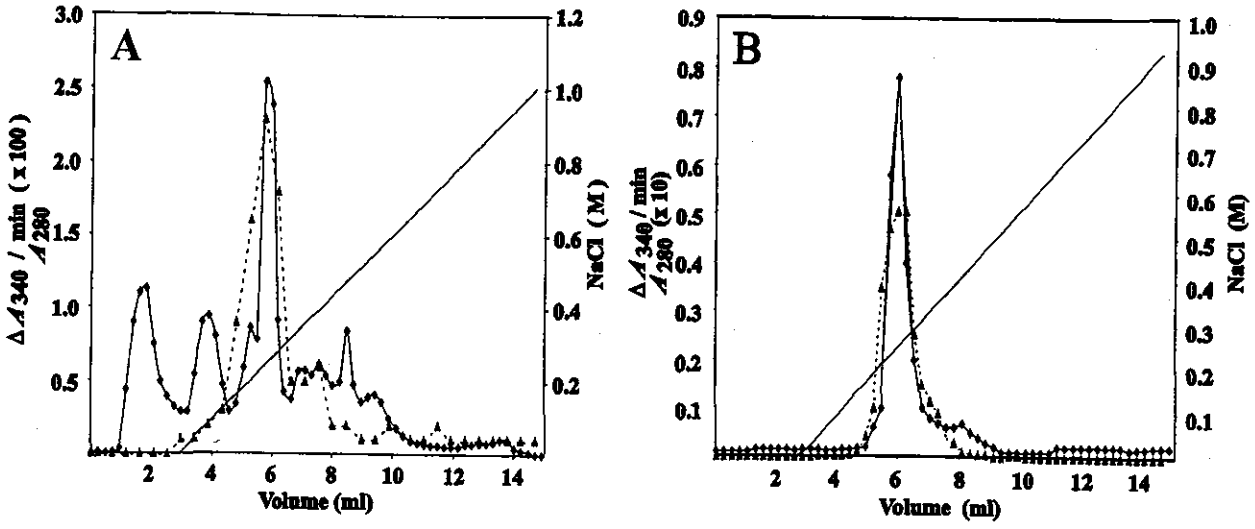


Figure 3 Separation of the native EhGDH from the *E. histolytica* trophozoites and rEhGDH by Mono Q anion-exchange chromatography

(**A**) Elution profile of the native EhGDH. The total lysate of *E. histolytica* trophozoites was separated on the anion-exchange column at pH 8.0 with a linear gradient of NaCl (0–1.0 M). (**B**) Elution profile of the recombinant GDH protein. The rEhGDH protein was dialysed against the binding buffer and fractionated under identical conditions. The plots shown by diamonds and a solid line represent absorbance at 280 nm (A_{280}). The plots drawn with triangles and a broken line indicate EhGDH activity, shown as the decrease in the absorbance at 340 nm/min (ΔA_{340}/min, 100-fold). A solid line represents the NaCl concentration of a linear gradient.

GDH activity was eluted at the predicted molecular size of 75–80 kDa (results not shown). This observation is consistent with the notion that rEhGDH exists as a dimer (monomer 35.8 + 2.6 kDa). In addition, separation of the amoebic crude lysate on the gel-filtration column showed that native GDH activity was also eluted at the position compatible with the native GDH being a dimer (results not shown). These data suggest that both the native and recombinant enzymes exist in their homodimeric forms, as found in other species [26–28]. Our attempt to purify the native GDH to homogeneity in order to perform peptide sequencing failed due to instability of the enzyme (results not shown).

DISCUSSION

Presence of GDH and the non-phosphorylated pathway in serine degradation in the anaerobic parasitic protist

In the present study, we have demonstrated that the enteric protozoan parasite *E. histolytica* possesses one of the key enzymes of serine metabolism. As far as we are aware, this is the first demonstration of GDH in unicellular eukaryotes, including parasitic and non-parasitic protists. GDH has been implicated in having an essential role in the serine degradation pathway in humans, as demonstrated by genetic diseases caused by its deficiency [4,29]. We propose, on the basis of the following bioinformatic and biochemical evidence, that this enzyme also has a key role in maintenance of the intracellular serine concentration in this anaerobic parasitic protist.

First, on the basis of the presence of orthologues of GDH, GK, PGDH, and PSAT in the genome database (results not shown), *E. histolytica* probably possesses both phosphorylated and non-phosphorylated pathways for serine metabolism. A gene encoding GDH is absent in other parasitic and non-parasitic protists, including *Leishmania*, *Plasmodium*, *Giardia*, *Trypanosoma*, *Trichomonas* and *D. discoideum*. The presence of a non-phosphorylated pathway might be unique for *E. histolytica*, or a group of anaerobic protists including *E. histolytica*. This presents the possibility that GDH and the non-phosphorylated serine pathway may be involved in cellular metabolism associated with anaerobic metabolism (see below). Disclosure of the entire genome data for other anaerobic protists, e.g. *Trichomonas* and *Giardia*, should address this question.

Secondly, kinetic parameters determined for EhGDH also support the premise that the amoebic GDH functions in the direction of serine degradation. This amoebic GDH showed a strong preference towards NADPH and HP as substrates as compared with $NADP^+$ and D-glycerate. Furthermore, the amoebic GDH showed a $\approx$100-fold-higher affinity for $NADP^+$/NADPH than for NAD^+/NADH. Assuming that the cytosolic NADPH:$NADP^+$ ratio (approx. 100:1) [30] and the HP concentration ($\approx$5 μM) [7] are comparable between mammals and *E. histolytica*, we conclude that the forward reaction, which leads to serine degradation, is favourable in the amoeba. In *E. histolytica* trophozoites, most of the NADH in the cell is probably converted into NADPH during glycolysis by conversion of phosphoenolpyruvate into pyruvate via oxaloacetate and malate in a reaction catalysed by malate dehydrogenase and malic enzyme [31]. *E. histolytica* also possesses pyridine nucleotide transhydrogenase (EC 1.6.1.1) [32], which catalyses the hydrogen-exchange reaction between NADH and $NADP^+$, thereby reinforcing the predominance of NADPH over NADH in this parasite. Taken together, these findings support our premise that the amoebic GDH acts *in vivo* as an NADPH-dependent HP-reducing enzyme to produce D-glycerate, and is thus likely have a role in serine degradation, but not in serine biosynthesis.

Possible biological role of GDH in *E. histolytica*

The presence of a non-phosphorylated serine metabolic pathway in *E. histolytica* might be associated with the unique metabolism in this anaerobic/microaerophilic parasite. Since *E. histolytica* does not possess a functional tricarboxylic-acid cycle and pentose-phosphate pathway, the major source of energy is from glycolysis. In addition, due to the absence of lactate dehydrogenase, NADH formed during glycolysis is not reoxidized by the conversion

of pyruvate into lactate. Instead, acetyl-CoA is anaerobically reduced to ethanol and CO_2 [33,34], where NADH is reoxidized. NADH is also converted into NADPH by pyridine nucleotide transhydrogenase, as described above. Serine, together with pyruvate, is located at the 'gateway' of glycolysis, fermentation and amino acid metabolism in *E. histolytica*. Serine dehydratase is implicated in the microaerophilic energy metabolism of this parasite, since serine stimulates oxygen consumption and is converted into pyruvate in living cells and extracts [35]. This prompted us to dissect the serine metabolic pathway at the molecular level for a comprehensive understanding of energy metabolism in this parasite.

The physiological role of GDH has been well demonstrated by L-glyceric aciduria (hyperoxaluria type II) [4] in humans. GDH deficiency causes accumulation of HP and glyoxylate, which are converted into L-glycerate and oxalate respectively by lactate dehydrogenase, resulting in the abnormal excretion of L-glycerate and oxalate in the urine [4,6,7]. Therefore the key role of GDH in mammals is to maintain the intracellular concentration of both HP and glyoxylate during the catabolism of serine. Since *E. histolytica* lacks lactate dehydrogenase, and the amoebic GDH does not catalyse glyoxylate reduction, the fate of HP, when GDH is absent, is not known. However, it is conceivable that a high concentration of HP may be detrimental to the amoeba. To support this premise, serine (at a concentration of 5–20 mM) inhibited the trophozoite growth *in vitro* by 50–70% (M. Tokoro and T. Nozaki, unpublished work). Taken together, we propose that, in *E. histolytica*, GDH may have an important role in the maintenance of intracellular serine and HP concentrations, and therefore also in the control of cysteine biosynthesis located downstream of the serine metabolic pathway.

Peculiarity of the amoebic GDH

Whereas some oxidoreductases use both NADH and NADPH coenzymes, others are very specific for either NADH or NADPH. For example, lactate dehydrogenase and malate dehydrogenase only utilize NAD^+/NADH [20], whereas glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase and malic enzymes specifically require $NADP^+$/NADPH. Similarly, EhGDH and the rat-liver GDH [7] are very specific for $NADP^+$/NADPH, whereas bacterial GDH utilizes only NAD^+/NADH [29]. It is conceivable that the replacement of a charged amino acid (aspartate or glutamate), which is well conserved in GDH from plants (e.g. Asp^{196} of cucumber GDH), archaea (Asp^{179} of *A. fulgidus* GDH) and α- and β-proteobacteria (Asp^{178} of *H. methylovorum*; Asp^{177} of *N. meningitidis* GDH) with a non-charged hydroxy amino acid (Ser^{177} of EhGDH; Thr^{183} of human GDH) [5,7] is responsible for the observed high affinity of these enzymes towards $NADP^+$/NADPH. It should also be noted that the PGDH enzymes from a variety of organisms [3,36] possess the conserved aspartate residue, and that all of the PGDHs for which biochemical data are available have a strong preference for NAD^+/ NADH. An extra phosphate group present in $NADP^+$/NADPH might interfere with the interaction between the mammalian and amoebic GDHs and $NADP^+$/NADPH. The carboxylic group of asparate and negative charge of phosphate group at neutral pH produce a repulsion force that may reduce proper binding. Replacement of aspartate with an uncharged polar amino acid, as seen in the amoebic and mammalian GDHs, might enable the phosphate group of $NADP^+$/ NADPH to interact with this amino acid. Mutational analysis of the amoebic and mammalian GDHs should either prove or disprove this hypothesis.

We thank Shin-ichiro Kawazu and Shigeyuki Kano, International Medical Centre of Japan, for use of a fluorimeter. This work was supported by a grant for Precursory Research for Embryonic Science and Technology, Japan Science and Technology Corporation (to T. N.), a fellowship from the Japan Society for the Promotion of Science to V. A. (no. PB01155), a grant for research on emerging and re-emerging infectious diseases from the Ministry of Health, Labour and Welfare of Japan (to T. N.), Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan to T. N. (nos. 15019120 and 15590378), and a grant for Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation to T. N. (SA14706).

REFERENCES

1 Snell, K. (1986) The duality of pathways for serine biosynthesis is a fallacy. Trends Biochem. Sci. **11**, 241–243
2 Chen, K. S. and Lardy, H. A. (1988) Pathway of gluconeogenesis from D- and L-glycerate in rat hepatocytes. Arch. Biochem. Biophys. **265**, 433–440
3 Ho, C. L., Noji, M., Saito, M. and Saito, K. (1999) Regulation of serine biosynthesis in *Arabidopsis*. Crucial role of plastidic 3-phosphoglycerate dehydrogenase in non-photosynthetic tissues. J. Biol. Chem. **274**, 397–402
4 Giafi, C. F. and Rumsby, G. (1998) Kinetic analysis and tissue distribution of human D-glycerate dehydrogenase/glyoxylate reductase and its relevance to the diagnosis of primary hyperoxaluria type 2. Ann. Clin. Biochem. **35**, 104–109
5 Huang, T., Yang, W., Pereira, A. C., Craigen, W. J. and Shih, V. E. (2000) Cloning and characterization of a putative human D-2-hydroxyacid dehydrogenase in chromosome 9q. Biochem. Biophys. Res. Commun. **268**, 298–301
6 Chalmers, R. A., Tracey, B. M., Mistry, J., Griffiths, K. D., Green, A. and Winterborn, M. H. (1984) L-Glyceric aciduria (primary hyperoxaluria type 2) in siblings in two unrelated families. J. Inherit. Metab. Dis. **7**, 133–134
7 Van Schaftingen, E., Draye, J. P. and Van Hoof, F. (1989) Coenzyme specificity of mammalian liver D-glycerate dehydrogenase. Eur. J. Biochem. **186**, 355–359
8 WHO/PAHO/UNESCO Report (1997) A consultation with experts on amebiasis. Epidemiol. Bull. **18**, 13–14
9 Nozaki, T., Asai, T., Kobayashi, S., Ikegami, F., Noji, M., Saito, K. and Takeuchi, T. (1998) Molecular cloning and characterization of the genes encoding two isoforms of cysteine synthase in the enteric protozoan parasite *Entamoeba histolytica*. Mol. Biochem. Parasitol. **97**, 33–44
10 Nozaki, T., Asai, T., Sanchez, L. B., Kobayashi, S., Nakazawa, M. and Takeuchi, T. (1999) Characterization of the gene encoding serine acetyltransferase, a regulated enzyme of cysteine biosynthesis from the protist parasites *Entamoeba histolytica* and *Entamoeba dispar*. Regulation and possible function of the cysteine biosynthetic pathway in *Entamoeba*. J. Biol. Chem. **274**, 32445–32452
11 Nozaki, T., Tokoro, M., Imada, M., Saito, Y., Abe, Y., Shigeta, Y. and Takeuchi, T. (2000) Cloning and biochemical characterization of genes encoding two isozymes of cysteine synthase from *Entamoeba dispar*. Mol. Biochem. Parasitol. **107**, 129–133
11a Tokoro, M., Asai, T., Kobayashi, S., Takeuchi, T. and Nozaki, T. (2003) Identification and characterization of two isoenzymes of methionine γ-lyase from *Entamoeba histolytica*: A key enzyme of sulfur-amino acid degradation in an anaerobic parasitic protist that lacks forward and reverse transsulfuration pathways. J. Biol. Chem., DOI 10.1074/jbc.M212414200
12 Gillin, F. D. and Diamond, L. S. (1980) Attachment of *Entamoeba histolytica* to glass in a defined maintenance medium: specific requirement for cysteine and ascorbic acid. J. Protozool. **27**, 474–478
13 Gillin, F. D. and Diamond, L. S. (1981) *Entamoeba histolytica* and *Giardia lamblia*: effects of cysteine and oxygen tension on trophozoite attachment to glass and survival in culture media. Exp. Parasitol. **52**, 9–17
14 Diamond, L. S., Mattern, C. F. and Bartgis, I. L. (1972) Viruses of *Entamoeba histolytica*. Identification of transmissible virus-like agents. J. Virol. **9**, 326–341
15 Diamond, L. S., Harlow, D. R. and Cunnick, C. C. (1978) A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Trans. R. Soc. Trop. Med. Hyg. **72**, 431–432
16 Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**, 4673–4680
17 Wierenga, R. K., Terpstra, P. and Hol, W. G. (1986) Prediction of the occurrence of the ADP-binding beta alpha beta-fold in proteins, using an amino acid sequence fingerprint. J. Mol. Biol. **187**, 101–107
18 Goldberg, J. D., Yoshida, T. and Brick, P. (1994) Crystal structure of a NAD-dependent D-glycerate dehydrogenase at 2.4 Å resolution. J. Mol. Biol. **236**, 1123–1140

19 Birktoft, J. J. and Banaszak, L. J. (1983) The presence of a histidine–aspartic acid pair in the active site of 2-hydroxyacid dehydrogenases. X-ray refinement of cytoplasmic malate dehydrogenase. J. Biol. Chem. **258**, 472–482
20 Weaver, L. H., Kester, W. R. and Matthews, B. W. (1977) A crystallographic study of the complex of phosphoramidon with thermolysin. A model for the presumed catalytic transition state and for the binding of extended substances. J. Mol. Biol. **114**, 119–132
21 Dijkstra, B. W., Drenth, J. and Kalk, K. H. (1981) Active site and catalytic mechanism of phospholipase A_2. Nature (London) **289**, 604–606
22 Field, J., Rosenthal, B. and Samuelson, J. (2000) Early lateral transfer of genes encoding malic enzyme, acetyl-CoA synthetase and alcohol dehydrogenases from anaerobic prokaryotes to *Entamoeba histolytica*. Mol. Microbiol. **38**, 446–455
23 Nixon, J. E., Wang, A., Field, J., Morrison, H. G., McArthur, A. G., Sogin, M. L., Loftus, B. J. and Samuelson, J. (2002) Evidence for lateral transfer of genes encoding ferredoxins, nitroreductases, NADH oxidase, and alcohol dehydrogenase 3 from anaerobic prokaryotes to *Giardia lamblia* and *Entamoeba histolytica*. Eukaryot. Cell **1**, 181–190
24 Sugimoto, E., Kitagawa, Y., Hirose, M. and Chiba, H. (1972) Mechanisms of inhibition and activation of beef liver D-glycerate dehydrogenase by inorganic anions. J. Biochem. (Tokyo) **72**, 1317–1325
25 Coderch, R., Lluis, C. and Bozal, J. (1979) Effect of salts on D-glycerate dehydrogenase kinetic behavior. Biochim. Biophys. Acta **566**, 21–31
26 Greenler, J. M., Sloan, J. S., Schwartz, B. W. and Becker, W. M. (1989) Isolation, characterization and sequence analysis of a full-length cDNA clone encoding NADH-dependent hydroxypyruvate reductase from cucumber. Plant Mol. Biol. **13**, 139–150
27 Rosenblum, I. Y., Antkowiak, D. H., Sallach, H. J., Flanders, L. E. and Fahien, L. A. (1971) Purification and regulatory properties of beef liver D-glycerate dehydrogenase. Arch. Biochem. Biophys. **144**, 375–383
28 Izumi, Y., Yoshida, T., Kanzaki, H., Toki, S., Miyazaki, S. S. and Yamada, H. (1990) Purification and characterization of hydroxypyruvate reductase from a serine-producing methylotroph, *Hyphomicrobium methylovorum* GM2. Eur. J. Biochem. **190**, 279–284
29 Rumsby, G. (2000) Biochemical and genetic diagnosis of the primary hyperoxaluries: a review. Mol. Urol. **4**, 349–354
30 Veech, R. L., Eggleston, L. V. and Krebs, H. A. (1969) The redox state of free nicotinamide-adenine dinucleotide phosphate in the cytoplasm of rat liver. Biochem. J. **115**, 609–619
31 Lo, H. and Reeves, R. E. (1980) Purification and properties of NADPH:flavin oxidoreductase from *Entamoeba histolytica*. Mol. Biochem. Parasitol. **2**, 23–30
32 Weston, C. J., White, S. A. and Jackson, J. B. (2001) The unusual transhydrogenase of *Entamoeba histolytica*. FEBS Lett. **488**, 51–54
33 Reeves, R. E. (1984) Metabolism of *Entamoeba histolytica* Schaudinn, 1903. Adv. Parasitol. **23**, 105–142
34 McLaughlin, J. and Aley, S. (1985) The biochemistry and functional morphology of the *Entamoeba*. J. Protozool. **32**, 221–240
35 Takeuchi, T., Weinbach, E. C., Gottlieb, M. and Diamond, L. S. (1979) Mechanism of L-serine oxidation in *Entamoeba histolytica*. Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol. **62**, 281–285
36 Achouri, Y., Rider, M. H., Schaftingen, E. V. and Robbi, M. (1997) Cloning, sequencing and expression of rat liver 3-phosphoglycerate dehydrogenase. Biochem. J. **323**, 365–370

Received 29 April 2003/26 June 2003; accepted 23 July 2003
Published as BJ Immediate Publication 23 July 2003, DOI 10.1042/BJ20030630

JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2003, p. 3748–3756 Vol. 41, No. 8
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.8.3748-3756.2003

Geographic Diversity among Genotypes of *Entamoeba histolytica* Field Isolates

Ali Haghighi,[1,2] Seiki Kobayashi,[3] Tsutomu Takeuchi,[3] Nitaya Thammapalerd,[4] and Tomoyoshi Nozaki[1,5]*

Department of Parasitology, National Institute of Infectious Diseases,[1] and Department of Tropical Medicine and Parasitology, Keio University School of Medicine,[3] Shinjuku-ku, and Precursory Research for Embryonic Science and Technology, Japan Science and Technology Corporation, Tachikawa,[5] Tokyo, Japan; Department of Parasitology and Mycology, Shaheed Beheshti University of Medical Sciences, Tehran, Iran[2]; and Department of Microbiology and Immunology, Mahidol University, Bangkok, Thailand[4]

Received 26 December 2002/Returned for modification 10 February 2003/Accepted 27 May 2003

It has been known that only 5 to 10% of those infected with *Entamoeba histolytica* develop symptomatic disease. However, the parasite and the host factors that determine the onset of disease remain undetermined. Molecular typing by using polymorphic genetic loci has been proven to aid in the close examination of the population structure of *E. histolytica* field isolates in nature. In the present study, we analyzed the genetic polymorphisms of two noncoding loci (locus 1-2 and locus 5-6) and two protein-coding loci (chitinase and serine-rich *E. histolytica* protein [SREHP]) among 79 isolates obtained from different geographic regions, mainly Japan, Thailand, and Bangladesh. When the genotypes of the four loci were combined for all isolates that we have analyzed so far (overlapping isolates from mass infection events were excluded), a total of 53 different genotypes were observed among 63 isolates. The most remarkable and extensive variations among the four loci was found in the SREHP locus; i.e., 34 different genotypes were observed among 52 isolates. These results demonstrate that *E. histolytica* has an extremely complex genetic structure independent of geographic location. Our results also show that, despite the proposed transmission of other sexually transmitted diseases, including human immunodeficiency virus infection, from Thailand to Japan, the spectra of the genotypes of the *E. histolytica* isolates from these two countries are distinct, suggesting that the major *E. histolytica* strains prevalent in Japan at present were likely introduced from countries other than Thailand. Although the genetic polymorphism of the SREHP locus was previously suggested to be closely associated with the clinical presentation, e.g., colitis or dysentery and liver abscess, no association between the clinical presentation and the SREHP genotype at either the nucleotide or the predicted amino acid level was demonstrated.

Entamoeba histolytica is the causative agent of an estimated 40 million to 50 million cases of amebic colitis and liver abscess and is responsible for up to 100,000 deaths worldwide each year (6, 28, 33, 41). It has generally been granted that a majority of individuals infected with *E. histolytica* do not develop symptomatic disease (2, 11, 12, 15, 17, 18, 27). In recent cohort studies in Bangladesh, only about 3% of the *E. histolytica*-infected children developed symptoms attributable to amebic dysentery (16, 32). However, the parasite and the host factors that determine the onset of disease, i.e., whether or not amebae initiate tissue invasion and thus cause symptoms, remain undetermined (3, 41). A high degree of heterogeneity in virulence has been demonstrated previously. Interstrain variations in the adhesion of *E. histolytica* trophozoites to human epithelium have been demonstrated for two *E. histolytica* strains (1, 10), in which underrepresentation of the 35-kDa light subunit of the Gal-GalNAc lectin in the avirulent Rahman strain was shown to be correlated with a lack of cytopathic activity (1). Variations in cysteine proteinase expression between highly virulent and avirulent strains were also reported (23). In addition, marked differences in the levels of lipophosphoglycan-like and lipophosphopeptidoglycan molecules were demonstrated between virulent and avirulent strains of *E. histolytica* (24). Interstrain variations in the ability to produce liver abscesses in both gerbils and hamsters are also known. These interstrain variations in in vitro and in vivo virulence have prompted the World Health Organization's expert committee to recommend reinforced efforts through molecular epidemiological studies to determine whether some subgroups of *E. histolytica* are more likely than others to cause invasive disease (41). Another very puzzling question is why certain groups of infected individuals develop extraintestinal amebiasis without showing apparent intestinal symptoms. This observation also appears to be partially explained by interstrain variations in parasite virulence, i.e., tissue and organ tropisms, and host immune backgrounds, as suggested elsewhere (32). DNA typing of polymorphic genetic loci, recently developed by others (2, 7, 13, 43), helped us to closely examine the polymorphic structures of *E. histolytica* field isolates. While a majority of polymorphic genetic loci lack a correlation with virulent (or avirulent) phenotypes, Ayeh-Kumi et al. (2) recently showed that the serine-rich *E. histolytica* protein (SREHP) genotypes of clinical isolates from patients with liver abscesses were distinct from those of clinical isolates from patients with colitis and dysentery in Bangladesh, suggesting that an association between the SREHP genotypes (35) and clinical presentation may exist (2). Extensive genetic polymorphisms in both noncoding and cod-

* Corresponding author. Mailing address: Department of Parasitology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81-3-5285-1111, ext. 2733. Fax: 81-3-5285-1173. E-mail: nozaki@nih.go.jp.

TABLE 1. Background and genotypes of the *E. histolytica* isolates used in this study

No.	Isolate	Isolation		Clinical diagnosis	Serology result[a]	Zymodeme	DNA origin	Type		Chitinase	SREHP
		Location	Date					Locus 1-2	Locus 5-6		
1	TM19	Thailand	1987	Dysentery and colitis	+	II	Xenic	D	A6/Cv	C	12
2	TM20	Thailand	1987	Dysentery and colitis	+	II	Xenic	D	A6/Cv	C	12
3	TM21	Thailand	1987	Dysentery and colitis	+	II	Xenic	B	A6/Cv	C	6
4	TM23	Thailand	1987	Colitis	+	II	Xenic	D	A11v	A	5
5	TM24	Thailand	1987	Dysentery and colitis	+	II	Xenic	I	A7	C	8/16
6	TM25	Thailand	1987	Colitis	+	II	Xenic	D	A11v/A9	A/C	7
7	TM27	Thailand	1987	Colitis	+	II	Xenic	K	A5	C	14
8	TM28	Thailand	1987	Dysentery and colitis	+	II	Xenic	D	A7/Cv	C	9
9	TM29	Thailand	1987	Dysentery and colitis	+	II	Xenic	D	A7/Cv	C	ND
10	TM35	Thailand	NA[b]	ALA	+	ND[c]	Pus	B	A7	E	Irr[d]
11	TM36	Thailand	NA	ALA	+	ND	Pus	B	A6/Cv	C	Irr
12	TM37	Thailand	NA	ALA	+	ND	Pus	L	A7	B	Irr
13	TM51	Thailand	1992	ALA	+	II	Axenic	B	A6/Cv	C	3/F
14	TM53	Thailand	1988	Colitis	+	II	Xenic	D	A7/Cv	C	10/L
15	TM54	Thailand	1988	Colitis	+	II	Xenic	D	A7/Cv	C	10/L
16	TM55	Thailand	1988	Colitis	+	II	Xenic	D	A7/Cv	C	10/L
17	TM58	Thailand	1989	Colitis	+	II	Xenic	D	A7/Cv	C	10/L
18	TM59	Thailand	1989	Dysentery and colitis	+	II	Xenic	D	A7	C	G
18	TM60	Thailand	1989	Colitis	+	II	Xenic	B	A7	E	G
20	TM61	Thailand	1989	Colitis	+	II	Xenic	B	A7	C	G
21	TM62	Thailand	1989	Dysentery and colitis	+	II	Xenic	B	A7	E	G
22	TM63	Thailand	1989	Dysentery and colitis	+	II	Xenic	L	A6/Cv	C	G
23	TM64	Thailand	1989	Colitis	+	II	Xenic	L	A6/C7	C	10/L
24	TM65	Thailand	1989	Dysentery and colitis	+	II	Xenic	D	A9v/A7	E	10/L
25	TM67	Thailand	1989	Colitis	+	II	Xenic	D	A7/Cv	C/F	10/L
26	TM83	Thailand	2001	Dysentery and colitis	+	II	Xenic	D	A7/Cv	C	11
27	TM84	Thailand	2001	Colitis	+	II	Pus	D	A7	C/F	L
28	TM40	Bangladesh	2000	NA	+	ND	Xenic	D	A6	E	15
29	TM41	Bangladesh	2000	NA	+	ND	Xenic	D	A5	C	Mix[e]
30	TM42	Bangladesh	2001	NA	+	ND	Xenic	D	A6	E	13
31	TM43	Bangladesh	2001	NA	−	ND	Xenic	D	A9	C	13
32	TM44	Bangladesh	2001	NA	+	ND	Xenic	D	A8/A5	C	1/4
33	KU6	Ghana	1994	Asymptomatic	+	II	Xenic	L	Cv	C	F
34	KU12	Cambodia	1995	Asymptomatic	+	II	Xenic	D	A5	C	2/17/18
35	PK1	Indonesia	2002	Colitis	ND	ND	Stool	M	A9v/A7	G	H
36	KU33	Institution E[f]	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
37	KU34	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
38	KU35	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
39	KU36	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
40	KU37	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
41	KU38	Institution E	June, 2002	Asymptomatic	−	II	Xenic	F	A5v/Cv	C	K
42	KU39	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
43	KU40	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
44	KU41	Institution E	June, 2002	Asymptomatic	+	VII	Xenic	F	A5v/Cv	C	K
45	KU42	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K

[a] The gel diffusion precipitin test and enzyme-linked immunosorbent assay were used for serology.
[b] NA, not available.
[c] ND, not determined.
[d] Irr, irrelevant PCR fragments.
[e] Mixed, likely a mixture judged by sequencing.
[f] Institution E is located in Yamagata Prefecture, Japan.

ing loci, including the SREHP locus, were previously demonstrated among *E. histolytica* isolates obtained from two social populations (mentally handicapped individuals and homosexual men) in a limited geographic area (domestic cases only in Japan) (14). In the present study, we extend our previous study to answer three specific questions: (i) how polymorphic are the Southeast Asian *E. histolytica* isolates? (ii) how similar or dissimilar are the genotypes of the Southeast Asian strains in comparison to those of the Japanese strains? and (iii) does a correlation exist between the genotypes of the isolates and the clinical presentations that they cause? The results of the present study not only support the previous finding of extensive genetic diversity among *E. histolytica* isolates (2, 7, 13, 14, 43, 44) but also fail to demonstrate a notable association between SREHP genotypes and clinical presentation or geographic origin.

MATERIALS AND METHODS

Clinical specimens. A total of 79 *E. histolytica* isolates, including 45 that were newly isolated, were analyzed in this study (Table 1). Thirty-four strains reported previously (14) were also used in the present study for comparison. Among the 45 new isolates, 10 isolates were obtained from stool samples from asymptomatic but seropositive individuals, with one exception, in an institution for mentally handicapped individuals in Yamagata Prefecture, Japan. Twenty-seven isolates were collected from either stool or liver aspirates from patients who visited outpatient clinics of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Five specimens were kindly provided by Rashidul Haque, Dhaka, Bangladesh, through Mahidol University. Three additional strains were

also isolated from stool samples from two Japanese workers who previously worked in Ghana and Cambodia and a domestic patient from Manado, Indonesia. Four patients had amebic liver abscesses (ALAs), and 24 patients had amebic dysentery and/or colitis. Twelve patients did not show any notable symptoms and thus were considered asymptomatic cyst passers. Identification of individual isolates as *E. histolytica* and not *Entamoeba dispar* was verified as described previously (14) by PCR with *E. histolytica*- and *E. dispar*-specific oligonucleotide primers (see below). A past or present history of invasive amebiasis was verified for 40 patients by serology by the gel diffusion precipitation test (26) and enzyme-linked immunosorbent assay (36). The clinical status of patients infected with five isolates (isolates TM40 to TM44) were not determined. All clinical specimens were collected after informed consent was obtained from the patients.

Cultivation. Xenic and axenic in vitro cultures were established by using Robinson's medium and BI-S-33 medium, respectively, as described previously (9, 29). Most xenic and axenic strains were cryopreserved by the method of Diamond (8) after xenic and axenic cultures were established and were revived 1 to 3 months prior to the present study to minimize possible changes, if any, in the genotypes.

DNA preparation, PCR, and sequence analysis. Total genomic DNA from trophozoites and/or cysts was purified from either cultured trophozoites or clinical specimens as described previously (14). Identification of *E. histolytica* and exclusion of *E. dispar* were verified by PCR with two sets of primers (primers Hsp1 and Hsp2 for *E. histolytica* and primers Dsp1 and Dsp2 for *E. dispar*) under the conditions described previously (44). Individual *E. histolytica* isolates were classified by PCR amplification of four previously described loci, i.e., locus 1-2 and locus 5-6 (43) and the chitinase and SREHP loci (13), by using four sets of oligonucleotides under the PCR conditions described previously (14), except that an annealing temperature of 50°C was used for all four loci. Loci 1-2 and 5-6 are present as tandemly linked multicopies within a >20-kb region (43) and contain tRNA genes (C. G. Clark, personal communication). No polymorphism in the nucleotide sequences was found among individual repeat units in the genome database (data not shown), which is consistent with the finding that PCR fragments containing these loci are homogeneous. Chitinase and SREHP are each apparently present as a single copy per haploid genome; only one copy of chitinase and SREHP each was found in the HM1 genome database. Therefore, although the ploidy of *E. histolytica* has not been determined, each of these genetic markers can be considered to be present as a single copy (per haploid genome). PCR products containing these loci were directly sequenced with an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction II kit (PE Applied Biosystems, Foster City, Calif.) on an ABI PRISM 310 Genetic Analyzer. In cases in which multiple (more than one) bands were recognized after separation by 2% agarose gel electrophoresis, each DNA fragment was excised and sequenced separately, as described previously (14). The expected frequency of mutations with HotStar *Taq*DNA polymerase (Qiagen, Tokyo, Japan) was 2×10^{-5} (data not shown). We also tried to minimize the cycle numbers to avoid the accumulation of PCR products, which is known to increase the chance of introduction of mutations. Thus, when the lengths of the PCR fragments amplified in this study are considered (120 to 490 bp), the chance that mutations were introduced by PCR was negligible. The sequences obtained were manually edited and aligned by using DNASIS (version 3.7; Hitachi, Yokohama, Japan).

Restriction length polymorphism (RFLP) analysis of SREHP locus. Approximately 0.1 μg of the SREHP PCR products was digested with 3 U of *Alu*I (Takara, Tokyo, Japan) in a volume of 20 μl at 37°C for 2 to 16 h. About 5 μl of the *Alu*I-digested material was electrophoresed in 12% polyacrylamide gels (30). To visualize the DNA, the gels were stained by use of a silver staining kit (Pharmacia Biotech, Tokyo, Japan).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in the present work have been submitted to the GenBank/EMBL/DDBJ database under accession numbers AB096653 to AB096676.

RESULTS

High-resolution genotyping of *E. histolytica* field isolates and identification of new genotypes. It was previously shown that the levels of genetic polymorphism of the four polymorphic genetic loci mentioned above among *E. histolytica* isolates in Japan are extremely high (14). However, it is unknown if genetic polymorphisms also exist among the amebic isolates within the areas of developing countries where *E. histolytica* is endemic, e.g., Southeast Asian countries, and, if so, to what extent. Thus, we conducted high-resolution genotyping of these polymorphic loci for a large number of the *E. histolytica* isolates obtained from the area of endemicity. We attempted to answer the following questions: (i) how polymorphic are the Southeast Asian *E. histolytica* strains? (ii) how similar or dissimilar are the genotypes of the Southeast Asian ameba strains in comparison to those of the Japanese strains? and (iii) does any correlation exist between the genotypes of the isolates and the clinical presentations that they cause? We chose Thai isolates for analysis since it has been demonstrated that some of the human immunodeficiency virus (HIV) strains present in Japan were imported from Thailand (5, 20, 40). We amplified the four loci by PCR and sequenced individual fragments from 45 clinical isolates (27 isolates from Thailand; 5 isolates from Bangladesh; 10 isolates from Japan; and 1 isolate each from Cambodia, Indonesia, and Ghana). The profiles of the PCR fragments on agarose gels are shown in Fig. 1A for representative isolates (only data for new genotypes of SREHP are shown; see reference 14 for the previously identified genotypes). After sequencing, we identified among these 45 isolates 3 novel genotypes for locus 1-2 (genotypes K, L, and M), 2 novel genotypes for locus 5-6 (genotypes A11v and A9v), 1 novel genotype for chitinase (genotype G), and 18 novel genotypes for SREHP (genotypes 1 to 18) (a schematic diagram of all SREHP genotypes only is shown in Fig. 2; those of the other loci are not shown; all the sequence information was deposited in the GenBank/EMBL/DDBJ database). When these data are combined with previous data (14), we have identified among our 79 isolates and 4 previously reported isolates (13, 39) 13 different genotypes in locus 1-2, 15 different genotypes in locus 5-6, 9 different genotypes in the chitinase locus (data not shown), and 37 different genotypes in the SREHP locus (Fig. 2). The deduced peptide sequences of the chitinase and SREHP loci were also analyzed. The total number of SREHP genotypes based on the predicted amino acid sequences (31) was only slightly smaller than the number of SREHP genotypes based on the nucleotide sequences of individual PCR fragments (37) (data not shown), whereas the total number of chitinase genotypes was identical between the nucleotide and the predicted amino acid sequences. Among 27 Thai isolates, we identified 5, 7, 6, and 13 distinct genotypes for locus 1-2, locus 5-6, the chitinase locus, and the SREHP locus, respectively (4 isolates were excluded from the analysis of the SREHP locus for the reason explained in footnotes *c* and *d* of Table 1), suggesting that the extent of polymorphism is comparable between the isolates from Thailand and those from the Japanese homosexual men.

Heterozygosity of chitinase and SREHP. Although the chitinase locus was previously found to be homozygous, with one exception (type A/C) (14), we have found three additional isolates with two distinct chitinase genes (previously identified as genotype A/C and a new genotype, C/F). The demonstration of double SREHP genotypes in several isolates (i.e., isolates TM24, TM44, TM51 to TM58, and TM64 to TM67) strongly argues for the heterozygosity of this gene, as suggested previously (2, 13, 14). However, one isolate (isolate KU12) showed triple SREHP fragments on agarose gel electrophoresis corresponding to genotypes 2, 17, and 18.

Intergeographic differences in distributions of genotypes of each polymorphic locus. To examine the similarities and dif-

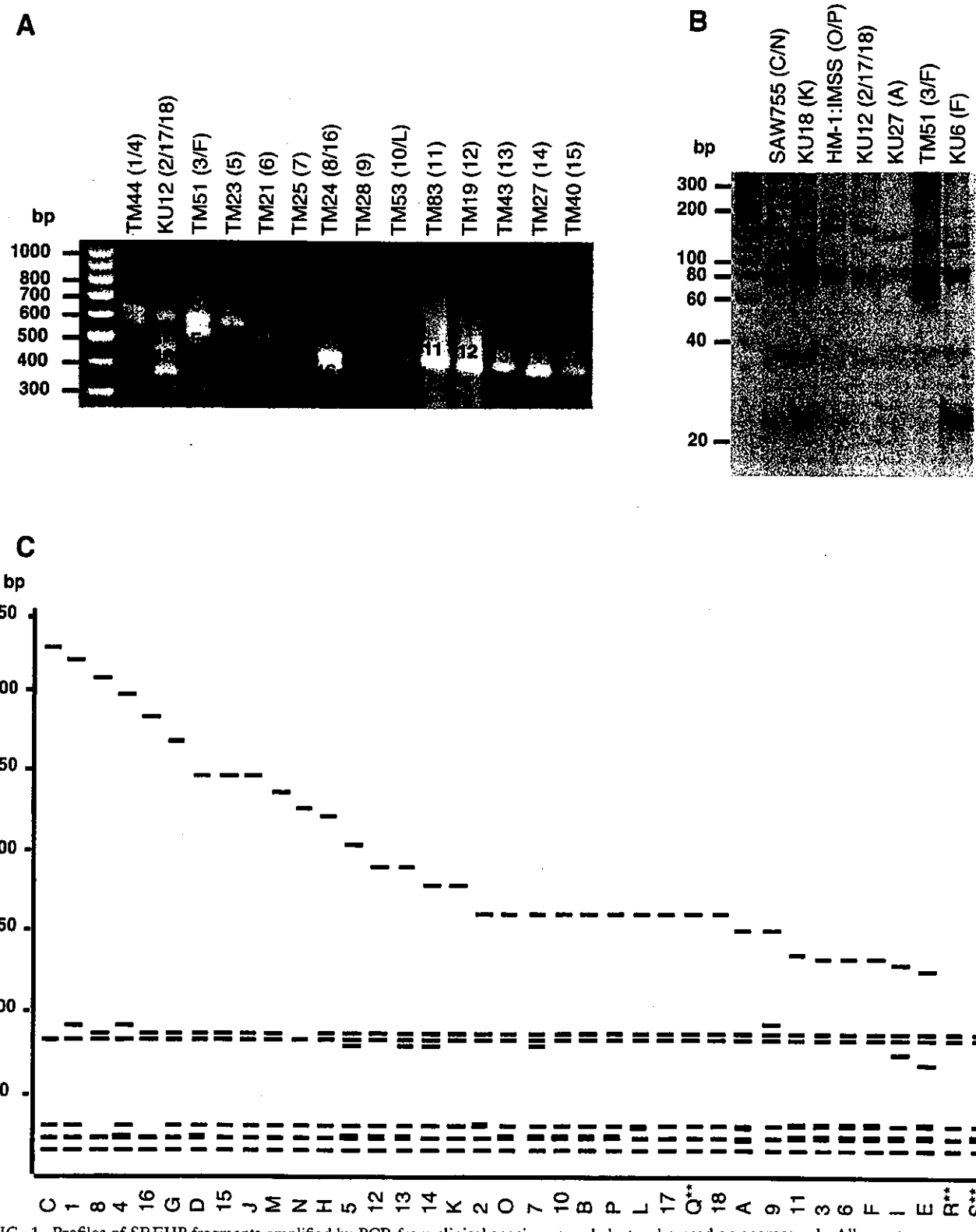
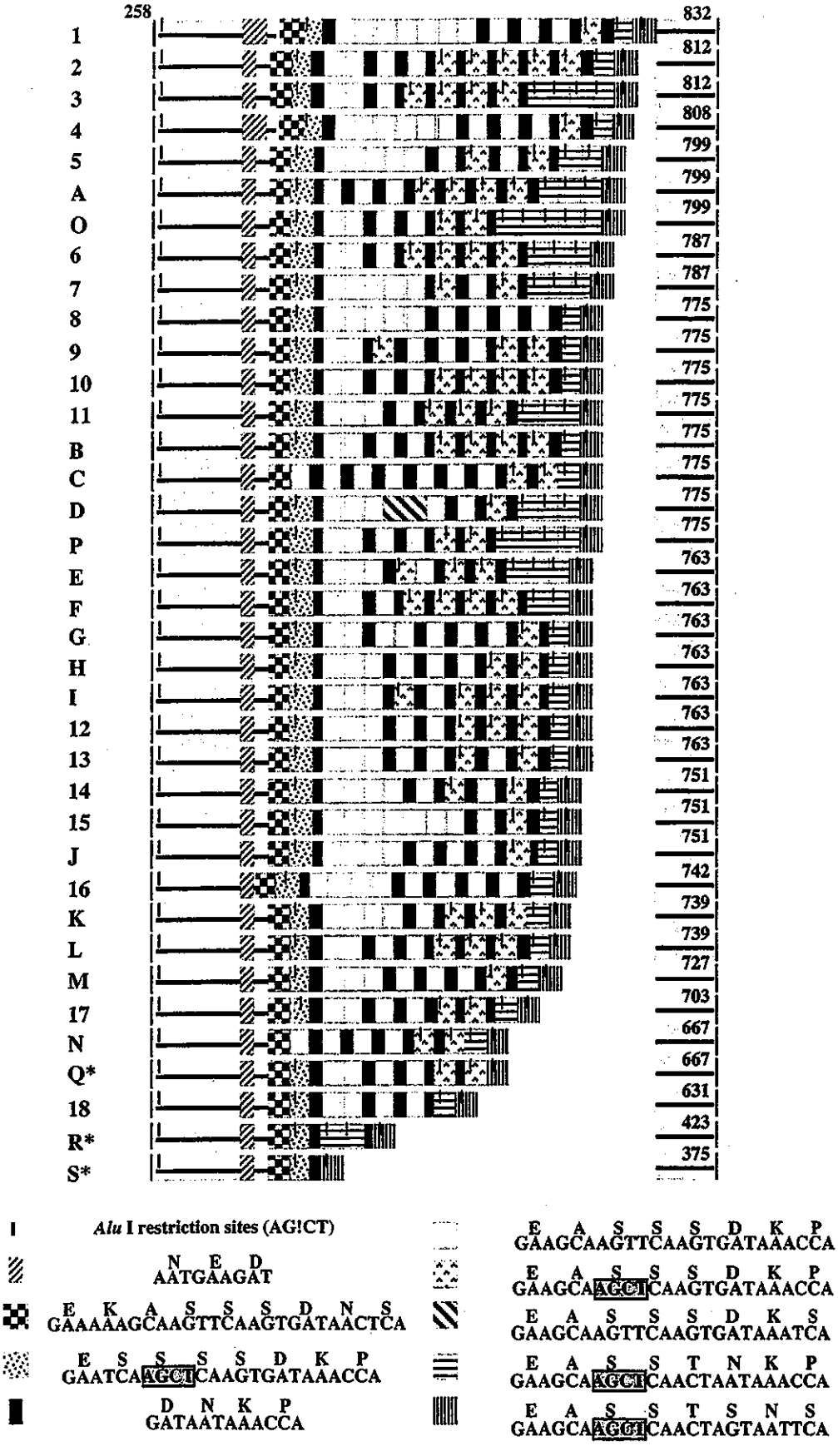


FIG. 1. Profiles of SREHP fragments amplified by PCR from clinical specimens and electrophoresed on agarose gels. All genotypes reported previously (14) and reported by Ghosh et al. (13) are given letter designations (A to S [14; this study]), and new genotypes designated in the present study are given number designations (1 to 18) in this and the other figures. (A) Agarose gel electrophoresis of undigested SREHP PCR fragments from representative *E. histolytica* isolates. Only the results for representative isolates that belong to each genotype (presented in parentheses) are shown. (B) Polyacrylamide gel electrophoresis of PCR-amplified and *Alu*I-digested SREHP fragments from selected isolates. (C) Schematic representation of *Alu*I digests of all genotypes. Note that the individual genotype is designated for each DNA fragment. Double asterisks indicate that more information can be found in reference 13.


258
832
1
812
2
812
3
808
4
799
5
799
A
799
O
787
6
787
7
775
8
775
9
775
10
775
11
775
B
775
C
775
D
775
P
763
E
763
F
763
G
763
H
763
I
763
12
763
13
751
14
751
15
751
J
742
16
739
K
739
L
727
M
703
17
667
N
667
Q*
631
18
423
R*
375
S*
I Alu I restriction sites (AG!CT)
N E D
AATGAAGAT
E K A S S S D N S
GAAAAAGCAAGTTCAAGTGATAACTCA
E S S S S D K P
GAATCAAGCTCAAGTGATAAACCA
D N K P
GATAATAAACCA
E A S S S D K P
GAAGCAAGTTCAAGTGATAAACCA
E A S S S D K P
GAAGCAAGCTCAAGTGATAAACCA
E A S S S D K S
GAAGCAAGTTCAAGTGATAAATCA
E A S S T N K P
GAAGCAAGCTCAACTAATAAACCA
E A S S T S N S
GAAGCAAGCTCAACTAGTAATTCA

ferences among the isolates from Japan, Thailand, and other countries, the distributions of the genotypes of the four loci for all 79 isolates analyzed in our previous and present studies, together with those of four strains reported by others (13, 43), were examined (Fig. 3). We excluded 16 of 21 Japanese isolates from mentally handicapped individuals from these analyses because the mass infections were likely attributable to single strains (14), and therefore, inclusion of these genotypes would likely bias the outcomes of the analyses; e.g., locus 1-2 genotype F, locus 5-6 genotype A5v/Cv, chitinase locus genotype C, and SREHP locus genotype K were found in 16 isolates from mentally handicapped individuals. Marked differences in the histograms of locus 1-2, locus 5-6, and the SREHP locus were readily recognized, whereas the distributions of the chitinase locus genotypes were similar among the three groups of isolates. Some differences were very striking; e.g., genotype B of locus 1-2 represents a dominant type among the Japanese isolates (about 40%), while genotype D of locus 1-2 is dominant among the isolates from Thailand and other countries (about 50%) (Fig. 3A). Marked differences in histograms were found not only in a homozygous locus, i.e., locus 1-2, but also in a heterozygous noncoding locus, i.e., locus 5-6. A histogram showing the frequencies of locus 5-6 (Fig. 3B) showed that locus 5-6 genotype A5v or A7 was detected in about 20% of the Japanese isolates, whereas genotypes A6/Cv, A7, and A7/Cv were dominant (about 30%) among the Thai isolates. The results were almost similar when the allelic types, but not combinations of alleles, of locus 5-6 were compared (data not shown). Locus 5-6 allelic genotypes A5, A5v, and A7 were dominant and were found in about 60% of the Japanese isolates, while allelic type A7 was dominant (45%) among the Thai isolates. In contrast to the notable differences in the genotype distributions of loci 1-2 and 5-6, the chitinase locus genotypes showed similar distributions among the three groups (Fig. 3C). The extent of genetic variation of the SREHP locus is much higher than those of the other three loci (Fig. 3D); only one SREHP locus genotype (genotype 7) was shared by the Japanese and Thai isolates.

RFLP analysis of *Alu*I digests of the SREHP locus. Ayeh-Kumi et al. (2) recently demonstrated, using RFLP analysis of the *Alu*I digests of the SREHP PCR fragments (7), polymorphic patterns among clinical isolates from Bangladesh. They also reported that the majority (92%) of isolates from liver abscesses showed patterns distinct from those of the intestinal isolates. On the basis of these data, they proposed that particular SREHP locus genotypes and RFLP patterns may be closely associated with virulence. To further test this hypothesis, we first conducted a computational RFLP analysis based on the nucleotide sequences of the SREHP loci of all isolates that we obtained (Fig. 1C; only representative patterns are shown). Notable differences in the RFLP patterns were seen among these genotypes; the number of the patterns, however, decreased significantly compared to the number obtained by genotyping based on nucleotide sequences (n = 34 to 24 patterns) (Fig. 2). We also found that the histograms of the RFLP patterns between Japanese and Thai isolates differed significantly (data not shown), which was similar to the observation for the comparisons at the nucleotide level. However, we were unable to find any RFLP patterns that correlated with clinical presentations (e.g., ALA, colitis, or cyst carrier), the backgrounds of the patients (e.g., homosexual men or mentally handicapped individuals), or geographic origin. These computational RFLP analyses of the SREHP locus were also verified by *Alu*I digestion and polyacrylamide gel electrophoresis analyses of the PCR fragments from several representative isolates (Fig. 1B).

DISCUSSION

Using high-resolution genotyping based on the nucleotide sequences of four polymorphic loci of *E. histolytica*, we were able to demonstrate that this parasite from an area of endemicity in Southeast Asia has an extremely polymorphic genetic structure; e.g., 21 different combinations of genotypes were found among the 27 isolates obtained from Thailand. In combination with previous results (14), 53 combinations of genotypes were observed among 63 isolates. (Note that 16 isolates from institutions for mentally handicapped individuals [e.g., KU13, KU19 to KU22, KU28, and KU29 [14] and KU34 to KU42] were excluded for the reason described in Results.) This, together with previous work (14), in which an extensive polymorphism of the amebic strains from Japanese homosexual men was shown, reinforces the premise that *E. histolytica* has an extremely complex genetic structure independent of geographic location.

On the basis of the close social and economic relationship between Japan and Thailand and the fact that (i) sexual intercourse between homosexual men is closely associated with both HIV and amebic infections and (ii) comparison of genotypes between Thai and Japanese HIV strains indicates that a proportion of Japanese HIV strains were imported from Thailand (19, 21, 25, 37, 40), we hypothesized that the Japanese and Thai *E. histolytica* isolates might reveal a similar spectrum of genotypes that is indicative of similarities in the population structures of the *E. histolytica* strains between the two countries. However, our results appeared to argue against this hypothesis. Although notable similarities in the genotypes of locus 1-2, locus 5-6, and the chitinase locus were found between the Japanese and Thai isolates, polymorphisms in the SREHP locus have been found to be very extensive: only one of the Thai isolates showed an SREHP genotype identical to that of the Japanese isolates. When the genotypes of all four loci were combined, none of the Thai isolates had genotypes identical to those of the Japanese strains. Extensive polymor-

FIG. 2. Schematic representation of the polymorphisms in the repeat-containing region of the SREHP gene demonstrated among all isolates analyzed in the previous (14) and present studies and reference strains. *Alu*I restriction sites are depicted by vertical lines. The numbers shown correspond to the nucleotides of strain HM1 (accession no. M80910). Gaps were manually introduced to optimize alignments. Conserved regions are highlighted with gray rectangles. The nucleotide and deduced amino acid sequences of tri-, tetra-, octa-, and nonapeptide repeats are shown below. Also note that a previously unidentified tripeptide repeat unit is also included here. Asterisks next to the genotypes indicate that more information can be found in reference 13.

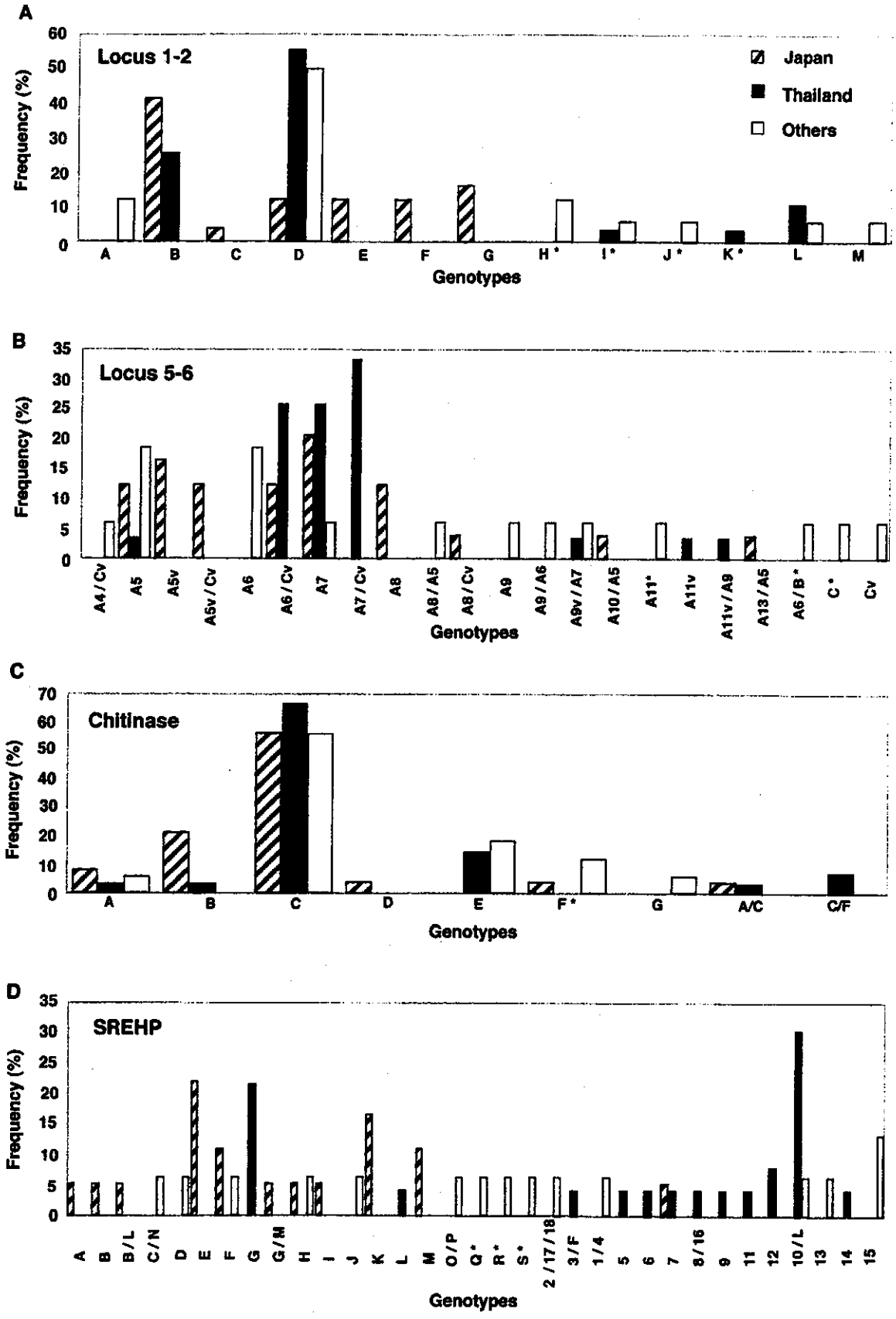


FIG. 3. Histograms showing genotype distributions of locus 1-2 (A), locus 5-6 (B), the chitinase locus (C), and the SREHP locus (D) from representative isolates from Japan, Thailand, and other countries. Only results for representative isolates of each genotype are shown. Asterisks indicate that more information can be found in references 13 and 43. Note that 16 of 21 Japanese isolates obtained from mentally handicapped individuals, which showed identical genotypes for all four loci, were excluded from these analyses (as explained in Results). Also note that 11 isolates showing either mixed or irrelevant PCR bands as judged by sequencing were excluded from analysis of the SREHP locus.

phism of the SREHP locus at the nucleotide level was shown previously (14); e.g., six distinct genotypes were found among 11 isolates from Japanese male homosexual men. Although genetic polymorphisms in a restricted geographic location in the area of endemicity have been reported by Ayeh-Kumi et al. (2), it is conceivable that those investigators underestimated the degree of polymorphism due to a lack of resolution of their analytical methods (PCR amplification of only the SREHP locus, followed by RFLP analysis on agarose gels). On the basis of the nucleotide sequences of all SREHP fragments and computational analyses of virtual RFLPs, we found that several SREHP genotypes would have been indistinguishable by RFLP analysis (e.g., genotypes D, 15, and J and genotypes 10, B, P, L, 17, Q, and 18; Fig. 1C). We should also mention that a histogram of the SREHP genotypes of the Thai isolates was also significantly different from that of the isolates from neighboring countries, including Bangladesh and Indonesia (Fig. 3D), although the number of isolates from those countries was too small to draw a definitive conclusion.

A high degree of genetic polymorphism in the repeat-containing region of SREHP raised a number of questions regarding the function of this protein and its association with the virulence and pathophysiology of *E. histolytica*. One of the most obvious questions is why the degree of polymorphism of SREHP is higher than those of the chitinase and noncoding loci. Although Ayeh-Kumi et al. (2) suggested that certain SREHP genotypes are more likely associated with ALA, this premise was not supported in the present study. We did not find any particular SREHP type, at either the nucleotide sequence level or the predicted amino acid sequence level, in association with clinical presentation. This is not likely due to either a lack of resolution of their analytical methods or differences in geographic backgrounds; the heterogeneity of RFLP patterns that Ayeh-Kumi et al. (2) reported (34 distinct patterns among 54 isolates) was comparable to the polymorphism of the SREHP locus at the nucleotide level that we report here. It has previously been demonstrated (31, 34) that SREHP is highly immunogenic because it possesses a number of conserved epitopes and that more than 80% of the individuals with ALA possess antibodies to SREHP, highlighting this protein as an important vaccine candidate. Together with the remarkable polymorphisms within the repeat-containing region of SREHP, as shown in this and other studies, these data strongly suggest that this polymorphism likely has a biological role, including immune evasion, as suggested elsewhere (34, 45, 46). However, the nucleotide and amino acid polymorphisms of the SREHP locus are too extensive to discuss the biological significance of these polymorphisms and their constraints on SREHP as a functional protein.

To verify the stability of the genotype observed for each isolate, we examined the nucleotide sequences of the four loci from four different isolates (isolates KU14, KU18, and KU26 [14] and isolate KU36) using xenic cultures, monoxenic cultures (cultivation with *Crithidia fasciculata*), and axenic cultures. We found no change in the genotypes of any of the four loci in these four strains (data not shown), indicating, together with previous findings (7, 44), that the nucleotide sequences of these loci are stable under a variety of conditions, e.g., long-term cultivation, axenization, cell cloning, and animal passage.

We should also note that the genotypes of the isolates obtained from institution E (locus 1-2, genotype F; locus 5-6, genotype A5v/Cv; the chitinase locus, genotype C; and the SREHP locus, genotype K) are identical to those of isolates from two other institutions for mentally handicapped individuals (institutions B and C) (14). These isolates were obtained from independent mass infection events at remote geographic locations (Kanagawa, Shizuoka, and Yamagata Prefectures in Japan, approximately 540 km apart) at different times (1994, 2000, and 2002). This finding further supports the premise that the genotypes of the *E. histolytica* isolates are stable after human transmission.

We also present further evidence of the heterozygosity of the chitinase and SREHP loci. The presence of multiple isoenzymes showing distinct affinities for substrates and the inhibitor allosamidin was demonstrated in *Entamoeba invadens* (38), posing the question of why only a small proportion (5%) of *E. histolytica* isolates possess multiple chitinase isoenzymes. The presence of multiple chitinase isoenzymes may be beneficial for the ameba since a broader substrate range may be covered by isoenzymes possessing distinct properties, as shown for two isoforms from *Serratia marcescens* (4), *E. invadens* (38), and *Plasmodium gallinaceum* (39). In contrast to chitinase genes, the SREHP locus was found to be heterozygous in approximately 29% of all isolates, suggesting the biological significance of heterozygosity in this gene. The presence of the triple SREHP genes in isolate KU12 cannot be due to a mixed culture or cross contamination since (i) none of these three bands were found in the other isolates and (ii) none of the other loci, i.e., locus 1-2, locus 5-6, and the chitinase locus, showed mixed patterns. This is inconsistent with the previous finding indicating that the SREHP gene appears to be present in a single copy (22). Thus, this isolate may represent a triploid or aneuploid, although the ploidy of reference strain HM1 was previously suggested to be at least four (42).

ACKNOWLEDGMENTS

We thank Rashidul Haque, International Center for Diarrheal Disease Research, Dhaka, Bangladesh, and Mihoko Imada, Japan International Cooperation Agency, Manado, Indonesia, for providing DNA from *E. histolytica* isolates; Yumiko Saito-Nakano and Yasuo Shigeta, National Institute of Infectious Diseases of Japan, for technical support; and Shin-ichiro Kawazu and Shigeyuki Kano, International Medical Center of Japan, for technical help in sequencing.

This work was partially supported by a fellowship (fellowship 200005) from the Japan Society for the Promotion of Science to A.H., a grant for research on emerging and reemerging infectious diseases from the Ministry of Health, Labour and Welfare of Japan to T.N., a grant (grant SA14706) for research on health sciences focusing on drug innovation from the Japan Health Sciences Foundation to T.N., and a grant for Precursory Research for Embryonic Science and Technology, Japan Science and Technology Corporation, to T.N.

REFERENCES

1. **Ankri, S., F. Padilla-Vaca, T. Stolarsky, L. Koole, U. Katz, and D. Mirelman.** 1999. Antisense inhibition of the light subunit (35 kDa) of the Gal/GalNac lectin complex inhibits *Entamoeba histolytica* virulence. Mol. Microbiol. 33: 327–337.
2. **Ayeh-Kumi, P. F., I. M. Ali, L. A. Lockhart, C. A. Gilchrist, W. A. Petri, Jr., and R. Hague.** 2001. *Entamoeba histolytica*: genetic diversity of clinical isolates from Bangladesh as demonstrated by polymorphisms in the serine-rich gene. Exp. Parasitol. 99:80–88.
3. **Blessmann, J., L. P. Van, P. A. Nu, H. D. Thi, B. Muller-Myhsok, H. Buss, and E. Tannich.** 2002. Epidemiology of amebiasis in a region of high incidence of amebic liver abscess in central Vietnam. Am. J. Trop. Med. Hyg. 66:578–583.
4. **Bruberg, M. B., I. F. Nes, and V. G. Eijsink.** 1996. Comparison studies of chitinases A and B from *Serratia marcescens*. Microbiology 142:1581–1589.

5. **Cassol, S., B. G. Weniger, P. G. Babu, M. O. Salminen, X. Zheng, M. T. Htoon, A. Delaney, M. O'Shaughnessy, and C. Y. Ou.** 1996. Detection of HIV type 1 *env* subtypes A, B, C, and E in Asia using dried blood spots: a new surveillance tool for molecular epidemiology. AIDS Res. Hum. Retrovir. **10**:1435–1441.
6. **Clark, C. G.** 2000. The evolution of *Entamoeba*, a cautionary tale. Res. Microbiol. **151**:599–603.
7. **Clark, C. G., and L. S. Diamond.** 1993. *Entamoeba histolytica*: a method for isolate identification. Exp. Parasitol. **77**:450–455.
8. **Diamond, L. S.** 1995. Cryopreservation and storage of parasitic protozoa in liquid nitrogen. J. Eukaryot. Microbiol. **42**:585–590.
9. **Diamond, L. S., D. R. Harlow, and C. C. Cunnick.** 1978. A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Trans. R. Soc. Trop. Med. Hyg. **72**:431–432.
10. **Flores-Romo, L., T. Estrada-Garcia, M. Shibayama-Salas, R. Campos-Rodriguez, K. Bacon, A. Martinez-Palomo, and V. Tsutsumi.** 1997. In vitro *Entamoeba histolytica* adhesion to human endothelium: a comparison using two strains of different virulence. Parasitol. Res. **83**:397–400.
11. **Gathiram, V., and T. F. H. G. Jackson.** 1985. Frequency distribution of *Entamoeba histolytica* zymodemes in rural South Africa population. Lancet **i**:719–721.
12. **Gathiram, V., and T. F. H. G. Jackson.** 1987. A longitudinal study of asymptomatic carriers of pathogenic zymodemes of *Entamoeba histolytica*. S. Afr. Med. J. **72**:669–672.
13. **Ghosh, S., M. Frisardi, L. Ramirez-Avila, S. Descoteaux, K. Sturm-Ramirez, O. A. Newton-Sanchez, J. I. Santos-Preciado, C. Ganguly, A. Lohia, S. Reed, and J. Samuelson.** 2000. Molecular epidemiology of *Entamoeba* spp.: evidence of a bottleneck (demographic sweep) and transcontinental spread of diploid parasites. J. Clin. Microbiol. **38**:3815–3821.
14. **Haghighi, A., S. Kobayashi, T. Takeuchi, G. Masuda, and T. Nozaki.** 2002. Remarkable genetic polymorphism among *Entamoeba histolytica* isolates from a limited geographic area. J. Clin. Microbiol. **40**:4081–4090.
15. **Haque, R., I. M. Ali, and W. A. Petri, Jr.** 1999. Prevalence and immune response to *Entamoeba histolytica* infection in preschool children in Bangladesh. Am. J. Trop. Med. Hyg. **60**:1031–1034.
16. **Haque, R., I. M. Ali, R. B. Sack, B. M. Farr, G. Ramakrishnan, and W. A. Petri, Jr.** 2001. Amebiasis and mucosal IgA antibody against the *Entamoeba histolytica* adherence lectin in Bangladeshi children. J. Infect. Dis. **183**:1787–1793.
17. **Haque, R., P. Duggal, I. M. Ali, M. B. Hossain, D. Mondal, R. B. Sack, B. M. Farr, T. H. Beaty, and W. A. Petri, Jr.** 2002. Innate and acquired resistance to amebiasis in Bangladeshi children. J. Infect. Dis. **186**:547–552.
18. **Jackson, T. F. H. G., V. Gathiram, and A. E. Simjee.** 1985. Seroepidemiological study of antibody responses to the zymodemes of *Entamoeba histolytica*. Lancet **i**:716–719.
19. **Joshi, M., A. S. Chowdhary, P. J. Dalal, and J. K. Maniar.** 2002. Parasitic diarrhoea in patients with AIDS. Natl. Med. J. India **15**:72–74.
20. **Kihara, M.** 1993. On the recent abrupt rise in the number of foreign females in the AIDS surveillance in Japan. Nippon Koshu Eisei Zasshi **40**:1001–1005.
21. **Law, C. L., J. Walker, and M. H. Qassim.** 1991. Factors associated with the detection of *Entamoeba histolytica* in homosexual men. Int. J. STD AIDS **2**:346–350.
22. **Li, E., C. Kunz-Jenkins, and S. L. Stanley, Jr.** 1992. Isolation and characterization of genomic clones encoding a serine-rich *Entamoeba histolytica* protein. Mol. Biochem. Parasitol. **50**:355–358.
23. **McGowan, K., C. F. Deneke, G. M. Thorne, and S. L. Gorbach.** 1982. *Entamoeba histolytica* cytotoxin: purification, characterization, strain virulence, and protease activity. J. Infect. Dis. **146**:616–625.
24. **Moody, S., S. Becker, Y. Nuchamowitz, and D. Mirelman.** 1997. Virulent and avirulent *Entamoeba histolytica* and *E. dispar* differ in their cell surface phosphorylated glycolipids. Parasitology **114**:95–104.
25. **Ohnishi, K., and M. Murata.** 1997. Present characteristic of symptomatic amebiasis due to *Entamoeba histolytica* in the east-southeast area of Tokyo. Epidemiol. Infect. **119**:363–367.
26. **Ouchterlony, O.** 1966. The antigenic pattern of immunoglobulins. G. Mal. Infett. Parasit. **18**(Suppl. 1):942–948.
27. **Petri, W. A., Jr.** 2002. Pathogenesis of amebiasis. Curr. Opin. Microbiol. **5**:443–447.
28. **Petri, W. A., Jr., R. Haque, D. Lyerly, and R. R. Vines.** 2000. Estimating the impact of amebiasis on health. Parasitol. Today **16**:320–321.
29. **Robinson, G. L.** 1968. Laboratory cultivation of some human parasitic amoebae. J. Gen. Microbiol. **53**:69–79.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Stanley, S. L., Jr.** 1997. Progress towards development of a vaccine for amebiasis. Clin. Microbiol. Rev. **10**:637–649.
32. **Stanley, S. L., Jr.** 2001. Protective immunity to amebiais: new insights and new challenges. J. Infect. Dis. **184**:504–506.
33. **Stanley, S. L., Jr., and S. L. Reed.** 2001. Microbes and microbial toxin: paradigms for microbial mucosal I interactions. VI. *Entamoeba histolytica*: parasite-host interactions. Am. J. Physiol. Gastrointest. Liver Physiol. **280**: G1049–G1054.
34. **Stanley, S. L., Jr., T. F. H. G. Jackson, S. L. Reed, J. Calderon, C. Kunz-Jenkins, V. Gathiram, and E. Li.** 1991. Serodiagnosis of invasive amebiasis using a recombinant *Entamoeba histolytica* protein. JAMA **266**:1984–1986.
35. **Stanley, S. L., Jr., A. Becker, C. Kunz-Jenkins, L. Foster, and E. Li.** 1990. Cloning and expression of a membrane antigen of *Entamoeba histolytica* possessing multiple tandem repeats. Proc. Natl. Acad. Sci. USA **87**:4976–4980.
36. **Takeuchi, T., H. Matsuda, E. Okuzawa, T. Nozaki, S. Kobayashi, and H. Tanaka.** 1988. Application of a micro enzyme-linked immunosorbent assay (ELISA) to detection of anti-amoebic antibody in various forms of amoebic infection. Jpn. J. Exp. Med. **58**:229–232.
37. **Takeuchi, T., Y. Miyahira, S. Kobayashi, T. Nozaki, S. R. Motta, and J. Matsuda.** 1990. High seropositivity for *Entamoeba histolytica* infection in Japanese homosexual men: further evidence for the occurrence of pathogenic strains. Trans. R. Soc. Trop. Med. Hyg. **84**:250–251.
38. **Villagomez-Castro, J. C., and E. Lopez-Romero.** 1996. Identification and partial characterization of three chitinase forms in *Entamoeba invadens* with emphasis on their inhibition by allosamidin. Antonie Leeuwenhoek **70**:41–48.
39. **Vinetz, J. M., J. G. Valenzuela, C. A. Specht, L. Aravind, R. C. Langer, J. M. Ribeiro, and D. C. Kaslow.** 2000. Chitinases of the avian malaria parasite *Plasmodium gallinaceum*, a class of enzymes necessary for parasite invasion of the mosquito midgut. J. Biol. Chem. **275**:10331–10341.
40. **Weniger, B. G., Y. Takebe, C.-Y. Ou, and S. Yamazaki.** 1994. The molecular epidemiology of HIV in Asia. AIDS **8**(Suppl. 2):S13–S28.
41. **WHO News and Activities.** 1997. *Entamoeba* taxonomy. Bull. W. H. O. **75**:291–292.
42. **Willhoeft, U., and E. Tannich.** 1999. The electrophoretic karyotype of *Entamoeba histolytica*. Mol. Biochem. Parasitol. **99**:41–53.
43. **Zaki, M., and C. G. Clark.** 2001. Isolation and characterization of polymorphic DNA from *Entamoeba histolytica*. J. Clin. Microbiol. **39**:897–905.
44. **Zaki, M., P. Meelu, W. Sun, and C. G. Clark.** 2002. Simultaneous differentiation and typing of *Entamoeba histolytica* and *Entamoeba dispar*. J. Clin. Microbiol. **40**:1271–1276.
45. **Zhang, T., and S. L. Stanley, Jr.** 1999. DNA vaccination with the serine-rich *Entamoeba histolytica* protein (SREHP) prevents amebic liver abscess in rodent models of disease. Vaccine **18**:868–874.
46. **Zhang, T., P. R. Ciesiak, L. Foster, C. Kunz-Jenkins, and S. L. Stanley, Jr.** 1994. Antibody to the serine-rich *Entamoeba histolytica* protein (SREHP) prevents amebic liver abscess in severe combined immunodeficient (SCID) mice. Parasite Immunol. **16**:225–230.

J. Parasitol., 90(?), 2004, pp. 000–000

AXENIC CULTIVATION OF *ENTAMOEBA DISPAR* IN NEWLY DESIGNED YEAST EXTRACT–IRON–GLUCONIC ACID–DIHYRDOXYACETONE–SERUM MEDIUM

Seiki Kobayashi, Eiko Imai, Ali Haghighi*, Shaden A. Khalifa, Hiroshi Tachibana†, and Tsutomu Takeuchi
Department of Tropical Medicine and Parasitology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. *e-mail: skobaya@sc.itc.keio.ac.jp*

ABSTRACT: Yeast extract–iron–gluconic acid–dihyrdoxyacetone-serum medium that allows axenic cultivation of *Entamoeba dispar* was designed based on casein-free yeast extract-iron-serum (YI-S) medium, and the usefulness of the medium was assessed. The main differences from YI-S medium are replacement of glucose by gluconic acid, addition of dihydroxyacetone and D-galacturonic acid monohydrate, and sterilization by filtration. This medium promoted the axenic growth of 5 strains of *E. dispar* (2 strains of nonhuman primate isolates and 3 strains of human isolates). In addition, to clarify the biological basis for the growth of *E. dispar* in this medium, analyses of relevant enzymes on the glycolytic pathway of the amoebae as well as of the protozoans that are the best culture supplement for amoebae are being performed.

ED: Please note that the abbreviation YIGADHA-S in lllle has been expanded. Please check. Author: This article has been lightly edited for grammar, style, and usage. Please compare it with your original document and make changes on these pages. Please limit your corrections to substantive changes that affect meaning. If no change is required in response to a question, please write "OK as set" in the margin. Copy editor

After axenic cultivation of *Entamoeba dispar* (strain: SAW760RRcloneAR) was reported (Clark, 1995) in casein-free yeast extract–iron–serum (YI-S) medium (Diamond et al., 1995) supplemented with gastric mucin, the efficacy of this axenic culture system was assessed for other *E. dispar* strains. However, despite its utility, the only *E. dispar* strain established as an axenic amoeba in YI-S medium thus far is SAW760RR clone A.

On the other hand, when a monoxenic culture system for *E. dispar* in biosate–cysteine–starch–iron–serum (BCSI-S) medium with *Pseudomonas aeruginosa* was developed (Kobayashi et al., 1998), a significant growth-promoting effect of dihydroxyacetone (DHA) was observed. DHA is a ketotriose and as a sugar source for *E. dispar* is thought to be directly metabolized to DHA phosphate (DHAP), which is an intermediary metabolite in the Embden–Meyerhof–Parnas glycolytic pathway.

Another significant result was that during characterization of the glycolytic pathway of *Crithidia fasciculata*, an effective culture associate of *E. dispar* in BI-S-33 medium (Diamond et al., 1978), approximately 35 times as much glucose-6-phosphate dehydrogenase (G6PDH) activity as that of *Trypanosoma cruzi* (Tulahuen strain) was detected. *Trypanosoma cruzi* can be used as a growth-promoting supplement for monoxenic culture of *E. histolytica*. However, it does not promote the growth of *E. dispar*, whereas *C. fasciculata* does (S. Kobayashi, unpubl.). G6PDH activity has not been detected in axenically grown *E. histolytica* (Reeves, 1972) or *E. dispar* (S. Kobayashi, unpubl.). G6PDH is an essential enzyme not only in the pentose-phosphate pathway but also in the Entner–Doudoroff pathway, which starts with G6P. G6PDH catalyzes the transformation of G6P to 6-phosphogluconate (6PG) via 6-phosphogluconolactone, and we focused attention on the glycolytic pathway after 6PG in *E. dispar* and examined the reactions after 6PG. Both the pentose-phosphate and Entner–Doudoroff glycolytic pathways can branch from 6PG. The activity of 6PG dehydratase (EC 4.2.1.12.) (Gottschalk and Bender, 1982; Nguyen and Schiller, 1989), which is one of the enzymes in the Entner–Doudoroff pathway, was detected in the lysates of both *E. histolytica* (HM-1:IMSS clone 6) and *E. dispar* (CYNO 16:TPC), and its activity in *E. dispar* was 2.87 times greater than in *E. histolytica*. By contrast, no 6PG dehydrogenase activity in the pentose-phosphate pathway was detected in either lysate (S. Kobayashi, unpubl.). On the other hand, DHA, which has a growth-promoting effect, is thought to be metabolized to G3P. through DHAP, which is converted to G3P by triose phosphate isomerase (EC 5.3.1.1.). Thus, if viable *C. fasciculata* actually supplies a growth-promoting substance like DHA, DHA may be preferable to viable *C. fasciculata* as a culture ingredient. On the basis of this information, we hypothesized the existence of some failure in the upstream portion of the glycolytic pathway in *E. dispar*, before DHAP or G3P production from glucose in YI-S medium. We, therefore, attempted to design an axenic culture medium for *E. dispar* by modifying the ingredients of YI-S medium, and we tested its usefulness.

Plea
G3P

MATERIALS AND METHODS

Reagents

All chemicals used in this study were of the highest quality commercially available unless otherwise stated.

Entamoeba dispar Isolates

Five strains of *E. dispar* were subjected to a trial of axenic cultivation in this study. Two strains (CYNO 09:TPC and CYNO 16:TPC) from nonhuman primates were isolated in Japan (Kobayashi et al., 1998) in Robinson's medium (Robinson, 1968), and 2 strains (AS 2 IR and AS 16 IR) were isolated from humans in Iran. An *E. dispar* strain, SAW 1734R clone AR (SAW1734RclAR), was used as the reference strain. The xenic *E. dispar* strains were transferred to monoxenic culture medium with viable *C. fasciculata* and antibiotics (as in the classic approach Diamond used for *E. histolytica*, Diamond, 1983) in BI-S-33 medium. The *C. fasciculata* was then replaced with antibiotic-sensitive *P. aeruginosa* in BCSI-S medium because *P. aeruginosa* is removed easily by antibiotics and antibiotic-resistant *C. fasciculata* usually survive in test medium for axenic culture even after 2–3 subcultures, and it promotes the growth of *E. dispar*.

Yeast–iron–gluconic acid–dihydroxyacetone–serum medium

Yeast–iron–gluconic acid–dihydroxyacetone (YIGADHA) broth was prepared by first dissolving the following ingredients in 880 ml of water distilled and purified with a Milli-Q column (Millipore Co., Billerica, Massachusetts): K_2HPO_4, 1 g; KH_2PO_4, 0.6 g; NaCl, 2.0 g; yeast extract (BBL, Becton Dickinson Co., Cockeysville, Maryland), 30 g; gluconic acid, 5.0 g; DHA (Sigma Chemical Co., St. Louis, Missouri), 1.0 g; D-galacturonic acid monohydrate, 0.2 g; L-cysteine hydrochloride, 1.0 g; ascorbic acid, 0.2 g; and ferric ammonium citrate (brown), 22.8 mg. The pH was adjusted to 6.5 with 1 N NaOH, and the solution was

Received 20 January 2004; revised 4 May 2004; accepted 4 May 2004.

* Present address: Department of Medical Parasitology and Mycology, School of Medicine Shaheed Beheshti University of Medical Sciences, Eveen, Teheran 19395, Iran.
† Present address: Department of Infectious Diseases, Tokai University School of Medicine, Ischara, Kanagawa 259-1193, Japan.

sterilized by filtration (Sartorius membrane filter; 0.2-μm pore size). An 88 ml volume of YIGADHA broth was aseptically dispensed into a 100-ml, screw-capped glass bottle and stored at −30 C.

To complete the YIGADHA-serum (YIGADHA-S) medium, 2 ml of vitamin mixture #18 prepared according to the instructions for PDM-805 medium (Diamond and Cunnick, 1991), and 16 ml of heat-inactivated bovine serum was aseptically added to 88 ml of the YIGADHA broth. A 5-ml volume of the complete medium was then dispensed into 13- × 100-mm, screw-capped borosilicate glass culture tubes (Asahi Techno Glass Co., Chuo-ku, Tokyo, Japan).

Establishment of the culture of *Entamoeba dispar* with sterilized *Crithidia fasciculata*

After removing *P. aeruginosa* by washing and addition of antibiotics (polymyxin B, 130 units/ml), trophozoites of the 5 strains of *E. dispar* were transferred to YIGADHA-S medium with sterilized *C. fasciculata* (2–3 million/ml, ReF-1:PRR, ATCC no. 50083) by fixation with 10% (v/v) formalin as described in the literature (Clark, 1995). All 5 strains of *E. dispar* adapted to the culture at 35.5 C in the YIGADHA-S medium with the associate within 2 wk after 3–4 whole-medium changes by centrifugation (275 *g*, 4 min) and eventually started to grow continuously. When the same growth-promoting effect was later found with autoclaved *C. fasciculata* in Hanks' solution as with the formalin-fixed *C. fasciculata*, we switched the method of sterilizing *C. fasciculata* to autoclaving (121 C, 15 min) because of its simplicity.

When the amoebae were subcultured, 0.6–0.8 ml of the amoeba suspension (2.5×10^4 to 5×10^4/ml) was usually transferred to the fresh medium, after the cultures were chilled for 5 min in an ice-water bath. They were then inverted several times to disperse the amoebae. Subcultures were successfully prepared at intervals of 3 and 4 days.

Growth kinetics of the amoebae

At 24-hr intervals for 96 hr, the number of amoebae in 5 μl of the homogeneous amoeba suspension diluted with known volume of YIGADHA-S medium were counted under a microscope after chilling the culture tubes for 5 min in an ice-water bath. The data were then converted to number per milliliter.

Zymodeme analysis and polymerase chain reaction

Zymodeme analysis (Sargeaunt, 1988) and polymerase chain reaction (PCR) analysis (Tachibana et al., 1991; Cheng et al., 1993) of the amoeba isolates were performed to characterize the amoebae grown in the axenic culture.

RESULTS

YIGADHA-S medium was designed, and the culture associates tested could be removed with no loss of *E. dispar* viability as a result of searching for the improved basic medium and condition for axenic cultivation of *E. dispar*, based on the casein-free YI-S medium.

YI-S medium was therefore modified as follows. Glucose was replaced by GA, DHA and D-galacturonic acid monohydrate were added because of the significant growth-promoting effects under the axenic culture conditions, and the medium was sterilized by filtration and adjusted to pH 6.5 with NaOH.

As a result of these modifications, trophozoites of *E. dispar* started to grow continuously in the absence of viable *C. fasciculata* or *P. aeruginosa* for the first time, although supplementation with the formalin-fixed organisms was still necessary for growth.

The growth-promoting ability of *C. fasciculata* was also maintained in this YIGADHA-S culture system even after it was autoclaved (121 C, 15 min) in Hanks' solution. There was no difference between the growth-promoting effect of autoclaved *C. fasciculata* and of formalin-fixed organisms. Because the procedure was easier, the amoebae were maintained in the medium with autoclaved *C. fasciculata*. The growth kinetics of the 5 strains of *E. dispar* in this culture system are shown in Figure 1.

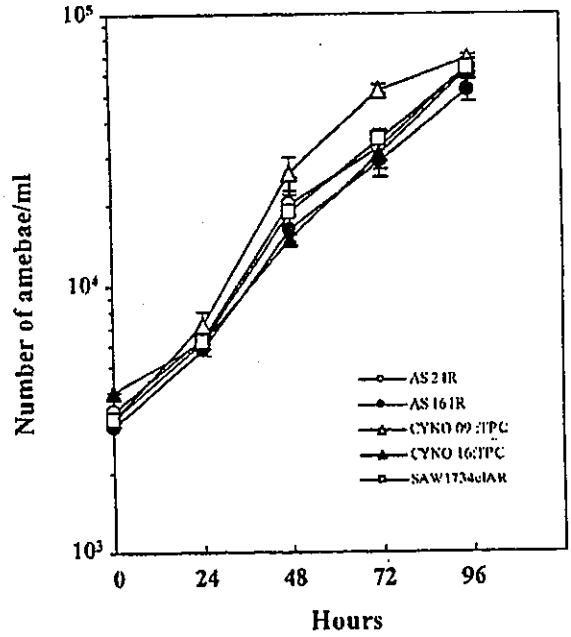


FIGURE 1. Growth kinetics of 5 strains of *Entamoeba dispar* in the 4-yr passaged cultures in YIGADHA-S medium supplemented with autoclaved *Crithidia fasciculata*. Mean numbers of amoebae in duplicate cultures are plotted.

After 3 mo of maintenance in this culture system, 1 strain of *E. dispar* (CYNO 16:TPC) started to grow without any supplement, e.g., without autoclaved *C. fasciculata*. However, addition of 6PG (Sigma P-7877, 67 μg/ml), an intermediary metabolite in the Entner–Doudoroff pathway (Fig. 3), was necessary to maintain this strain without the autoclaved organisms.

After culturing with autoclaved *C. fasciculata* for 1 yr and 4 yr, respectively, other *E. dispar* strains started to grow axenically in the YIGADHA-S medium without the addition of any culture associates. The growth kinetics of 5 strains of axenically grown *E. dispar* are shown in Figure 2. The clear differences in growth kinetics between monoxenic culture (Fig. 1) and axenic culture (Fig. 2), except for the CYNO 09:TPC strain, suggest the existence of some other growth-promoting factor in autoclaved *C. fasciculata*.

The CYNO 16:TPC and CYNO 09:TPC strains were usually subcultured by transferring 1 ml of amoeba suspension to fresh YIGADHA-S medium, and the AS 16 IR, AS 2 IR, and SAW1734RclAR strains were subcultured by transferring 1 ml of amoeba suspension concentrated to an adequate density for subculture (3×10^4 to 6×10^4 amoebae/ml) by centrifugation (275 *g*, for 4 min).

The zymodemes of the 5 strains grown in both the monoxenic and axenic YIGADHA-S media were all judged to be type I. All 10 DNA samples of the 5 strains used in the PCR analyses described above were amplified with *E. dispar*–specific primers

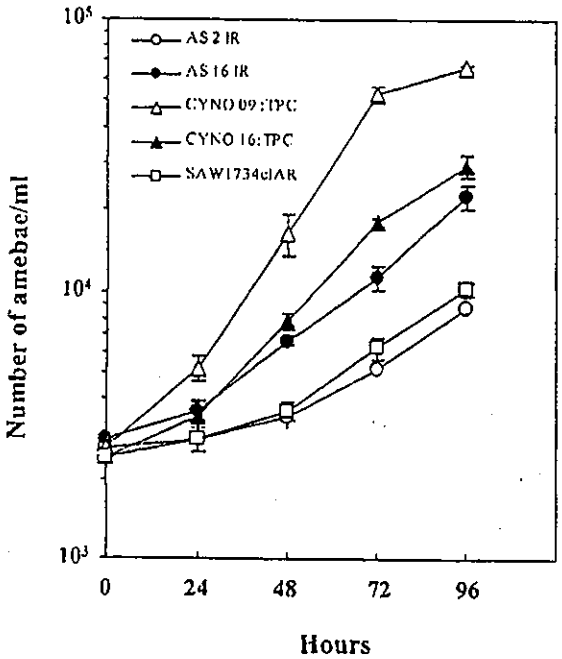


FIGURE 2. Growth kinetics of 5 strains of axenically grown *Entamoeba dispar* in 2-yr (CYNO 09: TPC), 3-yr (CYNO 16:TPC), and 2-mo (AS 2 IR, AS 16 IR, and SAW 1734RclAR) passaged cultures in YIGADHA-S axenic medium. Mean numbers of amoebae in duplicate cultures are plotted.

alone; the *E. histolytica*–specific primers did not elicit any DNA amplification. These findings are summarized in Table I.

DISCUSSION

We have previously reported a monoxenic culture system for *E. dispar* in BCSI-S medium in which glucose was replaced with starch and to which sterilized *C. fasciculata* were added after heat treatment at 56 C for 30 min followed by incubation in 1% hydrogen peroxide at 4 C for 24 hr (Kobayashi et al., 1998). The sterilized parasite appeared to be metabolically inactive as judged by the nuclear magnetic resonance spectra. Although the growth-promoting effect of *C. fasciculata* was

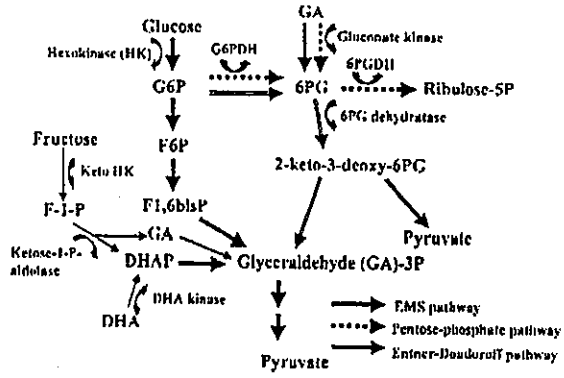


FIGURE 3. Metabolic map of 3 glycolytic pathways.

partially lost as a result of this sterilization step, the minimum essential effect persisted, which made it possible to establish a long-term monoxenic culture of *E. dispar* with metabolically inactive *C. fasciculata*. In this study, we succeeded in producing an axenic culture system of *E. dispar* by using YIGADHA-medium designed on the basis of YI-S medium from the monoxenic culture of the amoeba with sterilized *C. fasciculata*.

During attempts to improve the culture system, it was found that by replacing some kinds of sugar from starch, the growth of *E. dispar* was promoted significantly in the BCSI-S monoxenic culture system. First, a marginal growth-promoting effect of fructose was found, although its effect was insufficient for axenic cultivation of *E. dispar*, and a more useful compound, DHA, was used. The concentration (0.1%) of DHA required for the growth of *E. dispar* in YIGADHA-S medium was very critical: DHA concentrations below 0.05% and greater than 0.15% failed to effectively promote the growth of *E. dispar*. However, despite the clear effect of DHA and fructose on the growth of *E. dispar*, the results of our biochemical assays for phosphorylation of DHA by DHA kinase, ketohexokinase, and ketose-1-phosphate aldolase, the enzymes involved in the conversion of fructose or DHA to DHAP (Fig. 3), showed no significant activity of these enzymes in the crude lysates of axenic *E. dispar* (CYNO 16:TPC) or *E. histolytica* (HM-1:IMSS clone 6) (data not shown). The mechanism of growth-promoting effect of DHA and fructose is not yet understood.

Pimenta et al. (2002) demonstrated an interesting morpho-

TABLE I. Zymodeme and PCR analyses of 5 strains of *Entamoeba dispar*.

Strain	Xenic (date)	Zymodeme*		PCR†	
		Monoxenic (date)	Axenic (date)	Monoxenic	Axenic
SAW 1734RclAR	Z-III‡ (March 1985)	Z-I (January 2000)	Z-I (February 2003)	*E. dispar*	*E. dispar*
AS 2 IR	Z-I (June 1998)	Z-I (January 2000)	Z-I (February 2003)	*E. dispar*	*E. dispar*
AS 16 IR	Z-I (June 1998)	Z-I (January 2000)	Z-I (February 2003)	*E. dispar*	*E. dispar*
CYNO 09:TPC	Z-I (April 1992)	Z-I (January 2000)	Z-I (February 2003)	*E. dispar*	*E. dispar*
CYNO 16:TPC	Z-III (April 1992)	Z-I (January 2000)	Z-I (February 2003)	*E. dispar*	*E. dispar*

* Zymodemes type I and III are classified as *E. dispar*.
† PCR analysis using 2 sets of oligonucleotide primers each (p11 plus p12 and p13 plus p14, respectively) for amplification of the DNAs of *E. histolytica* and *E. dispar*. The zymodeme and PCR analyses were performed around the same time.
‡ Data cited from Mirelman et al. (1986).

logical characteristic of *E. dispar* by electron microscopy: some concomitant bacteria cells were free and viable in the cytoplasm of *E. dispar*, without being surrounded by a distinct phagosome membrane. This suggests that some bacterial species can survive in the cytoplasm and exist in a symbiotic relationship with *E. dispar*. If the Entner–Doudoroff pathway does indeed function in glycolysis in *E. dispar*, as reported in *E. histolytica* under xenic conditions (Hilker and White, 1959), the reactions upstream in the EMS pathway (Fig. 3) including a reaction regulating the transformation of fructose-6-phosphate to fructose-1,6-bisphosphate or vice versa, usually catalyzed by 6-phosphofructokinase (6-PFK) and fructose-bis-phosphatase (EC 3.1.3.11.) and affecting the both the glycolysis and glycogenesis pathways, may not function well in *E. dispar*. The regulatory reactions in *E. histolytica* are well known to be regulated by a single unique enzyme (ppi-dependent 6-PFK; EC 2.7.1.90.), and the reaction is reversible and has no apparent regulatory function (Reeves et al., 1974, 1976).

The clear growth-promoting effect of autoclaved *C. fasciculata* (or *P. aeruginosa*) indicated that they contain as yet unidentified heat-stable growth-promoting substances for *E. dispar*. If the substances can be identified, YIGADHA-S medium will be improved. Further analyses of the axenically grown *E. dispar* will enable us to further elucidate the biological properties of *E. dispar* that differ from those of pathogenic *E. histolytica*.

ACKNOWLEDGMENTS

The authors thank Louis S. Diamond, NIH, for supplying the reference strain of *E. dispar* (SAW1734RC1AR) and *C. fasciculata* (ReF-1: PRR). This work was supported by a Grant-in-Aid for Scientific Research (10670237) from the Ministry of Education of Japan, and also by a Grant-in-Aid from the Ministry of Health, Labor and Welfare of Japan.

LITERATURE CITED

CHENG, X.-J., H. TACHIBANA, S. KOBAYASHI, Y. KANEDA, AND M.-Y. HUANG. 1993. Pathogenicity of *Entamoeba histolytica* isolates from Shanghai, China. Parasitology Research 79: 608–610.

CLARK, C. G. 1995. Axenic cultivation of *Entamoeba dispar* Brumpt 1925, *Entamoeba insolita* Geiman and Wichterman 1937 and *Entamoeba ranarum* Grassi 1879. Journal of Eukaryotic Microbiology 42: 590–593.

DIAMOND, L. S. 1983. Lumen-dwelling protozoa: *Entamoeba*, trichomonads and *Giardia*. *In* In vitro cultivation of protozoan parasites, J. B. Jensen (ed.). CRC press, Boca Raton, Florida, p. 65–109.

———, C. G. CLARK, AND C. C. CUNNICK. 1995. YI-S, a casein-free medium for axenic cultivation of *Entamoeba histolytica*, related *Entamoeba, Giardia intestinalis* and *Trichomonas vaginalis*. Journal of Eukaryotic Microbiology 42: 277–278.

———, AND C. C. CUNNICK. 1991. A serum-free, partly defined medium, PDM-805, for axenic cultivation of *Entamoeba histolytica* Schaudinn, 1903 and other *Entamoeba*. Journal of Protozoology 38: 211–216.

———, D. F. HARLOW, AND C. C. CUNNICK. 1978. A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Transactions of Royal Society of Tropical Medicine and Hygiene 72: 431–432.

GOTTSCHALK, G., AND R. BENDER. 1982. D-Gluconate dehydratase from *Clostridium pasteurianum*, Vol. 90. *In* Methods in enzymology, W. A. Wood (ed.). Academic Press, New York, p. 283–287.

HILKER, D. M., AND A. G. C. WHITE. 1959. Some aspects of the carbohydrate metabolism of *Entamoeba histolytica*. Experimental Parasitology 8: 539–548.

KOBAYASHI, S., E. IMAI, H. TACHIBANA, T. FUJIWARA, AND T. TAKEUCHI. 1998. *Entamoeba dispar*: Cultivation with sterilized *Crithidia fasciculata*. Journal of Eukaryotic Microbiology 45: 3S–8S.

MIRELMAN, D., R. BRACHA, A. WEXLER, AND A. CHAYEN. 1986. Changes in isoenzyme patterns of a cloned culture of nonpathogenic *Entamoeba histolytica* during axenization. Infection and Immunity 54: 827–832.

NGUYEN, L. K., AND N. L. SCHILLER. 1989. Identification of a slime exopolysaccharide depolymerase in mucoid strains of *Pseudomonas aeruginosa*. Current Microbiology 18: 323–329.

PIMENTA, P. F. P., L. S. DIAMOND, AND D. MIRELMAN. 2002. *Entamoeba histolytica* Shaudinn, 1903 and *Entamoeba dispar* Brumpt, 1925: Differences in their cell surfaces and in the bacteria-containing vacuoles. Journal of Eukaryotic Microbiology 49: 209–219.

REEVES, R. E. 1972. Carbohydrate metabolism in *Entamoeba histolytica*. *In* Comparative biochemistry of parasites, H. Van den Bossche (ed.). Academic Press, New York, p. 351–358.

———, R. SERRANO, AND D. J. SOUTH. 1976. 6-Phosphofructokinase (pyrophosphate) properties of the enzyme from *Entamoeba histolytica* and its reaction mechanism. Journal of Biological Chemistry 251: 2958–2962.

———, D. J. SOUTH, H. J. BLYTTI, AND L. G. WARREN. 1974. Pyrophosphate: D-fructose 6-phosphate 1-phosphotransferase. A new enzyme with the glycolytic function of 6-phosphofructokinase. Journal of Biological Chemistry 249: 7737–7741.

ROBINSON, G. L. 1968. The laboratory diagnosis of human parasitic amoebae. Transactions of Royal Society of Tropical Medicine and Hygiene 62: 285–294.

SARGEAUNT, P. G. 1988. Zymodemes of *Entamoeba histolytica*. *In* Amebiasis: Human infection by *Entamoeba histolytica*, J. I. Radvin (ed.). John Wiley and Sons, Inc., New York, p. 370–387.

TACHIBANA, H., S. KOBAYASHI, M. TAKEKOSHI, AND S. IHARA. 1991. Distinguishing pathogenic isolates of *Entamoeba histolytica* by polymerase chain reaction. Journal of Infectious Diseases 164: 825–826.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Parasitology International 53 (2004) 247–254
www.elsevier.com/locate/parint

An experimental model for amoebic abscess production in the cheek pouch of the Syrian golden hamster, *Mesocricetus auratus*☆

M.A. Beg[a,*], S. Kobayashi[b], A.S. Hussainy[a], A. Hamada[c], E. Okuzawa[c], R.A. Smego Jr[a,1], R. Hussain[a]

[a]*Departments of Microbiology, Pathology and Medicine, The Aga Khan University Medical College, P.O. Box 3500, Stadium Road, Karachi 74800, Pakistan*
[b]*The Department of Tropical Medicine and Parasitology, Keio University, School of Medicine, Tokyo160-8582, Japan*
[c]*The Japanese Overseas Health Administration Centre (JOHAC), 3211 Kozukue-cho, Kohoku-ku, Yokohama, Japan*

Received 24 December 2003; accepted 5 February 2004

Abstract

A new experimental model was developed in hamsters for amoebic abscess caused by *Entamoeba histolytica*. *E. histolytica* trophozoites were cultured in a liquid axenic medium, and then injected intradermally into the cheek pouch of the Syrian golden hamster, *Mesocricetus auratus*. Inoculation consistently resulted in abscess formation at the site in 20 of 22 (91%) study animals. The amoebic nature of the abscesses was confirmed by light microscopy and histopathologic examination. Abscess formation was maximal at day 12 post-inoculation. Potential applications of this simple and reliable model include further elucidation of the pathogenesis of invasive amoebiasis, studies of the host response to amoebae, and in vivo evaluation of chemotherapeutic agents that show in vitro efficacy against *E. histolytica*.

Keywords: *Entamoeba histolytica*; Amoebic abscess; Animal model; Cheek pouch; Hamster

1. Introduction

Since the discovery of *Entamoeba histolytica* as the causative agent of invasive amoebiasis in humans by Losch in 1875, scientists have tried to duplicate the disease in laboratory animals. Although rodent models have been used extensively, no single animal has been found to reliably replicate the natural infection that occurs in humans. Experimentally, amoebic abscesses, particularly in the liver, have been produced in rodents by inoculating virulent trophozoites of *E. histolytica* through various routes [1]. Injection of parasites either directly into the liver parenchyma or via the portal vein have been the most widely used techniques for in vivo studies [2,3].

☆ Presented in part at the 50th Annual Meeting of the American Society for Tropical Medicine and Hygiene, Atlanta, November 11–15, 2001.

*Corresponding author. Tel.: +92-21-4859-4516; fax: +92-21-4934294/4932095.
E-mail address: masim.beg@aku.edu (M.A. Beg).
[1] *Present address:* Department of Medicine, University of North Dakota, 1919 Elm Street North Fargo, North Dakota 58102, USA.

1383-5769/04/$ - see front matter © 2004 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.parint.2004.02.002

Shibayama et al., [4] have demonstrated liver invasion and abscess formation by intraperitoneal inoculation of trophozoites in hamsters. Several unique characteristics of the golden hamster, *Mesocricetus auratus*, would seem to make it an ideal animal model for research work such as its immunogenetic tolerance to human parasites and its possession of paired eversible cheek pouches that can serve as natural windows for detailed microscopic investigation [5]. Early parasitologic research using the golden hamster includes studies involving *E. histolytica* [6,7], Leishmania spp [8,9], *Schistosoma mansoni* [10], *Hymenolepis nana* [11], and *Trichinella spiralis* [12,13]. More recently, the suitability of the cheek pouch of the golden hamster, as an intact living membrane, has been demonstrated in experimental *Trypanosoma cruzi* [14,15] and *Plasmodium berghei* [16] infections.

The physiologic relevance of this site for development of an amoebic abscess has been reported previously as examples of ameobiasis of the female genital tract [17] and penis [18]. Since both sites are lined by stratified squamous epithelium this suggests that the hamster cheek pouch is a good potential site for experimental amoebiasis.

A model for invasive amoebiasis, including abscess production, would be useful for further investigations of pathogenesis and potential therapeutic regimens. In this report, we describe a simple and reliable technique for producing amoebic abscesses in the cheek pouch of the golden hamster via intradermal inoculation of axenically-cultured *E. histolytica* trophozoites.

2. Materials and methods

2.1. Animals and preparation of inocula

Male golden hamsters (*M. auratus*) aged 4–6 weeks and each weighing approximately 40–50 g were used in all experiments; hamsters were given free access to water and standard feed. Individual animals were selected for time-sequence studies and killed at different points after inoculation of parasites. Highly virulent, axenic trophozoites of *E. histolytica* strain SAW755CR clone B (generously supplied Dr Diamond) were maintained in a liquid TYI-S-33 15% Bovine serum (BS) medium [19]. Inoculae were prepared from 72 h amoebic cultures in a late logarithmic phase of growth. Strain virulence was confirmed by inoculating trophozoites directly into the liver of a hamster with resultant amoebic liver abscess production within 1 week. Each hamster cheek pouch was injected with an inoculum containing 5×10^5 *E. histolytica* trophozoites in 0.2 ml of liquid medium. This dose was calculated after testing a range of concentrations of amoebae from 0.5 to 2.0×10^6 organisms per inoculum. Strains of *Entamoeba dispar* were maintained under monoxenic culture conditions in YIGADHA-S (yeast–iron–gluconic acid–dihydroxyacetone-serum) medium [20]. The maintenance and care of experimental animals complied with the Aga Khan University guidelines for the humane use of laboratory animals.

2.2. Induction of amoebiasis

2.2.1. Experiment 1

Two groups of hamsters (containing four animals each) were inoculated intradermally with axenically-grown trophozoites of either *E. histolytica* strain SW755CR clone B (Group 1) or non-pathogenic *E. dispar* CYNO16TPC (Group 2) [20]. Organisms were inoculated intradermally into the hamster's cheek pouch using a tuberculin needle ($26G\times\frac{1}{2}$) and syringe.

2.2.2. Experiment 2

The cheek pouches of 10 animals were inoculated intradermally with trophozoites of the *E. histolytica* SAW755CR clone B study strain using a tuberculin needle and syringe. Single study animals were killed at days 2 and 4 whilst pairs of animals were killed at days 7, 12 and 17 post-inoculation. The last pair was not sacrificed in order to observe the natural course of the infection.

2.2.3. Experiment 3

Twelve animals were divided into groups of four each: Group 1—the cheek pouches of all animals were inoculated intradermally with trophozoites of the *E. histolytica* SAW755CR clone B study strain using a tuberculin syringe. Group 2—the cheek

Table 1
Development of amoebic abscess after hamster cheek pouch inoculation

Experiment	Inoculation	No. of animals inoculated*	No. of animals with abscess production (%)
1	*E. histolytica*	4	4 (100)
	E. dispar	4	0 (0)
2	*E. histolytica*	10	9 (90)
3	*E. histolytica*	4	3 (75)
4	*E. histolytica*	4	4 (100)
	Medium (TYI-S-33)	4	0 (100)

* Inoculum: trophozoites of *E. histolytica*, *E. dispar* 5×10^5/0.2 ml of TYI-S-33 media 0.2 ml of TYI-S-33, 15% BS media was inoculated as control.

pouches of all animals were inoculated with trophozoites of the *E. histolytica* SAW755 CR clone B study strain using a blunt esophageal tube placed inside of the cheek pouch without trauma to the tissue; Group 3—the backs of all animals inoculated intradermally with trophozoites of the *E. histolytica* strain SAW755CR clone B study strain using a tuberculin syringe

2.2.4. Experiment 4

Four male hamsters were used as sham controls in this study. Each animal was injected with *E. histolytica* trophozoites 5×10^5/0.2 ml of media in the right cheek pouch and with 0.2 ml of TY I-S-33 15% BS media in the left cheek pouch. All animals were examined for presence of amoebic abscess 5–7 days post infection.

2.3. Cheek pouch preparation

Methods were followed as described in detail by Handler and Shepro [5]. For animals given intradermal injections, each hamster cheek pouch was inoculated with *E. histolytica* trophozoites in a dose of 5×10^5 in 0.2 ml of medium. Injection was carefully performed using a tuberculin syringe until a bleb appeared. For each animal, amoebic abscesses were examined macroscopically for signs of inflammation or ulcer formation. Exudate from abscesses was aspirated with a pipette, a wet mount preparation was examined using a light microscope, and the presence of *E. histolytica* trophozoites was recorded. For killed animals, the cheek pouch was dissected, removed, and preserved in 10% formalin, and representative sections of specimens were submitted for histopathologic examination. After an overnight processing of dehydration and clearing, tissue blocks were embedded in paraffin, and 5 μ thick sections were cut from these blocks and stained using hematoxylin and eosin.

3. Results

Intradermal inoculation with axenically cultured trophozoites of the *E. histolytica* study strain readily and consistently produced infection in the cheek pouch of study animals; overall, 20 of 22 (91%) hamsters developed amoebic abscesses at the injection site (Table 1).

In experiment 1, animals inoculated with *E. histolytica* produced multiple cheek pouch lesions, whitish-yellow in color and with irregular borders (Fig. 1). Abscesses were semisolid with a thick brownish exudates that could be easily expressed; gram stain examination of this material demonstrated abundant polymorph nuclear leucocytes. In addition, live motile trophozoites were also seen. All animals were killed on day 7 by ether inhalation. The hamsters inoculated with *E. dispar* trophozoites showed no signs of active amoebic infection (Table 1).

In experiment 2, inoculation with trophozoites of *E. histolytica* produced abscesses as in experiment 1, in nine of 10 hamsters; one animal killed at day 2 post-inoculation did not show evidence of abscess formation. For the other serially killed hamsters, the largest abscesses and most visible

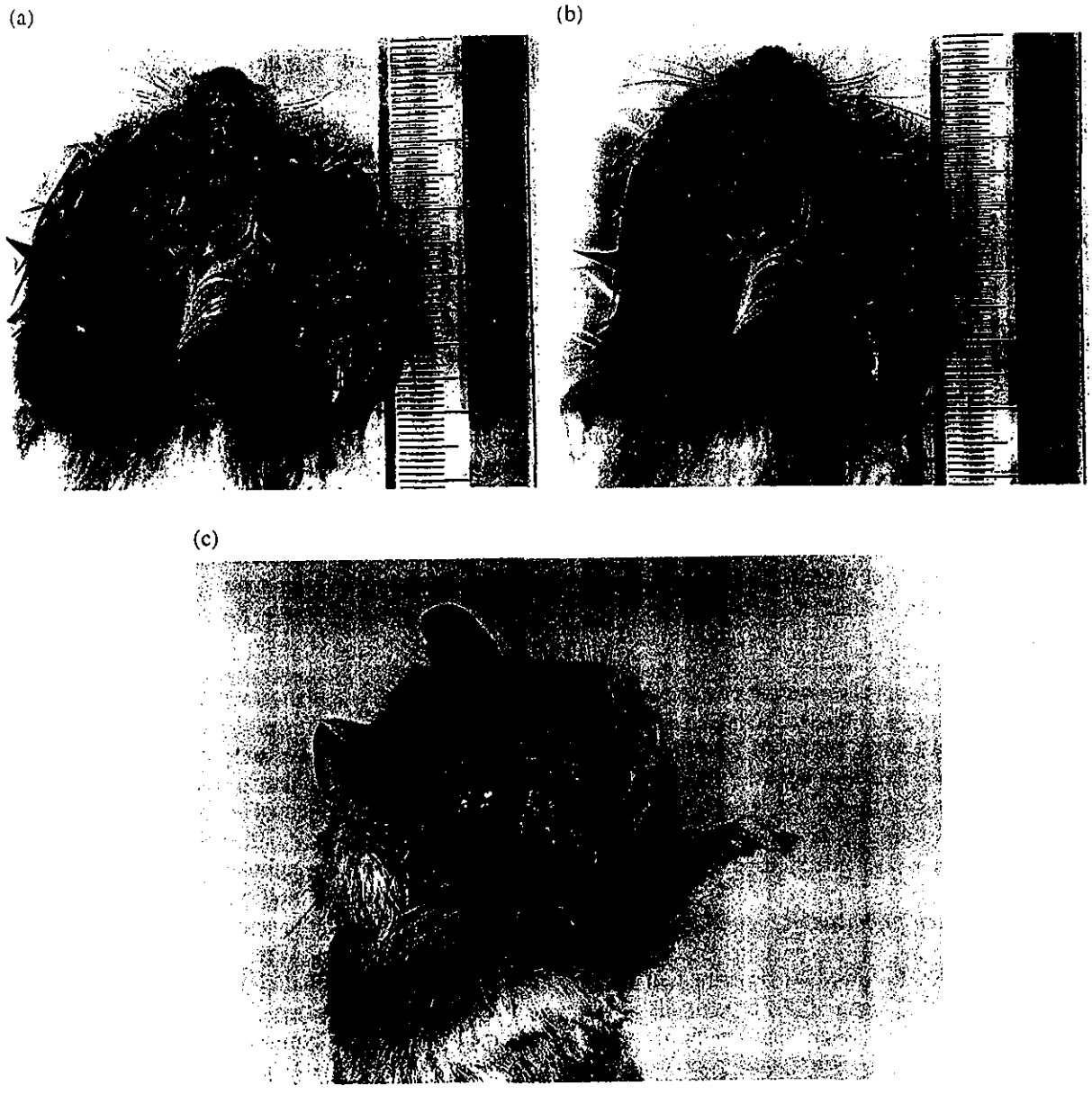


Fig. 1. Macroscopic abscesses of the hamster cheek pouch after intradermal injection of axenically grown *E. histolytica* trophozoites (a, day 7 with cheek pouch intact; b, day 7 with cheek pouch dissected away; c, day 12).

and motile trophozoites were seen on day 12 post-inoculation. At day 17, abscesses were smaller and no motile trophozoites were visible. Two animals died on day 20, at which time lesions had further resolved with residual scar tissue formation and no amoebic trophozoites seen on microscopy.

In experiment 3, hamsters injected with *E. histolytica* trophozoites into the cheek pouch demonstrated abscess formation. The abscesses produced in animals of group 1 showed maximal changes on day 12 post-inoculation, with motile trophozoites seen on light microscopy. One animal