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CASE REPORT

Raltegravir can be used safely in HIV-1-infected patients treated with warfarin

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Summary: Drug co-administration often affects the patient response to warfarin through various mechanisms. We describe here five HIV-1-infected patients on treatment with warfarin in whom the use of raltegravir was associated with a favourable outcome.

Keywords: HIV/AIDS, warfarin, raltegravir, etravirine, cytochrome P450, drug interaction, antiretroviral therapy

Drug co-administration often affects the patient response to warfarin through various mechanisms. For example, some drugs induce or inhibit liver enzymes, such as cytochrome P450 (CYP) isozymes responsible for warfarin metabolism;[1,2] others alter warfarin sensitivity by changing vitamin K synthesis or absorption, alter warfarin distribution or metabolism by increasing its affinity for receptor sites, or change the synthesis of functional coagulation factors. As the life expectancy of HIV-infected individuals is becoming longer, co-administration of warfarin with antiretrovirals needs to be assessed carefully. Nevirapine and lopinavir–ritonavir reduce serum concentrations of warfarin,[3,4] while efavirenz increases the concentration,[4] probably by the induction and inhibition of CYP2C9,[1,2] the main enzyme in warfarin metabolism. We reported previously the favourable effects of non-boosted fosamprenavir in patients treated with warfarin.[5] The clinical use of warfarin co-administered with raltegravir has not been described so far, though raltegravir seems to be a safe choice because it does not inhibit or induce CYP isoenzymes.[6] We describe here five HIV-1-infected patients on treatment with warfarin in whom the use of raltegravir was associated with a favourable outcome (Table 1). Cases 1–3 were Japanese men who had been treated with a stable dose of warfarin (mean daily dose, 3–4 mg) for underlying diseases, and their international normalized ratios (INR) were maintained within the optimal ranges (1.5–2.5 or 2.0–3.0) before the introduction of antiretroviral therapy (ART). Dose modification of warfarin was not necessary after starting ART containing raltegravir, as INRs remained within the optimal ranges. Case 4 was a 62-year-old Japanese man who had been treated with abacavir, lamivudine and non-boosted fosamprenavir (1400 mg twice daily). Based on his request, ART was switched to abacavir, lamivudine and raltegravir, and INR was maintained within the optimal range (1.5–2.5). Therefore, warfarin dose modification was not necessary. Case 5 was a 57-year-old Japanese man who had been treated with abacavir, lamivudine and lopinavir/ritonavir. He developed chronic atrial flutter. The initial dose of warfarin was 1 mg/day to maintain INR within the optimal range (1.5–2.5). Three months later, INR control became difficult at 4 mg/day of warfarin (INR; 0.70–0.91) and warfarin was terminated because it seemed ineffective. Non-boosted fosamprenavir could not be used because genotypic analysis showed resistance of HIV-1 to fosamprenavir. When raltegravir became available in Japan (9 months after discontinuation of warfarin), treatment was switched to ART comprising abacavir, lamivudine, raltegravir and etravirine, as well as warfarin (at initial dose of 1 mg/day). Three months later, INR was controlled within 1.46–2.49 at 3.5 mg of warfarin. The new regimen allowed maintenance of INR within the optimal range.

Cardiovascular events are increasing with the long-term use of ART. For patients treated with warfarin, raltegravir is a safe and clinically effective ART agent. Etravirine can potentially interact with warfarin by inducing CYP3A and mild inhibition of CYP2C9 and CYP2C19.[7] However, in Case 5, it was used successfully in combination with raltegravir. Such combinations may be helpful for the control of drug-resistant HIV-1 in warfarin-treated patients. Genetic polymorphisms in CYP2C9 may affect the response to warfarin,[8] though such data were not available in our five patients. The clinical introduction of raltegravir has expanded the ART options, though further clinical evidence is necessary in warfarin-treated patients.

Conflict of interest: None declared.

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Table 1 **HIV-1 infected patients with favourable outcome following treatment with raltegravir and warfarin**

No.	Age (years)	Sex	Underlying disease	ART regimen	Dose of warfarin (mean daily dose) (mg)	Maintained INR	Follow-up period (months)	Remarks
1	55	M	Chronic atrial flutter, cerebral embolism	RAL TDF FTC	3.5–4	1.69–2.64	15	–
2	57	M	Portal vein thrombosis	RAL ABC 3TC	3	1.68–2.31	2	–
3	59	M	Chronic atrial flutter, cerebral embolism	RAL ABC 3TC	3	2.03–2.94	3	
4	62	M	Chronic atrial flutter	RAL ABC 3TC	2.0–2.5	1.46–2.49	5	Switched non-boosted FPV to RAL.
5	57	M	Chronic atrial flutter	RAL ETV ABC 3TC	3.5	1.60–1.71	5	Switched LPV/RTV to RAL/ETV

RAL = raltegravir 800 mg/day; ETV = etravirine 400 mg/day; TDF = tenofovir 300 mg/day; FTC = emtricitabine 200 mg/day; ABC = abacavir 600 mg/day; 3TC = lamivudine 300 mg/day; FPV = fosamprenavir 2800 mg/day; LPV/RTV = lopinavir 800 mg/day and ritonavir 200 mg/day

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HIV/AIDS MAJOR ARTICLE

Prophylactic Effect of Antiretroviral Therapy on Hepatitis B Virus Infection

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Background. Hepatitis B virus (HBV) infection is common in individuals infected with human immunodeficiency virus, especially in men who have sex with men (MSM). Almost all currently used regimens of antiretroviral therapy (ART) contain lamivudine (LAM) or tenofovir disoproxil fumarate (TDF), both of which have significant anti-HBV activity. However, the prophylactic effect of ART on HBV infection has not been assessed previously.

Methods. Non-HBV-vaccinated HIV-infected MSM were serologically evaluated for HBV infection using stocked serum samples. Cases negative for HBV surface antigen (HBsAg), antibody to HBsAg (anti-HBs), and antibody to HBV core antigen (anti-HBc) in first serum samples were serologically followed until last available stocked samples. HBV genotype and LAM-resistant mutation (rtM204V/I) were analyzed in cases that became HBsAg-positive.

Results. The first stocked samples were negative for all analyzed HBV serological markers in 354 of 1434 evaluated patients. The analysis of their last samples indicated HBV incident infection in 43 of them during the follow-up period. The rate of incident infections was lower during LAM- or TDF-containing ART (0.669 incident infections in 100 person-years) than during no ART period (6.726 incident infections in 100 person-years) and other ART (5.263 incident infections in 100 person-years) ($P < .001$). Genotype A was most prevalent (76.5%), and LAM-resistant HBV was more frequent in incident infections during LAM-containing ART (50.0%) than in those during no ART and other ART (7.1%) ($P = .029$).

Conclusions. LAM- and TDF-containing ART regimens seem to provide prophylaxis against HBV infection, although drug-resistant strains seem to evade these effects.

Keywords. lamivudine; tenofovir disoproxil fumarate; resistant; chronic infection.

Patients with human immunodeficiency virus (HIV) infection are at high risk for both hepatitis B virus (HBV) infection and development of chronic infection [1–4]. Based on information from Western countries, the rate of coinfection varies according to risk categories; the highest rate is in men who have sex with men (MSM), with a slightly lower rate among intravenous drug users, and much lower in individuals infected through heterosexual contacts [5–8]. In Japan, HIV/HBV coinfection is also significantly associated with MSM [9, 10]. The progression of chronic HBV infection to cirrhosis, end-stage liver diseases, and/or hepatocellular carcinoma is more rapid in HIV-infected persons than in those with chronic HBV infection alone [11, 12]. Vaccination of non-HBV-immunized HIV-infected individuals is recommended to prevent HBV infection [13]. However, all current recommended antiretroviral therapy (ART) regimens contain lamivudine (LAM) or tenofovir disoproxil fumarate (TDF), both of which have significant anti-HBV activity [14]. Do these ART regimens provide any prophylaxis against HBV infection? This is an important question, as a positive answer could influence the strategy applied to prevent HBV infection in HIV-infected individuals. To delineate the hepatitis B prophylactic effect of ART, we used stocked samples for serological evaluation of HBV infection in HIV-infected MSM. The present

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study included those patients who had tested negative for hepatitis B surface antigen (HBsAg), antibody to HBsAg (anti-HBs), and antibody to hepatitis B core antigen (anti-HBc) using their first stocked blood samples, who were followed up serologically to identify new HBV incident infections among them. The other part of the study covered analysis of the relation between the frequency of incident infection and ART regimens.

METHODS

Patients

Since April 1997, we have stocked serum samples taken at routine clinical practice from HIV type 1 (HIV-1)–infected patients who visited the Outpatient Clinic of the AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan, under signed informed consent for use in virologic research. Every patient had been interviewed at the first visit by clinical nurse specialists at the HIV outpatient clinic using a structured questionnaire that includes items on sexuality and history of HBV vaccination. Most of the patients regularly visited our clinic every 1–3 months, and we had collected and stored their sera at almost all visits. The ethics committee of the National Center for Global Health and Medicine approved the collection and analysis of the samples. First, we selected HIV-1–infected MSM who met the following inclusion criteria: (1) the first visit to our clinic was between April 1997 and December 2009, (2) they had not received HBV vaccination before the first visit, and (3) at least 2 serum samples were available and collected at least 6 months apart. The first sample was defined as the baseline serum sample, and baseline clinical data were defined as those recorded on the date of sampling of the first stocked serum. Patients' baseline characteristics, including age, race, hepatitis C virus antibody, results of *Treponema pallidum* hemagglutination assay, and $CD4^+$ cell count were collected from the medical records.

HBV Analysis

In order to identify new HBV incident infection, we excluded patients with previously confirmed HBV infection. The baseline samples of the patients who met the inclusion criteria described above were serologically evaluated for HBsAg, anti-HBs, and anti-HBc using ARCHITECT HBsAg QT assay, anti-HBs assay, and anti-HBc assay, respectively (Abbott Laboratories, Chicago, Illinois) [15, 16]. Patients positive for any of HBsAg, anti-HBs, and anti-HBc at baseline were excluded from the serological follow-up. The remaining patients were considered to have never been infected with HBV before the baseline. Their last stocked sample taken before or in December 2010, or before HBV vaccination if performed during the follow-up period, was analyzed for HBsAg, anti-HBs, and anti-HBc. If the last sample was negative for all 3, the patient was considered to have never been infected with HBV up to the sampling date of the last stocked serum. If HBsAg, anti-HBs, or anti-HBc was positive in the last stocked serum, the patient was considered to have HBV incident infection during the follow-up period. In the latter case, the baseline samples were subjected to polymerase chain reaction (PCR) analysis for HBV DNA [17, 18], and all the stocked samples during the follow-up period were serologically analyzed to determine the date of HBV incident infection. The date of incident infection was defined as the sampling date of the first positive serum for any HBV serological marker. The time from the baseline to HBV incident infection was analyzed by the Kaplan-Meier method. The data were censored at the sampling date of the last stocked sample if it was negative for all analyzed HBV serological markers. Patients' age and $CD4^+$ cell count at the date of incident infection and alanine aminotransferase (ALT) values within 3 months of incident infection were collected. If an HBsAg-positive sample was available, HBV genotype and LAM-resistant mutation (rtM204V/I) were analyzed by PCR-invader assay [17–19]. The diagnosis of chronic HBV infection was considered when HBsAg was still positive in sera taken at 6 months or longer after the incident infection.

Antiretroviral Therapy

To determine the type of ART under which HBV incident infection occurred, the regimen information of ART was collected from medical records over the period spanning from the baseline to the incidence infection or to the end of follow-up. The treatment status was divided into 4 categories: (1) No ART, no treatment with any antiretroviral agent; (2) Other-ART, ART with regimens that did not contain LAM, TDF, or emtricitabine (FTC); (3) LAM-ART, ART with LAM-containing regimens that did not contain TDF or FTC; and (4) TDF-ART, ART with TDF-containing regimens with or without LAM or FTC. Data were censored on the sampling date of the last stocked sample if it was negative for all analyzed HBV serological markers. When the treatment category was modified, the data were censored on the date of category change for the previous treatment category and a new follow-up as a different case was initiated for the replacement treatment category.

Statistical Analysis

The time from the baseline to HBV incident infection was analyzed by the Kaplan-Meier method. The Cox proportional hazards regression analysis was used to assess the risk of HBV incident infections. The impact of patients' baseline characteristics, year of entry, the use of antiretroviral agents (any antiretroviral, and any of LAM, TDF, or FTC), and the frequency of changing ART regimen during the follow-up period was estimated with univariate analysis, and those with statistical significance were incorporated into multivariate analysis. The

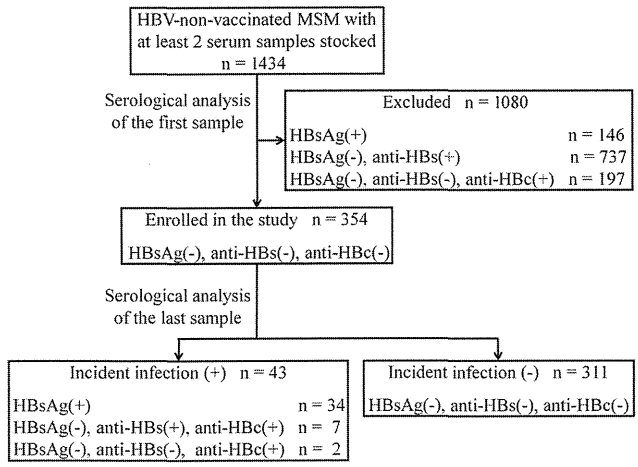

Figure 1. Patient selection process: 1434 patients met the inclusion criteria. Of these patients, 1080 were excluded because of positive hepatitis B virus serology in the first samples. The results of various serological tests are shown. The remaining 354 were enrolled for serological follow-up. Of these, 43 were positive in the last sample analysis. Their stocked samples were analyzed serologically and the results of HBV serology using the first positive samples are indicated. Abbreviations: anti-HBc, antibody to HBV core antigen; anti-HBs, antibody to HBsAg; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; MSM, men who have sex with men.

frequency and risk of HBV incident infection during each treatment category was also assessed by univariate Cox proportional hazards regression analysis. We used hazard ratios and 95% confidence intervals to estimate the impact of each variable on incident infection. Patients' age and $CD4^+$ cell count on the date of incident infection, and peak value of ALT within 3 months of incident infection were compared between transient infection and chronic infection with Wilcoxon rank-sum test. The differences in rates of HBV genotype A and rtM204V/I mutation were compared with χ^2 test (ie, the Fisher exact test). Statistical significance of difference was defined as a 2-sided *P* value of <.05. All statistical analyses were performed with the Statistical Package for Social Sciences version 17.0 (SPSS, Chicago, Illinois).

RESULTS

Figure 1 shows the patient selection procedure. A total of 1434 HIV-1–infected MSM met the inclusion criteria described in the Methods section. Of these, 146 patients (10.2%) were positive for HBsAg, 737 (51.4%) were positive for anti-HBs, and 197 (13.7%) were solely positive for anti-HBc using baseline samples. The remaining 354 patients (24.7%; negative for HBsAg, anti-HBs, and anti-HBc at baseline), who were considered to have never been infected with HBV, were enrolled for serological follow-up. Table 1 lists their baseline characteristics. Serological analysis of the last sample of each of these patients showed HBV incident infection during follow-up in 43 (12.1%). Their baseline samples were found to be PCR-negative for HBV DNA, confirming that the incident infection in these patients occurred during the follow-up period. All stocked samples of the 43 patients were analyzed serologically to determine the date of HBV incident infection. HBV incident infections occurred every year between 1997 and 2010 except in 1998. The median time period from the baseline to HBV incident infection was 1.6 years (interquartile range [IQR], 192–1151 days; range, 28–4068 days). The total observation period was 1607 person-years (median, 3.7 years [IQR], 1.9–6.5 years). Figure 2 shows the Kaplan-Meier curve for the HBV incident infection for the whole cohort of enrolled patients.

In order to assess the risk of HBV incident infections, patients' baseline characteristics, year of entry, the use of any antiretroviral agents, the use of any of LAM, TDF, or FTC, and the frequency of changing ART regimen during the follow-up

Table 1. Baseline Characteristics of the 354 Enrolled Patients

		Year of Entry			
Characteristic	Total (n = 354)	1997–2000 (n = 61)	2001–2003 (n = 79)	2004–2006 (n = 112)	2007–2009 (n = 102)
Age, y, median (IQR)	32.0 (27.0–38.0)	32.0 (27.8–37.3)	31.0 (27.0–37.8)	32.0 (27.0–38.0)	35.0 (27.0–42.0)
Race/ethnicity					
Japanese	340 (96.0)	59 (96.7)	78 (98.7)	109 (97.3)	94 (92.2)
Asian other than Japanese	4 (1.1)	0 (0.0)	0 (0.0)	1 (0.9)	3 (2.9)
Caucasian	10 (2.8)	2 (3.3)	1 (1.3)	2 (1.8)	5 (4.9)
HCV antibody, positive	8 (2.3)	1 (1.6)	2 (2.5)	1 (0.9)	4 (3.9)
TPHA positive	101 (28.5)	23 (37.7)	20 (25.3)	30 (26.8)	28 (27.5)
$CD4^+$ cell count, cells/mm^3, median (IQR)	277 (151–404)	277 (169–417)	313 (97–443)	316 (176–413)	252 (129–359)
HIV RNA, log_{10} copies/mL, median (IQR)	4.6 (3.8–5.2)	4.5 (3.6–5.2)	4.8 (3.9–5.4)	4.4 (3.8–4.9)	4.7 (3.9–5.2)

Data are No. (%) unless otherwise specified.

Abbreviations: HCV, hepatitis C virus; HIV, human immunodeficiency virus; IQR, interquartile range; TPHA, *Treponema pallidum* hemagglutination assay.

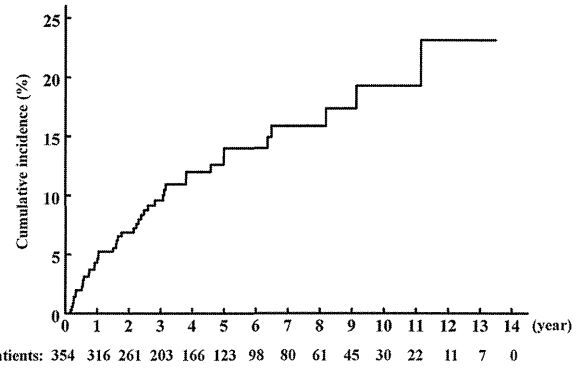

Figure 2. Kaplan-Meier curve showing the time to hepatitis B virus incident infection.

period were estimated using a proportional hazards model (Table 2). Younger age and higher $CD4^+$ cell count correlated positively, and use of any antiretroviral, use of LAM, TDF, or FTC, and the frequency of changing ART regimen correlated negatively with HBV incident infection, with statistical significance in univariate analysis. However, in multivariate analysis, the use of LAM, TDF, or FTC continued to show significant relation. Then, we focused on the relation between treatment status and HBV incident infection. The observation period in each patient was divided into 4 categories by treatment status: No ART, no treatment with any antiretroviral agent; Other-ART, ART with regimens that did not contain LAM, TDF, or FTC; LAM-ART, ART with LAM-containing regimens that did not contain TDF or FTC; or TDF-ART, ART with TDF-containing regimens with or without LAM or FTC. No participant received FTC single tablet (Emtriva). All the participants who took FTC received the combination tablet of TDF/FTC (Truvada), and therefore, such treatment status was categorized as TDF-ART. The total categorized observation period of No ART, Other-ART, LAM-ART, and TDF-ART was 446, 114, 814, and 233 person-years, respectively. The number of the HBV incident infections was 30 during the No ART period, 6 during Other-ART period, 7 during LAM-ART period, and 0 during TDF-ART period. No incident infection occurred at the time of changing ART regimen. The proportional hazards model showed a significantly lower frequency of HBV incident infection during LAM- or TDF-ART (0.669 incident infections per 100 person-years) compared with that during No ART (6.726 incident infections per 100 person-years), although there was no significant difference between Other-ART (5.263 incident infections per 100 person-years) and No ART, suggesting that ART regimens with anti-HBV activity can reduce HBV incident infections by 90% (Table 3). During LAM-ART, the HIV-1 load around the period of incident infection remained below the detection limit in all the 7 infected patients, indicating excellent adherence to ART.

Figure 3 shows peak ALT levels for the 43 HBV incident infections. Among the 36 incident infections observed the No ART and Other-ART groups, 16 infections (44.4%) were asymptomatic and not associated with significant increases in ALT (peak ALT, <60 IU/L). We were able to serologically follow 33 of the 36 cases for 6 months after the date of incident infection (TDF-ART was introduced within 6 months of incident infection in the other 3 cases). Among the 33 patients, 13 (39.4%) developed chronic infection (HBsAg was still positive 6 months after the date of incident infection). The median $CD4^+$

Table 2. Cox Proportional Hazards Regression Analysis for the Risk of Hepatitis B Virus Incident Infection

	Univariate Analysis		Multivariate Analysis	
Factors	Hazard Ratio (95% CI)	*P* Value	Hazard Ratio (95% CI)	*P* Value
Year of entry, per 1 y increase	.942 (.860–1.033)	.207		
Baseline characteristics				
Age, per 1 y increase	.921 (.879–.965)	.001	.958 (.917–1.001)	.054
Race (Japanese)	21.243 (.010–45 657.613)	.435		
HCV antibody	.048 (<.001–346.311)	.503		
TPHA	1.475 (.792–2.747)	.220		
$CD4^+$ cell count, per 100 cells/mm^3 increase	1.121 (1.008–1.246)	.035	.882 (.752–1.034)	.121
HIV RNA, per 1 log_{10} copies/mL increase	1.387 (.999–1.924)	.051		
Antiretroviral use during follow-up period				
Any antiretroviral	.097 (.052–.184)	<.001	.927 (.305–2.818)	.893
LAM, TDF, or FTC	.075 (.039–.146)	<.001	.110 (.031–.390)	.001
Frequency of changing regimen	.245 (.145–.414)	<.001	.700 (.385–1.270)	.240

Abbreviations: CI, confidence interval; FTC, emtricitabine; HCV, hepatitis C virus; HIV, human immunodeficiency virus; LAM, lamivudine; TDF, tenofovir disoproxil fumarate; TPHA, *Treponema pallidum* hemagglutination assay.

Table 3. Frequency and Hazard Ratio of Hepatitis B Virus Incident Infection in Each Treatment Status Category

ART	Observation Period (Person-Years)	Incident Infection	Hazard Ratio (95% CI)	*P* Value
No ART	446	30	1	. . .
Other-ART	114	6	.924 (.381–2.239)	.861
ART containing at least 1 of LAM, TDF, and FTC[a]	1047	7	.113 (.049–.261)	<.001
LAM-ART	814	7		
TDF-ART	233	0		

Abbreviations: ART, antiretroviral therapy; CI, confidence interval; FTC, emtricitabine; LAM, lamivudine; TDF, tenofovir disoproxil fumarate; LAM-ART, ART with LAM-containing regimens that did not contain TDF or FTC; Other-ART, ART with regimens that did not contain LAM, TDF, or FTC; TDF-ART, ART with TDF-containing regimens with or without LAM or FTC.

[a] No participant received FTC single tablet (Emtriva) during the observation period. All the participants who took FTC received the combination tablet of TDF/FTC (Truvada), and therefore, such treatment status was categorized into TDF-ART.

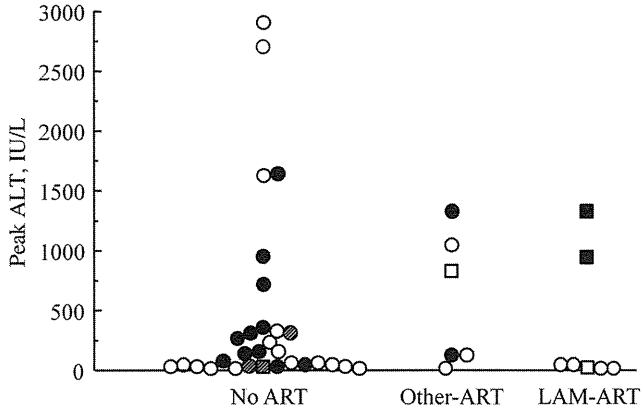

Figure 3. Peak alanine aminotransferase (ALT) values in hepatitis B virus (HBV) incident infections according to treatment regimen. Thirty, 6, and 7 HBV incident infections were observed during No antiretroviral therapy (ART), Other-ART, and lamivudine (LAM)–ART, respectively. No incident infection was identified during tenofovir disoproxil fumarate (TDF)–ART. No participant received emtricitabine (FTC) single tablet (Emtriva) during the observation period. All the participants who took FTC received the combination tablet of TDF/FTC (Truvada), and therefore, such treatment status was categorized into TDF-ART. Data are peak ALT values measured within 3 months of the date of incident infections. LAM-resistant mutation (rtM204V/I) was analyzed in 34 cases using the available hepatitis B surface antigen (HBsAg)–positive samples. Open squares: patients infected with LAM-resistant HBV. Closed circles and squares: patients who developed chronic infection (HBsAg-positive 6 months after the date of incident infection). Checked circles and squares: patients who received TDF-containing ART within 6 months of incident infection. Abbreviations: ALT, alanine aminotransferase; ART, antiretroviral therapy; LAM, lamivudine.

cell count was lower in the patients who developed chronic infection than in those with transient infection, although the difference was not significant ($P = .068$; Table 4), indicating that HIV-related immunodeficiency may play a role in the induction of chronic HBV infection. Among the 7 incident infections observed during LAM-ART, only 2 patients (28.6%) were symptomatic, had significant rise in ALT, and developed chronic HBV infection, and both of these infections were caused by LAM-resistant HBV (Table 5). The other 5 cases were asymptomatic and transient. Three of them were caused by LAM-sensitive strains and 1 was by LAM-resistant strain. HBsAg-positive serum sample was not available in the last case. LAM-resistant HBV was more frequently identified in analyzed incident infections during LAM-containing ART (50.0%) than in those during no ART and other ART (7.1%) ($P = .029$). Considered together, LAM seems to prevent acquisition of HBV infection, progression to symptomatic hepatitis, and development of chronic infection even after the development of infection, although these effects may be less pronounced in patients with LAM-resistant strains.

Among the 43 infection cases observed during total serological follow-up, HBsAg-positive samples were available in 34 cases and their HBV genotype was determined. Genotype A was the most frequent, as reported previously [10, 20–22], and genotypes B, G, and H were also identified. The rate of development of chronic infection was higher in genotype A than in other genotypes as previously reported [23], although the difference was not significant in our study. In the remaining 9 cases, only anti-HBc with (7 cases) or without (2 cases) anti-HBs were detected, although their samples were available and serologically analyzed at least every 3 months around the incident infection.

DISCUSSION

The results of this serological follow-up study indicated that LAM- and TDF-containing ART regimens protect against HBV incident infection. Furthermore, the results also suggested that LAM prevents progression to symptomatic hepatitis and development of chronic infection even after the development of HBV incident infection, provided such infection is caused by LAM-sensitive strains. However, it seems that LAM-resistant strains may evade this protective effect. One previous study that estimated the incidence of acute HBV infection among HIV-infected patients reported similar frequencies in patients receiving ART with and without LAM [5]. However, the authors defined immunoglobulin M anti-HBs positivity as a marker of HBV incident infection and did not exclude anti-HBc–positive patients at study entry. This probably made it difficult to distinguish incident infection from reactivation of chronic infection, as discussed in the report. In this study, we identified a

Table 4. Patient Characteristics and Clinical Features of Hepatitis B Virus Incident Infections in the No Antiretroviral Therapy (ART) and Other-ART Treatment Categories

Factors	Transient (n = 20)	Chronic[a] (n = 13)	Treated[b] (n = 3)	*P* Value[c]
Age, y, median (IQR)	31.0 (28.0–33.0)	29.0 (25.0–38.3)	25.0 (21.0–35.0)[d]	.406
$CD4^+$ cell count, cells/mm^3, median (IQR)	371 (308–518)	320 (235–383)	674 (206–1935)[d]	.068
Peak ALT level[e], U/L, median (IQR)	65 (30–573)	264 (115–774)	31 (15–314)[d]	.162
HBV genotype, No. (%)				.645
Genotype A	9 (45.0)	11 (84.6)	2 (66.7)	
Other genotypes	3 (15.0)	2 (15.4)	1 (33.3)	
Genotype unknown	8 (40.0)	0 (0.0)	0 (0.0)	
HBV rtM204V/I mutation, No. (%)				.480
Positive	1 (5.0)	0 (0.0)	1 (33.3)	
Negative	11 (55.0)	13 (100.0)	2 (66.7)	
Unknown	8 (40.0)	0 (0.0)	0 (0.0)	

Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis B virus; IQR, interquartile range.

[a] Hepatitis B surface antigen–positive 6 months after the date of incident infection.

[b] Treated cases with tenofovir disoproxil fumarate–containing antiretroviral therapy within 6 months of incident infection.

[c] *P* values between transient and chronic cases calculated with Wilcoxon rank-sum tests for continuous variables and χ^2 tests for proportions.

[d] Minimum and maximum values.

[e] Peak ALT level within 3 months of incident infection.

significant number of isolated anti-HBc–positive patients, a finding in agreement with previous reports [24–27], and excluded them from the serological follow-up to avoid improper inclusion of isolated anti-HBc–positive ones as HBV-naive [28, 29].

Table 5. Patient Characteristics and Clinical Features of Hepatitis B Virus Incident Infections During LAM-ART Treatment

Factors	Transient (n = 5)	Chronic[a] (n = 2)	*P* Value[b]
Age, y, median (IQR)	33.0 (30.3–36.5)	38.0 (33.0–43.0)[c]	.329
$CD4^+$ cell count, cells/mm^3, median (IQR)	430 (267–648)	362 (360–364)[c]	.699
Peak ALT level[d], U/L, median (IQR)	22 (14–51)	1133 (941–1325)[c]	.051
HBV genotype, No. (%)			>.999
Genotype A	3 (60.0)	1 (50.0)	
Other genotypes	1 (20.0)	1 (50.0)	
Genotype unknown	1 (20.0)	0 (0.0)	
HBV rtM204V/I mutation, No. (%)			.400
Positive	1 (20.0)	2 (100.0)	
Negative	3 (60.0)	0 (0.0)	
Unknown	1 (20.0)	0 (0.0)	

Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis B virus; IQR, interquartile range; LAM-ART, ART with LAM-containing regimens that did not contain TDF or FTC.

[a] Hepatitis B surface antigen–positive 6 months after the date of incident infection.

[b] *P* values calculated with Wilcoxon rank-sum tests for continuous variables and χ^2 tests for proportions.

[c] Minimum and maximum values.

[d] Peak ALT level within 3 months of incident infection.

HBV vaccination is recommended for individuals seeking evaluation or treatment for sexually transmitted diseases, HIV-infected patients, sexually active persons with >1 partner, and MSM [13]. However, the response and durability of adequate titers of anti-HBs are often reduced in HIV-infected patients [30–34]. Modified regimens of vaccination have been reported to improve anti-HBs response in HIV-infected patients, although the response rate was still low in those with low $CD4^+$ cell counts [35–37]. Our study demonstrated the HBV prophylactic effects of LAM- and TDF-containing ART regimens, suggesting that ART should be initiated before HBV vaccination, especially in those with low $CD4^+$ cell counts. Early introduction of ART was recommended recently not only for HIV-infected individuals, but also for prevention of transmission to others [38, 39]. Early introduction of treatment may also be recommended to prevent HBV infection to the patients themselves if they are HBV-naive. One randomized clinical trial reported the prophylactic effect of TDF combined with FTC in HIV prevention in seronegative MSM [40]. However, in that trial, HBV vaccination was offered to all susceptible participants, which made it impossible to estimate the prophylactic effect of the treatment on HBV prevention.

Our study carries certain limitations related to its retrospective nature. Patients on ART might have more opportunities to improve their behavior to prevent transmission of HIV to others, which could reduce HBV infection in themselves but

introduce bias in our analysis. However, the results suggest prophylaxis against potential HBV infection by oral medications, which could be useful for nonimmunized medical care providers.

Notes

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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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Prevalence and Risk Factors for Loss of Bone Mineral Density in Male Japanese Patients With HIV

To the Editors:

As the mortality of HIV-infected individuals was improved by combination antiretroviral therapy (cART), opportunistic infections were replaced by long-term complications including loss of bone mineral density (BMD).[1–9] It was first reported in 1999 and has emerged in the last decade[10,11] and has probably placed HIV-infected individuals at higher risk of bone fractures.[12,13] The causes are considered to be multifactorial and include HIV infection itself, body mass index (BMI), estimated glomerular filtration rate (eGFR), testosterone level, and hepatitis coinfection.[14–17] Some articles link the use of protease inhibitors and the tenofovir (TDF) with lower BMD.[18–20] Most of the articles associated with BMD loss in HIV-infected patients have been reported from North America, Europe, and Oceania. Accordingly, the BMD research on HIV-infected Asians is underrepresented. In this work, we focused on male Japanese HIV-infected patients and studied the prevalence and severity of BMD loss among them and attempted to determine clinical factors related to BMD loss.

Forty male Japanese HIV-infected individuals aged from 21 to 70 years who visited the Teikyo University Hospital were enrolled in this study. This study was approved by the ethical committee of the Teikyo University School of Medicine, and the patients gave written informed consent. The patients underwent BMD analyses from March 2010 to February 2012 with the same dual energy X-ray absorptiometry scan (Discovery SI, Hologic Inc, Bedford, MA). Thirty-nine patients were analyzed in both the lumbar spines and bilateral femoral necks, whereas 1 patient was limited to the bilateral femoral necks. The BMD data of the lumbar spines and the smaller BMD value of the femoral necks were employed to calculate T-scores that were calculated as comparison with young normal reference value expressed as SD units. Osteopenia was defined as a T-score of between −1 and −2.5SD, and osteoporosis was defined as that of ≥ −2.5 negative score following the World Health Organization classification.[21]

Clinical factors were collected including age, height, body weight, BMI, smoking status, use of corticosteroid, CD4 cell counts, durations of cART, medication of nucleoside/nucleotide reverse transcriptase inhibitors, serum creatinine, bone alkaline phosphatase (BAP), eGFR, serum, and urine N-terminal telopeptide (NTx). The eGFR for Japanese male patients were calculated by $194 \times \text{serum creatinine}^{-1.094} \times \text{age}^{-0.287}$ (mL/min per 1.73 m^2).[22]

To determine the high-risk groups, statistical differences of T-scores were calculated by using Wilcoxon signed ranks or Kruskal–Wallis test with the post hoc comparisons by the Dunnett test. The correlations between T-scores and each variable were examined by a linear regression employing the method of least squares. A multiple regression analysis was conducted by employing these variables with a P value <0.1 to predict BMD loss. All the analyses were performed by JMP version 8.0.1 (SAS Institute Inc, United States).

The median age of the patients was 39 years. Their median height and body weight were 169.3 cm and 65.0 kg, respectively. Twenty-three patients were ≤170 cm, and 14 were <60 kg. The median CD4 count was 396/μL. Twenty-seven were on cART, and median duration of cART was 2.9 years. Five have been continued on cART for >10 years. Twelve out of 27 took TDF/emtricitabine (FTC), and 13 took abacavir/lamivudine (3TC).

The median and interquartile ranges of BMD and T-scores in the lumbar spines and the femoral necks are summarized in Table 1. The median T-scores in the lumbar spines and the femoral necks were −0.8 and −1.2, respectively. The T-scores of the femoral necks were significantly lower than those of the lumbar spines (P = 0.0461). By employing the T-scores in the lumbar spines, the prevalence of osteopenia and osteoporosis was found to be 43.6% and 5.1%, respectively, and these percentages rose to 47.5% and 7.5%, respectively, if the least T-scores in the femoral necks were used. Moreover, the number (and percentage) of patients with osteopenia and osteoporosis were 21 (52.5%) and 4 (10%), respectively, in at least 1 of the T-score measured in the lumbar spines or in the femoral necks.

Subgroup analyses of patients' baseline characteristics revealed that the group of patients aged 50 years or older was the only group that showed significantly lower T-scores of the lumbar spines. In contrast, high-risk groups that showed significantly lower T-scores of the femoral necks were patients aged 40 years or older, ≤170 cm in height, weighed ≤60 kg, those on cART, particularly those continuing cART for ≥10 years. There were no significant differences between the 2 groups divided based on the medication of nucleotide reverse transcriptase inhibitors or use of corticosteroid.

TABLE 1. The Prevalence of BMD Loss

	The Lumbar Spines	The Femoral Necks
n	39	40
Median T-score (IQR)	−0.8 (−1.5 to 0.1)	−1.2* (−1.9 to −0.08), P = 0.0461*
Normal (%)	20 (51.3)	18 (45.0)
Osteopenia (%)	17 (43.6)	19 (47.5)
Osteoporosis (%)	2 (5.1)	3 (7.5)
BMD loss (%)	19 (48.7)	22 (55.0)

*Indicates statistical significance with $P < 0.05$.
IQR, interquartile range; n, number of examined patients.

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Correlation between BMD loss and clinical factors was analyzed by univariate analyses. Age, height, body weight, and duration of cART showed correlation with T-scores of both the lumbar spines and the femoral necks. In addition, serum creatinine, BAP, and eGFR were related to BMD loss measured in the femoral necks. Urinary phosphate excretion showed a tendency of correlation to T-scores in the lumbar spines (P = 0.0511). Serum NTx, 25-hydroxyvitamin D, cystatin C, free testosterone, urinary NTx, and smoking status were not correlated to BMD loss. Those variables that showed correlation or tendency of correlation were employed in a multiple regression analysis. Age, BAP, and eGFR were determined as independent clinical factors of T-scores of the femoral necks, whereas urinary phosphate excretion was the only independent variable to the T-score of the lumbar spines (Table 2).

Our study revealed that male Japanese HIV-infected patients are highly concomitant with low BMD. More than 60% of individuals were diagnosed with either osteopenia or osteoporosis. This is the first report from Asia, and the prevalence of low BMD in male Japanese HIV-infected patients is as high as that of previous reports from Europe, North America, or Oceania.[11] Increased age, short stature, and low body weight indicated from our study are well-known risk factors for BMD loss.[23] However, the age of these patients suffering from BMD loss is much younger, and the prevalence of BMD loss is significantly higher than that in the general population.

TABLE 2. Independent Risk Factors of BMD Loss

Variables	The Lumbar Spines: P	The Femoral Necks: P
Age	0.0593	0.0017*
Height	0.8958	0.7812
Body weight	0.8004	0.5926
BMI	0.8265	0.7650
Duration period of cART	0.9825	0.8133
Serum creatinine	0.0719	0.6006
BAP	0.4742	0.0234*
eGFR	0.3382	0.0413*
Urinary phosphate excretion	0.0110*	0.1185

Variables were examined by multiple regression analyses.
*Indicates statistical significance with $P < 0.05$.

It is reported previously that BMD in both the lumbar spines and the femoral necks decreases almost simultaneously in the general population of Japanese males.[24] In our study, the prevalence of osteopenia and osteoporosis diagnosed by the BMD in the femoral necks is higher than that in the lumbar spines. A meta-analysis by Bolland et al[25] showed consistent evidence of BMD loss especially among antiretroviral drug naive HIV-infected patients and that larger BMD loss were observed in the femoral necks than the lumbar spines. Osteoblast and osteoclast functions are influenced by a number of factors modulated during HIV infection and HIV itself or with other concomitant factors stimulate the progression of BMD loss.[26,27] It is possible that progression of BMD loss accelerated by HIV infection proceeds primarily targeting on cortical bone tissues such as the femoral necks.

A multiple regression analysis revealed that age, BAP, and eGFR are indicated as independent risk factors for BMD loss in the femoral necks. Decreased renal function is one of the risk factor for BMD loss among the general population. However, serum creatinine and eGFR remain within the normal range in many of our patients with BMD loss. We employed serum and urine NTx as markers for bone resorption and BAP as a marker for bone formation. Madeddu et al[28] reported that Italian HIV-infected patients with BMD loss in the lumbar spines had a higher mean BAP level, although it was not statistically significant in men. BAP might be a predictor of BMD loss in patients with HIV, implying an accelerated bone turnover. Interestingly, urinary phosphate excretion was determined as an independent variable of T-scores of the lumbar spines by a multiple regression analysis (P = 0.0110). Urinary phosphate excretion is mainly regulated by NaPi cotransporters that express in the brush border membrane of proximal tubular epithelia.[29] Although the mechanisms are unclear, it is hypothesized that HIV might have some effects on these transporters to stimulate excretion of phosphate. It has been investigated by both cross-sectional and longitudinal studies whether or not cART adds to the burden of metabolic bone disease in HIV infection with mixed conclusions.[20] In our study, no significant differences were observed in BMD loss between TDF/FTC group and abacavir/3TC group.

In conclusion, this is the first report on BMD loss in Asian HIV-infected patients. More than 60% of the patients are diagnosed as having osteopenia or osteoporosis. A multivariate regression analysis indicated that age, BAP, and eGFR are independent factors of BMD loss in the femoral necks, whereas urinary phosphate excretion is that of BMD loss of the lumbar spines. DXA scan examination not only in the lumbar spines but also in the femoral necks are recommended in patient more than 40 years old or those with increased BAP, decreased eGFR, or decreased urinary phosphate excretion.

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Pharmacological Assessment of Efavirenz Weight-Band Dosing Recommendations in HIV-Infected Thai Children

To the Editors:

Efavirenz (EFV) plus a dual–nucleoside reverse transcriptase inhibitor (NRTI) backbone combination is recommended for HIV-infected treatment-naive children aged 3 years or older.[1] EFV prescribing recommendations use weight-band dosing. Accumulating drug concentration data in HIV-infected children has raised concerns that the current dosing recommendations in the package insert, which is currently equivalent to the dosing recommendations in the US Department of Health and Human Services (US-DHHS) guidelines,[1] may lead to subtherapeutic concentrations.[2,3] It is unknown if the lower efavirenz clearance described in Thai adults compared with other populations[4] reduces the risk of underdosing in Thai children. We evaluated the steady-state pharmacokinetics of efavirenz prescribed according to conventional weight-band dosing guidelines in 40 HIV-infected Thai children.

The pharmacokinetic data reported were collected within an ongoing prospective, single-arm, open-label, multicenter trial investigating the safety and pharmacokinetics of a once daily regimen of tenofovir/lamivudine/efavirenz in virologically suppressed HIV-infected children. Eligible patients were aged between 3 and 18 years, weighing 15 kg or more, receiving a first-line antiretroviral regimen composed of 2 NRTIs (without tenofovir) plus non-NRTI and a plasma HIV-1 RNA <50 copies/mL within 6 months before study entry. Patients were enrolled after providing informed consent by their legal guardian(s). Assent from the child was obtained following local ethical guidelines. Patients were excluded if they had opportunistic infections or other significant medical diagnosis required ongoing therapy, had history of psychological or neurological illnesses, had impaired baseline renal function (defined as a creatinine clearance <60 mL/min and calculated using the Schwartz equation), or were pregnant. At enrollment, their antiretroviral regimen was modified to tenofovir/lamivudine/efavirenz once daily. The dosage for efavirenz was 250, 300, 350, 400, and 600 mg once daily for children with body weights of 15–20, 20–25, 25–32.5, 32.5–40, or ≥40 kg, respectively, as recommended in US-DHHS guidelines (February 23, 2009). Efavirenz was prescribed using STOCRIN 50 mg and 600 mg tablets and/or a generic 200 mg capsule [World Health Organization (WHO) prequalified product by Matrix Laboratories Limited, Maharashtra, India] titrated according to weight to the nearest 50 mg. Intensive 24-hour blood sampling for pharmacokinetic assessment was

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The authors have no conflicts of interest to disclose.
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Duodenal *Mycobacterium genavense* infection in a patient with acquired immunodeficiency syndrome

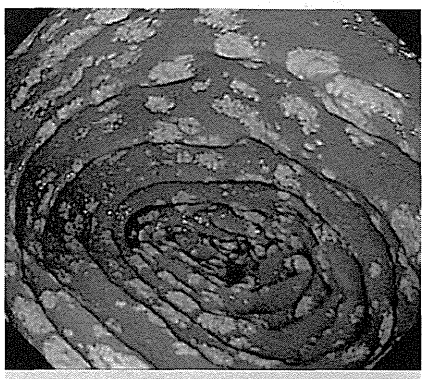

Fig. 1 Endoscopic view in the second portion of the duodenum in a 23-year-old man with known human immunodeficiency virus (HIV) infection showing widespread yellowish white nodules like xanthelasma.

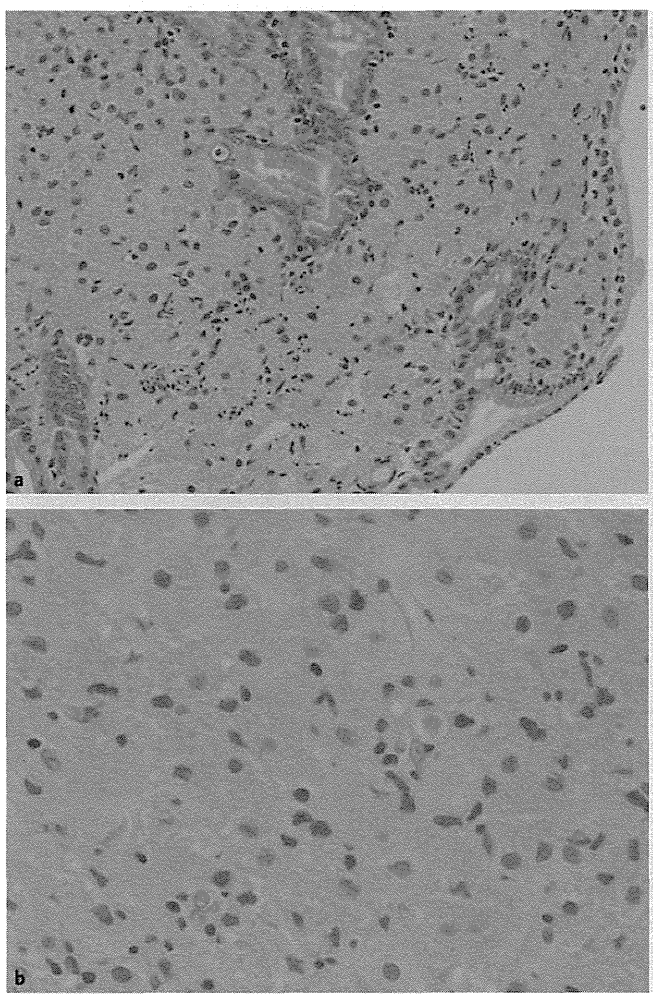

Fig. 2 Pathological appearance after hematoxylin and eosin (H&E) staining of the duodenal biopsy specimen showing an accumulation of macrophages in the lamina propria and submucosal layer: **a** in a low-power field; **b** in a high-power field.

Mycobacterial infection is sometimes fatal in patients with acquired immunodeficiency syndrome (AIDS). *Mycobacterium genavense*, a rare pathogen identified in 1992, causes about 10% of disseminated nontuberculous mycobacterial infections in patients with AIDS and mainly involves the small intestine [1–3]. The endoscopic findings of intestinal *M. genavense* infection are known to be nodules with a velvety appearance that is similar to that seen with *Mycobacterium avium-intracellulare* (*M. avium* complex [MAC]) [4].

A 23-year-old homosexual man with known human immunodeficiency virus (HIV) infection and a past history of hepatitis B and syphilis infections was referred to our hospital. Laboratory tests revealed his HIV RNA level to be 1.6×10^5 copies/mL and his CD4 count to be 11 cells/μL. He was admitted 2 months later with intermittent fever, general fatigue, and dry cough. A computed tomography (CT) scan of his chest showed a ground-glass appearance, suggestive of pulmonary infection.

A routine esophagogastroduodenoscopy performed 2 days after admission revealed widespread yellowish white nodules like xanthelasma in the second portion of the duodenum (◉ **Fig. 1**). Pathological examination of the biopsy specimen showed an accumulation of macrophages in the lamina propria and submucosal layer (◉ **Fig. 2**). Ziehl–Neelsen staining demonstrated numerous acid-fast bacteria being phagocytosed by macrophages (◉ **Fig. 3**). Cultures of bronchoalveolar lavage fluid and blood also detected acid-fast bacteria, which were finally identified as *M. genavense* by DNA amplification techniques. On the basis of these results, the patient was diagnosed as having disseminated *M. genavense* infection. Despite treatment with azithromycin, ethambutol, and levofloxacin, he died of respiratory failure.

Endoscopy_UCTN_Code_CCL_1AB_2AZ_3AZ

Competing interests: None

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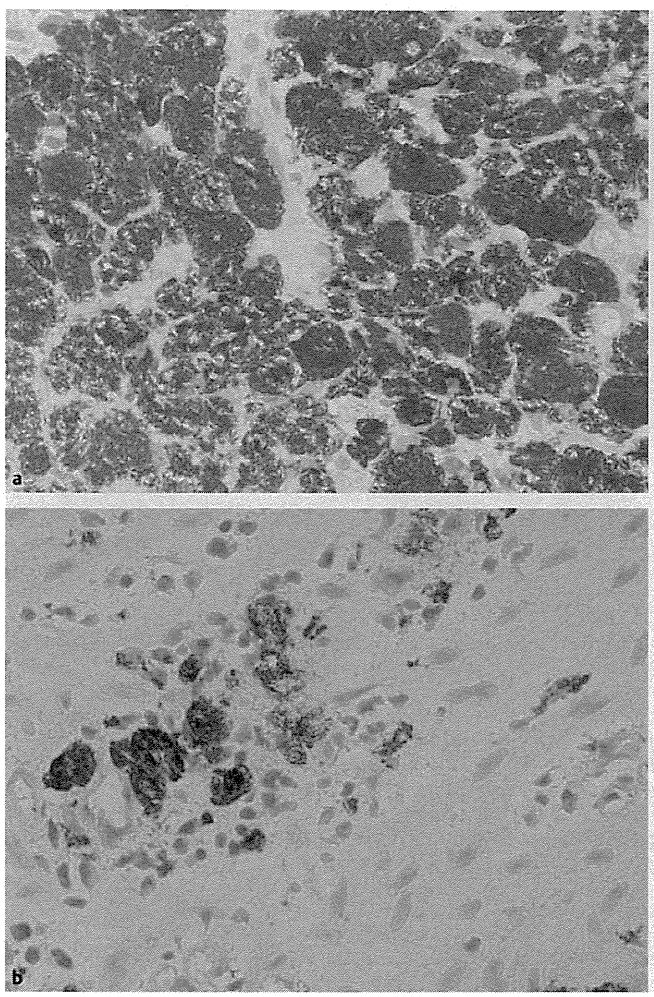

Fig. 3 Pathological appearance after Ziehl–Neelsen staining of the duodenal biopsy specimen showing numerous acid-fast bacteria being phagocytosed by the macrophages: **a** in a low-power field; **b** in a high-power field.

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Clostridium difficile flagellin stimulates toll-like receptor 5, and toxin B promotes flagellin-induced chemokine production via TLR5

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ABSTRACT

Aims: Clostridium difficile is an important pathogen in nosocomial infections. Although *C. difficile* toxins are considered to be major virulence factors, pathogenesis of *C. difficile* associated diseases remains to be determined. In this study, we investigated whether *C. difficile* flagellin is involved in the pathogenesis of *C. difficile*-associated diseases.

Main methods: C. difficile flagellin was extracted from bacterial body by using a combination of ultracentrifugation and low speed centrifugation. Extracted *C. difficile* flagellin was added to HEK293T cells transiently transfected with pUNO-mcs (empty vector) or pUNO-hTLR5, and NF-kappaB activation was compared by a dual-luciferase assay. The amount of *C. difficile* flagellin-induced inflammatory mediators such as interleukin-8 and CCL20 was measured by ELISA assay in the culture media of intestinal epithelial cell lines, HT29 cells and Caco-2 cells. Flagellin induced phosphorylation of p38 mitogen-activated protein kinase was examined by Western blotting analysis in Caco-2 cells. The amount of *C. difficile* flagellin-induced inflammatory mediators in the presence, or absence of *C. difficile* toxin B was also measured by ELISA assay.

Key findings: C. difficile flagellin induced activation of NF-kappaB in HEK293T cells via toll-like receptor 5. *C. difficile* flagellin also induced activation of p38 mitogen-activated protein kinase, and promoted the production of interleukin-8 and CCL20 in intestinal epithelial cells via toll-like receptor 5. Pretreatment with toxin B enhanced flagellin-induced cytokine productions.

Significance: Our results indicate that toxin B promotes flagellin-induced activation of intestinal epithelial cells, and that *C. difficile* flagellin may play a role in the occurrence of *C. difficile*-associated diseases.

Introduction

Clostridium difficile is an anaerobic gram-positive bacillus. This bacterium is the leading cause of infectious diarrhea in hospitals worldwide because of its virulence, spore-forming ability and persistence (Kelly and LaMont, 2008). *C. difficile* infection causes 15–25% of antibiotic-associated diarrhea, the severity of which ranges from mild diarrhea to fulminant pseudomembranous colitis, and can result in death (Bartlett and Gerding, 2008). The incidence of *C. difficile*-associated diseases (CDAD) is increasing, and severe cases are becoming more common. The mortality of CDAD has also been reported to be increasing in Western countries (Redelings et al., 2007).

C. difficile has two major toxins, toxin A and toxin B, which have been intensively studied as major virulence factors of *C. difficile* infection (Lyerly et al., 1988). Toxin A and toxin B are glucosyltransferases that inactivate Rho, Rac, and Cdc42 within target cells. The action of these toxins results in actin condensation, consequent rounding of the cells, membrane blebbing of intestinal epithelial cells and eventual apoptosis and death of the target cells (Voth and Ballard, 2005). Previous studies using a hamster infection model showed that purified toxin A alone causes the symptoms of CDAD (Du and Alfa, 2004; Sambol et al., 2001). However, it was recently reported that toxin B was essential, and a key virulence determinant, for CDAD (Lyras et al., 2009).

Toll-like receptors (TLRs) are key proteins in immune systems that recognize a variety of microbial components and induce innate immune responses (Takeda and Akira, 2007). It is known that intestinal epithelial cells express low levels of TLR2 and TLR4 and are poorly responsive to the ligands of these receptors, lipotechoic acid and lipopolysaccharide, respectively. In contrast, TLR5 is expressed in the large intestinal tract (Abreu et al., 2005), and plays a pivotal role in bacterial infections of the large intestine. Intestinal epithelial cells are activated via TLR5 by flagellin. Flagellin is one of the structural components of flagella that many bacterial strains possess (Reichhart, 2003). Stimulation of cells by flagellin leads to activation of NF-kappaB and mitogen-activated protein kinases (MAPK), and the production of inflammatory cytokines (Hayashi et al., 2001; Tallant et al., 2004; Yu et al., 2003). To date, it has been shown that flagellin is involved in the pathogenesis of

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infection of several bacterial species including *Salmonella* species, *Listeria monocytogenes*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Vibrio vulnificus* and *Vibrio cholera* (Bandyopadhaya et al., 2008; Donnelly and Steiner, 2002; Gewirtz et al., 2001; Harrison et al., 2008; Hayashi et al., 2001; Khan et al., 2004; Lee et al., 2006; Murthy et al., 2004; Schmeck et al., 2007; Smith et al., 2003; Steiner, 2007; Tallant et al., 2004; Yu et al., 2003; Zhang et al., 2007). TLR5 is mostly expressed on the basolateral side of intestinal epithelial cells, although a few TLR5 are on the apical side (Gewirtz et al., 2001). Therefore, flagellin interacts with TLR5 when flagellated pathogens invade the epithelium by transcytosing their flagellin across the epithelium, or when they approach the basolateral side of the intestinal membrane due to loss of epithelial barriers (Lyons et al., 2004).

C. difficile also has flagella that allow the bacteria to be highly motile (Tasteyre et al., 2000). We speculated that *C. difficile* flagellin may be recognized by TLR5 and play an important role in the occurrence of CDAD. In this study, we investigated the mechanisms by which *C. difficile* flagellin is involved in activation of intestinal epithelial cells.

Materials and methods

Cell culture and measurement of transepithelial electrical resistance

The HT29 and Caco-2 human intestinal epithelial cell lines and the HEK293T kidney cells were purchased from the American Type Culture Collection (Manassas, VA), and cultured in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) with 4 mM glutamine (Gibco Invitrogen, Carlsbad, CA), 10% fetal calf serum, 100 units/ml penicillin G and 100 μg/ml streptomycin (MP Biomedicals, Irvine, CA) at 37 °C in a humidified 5% CO_2 atmosphere. These cells were usually used in regular wells as non-polarized monolayers. As a tightly grown polarized monolayer, Caco-2 cells were cultured on transwell for 3 weeks. Transepithelial electrical resistance (TEER) represents the electrical resistance of tight junctions of a monolayer of cells. TEER was measured using millicel ERS (Millipore, Billerica, MA). Tightly grown monolayers displayed over 400 Ωcm^2 of TEER.

Flagellin purification

Salmonella typhimurium flagellin was purchased from InvivoGen (San Diego, CA). The *C. difficile* mutant which does not produce both toxin A and toxin B was purchased from the American Type Culture Collection. *C. difficile* flagellin was isolated essentially using the procedures described by Delmee et al. (1990). The strains were grown anaerobically in GAM buillon bacterial broth (NISSUI, Tokyo, Japan) for 48 h. Bacterial numbers were then measured by determination of the optical density of the culture at 600 nm (OD_{600}). The bacteria were harvested in 200 ml of liquid medium. Flagellin was then purified as follows: the medium containing *C. difficile* was strongly shaken for 2 min and centrifuged at 5000×*g* for 30 min at 4 °C. The supernatants were centrifuged at 25,000×*g* for 1 h at 4 °C. The pellets were suspended in 2 ml of phosphate-buffered saline (pH 7.4), and heated at 70 °C for 20 min to ensure flagellin monomers. Protein concentrations were quantified using a Micro BCA Protein Assay kit (Thermo Scientific, Rockford, IL).

SDS-PAGE and Western blot analysis

SDS-PAGE was performed as described by Laemmli (1970). The gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA) or were electrically transferred onto nitrocellulose membranes for immunoblotting. The membrane was blocked in blocking buffer containing 3% bovine serum albumin (Millipore) for 1 h. The membrane was then incubated with a primary antibody, followed by incubation with a HRP-labeled anti-immunoglobulin antibody. Antibodies against the following proteins were used: anti-p38, anti-ERK1/2, anti-phospho-ERK1/2 (all from Santa Cruz Biotechnology, Santa Cruz, CA) and anti-phospho-p38 (Thr-180/Tyr-182) (Cell Signaling, Danvers, MA). The protein bands were then visualized using the chemiluminescence reagent, Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Transfection and cell stimulation

Transient transfection of cells was performed using the FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. HEK293T cells were plated at a density of 4×10^4 cells/well in 12-well culture plates and were transiently transfected with the human TLR5-expressing vector, pUNO-hTLR5, or the control, empty vector, pUNO-mcs (InvivoGen). Forty-eight hours after transfection, flagellin was added at the indicated concentrations for 6 h. Flagellin was also added at the indicated concentrations to the culture medium of HT-29 and Caco-2 cells grown to 95–100% confluence and was incubated with the cells for 5 and 16 h, respectively. To block TLR5–flagellin interaction, 1.0 ng/ml of neutralizing, anti-TLR5 antibody (InvivoGen) was added to the culture medium 1 h before flagellin stimulation. Human IgA2 Ab (InvivoGen) was used as a control. For inhibition of p38 activity, 10 μM of SB203580 (Cell Signaling) was added to the culture medium 2 h before flagellin stimulation. *C. difficile* toxin B, purchased from List Biological Laboratories, Inc., (Campbell, CA) was added to the culture medium at a concentration of 0.25 μg/ml for the indicated times.

Dual luciferase reporter assay

HEK293T cells were plated in 12-well culture plates at a density of 4.0×10^4 cells/well, and cDNA plasmids were transiently transfected 48 h later using the FuGENE 6 transfection reagent. The cells were co-transfected with 0.5 μg of the NF-kappaB firefly luciferase reporter plasmid, pNF-kappaB-Luc, (Stratagene, Santa Clara, CA), 0.05 μg of a reporter plasmid that expresses constitutively active *Renilla* luciferase, pRL-TK (Promega, Madison, WI), and 0.5 μg of a TLR5-expressing plasmid or a control vector. Forty-eight hours after transfection firefly luciferase and *Renilla* luciferase activities were measured using a dual-luciferase reporter assay system (Promega). Relative luciferase activity is expressed as the ratio of firefly luciferase activity to *Renilla* luciferase activity.

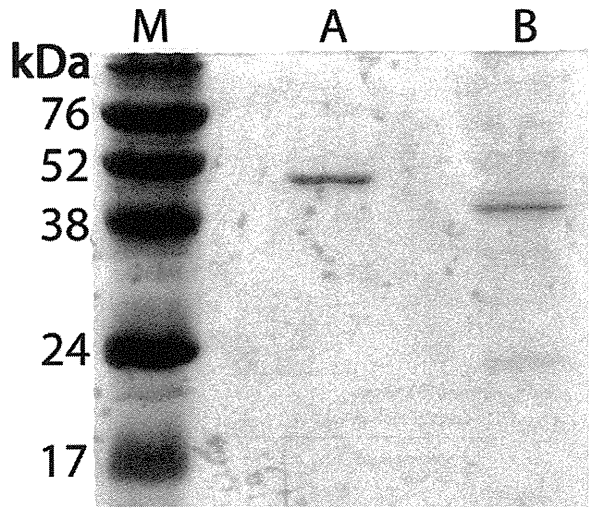

Fig. 1. SDS-PAGE analysis of extracted *C. difficile* flagellin. Flagellin of *Salmonella typhimurium* (lane A) and flagellin extracted from *C. difficile* (lane B) were analyzed by SDS-PAGE gel electrophoresis using a 12% separating gel, followed by Coomassie Brilliant Blue R-250 staining. Lane M; molecular weight markers.

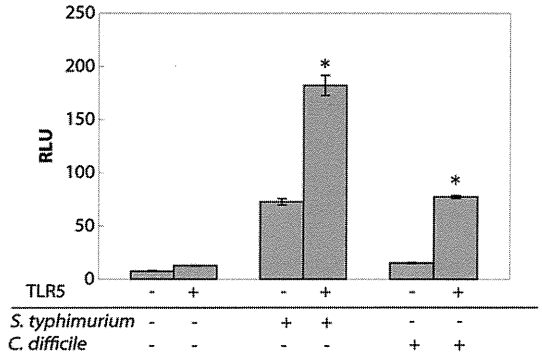

Fig. 2. Dual-luciferase assay of the effect of *C. difficile* flagellin on NF-kappaB activation. HEK293T cells were transiently transfected with pUNO-mcs (empty vector; −) or the human TLR5-expressing vector pUNO-hTLR5 (+) and were co-transfected with an NF-kappaB luciferase reporter and control vectors. The cells were then stimulated with vehicle, 100 ng/ml of *S. typhimurium* flagellin (positive control) or 1.0 mg/ml of *C. difficile* flagellin. NF-kappaB activation was then determined based on luciferase activity, which is expressed as relative light units (RLU). Error bars indicate the standard error of the mean. The asterisk, *, indicates a significant difference between the RLU of HEK293T cells that were transiently transfected with pUNO-hTLR5 and stimulated with *C. difficile* or *S. typhimurium* flagellin and the RLU of cells transfected with pUNO-mcs that were similarly stimulated.

ELISA assay

After cell stimulation with flagellin, cell culture supernatants were collected and stored at −80 °C until ELISA assay was performed. Flagellin-induced interleukin(IL)-8 or CCL20 production was determined using the Pierce human IL-8 ELISA Kit (Thermo Scientific, Waltham, MA) or a human CCL20 ELISA Kit (R&D Systems, Minneapolis, MN) according to the manufacturers' instructions.

Statistical analyses

All quantitative experiments were repeated at least three times, and each experiment was performed in triplicate. Calculations of means and standard errors were analyzed using one way ANOVA. P values less than 0.05 were considered significant. All analyses were performed with SPSS software for Windows (Ver.10.1) (SPSS Inc., Chicago, IL).

Results

Flagellin was extracted from C. difficile

C. difficile flagellin was extracted as described in Materials and methods and analyzed by gel electrophoresis. The molecular weight

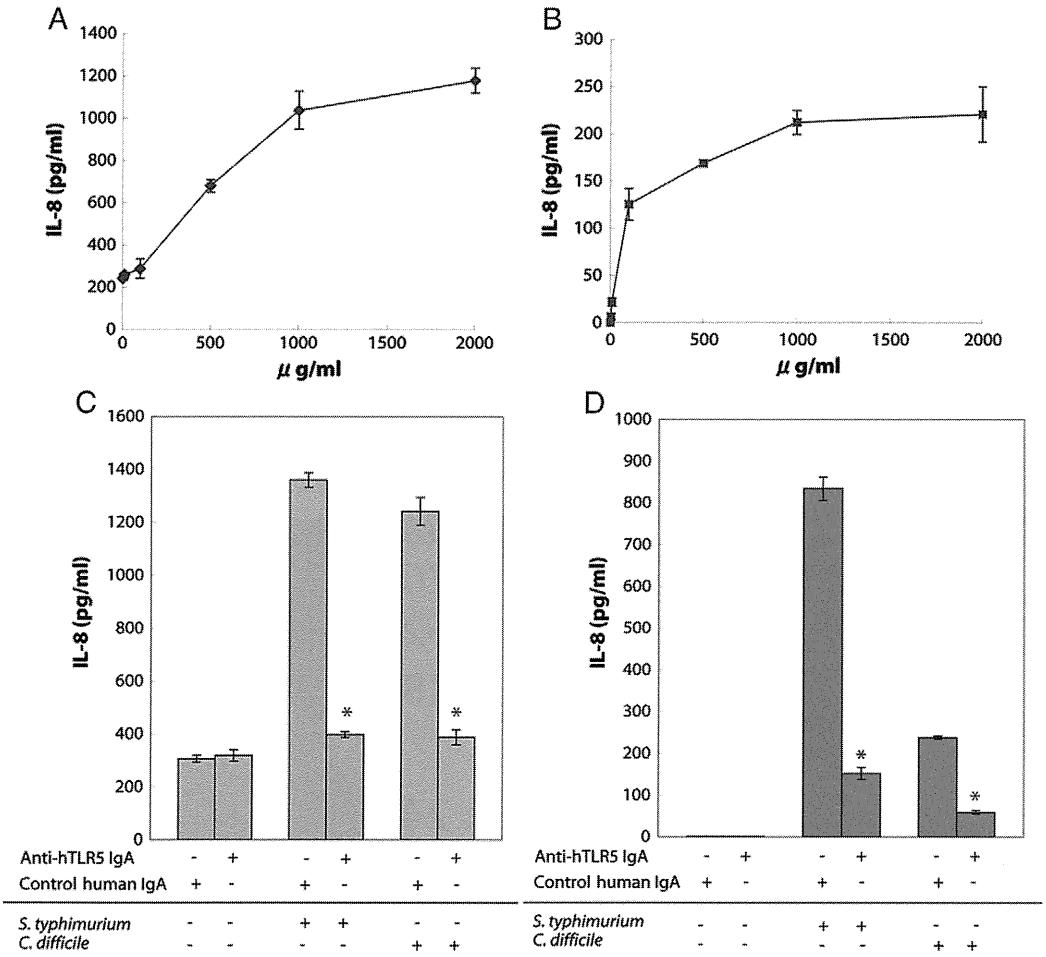

Fig. 3. ELISA assay of the effect of *C. difficile* flagellin on IL-8 production in intestinal epithelial cells. IL-8 produced in intestinal epithelial cells under the following conditions was measured using an ELISA. (A, B) HT29 (A) or Caco-2 (B) cells were stimulated with the indicated concentrations of *C. difficile* flagellin. (C, D) HT29 (C) or Caco-2 (D) cells were stimulated with vehicle, 100 ng/ml of *S. typhimurium* flagellin (positive control) or 1.0 mg/ml of *C. difficile* flagellin in the presence of a TLR5-neutralizing anti-TLR5 antibody or control human-IgA. Error bars indicate the standard error of the mean. The asterisk, *, indicates a significant difference between IL-8 production of HT29 or Caco-2 cells treated with anti-TLR5 antibody and cells treated with control human-IgA.

of the extracted protein was approximately 39 kDa (Fig. 1), similar to that reported by Delmee et al. (1990), indicating that the extracted protein was *C. difficile* flagellin.

C. difficile flagellin induced NF-kappaB activation and chemokine production via TLR5

We next assayed if *S. typhimurium* flagellin induced activation of NF-kappaB via TLR5 using a dual luciferase NF-kappaB reporter assay. HEK293T cells, which do not express endogenous TLR5 were transiently transfected with human TLR5 or a control vector and were co-transfected with an NF-kappaB luciferase reporter or control plasmids. The cells were then stimulated with *C. difficile* flagellin, *S. typhimurium* flagellin used as a positive control or vehicle. Only basal levels of luciferase activity were observed in non-stimulated control HEK293T cells (Fig. 2). Stimulation of non-TLR5-transfected cells with *S. typhimurium* flagellin increased luciferase activity. However, this activity was significantly enhanced in HEK293T cells expressing human TLR5 compared with control cells. In addition, the luciferase activity induced by *C. difficile* flagellin was significantly enhanced in HEK293T cells expressing human TLR5 compared with control HEK293T cells. These results indicated that *C. difficile* flagellin induced activation of NF-kappaB via TLR5.

Induction of IL-8 production by *C. difficile* flagellin, as well as the role of TLR5 in this induction, was assayed using HT29 and Caco-2 cells, which express endogenous cell surface TLR5. These cells were first stimulated with various concentrations of *C. difficile* flagellin, and IL-8 production was measured using ELISA. Stimulation with *C. difficile* flagellin promoted IL-8 production in both HT29 and Caco-2 cells in a dose dependent manner (Fig. 3A, B). To assess the role of TLR5 in this induction, TLR5 expressed on the surface of these cells was neutralized by treatment with an anti-TLR5 neutralizing antibody or control, human-IgA. These treated cells were then stimulated with *C. difficile* flagellin, *S. typhimurium* flagellin or vehicle, and IL-8 production was assayed. Stimulation with *C. difficile* or *S. typhimurium* flagellin, but not with vehicle induced IL-8 production in both HT29 and Caco-2 cells in the presence of control, human-IgA (Fig. 3C, D). However, flagellin-induced IL-8 production was significantly inhibited in cells treated with anti-TLR5 neutralizing antibody. These results demonstrated that *C. difficile* flagellin-induced IL-8 production and that this production is mediated by TLR5.

Induction of CCL20 production by *C. difficile* flagellin, and the role of TLR5 in this induction, was assayed in a similar manner to that of IL-8. Stimulation of HT29 cells and Caco-2 cells with various concentrations of *C. difficile* flagellin promoted CCL20 production in both cell types in a dose dependent manner (Fig. 4A, B). CCL20 was also

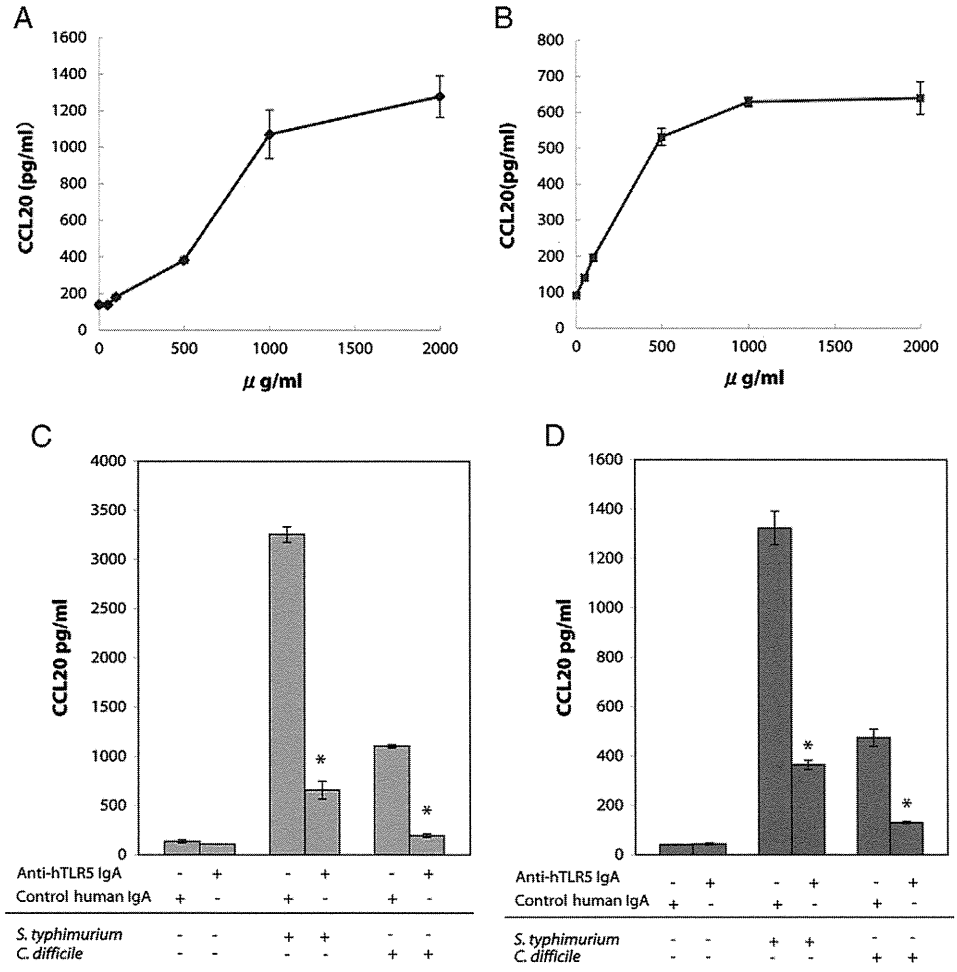

Fig. 4. ELISA assay of the effect of *C. difficile* flagellin on CCL20 production in intestinal epithelial cells. CCL20 produced in intestinal epithelial cells under the following conditions was measured using an ELISA. (A, B) HT29 (A) or Caco-2 (B) cells were stimulated with the indicated concentrations of *C. difficile* flagellin. (C, D) HT29 (C) or Caco-2 (D) cells were stimulated with vehicle, 100 ng/ml of *S. typhimurium* flagellin (positive control) or 1.0 mg/ml of *C. difficile* flagellin in the presence of a TLR5-neutralizing anti-TLR5 antibody or control human-IgA. Error bars indicate the standard error of the mean. The asterisk, *, indicates a significant difference between CCL20 production of HT29 or Caco-2 cells treated with anti-TLR5 antibody and cells treated with control human-IgA.