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

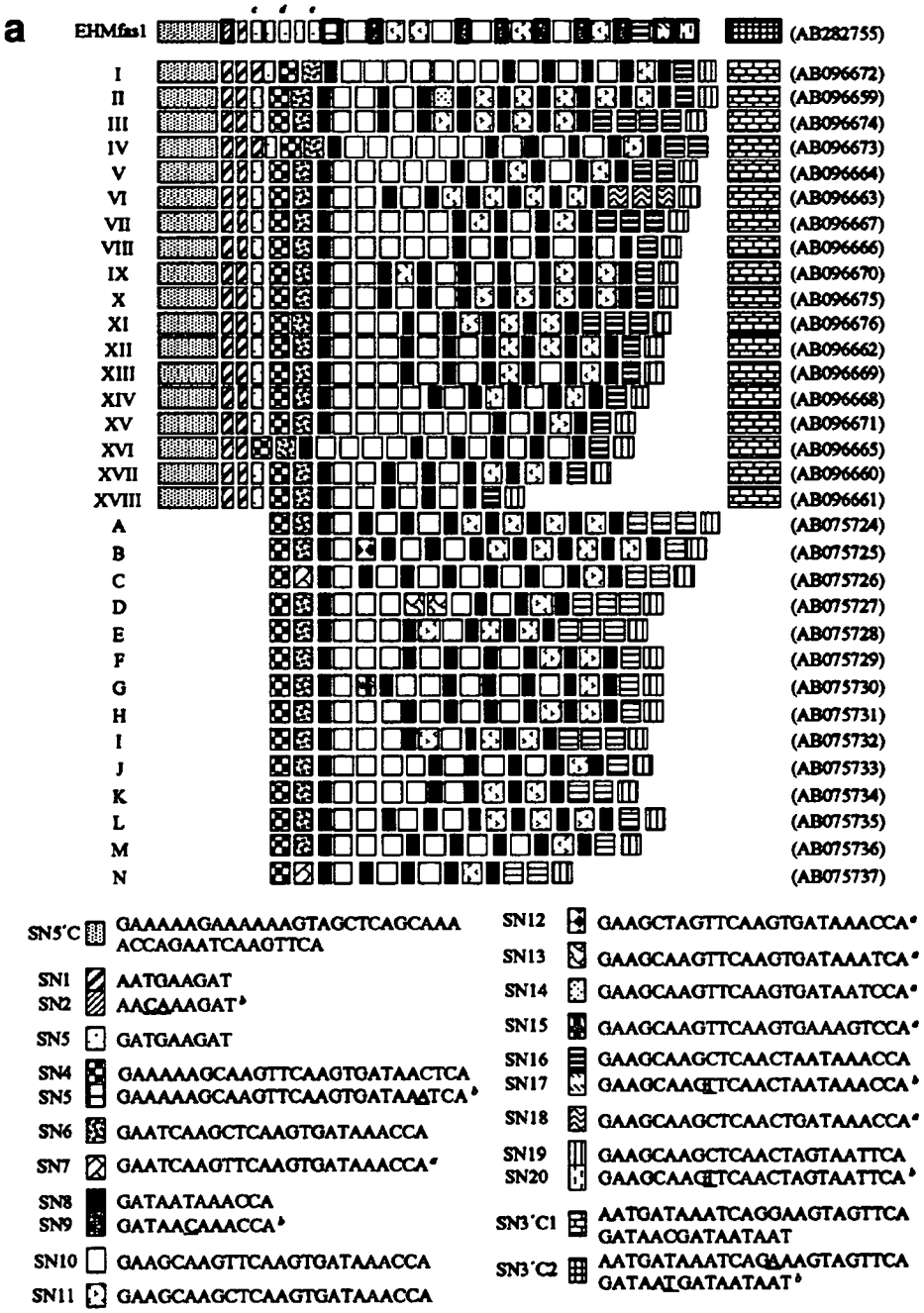

Fig. 2 Schematic representation of polymorphism in the repeat-containing region of the SREHP gene among EHMfas1 and human isolates. Nucleotide sequences pattern (**a**) and deduced amino-acid sequences pattern (**b**) were shown. Each nucleotide and deduced amino-acid sequence of unit was tentatively 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)

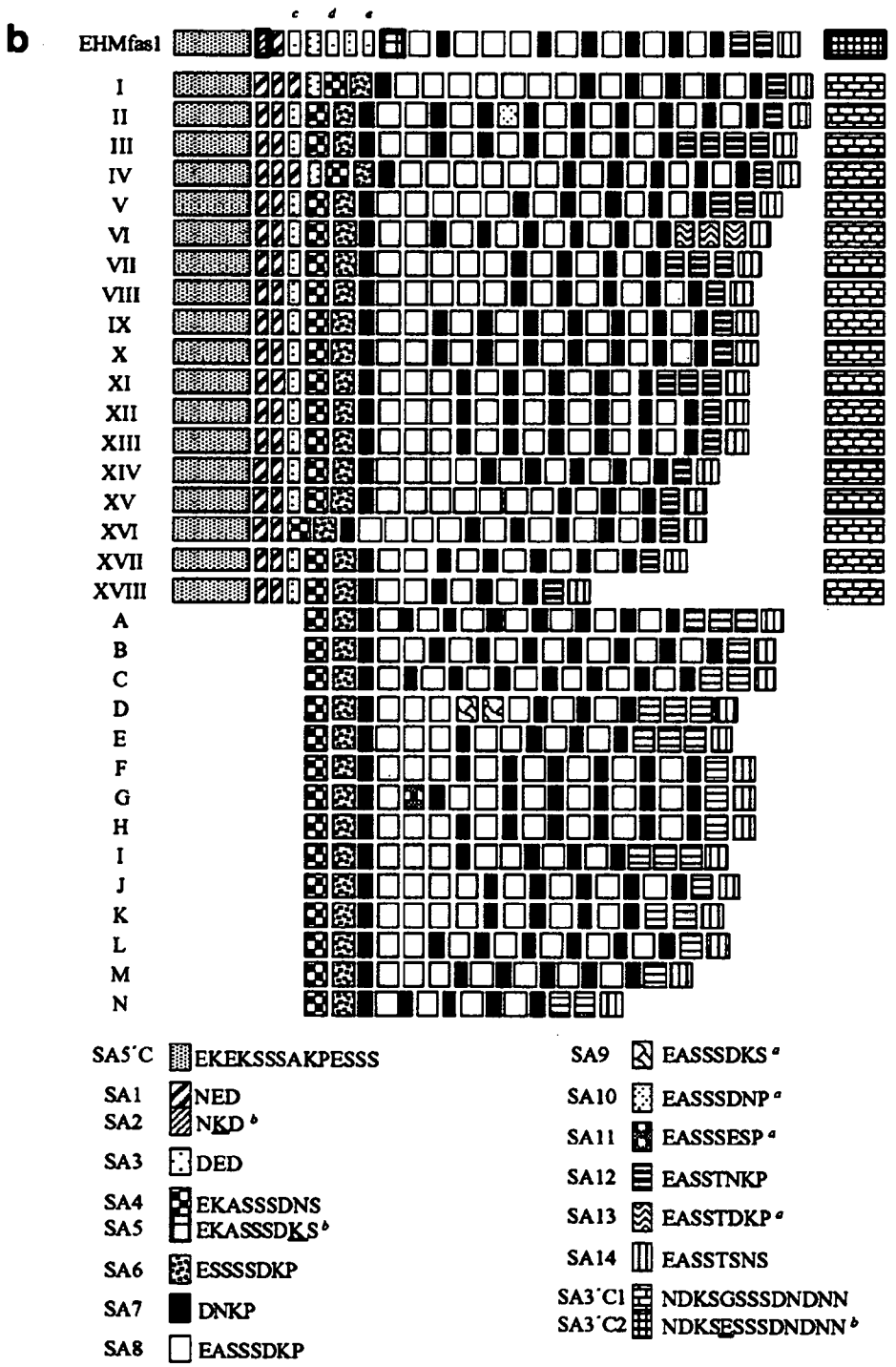

Fig. 2 (continued)

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.

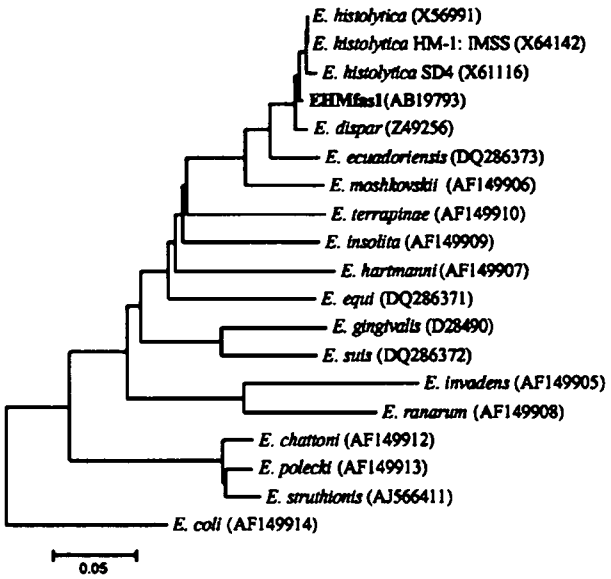

Fig. 3 Phylogenetic analysis of 16S-like SSUrDNA sequences among EHMfas1 and other *Entamoeba* species. Branch lengths are proportional to estimated number of substitutions per site, which represent the evolutional distance

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.

Acknowledgments We are grateful to Dr. Seiki Kobayashi for analyze of zymodeme pattern of EHMfas1. This work was supported by a Grant-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science and a grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare of Japan.

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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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(*Received 29 May 2007; revised 25 June 2007; accepted 4 July 2007; first published online 6 September 2007*)

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 110000 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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doi:10.1017/S0031182007003459 Printed in the United Kingdom

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, $P<0·01$;

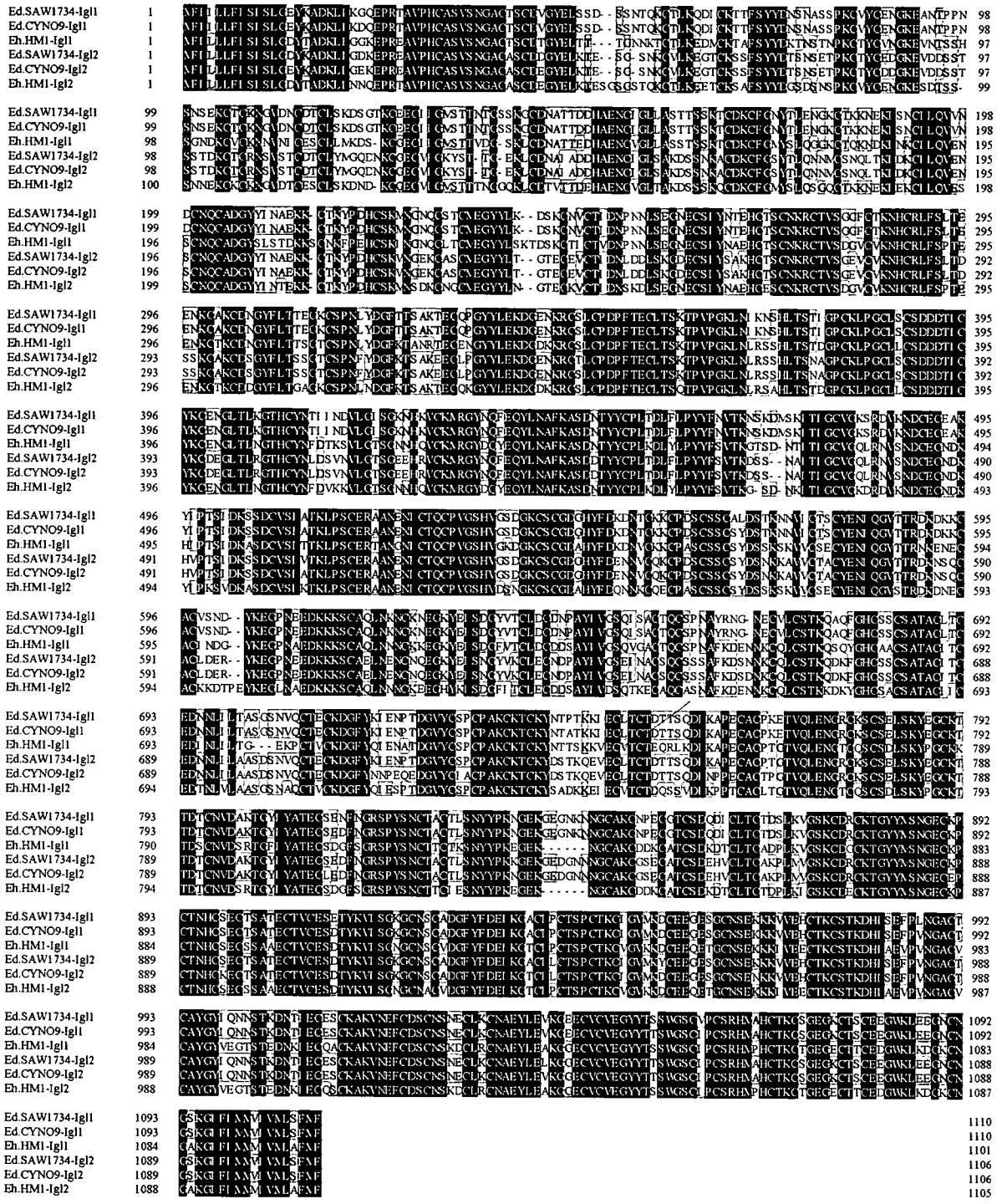


Fig. 1. Alignment of the deduced amino-acid sequences of the *Igl1* and *Igl2* genes from *Entamoeba dispar* SAW1734RclAR (Ed.SAW1734), *E. dispar* CYNO9:TPC (Ed.CYNO9) and *E. histolytica* HM-1:IMSS (Eh.HM1). Identical and conserved amino-acid residues are highlighted in black and grey, respectively.

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

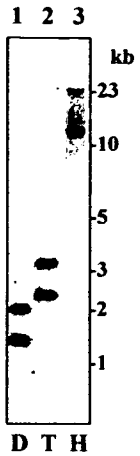


Fig. 2. Southern blot analysis of *Igl* genes in *Entamoeba dispar* SAW1734RclAR. Genomic DNA was digested with *Dra*I (D, lane 1), *Taq*I (T, lane 2) and *Hin*dIII (H, lane 3) and hybridized with the probe. The blot is representative of 2 independent experiments. Numbers to the right indicate the sizes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

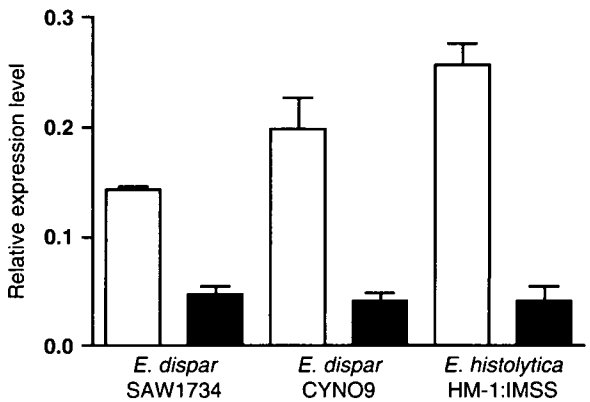
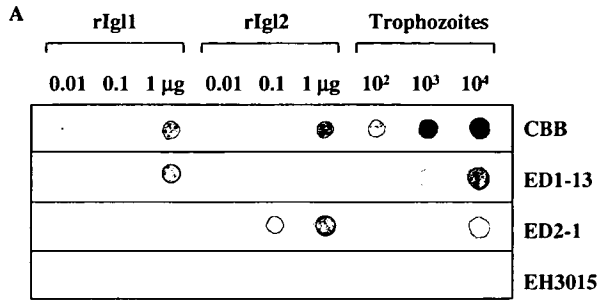


Fig. 3. Real-time reverse transcription PCR analysis of *Igl* genes from *Entamoeba dispar* and *E. histolytica*. Expression levels of *Igl1* (open bars) and *Igl2* (filled bars) in trophozoites from *E. dispar* SAW1734RclAR, *E. dispar* CYNO9:TPC and *E. histolytica* HM-1:IMSS are expressed as values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



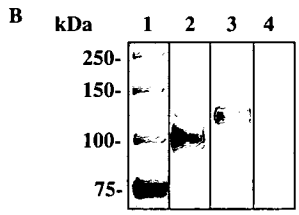


Fig. 4. (A) Reactivity of anti-Igl monoclonal antibodies to recombinant Igls and crude antigens of *Entamoeba dispar* in dot blot analysis. Various concentrations (0·01, 0·1 and 1 μg) of recombinant Igl1 (rIgl1) and recombinant Igl2 (rIgl2) and various numbers (10^2, 10^3 and 10^4) of sonicated trophozoites 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

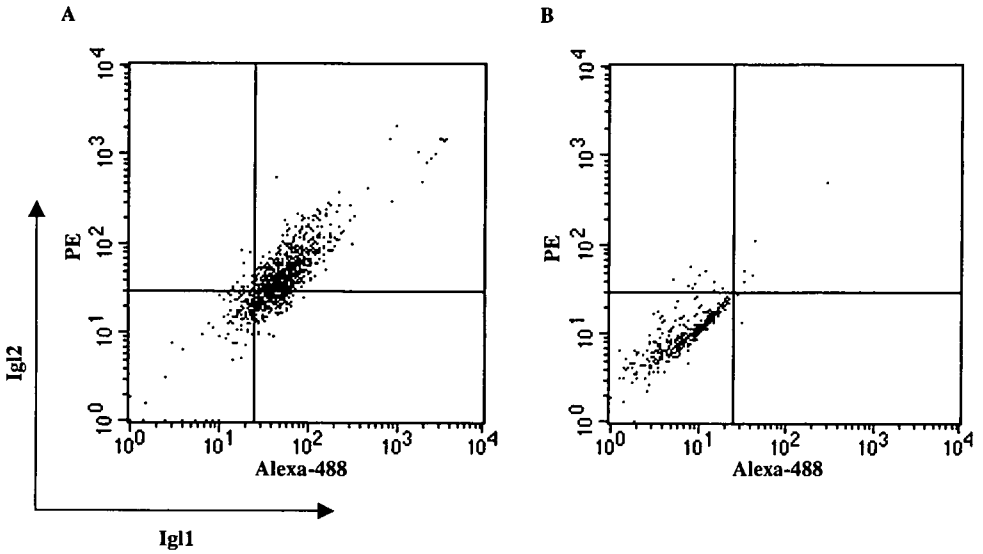


Fig. 5. Flow cytometric analysis of phenotypic expression of Igl1 and Igl2 on the surface of *Entamoeba dispar* trophozoites. Intact trophozoites from the SAW1734RclAR strain were double stained with monoclonal antibodies ED1-13 (specific for Igl1, IgG2b) and ED2-1 (specific for Igl2, IgG1), followed by Alexa Fluor 488-labelled goat anti-mouse IgG2b-specific antibody and PE-labelled goat anti-mouse IgG1-specific antibody (A). The control was stained only with secondary antibodies (B). The results are representative of 3 independent experiments.

trophozoites expressed both Igl1 and Igl2 on the cell surface.

Localization of Igls on trophozoites

Localization of Igl1 and Igl2 on *E. dispar* trophozoites was examined by confocal laser scanning microscopy using specific mAbs. Both Igls were localized on the plasma membrane and in cytoplasm in all trophozoites (Figs 6 and 7). However, the amount of each Igl in the trophozoites was variable, especially in the cytoplasm; that is, Igl1- and Igl2-dominant cells were present (arrows and arrowheads in Fig. 6). In addition, Igl1- and Igl2-dominant vacuoles were also observed within a single trophozoite (arrow and arrowhead in Fig. 7).

DISCUSSION

Comparison of the 2 *Igl* genes cloned from *E. dispar* with those from *E. histolytica* indicated differences in the sequences of the corresponding proteins in *E. dispar* and *E. histolytica* and in the 2 strains of *E. dispar*. However, all the cysteine residues, including the CXXC motifs, were conserved between species and between strains, which suggests that the fold of the protein is maintained and is important for its function. Amino acid identities of Igl1 and Igl2 within species (79% in *E. dispar* and 81% in *E. histolytica*; Cheng *et al.* 2001) were higher than those for each Igl between the 2 species (75–76% for Igl1 and 73–74% for Igl2). Insertions of 6 amino acids around position 840 in *E. dispar* were present in both Igl1 and Igl2, as shown in Fig. 1. Nucleotide identities of the 2 *Igl* genes were also higher within species, compared to the respective identities of the *Igl* genes between *E. dispar* and *E. histolytica*. These results suggest that duplication of the genes may have occurred after divergence of the species.

The properties of the Gal/GalNAc lectin of *E. histolytica* were demonstrated in a 150 kDa fraction purified by affinity chromatography using the *E. histolytica*-specific mAb EH3015 (Cheng *et al.* 1998). In addition, Igl1 and Igl2 of *E. histolytica* have been detected, in addition to Hgl and Lgl, in the protein fraction that binds specifically to GalNAc-BSA-coated magnetic beads (McCoy and Mann, 2005). However, when we performed a preliminary examination of the reactivity of recombinant Igls from *E. histolytica* and *E. dispar* with $GalNAc_{27}$-BSA by dot blot analysis and surface plasmon resonance, we could not prove that the recombinant proteins had sugar-binding properties (data not shown). Therefore, the Igls may exist as part of the lectin complex, perhaps with non-covalent association to another protein containing a sugar-binding site, with this association occurring either directly or being mediated by a third protein. Recently, it has been reported that Igl of *E. histolytica* is found in the protein fraction that interacts with purified brush border from human enterocytes (Seigneur *et al.* 2005). It has also been demonstrated that the 140 kDa fibronectin-binding molecule of *E. histolytica* (Talamas-Rohana *et al.* 1992) is identical with Igl2 (Hernandez-Ramirez *et al.* 2007). These observations indicate that Igls are important proteins for amoebic adherence to host cells, and since Igls are also expressed in the non-pathogenic amoeba, it seems likely that these proteins are important for colonization of amoebae in the large intestine.

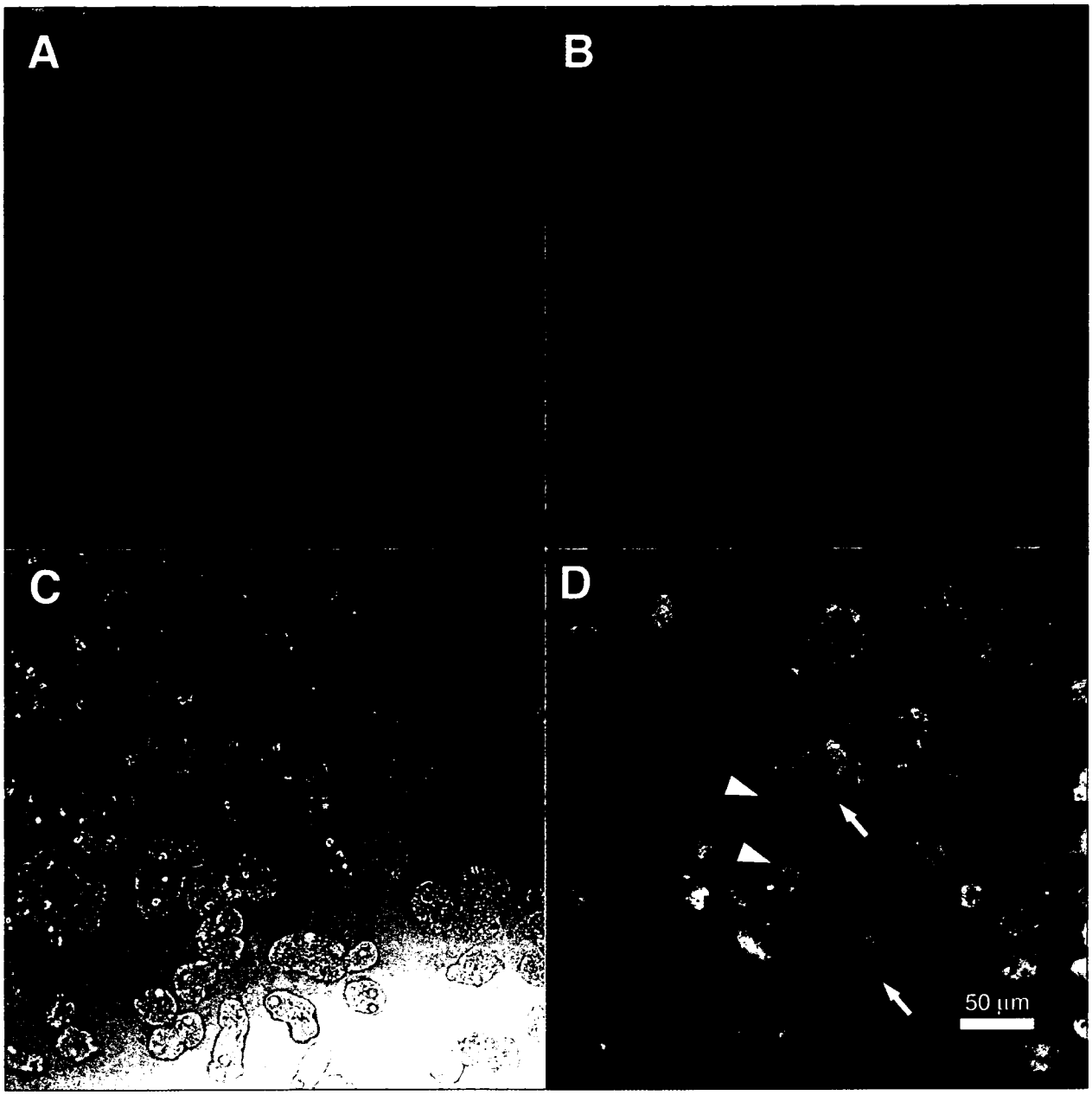


Fig. 6. Localization of Igl1 and Igl2 on trophozoites of *Entamoeba dispar* SAW1734RclAR observed by confocal laser scanning microscopy. Fixed trophozoites were stained with ED1-13 specific for Igl1 and a secondary Alexa Fluor 488-labelled anti-mouse IgG2b antibody (green) (A) or ED2-1 specific for Igl2 and a secondary Alexa Fluor 594-labelled anti-mouse IgG1 antibody (red) (B). Differential interference contrast microscopy is shown in (C). A merged image is shown in (D). Arrows and arrowheads indicate Igl1- and Igl2-dominant cells, respectively.

One of the interesting observations in this study is the difference in expression between Igl1 and Igl2. Since higher expression of Igl1 was observed at both protein and mRNA levels, the difference between the isoforms seems to be regulated mostly at the transcriptional level. The expression level of *Igl1* was also lower in *E. dispar* than in *E. histolytica*, whereas that of *Igl2* was comparable in the two species. It has been demonstrated that expression of *Hgl* (*Hgl2*) and *Lgl* (*Lgl1*) in *E. dispar* is lower than in *E. histolytica* (Pillai *et al.* 1997, 2001); therefore, Igl1 may be more closely associated with Hgl and Lgl. However, it is unknown whether the 2 Igl isoforms are associated with different isoforms of Hgl or Lgl. DNA microarray analyses have shown that a large number of genes are expressed differently in *E. histolytica* and *E. dispar*, and that there is a difference in gene expression between strains of *E. histolytica* of high and low virulence (Shah *et al.* 2005; MacFarlane and Singh, 2006; Davis *et al.* 2007). Lower expression of *Hgl* genes in *E. dispar* compared to *E. histolytica* has also been confirmed (MacFarlane and Singh, 2006), but *Lgl3* expression was found to be higher in an *E. histolytica* strain of low virulence compared to a strain of high virulence (Davis *et al.* 2007). *Igl* genes are down-regulated by

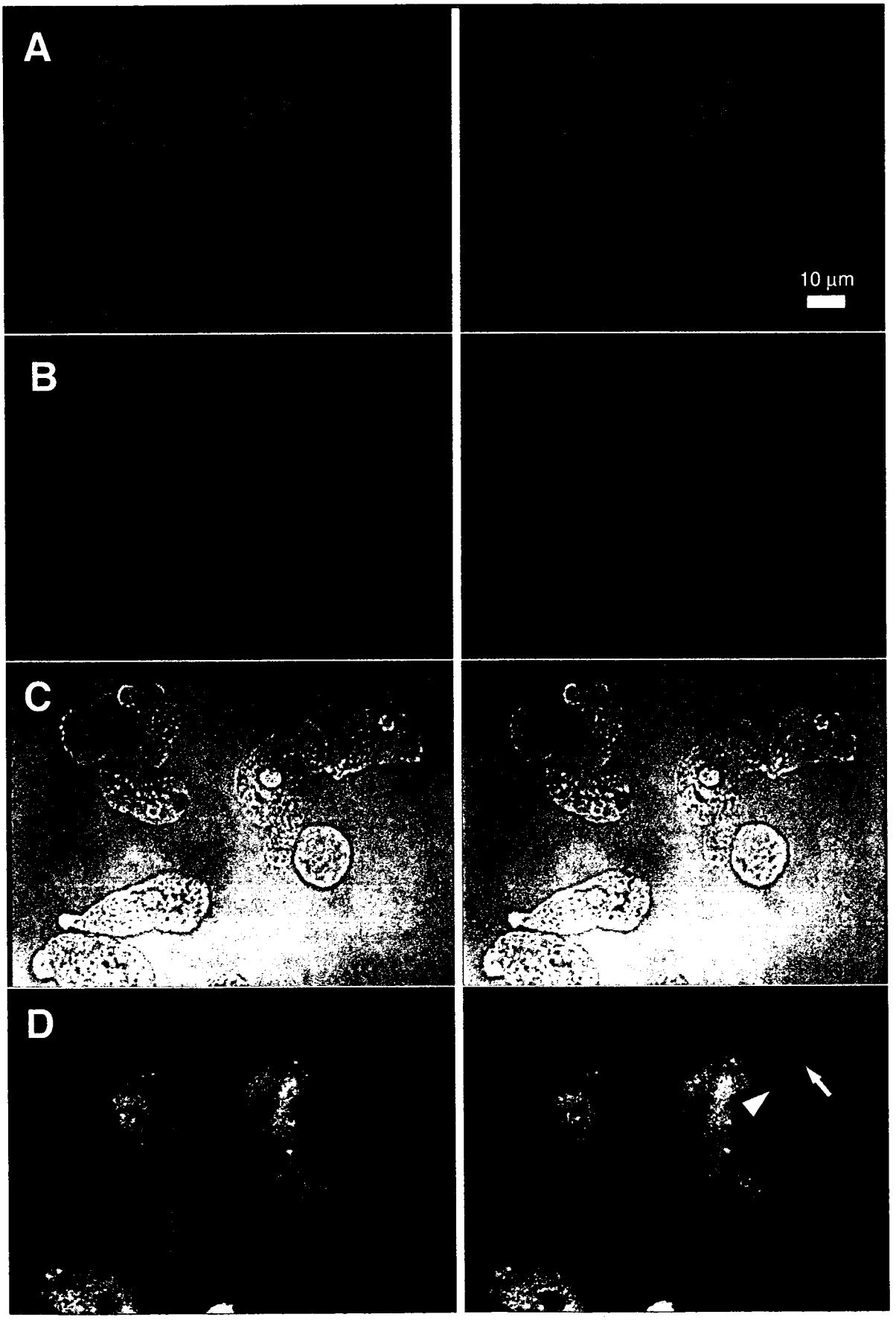


Fig. 7. Stereo images of localization of Igl1 (A) and Igl2 (B) on *Entamoeba dispar* trophozoites observed by confocal laser scanning microscopy. Fixed trophozoites were stained as described in Fig. 6. Differential interference contrast microscopy is shown in (C). A merged image is shown in (D). Arrow and arrowhead indicate single localization of Igl1 and Igl2, respectively.

heat shock stress, as are most *Hgl* and *Lgl* genes (Weber *et al.* 2006). Collectively, these observations suggest that the lectin as a whole is important for adherence and subsequent pathogenesis.

We also compared phenotypic expression of the two Igls in each trophozoite using flow cytometry and confocal microscopy. Interestingly, some trophozoites expressed Igl1 or Igl2 dominantly, although most cells expressed the two Igl proteins to a comparable extent, suggesting that both Igls are important for the amoeba. Igls were also localized in intracellular vacuoles of *E. dispar*. Recently, it has been demonstrated that Igl is contained in phagosomes of *E. histolytica* and that the quantity of Igl varies during phagosome maturation (Okada *et al.* 2005, 2006). The different localization of Igl1 and Igl2 in vacuoles suggests that the functions of the two Igls may differ or that their expression may vary during maturation of phagosomes or depending on certain cellular conditions. However, there may also be differences in the involvement of microtubules and proteases in phagosome maturation and degradation in *E. histolytica* and *E. dispar* (Mitra *et al.* 2005). In *E. histolytica*, different subcellular localization of the two Igl isoforms has yet to be shown.

In conclusion, this is the first study of the differences between Igl1 and Igl2 of *E. dispar*. Igl seems to be a vaccine candidate for amebiasis and may also be a useful antigenic molecule for specific serodiagnosis of amoebiasis (Cheng and Tachibana, 2001; Tachibana *et al.* 2004). Therefore, further studies of Igl are required to clarify its role in the host-parasite relationship.

This work was supported by a Grant-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science, and a grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare of Japan. X.-J. C. is a recipient of a Postdoctoral Fellowship for Foreign Researchers from the Japanese Society for the Promotion of Science.

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Am. J. Trop. Med. Hyg., 77(5), 2007, pp. 825–828

Short Report: Seroprevalence of *Entamoeba histolytica* Infection in HIV-Infected Patients in China

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Abstract. Seroprevalence of *Entamoeba histolytica* infection in HIV-infected individuals from Shanghai city, Anhui province, and Henan province, China, was examined by enzyme-linked immunosorbent assay using crude antigen and a recombinant surface antigen, C-Igl, of the parasite. In 215 HIV-infected individuals, the positive rates for these antigens were 12.1% and 7.9%, respectively; these rates were significantly higher than the rates of 3.1% and 0.5%, respectively, in 191 patients with gastrointestinal symptoms who were not infected with HIV. There was no significant difference in seropositivity to *E. histolytica* between men and women. Seropositivity in HIV-infected individuals was higher in patients with a $CD4^+$ T cell count of < 200/μL. This is the first report showing a higher seroprevalence of *E. histolytica* infection in HIV-infected patients in China. Our results also suggest that HIV infection is a risk factor for infection with *E. histolytica*.

Amebiasis caused by infection with *Entamoeba histolytica* is one of the most problematic parasitic diseases in developing and developed countries.[1] *E. histolytica* infection is also related to diarrhea in patients with HIV infection and AIDS, as well as those infected with opportunistic protozoan parasites such as *Cryptosporidium* spp., *Isospora belli*, and microsporidia.[2] Recently, a high prevalence of *E. histolytica* infection has been reported in HIV/AIDS patients in Japan and Taiwan.[3–5] In China, it is estimated that 650,000 individuals are infected with HIV, and HIV/AIDS is becoming a major public health problem (http://data.unaids.org/Media/PressReleases03/PR_china_060125_en.pdf). However, there is no information concerning the correlation between *E. histolytica* and HIV infections, and the prevalence of *E. histolytica* and *E. dispar*, which is morphologically indistinguishable from *E. histolytica* but is non-pathogenic, is not known in China. Because *E. dispar* is non-invasive, detection of high specific antibody titers indicates possible infection with *E. histolytica*.[6] Therefore, this preliminary study was undertaken to estimate the seroprevalence of *E. histolytica* infection in HIV-infected persons in the Chinese population.

This study was approved by the Ethics Committees of Fudan University School of Medicine and Shanghai Public Health Center, which is affiliated with Fudan University. A total of 466 peripheral blood samples were collected from June to August 2005. Samples from 215 individuals with HIV/AIDS were obtained at Shanghai Public Health Center and at treatment centers for AIDS in Henan province and Anhui province. The subjects were selected randomly, but all were receiving anti-retroviral therapy. Symptomatic gastrointestinal complaints or disorders were not recorded at the time the blood samples were collected. For comparison, serum samples from 191 individuals with gastrointestinal symptoms but no HIV infection were obtained at Huashan Hospital, Shanghai. Gastrointestinal symptoms in these individuals were caused by acute gastritis, giant hypertrophic gastropathy, gastric ulcer, duodenal ulcer, acute hemorrhagic necrotizing enteritis, ulcerative colitis, and intestinal obstruction, but many cases did not have a final diagnosis. No specific pathogens have been detected in culture or microscopy of stool samples examined to date. Serology to HIV was examined using a Cambridge Biotech HIV1 Western blot kit (Cambridge Biotech Corp, Rockville, MD). Serum samples from 60 healthy individuals without a history of amebiasis were used as negative controls. All serum samples were inactivated at 56°C for 30 minutes and stored at −30°C or −80°C until use.

Anti-*E. histolytica* serology was examined by enzyme-linked immunosorbent assay (ELISA) using crude *E. histolytica* antigen and the recombinant fragment of C terminus of intermediate subunit of galactose and *N*-acetyl-D-galactosamine-inhibitable lectin of *E. histolytica* (C-Igl), as previously described.[7] The cut-off for a positive result was defined as an ELISA value > 3 SD above the mean for healthy negative controls. Optical density (OD) values were plotted and analyzed using Prism ver. 4.0, and statistical analysis was performed with Stata ver. 7.0. A descriptive exploratory analysis of the data was performed to assess the distribution variables in the HIV-infected and HIV-uninfected groups. Categorical variables were compared using a Pearson χ^2 test.

Quantification of $CD4^+$ lymphocytes in individuals with HIV/AIDS was performed by cytofluorometry using a MultiTEST CD3 FITC/CD8 PE/CD45 PerCP/ CD4 APC (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions.

ELISA reactivities of sera from HIV-infected and HIV-uninfected patients are shown in Figure 1. The cut-off OD values were 0.412 for crude antigen and 0.402 for C-Igl antigen. In HIV-infected individuals, seropositivity to these antigens was 12.1% and 7.9%, respectively (Table 1). These values were significantly higher than those for HIV-uninfected individuals, which were 3.1% and 0.5%, respectively ($P < 0.005$ and $P < 0.001$, respectively). There was no significant difference in seropositivity to *E. histolytica* between HIV-infected men and women. Positive serology to C-Igl antigen in HIV-infected individuals was similar (6.8%–9.1%) in every age category, except for patients < 20 years old, for whom there were a limited number of samples. Seropositivity to C-Igl in HIV-infected individuals from Shanghai was higher

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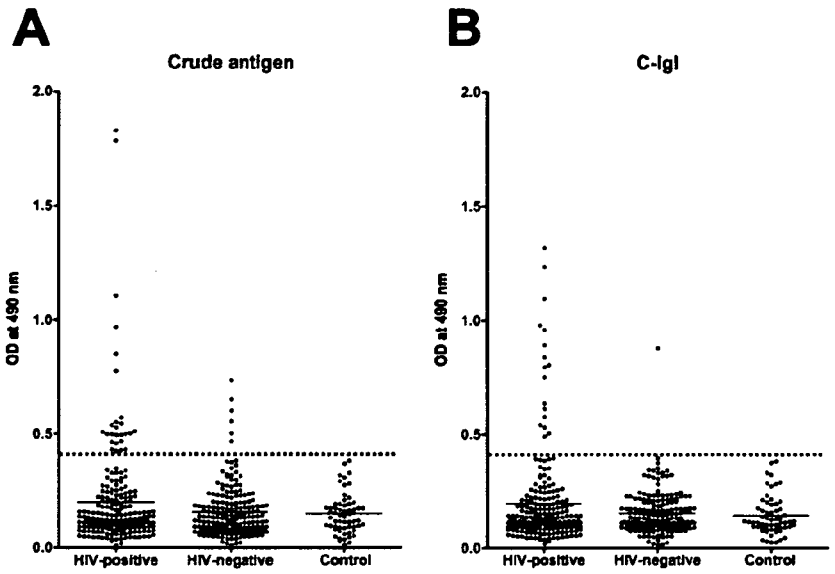

FIGURE 1. ELISA reactivities of sera from HIV/AIDS patients against crude antigen (**A**) and C-Igl (**B**). ELISA plates were coated with 1 μg per well of crude antigen or 100 ng per well of C-Igl. Serum samples from HIV/AIDS patients (HIV-positive, $N = 215$); patients with gastrointestinal symptoms, but not infected with HIV (HIV-negative, $N = 191$); and healthy controls without a history of amebiasis (control, $N = 60$) were used at 1:400 dilution. Horizontal bars indicate the arithmetic means of the groups and the dashed lines indicate the cut-off values.

than those in samples from Anhui and Henan, but the differences were not significant ($P = 0.140$ and $P = 0.122$, respectively). Seroprevalence to *E. histolytica* was higher in patients with a CD4⁺ T-cell count of < 200/μL compared with those with a count of ≥ 200/μL ($P < 0.005$). Stool samples from patients with positive serology to *E. histolytica* were obtained and examined microscopically using a direct smear and subsequent formalin-ether sedimentation. However, we were unable to detect *E. histolytica*/*E. dispar*, and no obvious symptoms of amebiasis were apparent at the time of serum collection.

This is the first report showing higher seroprevalence of *E. histolytica* infection in HIV/AIDS patients in China. In Japan, it is well known that *E. histolytica* infection is common in homosexual men.[8,9] Ohnishi and others[3] have reported that, in 58 patients (including 55 men) with this infection from three large cities in Japan, 56% of the men were homosexual, and antibodies to HIV were detected in 45% of tested pa-

TABLE 1
Seropositivity to *E. histolytica* in HIV/AIDS patients in China

Characteristics	HIV-positive			HIV-negative with gastrointestinal symptoms			Healthy controls		
	No. of samples	Anti-*E. histolytica* antibody-positive (%)		No. of Samples	Anti-*E.histolytica* antibody-positive (%)		No. of samples	Anti-*E. histolytica* antibody-positive (%)	
		Crude Ag	C-Igl		Crude Ag	C-Igl		Crude Ag	C-Igl
Total	215	26 (12.1)	17 (7.9)	191	6 (3.1)	1 (0.5)	60	0 (0)	0 (0)
Sex									
Male	144	18 (12.5)	11 (7.6)	102	6 (5.9)	1 (1.0)	35	0 (0)	0 (0)
Female	71	8 (11.3)	6 (8.5)	89	0 (0)	0 (0)	25	0 (0)	0 (0)
Age (years)									
< 20	3	0 (0)	0 (0)	0	0 (0)	0 (0)	0	0 (0)	0 (0)
20–29	11	3 (27.3)	1 (9.1)	13	0 (0)	0 (0)	60	0 (0)	0 (0)
30–39	66	8 (12.1)	5 (7.6)	87	1 (1.1)	1 (1.1)	0	0 (0)	0 (0)
40–49	80	11 (13.8)	7 (8.8)	61	3 (4.9)	0 (0)	0	0 (0)	0 (0)
50–59	44	3 (6.8)	3 (6.8)	22	2 (9.1)	0 (0)	0	0 (0)	0 (0)
≤ 60	11	1 (9.1)	1 (9.1)	8	0 (0)	0 (0)	0	0 (0)	0 (0)
District									
Shanghai	81	12 (14.8)	10 (12.3)	73	2 (2.7)	0 (0)	20	0 (0)	0 (0)
Anhui	86	12 (14.0)	5 (5.8)	2	1 (50.0)	0 (0)	12	0 (0)	0 (0)
Henan	48	2 (4.2)	2 (4.2)	3	0 (0)	0 (0)	14	0 (0)	0 (0)
Others	0	0 (0)	0 (0)	113	3 (2.7)	1 (0.9)	14	0 (0)	0 (0)
CD4⁺ lymphocytes/μL									
< 200	64	11 (17.2)	11 (17.2)	NA	NA	NA	NA	NA	NA
≤ 200	151	15 (9.9)	6 (4.0)	NA	NA	NA	NA	NA	NA

NA, not applicable.

tients. Recently, a similar trend has been found in HIV-infected persons in Taiwan.[10] In contrast, in Mexico, where both *E. histolytica* and *E. dispar* are endemic, *E. histolytica* prevalence is similar in HIV/AIDS patients and uninfected patients, although the prevalence of *E. dispar* is higher in HIV/AIDS patients.[11] In this study, the absence of a difference in seropositivity to *E. histolytica* between male and female HIV patients suggests that transmission of *E. histolytica* was not caused by homosexual male activity. Indeed, most transmission of HIV in China is thought to be through drug injection, commercial sex, and transfusion (http://data.unaids.org/Media/Press-Releases03/PR_china_060125_en.pdf). Only one HIV-infected man with positive serology for *E. histolytica* was confirmed to be homosexual in this study.

The prevalence of *E. histolytica* and *E. dispar* infections in China is not well understood. A survey in two villages in Shandong province from 1977 to 1984 showed an infection rate of *E. histolytica/E. dispar* of 6.4% using microscopy of direct smears of stool samples, with incidences of amebic dysentery and liver abscess of 2.8% and 0.9%, respectively.[12] Another survey in a village in Hebei province in 1985 showed a positive serology rate of 13.2% and a positive rate for *E. histolytica/E. dispar* in stool of 9.4%.[13] These reports suggest that *E. histolytica* infection may be endemic in rural areas of China. However, it has been reported that the prevalence of *E. histolytica/E. dispar* infection in Henan and Anhui provinces is 0.59% and 0.57%, respectively, using direct microscopy of stool samples.[14] Furthermore, the prevalence of *E. histolytica/E. dispar* infection in Shanghai has recently been estimated to be < 0.1% (Xu and others, personal communication). Because microscopy has low sensitivity and specificity for detection of amebiasis, it is possible that these prevalence rates are underestimates. However, the low seroprevalence to *E. histolytica* in non-HIV patients from Shanghai in this study is in good agreement with these data. In addition, our previous observation that two of three *E. histolytica/E. dispar* isolates obtained from patients with diarrhea in Shanghai were not *E. histolytica* but *E. dispar* also supports the low seropositivity in the non-HIV group.[15] Therefore, it is likely that the higher seroprevalence among HIV-infected persons is related to HIV infection and characteristics, rather than background seroprevalence because of inclusion of subjects from different districts. However, the reason why seropositivity in HIV patients from Shanghai was higher than in HIV patients from the other two provinces is unclear.

In China, HIV/AIDS patients with diarrhea are commonly treated with metronidazole, trimethoprim, and sulfamethoxazole, even if pathogenic protozoan parasites are not detected in the stool. Therefore, it is likely that the positive serology may be caused by past infection with *E. histolytica*.

It is unclear whether HIV infection is a risk factor for *E. histolytica* infections.[10,11,16] However, the higher seropositivity in patients with a $CD4^+$ cell count of < 200/μL in this study suggests that a deficiency in cellular immunity caused by HIV affects the chance of infection with *E. histolytica*. This observation is in accordance with a study in the United States, which estimated the risk ratios of *E. histolytica/E. dispar* infection in HIV-infected patients with $CD4^+$ T cell counts of 0–99 and 100–199/μL as 1.7 and 1.5, respectively, compared with HIV patients with a $CD4^+$ T cell count ≥ 200/μL.[16] It remains unclear how immunodeficiency affects the chance of *E. histolytica* infection. However, it is likely that patients with lower $CD4^+$ T cell counts may be subjected to more testing or that a lower $CD4^+$ T cell count may reflect an increased accumulated risk for amebiasis caused by reactivation of latent infection or increased susceptibility to new infection.[16]

We have recently identified Igl on the surface of trophozoites of *E. histolytica*,[17,18] and it has been shown that an ELISA using C-Igl is more specific than an ELISA using crude antigen.[7] In this study, the seropositive rate in the ELISA using crude antigen was higher than that in the ELISA using C-Igl. All 17 samples that were positive for C-Igl were also positive with crude antigen. The higher seropositivity to crude antigen than to C-Igl antigen seems to be caused by its lower specificity, as previously reported.[7] Therefore, the nine serum samples that were positive with crude antigen but negative with C-Igl may actually be false positives. Overall, our results show that the seroprevalence of *E. histolytica* infection in HIV/AIDS patients in China was 7.9%, which is a higher rate than in HIV-uninfected individuals. A further study will provide a better understanding of the correlation between these infectious diseases.

Received March 13, 2007. Accepted for publication July 2, 2007.

Acknowledgments: The authors thank Xiaozhang Pan for his encouragement of this study.

Financial support: This work was supported by a grant from SMHB (2001411), a Grant-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science, and a grant from the Ministry of Health, Labor and Welfare of Japan.

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