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Entamoeba histolytica-secreted cysteine proteases induce IL-8 production in human mast cells via a PAR2-independent mechanism

Young Ah Lee[1], Young Hee Nam[1], Arim Min[1], Kyeong Ah Kim[1], Tomoyoshi Nozaki[2], Yumiko Saito-Nakano[2], David Mirelman[3], and Myeong Heon Shin[1,*]

[1] Department of Environmental Medical Biology, Institute of Tropical Medicine, and Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul 120-752, Korea
[2] Department of Parasitology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo, Japan
[3] Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel

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Abstract – *Entamoeba histolytica* is an extracellular tissue parasite causing colitis and occasional liver abscess in humans. *E. histolytica*-derived secretory products (SPs) contain large amounts of cysteine proteases (CPs), one of the important amoebic virulence factors. Although tissue-residing mast cells play an important role in the mucosal inflammatory response to this pathogen, it is not known whether the SPs induce mast cell activation. In this study, when human mast cells (HMC-1 cells) were stimulated with SPs collected from pathogenic wild-type amoebae, interleukin IL-8 mRNA expression and production were significantly increased compared with cells incubated with medium alone. Inhibition of CP activity in the SPs with heat or the CP inhibitor E64 resulted in significant reduction of IL-8 production. Moreover, SPs obtained from inhibitors of cysteine protease (ICP)-overexpressing amoebae with low CP activity showed weaker stimulatory effects on IL-8 production than the wild-type control. Preincubation of HMC-1 cells with antibodies to human protease-activated receptor 2 (PAR2) did not affect the SP-induced IL-8 production. These results suggest that cysteine proteases in *E. histolytica*-derived secretory products stimulate mast cells to produce IL-8 via a PAR2-independent mechanism, which contributes to IL-8-mediated tissue inflammatory responses during the early phase of human amoebiasis.

Key words: *Entamoeba histolytica*, Cysteine protease, Mast cell, IL-8, Protease-activated receptor 2 (PAR2).

Résumé – Les cystéine protéases secrétées par *Entamoeba histolytica* induisent la production d'IL-8 chez les mastocytes humains par un mécanisme indépendant de PAR2. *Entamoeba histolytica* est un parasite extracellulaire des tissus provoquant des colites et occasionnellement des abcès du foie chez l'homme. Les produits de sécrétion dérivés d'*E. histolytica* (SPs) contiennent de grandes quantités de cystéine-protéases (CPs), l'un des principaux facteurs de virulence amibiens. Bien que les mastocytes tissulaires jouent un rôle important dans la réponse inflammatoire de la muqueuse à ce pathogène, on ne sait pas si les SPs induisent l'activation des mastocytes. Dans cette étude, lorsque des mastocytes humains (cellules HMC-1) ont été stimulés avec des SPs recueillis à partir d'amibes pathogènes de type sauvage, l'expression et la production de l'interleukine IL-8 ont été significativement augmentées par rapport à des cellules incubées avec du milieu seul. L'inhibition de l'activité des CPs dans les SPs avec la chaleur ou avec E64, un inhibiteur de CP, a entraîné une réduction significative de la production d'IL-8. En outre, les SPs obtenus à partir d'amibes surexprimant l'inhibiteur de protéases à cystéine (ICP) à faible activité de CP ont montré des effets stimulants plus faibles sur la production d'IL-8 que le contrôle de type sauvage. La pré-incubation des cellules HMC-1 avec des anticorps contre le récepteur 2 activé par la protéase humaine (PAR2) n'a pas affecté la production d'IL-8 induite par SPs. Ces résultats suggèrent que les cystéine-protéases des produits de sécrétion dérivés d'*E. histolytica* stimulent les mastocytes pour produire de l'IL-8 par l'intermédiaire d'un mécanisme indépendant de PAR2, ce qui contribue à la réponse inflammatoire tissulaire médiée par IL-8 au cours de la phase précoce de l'amibiase humaine.

*Corresponding author: myeong@yuhs.ac

Introduction

The enteric protozoan parasite *Entamoeba histolytica* Schaudinn, 1903 [25] is the causative agent of human amoebiasis. *E. histolytica* trophozoites bind colonic mucin via amoebic Gal-lectin and subsequently cause mucin degradation and colonic epithelial cell death through apoptosis or necrosis [19, 20]. *E. histolytica* releases various proteases into the extracellular milieu. In particular, cysteine proteases (CPs) are abundant in *E. histolytica*-derived secretory products (SPs). CPs are important for host mucin and extracellular matrix (ECM) degradation [11], cytopathic effects on the host cell, and activation of IL-1 β or IL-18.

E. histolytica-induced colon cell death promotes IL-8-mediated acute tissue inflammation at the site of infection [26]. IL-8 is a potent chemoattractant and activator of neutrophils and can cause non-specific tissue damage after activation [9]. Although neutrophils are the primary target cells of IL-8, IL-8 has other effects on various kinds of cells such as endothelial cells, other granulocytes, macrophages, and mast cells. The infiltration of immune cells including neutrophils, macrophages, and mast cells at the mucosal surface was observed during *E. histolytica* intestinal amoebiasis, suggesting that these cells might be important in host defense against this parasite [13]. Moreover, an increase in degranulation and disruption of mast cells was reported in *E. histolytica*–infected mice [16], suggesting that mast cells play a role in *E. histolytica*–induced tissue inflammation at the inflamed site. However, the precise role of amoebic CPs in mast cell activation is poorly understood.

Mast cells contribute to the innate and adaptive host defense mechanisms through the release of an arsenal of inflammatory mediators upon activation by various stimuli [3, 12]. Tissue-residing mast cells are major players in the mucosal inflammatory response to various bacterial and parasitic infections [6]. Activated mast cells release various proinflammatory mediators including histamine, IL-6, IL-8, IL-13, prostaglandin D2, leukotriene C4 (LTC4), and tumor necrosis factor-α (TNF-α) in response to various stimuli [14]. The essential role of mast cells in the host control of infection has been shown in animal models infected with various bacterial and parasitic pathogens [2, 4, 28]. However, little is known about the effect of amoebic SPs on IL-8 secretion in mast cells.

In this study, mast cells were stimulated with *E. histolytica*-derived SPs from *E. histolytica* wild-type or mutant strains to ascertain if SPs directly induce IL-8 production. The results of this work show that amoebic CPs participate in SP-induced IL-8 production in HMC-1 cells.

Materials and methods

Reagents

Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Mouse monoclonal antibody (Ab) against human protease-activated receptor 2 (PAR2) (SAM 11) and normal mouse IgG_{2a} were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fluorescent isothiocyanate (FITC)-labeled annexin V, PE-conjugated anti-human CD63, and PE-conjugated anti-mouse $IgG_1\kappa$ were purchased from BD Pharmingen (San Diego, CA, USA). Dichlorodihydrofluorescein diacetate (H_2DCFDA) was purchased from Molecular Probes (Eugene, OR, USA).

Cultivation of human HMC-1 cells

The HMC-1 human mast cell line was grown in Iscove's Modified Dulbecco's medium (IMDM) (Invitrogen) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 0.5% penicillin-streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. HMC-1 cell viability, as judged by trypan blue exclusion testing, was 99%.

Culture of bone marrow-derived murine mast cells (BMMCs) and ethics

BMMCs from BALB/c mice (Orient Bio, Seoul, Korea) were obtained by culturing mouse femoral bone marrow cells in RPMI containing 10% (v/v) heat-inactivated fetal bovine serum, 0.5% penicillin-streptomycin, 50 μM β-mercaptoethanol, and 20 ng/mL IL-3 (PeproTech, Rocky Hill, NJ, USA) for 4 weeks. At that time, the cells were > 98% c-kit^{high} FcεRI^{high}, according to flow cytometric analysis using PE anti-mouse FcεRIa (MAR-1) (eBioscience, San Diego, CA, USA) and FITC anti-mouse c-kit/CD117 (2D8) (eBioscience, San Diego, CA, USA). The present study was approved by the Yonsei University Animal Research Ethics Committee (No. 2013-0297).

Construction of *E. histolytica* cell lines overexpressing a GFP-tagged inhibitor of cysteine protease 1 (ICP1$^{+/+}$)

To create the ICP1-overexpressing *E. histolytica* cell line (ICP1$^{+/+}$), a full-length *E. histolytica ICP1* gene was amplified by PCR from cDNA using sense and antisense oligonucleotides containing appropriate restriction sites (underlined) at the 5′-end: *icp1* 5′-aatcccgggATGTCATTAACTGAAGATAATAAC AACACAAC-3′ (*Sma*I); and 5′-gggctcgagTTACTG GACAT TAACTTTTAAAGTAAAAG-3′ (*Xho*I). The PCR-amplified DNA fragments were digested and ligated into the same restriction sites of the overexpression vector, pKT-MG. This vector allows for the expression of a gene of interest as a N-terminal fusion with GFP. The plasmids were introduced into *E. histolytica* HM-1:IMSS trophozoites by liposome-mediated transfection as previously described [22], and stable transformants were cultured in medium containing 8 μg/mL G418 (for ICP1$^{+/+}$ and pKT-MG as a vector control).

Cultivation of *E. histolytica* trophozoites and preparation of secretory products

Trophozoites of the *E. histolytica* virulent HM-1:IMSS strain, the avirulent *E. histolytica* Rahman strain, and the hypo-CP strain ICP1$^{+/+}$ were grown at 37 °C in TYI-S-33 medium as described previously [10]. After cultivation for 48–72 h, logarithmic growth phase trophozoites were harvested by incubation on ice for 10 min, followed by centrifugation at 1000 rpm at 4 °C for 5 min. To collect SPs, trophozoites from the various strains were incubated in Hanks' balanced salt solution (GIBCO Laboratories, Grand Island, NY, USA) for 2 h at 37 °C at a final concentration of 1×10^7 amoebae per mL. The viability of *Entamoeba* trophozoites after incubation in Hank's balanced salt solution was 99% as determined by the trypan blue exclusion assay. Protein concentration was measured by the BCA protein assay using bovine serum albumin as a standard.

Measurement of cell viability and cell death

HMC-1 cell viability and cell death were measured by trypan blue and annexin V/propidium iodide (PI) double staining, respectively. HMC-1 cells (5×10^5 cells/sample) stimulated with SPs were incubated for 2 h at 37 °C in a humidified CO_2 incubator (5% CO_2 and 95% air). After incubation, the reaction was stopped by brief centrifugation, and the cells were washed with cold PBS twice and stained with FITC-conjugated annex in V and 1 μg/mL PI. Flow cytometric analysis was performed with a FACScan on at least 10,000 cells from the host cell fraction.

Measurement of exocytosis in HMC-1 cells or BMMCs

To elucidate the exocytosis of HMC-1 cells or BMMCs induced by the SPs, HMC-1 cells or BMMCs (5×10^5 cells/sample) were incubated with SP (30 or 100 μg/mL) for 2 h, or platelet-activating factor (PAF) (5 μM) for 1 h or phorbol 12-myristate 13-acetate (PMA) for 2 h. For positive control, BMMCs were treated with monoclonal anti-DNP IgE (250 ng/mL) for 4 h, and then the cells were challenged with DNP-HSA (250 ng/mL) for 1 h to trigger mast cell degranulation. After incubation, the cells were washed twice with washing buffer (0.1% sodium azide and 1% FBS in PBS) and stained with PE-conjugated anti-human or anti-mouse CD63. PE-conjugated anti-mouse IgG_1 or anti-rat IgG_{2a} was used as an isotype control. A flow cytometric analysis was performed using a FACScan on at least 10,000 cells from each sample.

Quantitative real-time PCR for IL-8 mRNA expression

HMC-1 cells (1×10^6/sample) were incubated with or without SPs for 30 min at 37 °C in a CO_2 incubator. After incubation, total RNA was obtained from HMC-1 cells (1×10^6/sample) incubated with or without SPs using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA) and was reverse-transcribed using a ProSTAR first-strand RT-PCR kit (Stratagene, La Jolla, CA, USA). Obtained cDNA was amplified using a SYBR® Green PCR Master Mix. The primers used were as follows: human IL-8; 5′-TCT GCA GCT CTG TGT GAA GGT G-3′ and 5′-AAT TTC TGT GTT GGC GCA GTG-3′, human GAPDH; 5′-GAA GGT GAA GGT CGG AGT C-3′ and 5′-GAA GAT GGT GAT GGG ATT TC-3′. IL-8 gene expression was analyzed using the Applied Biosystems 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The relative amount of mRNA for the genes of interest was determined by subtracting the threshold cycle (Ct) values for the gene from the Ct value for the internal control gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ΔCt). Each measurement of a sample was performed in triplicate. The data represent IL-8 mRNA fold induction.

IL-8 ELISA

For measurement of IL-8 production, HMC-1 cells (5×10^5/well) were seeded in 24-well tissue culture plates and then directly incubated for the indicated times with or without native or modified SPs for 12 h at 37 °C in a CO_2 incubator. After incubation, culture supernatants were collected from HMC-1 cells. To evaluate the involvement of PAR2 in SP-induced IL-8 production, HMC-1 cells (5×10^5/well) were preincubated with anti-PAR2 (10 μg/mL) or mouse IgG_{2a} (10 μg/mL) for 30 min. After preincubation, the cells were incubated for the indicated times with or without native SPs for 12 h at 37 °C in a CO_2 incubator. Then, culture supernatants were collected from HMC-1 cells, and the amount of IL-8 production was measured with a specific human IL-8 screening kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Measurement of cysteine protease activity

Native SPs obtained from wild-type amoebae were pretreated with or without protease inhibitor (E64 or PMSF) or heat-modified SPs (100 °C for 10 min), and then CP assays were performed. In addition, ICP1$^{+/+}$, vector control, or Rahman-derived native SPs were also measured for CP activity. Briefly, *E. histolytica* trophozoites (4×10^5/well) were incubated in 100 mL Opti-MEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 137 mM cysteine and 19 mM ascorbic acid, pH 6.8 in a 96-well tissue culture plate at 37 °C for 1 h. After incubation, the culture supernatant for secreted CP activity was collected and remaining trophozoites for intracellular CP activity were obtained by centrifugation. CP activity was measured according to the cleavage of a z-Arg-Arg-pNA2 HCl synthetic peptide substrate, which was monitored spectrophotometrically as described previously [21, 23].

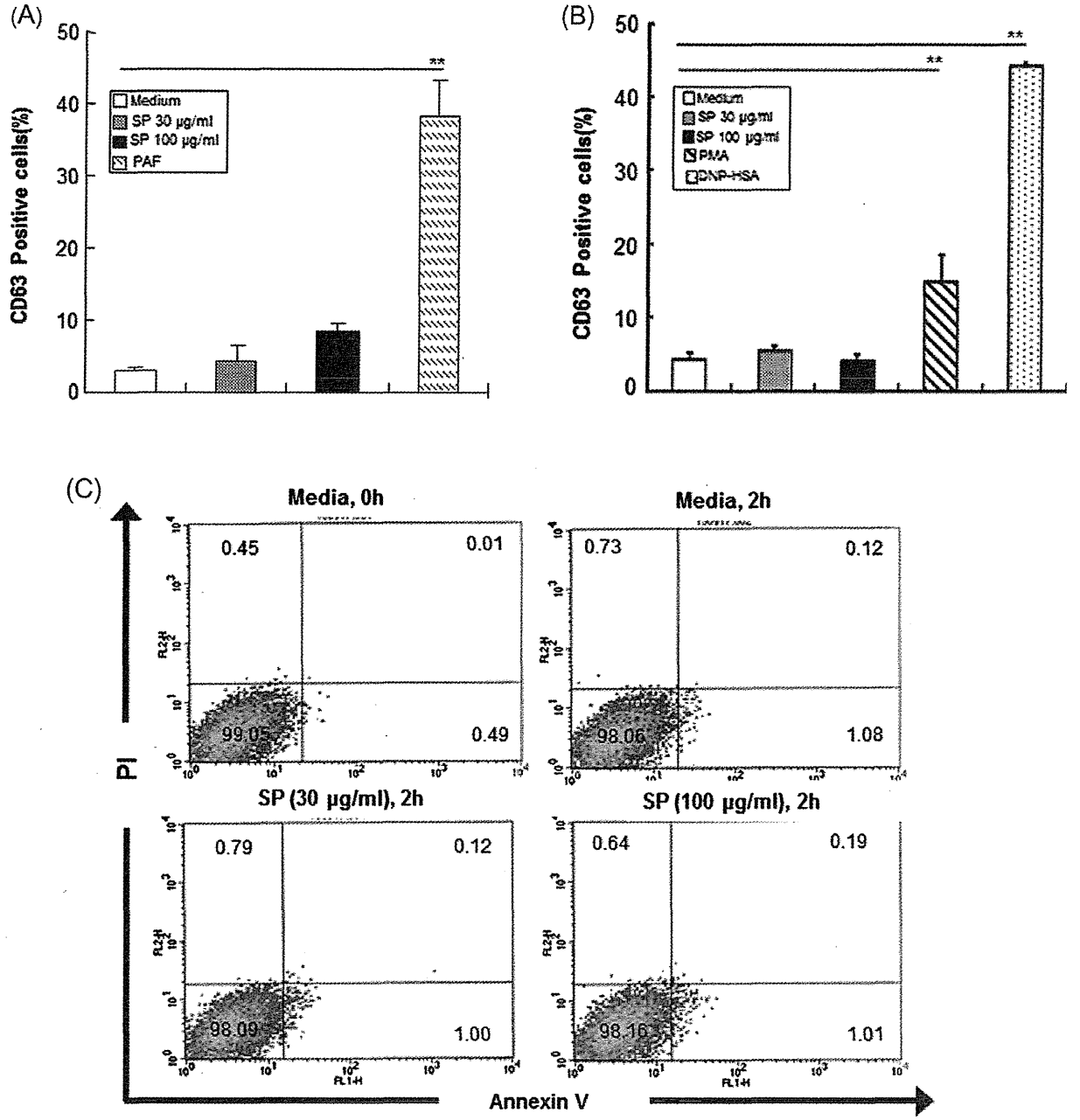

Figure 1. *E. histolytica*-derived secretory products (SPs) of pathogenic amoebae do not induce cell death or exocytosis in mast cells. (A) Percentage of CD63-positive cells in HMC-1 cells incubated with SPs or platelet-activating factor (PAF) (5 μM). (n = 3) (B) Percentage of CD63-positive cells in bone marrow-derived murine mast cells incubated with SPs, phorbol 12-myristate 13-acetate (PMA) (50nM) or DNP-HSA (250 ng/mL). (n = 4). (C) Flow cytometry analysis of HMC-1 cell death after stimulation with or without SPs. (n = 4). All reactions were performed in triplicate measurements of each experiment. All data are presented as the mean ± SEM of at least three independent experiments. The asterisks indicate the results of comparisons with the controls (**$p < 0.01$).

Measurement of ROS generation in HMC-1 cells

Intracellular ROS accumulation in HMC-1 cells was measured by staining cells with the green fluorescence probe H_2DCFDA, which is rapidly oxidized to highly fluorescent DCF in the presence of intracellular H_2O_2, and analyzed spectrofluorometrically (model Axiovert 200). Briefly, HMC-1 cells (1×10^5 cells/sample) were preloaded with 5 μM H_2DCFDA for 30 min and washed twice with culture medium. The washed cells were incubated with or without SPs or PAF for up to 30 min at 37 °C in a CO_2 incubator. The production of intracellular ROS was determined on a Perkin Elmer LS50B

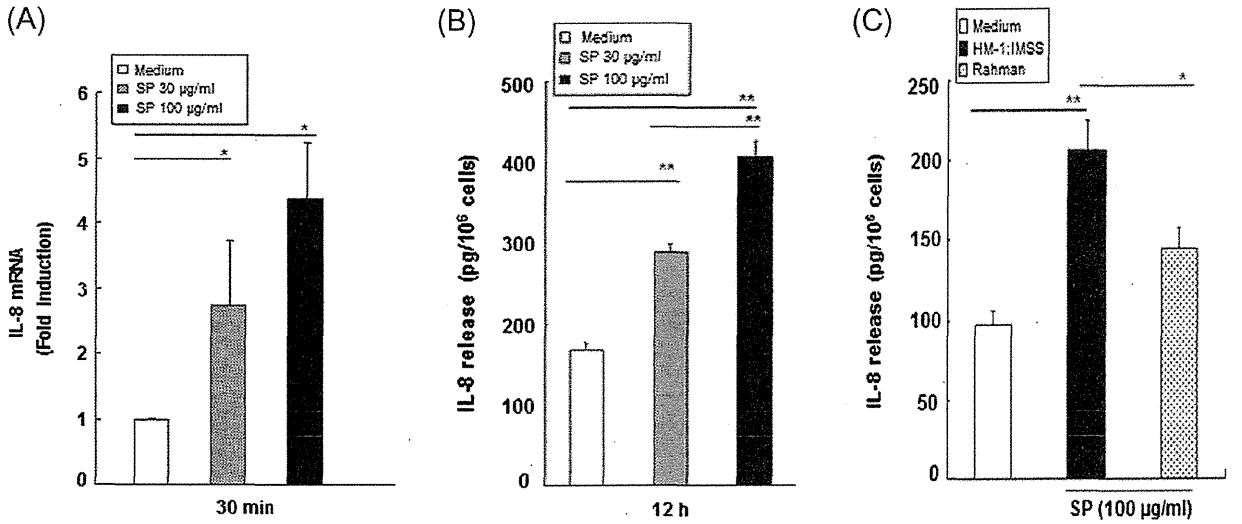

Figure 2. *E. histolytica*-derived secretory products (SPs) from the pathogenic wild-type HM-1:IMSS strain induce IL-8 gene expression and protein secretion in HMC-1 cells. (A) Increased *IL-8* mRNA expression in HMC-1 cells induced by SPs. (n = 3) (B) IL-8 production in SPs-stimulated HMC-1 cells. (n = 6) (C) Comparison of IL-8 production in HMC-1 cells stimulated with SPs obtained from wild-type or non-pathogenic Rahman strains. (n = 4). All reactions were performed in triplicate measurements of each experiment. Data are presented as the mean ± SEM of at least three independent experiments. The asterisks indicate the results of comparisons with the controls (*$p < 0.05$, **$p < 0.01$).

spectrofluorometer using excitation and emission wavelengths of 485 and 530 nm, respectively. All background fluorescence was subtracted using the appropriate controls.

Statistical analysis

All reactions were performed in triplicate measurements of each experiment. Results are presented as the mean ± SEM of 3 to 6 independent experiments. One-way analysis of variance (ANOVA) was performed using the statistical software package SPSS version 20 for Windows. The post hoc comparisons of means from different groups were made by the Bonferroni post hoc test. Values were considered statistically significant when the p-value ≤ 0.05.

Results

E. *histolytica*-derived secretory products do not induce mast cell degranulation

HMC-1 cells were treated with various concentrations (30 or 100 μg/mL) of SPs for 2 h to examine whether SPs could induce mast cell activation. CD63 expression is generally used as a surface marker for degranulation via exocytosis in mast cells [24]. When HMC-1 cells or BMMCs were treated with 100 μg/mL SP for 2 h, the percentage of CD63-positive cells was 8.1 ± 1.7 and 4.0 ± 0.6, respectively. However, HMC-1 cells treated with 5 μM PAF or BMMCs treated with DNP-HSA as a positive control showed a marked increase in CD63 expression (Figure 1A and 1B). At that time, there was no difference in cell viability after incubation with SPs for 2 h compared with the results for HMC-1 cells (Figure 1C) or BMMCs (data not shown) incubated with medium alone.

Stimulation with secretory products increased IL-8 mRNA expression and protein secretion in HMC-1 cells

To examine whether SPs could induce secretion of IL-8 in HMC-1 cells, real-time PCR and ELISA analyses were performed. The real-time PCR analysis revealed that HMC-1 cells stimulated with SPs for 30 min resulted in a 3- to 4-fold increase in IL-8 mRNA as compared with cells treated with medium alone (Figure 2A). As shown in Figure 2B, SP-stimulated HMC-1 cells released IL-8 protein in a dose-dependent manner, and HMC-1 cells stimulated with SPs for 12 h resulted in a 1.7- and 2.4-fold increase in IL-8 protein at 30 and 100 μg/mL SP, respectively, compared with medium-treated cells. There was no difference in the number of annexin V-positive HMC-1 cells after incubation with SPs for 12 h compared with the results for cells incubated with medium alone (data not shown). Interestingly, SPs from the non-pathogenic Rahman strain showed a 30% reduction compared with SPs from HM-1:IMSS amoebae (Figure 2C).

Cysteine protease activity is required for IL-8 production in HMC-1 cells induced by secretory products

To determine if amoebic CPs obtained from wild-type HM-1:IMSS SPs are responsible for SP-triggered IL-8

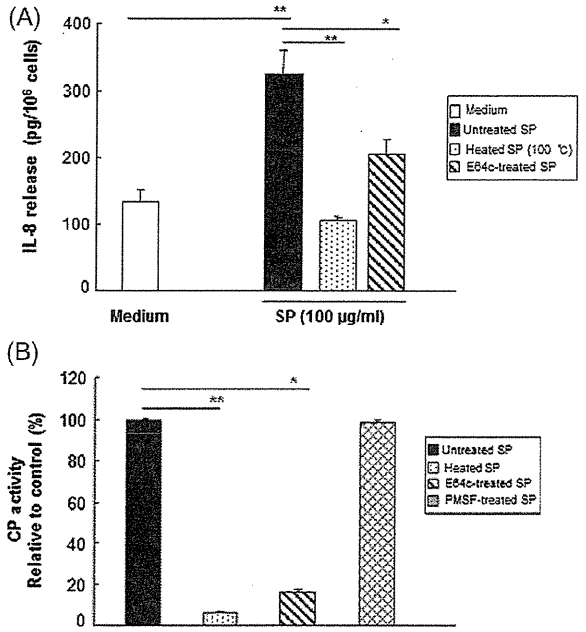

Figure 3. Amoebic cysteine protease activity is required for IL-8 production in HMC-1 cells induced by *E. histolytica*-derived secretory products (SPs). (A) IL-8 production in HMC-1 cells stimulated with SPs from native or modified wild-type SPs either heat-treated (100 °C, 10 min) or treated with 25 µM protease inhibitor (E64c). (n = 4) (B) CP activity from native or modified wild-type SPs with heat- (100 °C, 10 min) or 25 µM protease inhibitor (E64c or PMSF) treatment were measured using z-Arg-Arg-pNA·2 HCl as a substrate. The level of activity is shown as a percentage relative to the control. (n = 3). All reactions were performed in triplicate measurements of each experiment. Data are presented as the mean ± SEM of at least three independent experiments. The asterisks indicate the results of comparisons with the controls (*p < 0.05. **p < 0.01).

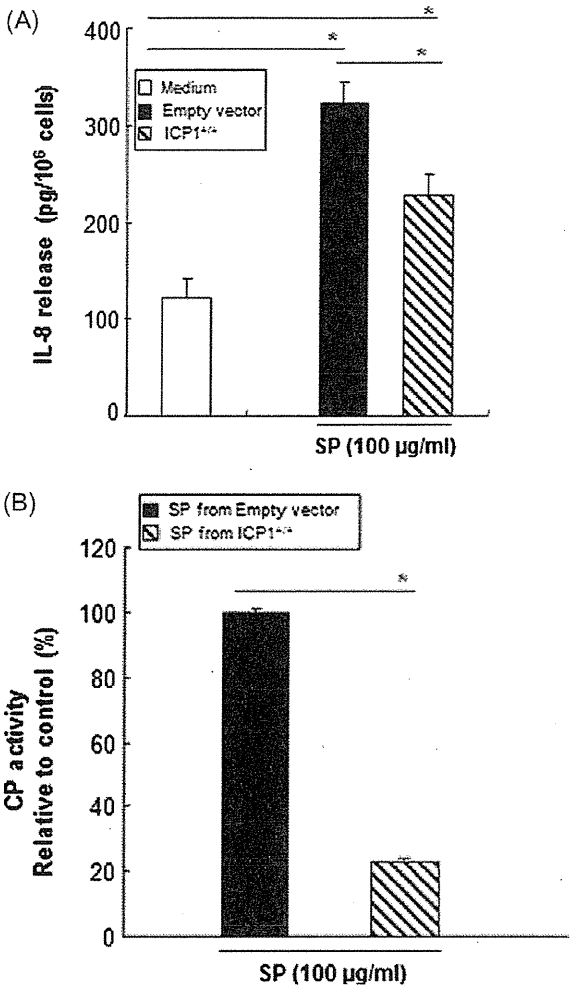

Figure 4. *E. histolytica*-derived secretory products (SPs) from the inhibitor of cysteine protease 1 ($ICP1^{+/+}$) strain show a reduction in IL-8 protein secretion and cysteine protease activity in HMC-1 cells. (A) IL-8 production in HMC-1 cells stimulated with SPs from the $ICP1^{+/+}$ or vector control strain. (n = 4) (B) The CP activity of SPs from the $ICP1^{+/+}$ or vector control strain was measured using z-Arg-Arg-pNA·2 HCl as a substrate. (n = 3). All reactions were performed in triplicate measurements of each experiment. Data are presented as the mean ± SEM of at least three independent experiments. The asterisks indicate the results of comparisons with the controls (*p < 0.05).

production in mast cells, modified SPs were incubated with HMC-1 cells. As shown in Figure 3A, the IL-8 production induced by SPs was abolished by pretreatment of the SPs with heat, suggesting that *Entamoeba*-secreted heat-labile protein components may participate in IL-8 production in HMC-1 cells. SP-induced IL-8 production in HMC-1 cells was significantly reduced by pretreatment of the SPs with the cysteine protease inhibitor E64c. Next, we compared the CP activity of native or modified SPs by treatment with specific protease inhibitors. As shown in Figure 3B, cysteine protease inhibitor E64c-treated SPs and heat-treated SPs from the wild-type significantly reduced CP activity as compared with untreated SPs. In contrast, serine protease inhibitor PMSF-treated SPs had no inhibitory effect on CP activity. To demonstrate that amoebic CP is responsible for SP-triggered IL-8 production in mast cells, we observed the IL-8 production in HMC-1 cells by SPs from the $ICP1^{+/+}$ strain. SPs derived from the $ICP1^{+/+}$ strain resulted in a decrease in IL-8 production compared with its transfectant control (Figure 4A). In addition, a marked reduction of CP activity was observed in SPs obtained from the $ICP1^{+/+}$ strain compared with its vector control amoebae (Figure 4B).

Secretory products-induced IL-8 production in HMC-1 cells occurs through a PAR2- and ROS-independent mechanism

Next, we investigated whether PAR2, which is a known G-coupled receptor, is involved in IL-8 production induced by SPs. HMC-1 cells were preincubated with monoclonal Ab (10 µg/mL) to PAR2 or the isotype control IgG_{2a} for 30 min at room temperature and subsequently incubated for 12 h with or without SPs. As shown in Figure 5A, Ab to PAR2 did not

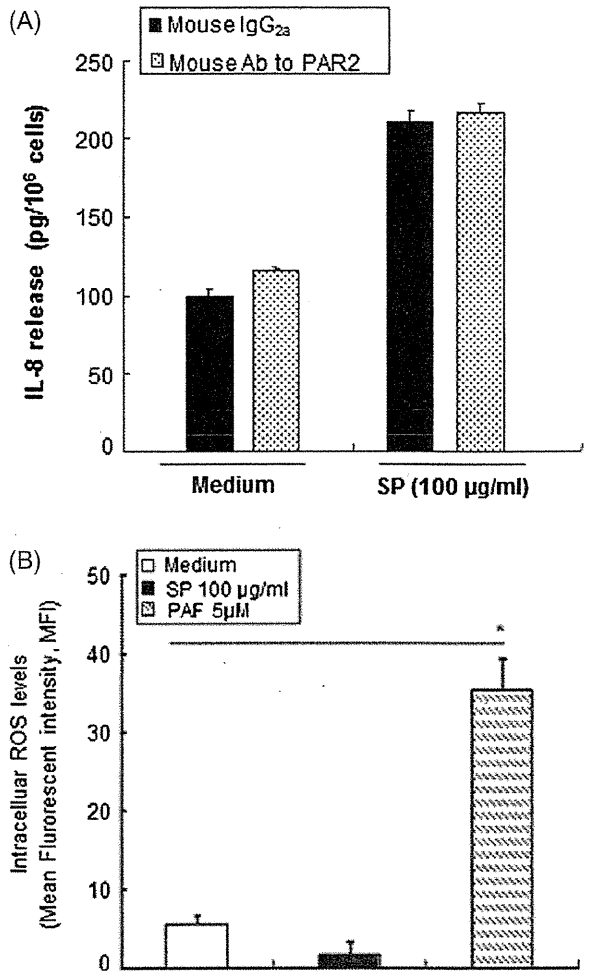

Figure 5. *E. histolytica*-derived secretory product (SP)-induced IL-8 production in HMC-1 cells occurs through a protease-activated receptor 2 (PAR2) or ROS-independent mechanism. (A) The effect of anti-PAR2 on SP-stimulated IL-8 production. ($n = 4$) (B) The effect of SPs on induction of ROS generation at 30 min in HMC-1 cells. ($n = 3$). All reactions were performed in triplicate measurements of each experiment. Data are presented as the mean ± SEM of at least three independent experiments. The asterisks indicate the result of comparisons with the control ($^*p < 0.05$).

inhibit SP-induced IL-8 production in HMC-1 cells. Next, we investigated whether ROS were involved in the SP-induced IL-8 production in HMC-1 cells. As shown in Figure 5B, intracellular ROS were not detected in SP-stimulated HMC-1 cells within 30 min. In contrast, intracellular ROS levels were strongly increased in HMC-1 cells stimulated with PAF as a positive control.

Discussion

In this study, our results show that amoebic CP participates in IL-8 production through a PAR2-independent pathway in HMC-1 cells. *E. histolytica*-derived SPs containing serine or amoebic CPs have been shown to constantly secrete CPs into the extracellular environment [1, 18]. The SPs of the Rahman and ICP1$^{+/+}$ strains resulted in a significant decrease in IL-8 production in HMC-1 cells compared with the SPs of wild-type *Entamoeba,* suggesting that a reduction in CP activity may result in a significant reduction of SP-induced IL-8 production in HMC-1 cells. Our data indicate that amoebic CP might be involved in IL-8 production in HMC-1 cells.

Recent work has shown that *E. histolytica* SPs can markedly increase IL-8 mRNA expression and protein production in colonic epithelial cells [9]. In addition, recent studies have provided evidence that chemokines such as IL-8 are crucial mediators in inflammation and in tissue injury in intestinal inflammation. IL-8 is a small, 8- to 11-kDa secreted protein that may participate in immune and inflammatory responses through chemoattraction and activation of neutrophils or leukocytes [1]. *E. histolytica* invades the intestinal mucosa and causes amoebic colitis and severe ulceration. Analysis of the inflammatory response during intestinal amoebiasis in human and animal models of the disease has revealed an important regulatory role for chemokines and cytokines. Recruitment and activation of inflammatory cells are modulated by secreted amoebic factors. SPs contain many components including cysteine protease (CP), serine protease, other proteases, phosphatases and prostaglandin E2 (PGE2).

In our preliminary experiment, we got IL-8 results of HMC-1 cells stimulated with five various concentrations (0, 10, 30, 100, and 200 μg/mL) of SP. However, no effect of 10 μg/mL of SP on IL-8 production was observed. In addition, the highest concentration of SP (200 μg/mL) showed a cytotoxic effect on HMC-1 cells (about 10% of cells were dead for 12 h), although the amount of IL-8 release in SP (200 μg/mL)-stimulated cells showed a similar level compared with the result of stimulation with 100 μg/mL (data not shown). As a result, we chose 30 and 100 μg/mL concentrations for all experiments.

In the present study, the amount of IL-8 production in HMC-1 cells induced by heat-treated SPs (100 °C for 10 min) abrogated IL-8 production, suggesting that heat-labile proteins participate in IL-8 production in HMC-1 cells. Interestingly, this result is consistent with the fact that the RGD motif in promature CP5 (PCP) binds to integrin of colon cells and induces NF-kB-mediated IL-8 production in Caco-2 cells [15]. In addition, SPs from the Rahman strain resulted in a significant decrease in IL-8 production in HMC-1 cells, whereas IL-8 production by the ICP1$^{+/+}$ strain, which is deficient in CP, was slightly diminished. This result is in agreement with the report that the Rahman strain decreased CP expression. According to a previous report [9], PGE2 participation in SP-induced IL-8 production was demonstrated in colon cells, where boiled amoebic secretory product (100 °C for 30 min) abolished SP-stimulated IL-8 production. However, involvement of the lipid mediator PGE2 in SP-stimulated IL-8 production in HMC-1 cells was not investigated in this study.

There is no information on how SPs can induce mast cell activation such as IL-8 release. G-coupled receptors or Toll-like receptors (TLR) residing on the mast cell surface may act as the biological sensor for various infectious agents during the process of mast cell activation. For example, certain proteases, including serine protease and trypsin, are signaling molecules

that regulate cells by cleaving and triggering PARs [27]. Accordingly, involvement of the PAR2 receptor that is activated by serine protease and occasionally by cysteine protease was tested. In particular, PAR2 is closely related to inflammation [8]. Although SPs contain many kinds of proteases including serine, cysteine and aspartic proteases, SP-induced IL-8 production in mast cells did not occur via the PAR2 receptor. Also, it is well known that TLRs act as biological sensors of various infectious agents (i.e., viruses, bacteria, or fungi) or their products (such as lipopolysaccharide, lipoteichoic acid, and peptidoglycan) and are expressed by various innate immune cells (i.e., macrophages, neutrophils, or dendritic cells) [7, 17]. In addition to recognizing external dangers, TLRs also regulate the immune response by recognizing endogenously produced danger signals including necrotic cells, heat shock proteins, or ECM breakdown products [5]. As such, TLRs may participate in SP-induced mast cell activation; however, more experimentation is needed to investigate TLR involvement in response to SPs.

In conclusion, we have demonstrated that CPs present in SPs contribute to IL-8 production in human mast cells. Additionally, non-pathogenic Rahman and the CP-deficient ICP1$^{+/+}$ strains showed a decrease in IL-8 production, suggesting the involvement of amoebic CP in the host cell response induced by *E. histolytica* infection, helping us to understand the mechanism of pathogenesis in *E. histolytica*.

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MAJOR ARTICLE

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

Koji Watanabe,[1,2] Takahiro Aoki,[1] Naoyoshi Nagata,[3] Junko Tanuma,[1] Yoshimi Kikuchi,[1] Shinichi Oka,[1,2] and Hiroyuki Gatanaga[1,2]

[1]AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan; [2]Center for AIDS Research, Kumamoto University, Kumamoto, Japan and [3]Department of Gastroenterology and Hepatology, National Center for Global Health and Medicine, Tokyo, Japan

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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Correspondence: Dr Hiroyuki Gatanaga, AIDS Clinical Center, National Center for Global Health and Medicine, 1-21-1, Toyama, Shinjuku-ku, Tokyo 162-8655, Japan (higatana@acc.ncgm.go.jp).

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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 $\geq$×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,

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

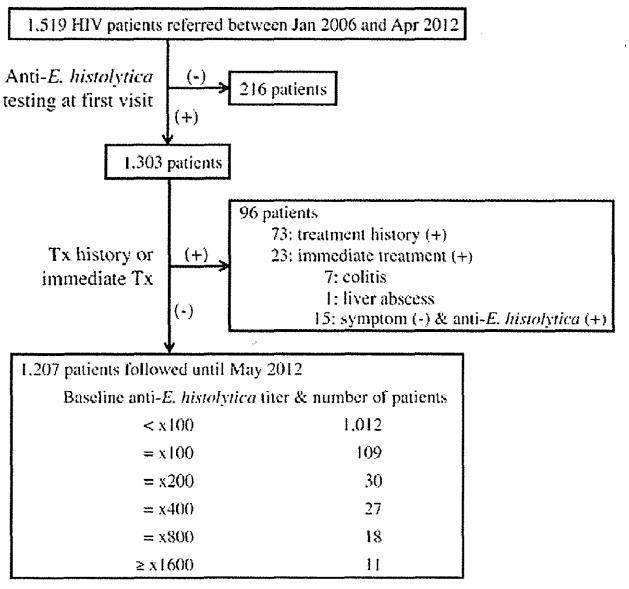

Figure 1. Flow diagram of patient recruitment process. Abbreviations: Anti-*E. histolytica*, anti-*Entamoeba histolytica* antibody; IA, invasive amebiasis; Tx, treatment.

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
HCV Ab-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
HCV Ab-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.

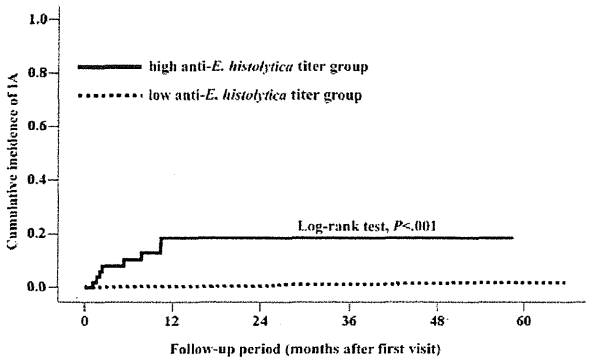

Figure 2. Incidence of invasive amebiasis in low and high anti-*E. histolytica* titer groups. Differences in the time from first visit to the diagnosis of invasive amebiasis (IA) between the low anti-*E. histolytica* titer group (≤×200 at baseline) and high anti-*E. histolytica* titer group (≥×400 at 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.

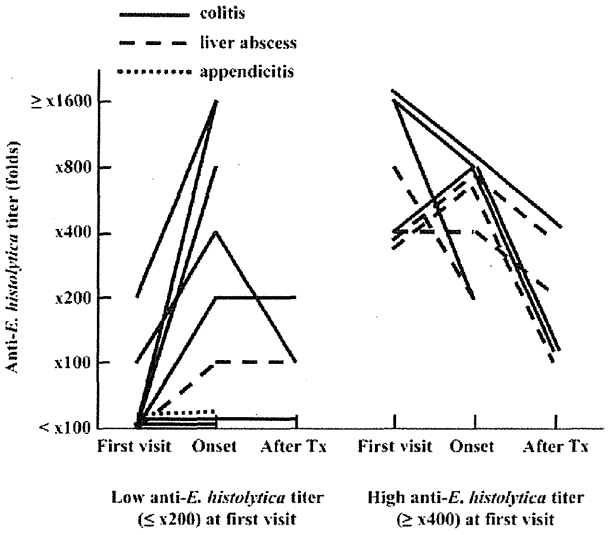

Figure 3. Anti-*E. histolytica* titer before and after diagnosis of invasive amebiasis. Anti-*E. histolytica* titer at the onset of IA was compared to that at baseline (first visit to the clinic) by Wilcoxon signed-rank test. Anti-*E. histolytica* titers after treatment were measured at 219 days [range: 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

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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Species-Specific Immunity Induced by Infection with *Entamoeba histolytica* and *Entamoeba moshkovskii* in Mice

Chikako Shimokawa[1], Richard Culleton[2], Takashi Imai[3], Kazutomo Suzue[3], Makoto Hirai[3], Tomoyo Taniguchi[3], Seiki Kobayashi[4], Hajime Hisaeda[3], Shinjiro Hamano[1,5*]

1 Department of Parasitology, Nagasaki University, Nagasaki, Japan, **2** Malaria Unit, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Nagasaki, Japan, **3** Department of Parasitology, Graduate School of Medicine, Gunma University, Gunma, Japan, **4** Department of Tropical Medicine and Parasitology, Keio University School of Medicine, Shinjuku, Tokyo, Japan, **5** Global COE Program, Nagasaki University, Nagasaki, Japan

Abstract

Entamoeba histolytica, the parasitic amoeba responsible for amoebiasis, causes approximately 100,000 deaths every year. There is currently no vaccine against this parasite. We have previously shown that intracecal inoculation of *E. histolytica* trophozoites leads to chronic and non-healing cecitis in mice. *Entamoeba moshkovskii*, a closely related amoeba, also causes diarrhea and other intestinal disorders in this model. Here, we investigated the effect of infection followed by drug-cure of these species on the induction of immunity against homologous or heterologous species challenge. Mice were infected with *E. histolytica* or *E. moshkovskii* and treated with metronidazole 14 days later. Re-challenge with *E. histolytica* or *E. moshkovskii* was conducted seven or 28 days following confirmation of the clearance of amoebae, and the degree of protection compared to non-exposed control mice was evaluated. We show that primary infection with these amoebae induces a species-specific immune response which protects against challenge with the homologous, but not a heterologous species. These findings pave the way, therefore, for the identification of novel amoebae antigens that may become the targets of vaccines and provide a useful platform to investigate host protective immunity to *Entamoeba* infections.

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* E-mail: shinjiro@nagasaki-u.ac.jp

Introduction

Amoebiasis, an infectious disease caused by the parasitic protozoan *Entamoeba histolytica* is responsible for over 50 million cases in tropical regions and nearly 100,000 deaths worldwide each year. Infection is initiated through the ingestion of cysts in contaminated food or water. *E. histolytica* primarily infects the intestine, and may cause a wide range of symptoms from mild diarrhea to serious dysentery. If untreated, the parasite can cause life-threatening hemorrhagic colitis and/or extra-intestinal abscesses [1-5].

E. histolytica trophozoites are able to colonize the human intestine by adhering to colonic mucins and subsequently to epithelial cells via cell surface lectin [6]. This lectin is important for colonic colonization by *E. histolytica*. A colonization-blocking vaccine targeting this parasite lectin could prevent trophozoite adherence and thus provide protection against subsequent invasive disease [7]. Furthermore, recently, it has also been reported that there is a correlation between the presence of anti-lectin fecal immunoglobulin A (IgA) antibodies and protection from parasitic colonization in humans and mice [7-9]. These reports suggest that amoebiasis can be controlled by acquired immunity.

Entamoeba moshkovskii is closely related to *Entamoeba dispar* and *E. histolytica* and is microscopically indistinguishable from them in its cyst and trophozoite forms [10]. Recently, we reported that *E. moshkovskii* causes diarrhea, colitis and weight loss in mice, and that in

Bangladeshi children, acquisition of *E. moshkovskii* infection was associated with diarrhea [11].

Here, using *E. histolytica* and *E. moshkovskii* infections in mice, we evaluate whether the immunity against reinfection that occurs following a primary infection is species-specific. We find that, following a primary infection with either *E. histolytica* or *E. moshkovskii*, mice are protected from re-challenge with a homologous species, but remained susceptible to a heterologous species. These results show, for the first time, that the immunity acquired during primary infection with *Entamoeba* spp. confers species-specific protective immunity.

Materials and Methods

Mice

Male CBA/J mice were purchased from Jackson Laboratories. Animals were maintained under specific pathogen free conditions at the Animal Research Center for Tropical Infectious Diseases, Nagasaki University, and were challenged when they were 5-8 weeks old. All experiments that involved mice were reviewed and approved by the Committee for Ethics on Animal Experiments of the Graduate School of Nagasaki University, and were conducted under the control of the Guidelines for Animal Experiments in the Graduate School of Medicine, Nagasaki University, and the Law (No. 105) and Notification (No. 6) of the Japanese Government pertaining to the use of experimental animals.

Parasite Culture and Infection

Trophozoites of *E. histolytica*, originally laboratory strain HM1:IMSS (American Type Culture Collection, Manassas, VA), were from Prof. Eric Houpt, University of Virginia, and were serially passaged *in vivo* through the ceca of mice [12]. Trophozoites of the *E. moshkovskii* Laredo strain, were a gift from Dr. Seiki Kobayashi, Keio University, School of Medicine (originally from the late professor Louis S. Diamond, NIH, Bethesda, Maryland). Cecal contents were cultured at 37°C and 25°C, respectively, in BIS-33 medium supplemented with heat-inactivated 10% adult bovine serum, 25U/ml penicillin and 25 mg/ml streptomycin [13]. Trophozoites in the logarithmic growth phase were used in the experiments.

Intracecal inoculation of *Entamoeba* spp

Trophozoites of *E. histolytica* HM1:IMSS and *E. moshkovskii* Laredo strain were collected after incubating the tubes on ice for 5-10 minutes. Then, the number of trophozoites was counted. We anesthetized mice with Domitor (medetomidine hydrochloride: 0.1mg/kg) and Dormicum (midazolam: 0.1 mg/kg), shaved their abdomens to incise the skin, exteriorized each cecum from the peritoneum, and injected 150μl of 1×10^6 trophozoites into the apical sites of cecum. Then, the cecum was blotted and the peritoneum and the skin were sutured. Mice were kept on warming blankets at 37°C throughout surgery. Survival rates were ≥90% in all mice.

Detection of each *Entamoeba* spp. by PCR using DNA extracted from stool of mice

For isolation of *Entamoeba* DNA from mouse stools, QIAamp DNA Stool Kits (QIAGEN, Valencia CA) were used according to manufacturer's instructions. The primer sequences used for PCR are as previously described [14].

Administration of metronidazole

For *in vivo* studies, stock solutions of metronidazole (Sigma Aldrich, St. Louis, MO) were prepared in 100% dimethyl sulfoxide at a concentration of 10 mg/mL and stored at 4°C. The stock solution was diluted 32 times with distilled water to 0.3125 mg/mL, in which the concentration of DMSO was 3.125%. Mice were treated orally with metronidazole at a dose of 12.5mg/kg of body weight. To cure primary infections with *E. histolytica* or with *E. moshkovskii*, all of mice challenged with *E. histolytica* or with *E. moshkovskii* were treated with 1 mL of metronidazole orally (0.3125 mg/mL) using gastric intubation on day 14 post-infection. Naïve mice were also administered with metronidazole and used as control.

Statistical analysis

Differences between groups were analyzed for statistical significance with unpaired Student's *t*-test and χ^2 test. All of these were performed using Excel software. Probabilities below 0.05 were considered statistically significant.

Results

E. *moshkovskii* infections were resolved earlier than *E. histolytica* infections

We have previously demonstrated that C3H/HeN, C3H/HeJ and CBA/J mice allow the establishment of *E. histolytica* and *E. moshkovskii* infections, while many strains of mice including C57BL/6 and BALB/c mice do not, indicating that susceptibility to *E. histolytica* and *E. moshkovskii* infection is dependent on the genetic background of the host [11,12,15-17]. Trophozoites of *E. histolytica* and *E. moshkovskii* were intracecally inoculated into CBA/J mice. As expected, both *E. histolytica* and *E. moshkovskii* succeeded in infecting CBA/J mice after challenge (Figure 1). *E. histolytica* infected the ceca in approximately 80% of CBA/J mice (16 of 20) as confirmed by both culture and PCR of intracecal contents two days after challenge. In contrast, *E. moshkovskii* infected the ceca of CBA/J mice in approximately 65% of mice (13 of 20) at the same point. At day 14 post-challenge, the infection rate of *E. histolytica* was approximately 60% (12 of 20 mice positive), though that of *E. moshkovskii* was approximately 5% of mice (1 of 20). At 21 days post-challenge, the infection rate of *E. histolytica* was approximately 58% (11 of 19) and that of *E. moshkovskii* was 0%.

Metronidazole Sensitivity in E. *moshkovskii*

So as to treat mice infected with *E. histolytica* and *E. moshkovskii*, the effect of metronidazole on the growth and survival of *E. histolytica* and *E. moshkovskii* trophozoites was evaluated *in vitro*. The number of viable cells in glass tubes

Figure. 1

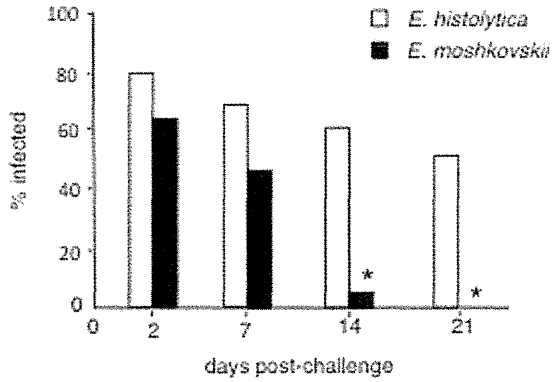

Figure 1. Entamoeba moshkovskii infections were resolved earlier than *Entamoeba histolytica* infections. CBA/J mice were intracaecally infected with 1×10^6 trophozoites of *E. histolytica* and *E. moshkovskii*. Infection rate was monitored by detecting amoebae in caecal content and by amplifying the amoeba gene from faecal DNA on days 2, 7, 14, and 21. Infection rate of mice with *E. histolytica* and *E. moshkovskii* was shown as open and closed columns, respectively. Values show the representative result out of 3 individual experiments. Asterisks indicate statistical significance with $p < 0.05$ between mice infected with *E. histolytica* and *E. moshkovskii* by χ^2 test.

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Figure 2

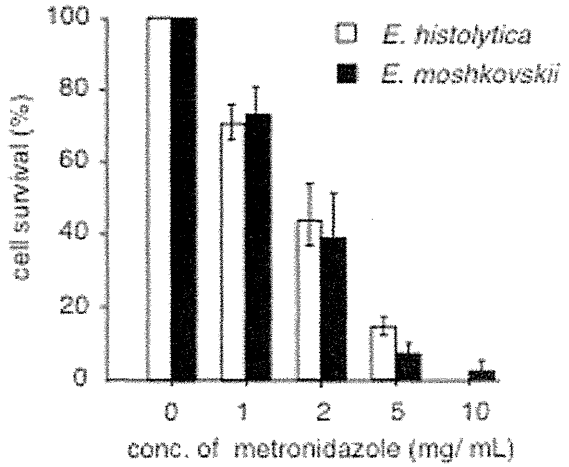

Figure 2. The effect of metronidazole on the growth and survival of *Entamoeba histolytica* and *Entamoeba moshkovskii* trophozoites was evaluated *in vitro*. *E. histolytica* and *E. moshkovskii* were incubated with various concentrations of metronidazole for 48h. Then, the number of viable cells was counted and the proportion of it versus initial number was shown as open and closed columns, respectively.

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was counted after incubation of *E. histolytica* and *E. moshkovskii* with various concentrations of metronidazole for 48h. The numbers of *E. histolytica* and *E. moshkovskii* treated with metronidazole decreased significantly in a dose dependent manner (Figure 2).

Amoebic infection induced species-specific protective immunity

In order to examine whether protection against re-infection can be induced by primary infection, the mice that allowed the establishment of the primary infection with *E. histolytica* or *E.*

Figure 3

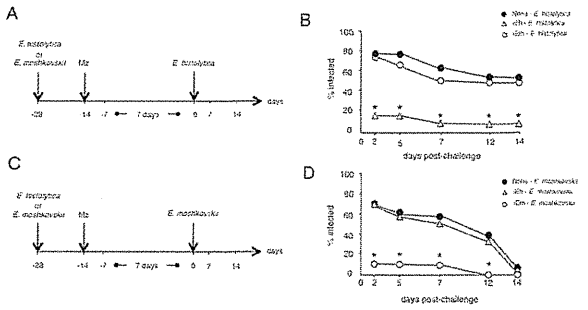

Figure 3. Amoebic infection induced species-specific protective immunity. Mice were infected with 1 × 10^6 trophozoites of *Entamoeba histolytica* (open triangle) or *Entamoeba moshkovskii* (open circle) and treated with metronidazole (Mz) on day 14 following induction of the primary infection. The clearance of amoeba was confirmed seven days after treatment by PCR. Mice were kept without any intervention for an additional week, and then re-challenged with 1 × 10^6 trophozoites of *E. histolytica* (A, B) or *E. moshkovskii* (C, D) at 14 days after treatment. The number of mice used was as follows: for naïve→*E. histolytica*, N=16: for *E. histolytica*→*E. histolytica*, N=20: for *E. moshkovskii* →*E. histolytica*, N=20 (A, B); for naïve→*E. moshkovskii*, N=20: for *E. histolytica*→*E. moshkovskii*, N=20: for *E. moshkovskii* →*E. moshkovskii*, N=20 (C, D). Asterisks indicate statistical significance with $p < 0.05$ by χ^2 test between mice infected with *E. histolytica* and *E. moshkovskii* in the primary infection.

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moshkovskii were treated with metronidazole on day 14 and used for the secondary challenge. The clearance of amoeba was confirmed seven days after treatment by PCR. Mice were kept without any intervention for an additional week, and then re-challenged with *E. histolytica* or *E. moshkovskii* a total of 14 days after treatment (Figure 3A, C). The mice infected with *E. histolytica* and treated with metronidazole showed resistance to homologous re-challenge infection (Figure 3B), but allowed establishment of infection with the heterologous species *E. moshkovskii* in a manner similar to that seen in naive mice (Figure 3D). Similarly, mice infected with *E. moshkovskii* and treated with metronidazole showed resistance to homologous re-challenge infection with *E. moshkovskii* (Figure 3D), but allowed the establishment of infection with the heterologous species *E. histolytica* (Figure 3B). Thus, mice that experienced primary amoebic infection acquired resistance to secondary homologous species infection. However, primary amoebic infection did not confer protection against heterologous species secondary infection. These results show that intestinal amoebic infection induces species-specific protective immunity.

The protection induced by primary infection lasts more than four weeks

To examine how long the protection observed against secondary infection lasts, mice were re-challenged with homologous or heterologous amoebae on day 35 after treatment with metronidazole 14 days after primary infection. The clearance of amoeba was confirmed seven days after treatment by PCR (Figure 4A, C). As shown in Figure 4B and 4D, 35 days after the treatment of the primary infection, mice were resistant to homologous re-challenge, but were susceptible to heterologous species infection. Mice kept for 35 days after the treatment of the primary infection showed increased infection rates compared to those kept just for 14 days, a phenomenon that was most apparent on day 2 post re-challenge in the case of *E. histolytica* and on days 2 and 5 post challenge with *E. moshkovskii* (Figure 4B and 4D). These results suggest that the protection induced by primary infection may include not only memory responses but also remaining primary immune responses, both of which are species specific.

Infection-induced species-specific immunity protects mice from weight loss

During the primary infection, mice infected with *E. moshkovskii* suffered severe symptoms. Following re-challenge with *E. moshkovskii* (homologous species) 14 days after treatment of the primary infection, mice did not show any weight loss (Figure 5A). Slight weight loss was observed, however, in mice re-challenged 35 days after treatment, but the severity of weight loss was much smaller than that observed during the primary infection (Figure 5B). The weight loss was also ameliorated in mice re-infected with *E. histolytica*, when having been given a primary infection with the homologous species (data not shown).

Discussion

Our results clearly demonstrate that exposure to, and subsequent drug clearance of, the parasitic amoebae *E. histolytica* and *E. moshkovskii* invokes a strong immune response that protects mice from subsequent infection with a homologous species. This protection is species specific, affording little to no protection against a heterologous species challenge. The fact that this strong species-specific immunity was shown to last at least 35 days following the treatment of the initial infection suggests that memory responses are involved.