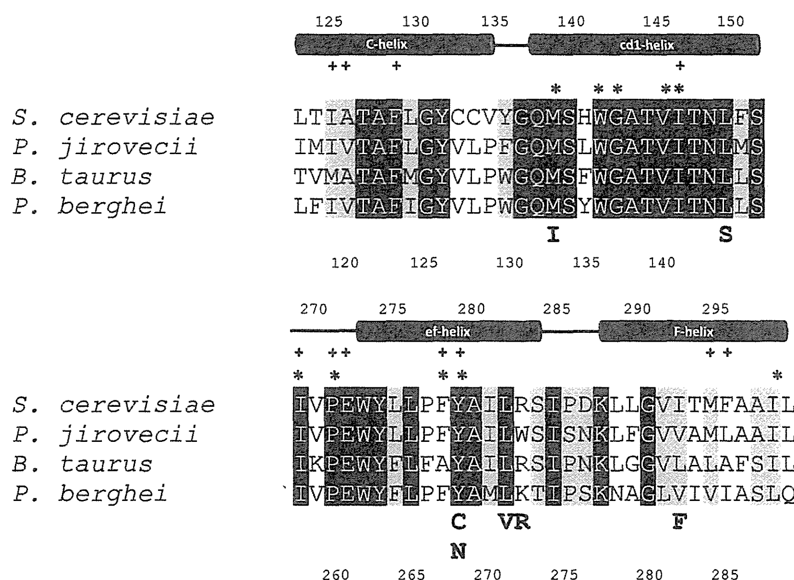

Fig. 4. Sequence alignment of the cytochrome *b* residues consisting of the Q_o site. Secondary structure informations are deduced from *S. cerevisiae* structure. +: residues involved in stigmatellin binding, *: residues involved in HHDBT binding, X: atovaquone resistance mutation sites in this study. Accession numbers are: *S. cerevisiae*, P03873; *P. jirovecii*, AFV57344; *Bos taurus* (*B. taurus*), CAA24007; *P. berghei*, O99253.

group of atovaquone is bulkier than that of ubiquinol and has more functional groups (Fig. 3c).

Comparison between the inhibition mechanism based on the crystal structure complexed with inhibitor and the actual IC_{50} value is helpful in elucidating the functions of active site residues during quinol oxidation by the cytochrome bc_1 complex. The residues interacting with HHDBT in the yeast cytochrome bc_1 structure, Trp^{142}, Gly^{143}, Ile^{269}, Pro^{271} and Tyr^{279}, have been proven to be important for ubiquinol oxidation by the point mutation analysis [27,28]. By aligning the conserved region of cyt *b* between yeast and malaria parasite, atovaquone-resistant clones in the malaria parasites do not carry those corresponding four mutations (Trp^{142}, Gly^{143}, Ile^{269}, Pro^{271}) except for yeast Tyr^{279}. Therefore, the basic mechanism of quinol oxidation in the malaria cytochrome bc_1 seems not similar to that in the yeast bc_1 complex. In this study, it became clear that the mechanism of atovaquone inhibition at the Q_o site of malaria cytochrome bc_1 complex could not be modeled based on the previous results in yeast or photosynthetic bacteria. Malaria parasites may devise a strategy to finely tune an ubiquinol oxidation in their mitochondria. The findings indicate the importance of the mutation in the quinone binding site of the *cyt b* gene and provide a direct evidence for the atovaquone inhibitory mechanism in the parasite cytochrome bc_1 complex.

4. Note added in proof

During the revision of this manuscript, a report on the co-crystal structure analysis of yeast bc_1 complex with atovaquone has been appeared (Nature Comm., DOI: http://dx.doi.org/10.1038/ncomms5029). The crystal structure is quite similar to that of our hypothetical binding model of bound atovaquone to yeast bc_1 complex. Although most of the data described in the paper are consistent with our result, we need to be careful in discussing the atovaquone inhibitory mechanism in the *Plasmodium* cytochrome bc_1 complex based on the model systems such as yeast and bacterial enzymes as shown in our present study.

Acknowledgment

This paper is dedicated to Professor Kazuyuki Tanabe who has passed away on August 12, 2013. He has been a leading scientist in the field of malaria research and always encouraging us. This work was supported initially by a grant from the Indonesian government through the Ministry of Research and Technology and subsequently from the Japan Society for the Promotion of Sciences (JSPS) to JES, through JSPS fellowship for Ph.D. RONPAKU program (to JES), and was supported by Grant-in-aid for Scientific Research (26253025) to KK from the Japanese Society for the Promotion of Science. We also acknowledge the support from the Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries and Food Industry and JST/JICA, SATREPS (Science and Technology Research Partnership for Sustainable Development) (10000284) to KK.

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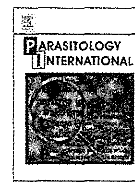

Short communication

Lactate retards the development of erythrocytic stages of the human malaria parasite *Plasmodium falciparum*

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ABSTRACT

The intraerythrocytic form of the human malaria parasite *Plasmodium falciparum* relies on glycolysis for its energy requirements. In glycolysis, lactate is an end product. It is therefore known that lactate accumulates in *in vitro* culture; however, its influence on parasite growth remains unknown. Here we investigated the effect of lactate on the development of *P. falciparum* during *in vitro* culture under lactate supplementation in detail. Results revealed that lactate retarded parasite development and reduced the number of merozoites in the schizont stage. These findings suggest that lactate has the potential to affect parasite development.

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Malaria is one of the major global public health problems, annually truncating several hundred thousand lives, mostly in sub-Saharan African countries [1]. The disease is caused by parasitic protozoa, belonging to the *Plasmodium* genus. In this genus, *Plasmodium falciparum*, the human malaria parasite, causes cerebral malaria (often referred to as falciparum malaria), with a remarkably high mortality. *P. falciparum* has a complicated life cycle, in which there are two major cycles: asexual multiplication in humans and sexual multiplication in mosquitoes [2]. In humans, an erythrocytic cycle is composed of the ring form, trophozoite, and schizont stages. The erythrocytic stages can be cultured *in vitro* using human red blood cells [3]. In the early 1980s, many researchers tried to establish a culture method for high-efficiency production of the parasite. Some of these studies have reported that lactate accumulated in the supernatant of the culture medium and had a deleterious effect on parasite growth [4,5]. In the erythrocytic stages, ATP production by the parasite is highly dependent on glycolysis [6]. Lactate is an end product of the ATP production pathway by glycolysis; hence, it accumulates in the supernatant. Apart from the deleterious influence of lactate on parasite growth *in vitro*, hyperlactatemia has been used as an indicator of disease severity for malaria patients [7]. In most clinical studies on falciparum malaria, compared with the normal plasma lactate level (0.3 to 1.3 mM) [8], the levels of plasma lactate tend to be high and indicate significant variability, ranging from 2 to 26.7 mM [7, 9]. Consequently, the malaria parasite is often exposed to high concentrations of lactate in malaria patients and may be subjected to the adverse influence of lactate in the infected human body as well as in *in vitro* culture. Lactate is thus considered to be an intriguing molecule for parasite growth, although the mechanism by which lactate affects the parasite remains unclear. In this study, we conducted a cell biological study to assess the effect of lactate on the development of the human malaria parasite *P. falciparum* using an *in vitro* culture system under lactate supplementation.

The human malaria parasite *P. falciparum* 3D7 strain was synchronized to the ring form stage using the single-step sorbitol method [10] and was cultured as described previously [3] with various concentrations (0, 1, 5, 10, 20, 30, and 60 mM) of sodium L-lactate (Sigma). As a result, the growth of synchronous parasites was suppressed by lactate in a dose-dependent manner (Fig. 1A). This is not caused by pH fluctuation because lactate supplementation did not change the pH of the culture medium (data not shown). Parasitemia of cultures supplemented with more than 10 mM lactate for 48 h was significantly lower than that of a culture without lactate ($P < 0.01$). When culturing parasites for 24 h, the percentage of each developmental stage was affected by lactate supplementation (Supplementary Fig. 1A). In these cultures, parasite development had a tendency to be retarded by each lactate concentration (*e.g.*, the percentage of the ring form stage: 22% in 10 mM lactate; 13% in 20 mM lactate; 8% in 30 mM lactate; 3% in 60 mM lactate). In parasites cultured for 48 h, lactate supplementation at concentrations of 20, 30, and 60 mM affected the percentage of each developmental stage more remarkably (*e.g.*, the percentage of the ring form stage: 65% in 0 mM lactate; 87% in 20 mM lactate; 93% in 30 mM lactate; 28% in 60 mM lactate) (Supplementary Fig. 1B). These results suggest that lactate would retard parasite development and consequently suppress parasite multiplication. To further investigate the parasite stages that are most affected by lactate, we prepared

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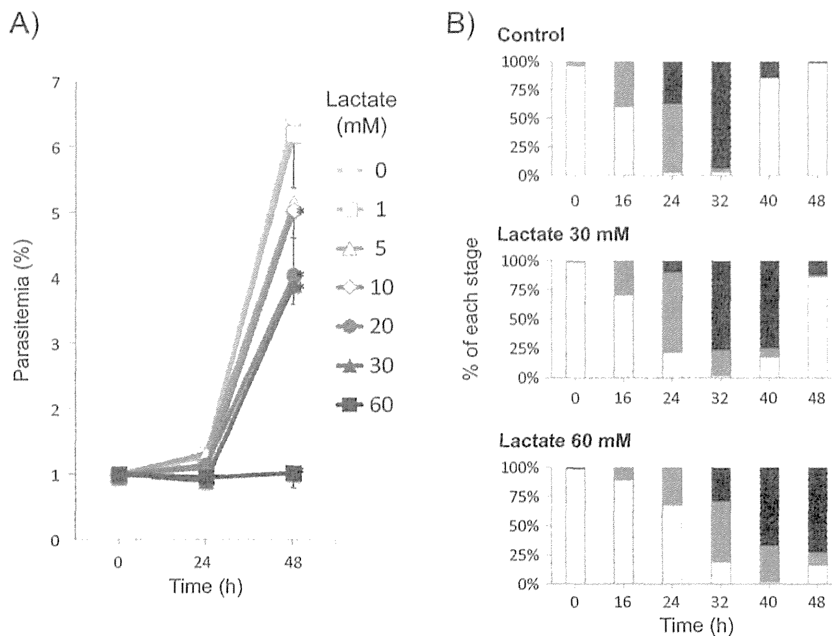

Fig. 1. Growth suppression and developmental retardation of *Plasmodium falciparum* by lactate. (A) Parasite cultures synchronized with 5% (w/v) D-sorbitol were incubated at lactate concentrations of 0, 1, 5, 10, 20, 30, and 60 mM. Parasitemia at 0 h was 1.0% in each group. All experiments were performed in triplicate, and all data are represented as mean values and standard errors. *P < 0.01 compared with parasitemia of a culture not supplemented with lactate (0 mM). (B) Parasite cultures synchronized with sorbitol and Percoll density gradients were incubated at lactate concentrations of 0, 30, and 60 mM. White, gray, and black bars indicate percentages of ring form, trophozoite, and schizont, respectively. In a control culture, to which no lactate is added, it took 48 h for the parasite to develop from a ring form to the next ring form. Developmental retardation of the parasites was observed from 24 h to 48 h after supplementation with 30 mM lactate. When supplemented with 60 mM lactate, the retardation was even more marked.

the parasites whose development was tightly synchronized to the ring form stage by sorbitol and Percoll (GE healthcare) density gradients [11]. As a result, when the parasites were cultured with lactate at 30 and 60 mM, a significant number of parasites remained in the schizont stage (Fig. 1B). This result implies that the schizont stage may be susceptible to the effect of lactate.

In addition to the growth suppression, the effect of lactate on parasite morphology was observed. As shown in Supplementary Fig. 2, the ring form and trophozoite stages showed a normal shape even when these stages of parasites were cultured in the highest concentration of lactate (60 mM). In sharp contrast, when the schizont stage of the parasite was exposed to a high concentration of lactate at 60 mM but not at 0 and 30 mM, it showed an abnormal morphology: a smaller schizont in size and fewer purple dots in the schizont. Besides the morphological change in the schizont stage, we found a remarkable variation in the number of merozoites in a segmenter (the late stage of the schizont stage). To confirm this difference, we counted the number of merozoites using 50 representative segmenters in each group. In a culture not supplemented with lactate (control), approximately 19 merozoites were found in a segmenter (Fig. 2). When lactate was added to the culture at the concentrations of 30 and 60 mM, 16 and 10 merozoites were found in a segmenter, respectively. These numbers were significantly lower than those in the control culture, suggesting that lactate reduced the number of *P. falciparum* merozoites formed as well as retarded their development.

In this study, we demonstrated that lactate, which is an end product of glycolysis, had an adverse impact on *P. falciparum* growth when it was present in excess in culture medium. In the erythrocytic stages, a trophozoite stage parasite has been reported to produce the largest amount of lactate [4]. It is thus reasonable to infer that the trophozoite may need to extrude lactate to the outside using a mechanism such as an efflux pump. In general, lactate is transported *via* the monocarboxylate transporter (MCT) family [12]. In *P. falciparum*, two genes encoding MCTs (PF3D7_0210300 and PF3D7_0926400) are identified in its

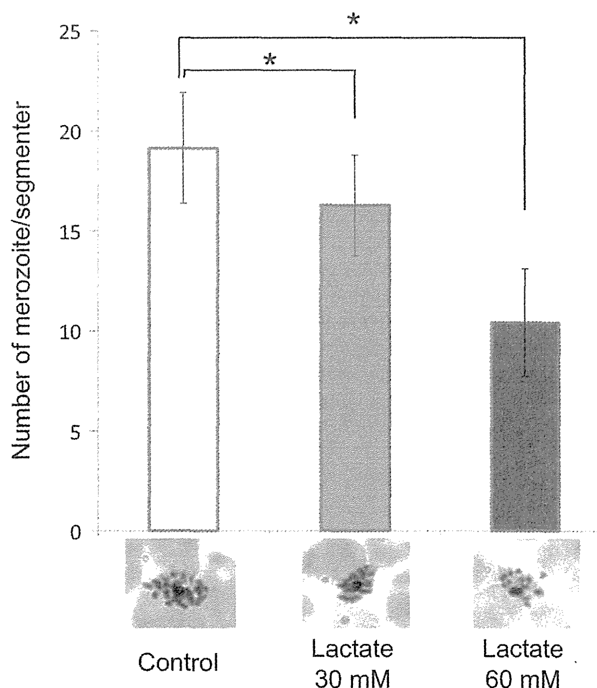

Fig. 2. Comparison of the number of merozoites contained in one segmenter. In total, 50 segmenters were counted. *P < 0.01 compared with culture not supplemented with lactate (control). Bottom figures indicate representative segmenters, which were stained using Giemsa stain solution.

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