to be significant for discrimination of the monkey strain from *E. histolytica*.

Genetic differences between the P19-061405 strain and *E. histolytica* were also demonstrated in related proteins using zymodeme analysis. In this analysis, HXK is a key enzyme for discrimination between *E. histolytica* and *E. dispar* [27]. The primary structures of two proteins, HXK 1 and 2, in the monkey strain are clearly different from those of *E. histolytica* and *E. dispar* [28]. Recently, it has also been demonstrated that zymodemes of *E. histolytica*, Z-II, -IIα-, -XIV, and -XIX, are expressed by six alleles of *GPI* genes [29]. Two *GPI* genes in the monkey strain encoded proteins that differed from three GPI proteins in *E. histolytica*. Indeed, zymodeme analysis of the monkey strain demonstrated unique patterns for HXK and GPI. The position of the slowly migrating band in HXK is explained by the different p*I* of HXK 1 from the monkey strain, compared to that of *E. histolytica* HXK 1. The appearance of a γ band for GPI is also reasonable, since the two GPIs from the monkey strain had identical p*I*s that differed from those of the three *E. histolytica* GPIs. These differences in the monkey strain do not seem to be intraspecific variations of *E. histolytica* isolates.

We have reported that PCR analysis of *PRX* genes is useful for discrimination between *E. histolytica* and *E. dispar*, and we have also detected *E. histolytica* in clinical samples such as abscesses [18,30–32]. Although the PCR analysis was unable to discriminate between the monkey strain and *E. histolytica*, the protocol still seems to be valuable because the monkey strain was virulent. The small sequence differences in *PRX* genes between the monkey and human isolates allow design of primers specific for the monkey strain. Comparison of the sequences of *PRX* genes from *E. histolytica*, *E. dispar*, and *E. moshkovskii* indicate large differences in the N-terminus of the protein: an insertion in *E. dispar* and a deletion in *E. moshkovskii* [33,34]. However, size differences of PRX were not detected between the monkey strain and *E. histolytica*. Therefore, the common primary structure of PRX in these two strains may suggest an important role of the protein in pathogenesis, in addition to a housekeeping role [35,36].

We have previously isolated *E. histolytica* from a Japanese macaque, *Macaca fuscata fuscata* [12]. However, no significant differences at the genetic level were detected between the isolate and the *E. histolytica* reference strain in preliminary experiments (Tachibana et al., unpublished data). Recently, an *E. histolytica*-like strain has also been isolated from a cynomolgus monkey, *Macaca fascicularis*, which was imported into Japan from China for research use [14]. Sequence analysis of the serine-rich protein gene in the strain revealed unique codon usages in comparison with *E. histolytica* human isolates (Takano et al., unpublished data). It has been shown that there are differences in codon usage between *E. histolytica* and *E. dispar* and also between high expression and low expression proteins of *E. histolytica* [37–39]. Since unique codon usage was also observed in the present study, it may be a common feature in monkey strains and strongly indicates that *E. histolytica*-like strains from monkeys are phylogenetically different to human isolates.

The host rhesus monkey that delivered the *Entamoeba* cysts seemed to be asymptomatic, since the stool was not loose or bloody. However, the monkey strain was virulent when trophozoites were inoculated into hamsters. Therefore, we cannot exclude the possibility that the monkey strain is infective to humans and could cause human amebiasis. It would be interesting to investigate whether the monkey strain is dominant in monkey colonies, to determine if an infection with *E. histolytica* identical to human isolates exists in the colony, and to find evidence for human cases of natural infection with the monkey strain. Epidemiological studies are required to answer these questions.

Invasive amebiasis diagnosed by histopathology has been reported in captive and in wild-trapped non-human primates [40–43]. The present study suggests that we cannot exclude the possibility that these cases were due to the P19-061405 type of *Entamoeba*, but misdiagnosed as *E. histolytica*. In older literature, the name *Entamoeba nuttalli* was proposed for a *E. histolytica*-like species found in a liver abscess in a *Macacus pileatus* monkey in Colombo, Ceylon [44]. Furthermore, several *E. histolytica*-like amebas in monkeys have been described as *E. duboscqi*, *E. chattoni*, *E. ateles*, and *E. cynomolgi*. Except for *E. chattoni*, which is single nucleate, these species may really be *E. nuttalli*, which has been thought of as synonymous to *E. histolytica* [45,46].

In conclusion, the *E. histolytica*-like strain isolated from rhesus monkey is virulent, but genetically different from *E. histolytica* human isolates. We propose that the organism be separated from *E. histolytica* by revival of the name *E. nuttalli* Castellani [44].

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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

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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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Comparison of *Entamoeba histolytica* DNA isolated from a cynomolgus monkey with human isolates

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Abstract Three protein-coding loci in DNA of an *Entamoeba histolytica* strain (EHMfas1) isolated from cynomolgus monkey (*Macaca fascicularis*) were sequenced; these loci corresponded to the genes for chitinase, the serine-rich *E. histolytica* protein (SREHP), and the 16 S-like small subunit ribosomal RNA (16S-like SSUrRNA). The nucleotide and deduced amino-acid sequences of chitinase and SREHP were compared with sequences from human isolates. EHMfas1 had several specific mutations in units in the polymorphic regions of the chitinase and SREHP loci, with some repetition of these mutated units. The sequence of the 16S-like SSUrRNA gene (16S-like SSUrDNA) was compared with other *Entamoeba* species. In phylogenetic analysis, EHMfas1 was not categorized in the *E. histolytica* cluster but between *E. histolytica* and *E. dispar*. To our knowledge, this is the first molecular characterization of *E. histolytica* isolated from cynomolgus monkey, and our results indicate that EHMfas1 may be a subspecies of *E. histolytica* that infects cynomolgus monkey.

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Introduction

Amoebiasis is caused by infection with *Entamoeba histolytica* and is one of the most important parasitic diseases in humans. In nonhuman primates, amoebiasis has been reported in captive and in wild-trapped nonhuman primates (Amyx et al. 1978; Beaver et al. 1988), but most of these reports did not distinguish *E. histolytica* from *E. dispar*, which is morphologically similar to *E. histolytica* but nonpathogenic. One case of *E. histolytica* infection in Japanese macaque (*Macaca fuscata*) was determined by isoenzyme analysis (Tachibana et al. 1990), and PCR tests have been used to identity six cases of infections in three species of Old World monkeys and three species of New World monkeys (Verweij et al. 2003) and, in one case, in cynomolgus monkey (*M. fascicularis*; Takano et al. 2005). However, it is unclear whether the *E. histolytica* strains in nonhuman primates originated from humans or are species-specific strains.

The genes for chitinase (de la Vega et al. 1997; Ghosh et al. 2000; Haghighi et al. 2002, 2003) and serine-rich *E. histolytica* protein (SREHP; also known as K2; Li et al. 1992; Clark and Diamond 1993; Kohler and Tannich 1993; Stanley et al. 1990; Ayeh-Kumi et al. 2001; Haghighi et al. 2002, 2003) have highly polymorphic DNA loci, including the protein-coding sequences. These polymorphic loci have been used to study the molecular epidemiology and geographic diversity within human isolates of *E. histolytica*. Genes for the 16 S-like small subunit ribosomal RNA (16S-like SSUrDNA; Medlin et al. 1988) have been used for identification of species (Clark and Diamond 1991; Novati et al. 1996), and phylogenetic analysis of the genus *Entamoeba* has been performed using riboprinting (Clark and Diamond 1997) and nucleotide sequencing (Silberman et al. 1999; Clark et al. 2006).

In this study, we determined the DNA sequences of the chitinase and SREHP loci of the *E. histolytica* strain EHMfas1 and compared these sequences with those from human isolates. We also examined the 16S-like SSUrDNA gene sequence to compare EHMfas1 with other *Entamoeba* species.

Materials and methods

E. histolytica from cynomolgus monkey

The *E. histolytica* strain EHMfas1 was isolated from a healthy cynomolgus monkey imported into Japan from China (Takano et al. 2005).

DNA preparation and sequencing

Total genomic DNA from trophozoites was extracted using a QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's directions. Chitinase and SREHP were amplified by PCR using previously described primer sets: 5′-GGA ACA CCA GGT AAA TGT ATA-3′ and 5′-TCT GTA TTG TGC CCA ATT-3′ for the chitinase forward and reverse primers, respectively, and 5′-GCT AGT CCT GAA AAG CTT GAA GAA GCT G-3′ and 5′-GGA CTT GAT GCA GCA TCA AGG T-3′ for the SREHP forward and reverse primers, respectively (Ghosh et al. 2000). Polymerase chain reaction (PCR) was carried out in a 50-μl reaction mixture containing 2 μl of extracted DNA and 0.1 μg of bovine serum albumin per microliter using Takara Ex *Taq* (Takara). A total of 35 cycles of PCR were performed as follows: denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 45 s. An initial denaturation step at 94°C for 5 min and a final extension step at 72°C for 7 min were also performed.

16S-like SSUrDNA was also amplified using previously described primer sets (RD5 and RD3; Clark and Diamond 1997), with some modification, and newly designed internal primer sets to obtain accurate nucleotide sequencing: RD5, 5′-ATC TGG TTG ATC CTG CCA GT-3′ and 1r, 5′-TCG TCA CTA CCT CTT CAA TTC G-3′; 2f, 5′-CAC TTC TAA GGA AGG CAG CAG-3′ and 2r, 5′-AGC GAT CAT GGA TTT TCA CC-3′; 3f, 5′-AAA AGG AAC AAT TGG GGT GA-3′ and 3r, 5′-CCA TGC ACC ACT ACC CAA TA-3′; 4f, 5′-TGG TCA CAA GGC TGA AAC TT-3′ and 4r, 5′-CCA AGA TGT CTA AGG GCA TCA-3′; 5f, 5′-GGG AAA AAG AAA AAG GAA GCA-3′ and RD3, 5′-ATC CTT CCG CAG GTT CAC CT-3′). A total of 30 cycles of PCR were performed as follows: denaturation at 94°C for 1 min, annealing at 58°C for 30 s, and extension at 72°C for 1 min. An initial denaturation step at 94°C for 5 min and a final extension step at 72°C for 7 min were also performed.

Each PCR product of EHMfas1 was directly sequenced with an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems), according to the manufacturer's directions. Each DNA sequence was analyzed several times using independent DNA preparations.

DNA sequences of chitinase and SREHP were aligned using GENETYX ver. 7.0.11 (Genetyx). The GenBank accession numbers of the sequences used for comparison with the chitinase and SREHP genes are shown in Figs. 1 and 2.

Phylogenetic analysis

Analysis and multiple alignment of DNA sequences of 16S-like SSUrDNA were performed with ClustalX (Thompson et al. 1997), and the phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987). The GenBank accession numbers and length of the sequences used in the phylogenetic analysis were as follows: X56991 (*E. histolytica*: 1,911 bp), X64142 (*E. histolytica* strain HM-1:IMSS: 1,910 bp), X61116 (*E. histolytica* strain SD4: 1,907 bp), Z49256 (*E. dispar*: 1,913 bp), AF149906 (*E. moshkovskii*: 1,911 bp), AF149910 (*E. terrapinae*: 1,910 bp), AF149909 (*E. insolita*: 1,909 bp), AF149907 (*E. hartmanni*: 1,927 bp), D28490 (*E. gingivalis*: 1,910 bp), AF149905 (*E. invadens*: 1,932 bp), AF149908 (*E. ranarum*: 1,901 bp), AF149912 (*E. chattoni*: 1,830 bp), AF149913 (*E. polecki*: 1,825 bp), AF149914 (*E. coli*: 2,068 bp), DQ286371 (*E. equi*: 1,886 bp), DQ286372 (*E. suis*: 1,970), DQ286373 (*E. ecuadoriensis*: 1,908 bp) and AJ566411 (*E. struthionis*: 1,830 bp). Comparisons were made over 1,911 bp of EHMfas1. However, as the submitted *E. suis* sequence lacked some homologous base pairs at the 5′ and 3′ termini, the GenBank sequences were aligned after deleting some terminal base pairs to allow a direct alignment.

Nucleotide sequence accession numbers

The nucleotide sequence data reported here have been submitted to the GenBank/European Molecular Biology Laboratory/DNA Data Bank of Japan database under accession numbers AB282755 (chitinase), AB197935 (SREHP), and AB197936 (16S-like SSUrRNA).

Results

Polymorphism in the chitinase and SREHP loci of EHMfas1

The chitinase and SREHP genes of EHMfas1 were sequenced and compared with human isolates, which were

genotyped and classified by the combination pattern of repeating units in the polymorphic region (Haghighi et al. 2002, 2003; Figs. 1 and 2).

The 5′-conserved region, the repeat-containing polymorphic region, and the 3′-conserved region of the EHMfas1 chitinase gene were compared with those of human isolates. A number was tentatively assigned to each unit in the nucleotide and deduced amino-acid sequences (Fig. 1). In human isolates, the polymorphic region is constructed from combinations of one type of non-repeating unit, three types of repeating units, and two conserved regions (Haghighi et al. 2002). The nucleotide sequence of EHMfas1 consists of two types of non-repeating units (CN1 and CN7), three types of repeating units (CN2, CN3, and CN5), and the 5′- and 3′-conserved regions (CN5′C and CN3′C1; Fig. 1a). The CN1 and CN2 units were common to EHMfas1 and the human isolates, but CN3, CN5, CN7, and CN3′C2 were EHMfas1-specific mutated units; these units corresponded to the CN2, CN4, CN6, and CN3′C1 units, respectively, in human isolates, with a single-nucleotide substitution in each unit (Fig. 1a). In the deduced amino-acid sequence, only the CA3′C2 had a single amino-acid substitution compared to the corresponding human units (Fig. 1b).

The 5′-conserved region, the repeat-containing polymorphic region, and the 3′-conserved region of the EHMfas1 SREHP gene were also compared with those of human isolates. As for chitinase, a number was tentatively assigned to each unit in the nucleotide and deduced amino-acid sequences (Fig. 2). In human isolates, the polymorphic region of the SREHP gene contains combinations of four types of non-repeating units, five types of repeating units, and two conserved regions; some of the human isolates also have strain-specific mutated units (Haghighi et al. 2002, 2003). The nucleotide sequence of EHMfas1 consists of six types of non-repeating units (SN1, SN2, SN5, SN16, SN17, and SN20), four types of repeating units (SN3, SN9, SN10, and SN11), and the 5′- and 3′-conserved regions (SN5′C and

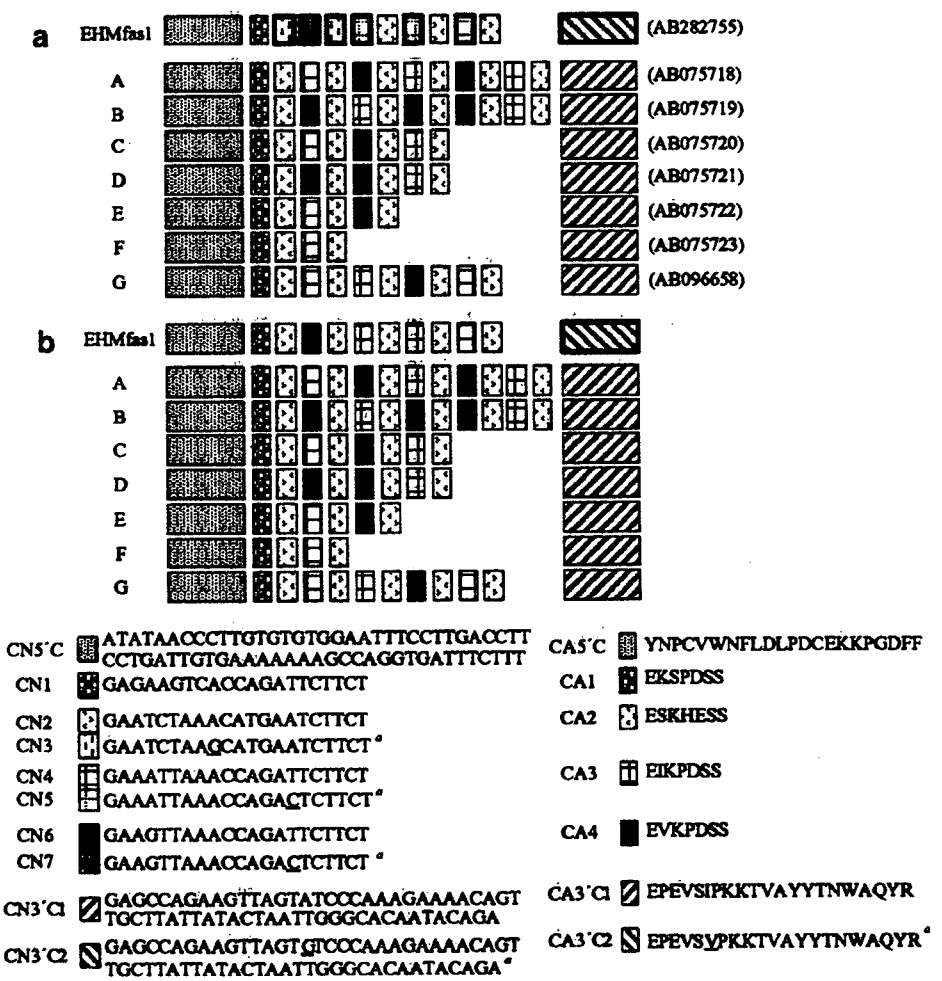

Fig. 1 Schematic representation of polymorphism in the repeat-containing region of the chitinase gene among EHMfas1 and human isolates. Nucleotide sequences pattern (**a**) and deduced amino-acid sequences pattern (**b**) were shown. Each nucleotide sequence of unit was tentatively given a number. Nucleotide and deduced amino-acid sequences of these units are also shown. *Enclosed units with bold line* were EHMfas1-specific units. EHMfas1-specific mutations in nucleotide and deduced amino-acid sequences are *underlined*. *Superscript letter a* EHMfas1-specific unit sequences

SN3′C; Fig. 2a). The SN1, SN3, SN10, SN11, and SN16 units were common to EHMfas1 and the human isolates, whereas SN2, SN5, SN9, SN17, SN20, and SN3′C2 were EHMfas1-specific mutated units; these units corresponded to the SN1, SN4, SN8, SN16, SN19, and SN3′C1 units, respectively, in human isolates, with a single- or double-nucleotide substitution in each unit (Fig. 2a). Furthermore, three EHMfas1-specific mutations were observed as block

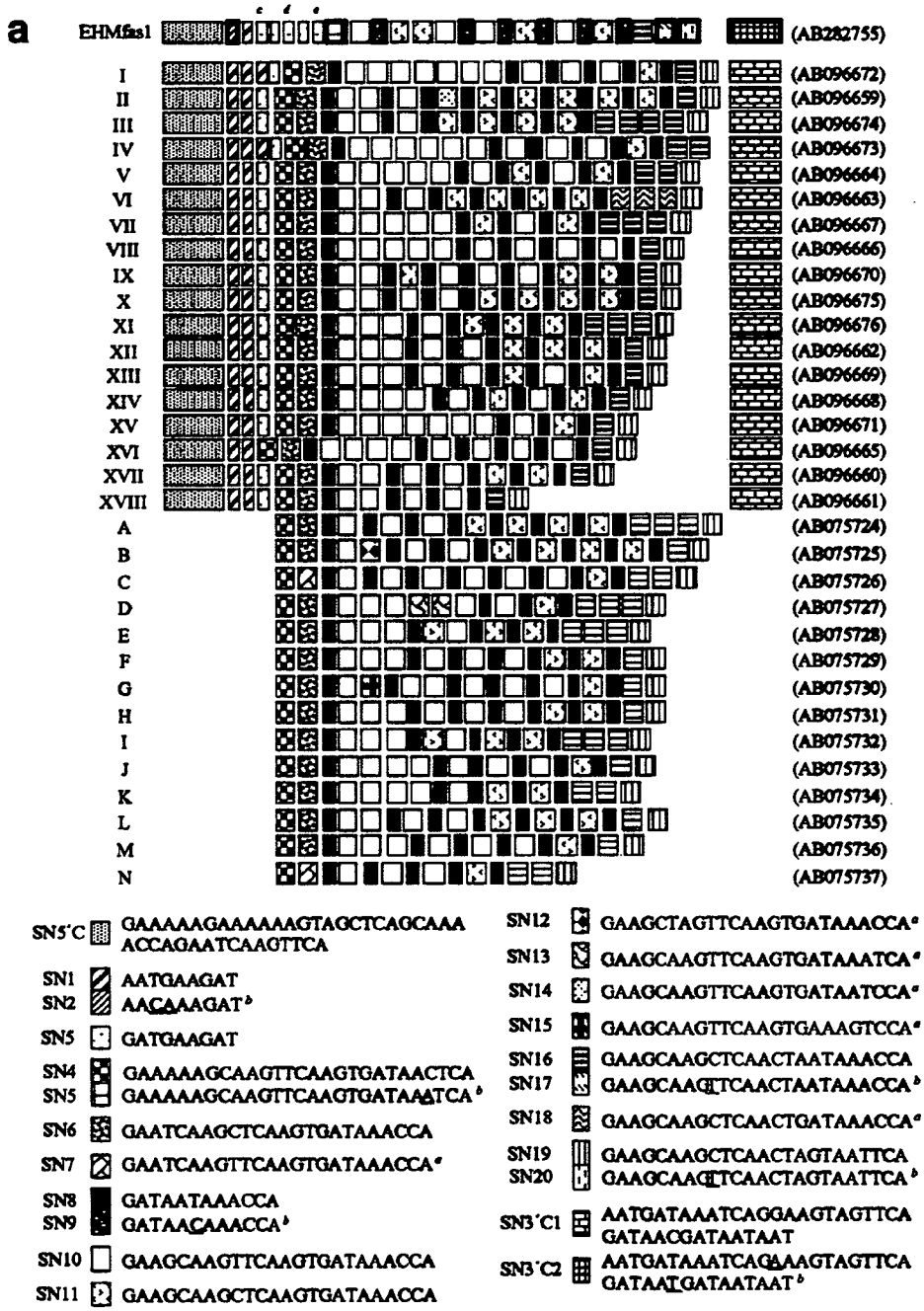

Fig. 2 Schematic representation of polymorphism in the repeat-containing region of the SREHP gene among EHMfas1 and human isolates. Nucleotide sequences pattern (**a**) and deduced amino-acid sequences pattern (**b**) were shown. Each nucleotide and deduced amino-acid sequence of unit was tentatively given a number. Nucleotide and deduced amino-acid sequences of these units are also shown. *Enclosed units with bold line* were EHMfas1-specific units. EHMfas1-specific mutations in nucleotide and deduced amino-acid sequences are *underlined*. *Superscript letter a* minor unit sequences observed as strain-specific unit in some human isolates. *Superscript letter b* EHMfas1-specific unit sequences. EHMfas1-specific block insertions, *superscript letter c* GAGGAA (EE), *superscript letter d* GATGAAGAA (DEE), *superscript letter e* GAGAAT (EN)

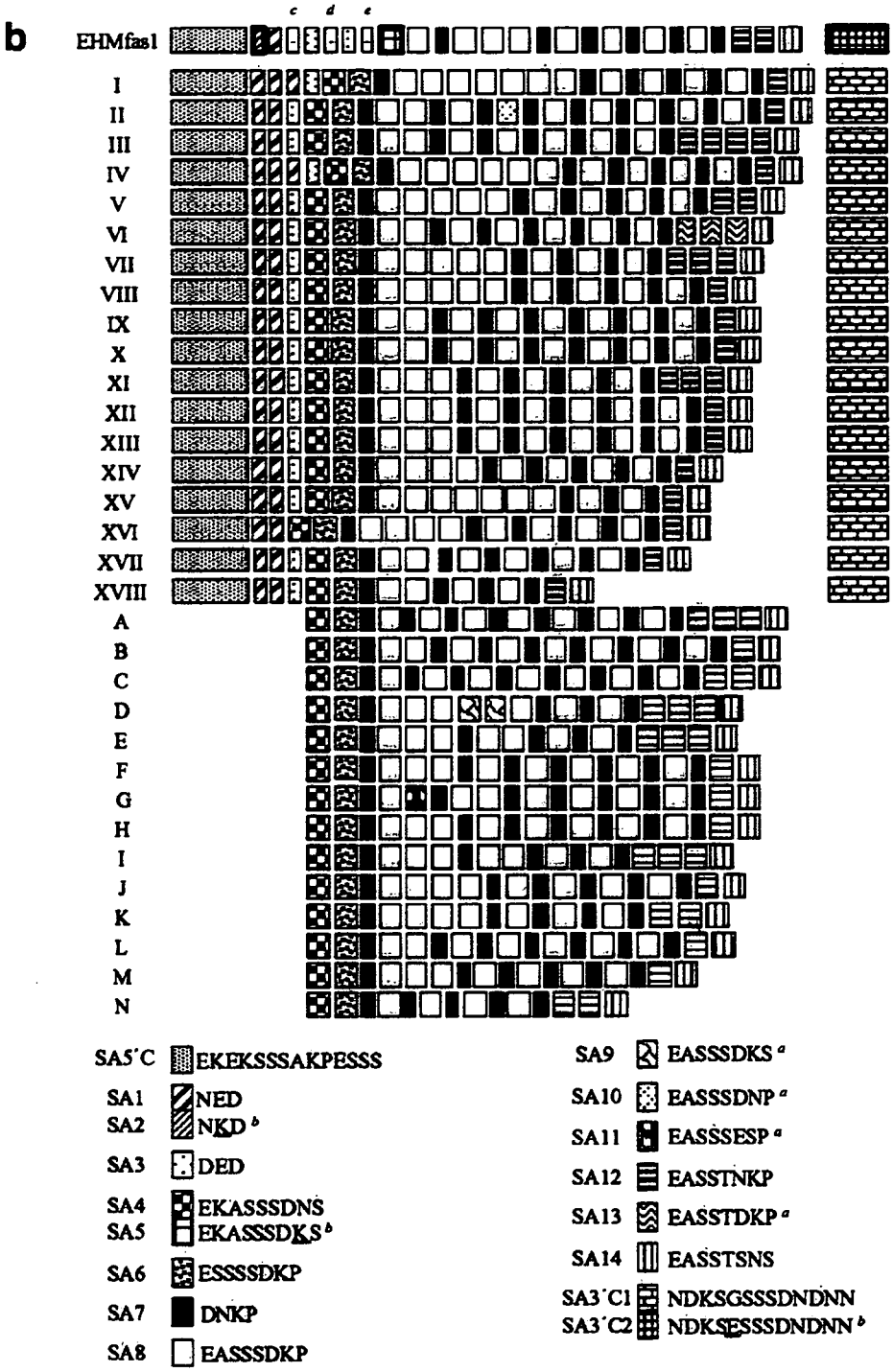

Fig. 2 (continued)

insertions in the repeat-containing region: in the third unit in polymorphic region GAGGAA (EE), the fifth unit in GATGAAGAA (DEE) and the seventh unit in GAGAAT (EN; Fig. 2a and b). In the deduced amino-acid sequence, two types of non-repeating units (SA2 and SA5) and SA3′C2 had a single amino-acid substitution compared to the corresponding human units (Fig. 2b). EHMfas1 lacked the SA6 unit found in human isolates.

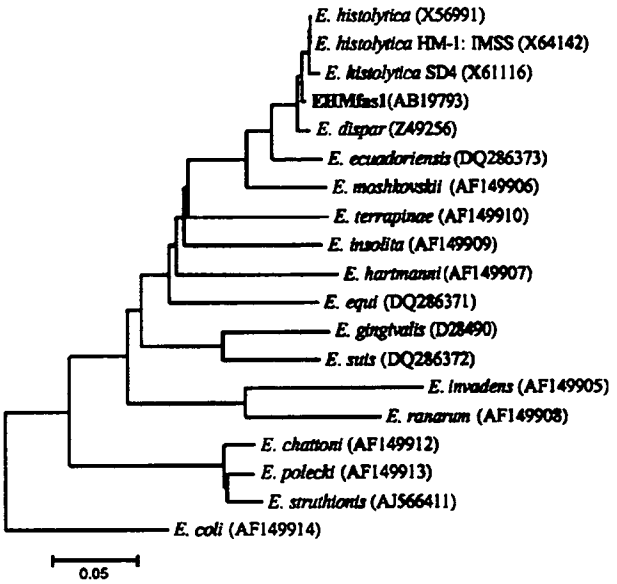

Fig. 3 Phylogenetic analysis of 16S-like SSUrDNA sequences among EHMfas1 and other *Entamoeba* species. Branch lengths are proportional to estimated number of substitutions per site, which represent the evolutional distance

Phylogenetic relationship of EHMfas1 among *Entamoeba* species

The 16S-like SSUrDNA of EHMfas1 was sequenced directly, and the phylogenetic relationship among *Entamoeba* species was reconstructed with inclusion of EHMfas1 (Fig. 3). In this phylogenetic tree, EHMfas1 was not categorized in the *E. histolytica* cluster, but between the *E. histolytica* cluster and the *E. dispar* branch.

Discussion

Three studies of *E. histolytica* infection in nonhuman primates have been performed (Tachibana et al. 1990; Verweij et al. 2003; Takano et al. 2005), using isoenzyme analysis, monoclonal antibody tests, and PCR. However, it is not clear if these infections were natural to the nonhuman primates or zoonotic infections from humans, i.e., whether *E. histolytica* is host-specific or a zoonotic agent. In this study, we sequenced three loci of EHMfas1 isolated from a cynomolgus monkey imported from China into Japan for use in medical research (Takano et al. 2005) and compared these data with human isolates.

The sequence of the chitinase gene was compared with those from human isolates to determine whether EHMfas1 could be categorized into any of the seven genotypes known to be present in humans (Haghighi et al. 2002, 2003). Chitinase genes from human isolates have been genotyped based on the combination pattern of units in the polymorphic region, but our results showed that the nucleotide sequence of the chitinase gene of EHMfas1 and the deduced amino-acid sequence did not fit with any of the known genotypes (Fig. 1a and b). Furthermore, the chitinase gene of EHMfas1 comprised several new units containing nucleotide substitutions from the corresponding units in human isolates; these mutated units suggest that EHMfas1 may be a subspecies of *E. histolytica* rather than a new genotype.

The sequence of the SREHP gene was also compared with those in human isolates to confirm the conclusions drawn from the chitinase sequence, as the SREHP gene is more diverse and shows more variation in the human isolates (Haghighi et al. 2002, 2003). Similarly to the chitinase gene, the SREHP gene of EHMfas1 could not be categorized into any known human genotype at the nucleotide and amino-acid levels (Fig. 2a and b), and this gene also included some new units containing nucleotide substitutions, compared to human sequences, and also included three block insertions and deletion of a common human unit. Therefore, comparison of the SREHP gene in EHMfas1 with those in human isolates also suggests that EHMfas1 is a subspecies of *E. histolytica*.

Some strain-specific mutated units have been observed in the SREHP gene in human isolates (SN7 for type C and type N, SN12 for type B, SN13 for type D, SN14 for type II, SN15 for type G, and SN18 for type VI); these strain-specific units were either non-repeated (SN7, SN12, SN14, SN15) or tandemly repeated (SN13 and SN18). From an evolutionary perspective, it is thought that non-repeated mutative units were derived from a common unit quite recently due to nucleotide substitutions and that tandemly repeated units were replicated after nucleotide substitution, as a result of geographical differentiation of strains. In contrast to the strain-specific units in human isolates, SN9 in EHMfas1 was dispersely repeated six times. This result indicates that SN9 divided from SN8 or from a common ancestral unit before unit amplification; in other words, EHMfas1 might have divided from human isolates before the effect of geographical differentiation in human isolates. In the same way, the CN3 and CN5 units in the chitinase gene, which were EHMfas1-specific mutated units, were dispersely repeated two and three times, respectively. Furthermore, insertions of three EHMfas1-specific blocks and deletion of the common unit SA6 support the hypothesis that EHMfas1 is a cynomolgus monkey-specific strain of *E. histolytica*. The results also suggest that EHMfas1 is a subspecies of *E. histolytica*, although it is possible that it is a new species. At present, the chitinase and SREHP genes have been sequenced from only a small number of isolates and are highly polymorphic between the various isolates. However, we believe the differences between codon usage in these two genes are useful to

distinguish EHMfas1, which was detected as *E. histolytica*, using species-specific PCR and antigen-capture enzyme-linked immunosorbent assay (Takano et al. 2005), together with 16S-like SSUrDNA analysis.

The 16S-like SSUrDNA was sequenced to investigate the relationship between EHMfas1 and the *Entamoeba* species, because 16S-like SSUrDNA have been used for previous phylogenetic analysis of *Entamoeba* (Clark and Diamond 1997; Silberman et al. 1999; Clark et al. 2006). Analysis of 16S-like SSUrDNA also suggested that EHMfas1 is a subspecies or a closely related new species of *E. histolytica*, as in the phylogenetic tree, EHMfas1 was categorized as intermediate between the *E. histolytica* cluster and the *E. dispar* branch (Fig. 3). In addition, isoenzyme analysis of EHMfas1 was also performed to compare EHMfas1 with human isolates. The zymodeme pattern of EHMfas1 (i.e., PGM: *E. dispar* type, ME: *E. histolytica*/*E. dispar* type, GPI: gamma band only, and HK: new type in which the faster running band indicated the *E. histolytica* type, whereas the slower running band was intermediate between the *E. histolytica* and *E. dispar* types) was not classified into any known patterns. Isoenzyme analysis also supports our hypothesis that EHMfas1 is a subspecies or a closely related new species of *E. histolytica* and *E. dispar*.

In the current paper, we have described one amoeba strain isolated from a cynomolgus monkey. *E. histolytica* infection is unusual in nonhuman primates (Smith and Meerovitch 1985; Jackson et al. 1990; Rivera and Kanbara 1999; Tachibana et al. 2000, 2001; Takano et al. 2005), and only a small number of isolates have been studied. Therefore, it will be necessary to examine more *E. histolytica* strains isolated from nonhuman primates to determine the true relationships among nonhuman primates and human isolates.

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Primary structure, expression and localization of two intermediate subunit lectins of *Entamoeba dispar* that contain multiple CXXC motifs

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SUMMARY

We have recently identified 2 surface proteins in *Entamoeba histolytica* as intermediate subunits of galactose- and *N*-acetyl-D-galactosamine-inhibitable lectin (EhIgl1 and EhIgl2); these proteins both contain multiple CXXC motifs. Here, we report the molecular characterization of the corresponding proteins in *Entamoeba dispar*, which is neither pathogenic nor invasive. Two *Igl* genes encoding 1110 and 1106 amino acids (EdIgl1 and EdIgl2) were cloned from 2 strains of *E. dispar*. The amino acid sequence identities were 79% between EdIgl1 and EdIgl2, 75–76% between EdIgl1 and EhIgl1, and 73–74% between EdIgl2 and EhIgl2. However, all the CXXC motifs were conserved in the EdIgl proteins, suggesting that the fold conferred by this motif is important for function. Comparison of the expression level of the *Igl* genes by real-time RT-PCR showed 3–5 times higher expression of *EdIgl1* compared to *EdIgl2*. Most EdIgl1 and EdIgl2 proteins were co-localized on the surface and in the cytoplasm of trophozoites, based on confocal microscopy. However, a different localization of EdIgl1 and EdIgl2 in intracellular vacuoles and a different level of phenotypic expression of the two Igls were also observed. These results demonstrate that Igls are important proteins even in non-pathogenic amoeba and that Igl1 and Igl2 may possess different functions.

Key words: *Entamoeba dispar*, *Entamoeba histolytica*, intermediate subunit of Gal/GalNAc lectin (Igl), cysteine-rich protein.

INTRODUCTION

It has been estimated that 480 million people worldwide are infected with *Entamoeba histolytica* or *Entamoeba dispar* (Walsh, 1986). *E. histolytica* is the causative agent of human amoebic colitis and liver abscess, which result in up to 110 000 deaths annually. *E. dispar* is morphologically indistinguishable from *E. histolytica*, but is non-pathogenic and non-virulent (Diamond and Clark, 1993). Adherence of *E. histolytica* trophozoites to host cells is an essential step in its pathogenicity, and it is well known that the 170 kDa heavy subunit of galactose- and *N*-acetyl-D-galactosamine (Gal/GalNAc)-inhibitable lectin (Hgl) is the key factor in adherence and subsequent pathogenesis of the amoeba (Petri *et al.* 2002). Hgl is a transmembrane protein that forms a heterodimer with a glycosylphosphatidylinositol (GPI)-anchored 35 kDa light subunit (Lgl) via disulfide bonds (Petri *et al.* 1989). Recently, we have identified a GPI-anchored 150 kDa intermediate subunit (Igl) of lectin, which is non-covalently associated with Hgl (Cheng *et al.* 1998, 2001). There are 2 isoforms of Igl, which consist of 1101 and 1105 amino acids and are referred to as Igl1 and Igl2, respectively; both are cysteine-rich proteins containing multiple CXXC motifs. A mouse monoclonal antibody (mAb) to Igl significantly inhibits adherence and cytotoxicity of trophozoites to mammalian cells *in vitro* and also inhibits liver abscess formation in hamsters (Tachibana *et al.* 1997; Cheng *et al.* 1997, 1999). Antibodies to Igl have been detected not only in symptomatic patients with amoebiasis but also in asymptomatic cyst passers of *E. histolytica* (Tachibana *et al.* 2004). Immunization of hamsters with affinity-purified Igl can prevent amoebic liver abscess formation (Cheng and Tachibana, 2001), and Igl has also been detected in the *E. histolytica* fraction that interacts with the brush border of enterocytes (Seigneur *et al.* 2005). Therefore, Igl seems to be one of the key molecules in amoebic adherence to host cells and pathogenicity; however, the correlation of these effects with

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each Igl isoform and the differences between the isoforms are not known.

Comparison of *E. histolytica* and *E. dispar* is also of importance for understanding the pathogenicity of amoeba. One well-known difference between the two species is associated with the family of cysteine proteases that are pathogenic factors in *E. histolytica*; in *E. dispar*, the *EhCP1* gene is absent and *EhCP5* is degenerate (Bruchhaus *et al.* 1996; Willhoeft *et al.* 1999). Concerning lectins, it has been reported that Hgl is present in *E. dispar*, but that its expression level is lower than that in *E. histolytica* (Pillai *et al.* 1997, 2001). Therefore, it is of interest to determine if Igl1 and Igl2 are expressed in *E. dispar*. We report here the primary structure of the 2 Igl isoforms in 2 *E. dispar* strains isolated from human and cynomolgus monkey, respectively. We also compared the expression levels of *Igl* genes between *E. dispar* and *E. histolytica*, and examined the expression and localization of Igl1 and Igl2 in *E. dispar*.

MATERIALS AND METHODS

Cultivation of parasites

Trophozoites of the *E. dispar* SAW1734RclAR strain were grown axenically or monoxenically with sterilized *Crithidia fasciculata* in YIGADHA-S medium supplemented with 15% adult bovine serum at 37 °C (Kobayashi *et al.* 2005; Khalifa *et al.* 2006). Trophozoites of the *E. dispar* CYNO9:TPC strain were axenically cultured in the YIGADHA-S medium. Trophozoites of *E. histolytica* HM-1:IMSS were axenically cultured in TYI-S-33 medium supplemented with 15% adult bovine serum at 37 °C (Diamond *et al.* 1978). Cultured trophozoites were harvested in the logarithmic phase of growth and used in subsequent experiments.

Construction of a cDNA library and cloning of the Igl *gene*

Poly(A) RNA of *E. dispar* SAW1734RclAR trophozoites was isolated using a QuickPrep mRNA purification kit (Amersham Pharmacia). A cDNA library was constructed from 5 μg of poly(A) RNA using a cDNA synthesis kit (Amersham Pharmacia) and a λgt11 vector kit (Stratagene). The library was screened with a 657 bp probe using the Gene Images AlkPhos Direct labelling and detection system (Amersham Pharmacia). The probe was prepared from plasmid DNA containing the *E. histolytica Igl1* gene by PCR amplification using primers EhIgl1-S877 (5′-CCC TCG AGT CAA ATG GTG AAT GTA AGC C-3′) and EhIgl1-AS1088 (5′-CCC TCG AGT TAA ATG CCT TTA GCT CCA TT-3′) (Tachibana *et al.* 2004). The positive clone containing the longest insert was subcloned into a pUC19 vector and sequenced. To extend the sequence of the 5′ end, rapid amplification of the cDNA end was performed with a 5′-Full RACE Core Set (Takara). For the cloning of the other *Igl* gene, the cDNA library was subjected to PCR using primers 5′-CAA TTT CAC TTG GTG AGT ACA AAG CTG-3′ (forward) and 5′-GAA AAT TCC TTT ACT TCC ATT GCA GTT TCC-3′ (reverse). These primers were prepared based on the sequence of the first cloned *E. dispar Igl* gene, with reference to the location of common sequences between the two *E. histolytica Igl* genes. The amplified genes were cloned using a TOPO TA Cloning Kit (Invitrogen) and sequenced. To extend the sequence of the 5′ and 3′ ends of the cloned DNA, a 5′-Full RACE Core Set and 3′-Full RACE Core Set were used (Takara). For the cloning of *Igl* genes from the CYNO9:TPC strain, genomic DNA isolated as previously described (Tachibana *et al.* 1991) was used as a template for PCR, using the forward primers 5′-ATG TTT ATT ATT CTT TTA TTC ATA TCA ATT TCA C-3′ (*Igl1*) and 5′-ATG TTT ATT CTT CTT TTA TTT ATA TCA ATT TCA C-3′ (*Igl2*), and the reverse primer 5′-TTA GAA CAT AAA TGA TAA CAT GAC TAT CAC CAT C-3′. Thirty-five cycles of PCR using *Pyrobest* DNA polymerase (Takara) were performed as follows: denaturation at 94 °C for 15 s (195 s in cycle 1), annealing at 58 °C for 30 s, and polymerization at 72 °C for 180 s (600 s in cycle 35). Amplified DNA was cloned using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen) and sequenced. Nucleotide sequence data were analysed using Genetyx-Mac ver. 11.

Southern blot analysis

Genomic DNA was isolated from *E. dispar* SAW-1734RclAR trophozoites as described previously (Tachibana *et al.* 1991). Three μg of genomic DNA was digested with restriction enzymes *Dra*I, *Taq*I and *Hin*dIII. The fragments were separated on a 1% agarose gel, transferred to a Hybond N^+ membrane (Amersham Pharmacia) by capillary action, and fixed by alkaline denaturation. The membrane was hybridized at 55 °C in buffer containing a Gene Images AlkPhos Direct-labelled probe (Amersham Pharmacia) prepared by PCR amplification of cloned cDNA. The primers used for amplification were 5′-AGA TGG ATT CTA TTT TGA TGA-3′ (forward) and 5′-CAT ATG TCT TGA ACA TGG-3′ (reverse). The blots were detected using a CDP-star detection reagent (Amersham Pharmacia) and exposed to autoradiography films.

Real-time RT-PCR analysis

Total RNAs of *E. dispar* and *E. histolytica* trophozoites isolated using an RNeasy mini kit (Qiagen) were used for cDNA synthesis with a GeneAmp RNA PCR kit (Applied Biosystems). Reaction mixtures for quantitative real-time PCR analysis were

prepared using SYBR Premix Ex *Taq* (Takara), specific primers, Rox dye, and the cDNAs. The primers used were as follows: 5′-TGA CAA AGA CAA TAC TTG TAA AAA GTG-3′ (forward) and 5′-ATT ACT AAC ACA TGC ACA TTT TTT GTC-3′ (reverse) for *E. dispar Igl1* genes; 5′-TCG ATG AAA ATA ATG TAT GCC AGA AAT-3′ (forward) and 5′-TCA TCA AGG CAA GCA CAT TGA CTG-3′ (reverse) for *E. dispar Igl2* genes; 5′-GTT CAC AGG TTG GTG CTT GTA CG-3′ (forward) and 5′-ACA GTA CAT GGC TTT TCT CCG GTA-3′ (reverse) for *E. histolytica Igl1* genes; 5′-GAT TCA CAA ACA AAG GAG TGT GCC-3′ (forward) and 5′-GTG CAT TTG AAC CAC TAG CAG CAA-3′ (reverse) for *E. histolytica Igl2* genes; and 5′-CCA GCT ATG TAT GTT GGA ATT CAA G-3′ (forward) and 5′-GAT CAA GTC TAA GAA TAG CAT GTG G-3′ (reverse) for *actin* genes. Forty cycles of amplification with recording of fluorescence intensity in each cycle were performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). After initial denaturation at 95 °C for 10 sec, a shuttle PCR protocol consisting of denaturation at 95 °C for 5 sec and annealing-extension at 60 °C for 30 sec was applied. Relative quantitation with data from the ABI PRISM 7700 Sequence Detection System software version 1.7 was performed by the comparative C_T method, using the actin gene as an internal standard. The experiments were repeated 3 times, including the steps of culture of trophozoites and isolation of RNA.

Expression of recombinant Igl1 *and* Igl2

DNA fragments encoding full-length Igls, except for the N-terminus and C-terminus signal sequences, were obtained by PCR amplification of cloned *Igl* genes. Primers EdIgl1-S14-Xho (5′-CCC TCG AGG AGT ACA AAG CTG ATA AAC T-3′) and EdIgl-AS-Xho (5′-CCC TCG AGT TAA ATT CCT TTA CTT CCA TT-3′) were used for amplification of the *Igl1* gene of SAW1734RclAR. For amplification of the *Igl2* gene of SAW1734RclAR, primers EdIgl2-S14-Xho (5′-CCC TCG AGG ATT ACA AAG CTG ATA AAC TCA TC-3′) and EdIgl-AS-Xho were used. PCR was performed as previously described (Tachibana *et al.* 2004). Each amplified DNA fragment was digested with *Xho*I, purified, and ligated with pET19b vector (Novagen). The plasmid was introduced into competent *Escherichia coli* JM109 cells and the direction and sequence of inserts were confirmed. *E. coli* BL21Star(DE3)pLysS cells (Invitrogen) were transformed with the cloned plasmids. Each clone was cultured in 400 ml of Luria-Bertani medium containing ampicillin until the culture reached an optical density of 0·6 at 600 nm. Isopropyl-β-D-thiogalactopyranoside was added to the cultures at a final concentration of 1 mM, and the cultures were incubated at 37 °C for 3 h. Preparation of inclusion bodies and refolding of the proteins were performed as previously described (Tachibana *et al.* 2004).

Production of specific mAbs

MAbs to Igl1 and Igl2 of *E. dispar* SAW1734RclAR were prepared as follows. Six-week-old male BALB/c mice were inoculated intraperitoneally with 10 μg of recombinant proteins in Freund's complete adjuvant and were inoculated again after 2 weeks. After an additional 3 weeks, the mice received only recombinant proteins. Four days later, spleen cells of immunized mice were isolated and fused with X63 Ag8.653 mouse myeloma cells using 50% polyethylene glycol 1500. Hybridomas secreting mAbs against *E. dispar* Igls were screened by immunofluorescent staining and ELISA, and were cloned by limiting dilution. Immunoglobulin isotypes of mAbs were determined by immunofluorescent staining using subtype-specific antibodies. Ascites was obtained by intraperitoneal inoculation of hybridomas into pristine-primed mice, and immunoglobulin was purified using an Affi-Gel protein A MAPS II kit (Bio-Lab).

Dot blot analysis

Recombinant Igls and sonicated trophozoites of *E. dispar* SAW1734RclAR were blotted on the nitrocellulose membrane using a Bio-Dot microfiltration apparatus (Bio-Rad). Filter strips were blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and reacted with mouse anti-*E. dispar* Igl mAbs for 30 min. After being washed with PBS containing 0·05% Tween-20 (PBS-Tween), the strips were incubated with horseradish peroxidase (HRP)-labelled goat anti-mouse IgG antibody (MP Biomedicals) for 30 min. The strips were then washed with PBS-Tween and developed with a Konica Immunostaining HRP-1000 kit.

SDS-PAGE and Western blot analysis

Recombinant Igl proteins or *E. dispar* trophozoites were treated with Laemmli's sample buffer (Laemmli, 1970) containing 2 mM phenylmethylsulfonyl fluoride, 2 mM *N*-α-*p*-tosyl-L-lysine chloromethylketone, 2 mM *p*-hydroxymercuriphenylsulfonic acid, and 4 μM leupeptin for 5 min at 95 °C and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blot analysis was performed as previously described (Tachibana *et al.* 2004).

Flow cytometry

Immunophenotypic surface staining of Igl1 and Igl2 using flow cytometry was performed on trophozoites of *E. dispar* SAW1734RclAR strain. Intact cells were incubated on ice with 3% BSA in PBS for 15 min, and then with a mixture of mAbs ED1-13 and ED2-1 for 15 min. After washing with ice-cold PBS, the cells were incubated with a mixture of Alexa Fluor 488-labelled goat anti-mouse IgG2b-specific antibody (Molecular Probes) and PE-labelled goat anti-mouse IgG1-specific antibody (Santa Cruz Biotechnology) for 15 min on ice. The cells were washed with ice-cold PBS and then fixed in 4% paraformaldehyde. Aliquots of approximately 5000 cells per sample were analysed using FACS Calibur (Becton Dickinson), with data analysis using CellQuest Software (BD Immunocytometry systems).

Confocal microscopy

E. dispar SAW1734RclAR trophozoites were fixed with 4% paraformaldehyde in PBS and attached to silane-coated glass slides using Shandon Cytospin 2. After washing with PBS, the glass slides were incubated with 10% sucrose in PBS for 1 h and then stored at −80 °C until use. For double staining of Igl1 and Igl2, fixed trophozoites on the slides were treated with 0·1% Triton X-100 in PBS for 5 min, blocked with 3% BSA in PBS for 30 min and then incubated for 1 h at room temperature with a mixture of 2 mAbs, ED1-13 and ED2-1. After washing, the slides were incubated with a mixture of Alexa Fluor 488-labelled goat anti-mouse IgG2b-specific antibody and Alexa Fluor 594-labelled goat anti-mouse IgG1-specific antibody (Molecular Probes) for 1 h. The stained trophozoites were mounted using glycerol containing 1·25 mg/ml 1,4-diazbicyclo(2,2,2)octane and 10% PBS, and the samples were observed using a Zeiss LSM510 META confocal laser scanning microscope.

RESULTS

Cloning of genes encoding E. dispar *Igl1 and Igl2*

Two *Igl* genes cloned from the cDNA library from the *E. dispar* SAW1734RclAR strain encoded proteins of 1110 and 1106 amino acids, respectively, with calculated molecular masses of 120·9 kDa and 120·3 kDa and theoretical p*I* values of 5·5 and 4·87, respectively (DDBJ, EMBL, and GenBank Accession numbers AB287423 and AB287424). Two *Igl* genes were also cloned from the genomic DNA of the *E. dispar* CYNO9:TPC strain. These genes also encoded proteins of 1110 and 1106 amino acids, respectively, with calculated molecular masses of 121·0 kDa and 120·4 kDa and theoretical p*I* values of 5·41 and 4·74, respectively (DDBJ, EMBL, and GenBank Accession numbers AB287425 and AB287426). Based on the similarity of the p*I* values to those of Igls of *E. histolytica* (5·52 for Igl1 and 5·17 for Igl2) (Cheng *et al.* 2001), the former protein was designated as Igl1 and the latter as Igl2 in both *E. dispar* strains. Multiple alignments of amino acid sequences among these Igl proteins and other *E. histolytica* Igls are shown in Fig. 1; the amino acid identity between *E. dispar* Igl1 and Igl2 was 79% in both strains. Differences in amino acids between the proteins in the two *E. dispar* strains were greater for Igl2 than for Igl1. In comparison with the *E. histolytica* Igl isoforms, the amino acid sequence identities were 75–76% for Igl1 and 73–74% for Igl2. Insertions of 6 amino acids in Igl1 and Igl2 are present in the two *E. dispar* strains around position 840. However, all cysteine residues found in *E. histolytica* Igl proteins were conserved in the *E. dispar* proteins. Both *E. dispar* Igl isoforms also contained hydrophobic amino- and carboxy-terminal signal sequences consistent with a GPI-anchored plasma membrane protein, and all Igl sequences contained a signature epidermal growth factor-like domain close to the C-terminus. The nucleotide sequence identities were 88% between the *E. dispar Igl1* and *Igl2* genes, 83% between the *E. dispar and E. histolytica Igl1* genes, and 82% between the *E. dispar* and *E. histolytica Igl2* genes.

A BLAST search of *E. dispar* SAW760 strain genomic sequences <http://www.ncbi.nlm.nih.gov/BLAST/>was performed, although the *E. dispar* genome project is incomplete. An identical sequence to that of the *Igl1* gene of the SAW1734-RclAR strain was found (Genbank Accession no. AANV01000026), and 2 sequences (AANV01000644 and AANV01001389) were identified that showed 99% identity with the *Igl2* gene of SAW1734RclAR in partially overlapping regions.

Southern blot analyses of Igl *genes*

Southern blot hybridization using a 420-bp PCR product as a probe was performed on *E. dispar* SAW1734RclAR genomic DNA digested with *Dra*I, *Taq*I and *Hin*dIII (Fig. 2). The results indicated the presence of 2 *Igl* genes in the *E. dispar* genomic DNA.

Real-time RT-PCR analysis of Igl *genes*

Expression levels of *Igl1* and *Igl2* are compared in Fig. 3. In the SAW1734RclAR strain, expression of *Igl1* was 3 times higher than that of *Igl2* ($P<0·001$), and in the CYNO9:TPC strain, expression of *Igl1* was 5 times higher than that of *Igl2* ($P<0·01$). Higher expression of *Igl1* compared to *Igl2* was also observed in *E. histolytica* ($P<0·001$). Expression of *Igl1* was lower in *E. dispar* than in *E. histolytica* (SAW1734RclAR *vs* HM-1:IMSS, $P<0·01$;

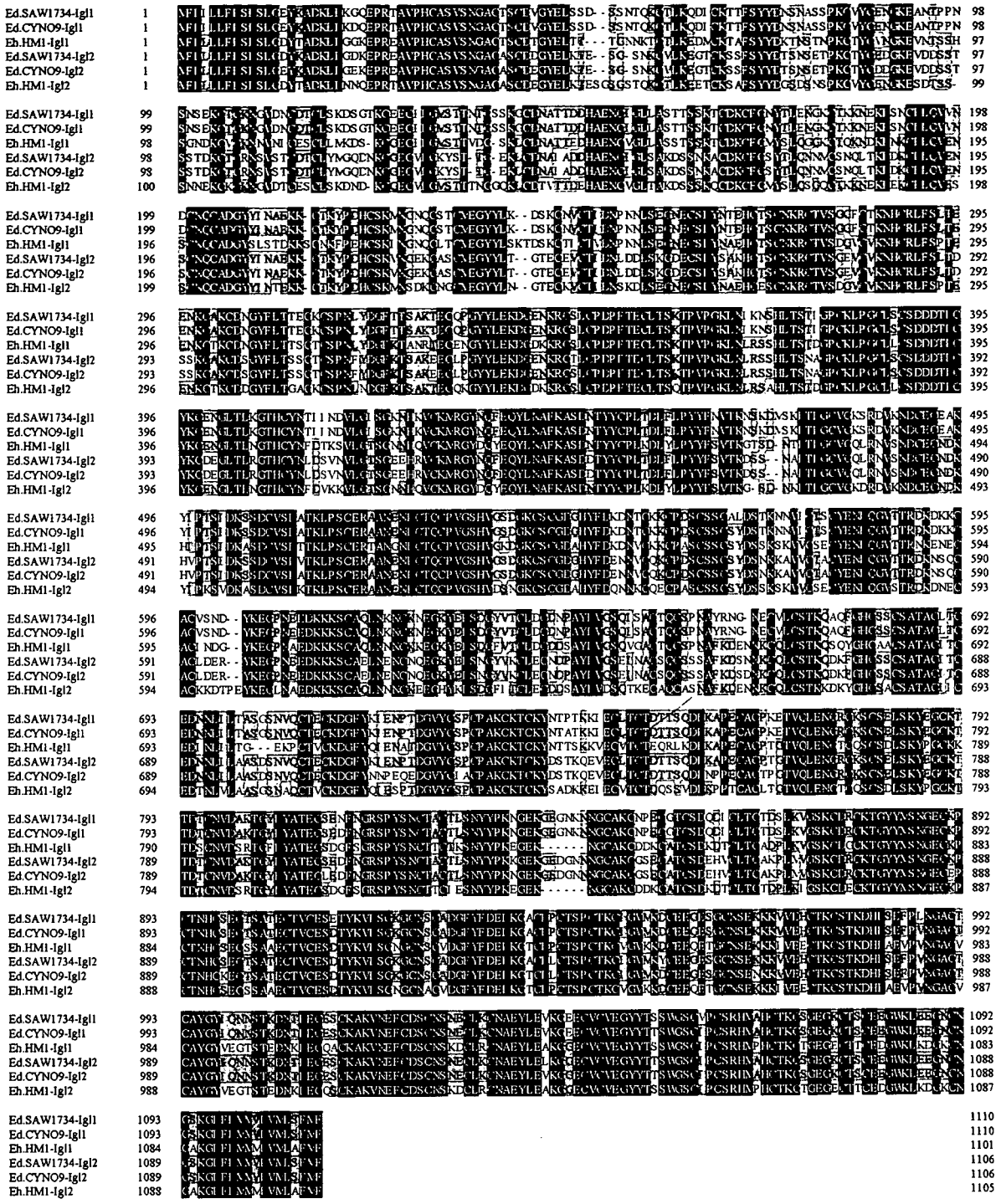

Fig. 1. Alignment of the deduced amino-acid sequences of the *Igl1* and *Igl2* genes from *Entamoeba dispar* SAW1734RclAR (Ed.SAW1734), *E. dispar* CYNO9:TPC (Ed.CYNO9) and *E. histolytica* HM-1:IMSS (Eh.HM1). Identical and conserved amino-acid residues are highlighted in black and grey, respectively.

CYNO9:TPC *vs* HM-1:IMSS, not significant), whereas *Igl2* expression was similar in the two species.

Dot blot and Western blot analyses of Igls

Full length (except for the signal sequence) recombinant *E. dispar* Igls from the SAW1724RclAR strain were prepared in *Escherichia coli*. The apparent molecular weight of the recombinant protein with the leader peptide was slightly larger for Igl2 (190 kDa) than for Igl1 (170 kDa) in SDS-PAGE under reducing conditions (data not shown). Recombinant Igl1 and Igl2 proteins were used for immunization of mice to prepare mAbs specific for each Igl. In dot blot analysis, mAb ED1-13 reacted specifically with recombinant Igl1, but not with recombinant Igl2; in contrast, mAb ED2-1 was

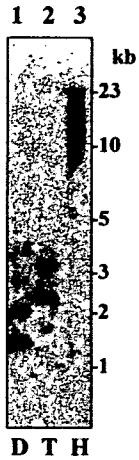

Fig. 2. Southern blot analysis of *Igl* genes in *Entamoeba dispar* SAW1734RclAR. Genomic DNA was digested with *Dra*I (D, lane 1), *Taq*I (T, lane 2) and *Hin*dIII (H, lane 3) and hybridized with the probe. The blot is representative of 2 independent experiments. Numbers to the right indicate the sizes of DNA markers (in kilobases).

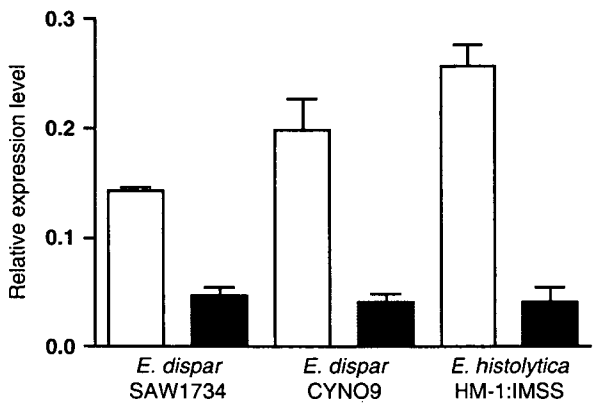

Fig. 3. Real-time reverse transcription PCR analysis of *Igl* genes from *Entamoeba dispar* and *E. histolytica*. Expression levels of *Igl1* (open bars) and *Igl2* (filled bars) in trophozoites from *E. dispar* SAW1734RclAR, *E. dispar* CYNO9:TPC and *E. histolytica* HM-1:IMSS are expressed as values relative to the expression level of *actin*. Vertical bars indicate the S.E. of the mean from 3 experiments.

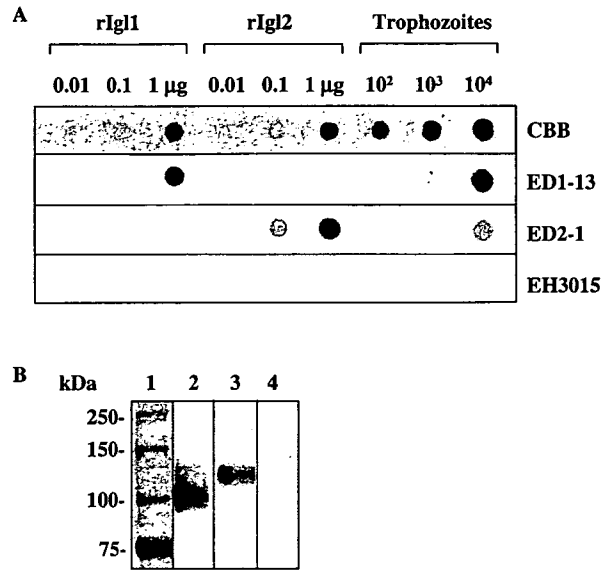

Fig. 4. (A) Reactivity of anti-Igl monoclonal antibodies to recombinant Igls and crude antigens of *Entamoeba dispar* in dot blot analysis. Various concentrations (0·01, 0·1 and 1 μg) of recombinant Igl1 (rIgl1) and recombinant Igl2 (rIgl2) and various numbers (10^2, 10^3 and 10^4) of sonicated trophozoites from strain SAW1734RclAR were spotted on nitrocellulose membranes. One strip was stained with Coomassie brilliant blue (CBB). Other strips were treated with anti-*E. dispar* Igl monoclonal antibodies (ED1-13, ED2-1) and anti-*E. histolytica* Igl monoclonal antibody (EH3015). HRP-conjugated goat antibody to mouse IgG was used as a secondary antibody. The blot is representative of 2 independent experiments. (B) Western immunoblot analysis of native Igls of *E. dispar*. Lysates of SAW1734RclAR trophozoites were subjected to SDS-PAGE in a 7·5% gel under non-reducing conditions and transferred to polyvinylidene difluoride membranes. Protein bands of the size marker in lane 1 were stained with Coomassie brilliant blue. The strips were treated with monoclonal antibodies as follows: lane 2, ED1-13; lane 3, ED2-1; and lane 4, EH3015. HRP-conjugated goat antibody to mouse IgG was used as a secondary antibody. The blot is representative of 3 independent experiments. The numbers to the left indicate molecular masses (in kilodaltons).

reactive specifically with recombinant Igl2 (Fig. 4A). In conditions under which the reactivity of these mAbs to *E. dispar* trophozoites was comparable, differences in reactivity to equal amounts of recombinant Igls were observed. The reactivity of mAb ED1-13 to 10^4 trophozoites was similar to the reactivity to 1 μg of EdIgl1, whereas the reactivity of mAb ED2-1 to 10^4 trophozoites were comparable to the reactivity to 0·1 μg of EdIgl2, suggesting that the amount of Igl2 was approximately one-tenth that of Igl1 in the trophozoites. In Western blot analysis using trophozoites from the SAW1734RclAR strain, mAbs ED1-13 and ED2-1 recognized a 100 kDa and a 120 kDa band, respectively, under non-reducing conditions (Fig. 4B). No bands were detected in the Western blot under reducing conditions, indicating that the mAbs recognized discontinuous epitopes on the Igl proteins.

Phenotypic expression of Igls on the surface of trophozoites

To compare the amounts of Igl1 and Igl2 expressed on the surface of trophozoites from the *E. dispar* SAW1724RclAR strain, flow cytometric analysis was performed using specific mAbs for each Igls (Fig. 5). The results demonstrated that almost all