A

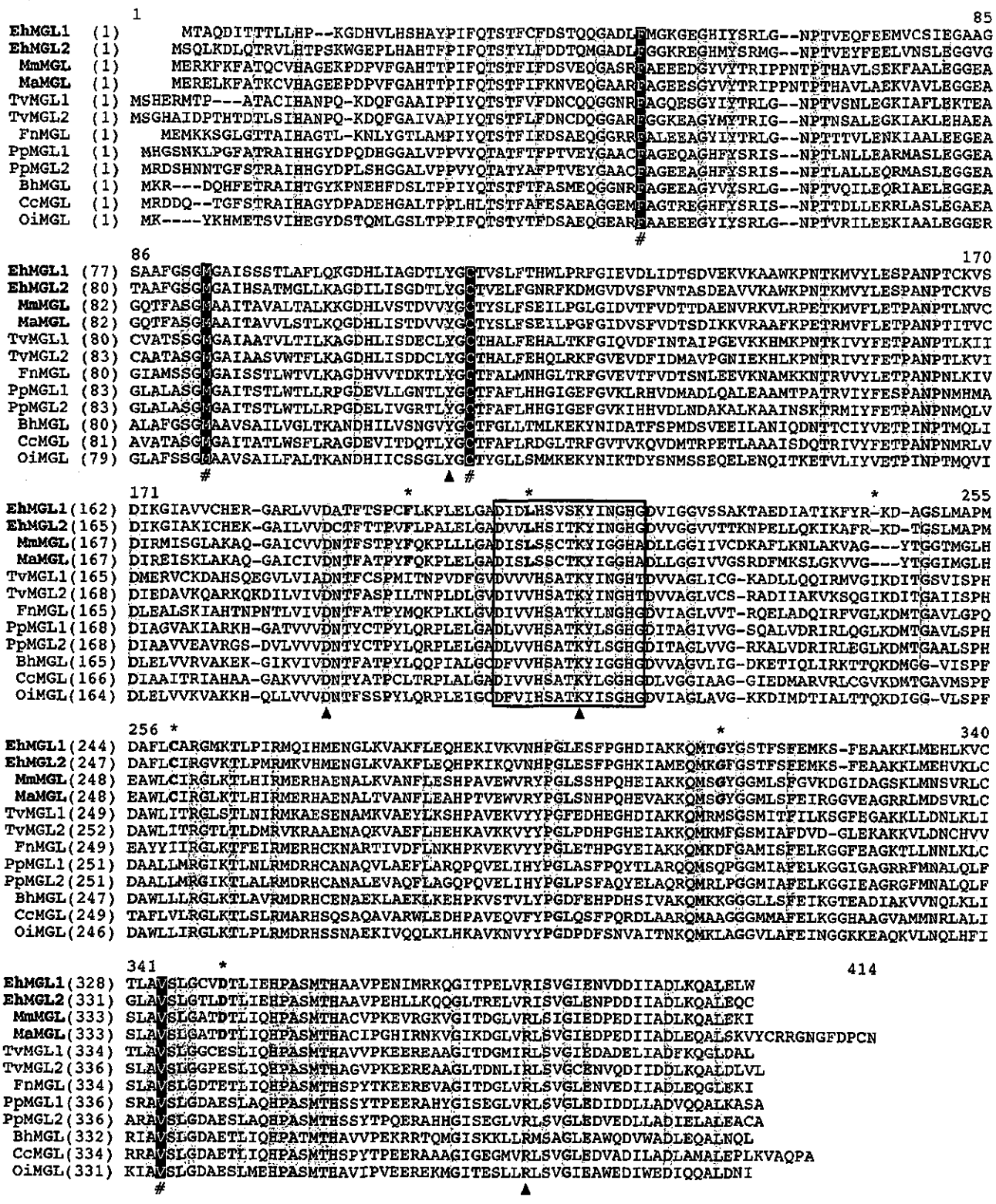

FIG. 2. **Amino acid comparison of MGLs and phylogenetic analysis of γ-subfamily of PLP enzymes.** *A*, comparison of deduced amino acid sequences of MGL from *Entamoeba* and other organisms. Pair-wise alignment was performed as described under "Experimental Procedures". Computer-generated gaps are indicated by *hyphens*. Residues that are conserved among all MGL sequences as well as other members of the γ-subfamily of PLP enzymes are shown *on a gray background*, and "MGL signature-like residues" conserved in MGLs, but not in other members of the γ-subfamily of PLP enzymes, are shown as *reversed letters* on *a black background* and marked with *sharps*. The two amebic and two archaeal MGLs are labeled in *bold*, and their conserved residues are shown on *a gray background* marked with *asterisks*. A region corresponding to the PLP attachment site in cysteine/methionine-metabolizing enzymes (PROSITE accession number: PS00868) is marked with an *open rectangle*. Active-site residues depicted from a crystal structure of PpMGL (33) are indicated with *arrowheads*. Protein accession numbers are shown in Fig. 2*B*. *B*, phylogenetic reconstruction of MGLs and other related PLP enzymes belonging to the γ-subfamily. An unrooted NJ tree, which is representative of the results of three independent methods for phylogenetic reconstruction, is shown. The root is arbitrarily placed using EhCSs (β-family of PLP enzyme) as the outgroup[c], and numbers beside the nodes indicate bootstrap values from 1000 replicates. The horizontal length of each branch is proportional to the estimated number of amino acid substitutions. EhMGL[a] accession numbers are shown in the DDBJ footnote, and other accession numbers are shown in parentheses (NCBI protein data base except [b]Swiss-Prot accession number). Abbreviations are defined in Footnote 2.

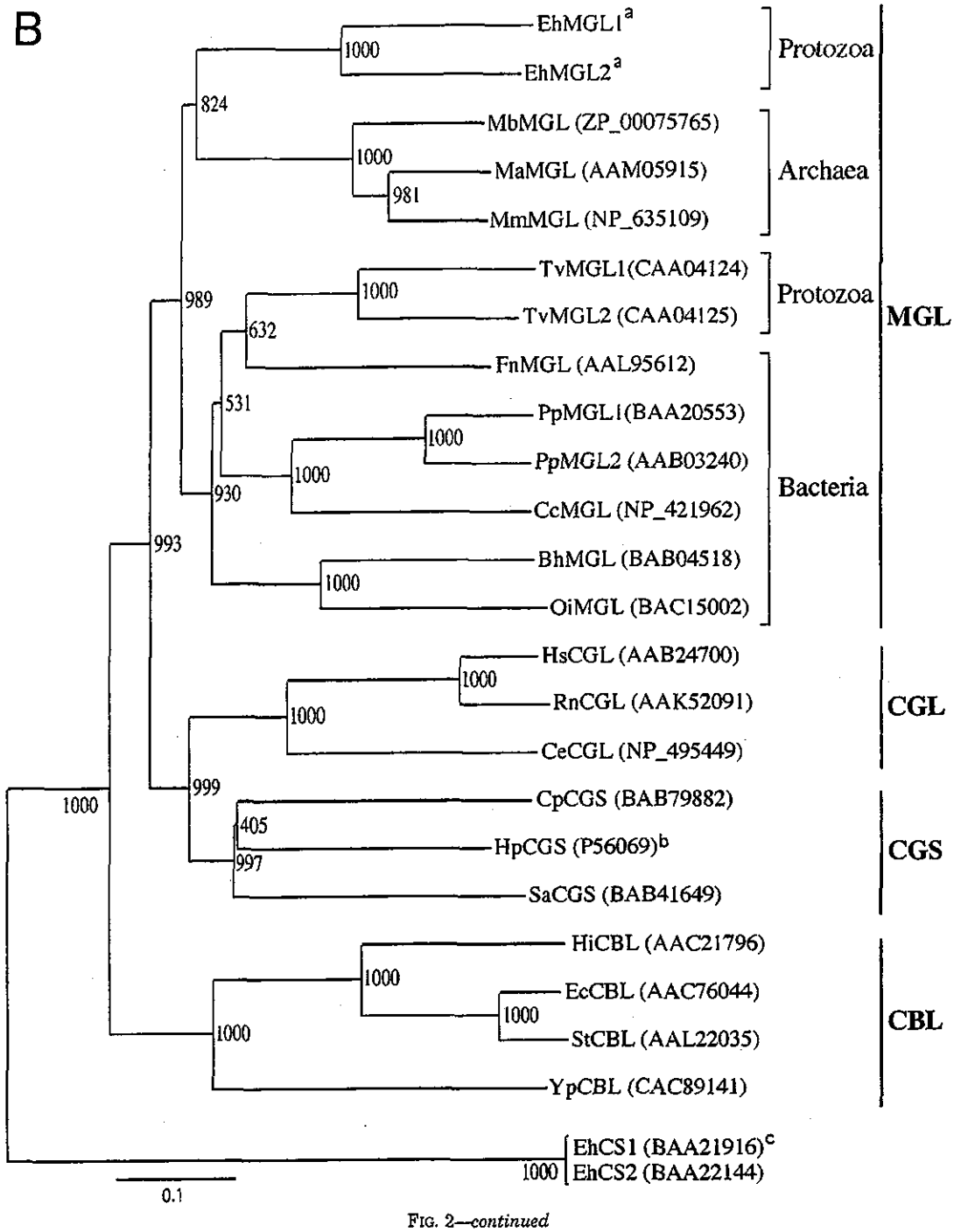

FIG. 2—*continued*

period of the cultures were chosen to maintain the control trophozoites in the late-logarithmic growth phase throughout the experiment, and also to allow the measurement of optical density in the linear portion of the curves (between 4×10^3 to 2.0×10^5 cells/ml).

RESULTS AND DISCUSSION

Data Base Search of the PLP-dependent Enzymes—In an attempt to obtain genes encoding the PLP-dependent enzymes involved in the metabolism of sulfur-containing amino acids, we searched the genome data base for putative proteins that possessed a conserved PLP-binding domain as described under "Experimental Procedures." Two independent contigs were found in the genome data base. These contigs (Contig 315785 and 316820, TIGR) contained two similar but not identical ORFs that encode proteins possessing a region containing the PLP-binding motif of the γ-subfamily of PLP enzymes. Non-coding flanking regions within these contigs also showed significant variations (data not shown). All other contigs or singletons showing significant identity to these two contigs perfectly overlapped them, which is consistent with the notion that these fragments are present as a single copy in the genome.

We also searched for genes containing the PLP-binding site of the β-family of PLP enzymes using both the amebic CS and the yeast and mammalian CBS. However, after eliminating contigs and singletons that contain genes encoding the two CS isotypes described previously (14), no contig or singleton was found to contain this motif. This suggests that *E. histolytica* possesses two uncharacterized genes encoding proteins that belong to the γ-subfamily of PLP enzymes, and lacks the β-family of PLP enzymes (*i.e.* CBS) known to be involved in the

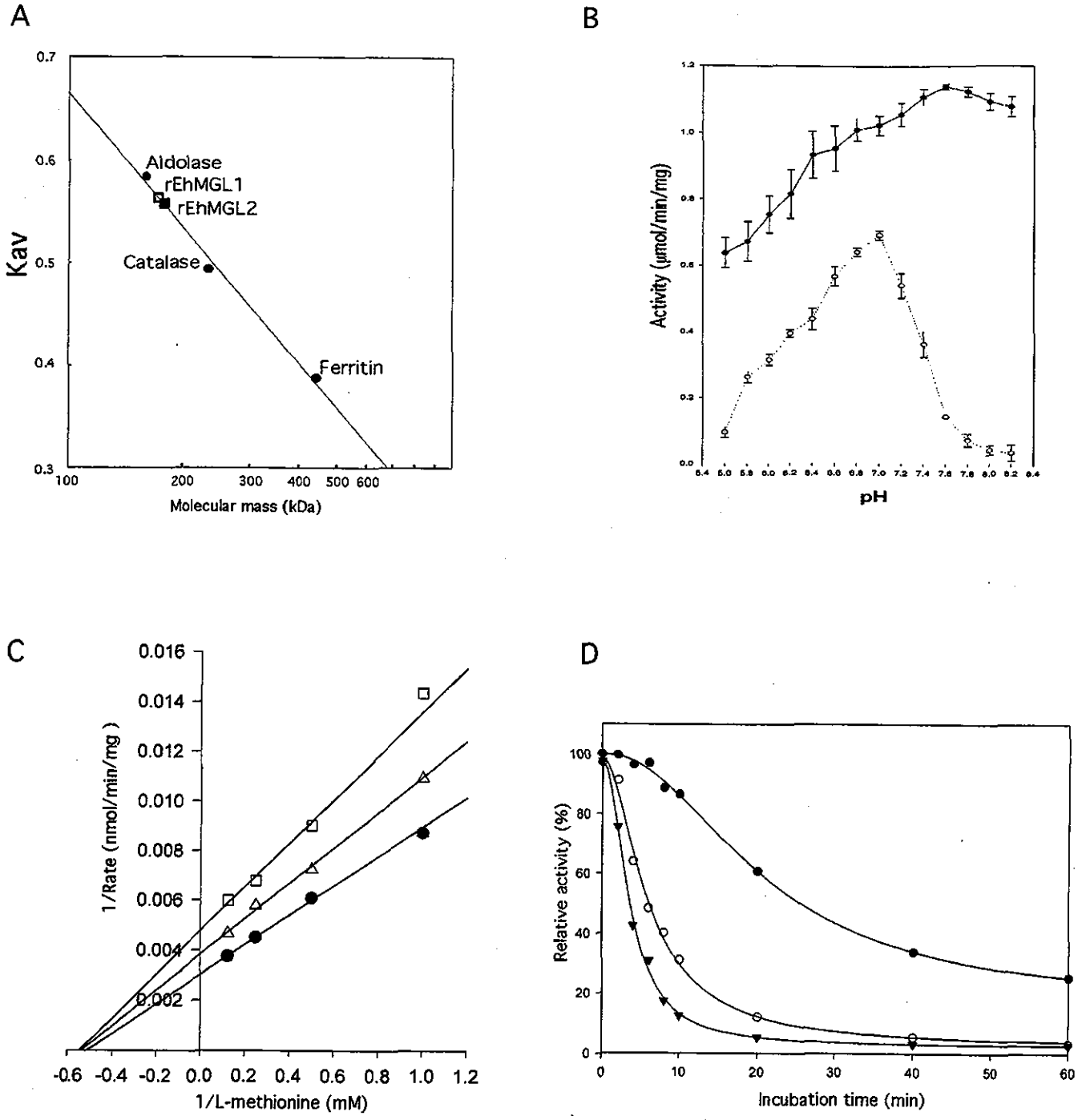

FIG. 3. **Enzymological characteristics of the recombinant EhMGL isotypes.** *A*, Size exclusion chromatography of the recombinant EhMGLs. rEhMGL1 (□) and 2 (■)were applied to a column of Toyopearl HW-65S and detected as described under "Experimental Procedures." Standard calibration of this column was performed with blue dextran (2000 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa). The K_{av} of each protein was calculated using the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$ (V_e, elution volume; V_t, total bed volume; V_0, column void volume). *B*, pH optimum of rEhMGLs. The optimum pH was determined at 37 °C with 50 mM MES buffer (pH 5.6–6.2), MOPS buffer (pH 6.4–7.4), and HEPES buffer (pH 7.4–8.2). Activities of rEhMGL1 (○) and 2 (●) were monitored by hydrogen sulfide assay for 5 min using 5 mM DL-homocysteine and 1 μg of each rEhMGL in a 200 μl reaction mixture as described under "Experimental Procedures." Data shown are means ± S.D. of triplicates. *C*, Lineweaver-Burk double reciprocal plot of MGL showing non-competitive inhibition of rEhMGL2 by DL-propargylglycine (PPG). rEhMGL2 was preincubated with 0 (●), 10 (△), or 20 μM (□) PPG in a reaction mixture without L-methionine at 36 °C for 5 min. Reactions were initiated by adding appropriate concentrations of L-methionine, and pyruvate production was assayed after 5 min. *D*, slow binding inhibition of rEhMGL2 by PPG. rEhMGL2 was preincubated with 0 (●), 10 (○), or 20 μM (▲) PPG in a reaction mixture without L-methionine at 36 °C for each period indicated. Reactions were initiated by adding L-methionine and carried out for 5 min. Percentages of MGL activity relative to the untreated control are shown.

reverse trans-sulfuration pathway in other organisms. We also tentatively concluded that the trans-sulfuration sequences in both the forward and reverse orientation are incomplete since the amebic genome lacks putative genes for CBL, CGS, and CGL (also see below). In addition, the major pathways for cysteine degradation of sulfur amino acids present in mammals, *i.e.* the cysteine sulfinic acid (cysteine dioxygenase as a key enzyme), 4′-phosphopanthetheine (leading to synthesis of coenzyme A and cysteamine), and mitochondrial mercaptopyruvate pathways, are apparently absent in this organism (data

TABLE I

Products of L-methionine degradation by the amebic crude extract and recombinant EhMGLs

Rates of product formation were determined from three independent experiments, and results of one representative experiment are shown. Assays to measure individual products were performed as described under "Experimental Procedures." Reactions were carried out with 10 mM L-methionine at 37 °C for 30 min with 200 μg of crude extract or for 10 min with 3 μg of recombinant enzyme in a 200 μl of reaction mixture. Crude extracts from trophozoites cultured for 48 hrs in BI-S-33 medium supplemented with or without 20 μM PPG were also used. Methanethiol and nitrogen could not be measured when the crude extract was used as a source of MGL because the backgrounds due to endogenous organic and inorganic thiols and nitrogen were not negligible.

Product	Rate of production (nmol/min/mg)			
	Crude extract		Recombinant enzyme	
	BI-S-33	BI-S-33 + 20 μM PPG	rEhMGL1	rEhMGL2
Methanethiol	n.d.[a]	n.d.	524.7	738.7
α-Buryric acid	0.157	0.004	288.7	468.7
Nitrogen	n.d.	n.d.	385.3	793.3

[a] n.d., not determined.

not shown). Therefore, *E. histolytica* must have alternative enzymes involved in the degradation of toxic sulfur-containing amino acids, e.g. homocysteine, which is implicated in the well characterized human genetic disease homocysteinuria and its cytotoxicity (30).

Identification and Features of EhMGL Genes and Their Proteins—The nucleotide sequences of the 1170- and 1179-bp ORFs recognized in the contigs described above were homologous to those of the γ-subfamily of PLP-dependent enzymes, including MGL, CBL, CGS, and CGL. These putative genes encode 389- and 392-amino acid polypeptides with predicted molecular masses of 42.3 and 42.7 kDa and predicted pIs of 6.01 and 6.63, respectively. We designated these genes *EhMGL1* and *EhMGL2* since their predicted proteins showed highest similarity to methionine γ-lyase (MGL; EC 4.4.1.11) from the Archaea. The deduced amino acid sequences of EhMGL1 and 2 are 69% identical to each other. The EhMGL1 and 2 proteins showed 44–49/44–48% identity to MGL from three archael species, *i.e. Methanosarcina mazei* (MmMGL)[2] *Methanosarcina acetivorans* (MaMGL), and *Methanosarcina barkeri* (MbMGL), respectively. EhMGL1 and 2 also showed 39–46/40–43% identity to MGL from bacteria, *i.e. Fusobacterium nucleatum* (FnMGL, 46/43%) and *Pseudomonas putida* (PpMGL1, 39/40%), and 45/43% identity to MGL from another eukaryotic protozoan parasite, *i.e. T. vaginalis* (TvMGL1). In addition, EhMGL1 and 2 showed 39/41% identity to CGS from *Helicobacter pylori* (HpCGS), 37/36% identity to human CGL (HsCGL), and 26/28% identity to *E. coli* CBL (EcCBL).

Comparison of the deduced amino acid sequences of these MGLs as well as 18 other PLP enzymes (Fig. 2*A*, only MGLs are shown) revealed conserved amino acids as well as residues unique to MGLs. Phe^{44}, Met^{84}, Cys^{110}, and Val^{331} (amino acid numbers are based on EhMGL1) were conserved among all MGLs and absent in all other PLP enzymes. Conservation of Cys^{110} was previously reported for MGL from *P. putida* (31) and *T. vaginalis* (32). Amino acid residues implicated in substrate binding and catalysis from the crystal structure of MGL from *P. putida* (Tyr^{108}, Asp^{180}, Lys^{205}, Arg^{367}) (33) were also conserved in EhMGLs, although these residues were also shared by other PLP-dependent enzymes.

Considering these MGL-specific residues, the *MmMGL* and *MbMGL* genes, which were initially deposited in the data base as CGS (NP_635109) and a hypothetical protein (NZ_AAAR-01001136), respectively, likely encode MGL since all the important residues that were shown to be unique to and shared by biochemically characterized MGLs from other organisms, including the amebic MGL, were completely conserved in these 2 sequences. In addition, amino acid residues of Phe^{188}, Leu^{200}, Cys^{248}, Gly^{302}, and Asp^{337}, and a deletion (at position 232–233) were uniquely conserved among the two EhMGL isozymes and all the archaeal MGLs.

Phylogenetic Analyses of EhMGLs—Phylogenetic reconstruction of the 23 protein sequences that belong to the γ-subfamily of PLP enzymes from a variety of organisms, together with two EhCS isotypes (β-family of PLP enzymes) as the outgroup, was performed with the NJ, MP, and ML methods as described under "Experimental Procedures." These analyses (only the result of the NJ method is shown in Fig. 2*B*) revealed that PLP enzymes involved in sulfur amino acid metabolism were clearly divided into four distinct groups, *i.e.* MGL, CGL, CGS, and CBL, which was supported by high bootstrap proportions (98.9–100%). In the MGL clade, a monophyletic relationship among the MGLs from *E. histolytica* and three Archaea, *i.e. M. acetivorans*, *M. mazei*, and *M. barkeri*, was confirmed (bootstrap proportion 82.4%), while bacterial MGLs and TvMGLs formed an independent clade (93.0%). Therefore, MGL appeared to be subdivided into the *Entamoeba-Archaea* and the *Trichomonas-Bacteria* groups. TvMGLs and EhMGLs did not form a statistically supported clade with any of the three independent analytical methods (data not shown). That is, despite a predicted close relationship between these two protozoan organisms based on several biological and biochemical characteristics (*e.g.* anaerobic metabolism and a lack of mitochondria and the glutathione system), they do not likely share a common ancestor for their MGLs.

Evolutionary Distribution of MGL—The presence of an MGL gene or its encoded protein has been demonstrated in only a fraction of bacteria, including *Clostridium sporogenes* (34), *P. putida* (= *ovalis*) (35), *Pseudomonas taetrolenz* (36), *Bacillus halodurans* (37), *Aeromonas sp.* (38), *Citrobacter intermedius* (39), and *Brevibacterium linenese* (40), and only two eukaryotic organisms, *T. vaginalis* (41) and *E. histolytica* (this study). Coombs *et al.* (41, 42) also reported that MGL activity was not detected in crude extracts from the other anaerobic protozoan parasites *Entamoeba invadens, Trichomonas fetus, Trichomitus batrachorum,* and *Giardia lamblia*, suggesting that the presence of MGL is not directly associated with anaerobic metabolism. In addition, our search for a putative MGL gene in 23 archaeal genome databases available at NCBI revealed that only 3 Archaea, *M. mazei*, *M. acetivorans,* and *M. barkeri*, possess orthologous genes. We were also unable to find a MGL ortholog in other eukaryotes including yeasts, fungi, slime mold, and higher eukaryotes. This unique distribution of MGL strongly supports the premise that two distinct MGL subgroups have been horizontally transferred, *i.e.* from a subgroup of Archaea to *E. histolytica* and from a subgroup of bacteria to *T. vaginalis*.

Molecular and Structural Characterization of Recombinant EhMGL Isoenzymes—In order to understand the biochemical properties of the two EhMGL isoenzymes, recombinant proteins were produced. The recombinant proteins (rEhMGL1 and 2) were assessed to be >95% pure with Coomassie-stained

[2] Names of organisms: Eh, *Entamoeba histolytica*; Mm, *Methanosarcina mazei Goe1*; Tv, *Trichomonas vaginalis*; Fn, *Fusobacterium nucleatum subsp. C2A nucleatum ATCC 25586*; Ma, *Methanosarcina acetivorans*; Mb, *Methanosarcina barkeri*; Pp, *Pseudomonas putida*; Hs, *Homo sapiens*; Ec, *Escherichia coli K12*; Bh, *Bacillus halodurans*; Cc, *Caulobacter crescentus CB15*; Oi, *Oceanobacillus iheyensis*; Rn, *Rattus norvegicus*; Ce, *Caenorhabditis elegans*; Cp, *Clostridium perfringens*; Hp, *Helicobacter pylori*; Sa, *Staphylococcus aureus subsp. aureus N315*; Hi, *Haemophilus influenzae Rd*; St, *Salmonella typhimurium LT2*; Yp, *Yersinia pestis*.

TABLE II

Relative activities and kinetic properties of rEhMGLs

Relative activities and kinetic parameters were determined by using α-keto acid or nitrogen assays. Reactions were performed for 10 min at 36°C with 5 mM (for relative activities) or 0.2-10 mM (for kinetics) of each substrate and an appropriate amount of enzyme. Kinetic parameters were determined by α-keto acid assay for all the substrates except DL-homocysteine and *O*-acetyl-L-serine, which were monitored by nitrogen assay. For L-methionine, both assays were used. Experiments were repeated three times, and results of one representative experiment are shown (means ± S.D.). To determine kinetic constants, at least 7 different substrate concentrations were used. Lineweaver-Burk plots were obtained to calculate parameters using Sigma Plot 2000 software (SPSS Inc., Chicago, IL) with the Enzyme Kinetics module (version 6.0, Hulinks, Inc., Tokyo, Japan).

Substrate	Relative activity		K_m		V_{max}	
	rEhMGL1	rEhMGL2	rEhMGL1	rEhMGL2	rEhMGL1	rEhMGL2
	%		mM		μmol/min/mg	
L-Methionine	100.0	181.6	0.94 ± 0.16	1.90 ± 0.15	0.36 ± 0.20	0.44 ± 0.30
DL-Homocysteine	112.2	294.7	3.40 ± 0.67	1.87 ± 0.77	0.98 ± 0.11	1.31 ± 0.24
L-Cystathionine	<10	<10	ND[a]	ND	ND	ND
L-Cysteine	19.7	160.1	n.d.[b]	2.30 ± 0.10	n.d.	1.26 ± 0.81
O-Acetyl L-serine	11.1	33.8	n.d.	0.89 ± 0.23	n.d.	0.085 ± 0.05

[a] ND, not detectable.
[b] n.d., not determined.

SDS-PAGE gel (data not shown). The apparent molecular masses of rEhMGL1 and 2 (Fig. 4*A*, immunoblots using antibodies against rEhMGL1 and 2 are shown) agreed well with the predicted values (43.3 and 43.7 kDa, respectively, with 10 extra amino acids attached at the N terminus). Two-dimensional PAGE (Fig. 4*C*, *upper two panels*; also see below) showed that these rEhMGL isoenzymes had pIs consistent with those calculated (5.9 and 6.5, respectively). Gel filtration chromatography showed that the molecular mass of the native forms of rEhMGL1 and 2 was 171–177 kDa (Fig. 3*A*), indicating that both rEhMGL proteins form a homotetramer.

Enzymological Characterization of EhMGL Isoenzymes—Both rEhMGL1 and 2 catalyzed α-, γ- or α-, β-elimination of L-methionine, DL-homocysteine, L-cysteine, and OAS, but not L-cystathionine, to form α-keto acid, ammonia, and hydrogen sulfide (from cysteine), methanethiol (from methionine) or acetate (from OAS) (Tables I and II). The specific activity of rEhMGL1 and 2 (*e.g.* 0.36 and 0.44 μmol/min/mg toward L-methionine, respectively) was significantly lower than that of recombinant MGLs from other organisms (*e.g.* rPpMGL, 45.3 μmol/min/mg (43) and rTvMGL1/2, 10.4 ± 0.31/0.67 ± 0.05 μmol/min/mg (32), while the K_ms for substrates were comparable (0.9–3.4 mM) (Table II). Although EhMGL1 and 2 showed moderate homology in their amino acid sequences to EcCBL, HsCGL and HpCGS (shown above), L-cystathionine degradation by either rEhMGL1 or rEhMGL2 was negligible.

Marked differences in substrate specificity and specific activity exist between EhMGL isotypes. rEhMGL1 preferentially degraded L-methionine and DL-homocysteine and showed less activity toward cysteine and OAS, whereas rEhMGL2 catalyzed the degradation of these four amino acids with a comparable efficiency. That is, the ratio of rEhMGL1 activity toward L-cysteine to that toward L-methionine was 0.20 whereas that of rEhMGL2 was 0.88. In addition, rEhMGL2 generally showed 1.8- to 8-fold higher level of specific activity than rEhMGL1, independent of substrates (Table II).

Inter-isotype differences in substrate specificity were previously reported for two MGL isozymes from *T. vaginalis* (32). rTvMGL1 was shown to possess a broader substrate range than rTvMGL2; rTvMGL1 prefers methionine whereas rTvMGL2 is able to utilize methionine, homocysteine, OAS, and cysteine at comparable levels (*e.g.* the ratio of activity toward L-cysteine to that toward L-methionine was 0.58 for rTvMGL1 whereas for rTvMGL2 it was 1.58). The most striking difference between the amebic and trichomonal MGLs was that the latter have a strong preference toward homocysteine (more than 30-fold higher activity for homocysteine than both methionine and cysteine). Reactivity toward L-cystathionine was absent in both the amebic and trichomonal MGLs. This is in good contrast to a recombinant *Pseudomonas* MGL (43). It should be noted that the recombinant TvMGLs and a native form *P. putida* MGL (44) also lacked reactivity for L-cystathionine, which may suggest that reactivity for L-cystathionine is easily lost during purification or in case of ectopic expression using the bacterial system.

The pH optima for the two EhMGL isoenzymes were also significantly different (Fig. 3*B*). Such isotype-dependent differences in the optimum pH have not previously been described for MGLs in other organisms. This, together with the fact that the two EhMGL isotypes show only 69% identity and have distinct pI values, indicates that they may interact with different proteins and also may be localized in distinct subcellular compartments. Immunolocalization of each MGL isotype in amebic transformants expressing epitope-tagged EhMGL1 and 2, which is now underway, should help to further clarify these possibilities.

Next, in order to verify the stoichiometry of the reactions catalyzed by rEhMGLs, individual products of L-methionine catabolism, *i.e.* methanethiol, α-butyric acid, and ammonia, were measured. Approximately equal amounts (within a range of 2-fold) of these compounds were detected (Table I), verifying that rEhMGLs possess comparable α- and γ-lyase activities against methionine.

We also examined whether EhMGL catalyzed the formation of cystathionine from cysteine and, as a source of the homocysteine moiety, *O*-acetyl-L-homoserine or *O*-succinyl-L-homoserine (45), in a reaction known to be catalyzed by cystathionine γ-synthase. However, neither rEhMGL1 nor 2 catalyzed these reactions (data not shown); instead, both enzymes used L-cysteine as substrate for α-, β-elimination. Thus, given our failure to find CBS, CGL, CGS, and CBL homologs in the genome data base, we concluded that, unlike other organisms, *E. histolytica* lacks several key enzymes and their genes involved in the forward and reverse trans-sulfuration reactions.

Inhibition of MGL by PPG—To better understand the biochemical characteristics of EhMGL, we evaluated effects of DL-propargylglycine (PPG), a potent inhibitor of the γ-subfamily of PLP-dependent enzymes (46, 47), on the recombinant EhMGLs. Activity of both rEhMGL1 and 2 was inhibited by PPG in an irreversible and slow-binding manner (Fig. 3, *C* and *D*) with an apparent K_i of 35 μM (for rEhMGL2) with 5 min of preincubation, which agreed well with a previous report on human CGL (47).

We further assessed the effect of PPG on the parasite's MGL in cultures (Table I). The MGL activity of trophozoites was almost completely inhibited (97.5%) when they were cultivated in the BI-S-33 medium supplemented with 20 μM PPG, while control CS activity was not affected by PPG; control CS activity

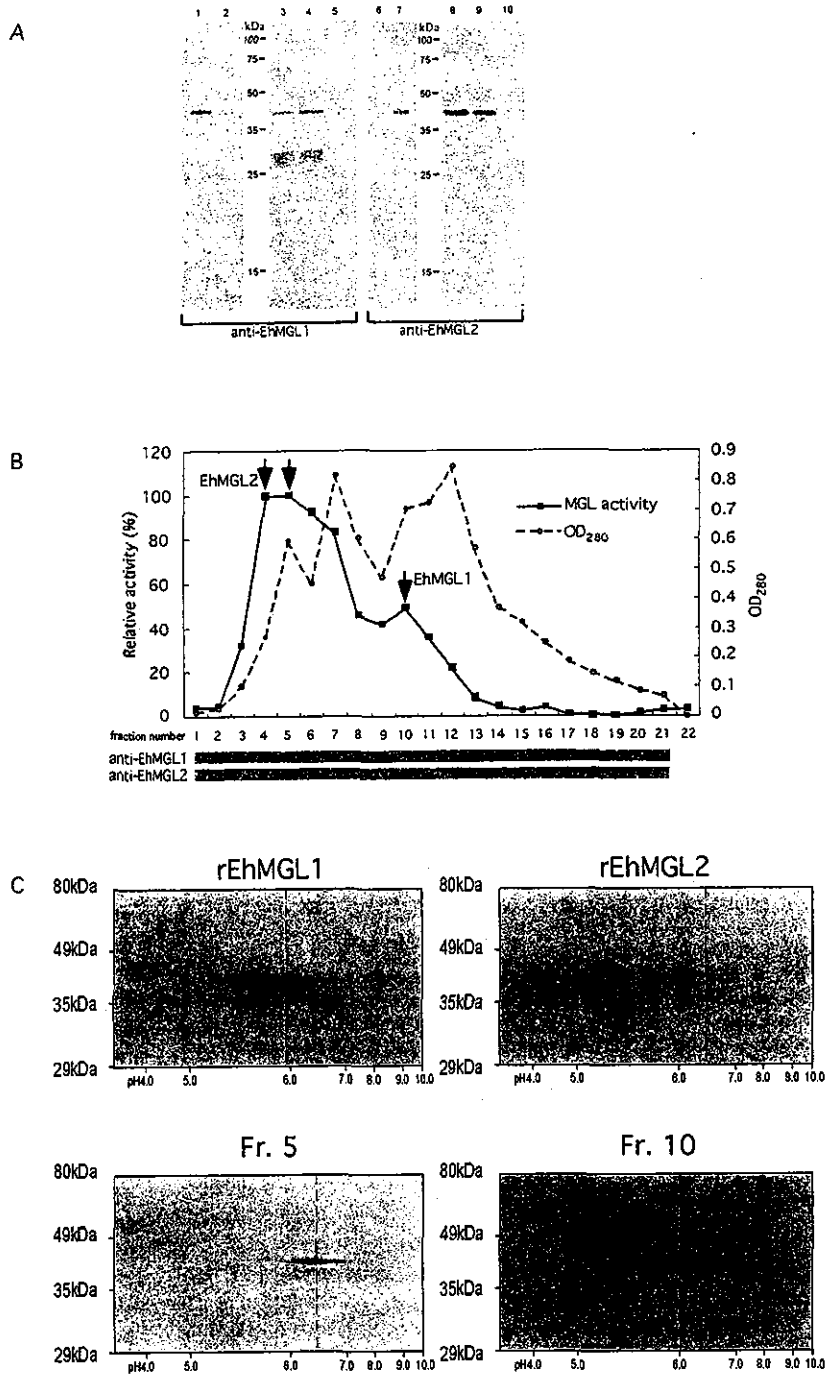

FIG. 4. **Immunoblot analyses of the native and recombinant EhMGL.** *A*, immunoblot analysis showing relative abundance and subcellular localization of two isotypes of EhMGLs. Recombinant EhMGL1 (*lanes 1* and *6*, 5 ng), recombinant EhMGL2 (*lanes 2* and *7*, 5 ng), a whole trophozoite lysate (*lanes 3* and *8*, 20 μg), a soluble fraction (*lanes 4* and *9*, 20 μg), and a pellet fraction (*lanes 5* and *1*, 20 μg) were electrophoresed on 10% SDS-PAGE gel, and subjected to immunoblot analyses with either anti-EhMGL1 (*lanes 1–5*) or anti-EhMGL2 (*lanes 6–10*) antibody as described under "Experimental Procedures." *B*, elution profile of the native EhMGL1 and EhMGL2 obtained by DEAE anion exchange chromatography. *Upper panel* shows MGL activities and A_{280} of individual fractions. *Arrows* indicate fractions in which EhMGLs were recognized with the antisera. *Middle* and *lower panels* show immunoblots of each fraction with anti-EhMGL1 (*middle*) and anti-EhMGL2 (*lower*) antibodies. *C*, two-dimensional PAGE analyses of the native and recombinant EhMGLs. *Upper panels*, one-hundred nanograms of rEhMGL1 or rEhMGL2 was subjected to two-dimensional PAGE and immunoblot analysis. *Lower panels*, two major MGL-containing peak fractions that were eluted from the DEAE column (*frs. 5* and *10*) were subjected to two-dimensional PAGE, followed by immunoblot analyses with the anti-EhMGL1 (*fr. 10*) or anti-EhMGL2 (*fr. 5*) antibody.

from the trophozoites cultured without PPG (72.8 nmol/min/mg) was comparable to that with PPG (69.4 nmol/min/mg). Interestingly, growth inhibition of trophozoites was negligible under these conditions (data not shown). Further, the growth inhibition was less than 5% even at higher PPG concentrations (up to 0.5 mM) (Fig. 5*B*).

These results suggest that MGL may not be essential for the amebae cultured *in vitro* using rich media. Considering the fact that this parasite possesses only incomplete methionine-cysteine conversion (*i.e.* trans-sulfuration) pathways and also lacks the cysteine degradation pathways present in other organisms, *e.g.* mammals, as described above, it is not understood how toxic sulfur-containing amino acids are degraded in the absence of MGL. A trace amount of MGL, together with other unidentified enzymes, may compensate for the decrease of MGL activity. It is also conceivable that the production of α-keto acids, *i.e.* pyruvate and butyrate, by MGL may not be essential in amebae in a nutrient-rich environment despite the fact that these products are used to form acetyl-CoA and α-propionic acid in a reaction catalyzed by pyruvate:ferredoxin oxidoreductase, and thus play a critical role in energy production in anaerobic protozoa (48). Furthermore, the other products of MGL, *i.e.* methanethiol and hydrogen sulfide, which have been implicated in the pathogenesis of oral microorganisms (49, 50), may not be required for *in vitro* growth of the amebic trophozoites. We are currently testing whether MGL shows more detrimental effects on amebae in nutrient-limited xenic cultures and also in animal intestine and liver models.

Characterization of Native Form EhMGL Isotypes in E. histolytica Trophozoites—Immunoblot analysis of the fractionated trophozoite lysate using specific antibody against rEhMGL1

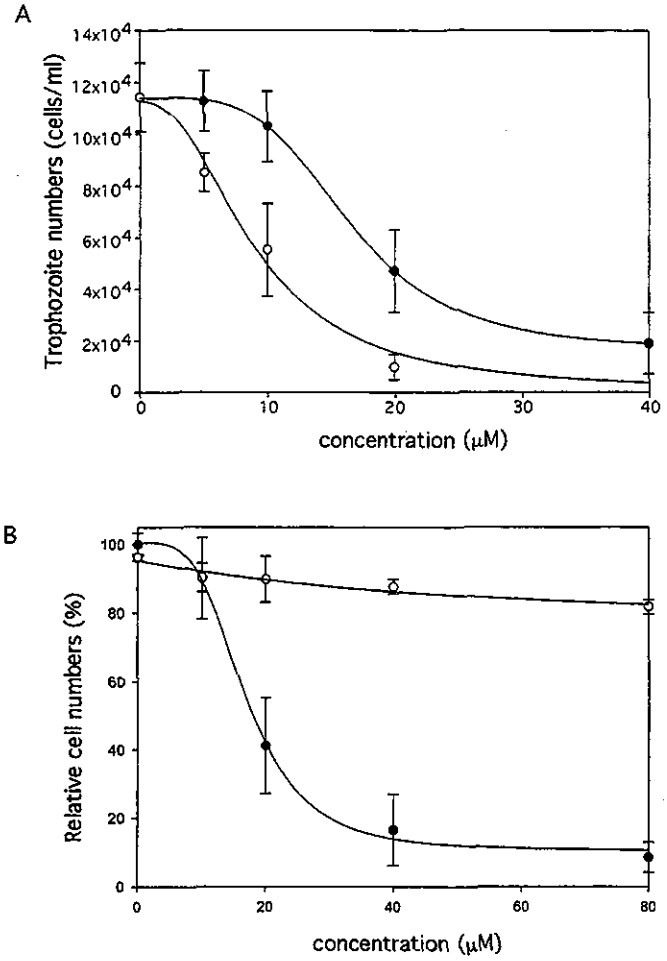

FIG. 5. **Effects of TFMET on the *E. histolytica* trophozoites.** *A*, cytotoxic effects of TFMET and metronidazole on the *E. histolytica* trophozoites. Trophozoites (1×10^5 cells/ml) were cultured with various concentrations of TFMET (●) and metronidazole (○) in BI-S-33 for 18 h. Numbers of live cells were assessed as described under "Experimental Procedures." Data shown are means ± S.D. of four independent experiments. *B*, amebicidal effect of TFMET was abolished by PPG. The trophozoites were cultivated with various concentrations of TFMET with (○) or without (●) 0.5 mM PPG. Percentages of the trophozoites relative to the untreated control are shown. Data shown are means ± S.D. derived from four independent experiments.

or rEhMGL2 showed that both of the native EhMGL isotypes were predominantly present in the cytosol fraction of the *E. histolytica* trophozoites (Fig. 4*A*). A quantitative estimate using densitometric measurements of native EhMGL in the trophozoite lysate with recombinant EhMGLs as controls revealed that EhMGL1 and 2 constitute ~0.05–0.1% of the total soluble protein of the cell (Fig. 4*A*, data for estimation not shown), which agreed well with the activity in the crude extract (Table I).

Fractionation of the whole trophozoite lysate by anion exchange chromatography, followed by the measurement of MGL activity in each fraction, revealed two major peaks possessing MGL activity (Fig. 4*A*, fractions (*frs.*) *4–7* and *9–11*). Immunoblot analyses using anti-EhMGL1 and 2 antibodies identified the first and second peaks as EhMGL2 and EhMGL1, respectively. To correlate the recombinant and native form EhMGLs further, we subjected concentrated samples corresponding to the first and second DEAE peaks (frs. 5 and 10) to two-dimensional gel electrophoresis and immunoblotting. We identified a single spot for each native EhMGL isotype on a two-dimensional gel with measured pIs that agreed well with theoretical pIs (6.01 for rEhMGL1, 6.66 for rEhMGL2). We also examined, by size exclusion chromatography, the subunit structure of these native EhMGL isotypes obtained from the DEAE columns. The apparent molecular mass of the native EhMGL1 and 2 was determined to be ~170 kDa (data not shown). These results suggest, based on the size of the EhMGL monomers observed on SDS-PAGE and two-dimensional gels, that the native EhMGL1 and EhMGL2, similar to the recombinant proteins, also form a homotetramer. This contrasts well to a native MGL from *T. vaginalis*, which was suggested to form a heterotetramer (*i.e.* 2 molecules each of MGL1 and MGL2) (32).

Toxic Effects of TFMET on the Amebic Trophozoites—To exploit MGL as a potential target to develop a new therapeutic against the ameba, we tested if the methinonine analog trifluoromethionine (TFMET) shows an inhibitory effect on trophozoite growth. TFMET, a fluorine substitution-containing analog of methionine, is presumed to be catabolized by MGL to form α-keto butyrate, ammonia, and trifluoromethanethiol. Trifluoromethanethiol is non-enzymatically converted to carbonothionic difluoride (CSF_2), a potent cross-linker of primary amine groups (51).

TFMET caused significant growth inhibition in the trophozoites at concentrations as low as 20 μM. In addition, TFMET showed not only a growth inhibitory effect, but also a notable cytolytic effect on the trophozoites under the same condition; *e.g.* trophozoites cultivated in the presence of 20 μM of TFMET were completely lysed within 72 h. The IC_{50} of TFMET (for the growth inhibition) was determined to be 18 μM, which is slightly higher than that of the most commonly used antiamebic drug metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole], which showed an IC_{50} of 7 μM under the same conditions (Fig. 5*A*). This amebicidal effect of TFMET was completely abolished when the trophozoites were co-incubated with 0.5 mM of PPG, an inhibitor of EhMGL (Fig. 5*B*). Under the same conditions, PPG did not abolish the growth inhibition by metronidazole (data not shown). These results strongly support the premise that catabolism of TFMET by MGL is a part of the cytotoxic mechanism of TFMET in the trophozoites. We should also note that the activity of CS, which is the major PLP-dependent enzyme of this parasite, is not inhibited by up to 100 μM PPG (data not shown), further supporting the premise that MGL is a major target of TFMET. The cytotoxic effect of TFMET was also reported for *T. vaginalis* (52).

Finally, the lack of MGL in higher eukaryotes including humans also highlights this enzyme as a suitable and attractive target for the development of a novel chemotherapeutic agent against amebiasis. Combining conventional metronidazole and a new potent drug, *e.g.* TFMET, for the chemotherapy of amebiasis patients should prevent the rise of metronidazole resistance (53).

Acknowledgments—We thank Masanobu Tanabe, Keio University, for technical assistance on two-dimensional gel electrophoresis and Cyrus J. Bacchi, Pace University, for generously donating TFMET. The data base search was conducted with a 7× *E. histolytica* genome data base available at The Institute for Genomic Research (TIGR) and Sanger Institute with financial support from National Institute of Allergy and Infectious Diseases and The Wellcome Trust.

REFERENCES

1. Behbehani, K. (1998) *Bull World Health Organ.* **76**, Suppl. 2, 64–67
2. Ravdin, J. I. (2000) *AMEBIASIS Series on Tropical Medicine and Practice, Vol. 2*, pp. 1–45 Imperial College Press, Covent Garden, London, UK
3. Clark, C. G., and Roger, A. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 6518–6521
4. Mazzuco, A., Benchimol, M., and De Souza, W. (1997) *Micron.* **28**, 241–247
5. Mai, Z., Ghosh, S., Frisardi, M., Rosenthal, B., Rogers, R., and Samuelson, J. (1999) *Mol. Cell. Biol.* **19**, 2198–2205
6. Müller, M. (1992) *Biosystems* **28**, 33–40
7. Reeves, R. E. (1984) *Adv. Parasitol.* **23**, 105–142
8. Fahey, R. C., Newton, G. L., Arrick, B., Overdank-Bogart, T., and Aley, S. B. (1984) *Science* **224**, 70–72
9. Rosenthal, B., Mai, Z., Caplivski, D., Ghosh, S., de la Vega, H., Graf, T., and

Samuelson, J. (1997) *J. Bacteriol.* **179,** 3736–3745
10. Field, J., Rosenthal, B., and Samuelson, J. (2000) *Mol. Microbiol.* **38,** 446–455
11. Diamond, L. S., Harlow, D. R., and Cunnick, C. C. (1978) *Trans. R. Soc. Trop. Med. Hyg.* **72,** 431–432
12. Gillin, F. D., and Diamond, L. S. (1980) *J. Protozool.* **27,** 474–478
13. Walker, J., and Barrett, J. (1997) *Int. J. Parasitol.* **27,** 883–897
14. Nozaki, T., Asai, T., Kobayashi, S., Ikegami, F., Noji, M., Saito, K., and Takeuchi, T. (1998) *Mol. Biochem. Parasitol.* **97,** 33–44
15. Nozaki, T., Asai, T., Sanchez, L. B., Kobayashi, S., Nakazawa, M., and Takeuchi, T. (1999) *J. Biol. Chem.* **274,** 32445–32452
16. Diamond, L. S., Mattern, C. F., and Bartgis, I. L. (1972) *J. Virol.* **9,** 326–341
17. Mehta, P. K., and Christen, P. (2000) *Adv. Enzymol. Relat. Areas. Mol. Biol.* **74,** 129–184
18. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680
19. Page, R. D. (1996) *Comput. Appl. Biosci.* **12,** 357–358
20. Kuhner, M. K., and Felsenstein, J. (1994) *Mol. Biol. Evol.* **11,** 459–468
21. Adachi, J., and Hasegawa, M. (1996) *Computer Science Monographs, Institute of Statistical Mathematics,* Tokyo, Japan **28,** 1–150
22. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: a Laboratory Manual,* 3rd Ed., pp. 18.62–18.74, Cold Spring Harbor Laboratory Press, *Cold Spring Harbor,* NY
23. Soda, K. (1967) *Agr. Biol. Chem.* **31,** 1054–1060
24. Thompson, J. F., and Morrison, G. R. (1951) *Anal. Chem.* **23,** 1153–1157
25. Siegel, L. M. (1965) *Anal. Biochem.* **11,** 126–132
26. Schneider, D., Jaschkowitz, K., Seidler, A., and Rogner, M. (2000) *Indian J. Biochem. Biophys* **37,** 441–446
27. Jaschkowitz, K., and Seidler, A. (2000) *Biochemistry* **39,** 3416–3423
28. Laakso, S., and Nurmikko, V. (1976) *Anal. Biochem.* **72,** 600–605
29. Kashiwamata, S., and Greenberg, D. M. (1970) *Biochim. Biophys. Acta.* **212,** 488–500
30. Starkebaum, G., and Harlan, J. M. (1986) *J. Clin. Investig.* **77,** 1370–1376
31. Nakayama, T., Esaki, N., Tanaka, H., and Soda, K. (1988) *Biochemistry* **27,** 1587–1591
32. McKie, A. E., Edlind, T., Walker, J., Mottram, J. C., and Coombs, G. H. (1998) *J. Biol. Chem.* **273,** 5549–5556
33. Motoshima, H., Inagaki, K., Kumasaka, T., Furuichi, M., Inoue, H., Tamura, T., Esaki, N., Soda, K., Tanaka, N., Yamamoto, M., and Tanaka, H. (2000) *J. Biochem. (Tokyo)* **128,** 349–354
34. Kreis, W., and Hession, C. (1973) *Cancer Res.* **33,** 1862–1865
35. Ito, S., Nakamura, T., and Eguchi, Y. (1976) *J. Biochem. (Tokyo)* **80,** 1327–1334
36. Zanin, V. A., Lukina, V. I., and Berezov, T. T. (1989) *Vopr. Med. Khim.* **35,** 84–89
37. Cuhel, R. L., Taylor, C. D., and Jannasch, H. W. (1981) *J. Bacteriol* **147,** 340–349
38. Nakayama, T., Esaki, N., Sugie, K., Beresov, T. T., Tanaka, H., and Soda, K. (1984) *Anal. Biochem.* **138,** 421–424
39. Faleev, N. G., Troitskaya, M. V., Paskonova, E. A., Saporovskaya, M. B., and Belikov, V. M. (1996) *Enzyme and Microbial Technology* **19,** 590–593
40. Dias, B., and Weimer, B. (1998) *Appl. Environ Microbiol* **64,** 3327–3331
41. Thong, K. W., Coombs, G. H., and Sanderson, B. E. (1987) *Mol. Biochem. Parasitol* **23,** 223–231
42. Lockwood, B. C., and Coombs, G. H. (1991) *Biochem. J.* **279,** 675–682
43. Hori, H., Takabayashi, K., Orvis, L., Carson, D. A., and Nobori, T. (1996) *Cancer Res.* **56,** 2116–2122
44. Esaki, N., and Soda, K. (1987) *Methods Enzymol.* **143,** 459–465
45. Shimizu, H., Yamagata, S., Masui, R., Inoue, Y., Shibata, T., Yokoyama, S., Kuramitsu, S., and Iwama, T. (2001) *Biochim Biophys Acta.* **1549,** 61–72
46. Johnston, M., Jankowski, D., Marcotte, P., Tanaka, H., Esaki, N., Soda, K., and Walsh, C. (1979) *Biochemistry* **18,** 4690–4701
47. Steegborn, C., Clausen, T., Sondermann, P., Jacob, U., Worbs, M., Marinkovic, S., Huber, R., and Wahl, M. C. (1999) *J. Biol. Chem.* **274,** 12675–12684
48. Upcroft, J. A., and Upcroft, P. (1999) *J. Eukaryot. Microbiol* **46,** 447–449
49. Lancero, H., Niu, J., and Johnson, P. W. (1996) *J. Dent. Res.* **75,** 1994–2002
50. Kapatral, V., Anderson, I., Ivanova, N., Reznik, G., Los, T., Lykidis, A., Bhattacharyya, A., Bartman, A., Gardner, W., Grechkin, G., Zhu, L., Vasieva, O., Chu, L., Kogan, Y., Chaga, O., Goltsman, E., Bernal, A., Larsen, N., D'Souza, M., Walunas, T., Pusch, G., Haselkorn, R., Fonstein, M., Kyrpides, N., and Overbeek, R. (2002) *J. Bacteriol.* **184,** 2005–2018
51. Alston, T. A., and Bright, H. J. (1983) *Biochem. Pharmacol.* **32,** 947–950
52. Coombs, G. H., and Mottram, J. C. (2001) *Antimicrob. Agents Chemother.* **45,** 1743–1745
53. Wassmann, C., Hellberg, A., Tannich, E., and Bruchhaus, I. (1999) *J. Biol. Chem.* **274,** 26051–26056
54. Tovar, J., Fischer, A., and Clark, C. G. (1999) *Mol. Microbiol.* **32,** 1013–1021

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Parasitology International 52 (2003) 169–173
www.elsevier.com/locate/parint

Research note

Induction of permeability changes and death of vertebrate cells is modulated by the virulence of *Entamoeba* spp. isolates

James A. Dvorak[a,*], Seiki Kobayashi[b], Tomoyoshi Nozaki[c,d], Tsutomu Takeuchi[b], Chizuko Matsubara[e]

[a]*Laboratory of Malaria and Vector Biology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892-0425, USA*
[b]*Department of Tropical Medicine and Parasitology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan*
[c]*Department of Parasitology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 160-8640, Japan*
[d]*Precursory Research for Embryonic Science and Technology, Japan Science and Technology Corporation, Kawaguchi, Japan*
[e]*Leica Microsystems K.K., East Tower 7F, Gate City Osaki, 1-11-2, Osaki, Shinagawa-ku, Tokyo 141-0032, Japan*

Received 18 October 2002; received in revised form 19 November 2002; accepted 19 November 2002

Abstract

Although *Entamoeba histolytica* is capable of inducing an apoptotic response in vertebrate cells in vitro (Cell. Microbiol. 2 (2000) 617), it is not known whether vertebrate cell death requires direct amoeba–vertebrate cell contact or simply the presence of amoebae in the area of the vertebrate cells. In addition, *Entamoeba* spp. vary in their virulence and pathogenicity. The potential effects of these critical parameters also have not been elucidated. We tested the virulent HM-1:IMSS isolate and the non-virulent Rahman isolate of *E. histolytica*, and the non-virulent *E. dispar* CYNO16:TPC isolate against two vertebrate cell lines, HeLa and Chinese hamster ovary cells in vitro using ethidium homodimer as a fluorescent indicator of changes in vertebrate cell permeability. Fluorescence appeared in vertebrate cell nuclei within approximately 2–3 min of contact between HM-1 amoebae and vertebrate cells independent of vertebrate cell type. However, vertebrate cells in the immediate vicinity of but not contacted by HM-1 amoebae were not affected. In contrast, although both *E. histolytica* Rahman and *E. dispar* CYNO16 amoebae moved freely among and contacted vertebrate cells, the nuclei of the vertebrate cells never fluoresced implying that the cells remained alive and impermeant to the ethidium homodimer. This is the first demonstration that direct contact between virulent amoebae and vertebrate cells is required to kill vertebrate cells and that the process is restricted to virulent *Entamoeba* isolates. An understanding at the molecular level of the processes involved could help to reduce the pathology associated with this parasite.

Keywords: *Entamoeba histolytica*; *E. dispar*; Vertebrate cells; Ethidium homodimer; Cell death

*Corresponding author. Tel.: +1-301-496-4880; fax: +1-301-480-1438.
E-mail address: jdvorak@niaid.nih.gov (J.A. Dvorak).

1383-5769/03/$ - see front matter © 2002 Elsevier Science Ireland Ltd. All rights reserved.
doi:10.1016/S1383-5769(02)00090-9

Amoebiasis caused by *Entamoeba histolytica* is a major public health problem in both developing and developed countries worldwide [1,2]. However, there are marked differences in the virulence and pathogenicity of *E. histolytica* isolates [3,4]. Furthermore, *E. dispar*, a non-pathogenic species, also exists [5]. Various operational definitions of virulence and pathogenicity of *Entamoeba* spp. have been reported [6–8]. Recently, there has been a focus on the ability of *E. histolytica* to induce an apoptotic response in vertebrate cells [9]. In the report presented here, we demonstrate that the ability to induce permeability changes in vertebrate cells, indicative of cell death as demonstrated in other host-parasite interactions [10], requires amoeba–vertebrate cell contact and is dependent upon the known virulence of the *Entamoeba* isolate.

We used two isolates of *E. histolytica*, HM-1:IMSS (ATCC 30459) clone 6 [11,12], and Rahman (ATCC 30886) [13] and one isolate of *E. dispar* CYNO16:TPC [14]. Amoebae were maintained in BI-S-33 medium [15] supplemented with 15% bovine serum for the HM-1:MSS isolate and 10% bovine serum for the Rahman isolate. *E dispar* was maintained in YIGADHA-S medium supplemented with 15% bovine serum [16]. We used two vertebrate cell lines, HeLa and Chinese hamster ovary (CHO) cells grown in a humidified atmosphere at 37 °C, 5% CO_2. HeLa cells were maintained in Dulbecco's medium and CHO cells were maintained in MEM medium and both media were supplemented with 10% fetal calf serum. For microscopy experiments, vertebrate cells were seeded into culture chambers (Lab-Tek, Cat. No. 155379; Nalge Nunc International, Tokyo, Japan) using 5–10% serum depending upon the amount of cell growth required for the culture to be subconfluent at the time of the experiment. In preliminary experiments, we determined that both amoebae and vertebrate cells survived with no apparent morphologic changes and normal amoeba motility for at least 2 h in a 1:1 mixture of amoeba and vertebrate cell culture media containing 10 μM ethidium homodimer (Cat No. E-3599, Molecular Probes Inc., Eugene, OR), a live cell-impermeant dye. For assay by microscopy, amoeba were detached from the walls of the culture tube in which they were growing by briefly cooling the tube in an ice bath. Following detachment, an equal volume of vertebrate cell culture medium was added to 5000–10 000 amoeba in amoeba culture medium and 10 μM ethidium homodimer was added to the mixture. Culture medium was removed by aspiration from the vertebrate cells in the culture chambers and the amoeba–vertebrate cell culture medium mixture containing amoebae and ethidium homodimer was added to the chamber, the time was recorded, and the cells were observed for a period of approximately 2 h. Microscopy was performed with a Leica DM IRE 2 inverted microscope equipped with a vertical fluorescence illuminator and Leica DC350F digital camera. Image analyses were performed using ImagePro version 4.5 (Media Cybernetic, Silver Spring, MD).

Amoeboid movement resumed after a minimum of 5 min; the amoeba moved actively among and on top of the vertebrate cells. However, there was a remarkable difference in the interaction between amoebae and vertebrate cells dependent upon amoeba isolate. Within 45 min of mixing vertebrate cells with the virulent *E. histolytica* HM-1:IMSS isolate, the nuclei of vertebrate cells began to display a red fluorescence indicating an uptake of the ethidium homodimer (Fig. 1a and b). The fluorescence appeared within approximately 2–3 min of contact between amoebae and vertebrate cells. This observation was repeatable and independent of the vertebrate cell type (data not shown). Vertebrate cells in the immediate vicinity of amoebae but not contacted by amoebae did not display fluorescent nuclei. Following the induction of nuclear fluorescence, the vertebrate cells slowly became detached from the substrate (Fig. 1c–e) and were eventually ingested by the amoebae (Fig. 1f). In contrast, although amoebae of both the *E. histolytica* Rahman isolate and the *E. dispar* isolate moved freely among and over the vertebrate cells, no observable changes in the vertebrate cells occurred over a 2 h observation period; the nuclei of the vertebrate cells never fluoresced implying that the cells remained alive and impermeant to the ethidium homodimer (data not shown).

This is the first demonstration of amoeba-induced changes in the permeability of vertebrate

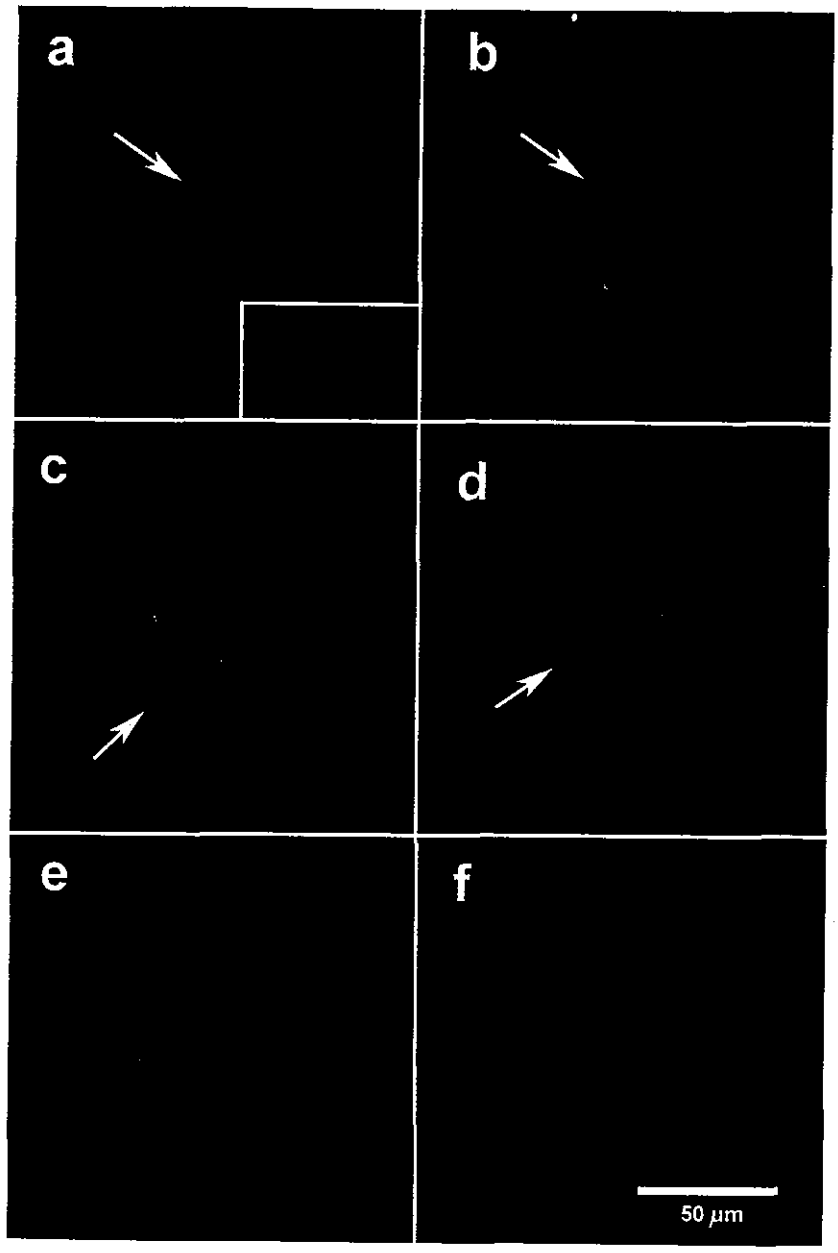

Fig. 1. Representative examples of the interaction between *Entamoeba histolytica* trophozoites and vertebrate cells. (a) An amoeba (arrow) contacts three vertebrate cells. One of the cells at the trailing edge of the amoeba was contacted first and the other two cells at the leading edge of the amoeba are in the process of being contacted. The insert, at a two-fold higher magnification, shows the presence of granules in the hyaline region of the advancing amoeba pseudopod at the point of contact between an amoeba and a vertebrate cell. (b) same cells shown in a (above) but approximately 2 min later. The nucleus of two of the vertebrate cells, the one in focus and the other out of focus have taken up the ethidium homodimer. The third vertebrate cell shown in the insert in figure being contacted by the amoeba (arrow) but out of focus in this image is just beginning to display nuclear fluorescence; (c) and (d) amoebae (arrows) in contact with vertebrate cells which are beginning to display ethidium homodimer fluorescence and retract from the floor of the culture chamber; (e) amoeba with the attached remnants of a vertebrate cell displaying ethidium homodimer fluorescence. The floor of the culture chamber with attached vertebrate cells is out of focus; (f) living and motile amoeba with fluorescent phagosome showing that the amoeba has ingested a dead vertebrate cell.

cells resulting in the death of the vertebrate cells following amoeba–vertebrate cell contact. The exact mechanism of vertebrate cell death remains to be determined. However, preliminary observations appear to indicate that as an amoeba touched a vertebrate cell, granules appeared in the hyaline region of the advancing amoeba pseudopod (cf. Fig. 1, insert). Shortly thereafter, the nucleus of the vertebrate cell began to fluoresce indicating an uptake of ethidium homodimer. These observations demonstrate that the cell was no longer impermeant to ethidium homodimer and was dying. It is assumed that these granules might contain virulence factors of *E. histolytica* including amebapore [17] and/or cysteine proteinases [18,19]. In light of the marked metabolic differences (e.g. anaerobic vs. aerobic) between *Entamoeba* spp. and vertebrate cells [20–22] it is reasonable to propose that it might be detrimental for amoebae to ingest a living, aerobic cell which might damage its own respiratory processes. Thus, virulent amoebae may have to 'kill or biochemically inactivate their prey' as often occurs with higher animals to protect themselves in this case from the potentially toxic effects of their prey. If the same sequence of events occurs in vivo as we observed in vitro it might help to explain the localized lesions in the liver and bowel resulting from infection with a virulent form of *E. histolytica* and the absence of equivalent pathology when infection occurs with a non-virulent form of *E. histolytica* or *E. dispar*. An understanding at the molecular level of the processes involved in localized vertebrate cell death resulting from contact with virulent amoeba could help to reduce the pathology associated with infection by this parasite.

Acknowledgments

The authors express appreciation to Dr Yasushi Miyahira, Juntendo University School of Medicine for providing the vertebrate cell cultures used in these experiments and to Mr Hiroshi Suzuki for his kindness and generosity in permitting us to use the facilities of Leica Microsystems, K.K. for the microscopy phase of our research. This work was supported in part by a Japan Society for the Promotion of Science fellowship to J.A.D.

References

[1] WHO/PAHO/UNESCO Report, A consultation with experts on amebiasis, Epidemiol Bull 1997;18:13–14.
[2] Nozaki T. Current problems of Amebiasis in Japan and recent advances in amebiasis researches. Jpn J Infect Dis 2000;53:229–37 (Review).
[3] De Menezes Feitosa L, Salgado LM, Rodriguez MA, Vargas MA, Orozco E. Phenotype variability and genetic polymorphism in *Entamoeba histolytica* clonal populations. Arch Med Res 1997;28:27–9.
[4] Flores-Romo L, Estrada-Garcia T, Shibayama-Salas M, et al. In vitro *Entamoeba histolytica* adhesion to human endothelium: a comparison using two strains of different virulence. Parasitol Res 1997;83:397–400.
[5] Diamond LS, Clark CG. A redescription of *Entamoeba histolytica* Schaudinn, 1903 (Emended Walker, 1911) separating it from *Entamoeba dispar* Brumpt, 1925. J Eukaryot Microbiol 1993;40:340–4.
[6] Clark CG. Amoebic disease. *Entamoeba dispar*, an organism reborn. Trans R Soc Trop Med Hyg 1998;92:361–4.
[7] Petri WA Jr., Haque R, Mann BJ. The bittersweet interface of parasite and host: Lectin-carbohydrate interactions during human invasion by the parasite *Entamoeba histolytica*. Annu Rev Microbiol 2002;56:39–64.
[8] Espinosa-Cantellano M, Martinez-Palomo A. Pathogenesis of intestinal amebiasis: from molecules to disease. Clin Microbiol Rev 2000;13:318–31.
[9] Meerovitch E, Chadee K. In vivo models for pathogenicity in amebiasis. In: Ravdin JI, editor. Amebiasis human infection by *Entamoeba histolytica*.Wiley, 1988. p. 177–90.
[10] Huston CD, Houpt ER, Mann BJ, Hahn CS, Petri WA Jr. Caspase 3-dependent killing of host cells by the parasite *Entamoeba histolytica*. Cell Microbiol 2000;2:617–25.
[11] Zieler H, Dvorak JA. Invasion in vitro of mosquito midgut cells by the malaria parasite proceeds by a conserved mechanism and results in death of the invaded midgut cells. Proc Nat Acad Sci 2000;97:11516–21.
[12] Diamond LS, Mattern CF, Bartgis IL. Viruses of *Entamoeba histolytica*. I Identification of transmissible virus-like agents. J Virol 1972;9:326–41.
[13] Diamond LS, Phillips BP, Bartgis IL. The clawed jird (*Meriones unguiculatus*) as an experimental animal for the study of hepatic amebiasis. Arch Invest Med (Mex) 1974;5(suppl 2):465–70.
[14] McGowan K, Deneke CF, Thorne GM, Gorbach SL. *Entamoeba histolytica* cytotoxin: purification, characterization, strain virulence, and protease activity. J Infect Dis 1982;146:616–25.
[15] Pillai DR, Kobayashi S, Kain KC. *Entamoeba dispar*: molecular characterization of the galactose/*N*-acetyl-*d*-galactosamine lectin. Exp Parasitol 2001;99:226–34.
[16] Diamond LS, Harlow DR, Cunnick CC. A new medium for the axenic cultivation of *Entamoeba histolytica* and

other *Entamoeba*. Trans R Soc Trop Med Hyg 1978;72:431–2.

[17] Kobayashi S, Imai E, Haghighi A, Tachibana H, Takeuchi T. Cultivation of *Entamoeba dispar*: growth-promoting effect of ferredoxin. Arch Med Res 2000;31(4):S210–211.

[18] Leippe M, Ebel S, Schoenberger OL, Horstmann RD, Muller-Eberhard HJ. Pore-forming peptide of pathogenic *Entamoeba histolytica*. Proc Natl Acad Sci 1991;88:7659–63.

[19] Keene WE, Petitt MG, Allen S, McKerrow JH. The major neutral proteinase of *Entamoeba histolytica*. J Exp Med 1986;163:536–49.

[20] McKerrow JH, Sun E, Rosenthal PJ, Bouvier J. The proteases and pathogenicity of parasitic protozoa. Ann Rev Microbiol 1993;47:821–53.

[21] Reeves RE. Metabolism of *Entamoeba histolytica* Schaudinn 1903. Adv Parasitol 1984;23:105–42.

[22] McLaughlin J, Aley S. The biochemistry and functional morphology of the *Entamoeba*. J Protozool 1985;32:221–40.

CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Jan. 2004, p. 216–218 Vol. 11, No. 1
1071-412X/04/$08.00+0 DOI: 10.1128/CDLI.11.1.216-218.2004

Bacterial Expression of a Human Monoclonal Antibody-Alkaline Phosphatase Conjugate Specific for *Entamoeba histolytica*

Hiroshi Tachibana,[1]* Masataka Takekoshi,[2] Xun-Jia Cheng,[1] Yuta Nakata,[1] Tsutomu Takeuchi,[3] and Seiji Ihara[2]

Departments of Infectious Diseases[1] and Molecular Life Sciences,[2] Tokai University School of Medicine, Isehara, Kanagawa 259-1193, and Department of Tropical Medicine and Parasitology, School of Medicine, Keio University, Shinjuku-ku, Tokyo 160-8582,[3] Japan

Received 1 August 2003/Returned for modification 14 September 2003/Accepted 2 October 2003

We previously produced human monoclonal antibody Fab fragments specific to *Entamoeba histolytica* in *Escherichia coli*. In order to use these Fab fragments for diagnostic purposes, an expression vector to produce a fusion protein of Fab and alkaline phosphatase (PhoA) in *E. coli* was designed and constructed. The *E. coli* PhoA gene was fused to the 3′ terminus of the gene encoding the heavy-chain Fd region. The kappa and Fd genes from a previously prepared antibody clone, CP33, which is specific for the 260-kDa lectin of *E. histolytica*, were used as human antibody genes. When the fusion protein of CP33 and PhoA was incubated with paraformaldehyde-fixed trophozoites of *E. histolytica* and developed with a substrate, the trophozoites appeared to be stained. These results demonstrate the feasibility of bacterial expression of a human monoclonal antibody-PhoA conjugate specific for *E. histolytica* and that the antibody can be used to detect *E. histolytica* antigen without the use of chemically conjugated secondary antibodies.

Amebiasis caused by infection with *Entamoeba histolytica* is one of the most important parasitic diseases not only in developing countries but also in developed countries. It has been estimated that 50 million people develop amebic colitis and extraintestinal abscesses, resulting in 40,000 to 100,000 deaths annually (3). Laboratory diagnosis of intestinal amebiasis is usually based on the microscopic detection of the organism in stool samples. However, nonpathogenic commensal *Entamoeba dispar*, which is morphologically identical with but genetically distinct from *E. histolytica*, has been identified recently as a separate species (9). Since treatment of *E. dispar* infection is not required, accurate diagnostic tools to discriminate between the two species are needed (3).

The application of monoclonal antibodies (MAbs) is one of several strategies for specific and sensitive diagnoses of infectious diseases. A number of MAbs which react specifically with *E. histolytica* or *E. dispar* have been produced by hybridoma technology (14, 16, 19–21). It has been reported that some MAbs were useful for detecting *E. histolytica* antigen in fecal and serum samples by sandwich enzyme-linked immunosorbent assay (1, 2, 11, 12). Recently, a new technology to produce a Fab fragment or single-chain Fv fragment in *Escherichia coli* has been established (4, 6, 15). The construction of vectors for the production of Fab in *E. coli* has also been reported (22, 24). When mouse immunoglobulin genes derived from a hybridoma producing *E. histolytica*-specific MAbs were expressed in this system, the specificity of the recombinant mouse Fab was comparable to that of the parent antibody (22). More recently, recombinant human MAb Fab fragments specific for *E. histolytica* have also been prepared from peripheral lymphocytes of a patient with an amebic liver abscess and of an asymptomatic cyst carrier (8, 18, 23). In order to use these human Fabs for diagnostic purposes, we report here the bacterial expression of a human Fab-alkaline phosphatase (PhoA) conjugate specific for *E. histolytica*.

The phagemid vector pRPLS/Fab1 (24) was digested with restriction enzymes *Not*I and *Eco*RI. A synthetic DNA linker consisting of two oligonucleotides (5′-GGCCGCAGGTGGC GGAGGTTCTGGTGGCGGAGGTTCTGGTGGCGGAGG TTCTAGACTCGAGTAAG-3′ and 5′-AATTCTTACTCGAG TCTAGAACCTCCGCCACCAGAACCTCCGCCACCAG AACCTCCGCCACCTGC-3′) was inserted into the *Not*I/*Eco*RI site of pRPLS/Fab1, thus creating a 15-mer Gly4Ser linker and recognition site for *Xba*I and *Xho*I. The resulting phagemid, pFab1-L, was digested with restriction enzymes *Xba*I and *Xho*I. To clone the *E. coli* PhoA gene, *E. coli* XL1-Blue was alkali lysed, neutralized, and subjected to PCR amplification. Two synthetic primers (5′-CCTCTAGAGGTACCCCAGAAATGCCTGTT CTAGAAA-3′ and 5′-GGCTCGAGTTTAAGCCCCAGAG CGGC-3′) were used to amplify 1.45 kb of the PhoA gene (17). The amplified gene was digested with *Xba*I and *Xho*I and subcloned into the *Xba*I/*Xho*I site of pFab1-L, resulting in phagemid pFab1-PhoA. This phagemid, pFab1-PhoA, was digested with *Xho*I and *Eco*RI. A synthetic DNA linker consisting of two oligonucleotides (5′-TCGAGGGTGGCGGAGGTTCT CATCACCATCACCATCACTAAG-3′ and 5′-AATTCATGG TGATGGTGATGGTGATGAGAACCTCCGCCACCC-3′) was inserted into the *Xho*I/*Eco*RI site of pFab1-PhoA, thus creating a 15-mer Gly_4Ser linker and a His_6 tag. The resulting plasmid was named pFab1-PhoA-H (Fig. 1).

As the source of human immunoglobulin genes, the kappa and Fd genes from a previously prepared antibody clone, CP33 (23), which is specific for the 260-kDa lectin of *E. histolytica*, were used for production of the fusion protein. The DNA fragment containing light- and heavy-chain genes was obtained by *Nhe*I/*Not*I digestion of pFab1-His2. The fragment was ligated with pFab1-PhoA-H and then introduced into compe-

* Corresponding author. Mailing address: Department of Infectious Diseases, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa 259-1193, Japan. Phone: 81 (463) 93-1121. Fax: 81 (463) 95-5450. E-mail: htachiba@is.icc.u-tokai.ac.jp.

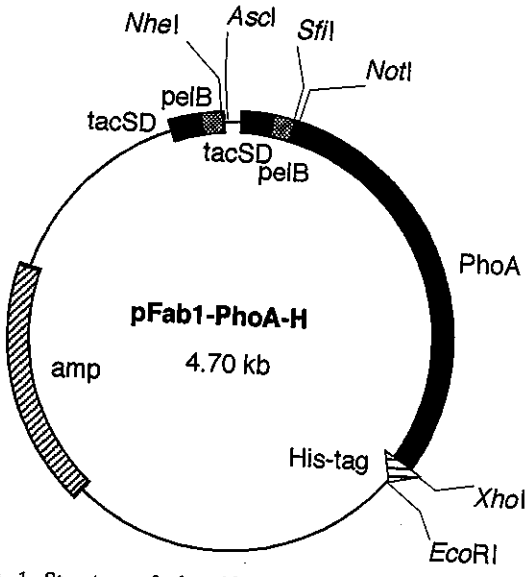

FIG. 1. Structure of plasmid vector pFab1-PhoA-H used for expression of the fusion protein of Fab and alkaline phosphatase. Genes encoding the light chain and the Fd region of the heavy chain are ligated into the *NheI*/*Asc*I and *Sfi*I/*Not*I sites, respectively. tacSD, tac promoter Shine-Dalgarno sequence; pelB, signal sequence of pectate lyase of *Erwinia carotovora*; PhoA, gene for alkaline phosphatase; His-tag, gene for hexahistidine tag; amp, gene for ampicillin resistance.

tent *E. coli* JM109. The bacteria were spread on Luria-Bertani plates containing 50 μg of ampicillin per ml, and the vector with the inserts was selected. The positive clone was cultured in 1 liter of super broth (30 g of tryptone, 20 g of yeast extract, 10 g of MOPS [morpholinepropanesulfonic acid] per liter [pH 7]) containing ampicillin at 37°C until an optical density at 600 nm of 0.5 was achieved. Isopropyl-β-D-thiogalactopyranoside was added to the cultures to a final concentration of 100 μM, and the cultures were then incubated at 30°C for 12 h to achieve optimal expression. The bacteria were pelleted by centrifugation at 6,000 × *g* for 20 min, suspended in 20 ml of phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride, and then sonicated. The lysates were centrifuged at 12,000 × *g* for 30 min, and the supernatant was filtered through 0.2-μm-pore-size syringe filters (Iwaki, Tokyo, Japan). Purification of the fusion protein from the supernatant was performed by affinity chromatography with His•Bind resin (Novagen, Madison, Wis.) in accordance with the manufacturer's instructions. Purified fusion protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (22). Western immunoblot analysis was also performed as previously described (22). The horseradish peroxidase (HRP)-conjugated goat immunoglobulin G (IgG) fraction specific to the human kappa chain (Organon Teknica, Durham, N.C.) and HRP-conjugated rabbit IgG fraction specific to alkaline phosphatase (Rockland, Gilbertsville, Pa.) were used for detection.

Approximately 2 × 10^5 trophozoites of *E. histolytica* HM-1:IMSS cultured axenically in BI-S-33 medium (10) were incubated on acetone-washed coverslips at 37°C for 30 min. The trophozoites were fixed with 4% paraformaldehyde in PBS for 30 min and then washed three times with PBS. After blocking with 5% bovine serum albumin was conducted for 15 min, the cells were incubated with the recombinant protein (50 μg/ml) for 30 min. After the cells were washed with PBS, development was conducted with a Vector red alkaline phosphatase substrate kit I (Vector Laboratories, Burlingame, Calif.) for 30 min in accordance with the manufacturer's instructions. Microscopic observation of the cells was performed under bright-field and fluorescent conditions by using a Nikon (Tokyo, Japan) XF-EFD2 fluorescence microscope.

SDS-PAGE analysis of the purified fusion protein of CP33 and PhoA revealed the expected sizes of two bands with apparent molecular masses of 25 and 75 kDa, although minor bands with apparent molecular masses of 50 kDa were also present (Fig. 2A). With Western immunoblot analysis, the 25-kDa band was recognized by an anti-human kappa chain goat antibody (Fig. 2B, lane 1). On the other hand, the 75-kDa band was detected by an anti-PhoA rabbit antibody, indicating that the molecule was a fusion protein of the Fd fragment and PhoA (Fig. 2B, lane 2). When the fusion protein of Fab-PhoA was incubated with paraformaldehyde-fixed trophozoites of *E. histolytica* and developed with the substrate, the surfaces of the trophozoites were stained clearly under both bright-field and fluorescent conditions (Fig. 3).

Recombinant human antibodies have been developed re-

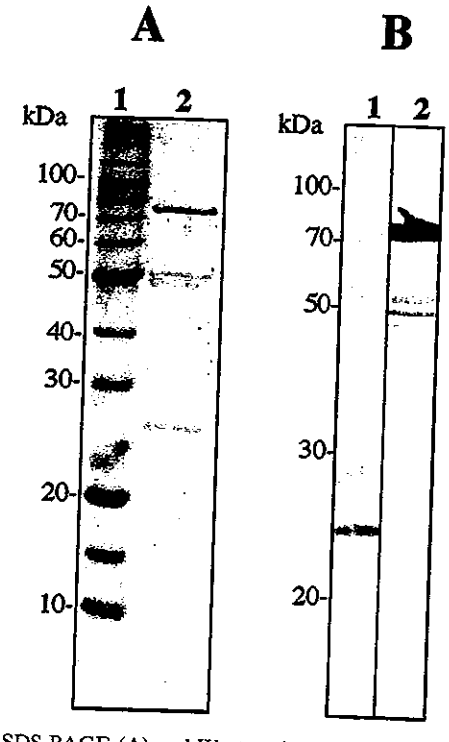

FIG. 2. SDS-PAGE (A) and Western immunoblot (B) analyses of a purified fusion protein of human Fab CP33 and alkaline phosphatase. (A) Two micrograms of the protein was subjected to analysis in 10% gel under reducing conditions and then stained with Coomassie brilliant blue. Lane 1, molecular size markers (BenchMark protein ladder; Life Technologies, Gaithersburg, Md.); lane 2, purified CP33-PhoA. Numbers to the left indicate molecular masses of the markers (in kilodaltons). (B) Protein bands were transferred to a polyvinylidene difluoride membrane. Lane 1 was treated with HRP-labeled anti-human kappa chain goat antibody. Lane 2 was treated with HRP-labeled anti-PhoA rabbit antibody.

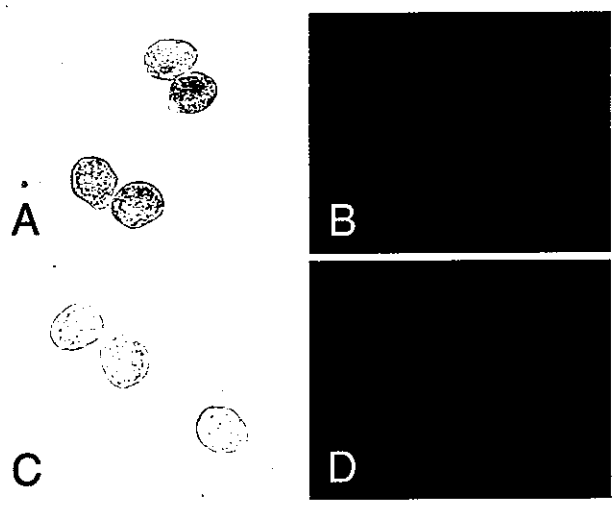

FIG. 3. Immunocytochemistry of *E. histolytica* with a fusion protein of human Fab CP33 and alkaline phosphatase. Paraformaldehyde-fixed trophozoites were treated with the fusion protein (A and B) and then with the substrate Vector red. As the controls (C and D), trophozoites were treated with a supernatant of *E. coli* lysates (vector control). (A and C) Bright-field microscopy; (B and D) fluorescence microscopy with a green filter. Magnification, ×360.

cently for application in immunoprophylaxis, or the treatment of infectious diseases. Although such human antibodies would also be useful for diagnostic purposes, one of the disadvantages of the use of human antibodies for the detection of pathogens in human samples might be reactivity of endogenous immunoglobulins with the secondary anti-human antibodies used in indirect methods. Therefore, direct labeling of the human antibody with enzymes is needed to reduce nonspecific binding of the second antibody. It was reported recently that immunoglobulin genes derived from murine hybridoma cells could be expressed in *E. coli* as fusion protein Fab-PhoA (7, 25) or scFv-PhoA (5, 7, 13, 17). The present study demonstrates that the bacterial expression of a human MAb-PhoA conjugate specific for *E. histolytica* is also possible. In addition to the advantage of using the antibody to detect the *E. histolytica* antigen without the need for chemically conjugated secondary antibodies, there is no requirement for experimental animals or reagents and equipment for the culture and cryopreservation of hybridoma cells. Accordingly, the use of this human recombinant antibody also provides an economic benefit.

The antigen recognized with CP33 was the heavy subunit of the galactose- and *N*-acetyl-D-galactosamine-inhibitable lectin of *E. histolytica* (23). It is well known that this lectin molecule is suitable as a target antigen for the detection of *E. histolytica* in fecal and serum samples (1, 2, 11, 12). In conclusion, we propose here that the human Fab-PhoA fusion protein can be used in the diagnosis of amebiasis.

This work was supported by a grant-in-aid for scientific research from the Japanese Society for the Promotion of Science and grants from the Ministry of Health, Labor, and Welfare of Japan.

REFERENCES

1. **Abd-Alla, M. D., T. F. H. G. Jackson, V. Gathiram, A. M. El-Hawey, and J. I. Ravdin.** 1993. Differentiation of pathogenic *Entamoeba histolytica* infections from nonpathogenic infections by detection of galactose-inhibitable adherence protein antigen in sera and feces. J. Clin. Microbiol. **31**:2845–2850.
2. **Abd-Alla, M. D., A. A. Wahib, and J. I. Ravdin.** 2000. Comparison of antigen-capture ELISA to stool-culture methods for the detection of asymptomatic *Entamoeba* species infection in Kafer Daoud, Egypt. Am. J. Trop. Med. Hyg. **62**:579–582.
3. **Anonymous.** 1997. WHO/PAHO/UNESCO report. A consultation with experts on amoebiasis. Mexico City, Mexico 28–29 January, 1997. Epidemiol. Bull. **18**:13–14.
4. **Better, M., C. P. Chang, R. R. Robinson, and A. H. Horwitz.** 1988. *Escherichia coli* secretion of an active chimeric antibody fragment. Science **240**:1041–1043.
5. **Bourin, P., A. Servat, J. J. Lataillade, M. Goyffon, D. Vaux, and P. Billiald.** 2000. Immunolabeling of CD3-positive lymphocytes with a recombinant single-chain antibody/alkaline phosphatase conjugate. Biol. Chem. **381**:173–178.
6. **Burton, D. R., and C. F. Barbas III.** 1994. Human antibodies from combinatorial libraries. Adv. Immunol. **57**:191–280.
7. **Carrier, A., F. Ducancel, N. B. Settiawan, L. Cattolico, B. Maillere, M. Leonetti, P. Drevet, A. Menez, and J. C. Boulain.** 1995. Recombinant antibody-alkaline phosphatase conjugates for diagnosis of human IgGs: application to anti-HBsAg detection. J. Immunol. Methods **181**:177–186.
8. **Cheng, X.-J., S. Ihara, M. Takekoshi, and H. Tachibana.** 2000. *Entamoeba histolytica*: bacterial expression of a human monoclonal antibody which inhibits *in vitro* adherence of trophozoites. Exp. Parasitol. **96**:52–56.
9. **Diamond, L. S., and C. G. Clark.** 1993. A redescription of *Entamoeba histolytica* Schaudinn, 1903 (Emended Walker, 1911) separating it from *Entamoeba dispar* Brumpt, 1925. J. Eukaryot. Microbiol. **40**:340–344.
10. **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.
11. **Haque, R., K. Kress, S. Wood, T. F. Jackson, D. Lyerly, T. Wilkins, and W. A. Petri, Jr.** 1993. Diagnosis of pathogenic *Entamoeba histolytica* infection using a stool ELISA based on monoclonal antibodies to the galactose-specific adhesin. J. Infect. Dis. **167**:247–249.
12. **Haque, R., N. U. Mollah, I. K. M. Ali, K. Alam, A. Eubanks, D. Lyerly, and W. A. Petri, Jr.** 2000. Diagnosis of amebic liver abscess and intestinal infection with the TechLab *Entamoeba histolytica* II antigen detection and antibody tests. J. Clin. Microbiol. **38**:3235–3239.
13. **Mousli, M., M. Goyffon, and P. Billiald.** 1998. Production and characterization of a bivalent single chain Fv/alkaline phosphatase conjugate specific for the hemocyanin of the scorpion *Androctonus australis*. Biochim. Biophys. Acta **1425**:348–360.
14. **Petri, W. A., Jr., T. F. H. G. Jackson, V. Gathiram, K. Kress, L. D. Saffer, T. L. Snodgrass, M. D. Chapman, Z. Keren, and D. Mirelman.** 1990. Pathogenic and nonpathogenic strains of *Entamoeba histolytica* can be differentiated by monoclonal antibodies to the galactose-specific adherence lectin. Infect. Immun. **58**:1802–1806.
15. **Skerra, A., and A. Pluckthun.** 1988. Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. Science **240**:1038–1041.
16. **Strachan, W. D., P. L. Chiodini, W. M. Spice, A. H. Moody, and J. P. Ackers.** 1988. Immunological differentiation of pathogenic and non-pathogenic isolates of *Entamoeba histolytica*. Lancet **i**:561–563.
17. **Suzuki, C., H. Ueda, E. Suzuki, and T. Nagamune.** 1997. Construction, bacterial expression, and characterization of hapten-specific single-chain Fv and alkaline phosphatase fusion protein. J. Biochem. (Tokyo) **122**:322–329.
18. **Tachibana, H., X.-J. Cheng, K. Watanabe, M. Takekoshi, F. Maeda, S. Aotsuka, Y. Kaneda, T. Takeuchi, and S. Ihara.** 1999. Preparation of recombinant human monoclonal antibody Fab fragments specific for *Entamoeba histolytica*. Clin. Diagn. Lab. Immunol. **6**:383–387.
19. **Tachibana, H., S. Kobayashi, X.-J. Cheng, and E. Hiwatashi.** 1997. Differentiation of *Entamoeba histolytica* from *E. dispar* facilitated by monoclonal antibodies against a 150-kDa surface antigen. Parasitol. Res. **83**:435–439.
20. **Tachibana, H., S. Kobayashi, Y. Kaneda, T. Takeuchi, and T. Fujiwara.** 1997. Preparation of a monoclonal antibody specific for *Entamoeba dispar* and its ability to distinguish *E. dispar* from *E. histolytica*. Clin. Diagn. Lab. Immunol. **4**:409–414.
21. **Tachibana, H., S. Kobayashi, Y. Kato, K. Nagakura, Y. Kaneda, and T. Takeuchi.** 1990. Identification of a pathogenic isolate-specific 30,000-M_r antigen of *Entamoeba histolytica* by using a monoclonal antibody. Infect. Immun. **58**:955–960.
22. **Tachibana, H., M. Takekoshi, X.-J. Cheng, F. Maeda, S. Aotsuka, and S. Ihara.** 1999. Bacterial expression of a neutralizing mouse monoclonal antibody Fab fragment to a 150-kilodalton surface antigen of *Entamoeba histolytica*. Am. J. Trop. Med. Hyg. **60**:35–40.
23. **Tachibana, H., K. Watanabe, X.-J. Cheng, H. Tsukamoto, Y. Kaneda, T. Takeuchi, S. Ihara, and W. A. Petri, Jr.** 2003. VH3 gene usage in neutralizing human antibodies specific for the *Entamoeba histolytica* Gal/GalNAc lectin heavy subunit. Infect. Immun. **71**:4313–4319.
24. **Takekoshi, M., F. Maeda, H. Tachibana, H. Inoko, S. Kato, I. Takakura, T. Kenjyo, S. Hiraga, Y. Ogawa, T. Horiki, and S. Ihara.** 1998. Human monoclonal anti-HCMV neutralizing antibody from phage display libraries. J. Virol. Methods **74**:89–98.
25. **Weiss, E., and G. Orfanoudakis.** 1994. Application of an alkaline phosphatase fusion protein system suitable for efficient screening and production of Fab-enzyme conjugates in *Escherichia coli*. J. Biotechnol. **33**:43–53.

INFECTION AND IMMUNITY, Aug. 2003, p. 4313–4319
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.8.4313–4319.2003

Vol. 71, No. 8

VH3 Gene Usage in Neutralizing Human Antibodies Specific for the *Entamoeba histolytica* Gal/GalNAc Lectin Heavy Subunit

Hiroshi Tachibana,[1]* Katsuomi Watanabe,[1] Xun-Jia Cheng,[1] Hideo Tsukamoto,[2] Yoshimasa Kaneda,[1] Tsutomu Takeuchi,[3] Seiji Ihara,[4] and William A. Petri, Jr.[5]

Departments of Infectious Diseases[1] and Molecular Life Sciences[4] and Laboratory for Molecular Science Research,[2] Tokai University School of Medicine, Isehara, Kanagawa 259-1193, and Department of Tropical Medicine and Parasitology, School of Medicine, Keio University, Tokyo 160-8582,[3] Japan, and Departments of Medicine, Microbiology, and Pathology, University of Virginia, Charlottesville, Virginia 22908-1340[5]

Received 13 December 2002/Returned for modification 13 March 2003/Accepted 29 April 2003

A combinatorial human immunoglobulin gene library was constructed from peripheral lymphocytes of an asymptomatic *Entamoeba histolytica* cyst passer and screened for the production of Fab antibody to the parasite. One of the Fab clones, CP33, recognized the 260-kDa galactose- and *N*-acetyl-D-galactosamine (Gal/GalNAc)-specific lectin of *E. histolytica*. By shuffling the heavy and light chains of CP33 with the heavy and light chains of two libraries derived from the cyst passer and a liver abscess patient, 18 additional clones were obtained. Sequence analysis of the heavy-chain genes, including CP33-H, revealed that all the nearest V-segment germ lines belonged to the VH3 family (VH3-21, VH3-30, VH3-48, and VH3-53), but the levels of homology were only 85 to 95%. The closest D-segment germ line was D2-2 or D6-6, and for the J-segment the closest germ line was JH4b or JH6b. On the other hand, all the light-chain genes, including CP33-L, belonged to the Vκ1 family, in which the closest Vκ germ line gene was 02/012 or L5, with the Jκ1, Jκ2, Jκ4, or Jκ5 segment. CP33 and three other Fabs obtained by light-chain shuffling were purified and analyzed further. All of these Fabs recognized the cysteine-rich domain of the 170-kDa heavy subunit of the Gal/GalNAc lectin. Preincubation of *E. histolytica* trophozoites with these Fabs significantly inhibited amebic adherence to Chinese hamster ovary cells and also inhibited erythrophagocytosis. The ability of the neutralizing antibodies to block erythrophagocytosis for the first time implicates the lectin in phagocytosis and VH3 antibodies in defense against parasitic infections. These results demonstrate the utility of a combinatorial human immunoglobulin gene library for identifying and characterizing neutralizing antibodies from humans with amebiasis.

Worldwide, the intestinal protozoan parasite *Entamoeba histolytica* causes an estimated 50 million cases of amebic colitis and liver abscess annually, resulting in up to 110,000 deaths (48). The ability of *E. histolytica* trophozoites to invade the colon and other tissues depends on several pathogenic factors. One of the most important factors is the galactose (Gal)- and *N*-acetyl-D-galactosamine (GalNAc)-inhibitable cell surface lectin of the ameba. The lectin mediates adherence of trophozoites to human colonic mucins, colonic epithelial cells, neutrophils, and erythrocytes (4, 7, 18, 24, 37, 38). The Gal/GalNAc lectin is also important in the cytolytic event that follows adherence. The amebic lectin is a 260-kDa hetrodimeric glycoprotein composed of 170-kDa heavy and 35- or 31-kDa light subunits (32). In addition, a 150-kDa intermediate subunit of the lectin also contributes to adherence (8, 12). Immunization of experimental animals with these lectin subunits provides protection from liver abscess formation (10, 25, 27, 34, 50). Such protection is also observed after passive immunization with a mouse monoclonal antibody to the intermediate subunit lectin (11).

Recently, it has been shown that *E. histolytica*-specific human monoclonal antibody Fab fragments can be prepared from the peripheral lymphocytes of a patient with an amebic liver abscess (9, 42). One of the clones which recognized the 260-kDa Gal/GalNAc lectin inhibited the adherence of trophozoites to mammalian cells (9). Successful cloning of the active fragments might be due to the patient's high antibody titer to *E. histolytica*.

There are also clinically asymptomatic individuals, which pass *E. histolytica* cysts in their stools. Most of the cyst passers have a positive serology for the ameba, although their antibody titers are low (44). In such cyst passers, there may be protective antibodies that block invasion of trophozoites into tissues. However, little is known about the immune response to *E. histolytica* in asymptomatic cyst passers. Therefore, molecular analysis of the immune response to the amebic lectin is important for understanding protective immunity and for vaccine development. We report here molecular cloning of immunoglobulins specific for the *E. histolytica* Gal/GalNAc lectin. The clones were derived from the peripheral lymphocytes of an asymptomatic cyst passer. We also report possible recombination and bacterial expression of antibody genes from both aymptomatic and symptomatic individuals.

MATERIALS AND METHODS

Cultivation of parasites. Trophozoites of 10 strains of *E. histolytica* (HM-1:IMSS, HK-9, 200:NIH, HB-301:NIH, H-302:NIH, H-303:NIH, DKB, C-3-2-1, SAW1627, and SAW755CR) were axenically grown in BI-S-33 medium (16). Trophozoites of *Entamoeba dispar* SAW1734RclAR were cultured monoxenically with *Pseudomonas aeruginosa* in BCSI-S medium (22). Trophozoites were washed three times with ice-cold 10 mM phosphate-buffered saline (PBS) (pH 7.4) before they were used.

* Corresponding author. Mailing address: Department of Infectious Diseases, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan. Phone: 81 (463) 93-1121. Fax: 81 (463) 95-5450. E-mail: htachiba@is.icc.u-tokai.ac.jp.

Construction of immunoglobulin gene library. Ten milliliters of peripheral blood was collected from the asymptomatic cyst passer. Cysts detected in the feces had been identified specifically as *E. histolytica* by PCR. The serum was positive for *E. histolytica* with a titer of 1:64 (borderline positive), as determined by an indirect fluorescent-antibody (IFA) test. Lymphocytes were separated from the blood by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. RNA was isolated from the lymphocytes with an RNeasy total RNA purification kit (QIAGEN GmbH, Hilden, Germany). Reverse transcriptase PCR was performed with a GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, Conn.) according to the manufacturer's instructions. An oligo$(dT)_{16}$ primer was used for cDNA synthesis. Genes encoding the κ and λ light chains and the Fd region of the γ heavy chain were amplified as previously described (42). Thirty-five cycles of PCR were performed as follows: denaturation at 94°C for 1 min (5 min in cycle 1), annealing at 50°C for 2 min, and polymerization at 72°C for 3 min (10 min in cycle 35). The light-chain genes were first ligated with an expression vector, pFab1-His2 (46), and then introduced into XL1-Blue Epicurian Coli (Stratagene, La Jolla, Calif.). The vector with inserts was selected, and then the Fd heavy-chain genes were ligated into the vector and introduced into the bacteria.

Expression of immunoglobulin genes and screening of clones producing anti-*E. histolytica* antibodies. Screening of positive clones was performed as previously described (9). The expression vector containing light-chain and Fd heavy-chain genes was introduced into competent *Escherichiacoli* JM109. Approximately 10^3 to 3×10^3 colonies per 90-mm plate were grown on Luria-Bertani agar plates containing 50 μg of ampicillin per ml at 37°C. Colonies were transferred to nitrocellulose filters and then incubated on fresh plates containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and ampicillin at 30°C for 6 h. Each filter was treated with chloroform vapor and then incubated with lysis buffer (100 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM $MgCl_2$, 1.5% bovine serum albumin, 1 μg of DNase per ml, 40 μg of lysozyme per ml) overnight. After washing with PBS containing 0.05% Tween 20 (PBS-Tween), the filter was incubated in PBS-Tween containing 5% skim milk and then with 400 μg of soluble *E. histolytica* antigen prepared from trophozoites of the HM-1:IMSS strain per ml. After washing, the filter was incubated with horseradish peroxidase (HRP)-conjugated antibody purified from the plasma of a patient with an amebic liver abscess. The filter was washed and then developed with a Konica immunostaining kit (HRP-1000; Konica Co., Tokyo, Japan). Positive clones were identified in original plates and then cultured in 5 ml of super broth (30 g of tryptone per liter, 20 g of yeast extract per liter, 10 g of morpholinepropanesulfonic acid [MOPS] per liter; pH 7) containing ampicillin until an optical density at 600 nm of 0.6 to 0.7 was obtained. IPTG, at a final concentration of 100 μM, was added to the bacterial cultures, which were then incubated at 30°C for 14 h. The bacteria were pelleted by centrifugation, suspended in 250 μl of PBS containing 1 mM phenylmethylsulfonyl fluoride, and then sonicated. The lysates were centrifuged at 12,000 × *g* for 10 min, and the supernatant was subjected to a second screening by performing an IFA test with intact trophozoites.

IFA test. The IFA test, with live intact trophozoites (45) or formalin-fixed trophozoites smeared on glass slides (43), was performed as previously described. Fluorescein isothiocyanate-conjugated goat immunoglobulin G (IgG) to human IgG Fab (Organon Teknica Co., Durham, N.C.) was used as the second antibody.

Shuffling of light- and heavy-chain genes and rescreening. The light-chain gene of the positive clone was replaced with light-chain genes from the library constructed from the asymptomatic cyst passer. The light-chain-shuffled plasmid containing the cloned Fd heavy chain was introduced into the bacteria and then screened again. The Fd heavy chain of the positive clone was also replaced with the library's heavy-chain genes and screened again. Another immunoglobulin library, prepared from a patient with an amebic liver abscess (9), was also used as a source of light- and heavy-chain genes. Positive clones were subjected to a second screening by the IFA test. Light- and heavy-chain genes of clones positive in the second screening were amplified by PCR, digested with *Mbo*I, *Rsa*I, *Dra*I, *Hin*fI, and *Hae*III and with *Mbo*I, *Rsa*I, and *Alu*I, respectively, and then compared by performing electrophoresis in an agarose gel.

ELISA. The soluble antigen derived from *E. histolytica* trophozoites, which was used for screening clones, was also used for an enzyme-linked immunosorbent assay (ELISA). The wells of the ELISA plates, containing 5 μg of antigen diluted with 50 mM sodium bicarbonate buffer (pH 9.6), were incubated overnight at 4°C. The plates were washed with PBS-Tween and then treated with PBS containing 3% skim milk for 1 h. For use in the ELISA, positive clones of bacteria were cultured in 10 ml of medium, and then 0.5 ml of the resultant supernatant was prepared as described above. One hundred microliters of the supernatant was added to the wells and incubated for 1 h at room temperature. After the wells were washed, they were incubated with 100 μl of HRP-conjugated sheep antibody to human IgG $F(ab')_2$ (ICN Pharmaceuticals, Aurora, Ohio) for 1 h at room temperature. After they were washed with PBS-Tween, they were incubated with 200 μl of a substrate solution (0.4 mg of *o*-phenylenediamine per ml in citric acid-phosphate buffer [pH 5.0] containing 0.001% hydrogen peroxide). After 30 min of incubation, the reaction was stopped by addition of 50 μl of 2.5 M H_2SO_4, and the optical density at 490 nm was determined with a model 550 microplate reader (Bio-Rad, Hercules, Calif.).

DNA sequencing. Cloned light-chain genes and Fd heavy-chain genes were recloned into sequencing vectors. Cycle sequencing in both directions was performed with Thermo Sequenase (Amersham Life Science, Cleavland, Ohio) by using M13 primers. Reactions were performed with a model 4000L automated DNA sequencer (LI-COR, Lincoln, Nebr.).

Purification of Fabs. Positive clones were cultured in 1 liter of medium. Twenty milliliters of each resultant supernatant, prepared as described above, was filtered through 0.22-μm-pore-size filters and used for purification of Fab fragments. Purification of Fabs was performed by affinity chromatography by using His·Bind Resin (Novagen, Madison, Wis.) according to the manufacturer's instructions.

SDS-PAGE and Western immunoblot analysis. Purified Fab fragments and trophozoites of *E. histolytica* HM-1:IMSS in PBS were solubilized with an equal volume of the sample buffer (23) containing 2 mM phenylmethylsulfonyl fluoride, 2 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 2 mM *p*-hydroxymercuriphenyl sulfonic acid, and 4 μM leupeptin for 5 min at 95°C. After centrifugation, the supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western immunoblot analysis was performed as previously described (43). HRP-conjugated sheep antibody to human IgG $F(ab')_2$ (ICN Pharmaceuticals) was used as the second antibody. Development was with a Konica HRP-1000 immunostaining kit.

Dot immunoblot analysis. Affinity-purified 260-kDa Gal/GalNAc lectin and the recombinant protein of the cysteine-rich domain in the 170-kDa heavy subunit of the lectin (rLecA), prepared as previously described (28), were used for a dot immunoblot analysis. One microgram of each of these antigens in PBS was spotted onto a nitrocellulose membrane and air dried. Each spot on the membrane was blocked for 30 min with PBS containing 3% skim milk and then incubated with 2 μg of each Fab for 1 h at room temperature. As controls, normal human Fab (OEM Concepts, Toms River, N.J.), antibody purified from the plasma of a patient with an amebic liver abscess, and rabbit antibody to the 260-kDa lectin were used. After the membrane was washed three times with PBS-Tween, it was incubated with HRP-conjugated sheep antibody to human IgG $F(ab')_2$ or HRP-conjugated goat antibody to rabbit IgG (ICN Pharmaceuticals). After washing, each antibody-bound dot was detected with a Konica HRP-1000 immunostaining kit.

Determination of affinity constant. Affinity measurement of purified Fabs by surface plasmon resonance was carried out by using a BIAcore 3000 instrument (Biacore AB, Uppsala, Sweden) according to the general procedure outlined by the manufacturer. Affinity-purified 260-kDa Gal/GalNAc lectin and rLecA were immobilized onto a CM5 chip (Biacore) surface at a low density by amine coupling chemistry. Affinity constants were determined by using the software provided by the manufacturer, BIAevaluation 3.1.

Adherence assay. Adherence of *E. histolytica* to Chinese hamster ovary (CHO) cells was evaluated as previously described (49). Briefly, trophozoites (10^4 cells) of the HM-1:IMSS strain were incubated with 100 μg of each Fab for 1 h at 4°C, washed with ice-cold PBS, and then suspended in Ham's F12 nutrient mixture containing 1% adult bovine serum. As a control, normal human Fab (OEM Concepts) was used. The trophozoites and CHO cells (2×10^5 cells) were suspended in 1 ml of the Ham's F12 nutrient mixture, centrifuged, and then incubated at 4°C for 2 h. After removal of 0.8 ml of supernatant, the pellet was gently vortexed, and the number of trophozoites with at least three adherent CHO cells was determined by examining 300 trophozoites.

Erythrophagocytosis assay. Erythrophagocytosis of *E. histolytica* was assayed as previously described (47). Briefly, trophozoites (10^4 cells) of HM-1:IMSS were exposed to 100 μg of each Fab at 37°C for 30 min and then washed with ice-cold PBS. Normal human Fab (OEM Concepts) was also used as a control. The erythrophagocytosis assay was performed by mixing the Fab-treated trophozoites with 10^6 human erythrocytes and then incubating the preparation at 37°C for 5 min. After lysis of free and adherent erythrocytes by addition of distilled water, the trophozoites were fixed and stained with a 3,3′-diaminobenzidine solution containing hydrogen peroxide. The number of erythrocytes ingested was determined by examining 300 trophozoites.

Nucleotide sequence accession number. The nucleotide sequence data reported here have been deposited in the DDBJ, EMBL, and GenBank databases under accession numbers AB095272 to AB095291.

	FR1	CDR1	FR2	CDR2
CP33-H	EVKLMESGGGVVQPGRSLRLSCAASGFRFS	TYAIH	WVRQAPGKGLEWVA	RISHDGSQTHYADSVQG
H-CP1	Q.Q.K.....L.K..G.....T..	DFSMN	.....S	S.TATSVYLK.....R.
H-CP4	D.Q.Q.....	.....	.....	.....
H-CP5	....LQ...---------...V.....TVN	SNHMS	.....S	I.YSA-GI.Y.....K.
H-CP6	....L....DLL...G.....T..	...MN	.....S	Y..SGSGTIY.....K.
H-LA5	.....	.....	.....	.....

	FR3	CDR3	FR4
CP33-H	RFGVSRDNSNYTAYVQLNSLRPDDTAVYFCAR	AYSSTP---DYGMDV	WGQGTAVTVSS
H-CP1	..TI.....DNSV.L.MTNVSGE.....Y...	DGGGKAASGY.....	.....T.....
H-CP4	.....	.....---.....	.....
H-CP5	..TI.....EN.LFL.M.....E.....Y...	GKY.PS--IG.YF.Y	.....L.....
H-CP6	..TI....AKNSVSL.M....GE.....Y...	GPRFEM----APF.Y	.....L.....
H-LA5	.....	.....---.....	.....

FIG. 1. Deduced amino acid sequences for genes coding for heavy-chain variable regions of human anti-*E. histolytica* Fab fragments. FR, framework regions. The dashes and dots indicate deletions and identical residues, respectively.

RESULTS

Cloning of recombinant Fab with *E. histolytica*. The combinatorial immunoglobulin gene library constructed from peripheral lymphocytes of an asymptomatic *E. histolytica* cyst passer contained 9.6×10^6 clones. When 6.32×10^4 clones were screened by colony blotting, 6 clones (0.0095%) showed positive signals. In the second screening performed by IFA with intact cells, two clones were positive. Since one of the positive clones, designated CP33, was reactive with the 260-kDa Gal/GalNAc lectin in a preliminary dot immunoblot analysis, this clone was analyzed further.

Chain shuffling of the recombinant Fab. To find other heavy- and light-chain genes which constitute antilectin Fab fragments with light- and heavy-chain genes of CP33, the libraries from the asymptomatic cyst passer (CP library) and the amebic liver abscess patient (LA library) were screened again after shuffling of heavy- and light-chain genes of CP33 with genes from the two libraries. Many positive signals were detected after chain shuffling. When the light-chain gene of CP33 was shuffled, the positive rates in the colony blot screening were 10- or 20-fold higher than those in the shuffling analysis of the heavy-chain genes (1.92 and 0.19%, respectively, for the LA library; 0.96 and 0.047%, respectively, for the CP library). In addition, when the LA library was used as the source of immunoglobulin genes, the positive rates were two- or fourfold higher than those obtained with the CP library (1.92 and 0.96%, respectively, for light-chain shuffling; 0.19 and 0.047%, respectively, for heavy-chain shuffling). All positive clones were secondarily screened with an IFA by using formalin-fixed trophozoites of *E. histolytica* HM-1:IMSS, and then the genes of IFA-positive clones were compared by restriction enzyme digestion. Based on the digestion patterns, 12 clones of the light-chain genes and four clones of the heavy-chain genes were identified as different from each other and also different from the light- and heavy-chain genes of CP33. Clones that showed the same digestion pattern as CP33 but were derived from the LA library were also selected. When more than two clones showed the same digestion pattern, the clone with the strongest IFA reactivity was selected. The reactivities of 18 selected clones to *E. histolytica* antigen were compared by ELISA. Three of 13 Fabs obtained by shuffling of light chains, CP33-H/L-CP17, CP33-H/L-CP26, and CP33-H/L-LA22, showed relatively high reactivity compared with the reactivity of CP33. The relative optical densities for these Fabs were 1.61, 1.26, and 1.42, respectively, when the optical density of CP33 was defined as 1. Two of the light chains (L-CP17 and L-CP26) were derived from the cyst passer, and one (L-LA22) was derived from the patient with a liver abscess. The reactivities of the five Fabs obtained by shuffling of the heavy chain were similar to or lower than the reactivity of CP33; the relative optical densities were between 0.71 and 0.95.

Gene analysis of recombinant Fabs. Six heavy-chain genes and 14 light-chain genes were sequenced, and the deduced amino acid sequences that they encode were compared. In the heavy chains, H-LA5 derived from the patient with a liver abscess was identical to CP33-H (Fig. 1). H-CP4 also was almost identical to CP33-H except for the FR1 region. In L-LA22, the sequence of complementarity-determining regions (CDRs) was identical to that in CP33-L (Fig. 2). The difference between L-CP17 and CP33-L in the CDRs was only one amino acid residue in CDR3. In L-CP26, three residues in CDR1 and four residues in CDR3 differed from residues in CP33-L. The sequence homology of these clones with germ lines was analyzed by using IgBLAST at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/igblast/) and V-QUEST at the international ImMunoGeneTics database (http://imgt.cines.fr:8104/textes/vquest/). As shown in Table 1, sequence analysis of the heavy-chain genes revealed that all of the most similar germ line of V-segments belonged to the VH3 family (VH3-21, VH3-30, VH3-48, or VH3-53). However, the level of amino acid homology with the germ line was low, 76% in CP33-H and H-LA5. The most similar germ line for the D-segment was D2-2 or D6-6, except for H-CP1, which was not identified, and the most similar germ line for the J-segment was either JH4b or JH6b. In contrast, all the light chains belonged to the Vκ1 family, in which the most similar

```
                  FR1                 CDR1          FR2          CDR2
CP33-L  DIVMTQTPSSLSASVGDRVTITC RASQSISSYLN WYQQRPGQAPKLLIY AASSLQS
L-CP9   ..EL..S..T.............. ....G...W.A ....K..K....... .......
L-CP13  ..Q...S...V......T..... ....T...... ....K..K...V... .......
L-CP17  A.Q...S.................. .......... ....K..K....... .......
L-CP26  ..QL..S...V.............. ....G...W.A ....K..K....... .......
L-LA2   ..Q...S...V.............. ....G..RW.V ....K..K....... G..T...
L-LA3   ...L..S...V.............. ....G...W.A ....K..K....... .......
L-LA8   ......S...V........A.... ....G..NW.A ....K..K..N...F ...T..T
L-LA10  ......S.................. .......... ....K..K....... .......
L-LA11  A.QL..S.................. .....V.TF.S ....K..K....... .......
L-LA12  E..L..S..YV.............. ....G.GRW.G ...HK.....N.... ...T..G
L-LA14  A.QL..S...A.....E....... ....G.NNW.G ....K..K....... .......
L-LA18  A.QL..S.................. .....VTT... ....K..K....... S......
L-LA22  ..E...S.................. .......... ....K..K....... .......

                  FR3                       CDR3        FR4
CP33-L  GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC QQSYSTFRTF GQGTRLDIKR
L-CP9   ............................... ..AN.F.L.. .G..KVE...
L-CP13  ............................... .......... ....KVE..G
L-CP17  ............................... .......H.. ....K.E...
L-CP26  ............................... ..ANNF.... ....KVE...
L-LA2   .....................N.........H. ..TD.L.L.. .G..KVE.R.
L-LA3   ..........................S.S... ..TN.F.I.. ......E...
L-LA8   ............................... ..AH.I.L.. .G..KVE...
L-LA10  ..........................A.... .......Y.. ....K.E...
L-LA11  .........A...................... .....I.Y.. ....K.E...
L-LA12  ..........................S.A... ..AN.F.... ....KVE...
L-LA14  ............................... ...N.F.... ....KVE...
L-LA18  ..............S.................. .......I.. ......E...
L-LA22  ............................... .......... ....KVE...
```

FIG. 2. Deduced amino acid sequences for genes coding for light-chain variable regions of human anti-*E. histolytica* Fab fragments. FR, framework regions. The dashes and dots indicate deletions and identical residues, respectively.

Vκ germ line was 02/012 or L5, with relatively high homology (Table 2). For the Jκ segments, Jκ1, Jκ2, and Jκ5 were combined with 02/012, and Jκ1, Jκ2, and Jκ4 were combined with L5.

Specificity of recombinant Fabs. CP33 and the three Fabs which showed high reactivity in ELISA (CP33-H/L-CP17, CP33-H/L-CP26, and CP33-H/L-LA22) were purified by His affinity column chromatography. SDS-PAGE demonstrated that the molar ratio of two bands with apparent molecular masses of 25 and 24 kDa was 1:1, suggesting the heterodimeric structure of Fab. These four Fabs reacted with all 10 strains of *E. histolytica* trophozoites, but not with *E. dispar* trophozoites, in IFA when fixed cells were used (data not shown). To identify the *E. histolytica* antigen recognized by these Fabs, a Western immunoblot analysis was performed. Under nonreducing conditions, four Fabs were reactive only with a 260-kDa antigen (Fig. 3). To identify the antigen, a dot immunoblot analysis was carried out. Four Fabs reacted with the affinity-purified 260-kDa Gal/GalNAc lectin and also with the cysteine-rich domain of the heavy subunit of the lectin (Fig. 4).

TABLE 1. Comparison of gene usage and structural homologies for heavy-chain variable regions of anti-*E. histolytica* Fab fragments

Clone	V-segment				D-segment	J-segment
	VH family	Closest germ line	% Homology with germline: DNA	% Homology with germline: Protein		
CP33-H	VH3	VH3-30	85	76	D2-2	JH6b
H-CP1	VH3	VH3-21	88	80	ND[a]	JH6b
H-CP4	VH3	VH3-30	85	78	D2-2	JH6b
H-CP5	VH3	VH3-53	92	87	D6-6	JH4b
H-CP6	VH3	VH3-48	95	88	D2-2	JH4b
H-LA5	VH3	VH3-30	85	76	D2-2	JH6b

[a] ND, not determined.

TABLE 2. Comparison of gene usage and structural homologies for light-chain variable regions of anti-*E. histolytica* Fab fragments

Clone	V-segment				J-segment
	VL family	Closest germ line	% Homology with germline: DNA	% Homology with germline: Protein	
CP33-L	Vκ1	02/012	98	95	Jκ5
L-CP9	Vκ1	L5	97	95	Jκ4
L-CP13	Vκ1	02/012	97	95	Jκ1
L-CP17	Vκ1	02/012	99	100	Jκ2
L-CP26	Vκ1	L5	97	93	Jκ1
L-LA2	Vκ1	L5	94	90	Jκ4
L-LA3	Vκ1	L5	98	94	Jκ5
L-LA8	Vκ1	L5	95	90	Jκ4
L-LA10	Vκ1	02/012	99	97	Jκ2
L-LA11	Vκ1	02/012	96	92	Jκ2
L-LA12	Vκ1	L5	93	85	Jκ1
L-LA14	Vκ1	L5	95	92	Jκ1
L-LA18	Vκ1	02/012	98	93	Jκ5
L-LA22	Vκ1	02/012	99	98	Jκ1

Affinity of recombinant Fabs. The affinity of the Fabs to the lectin was measured by surface plasmon resonance. The association constants for the four Fabs with the 260-kDa lectin ranged from 1.06×10^8 to 2.85×10^8 M^{-1} (Table 3). Although the affinity values for rLecA were low, they ranged from 6.43×10^7 to 1.29×10^8 M^{-1}. CP33-H/L-CP26 and CP33-H/L-LA22 showed higher affinity than CP33 and CP33-H/L-CP17 showed.

Neutralizing activity of recombinant Fabs. To evaluate the function of the four Fabs, their effects on amebic adherence to CHO cells were examined. In a normal human Fab-treated control, the level of adherence of trophozoites to CHO cells was 53.8%. After pretreatment of trophozoites with 100 μg of the antilectin Fabs, the level of adherence significantly decreased by 57 to 65% ($P < 0.001$). The effect of the Fabs on erythrophagocytosis by *E. histolytica* was also evaluated. When amebae were pretreated with 100 μg of the Fabs, the number of trophozoites ingesting erythrocytes and the number of erythrocytes ingested were significantly decreased (Table 4). No significant differences in inhibitory effects on adherence and erythrophagocytosis were seen among the four Fabs.

DISCUSSION

Our findings demonstrate that neutralizing human antibodies to amebic adherence and erythrophagocytosis can be prepared from an immunoglobulin gene library derived from an asymptomatic cyst passer infected with *E. histolytica* but not