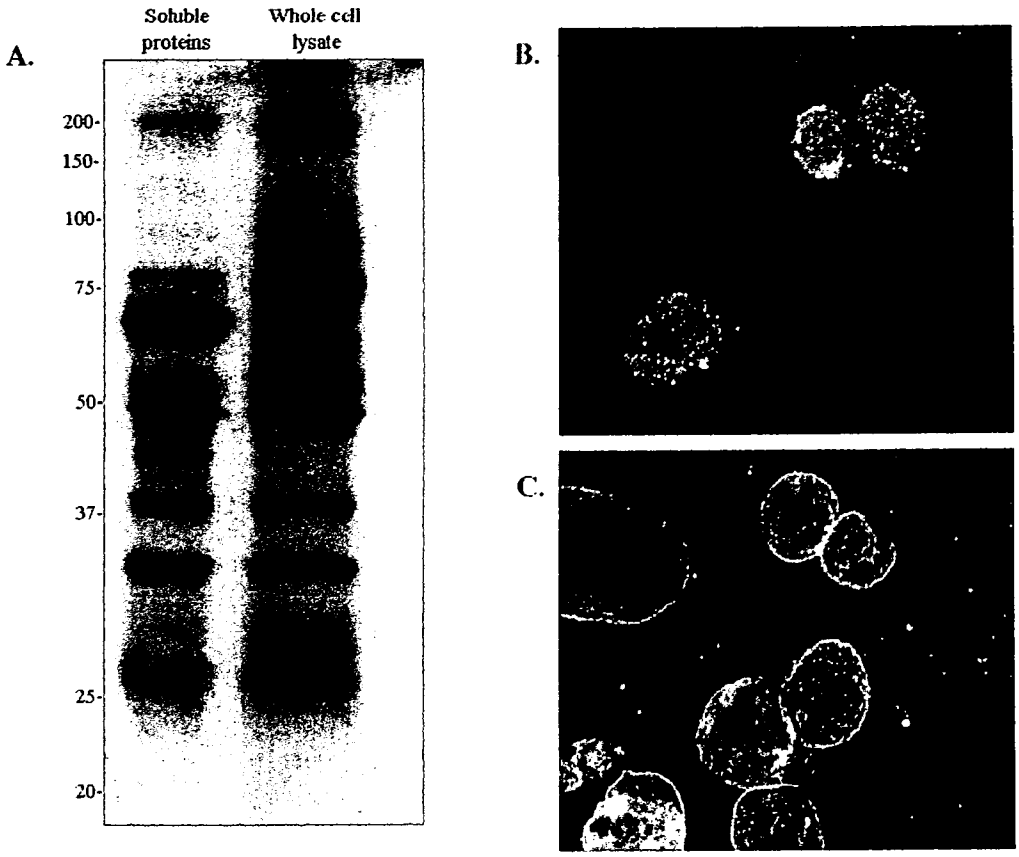

FIG. 5. Recognition of *Entamoeba histolytica* surface proteins by anti-ΔTMK96 rabbit serum. (A) Soluble trophozoite proteins as well as whole-cell lysates were analyzed by Western blots with anti-ΔTMK96 rabbit serum (1:5,000 dilution). Preimmune serum did not recognize any trophozoite proteins on Western blots (data not shown). (B) Confocal microscopy of permeabilized trophozoites with anti-ΔTMK96 rabbit serum. (C) Confocal microscopy of permeabilized trophozoites with anti-Gal/GalNAc lectin antibodies. No staining was seen with preimmune rabbit serum or in nonpermeabilized trophozoites with the anti-ΔTMK96 serum (data not shown). Magnification, ×400.

consistent with nonoverlapping biological functions for individual members of the TMK family.

The existence of multiple different extracellular domains of the TMKs suggests that each interacts with the host environment and signals into the parasite in distinct ways. The extracellular domains of the group B, C, and D TMKs had sequence similarity to Igl of *E. histolytica* (8), laminin LE domains (27, 57, 63), and VSPs of *Giardia lamblia* (1, 38). The sequence similarity is largely limited to a repeated CXXCXXGYY motif. In laminin the LE domains function as mini-globular folds arranged in tandem to form a rod-like structure. If the CXX CXXGYY motifs in the TMKs take on a similar conformation, then these motifs may function to help the extracellular domain of the TMKs project off the surface of the cell in a pilus-like manner. This may make them available for interaction with host cell factors. In each TMK subfamily there was one or more members expressed; however, most family members appeared not to be expressed under the conditions of laboratory culture. By RT-PCR we did observe that the expression of some TMKs varied between growth curves. This indicates that the expression of these genes may be dynamic. Whether the TMKs share with the *Giardia* VSPs the process of antigenic variation under different biological conditions remains to be determined.

The most significant feature of the TMKs is the kinase domain that, with the exception of two TMKs, is distinct from other known kinases. It is not possible, based on sequence analysis, to predict activity, as most have similarity to both the serine/threonine and tyrosine kinases. Interestingly, a closely related kinase, SplA from *D. discoideum*, is a dual-specificity kinase with both tyrosine and serine/threonine kinase activity (40). All of the essential kinase motifs were conserved, suggesting that these are functional kinases. We were not able to demonstrate kinase activity when the kinase domain was expressed in *E. coli*. Since all of the functional residues were conserved, the most likely explanation for this is that the kinase is not functional in *E. coli* or that the kinase domain is not able to phosphorylate the substrates we have used. It has been previously shown that some kinases were not functional when expressed in *E. coli* (18). Additionally, even if the kinase is functional, identification of a substrate is often the rate-limiting step in characterizing a kinase (5, 23). Further experimentation will be necessary to identify the substrate or interacting partners of the TMKs and determine if the TMKs are serine/threonine and/or tyrosine kinases. Phylogenetic and sequence analysis shows that there are six subfamilies of kinases with distinct motifs within the kinase domains. Some families had additional conserved motifs outside of the kinase domain. This

would imply that the subfamilies may represent functionally different families of kinases in sensing (differences in extracellular domains) and signaling (differences in kinase domains).

The focal staining pattern of the TMKs distinctly contrasts with the uniform plasma membrane staining pattern seen with the Gal/GalNAc lectin of *E. histolytica* or VSPs in *G. lamblia*, both of which lack cytoplasmic kinase domains (38, 42, 45). This localization suggests that the TMKs form a focal multimolecular signaling complex in the plasma membrane (31, 33).

In conclusion, the work presented here may begin to explain how *E. histolytica* is able to persist in the host for long periods of time despite immune surveillance, as well as sensing and responding to host stimuli. The large families of TMKs described here could serve in both biological sensing and antigenic variation. The distinct extracellular and kinase domains of the TMKs suggest that each TMK may sense or interact with different host factors and cause a distinct signaling event in response to that environmental cue.

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REVIEW ARTICLE

The Diversity of Clinical Isolates of *Entamoeba histolytica* in Japan

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In Japan, amebiasis is domestically transmitted by two major populations: male homosexuals and mentally handicapped persons, which is remarkably different from most other developed countries where *Entamoeba dispar* infection is predominantly observed. Here we briefly summarize epidemiology of amebiasis in Japan. We also review our current understanding of the diversity of *Entamoeba histolytica* clinical isolates in Japan, based on polymorphic genetic markers, clinical representations, and *in vivo* virulence, using an animal model. © 2005 IMSS. Published by Elsevier Inc.

Key Words: Amebiasis, Epidemiology, Heterogeneity, Sexually transmitted disease, Mentally handicapped persons.

Peculiarities of Amebiasis Endemic in Japan

The most unusual characteristic of amebiasis in Japan is that imported cases comprise only a minor proportion of all cases discovered in the country and cases attributable to domestic transmission dominate (1). We have two major populations infected by amebiasis: male homosexuals and mentally handicapped persons in institutions. We have approximately 500–600 cases of amebiasis reported to the Ministry of Health, Labour and Welfare, including three to four deaths annually. Approximately 90% of the reported cases are male. About 80% of cases have neither a history of traveling in endemic countries nor are they mentally handicapped. Thus, most of the reported cases are likely male homosexuals or bisexuals. Several groups previously reported a very high incidence of amebiasis among male homosexuals based on stool examination and serological tests (2–5). We recently observed sporadic cases of amebiasis transmitted through heterosexual intercourse (1) with an example of female commercial sex workers. Mentally handicapped persons are also severely affected by the disease (6–9). Mass infections of institutionalized mentally handicapped persons were often discovered during an onset of outbreaks as previously reported (6–9).

Recent Survey of Amebiasis in Institutions in Japan

Our recent survey to examine 484 individuals from six institutions [Institute B, C, D (10), E (11), and two other institutes (unpublished)] by a combination of microscopy, antigen capture ELISA, PCR, and serological tests showed that institutions were severely affected by amebiasis. Microscopic demonstration and antigen capture ELISA showed 9.7 and 12.3% overall positive, respectively. Serological tests, e.g., gel diffusion precipitin test and ELISA using whole parasite lysate as antigen showed 4.8 and 31.2% seropositive (unpublished). The positive rates of stool examination and serology varied significantly among institutions, suggesting that the intensity of infection varies among institutions or parasite strains spreading in each institution and vary in virulent competence (see below). Importantly, these cases are often unreported or underreported for several social reasons. In fact, the cases reported to the Ministry of Health, Labour, and Welfare included no cases of mentally handicapped persons. Thus, the number of amebiasis cases in Japan is largely underestimated. A very high incidence of *E. histolytica* infection in male homosexuals and mentally handicapped persons has not been reported in other developed countries (12–15) except for sporadic cases (16,17) and may be unique to Japan.

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Molecular Basis of the Diversity of *E. histolytica* Isolates in Japan

Genetic diversity among approximately 40 clinical strains isolated from either male homosexuals or mentally handicapped persons in Japan was determined by high-resolution genotyping based on nucleotide sequencing of two protein-coding (SREHP and chitinase) and non-coding regions (locus 1–2 and 5–6) (10,11). Remarkable differences existed in the degree of heterogeneity of genotypes between the two groups. The strains isolated from male homosexuals are extremely heterogeneous; all the isolates derived from male homosexuals showed distinct genotypes. In contrast, isotypes from mentally handicapped persons were less heterogeneous. Isolates obtained from an institute showed an identical genotype. Moreover, one genotype was detected at three institutions at different times (see below). Because the intensity of infection is low prior to mass infection, probably due to previous mass treatment, mass infection was likely caused by a single source. One genotype was isolated from three institutions [Institutions B, A (10) and E (11)] located in three prefectures (Kanagawa, Shizuoka, and Yamagata) in 1994, 2001, and 2002, respectively. The emergence of the same genotype coincided with the movement of a single infected individual. This case is a good example to demonstrate that molecular fingerprinting is indeed a very reliable tool to determine a source of infection and a route of transmission. In addition, this case also raised serious concern on the effective treatment of amebiasis.

The genotypes of Japanese isolates were distinct from four representative reference strains used worldwide (HM1:IMSS cl6, SAW755, SAW1627, and SAW1453) (10). In addition, none of 34 isolates from Thailand, Bangladesh, Cambodia, and Indonesia showed genotypes identical to Japanese isolates (11). Thus, the origin of Japanese strains is not understood and should be investigated in future studies.

Diversity of Clinical Manifestations and *In Vivo* Virulence among Japanese Strains

From a clinical point of view, heterogeneity of virulence attributable to genetic polymorphisms of the parasite likely exists. For instance, when we compared parasitological and serological results between Institutions D and E, which showed a similar rate of infection, notable differences in the serological marker for tissue invasion were observed. While parasitological stool examination gave a similar level of positive rate (28–30% positive) in these institutions, the seropositiveness evaluated by gel diffusion precipitin test significantly differed (0 or 16% in Institute D or E, respectively). The mean value of ELISA titer of the infected individuals was also significantly different between the two institutions (optical density at 405 nm of 0.13 or 0.50, respectively). The premise that this is not due to different rates of infection was also supported by the fact that the positive serology rate by ELISA was comparable between the two institutions (54–67%). These data strongly argue for the presence of genetic polymorphisms leading to distinct clinical manifestations.

Experimental animal infection using five isolates categorized into three representative genotypes from mentally handicapped persons also supported this premise. Hamsters were challenged with a direct inoculation of 5×10^4 trophozoites of KU13 (Institution A), KU19 (B), KU26 (C), KU27 (D), and KU33 (E), cultivated monoxenically with *Crithidia fasciculata* (18) to the liver, and abscess formation was evaluated a week later. All strains except for KU27 developed liver abscesses, while KU27 failed to cause abscess even in repeated attempts using a 4-times higher number of amebas (unpublished). These data agreed with the clinical manifestations in the patients infected with these strains and were consistent with the premise that a spectrum of virulence exists among the strains. *In vitro* virulence is conveniently assessed with the parasite's capacity to destroy a monolayer of mammalian cells (19). KU27 was incapable of destroying the monolayer of HeLa and Chinese hamster ovary cells, similar to *E. dispar* trophozoites (unpublished).

These avirulent phenotypes of KU27 are associated with a specific genotype of locus 1–2 type C and SREHP type A, neither of which was found among isolates examined in our laboratory, except two other isolates from the same institution (KU28 and KU29). Whether or not this specific marker is associated with the avirulent phenotype is not known. There is also no causal connection between this particular SREHP type and a lack of virulence. There are no notable differences in clinical manifestations of amebiasis between Japan and other countries. Hepatic, pulmonary, and brain abscesses are seen 5–20% of cases (almost exclusively in male). None of the four genetic markers was found to be associated with a tissue tropism.

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SHORT COMMUNICATION

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Entamoeba histolytica and *Entamoeba dispar* infections in cynomolgus monkeys imported into Japan for research

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Abstract Three hundred and three stool samples of cynomolgus monkeys (*Macaca fascicularis*) imported from China and the Philippines were examined for *Entamoeba histolytica*/*Entamoeba dispar* infections. Microscopy detected *E. histolytica*/*E. dispar* cysts in 41 samples. Positive rates were higher in the monkeys from China (37.5%) than in the monkeys from the Philippines (3.7%). PCR analysis of 25 samples successfully cultured from the cysts demonstrated that 24 were *E. dispar*, one of the samples from China was *E. histolytica*. The one sample was also identified as *E. histolytica* by an antigen detection kit, although the monkey was asymptomatic and serology was negative. To our knowledge, this is the first report of *E. histolytica* isolation from cynomolgus monkeys based on the discrimination between *E. histolytica* and *E. dispar*.

Introduction

Amoebiasis, caused by infection with *Entamoeba histolytica*, is one of the most important parasitic diseases of humans. In addition to symptomatic cases such as hemorrhagic colitis and liver abscesses, asymptomatic infections, in which only cysts are passed in the feces, also exist. Therefore, discrimination between *E. histolytica* and the morphologically indistinguishable but non-pathogenic *E. dispar* is requisite (WHO 1997).

Amoebiasis has also been reported in captive and in wild-trapped non-human primates (Amyx et al. 1978; Beaver et al. 1988). However, recent studies have demonstrated the prevalence of *E. dispar* infections, but not *E. histolytica*, in 17 species of captive non-human primates (Smith and Meerovitch 1985), baboons (Jackson et al. 1990), Japanese macaques (Rivera and Kanbara 1999), seven species of captive Old World *Macaca* monkeys (Tachibana et al. 2001) and chimpanzees (Tachibana et al. 2000). In Japan, cynomolgus monkeys (*Macaca fascicularis*) have been imported for experimental use in medical research. However, the prevalence of *E. histolytica*/*E. dispar* infections, based on discrimination between the two species, is unknown. In the present study, we surveyed imported monkeys for *E. histolytica* and *E. dispar* infections.

Materials and methods

Stool samples were obtained from 215 cynomolgus monkeys from the Philippines by five different shipments and from 88 cynomolgus monkeys from China by four different shipments from 2000 April to 2002 June. Microscopic observation of trichrome-stained stool smears was performed to detect *E. histolytica*/*E. dispar* cysts. Stools with cysts were cultured xenically in Robinson's medium (Robinson 1968). After 3 days of cultivation, trophozoites were collected by centrifugation using a Percoll-gradient, as described by Tachibana et al. (1990). Genomic DNA of the trophozoites was extracted by a single-tube PCR kit (Takara) and then subjected to PCR amplification using primer sets specific for *E. histolytica* (p11 plus p12) and for *E. dispar* (p13 plus p14), as described by Tachibana et al. (1991). An antigen-capture

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ELISA for *E. histolytica* was performed with the *E. histolytica* II kit (TechLab), using cultured trophozoites. Serodiagnosis for *E. histolytica*-infection was performed by an indirect immunofluorescent test, using Amoeba-Spot IF (bioMerieux).

Results

The results of the microscopic and PCR tests are summarized in Table 1. *E. histolytica/E. dispar* cysts were detected in 13.5% (41/303) of the stools. The positive rates varied among the different shipments and countries from 2.3 to 66.7%. The cyst-positive rate in the shipments from China, 37.5%, was much higher than that from the Philippines, 3.7%. When the 41 cyst positive samples were cultured in Robinson's medium, 25 samples were grown successfully. The main cause of the failure of culture in 16 samples was an outgrowth of *Blastocystis hominis* trophozoites. PCR analysis of the 25 samples revealed that 24 were *E. dispar* and one was *E. histolytica*. No mixed infections were found.

The trophozoites judged as *E. histolytica* by PCR showed a positive OD value of 1.78 in antigen detection ELISA, whereas all the other trophozoites, judged as *E dispar*, had negative OD values of less than 0.05. In the serological tests, none of the monkeys was scored as positive to *E. histolytica*, including the monkey with the *E. histolytica*-infection. The *E. histolytica*-positive monkey was judged to be asymptomatic.

Discussion

Recently, in Japan, *E. dispar* infections, but not *E. histolytica*, were reported in 43% of *M. fuscata* (Rivera and Kanbara 1999); in 66% of captive *Macaca* monkeys (Tachibana et al. 2001); and in 56% of chimpanzees (Tachibana et al. 2000). The dominance of *E. dispar* infections observed in the present study accords with these previous reports. However, PCR analysis in this study was done from cultured parasites and not directly from fecal samples. Recently, it has been shown that culture in particular underestimates *E. histolytica* infection (Blessmann et al. 2002). Therefore, we cannot exclude the possibility that the prevalence of *E. histolytica* might be biased in this study. The difference of positive rates between China and the Philippines may depend on the hygienic managements of the monkey colonies or may reflect different positive rates in wild macaques.

In the present study, one isolate was identified as *E. histolytica* based on PCR analysis of the peroxiredoxin gene and antigenicity of the surface lectin (Haque et al. 2000). It might be essential to discriminate the isolate with a closely related parasite, such as *Entamoeba chattoni* or *Entamoeba moshkovskii*. Since the cysts in stool smears had four nuclei, infection with *E. chattoni* was ruled out. In addition, the possibility of *E. moshkovskii* could be ruled out because the PCR analysis did not amplify peroxiredoxin genes of the parasite (Tachibana et al. 1991; Cheng et al. 2004). To date, a limited number of *E. histolytica* isolates from non-human primates has been reported, that is, in one Japanese macaque (Tachibana et al. 1990) and in three species of old world and three species of new world monkeys (Verweij et al. 2003). To our knowledge, this is the first report of the isolation of *E. histolytica* from cynomolgus monkeys based on the discrimination between *E. histolytica* and *E. dispar*.

Although the monkey infected with *E. histolytica* was asymptomatic and did not have a positive serology to *E. histolytica*, asymptomatic cyst passers can become symptomatic under immunosuppressive conditions. Furthermore, the *E. histolytica* cysts in the stool of infected monkeys represent a zoonotic hazard to the caretakers. Tests to differentiate between *E. histolytica* and *E. dispar*, followed by successful treatment to exclude *E. histolytica* from the monkeys, are essential for the safe use of monkeys in experiments.

Table 1 Detection of *E. histolytica/E. dispar* cysts in feces of cynomolgus monkeys and differentiation of both species by PCR analysis of cultured trophozoites

Country	Shipment	Number of monkeys	Number of positives by microscopy (%)		Number of successful cultures	Number of PCR positives	
						E. histolytica	*E. dispar*
Philippines	1	39	2	(5.1)	1	0	1
	2	44	2	(4.5)	1	0	1
	3	44	2	(4.5)	2	0	2
	4	44	1	(2.3)	1	0	1
	5	44	1	(2.3)	1	0	1
Subtotal		215	8	(3.7)	6	0	6
China	1	6	4	(66.7)	3	0	3
	2	50	20	(40.0)	7	0	7
	3	7	2	(28.6)	2	1	1
	4	25	7	(28.0)	7	0	7
Subtotal		88	33	(37.5)	19	1	18
Total		303	41	(13.5)	25	1	24

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ERRATUM

Koichi Koyama

Dendritic cell expansion occurs in mesenteric lymph nodes of B10.BR mice infected with the murine nematode parasite *Trichuris muris*

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Unfortunately, Table 1 was published with errors. The correct Table 1 is given here.

Table 1 Kinetics of $CD11c^+$ $B220^-$ cells in MLNs of *Trichuris muris*-infected B10.BR mice

Days p.i.	Percentage of positive staining cells[a]				Total cells isolated ($\times 10^7$)[b]
	$CD11c^+$ $B220^-$	$CD4^+$	$CD8^+$	$B220^+$	
Uninfected	1.0 ± 0.2[c]	39.2 ± 1.4	23.4 ± 2.5	32.0 ± 2.9	2.70 ± 0.77
14	0.9 ± 0.2	39.1 ± 2.5	21.6 ± 2.0	35.3 ± 4.9	4.05 ± 0.79*
20	2.0 ± 0.2**	30.7 ± 3.0**	18.0 ± 1.3**	44.3 ± 2.3**	6.40 ± 1.18**
25	1.0 ± 0.3	31.8 ± 3.2***	18.6 ± 2.3*	42.9 ± 4.1***	4.55 ± 0.65**
32	0.9 ± 0.1	36.7 ± 3.3	20.1 ± 2.2	38.6 ± 4.7*	4.76 ± 1.34*

[a]MLNCs were prepared from the MLNs of uninfected or *Trichuris muris*-infected B10.BR mice on days 14, 20, 25, and 32 p.i. MLNCs were stained with PE-anti-CD11c and FITC-anti-B220 MoAbs, or PE-anti-CD4 and FITC-anti-CD8 MoAbs. Stained cells were then analyzed using a FACScan
[b]Total numbers of mononuclear cells isolated from the MLNs of uninfected and infected mice
[c]Results represent the mean ± SD for five mice at each time-point and are representative of three independent experiments that gave the similar results
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with uninfected controls

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Entamoeba invadens: cysteine protease inhibitors block excystation and metacystic development

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Abstract

We examined the effects of six cysteine protease inhibitors on the excystation and metacystic development of *Entamoeba invadens*. Excystation, which was assessed by counting the number of metacystic amoebae after the induction of excystation, was inhibited by the cysteine protease inhibitors Z-Phe-Ala-DMK and E-64d in a concentration-dependent manner during incubation compared to the controls. Neither inhibitor had a significant effect on cyst viability; thus, their inhibitory effects were not due to the toxic effect on cysts. Metacystic development, when determined by the number of nuclei in amoeba, was also inhibited by these protease inhibitors, because the percentage of 4-nucleate amoebae was higher than in the controls on Day 3 of incubation. Although other cysteine protease inhibitors, Z-Phe-Phe-DMK, E-64, ALLM, and cathepsin inhibitor III, had a weak or little effect on the excystation, they inhibited cysteine protease activity in the lysates of *E. invadens* cysts. Broad bands with gelatinase activity of metacystic amoebae, as well as cysts and trophozoites, were detected in the gelatin substrate gel electrophores and were inhibited by Z-Phe-Ala-DMK. There was a difference in the protease composition between cysts and trophozoites, and the protease composition of metacystic amoebae changed from cyst-type to trophozoite-type during development. These results strongly suggest that cysteine proteases contribute to the excystation and metacystic development of *E. invadens*, which leads to successful infection.

Index Descriptors and Abbreviations: Entamoeba invadens; Protozoa; Cysteine protease; Excystation; Metacystic development; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

1. Introduction

Cysteine proteases are not only important virulence factors, but also play a role in the growth and differentiation in many protozoan parasites (McKerrow, 1989; Rosenthal, 1999; Sajid and McKerrow, 2002). This is also true of *Entamoeba histolytica*, because there is accumulating evidence for the potential role of cysteine proteases in the pathogenesis of invasive amebiasis as well as in the growth of the parasites (reviewed by Que and Reed, 2000). In *Entamoeba invadens*, which has been used as a model of encystation and excystation of *E. histolytica*, specific cysteine protease inhibitors significantly reduced the efficiency of encystation, although the effect of inhibition was secondary through decreased trophozoite multiplication (Sharma et al., 1996). However, no studies on the role of cysteine proteases in the excystation and metacystic development of *Entamoeba* have so far been reported.

Excystation and metacystic development are necessary for *Entamoeba* infection, and their processes have been described for *E. histolytica* (Cleveland and Sanders, 1930; Dobell, 1928). Since *E. histolytica* does not encyst

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efficiently in axenic culture, *E. invadens*, a reptilian parasite, has been commonly accepted as a model for the study of encystation and excystation (Eichinger, 1997; López-Romero and Villagómez-Castro, 1993). Excystation is the process through which the whole organism escapes from the cyst through a minute perforation in the cyst wall. Metacystic development is the process in which a hatched metacystic amoeba with four nuclei divides to produce eight amoebulae, which grow to become trophozoites (Cleveland and Sanders, 1930; Dobell, 1928; Geiman and Ratcliffe, 1936). The transfer of *E. invadens* cysts in an encystation medium to a growth medium induces in vitro excystation (Garcia-Zapien et al., 1995; Makioka et al., 2002; McConnachie, 1955; Rengpien and Bailey, 1975). In this study, we examined the effect of cysteine protease inhibitors on the excystation and metacystic development of *E. invadens*. Here, we report that cysteine proteases contribute to these processes of *E. invadens*.

2. Materials and methods

Trophozoites of the IP-1 strain of *E. invadens* were cultured in an axenic growth medium, BI-S-33 (Diamond et al., 1978), at 26 °C. To obtain cysts, trophozoites (5×10^5 cells/ml) were transferred to an encystation medium called 47% LG (LG is BI without glucose; Sanchez et al., 1994). After three days of incubation, the percentage of encystation reached 80% on average. The cells were harvested and treated with 0.05% sarkosyl (Sigma Chemical, St. Louis, MO) to destroy the trophozoites (Sanchez et al., 1994). The remaining cysts were washed with phosphate-buffered saline, counted, and then suspended in a growth medium. The viability of the cysts was determined by trypan blue dye exclusion, and the number of nuclei per cyst was determined after staining with modified Kohn's stain (Kumagai et al., 2001). Cyst preparation included 30% dead or denatured cysts and 70% viable cysts, where 4-nucleate cysts are 30% and 1- to 3-nucleate cysts are 70%. For the experiments on the excystation and metacystic development of *E. invadens*, duplicate cultures of 5×10^5 cysts/ml were incubated with inhibitors for three days. Metacystic amoebae were counted in a hemocytometer on Days 1 and 3, and their viability was determined by trypan blue dye exclusion. Viable metacystic amoebae and cysts were clearly distinguished as light yellow and light blue in color, respectively. The former was also identified by positive motility. The cysteine protease inhibitors used in this study, purchased from Calbiochem (San Diego, CA), are listed in Table 1. These inhibitors were previously used in cultures of human fibroblasts infected with *Toxoplasma gondii* to examine their effect on the intracellular development of parasites (Shaw et al., 2002). All of the inhibitors were dissolved in dimethyl sulfoxide (DMSO).

Table 1
Cysteine protease inhibitors used in the present study

Inhibitor	Specificity
Cysteine protease inhibitors	
Z-Phe-Ala-DMK	Cysteine proteases
Z-Phe-Phe-DMK	Cysteine proteases
E-64	Cysteine proteases
E-64d	Cysteine proteases
Calpain inhibitor 2 (ALLM)	Ca^{2+}-dependent cysteine proteases
Cathepsin (cysteine) protease inhibitor	
Cathepsin inhibitor III	Cathepsin proteases

Abbreviations: Z-Phe-Ala-DMK, Z-Phe-Ala-diazomethylketone; Z-Phe-Phe-DMK, Z-Phe-Phe-diazomethylketone; E-64d, (2S, 3S)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethylester; E-64, *N*-[*N*-(L-3*trans*-carboxirane-2-carbonyl)-L-leucyl]-agmatine; calpain inhibitor 2, *N*-acetyl-leu-leu-methioninal (ALLM); and cathepsin inhibitor III, Z-Phe-Gly-NHO-Bz-*p*OMe.

The control cultures received the same volume of DMSO.

Metacystic development was determined by the number of nuclei per amoeba. The cells were harvested on Days 1 and 3 in cultures with or without inhibitors and stained with modified Kohn's stain. The number of nuclei per amoeba was determined by the double-counting of least 100 amoebae.

For the assay of cysteine protease activity, cysts (2×10^7/ml) were harvested, washed, and subjected to three freeze–thaw cycles in a phosphate-buffered saline. After centrifugation, the supernatants were obtained as lysates. Protease activity was quantified by the cleavage of synthetic peptide substrate Z-Arg-Arg-AMC (benzyloxycarbonyl-arginine-arginine-4-amino-7-methyl-coumarin; Sigma) as previously described (Keene et al., 1986), and recorded as the initial velocity of the cleavage of the fluorescent 4-amino-7-methylcoumarin group/5 μl lysate. The lysates were preincubated for 15 min at room temperature with 10 and 50 μM each of the cysteine protease inhibitors as described above.

Protease gel activity was assessed by gelatin substrate gel electrophoresis as previously described (Keene et al., 1986). The cysts and trophozoites were solubilized using a Laemmli sample buffer (Laemmli, 1970) without a reducing agent, and the supernatants after centrifugation were used. To obtain metacystic amoebae with 4-nuclei and 1-nucleus separately, the cysts were transferred to the growth medium with or without 10 μg/ml aphidicolin (Sigma). The cultures with aphidicolin on Day 1 contained a higher percentage of 4-nucleate amoebae, while the cultures without the drug on Day 3 included that of 1-nucleate amoebae (Makioka et al., 2003). Metacystic amoebae in both cultures were lysed by treatment with a small volume of 0.05% sarkosyl, which had no effect on the cysts. The supernatants after centrifugation were then treated with the Laemmli sample buffer. In certain experiments, trophozoites were treated similarly as for metacystic amoebae. SDS–PAGE was conducted in non-

reducing conditions on 10% gels that had been copolymerized with 0.1% gelatin: equivalent to 5×10^4 loaded per lane. After electrophoresis, the gels was washed for 1 h in 2.5% Triton X-100 to remove SDS, rinsed twice in distilled water, and incubated in 100 mM Tris–HCl (pH 7.4) buffer containing 5 mM EDTA and 2 mM DTT with or without 1 mM Z-Phe-Ala-DMK for 12–18 h at 37 °C. After staining with Coomassie blue and several cycles of destaining, the gelatinase activity was detected as clear bands on the Coomassie blue-stained background of the control gels. All of the experiments of this study were performed at least three times and similar results were obtained. Therefore, the data presented in the results are representative.

3. Results

The effect of cysteine protease inhibitors on the number of metacystic amoebae of *E. invadens* after the transfer of cysts to a growth medium is shown in Fig. 1. Among the inhibitors tested, two cysteine protease inhibitors, Z-Phe-Ala-DMK and E-64d, were effective. For this reason, only the results of these inhibitors are shown. The number of metacystic amoebae in cultures with 10 μM Z-Phe-Ala-DMK during incubation was comparable to the controls, whereas it significantly decreased in cultures with 50 and 100 μM Z-Phe-Ala-DMK compared to the controls. Similarly, metacystic amoebae decreased in number during incubation in cultures with more than 50 μM E-64d. The effects of cysteine protease inhibitors on cyst viability are shown in Fig. 2. The number of viable cysts in the control cultures decreased during incubation. It is considered that most immature cysts contained in culture degenerate or die during incubation. The number of viable cysts in cultures containing 50 and 100 μM Z-Phe-Ala-DMK or E-64d during incubation was comparable to or greater than that of the controls, except for 100 μM E-64d on Day 3.

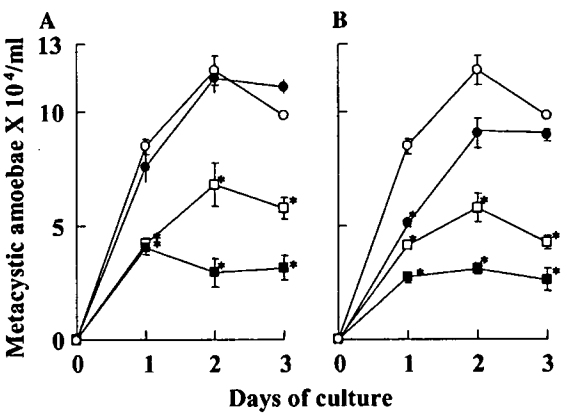

Fig. 1. Effect of cysteine protease inhibitors on the number of metacystic amoebae of *Entamoeba invadens*. Cysts were transferred to a growth medium containing various concentrations of cysteine protease inhibitors Z-Phe-Ala-DMK (A) and E-64d (B). The mean numbers ± SE of metacystic amoebae for the duplicate cultures are plotted (each asterisk indicates $P < 0.05$). Concentrations of 0, 10, 50, and 100 μM are indicated by the white circles, black circles, white squares, and black squares, respectively.

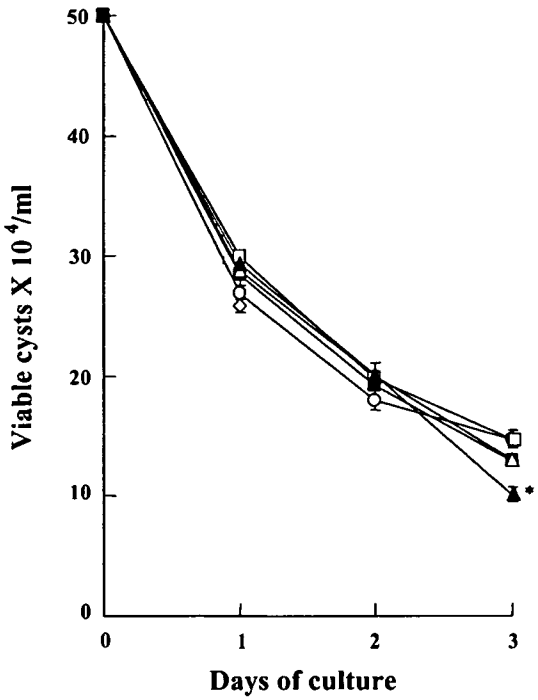

Fig. 2. Effect of cysteine protease inhibitors on the cyst viability of *E. invadens* in the growth medium. The experimental conditions were the same as those for Fig. 1. The mean numbers ± SE of viable cysts for the duplicate cultures are plotted (each asterisk indicates $P < 0.05$). Control (white circles), 50 μM Z-Phe-Ala-DMK (white squares), 100 μM Z-Phe-Ala-DMK (black squares), 50 μM E-64d (white triangles), and 100 μM E-64d (black triangles).

The effects of cysteine protease inhibitors on metacystic development were examined by counting the number of nuclei per cell. As shown in Fig. 3, 9% of the metacystic amoebae were 4-nucleate on Day 1 of incubation in the control cultures, whereas 29 and 34% of the amoebae were in cultures with 100 μM each of Z-Phe-Ala-DMK and E-64d, respectively. The percentage of 4-nucleate amoebae in the control cultures then decreased to 3% on Day 3, following the increased percentages of 1- to 3-nucleate amoebae. In contrast, the percentage of 4-nucleate amoebae in cultures with Z-Phe-Ala-DMK and E-64d was still 19 and 27% on Day 3, respectively, suggesting the inhibition of metacystic development due to these inhibitors.

Since there was a difference in the inhibitory effect on excystation among the cysteine protease inhibitors used, we examined the effects of these inhibitors on cysteine protease activity in cyst lysates. As shown in Fig. 4,

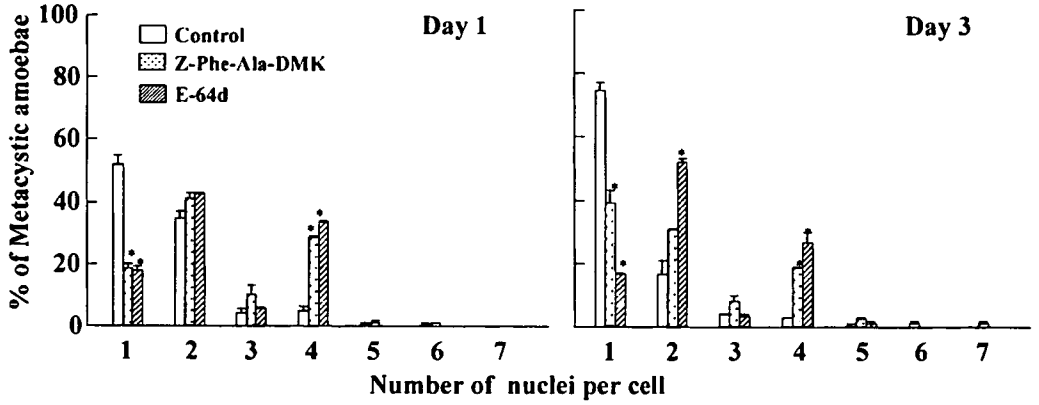

Fig. 3. The effect of cysteine protease inhibitors on the metacystic development of *E. invadens*. The cysts were transferred to a growth medium with or without 100 μM of Z-Phe-Ala-DMK or E-64d. The numbers of nuclei per metacystic amoeba stained with modified Kohn on Days 1 and 3 of incubation were counted, and the percentage of amoebae in each class (1- to 7-nucleate) was determined (each asterisk indicates $P < 0.05$).

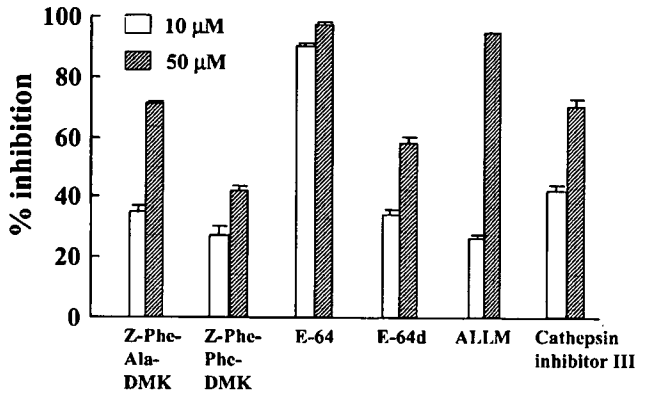

Fig. 4. The effect of cysteine protease inhibitors on cysteine protease activity in the lysates of *E. invadens* cysts. The lysates of *E. invadens* cysts (2×10^7/ml) were incubated with 10 or 50 μM each of the six inhibitors shown in Table 1. The percentages ± SE of inhibition against the control are plotted.

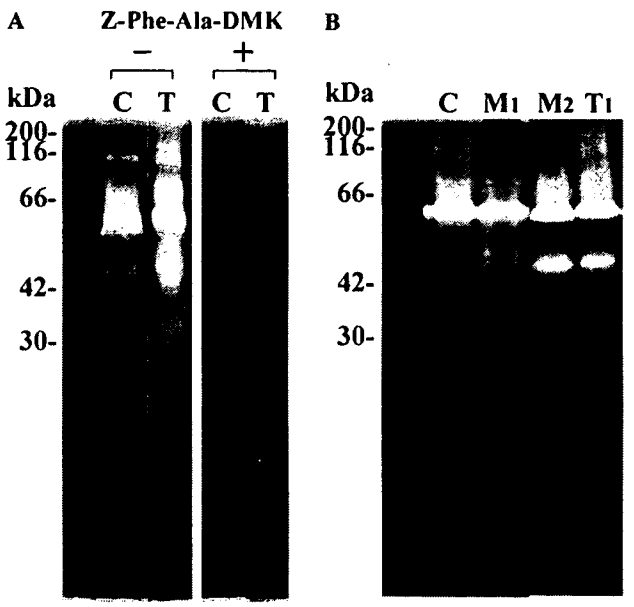

Fig. 5. Gelatin substrate SDS–PAGE of the lysates of *E. invadens* cysts, metacystic amoebae, and trophozoites. (A) Cysts (C); tophozoites (T). Following the removal of SDS, the gels were incubated in buffer alone (–) or with 1 mM Z-Phe-Ala-DMK (+). (B) M1 and M2 were metacystic amoebae from cultures with aphidicolin on Day 1, and those from cultures without the drug on Day 3, respectively. T1 was trophozoites treated similarly as those for metacystic amoebae.

cysteine protease activity in cyst lysates against synthetic peptide substrate Z-Arg-Arg-AMC was inhibited by all of the inhibitors, although there was a difference in their potency.

As shown in Fig. 5A, gelatin substrate SDS–PAGE indicated a major band of 56 kDa, broad bands of 58–66 kDa and 44–54 kDa, and a minor band of 43 kDa in cyst lysates (C). The 56 kDa band and these broad bands detected in cysts were also seen in trophozoite lysates (T). Additional broad bands of 29–41 kDa were also detected in the trophozoite lysates, suggesting a qualitative difference between these two forms. Most of these bands disappeared in the presence of Z-Phe-Ala-DMK. Newly hatched metacystic amoebae with four nuclei (M1) showed a band pattern similar to that of cysts, while more developed metacystic amoebae with one nucleus (M2) showed a band pattern similar to that of trophozoites (T1) (Fig. 5B).

4. Discussion

These results strongly suggest the participation of cysteine proteases in the excystation and metacystic development of *E. invadens*. As cyst viability was not affected by the two cysteine protease inhibitors, Z-Phe-Ala-DMK and E-64d, reduced excystation cannot be due to their toxic effect on cysts. Since other cysteine protease inhibitors used in the present study inhibited cysteine protease activity in cyst lysates, their failure to block

excystation must be due to their lower cell permeability. This is true for E-64, which had a strong inhibitory potency on the cysteine protease activity in cyst lysates. On the other hand, its inhibitory effect on excystation was much lower than E-64d, which is a membrane-permeable synthetic analog of E-64. The process of excystation includes the loosening and separation of amoeba from the cyst wall; the amoeba begins to move about within the cyst. The amoeba then flows back and forth through a small pore in the cyst wall and escapes from the cyst. Thus, cyst wall destruction is necessary for minute perforation of the cyst wall. Since the *Entamoeba* cyst wall contains a mix of protein and chitin (Arroyo-Begovich and Carbez-Trejo, 1982; Frisardi et al., 2000), it is conceivable that both protease and chitinase are essential for cyst wall destruction in the excystation process. The walls of *E. invadens* cysts are electron dense and have a uniform thickness of ~100 nm when observed by electron microscopy (Frisardi et al., 2000). Electron-dense materials were also present in the secretory vesicles and along the plasma membrane. Furthermore, the formation of a crescent-shaped space between the plasma membrane and the cyst wall was observed, and, frequently, some electron-dense bodies projected towards this newly formed space (Chavez-Munguia et al., 2003). Metacysts that endocytose the cyst wall residues were also observed. These observations suggest that secretory vesicles, including proteases and chitinase, are sent in close apposition to the plasma membrane. These enzymes are then secreted into the space between the plasma membrane and the cyst wall to destroy the cyst wall.

The hatched 4-nucleate metacystic amoeba grows rapidly and divides to form eight amoebulae. The results indicate that cysteine proteases are also involved in this metacystic development because the percentage of 4-nucleate amoebae was higher than in the controls on Day 3 of incubation. The results indicate the difference in the band pattern of protease activity between cysts and trophozoites, also changing the band pattern form from cyst-type to trophozoite-type during metacystic development. This is related to our previous results that suggest change in the expression of proteins during metacystic development (Makioka et al., 2003).

Regarding other proteases, we have previously demonstrated that lactacystin, a specific inhibitor of proteasome, had little effect on the excystation and metacystic development of *E. invadens*, suggesting the little contribution of proteasome to these processes (Makioka et al., 2002), although lactacystin inhibited the encystation in vitro of *E. invadens* (Gonzalez et al., 1999; Makioka et al., 2002).

It has recently been demonstrated that *E. histolytica* contains 20 cysteine protease (CP) genes, of which only a small subset is expressed during in vitro cultivation (Bruchhaus et al., 2003). Therefore, it is likely that at least some of these enzymes are required to infect the human host and/or complete the parasite life cycle (Bruchhaus et al., 2003). The gene that encodes CP5 is missing in the closely related but non-pathogenic *E. dispar*, suggesting the potential role of CP5 in the host tissue destruction of *E. histolytica*. Since cyst wall destruction is necessary for excystation by both amoebae, it appears that CP5 is not responsible for cyst wall destruction in either *E. histolytica* or *E. dispar*, or that other CP isoforms are used for it in *E. dispar*. Regarding CP genes in *E. invadens*, it has recently been demonstrated that among the 20 CP genes of *E. histolytica*, 14 homologous genes are found in this parasite (Wang et al., 2003).

Future study will focus on the identification and characterization of CP isoforms responsible for the excystation and metacystic development of *Entamoeba*, which will lead to a more accurate understanding of these processes and also to the identification of targets for vaccination and chemotherapy to inhibit *Entamoeba* infection.

Acknowledgments

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Laboratory and Epidemiology Communications

Genotyping of *Giardia* Isolates from Humans in Japan Using the Small Subunit Ribosomal RNA and Glutamate Dehydrogenase Gene Sequences

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The flagellate *Giardia intestinalis* (syn. *G. lamblia*, *G. duodenalis*) is a well-known intestinal parasite which causes enteric diseases in humans, livestock, and companion animals. Recent molecular studies have shown that *G. intestinalis* is composed of at least seven genetically distinct but morphologically identical assemblages (Assemblages A to G), and that most of these assemblages appear to have different host preferences, e.g., Assemblages C and D are found in dogs, Assemblage E in hoofed livestock, Assemblage F in cats, and Assemblage G in rats (1). Assemblage A, however, consists of isolates that can be classified into two genetic groups (1): genetic group A-I is isolated from a variety of animals including humans, while Assemblage A-II is isolated exclusively from humans. Assemblage B consists of a genetically diverse group of mostly human isolates, but some isolates from animals are included. Thus, the *G. intestinalis* isolates that have the potential for zoonotic transmission seem to be restricted within narrow genetic groups, specifically Assemblages A and B (1).

In Japan, giardiasis has been classified as a category V notifiable infectious disease in the National Epidemiological Surveillance of Infectious Diseases under the Law Concerning the Prevention of Infectious Diseases and Medical Care for Patients of Infections enacted in April of 1999. Although approximately one hundred cases of this infection were reported annually between 2000 and 2004 (http://idsc.nih.go.jp/iasr/virus/virus-e.html), the molecular epidemiology of *Giardia* in Japan remains unclear. To date, only two human isolates have been genotyped as Assemblage B in Japan (2,3). In the present study, we genotyped three isolates of *G. intestinalis* from humans in Japan using both small subunit ribosomal RNA and glutamate dehydrogenase gene sequences.

The three isolates (GH-125, GH-126 and GH-135) examined in the present study were isolated from Japanese individuals: isolates GH-125 and GH-126 were from asymptomatic individuals living in Osaka, and GH-135 came from a diarrheal HIV-positive patient in Tokyo. *Giardia* cysts were purified from each fecal sample by the sucrose centrifugal flotation method (4), and the genomic DNA was extracted and purified following the method reported previously (4,5). *Giardia* diagnostic fragments were amplified by polymerase chain reaction (PCR) with the following primer pairs targeting the different gene loci: RH11 and RH4 for the *Giardia* small subunit ribosomal RNA gene (SSUrDNA) (6) and GDH1 and GDH4 for the *Giardia* glutamate dehydrogenase gene (GDH) (7). PCR amplification of SSUrDNA was performed using LA Taq polymerase with 2X GC buffer I (LA Taq) (TaKaRa Shuzo Co., Ltd., Otsu, Japan), and amplicication of GDH using Ex Taq polymerase with 10X Ex Taq buffer (Ex Taq) (TaKaRa Shuzo) as reported previously (5). Sequencing of the PCR products and phylogenetic analysis were performed following the methods reported previously (2,8). The partial sequences of the SSUrDNA and GDH of each isolate were deposited in the GenBank database under accession numbers AB195219-AB195224.

SSUrDNA and GDH were successfully amplified in all isolates examined in the present study (data not shown). Partial sequences of the SSUrDNA of GH-125 and GH-126 were found to be identical to those of BAH40C11 and BAC2 that are known to belong to Assemblage A. Similarly, GH-135 had a sequence identical to those of BAH-12 and Ad-28 in Assemblage B (Fig. 1A). More precisely, analysis of GDH partial sequences (592 bp) made it possible to distinguish GH-125, which had a sequence identical to those of Ad-2 and Bris-136, from GH-126 by 3 bp differences, even though they were both classified into the anthroponotic genotype Assemblage A-II (Fig. 1B). Again, the GDH partial sequence of GH-135 was almost identical to that of BAH-12 with 2 bp differences and was grouped into zoonotic Assemblage B (Fig. 1B).

Recently, two human isolates of *Giardia*, GH-156 and GH-158, were genotyped as Assemblage B by phylogenetic analysis using GDH partial sequences in Japan (2,3). In addition, three distinct genotypes, pertaining to Assemblages A-I, D and E, have been isolated from a ferret, dogs and calves, respectively (2,5,8). Genotypes of Assemblage A-I are known to have wider range of host species and have the potential to infect humans, while Assemblages D and E are known to be host-specific and non-infective to humans. Based on the results reported in the present experiment together with those reported elsewhere (2,3,8), there are three *Giardia* genotypes present in Japan that are either of zoonotic (Assemblage A-I and Assemblage B) or anthroponotic (Assemblage A-II) potential for human infection. Further genetic analysis of both human and animal isolates of this microbe is needed to gain greater insight into the molecular epidemiology of endemic *G. intestinalis* in Japan.

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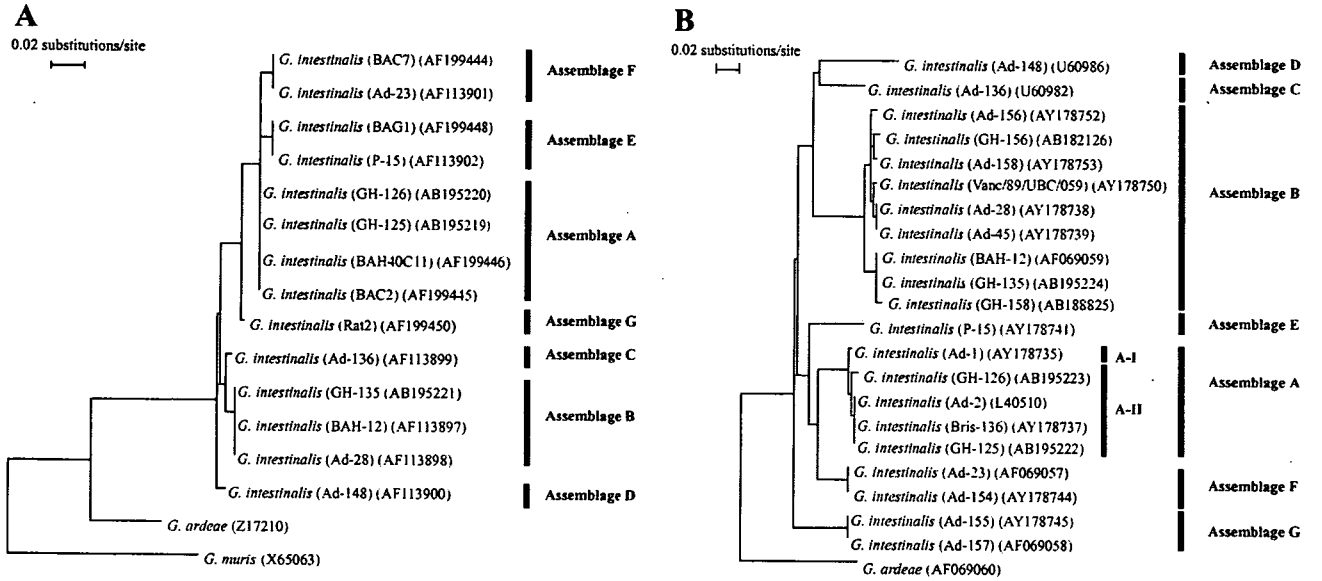

Fig. 1. Phylogenetic relationships of the isolates from humans examined in the present study to other *Giardia* spp. and *G. intestinalis* genotypes as inferred by neighbor-joining analysis, based on the nucleotide sequences of SSUrDNA (A) and GDH (B). Names of the isolates and accession numbers in GenBank are shown in parentheses.

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The flagellate *Giardia intestinalis* (syn. *G. lamblia*, *G. duodenalis*) is a well-known intestinal parasite which causes enteric diseases in humans, livestock, and companion animals. Recent molecular studies have shown that *G. intestinalis* is composed of at least seven genetically distinct but morphologically identical assemblages (Assemblages A to G), and that most of these assemblages appear to have different host preferences, e.g., Assemblages C and D are found in dogs, Assemblage E in hoofed livestock, Assemblage F in cats, and Assemblage G in rats (1). Assemblage A, however, consists of isolates that can be classified into two genetic groups (1): genetic group A-I is isolated from a variety of animals including humans, while Assemblage A-II is isolated exclusively from humans. Assemblage B consists of a genetically diverse group of mostly human isolates, but some isolates from animals are included. Thus, the *G. intestinalis* isolates that have the potential for zoonotic transmission seem to be restricted within narrow genetic groups, specifically Assemblages A and B (1).

In Japan, giardiasis has been classified as a category V notifiable infectious disease in the National Epidemiological Surveillance of Infectious Diseases under the Law Concerning the Prevention of Infectious Diseases and Medical Care for Patients of Infections enacted in April of 1999. Although approximately one hundred cases of this infection were reported annually between 2000 and 2004 (http://idsc.nih.go.jp/iasr/virus/virus-e.html), the molecular epidemiology of *Giardia* in Japan remains unclear. To date, only two human isolates have been genotyped as Assemblage B in Japan (2,3). In the present study, we genotyped three isolates of *G. intestinalis* from humans in Japan using both small subunit ribosomal RNA and glutamate dehydrogenase gene sequences.

The three isolates (GH-125, GH-126 and GH-135) examined in the present study were isolated from Japanese individuals: isolates GH-125 and GH-126 were from asymptomatic individuals living in Osaka, and GH-135 came from a diarrheal HIV-positive patient in Tokyo. *Giardia* cysts were purified from each fecal sample by the sucrose centrifugal flotation method (4), and the genomic DNA was extracted and purified following the method reported previously (4,5). *Giardia* diagnostic fragments were amplified by polymerase chain reaction (PCR) with the following primer pairs targeting the different gene loci: RH11 and RH4 for the *Giardia* small subunit ribosomal RNA gene (SSUrDNA) (6) and GDH1 and GDH4 for the *Giardia* glutamate dehydrogenase gene (GDH) (7). PCR amplification of SSUrDNA was performed using LA Taq polymerase with 2X GC buffer I (LA Taq) (TaKaRa Shuzo Co., Ltd., Otsu, Japan), and amplicication of GDH using Ex Taq polymerase with 10X Ex Taq buffer (Ex Taq) (TaKaRa Shuzo) as reported previously (5). Sequencing of the PCR products and phylogenetic analysis were performed following the methods reported previously (2,8). The partial sequences of the SSUrDNA and GDH of each isolate were deposited in the GenBank database under accession numbers AB195219-AB195224.

SSUrDNA and GDH were successfully amplified in all isolates examined in the present study (data not shown). Partial sequences of the SSUrDNA of GH-125 and GH-126 were found to be identical to those of BAH40C11 and BAC2 that are known to belong to Assemblage A. Similarly, GH-135 had a sequence identical to those of BAH-12 and Ad-28 in Assemblage B (Fig. 1A). More precisely, analysis of GDH partial sequences (592 bp) made it possible to distinguish GH-125, which had a sequence identical to those of Ad-2 and Bris-136, from GH-126 by 3 bp differences, even though they were both classified into the anthroponotic genotype Assemblage A-II (Fig. 1B). Again, the GDH partial sequence of GH-135 was almost identical to that of BAH-12 with 2 bp differences and was grouped into zoonotic Assemblage B (Fig. 1B).

Recently, two human isolates of *Giardia*, GH-156 and GH-158, were genotyped as Assemblage B by phylogenetic analysis using GDH partial sequences in Japan (2,3). In addition, three distinct genotypes, pertaining to Assemblages A-I, D and E, have been isolated from a ferret, dogs and calves, respectively (2,5,8). Genotypes of Assemblage A-I are known to have wider range of host species and have the potential to infect humans, while Assemblages D and E are known to be host-specific and non-infective to humans. Based on the results reported in the present experiment together with those reported elsewhere (2,3,8), there are three *Giardia* genotypes present in Japan that are either of zoonotic (Assemblage A-I and Assemblage B) or anthroponotic (Assemblage A-II) potential for human infection. Further genetic analysis of both human and animal isolates of this microbe is needed to gain greater insight into the molecular epidemiology of endemic *G. intestinalis* in Japan.

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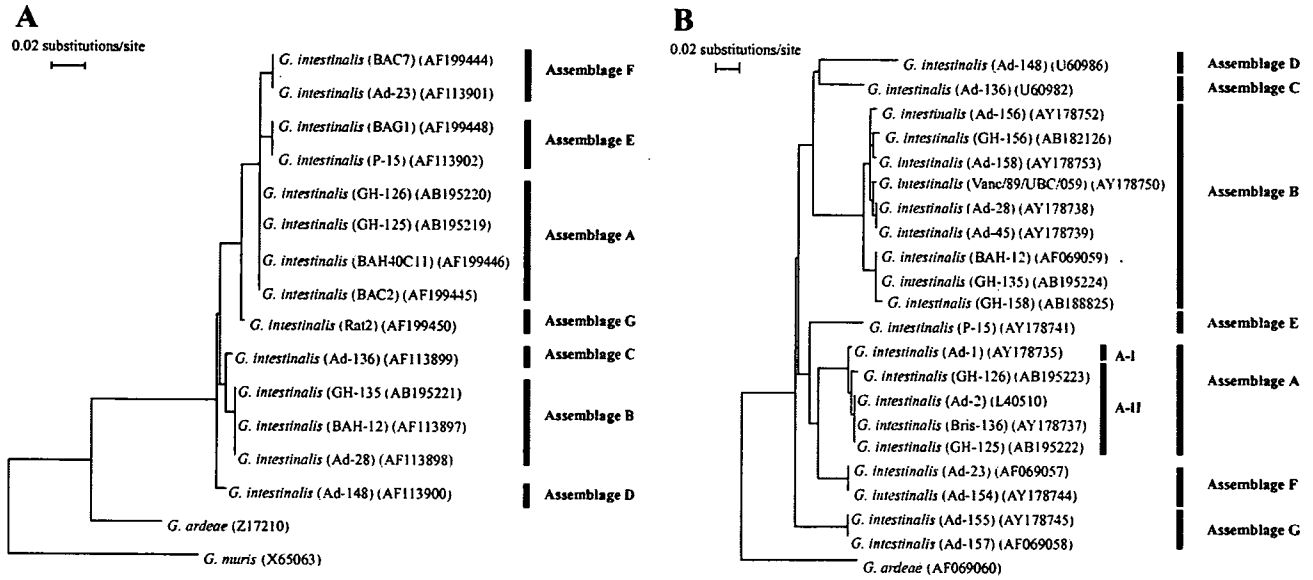

Fig. 1. Phylogenetic relationships of the isolates from humans examined in the present study to other *Giardia* spp. and *G. intestinalis* genotypes as inferred by neighbor-joining analysis, based on the nucleotide sequences of SSUrDNA (A) and GDH (B). Names of the isolates and accession numbers in GenBank are shown in parentheses.

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