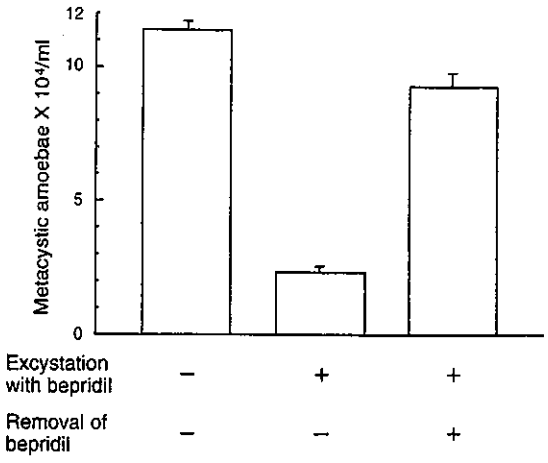


Fig. 7 Effect of removal of bepridil on excystation of *E. invadens*. After exposure of cysts to 100 μM bepridil in a growth medium for 1 day, the drug was removed by replacement of the medium with a drug-free growth medium and the cultures were further incubated for 3 days. Mean number + SE of metacystic amoebae for duplicate cultures are plotted

at 10–100 μM had no effect (Fig. 8B). TFP at 100 μM also reduced cyst viability during incubation compared to controls (Fig. 8C), whereas W-7 at 10–100 μM reduced cyst viability at day 1 (Fig. 8D).

Effect of TFP on metacystic development

As shown in Fig. 9, 12% of metacystic amoebae were four-nucleate and 84% of these were one- to three-nucleate at day 1 of incubation in control cultures, whereas 44% of amoebae were four-nucleate and 45% of these were one- to three-nucleate in cultures containing 100 μM TFP. At day 3, the percentage of four-nucleate amoebae decreased to 1% in control cultures, whereas it was still 16% in cultures with the drug.

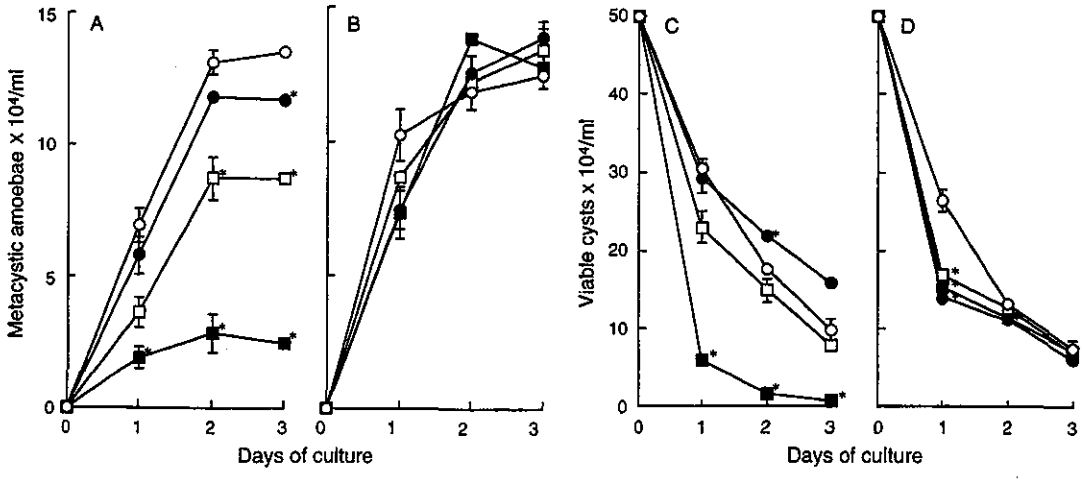
Reversibility of effect of TFP on excystation

To determine whether the inhibitory effect of TFP on excystation was reversible, spent medium containing 100 μM TFP for 1 day of incubation was replaced with a drug-free growth medium. After removal of the drug, the number of metacystic amoebae increased to 81% ($P < 0.05$) of the control (Fig. 10).

Discussion

The transfer of cysts from an encystation medium to a growth medium is necessary for the induction of excystation of *E. invadens* (Makioka et al. 2001b). This means that the signal of the medium change is transduced from the cyst wall to nuclei to trigger excystation. Since encystation medium (47% LG) was prepared from growth medium BI-S-33 by the removal of glucose and dilution with distilled water, the medium change would lead to an increase in Ca^{2+} in the medium. Depletion of Ca^{2+} in growth medium by EGTA or EDTA abrogates this increase, resulting in a failure to trigger excystation. In addition, the decrease in cyst viability by higher concentrations of EGTA or EDTA would also be related to a lower level of excystation. Metacystic development was delayed or became unusual with these chelators, indicating that this process is also dependent on Ca^{2+} concentration. Since the effect of EDTA on metacystic development was more severe than that of EGTA, other divalent cations than Ca^{2+} are also necessary for this process. In this regard, EGTA and EDTA were previously demonstrated to inhibit the growth of *E. histolytica* and *E. invadens*, with EDTA being more potent than EGTA (Makioka et al. 2001a). TMB-8 also inhibited excystation and metacystic development, suggesting the participation of the intracellular Ca^{2+} flux in

Fig. 8 Effect of calmodulin inhibitors trifluoperazine (TFP) (**A**, **C**) and W-7 (**B**, **D**) on the number of metacystic amoebae and cyst viability of *E. invadens*. Mean number ± SE of metacystic amoebae (**A**, **B**) and viable cysts (**C**, **D**) for duplicate cultures are plotted (*$P < 0.05$). Concentrations shown by *open circles*, *solid circles*, *open squares*, and *solid squares*, are 0, 10, 50, and 100 μM, respectively



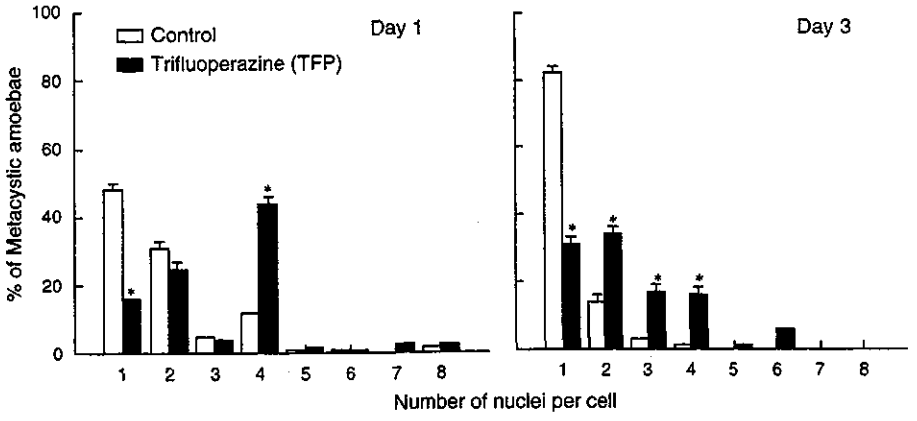


Fig. 9 Effect of TFP on metacystic development of *E. invadens*. Cysts were transferred to a growth medium with or without 100 μM TFP. The nuclei per metacystic amoeba stained with modified Kohn at day 1 and 3 of incubation were counted and the percentage of amoebae was determined (*$P<0.05$)

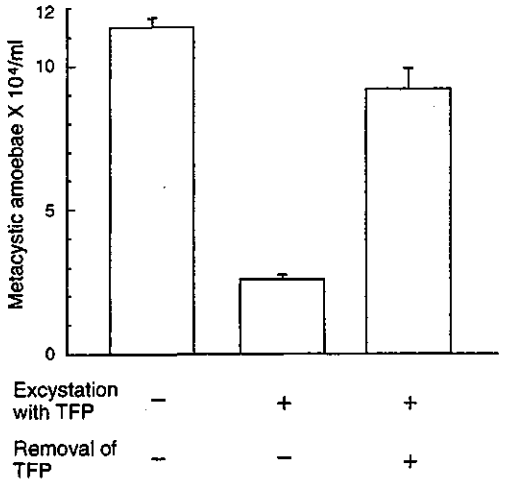


Fig. 10 Effect of removal of TFP on excystation of *E. invadens*. After exposure of cysts to 100 μM TFP in a growth medium for 1 day, the drug was removed by replacement of the medium with a drug-free growth medium and the cultures were further incubated for 3 days. Mean number + SE of metacystic amoebae for duplicate cultures are plotted

these processes. As cyst viability was not affected by TMB-8, reduced excystation is not due to its direct toxic effect on cysts. Since TMB-8 inhibits intracellular calcium transport (Malagodi and Chiou 1974), it could prevent the calcium-dependent gelation of amoebic actin (Meza et al. 1983), which is necessary for the function of amoebic filaments. Since our previous studies have demonstrated the importance of actin filament function for the excystation of *E. invadens* (Makioka et al. 2001b), it is possible that the inhibition of the calcium-dependent gelation of actin by TMB-8 also contributes to its inhibition of excystation.

Ravdin et al. (1982) demonstrated that the slow Na^+-Ca^{2+} channel blockers bepridil and verapamil inhibit the killing of target cells by trophozoites of *E. histolytica*, whereas the fast Na^+ channel blocker tetrodotoxin does not, suggesting the involvement of a transmembrane Ca^{2+} flux in the ameobic killing of target cells. They also demonstrated that bepridil alters the capacity of trophozoites to adhere to or kill target cells, whereas verapamil acts only on the target cell, making it less susceptible to killing by trophozoites. Bepridil also inhibited the excystation and metacystic development of *E. invadens*, whereas verapamil had little effect. These results suggest that, in accordance with the requirement of extracellular Ca^{2+}, Ca^{2+} flux into the amoeba is involved in excystation and metacystic development. Since bepridil appears to penetrate into cells to a greater extent than verapamil (Mras and Sperelakis 1982), this difference in penetrating activity between the two drugs may affect their potency. Bepridil may depress the calcium influx into the amoeba that is necessary for amoebic microfilament function. Viable cysts were reduced in number by higher concentrations of bepridil, which is also related to a lower level of excystation.

CaM contributes to the secretion of electron-dense granules containing collagenolytic activity by *E. histolytica* (Muñoz et al. 1991) and also to the interaction of trophozoites to fibronectin (Carbajal et al. 1996). The CaM inhibitor TFP inhibited excystation and metacystic development, suggesting that these processes are also CaM-dependent. W-7, another CaM inhibitor, had little effect at concentrations of up to 100 μM. Similar results have previously demonstrated that W-7 is less potent than TFP on the growth and encystation of *E. histolytica* and *E. invadens* (Makioka et al. 2001a). In this regard, TFP and W-7 inhibited excystation of *Giardia lamblia*, indicating involvement of the CaM-dependent process in the excystation as well (Bernal et al. 1998). CaM is known to regulate the assembly and disassembly of microtubules (Marcum et al. 1978). The antimicrotubule drug oryzalin inhibits the excystation and metacystic development of *E. invadens* (Makioka et al. 2002). Since metacystic development includes nuclear division, during which microtubules are seen, inhibition of this process by TFP would be related to its inhibition of regulation of microtubule assembly by CaM.

In summary, these results show that extracellular calcium ions, intracellular calcium flux, calcium channel, and CaM-dependent processes participate in the excystation and metacystic development of *Entamoeba*.

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ORIGINAL PAPER

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Effect of jasplakinolide on the growth, invasion, and actin cytoskeleton of *Plasmodium falciparum*

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Abstract The effect of jasplakinolide (JAS), an actin-polymerizing and filament-stabilizing drug, on the growth, invasion, and actin cytoskeleton of *Plasmodium falciparum* was examined. Jasplakinolide markedly decreased the parasitemia in a synchronized culture of *P. falciparum* strain FCR-3 in a time- and concentration-dependent manner. The decrease became evident at day 2 at concentrations of 0.3 μM and above, and parasites finally disappeared at day 4. Giemsa-stained smears of *P. falciparum*-infected erythrocytes demonstrated that there was no effect on the development of schizonts from ring forms. Merozoites were released from the infected erythrocytes in a normal manner with and without JAS. However, there were no ring form-infected erythrocytes when JAS was administered, even after the release of merozoites. This indicates that the merozoites exposed to JAS failed to invade erythrocytes. The inhibitory effect of JAS on the parasitemia was reversed by the removal of the drug after exposure to 1 μM of JAS for 1 day. Electron microscopy revealed that the merozoites treated with JAS showed a protrusion of the apical end which contained the microfilament structure. Immunoblot analysis indicated that the JAS treatment increased F-actin filaments of merozoites but had no effect on those of the trophozoites and schizonts. Therefore, this study demonstrated that JAS has an antimalarial activity.

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Introduction

Actin filaments in eukaryotic cells are responsible for cell motility, phagocytosis, and cell invasion. Actin-modifying agents such as cytochalasins, latrunculins, phalloidin, and jasplakinolide (JAS) have been used extensively to elucidate these microfilament functions. One class of agents, e.g., cytochalasins and latrunculins, inhibits actin polymerization, whereas another class, e.g., phalloidin and JAS, induces actin polymerization and stabilizes the filaments. Fluorescent phalloidin has been widely used to visualize actin filaments in permeabilized cells (Faulstich et al. 1988), but its inability to permeate the membranes of intact cells limits its use in living cells (Cooper 1987).

JAS, a naturally occurring cyclic peptide from the marine sponge *Jaspis* sp. (Crews et al. 1986), is a membrane permeable, actin-polymerizing and filament-stabilizing drug (Bubb et al. 1994). It has a wide range of biological functions, which include antifungal and antitumor activities (Scott et al. 1988; Stingl et al. 1992; Senderowicz et al. 1994). It is, therefore, considered to be a candidate chemotherapeutic. For the parasitic protozoa *Toxoplasma gondii*, which was examined in relation to the effect of JAS, the drug induced an acrosomal process and allowed better visualization of the actin filaments, demonstrating the conoid and apical complex as major sites of actin polymerization (Shaw and Tilney 1999). JAS has also been recently reported to inhibit both the growth and encystation of *Entamoeba*

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ORIGINAL PAPER

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Effect of proteasome inhibitors on the growth, encystation, and excystation of *Entamoeba histolytica* and *Entamoeba invadens*

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Abstract The effect of three proteasome inhibitors, lactacystin, *clasto*-lactacystin β-lactone, and MG-132, on the growth, encystation, and excystation of *Entamoeba histolytica* and *Entamoeba invadens* was examined. All of these drugs blocked *E. histolytica* growth in a concentration-dependent manner; lactacystin was most potent for the inhibition and MG-132 showed the inhibitory effect only at higher concentrations. *E. invadens* was more resistant to these drugs than *E. histolytica*. Encystation of *E. invadens* was also inhibited and was more sensitive to the drugs than was growth. β-Lactone was the most potent encystation inhibitor. The inhibitory effect of lactacystin and the β-lactone on encystation was slightly and little abrogated by the removal of the drug, respectively. Multinucleation occurred in *E. histolytica* trophozoites treated with these drugs, being most marked with lactacystin. In contrast, no multinucleation was observed in *E. invadens* treated with the drugs. Electron microscopy revealed that the treatment of *E. histolytica* trophozoites with lactacystin led to an increase in the number of cells with many glycogen granules in the cytoplasm. Lactacystin, β-lactone and MG-132 had no or little effect on the excystation and metacystic development of *E. invadens*. These results suggest that proteasome function plays an important role for *Entamoeba* growth and encystation, but has no obvious effect on excystation or metacystic development.

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Introduction

Protein degradation plays an essential role for a wide variety of cellular processes in eukaryotic cells. There are two major systems for proteolysis; one is the lysosomal and autophagic pathway and the other is the ubiquitin-proteasome pathway. The former is involved primarily in the degradation of membrane-associated proteins or extracellular proteins taken up by endocytosis, whereas the latter is responsible for the degradation of the bulk of the cytosolic and nuclear proteins in mammalian cells (Dice 1987; Lee and Goldberg 1998). In the ubiquitin-proteasome pathway, most proteins are targeted for degradation by covalent ligation to ubiquitin, a highly conserved small protein (Ciechanover 1994; Goldberg 1995). The conjugation of ubiquitin to a protein leads to its rapid degradation by the 26S proteasome, a 2,000-kDa ATP-dependent proteolytic complex (Coux et al. 1996). Studies with selective proteasome inhibitors such as lactacystin have demonstrated the possible contributions of protein degradation by the ubiquitin-proteasome pathway in various cellular processes including antigen presentation and the degradation of regulatory or membrane proteins (Lee and Goldberg 1998).

Lactacystin was isolated originally from actinomycetes by its ability to promote neurite outgrowth from cultured neurons and to block cell division (Omura et al. 1991). It was then identified as a specific inhibitor of proteasome function by binding covalently to the hydroxyl groups on the active site threonine of the β-subunits, resulting in the inactivation of the chymotrypsin- and trypsin-like activities with different kinetics (Fenteany et al. 1995; Craiu et al. 1997). Lactacystin shows high specificity for the proteasome, but it can also inhibit cathepsin A (Ostrowska et al. 1997; Geier et al. 1999). In aqueous solution, lactacystin is converted into its derivative *clasto*-lactacystin β-lactone, which is actually the active form of the inhibitor (Dick et al. 1996). On the other hand, the peptide aldehyde, MG-132, is a substrate analogue and potent transition-state

inhibitor primarily of the chymotrypsin-like activity of the proteasome (Rock et al. 1994; Lee and Goldberg 1996), although it also inhibits certain lysosomal cysteine proteases and the calpains (Lee and Goldberg 1996).

The presence of proteasome in *Entamoeba histolytica* (Scholze et al. 1996) and the sequence of its α-subunit gene have been reported (Ramos et al. 1997). Although it has also been demonstrated that lactacystin inhibits encystation of *Entamoeba invadens* (Gonzalez et al. 1997, 1999), our knowledge and understanding of proteasome function in *Entamoeba* are still limited. For other parasitc protozoa, there is evidence for proteasome function in the life and cell cycle progression of *Trypanosoma cruzi* and *Trypanosoma brucei* (Gonzalez et al. 1996; Mutomba et al. 1997; Mutomba and Wang 1998), in the development of exoerythrocytic and erythrocytic stages of *Plasmodium berghei* (Gantt et al. 1998), and in the intracellular growth of *Toxoplasma gondii* (Shaw et al. 2000). Therefore, in the present study, we examined whether proteasome inhibitors affect the growth and excystation as well as the encystation of *Entamoeba*.

Materials and methods

Trophozoites of the HM-1: IMSS strain of *E. histolytica* and strain IP-1 of *E. invadens* were cultured in axenic medium BI-S-33 (Diamond et al. 1978) at 35.5°C and 26°C, respectively. For experiments on the effect of proteasome inhibitors on cell growth of both amoebae, duplicate cultures containing 10^4 trophozoites/ml and 1–10 μM lactacystin and *clasto*-lactacystin β-lactone (hereafter termed β-lactone), or 10–100 μM MG-132 were incubated for 3 and 7 days, respectively. All of these drugs were purchased from Calbiochem (Eugene, Ore.) and were initially dissolved in dimethyl sulfoxide (DMSO). Control cultures contained the same volume of DMSO. The cells were counted in a hemocytometer and the viability was determined by trypan blue dye exclusion.

Since there is no available encystation medium for *E. histolytica* (López-Romero and Villagómez-Castro 1993), experiments on the effect of proteasome inhibitors on encystation were performed using an axenic encystation system of *E. invadens*. For this, trophozoites (5×10^5 cells/ml) were transferred to encystation medium called 47% LG (LG is BI without glucose; Sanchez et al.1994). Duplicate cultures including 1–10 μM lactacystin, β-lactone, or 10–100 μM MG-132 were incubated for 3 days. The cysts and trophozoites were counted to determine the percentage of cysts and the viability was determined by trypan blue dye exclusion.

The reversibility of the effects of lactacystin and β-lactone on encystation was studied in duplicate cultures of trophozoites (5×10^5/ml) with10 μM lactacystin or 5 μM β-lactone. Except for the control tubes, cells were washed twice with fresh encystation medium after 1 day incubation, and reincubated for another 2 days. The cysts and trophozoites were counted.

For light microscopy, trophozoites of *E. histolytica* and *E. invadens* cultured for 3 and 7 days, respectively, with 5 μM lactacystin, 10 μM β-lactone, or 100 μM MG-132, were washed once with phosphate-buffered saline (PBS), and stained with modified Kohn. One hundred cells were examined in duplicate to determine the number of nuclei per cell. For electron microscopy, trophozoites of *E. histolytica*, harvested after exposure to 5 μM lactacystin for 3 days, were fixed overnight at 4°C in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and then postfixed for 1 h with 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4. The cells were dehydrated using a graded series of ethanol and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined under a JEM-1200EX electron microscope.

Experiments on the excystation and metacystic development were performed only with *E. invadens* for the same reason as in the encystation experiments. Cysts and trophozoites in the encystation medium were harvested and treated with 0.05% sarkosyl (Sigma, St. Louis, Mo.) to destroy the trophozoites (Sanchez et al. 1994). The remaining cysts were washed with PBS, counted, and suspended in growth medium. Duplicate cultures including 5×10^5 cysts/ml and 1–10 μM lactacystin, β-lactone, or 10–100 μM MG-132 were incubated for 3 days. Control cultures contained the same volume of DMSO. The metacystic amoebae were counted daily and the viability was determined by trypan blue dye exclusion.

Results

Effect of proteasome inhibitors on growth

The effects of the three proteasome inhibitors on the growth of *E. histolytica* and *E. invadens* are shown in Fig. 1. For *E. histolytica* (Fig. 1A), 1, 5, and 10 μM lactacystin inhibited by 73%, 91%, and 100%, respectively, compared to the controls. Inhibition by β-lactone was slightly weaker than that of lactacystin at each concentration. Ten micromolars of MG-132 had no inhibitory effect on growth, whereas 50 and 100 μM of the drug inhibited by 73% and 97% respectively, compared to the controls. For *E. invadens* (Fig. 1B), 10 μM lactacystin and β-lactone inhibited by 45% and 36%, respectively, compared to the controls, whereas 100 μM MG-132 showed only a 28% inhibition. The time course of growth inhibition of *E. histolytica* by lactacystin is shown in Fig. 2. Control cultures showed a sharp increase in the number of cells from day 1 to day 3, whereas the rate of increase was diminished in the cultures with 1 μM of the drug. The number of trophozoites was merely retained with 5 μM and decreased with 10 μM of the drug.

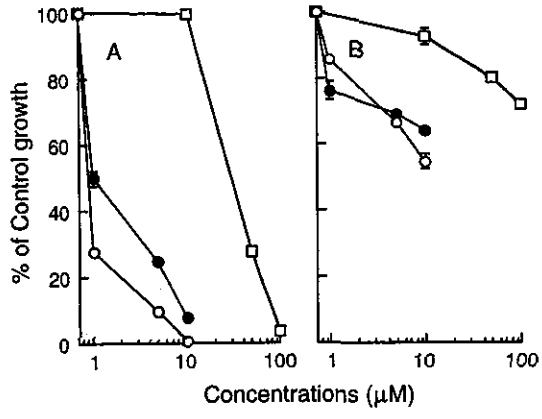


Fig. 1 Effect of proteasome inhibitors on the growth of *Entamoeba histolytica* (**A**) and *E. invadens* (**B**). Trophozoites were cultured in the presence of various concentrations of lactacystin (*white circles*), β-lactone (*black circles*), or MG-132 (*white squares*). Control growth was measured in the absence of the drug and expressed as 100%. Means ± SE for duplicate cultures are plotted at each concentration

Effect of proteasome inhibitors on encystation

The effects of proteasome inhibitors on the encystation of *E. invadens* are shown in Fig. 3. Control cultures without drugs produced an 80% cyst yield 3 days after the induction of encystation. There was no inhibitory effect on encystation with 1 μM lactacystin, whereas 5 and 10 μM of the drug inhibited by 64% and 97% relative to the control, respectively. At 1 μM of β-lactone, inhibition was 48% of the control encystation, and 5 and 10 μM inhibited encystation almost completely. Inhibition by 10, 50 and 100 μM of MG-132 was 0%, 41%, and 70%, respectively.

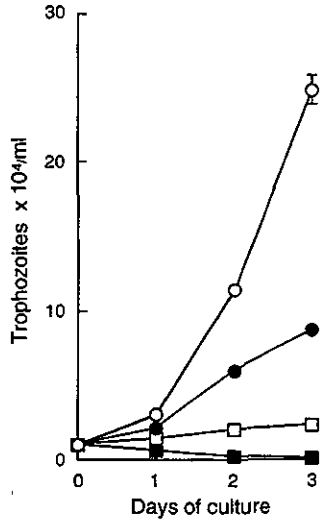


Fig. 2 Time course of growth inhibition of *E. histolytica* by lactacystin. Trophozoites of *E. histolytica* (10^4 cells/ml) were cultured in the absence or presence of lactacystin and cells were counted at day 1, 2, and 3 of culture. Means ± SE for duplicate cultures are plotted. Concentrations shown by *white circles*, *black circles*, *white squares*, and *black squares* are 0, 1, 5, and 10 μM, respectively

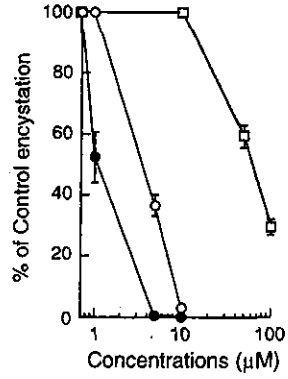


Fig. 3 Effect of proteasome inhibitors on the encystation of *E. invadens*. Trophozoites of *E. invadens* were transferred to encystation medium containing various concentrations of lactacystin (*white circles*), β-lactone (*black circles*), or MG-132 (*white squares*). Control encystation was measured in the absence of the drug and expressed as 100%. Means ± SE for duplicate cultures are plotted

Examination of the reversibility of the effects of lactacystin and β-lactone on encystation showed 28% and 0% of encystation after the removal of lactacystin and β-lactone, respectively (Fig. 4).

Effect of proteasome inhibitors on multinucleation

The effects of proteasome inhibitors on the multinucleation of the trophozoites of *E. histolytica* are shown in Fig. 5. Only 7% of the trophozoites in the control culture had more than two nuclei, whereas 41% of those grown with lactacystin had more than two nuclei. For β-lacton and MG-132, 18% of trophozoites grown in each drug had more than two nuclei. In contrast, no

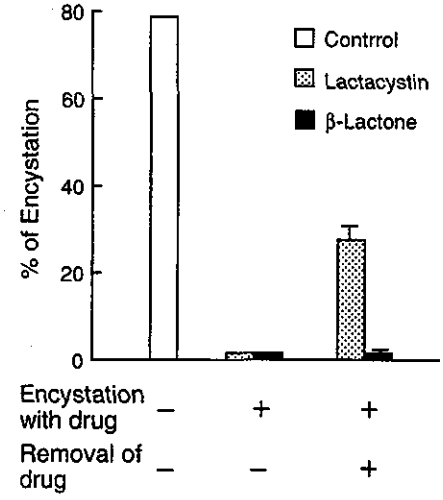


Fig. 4 Effect of removal of proteasome inhibitors on the encystation of *E. invadens*. After exposure to 10 μM lactacystin or 5 μM β-lactone in encystation medium for 1 day, the drugs were removed by replacement of the medium with drug-free encystation medium. Cysts and trophozoites were counted 2 days later. Means ± SE for duplicate cultures are plotted

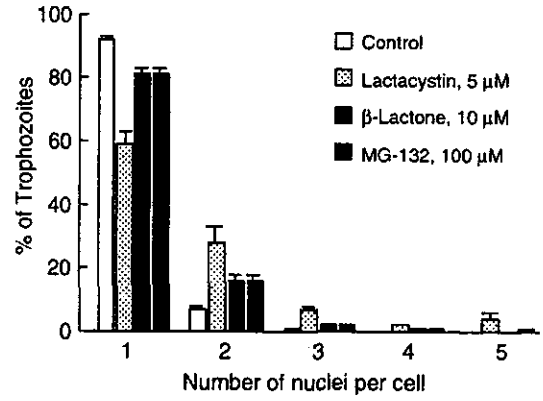


Fig. 5 Effect of proteasome inhibitors on multinucleation of *E. histolytica* trophozoites. Trophozoites were cultured for 3 days with or without 5 μM lactacystin, 10 μM β-lactone, or 100 μM MG-132, and the numbers of nuclei per cell were determined after staining with modified Kohn

multinucleation was observed in *E. invadens* trophozoites treated with these drugs (data not shown).

Electron microscopy of lactacystin-treated trophozoites

Since the treatment of *T. gondii* tachyzoites with lactacystin induced several membranous whorls as well as dense flocculent aggregates in the cytoplasm (Shaw et al. 2000), we observed trophozoites of *E. histolytica* treated with lactacystin by electron microscopy. We found that 56% of the drug-treated trophozoites had a number of glycogen granules in the cytoplasm (Fig. 6), whereas only 25% of the control trophozoites had such granules.

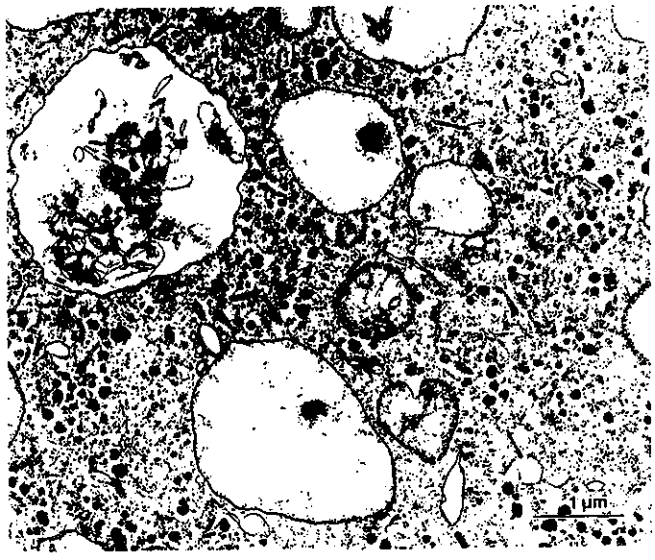


Fig. 6 Electron micrograph of part of a trophozoite of *E. histolytica* treated with lactacystin, showing appearance of a number of glycogen granules in the cytoplasm

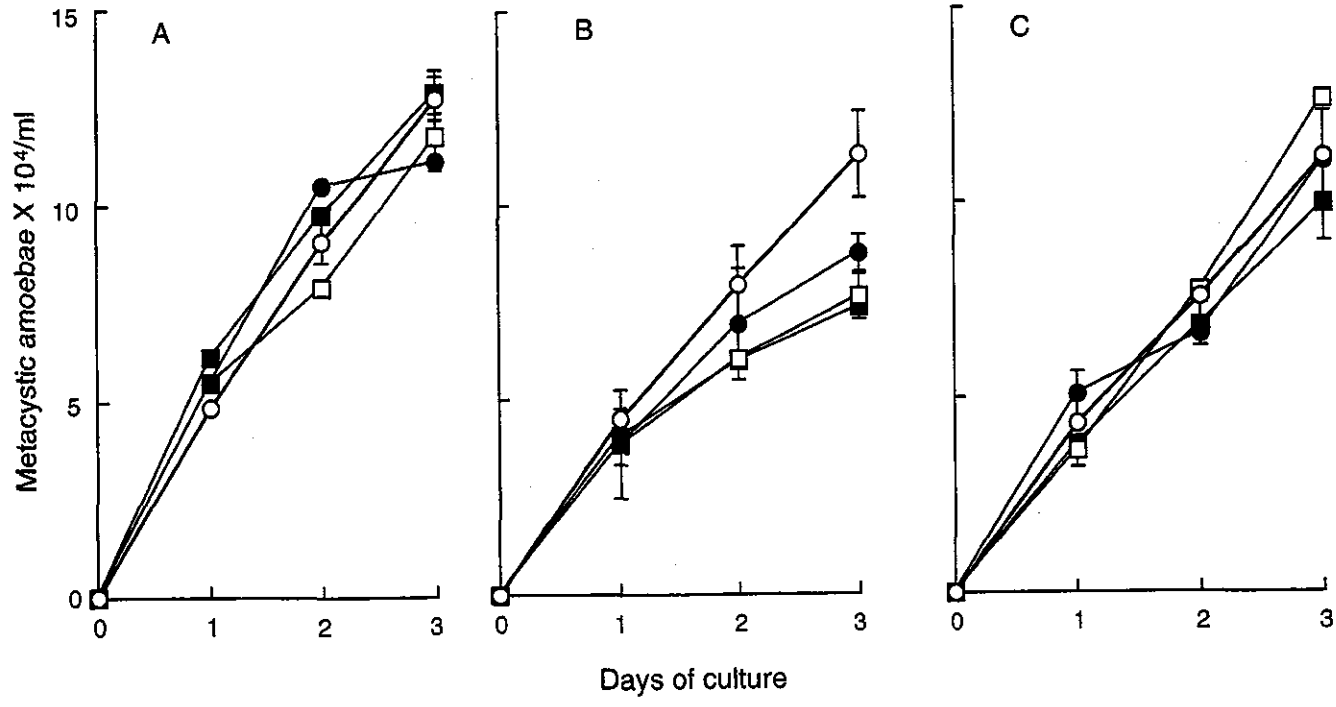
Effect of proteasome inhibitors on excystation and metacystic development

To determine whether the excystation and metacystic development of *E. invadens* are affected by proteasome inhibitors, prepared cysts were allowed to excyst in growth medium containing various concentrations of lactacystin, β-lactone, and MG-132. As shown in Fig. 7, an almost similar pattern of increase in the number of metacystic amoebae was observed in the cultures with lactacystin, β-lactone, and MG-132 as well as in the control cultures without the drug.

Discussion

The results indicate that three proteasome inhibitors affect the growth of *E. histolytica*. Since the evidence for the existence of 20S proteasomes in *E. histolytica* has been reported previously (Scholze et al. 1996), our results suggest the involvement of proteasome function in the growth of this parasite. *E. invadens* was less sensitive to these inhibitors than *E. histolytica*, suggesting a

Fig. 7A–C Effect of proteasome inhibitors on the excystation and metacystic development of *E. invadens*. Cysts were transferred to growth medium containing various concentrations of lactacystin (**A**), β-lactone (**B**), or MG-132 (**C**), and metacystic amoebae were counted at day 1, 2, and 3 of culture. Means ± SE for duplicate cultures are plotted. Concentrations shown by *white circles*, *black circles*, *white squares*, and *black squares*, respectively, are as follows. For **A** and **B**, 0, 1, 5, and 10 μM; for **C**, 0, 10, 50, and 100 μM



difference in sensitivity to these drugs among *Entamoeba* species. The time course studies on growth inhibition revealed that 1 and 5 μM lactacystin inhibit the cell division of *E. histolytica* and 10 μM is toxic to the parasite. The drug may affect the control of cell cycle progression in this parasite, as has been reported for a number of cell types (King et al. 1996) including *T. brucei* (Mutomba et al. 1997).

The encystation of *E. invadens* was also inhibited by these proteasome inhibitors in a concentration-dependent manner (Fig. 3). Gonzalez et al. (1997, 1999) previously reported that lactacystin inhibited *E. invadens* encystation and that the inhibition was complete with concentrations at or above 10 μM, whereas with up to 20 μM MG-132 this was not the case. We, however, found that β-lactone is more potent for the inhibition of encystation than lactacystin and that higher concentrations (50 and 100 μM) of MG-132 are effective. Concentrations of β-lactone that inhibited encystation were lower than those needed to inhibit growth. These results clearly demonstrate that proteasomes play an important role in the encystation process, as has been reported previously (Gonzalez et al. 1997, 1999). Our previous study demonstrated that encystation of *E. invadens* was dependent on the cell cycle of parasite (Kumagai et al. 1998) so that it is probable that the inhibition of encystation by proteasome inhibitors is also related to a failure to control cell cycle progression. Although higher concentrations of MG-132 were required for the inhibition of *E. invadens* encystation, concentrations of the drug as low as those of lactacystin had inhibitory effects on the transformation of *T. cruzi* trypomastigotes into amastigotes (Gonzalez et al. 1996). This suggests that the sensitivity to the drug is dependent on the differentiating cell types in parasitic protozoa. In this regard, MG-132, the peptide aldehyde, also inhibits certain lysosomal cysteine proteases and the calpains. Thus, studies on proteasome function in cells with these peptide aldehydes need suitable controls (Lee and Goldberg 1998).

Virtually no recovery of encystation was observed after the removal of β-lactone and lactacystin from the encystation medium. The irreversibility of the effect of β-lactone in the encystation medium suggests that the ability of the trophozoites to encyst is lost during exposure to the drug. The reason for this effect remains unresolved.

The results indicate that multinucleation occurred in *E. histolytica* trophozoites treated with these proteasome inhibitors, suggesting that cytoplasmic division was blocked by the drugs. How proteasome function is related to this process remains unresolved. Electron microscopy revealed an increased number of trophozoites with glycogen granules in the cytoplasm after treatment with lactacystin. This suggests that proteasome function is related to glycogen metabolism in this parasite. In this regard, there is a difference in the morphological changes induced by lactacystin among parasitic protozoa. Unlike *E. histolytica*, treatment of *T. gondii* tachyzoites with lactacystin produced a cytoplasmic accumulation of misfolded proteins reported in mammalian cells (Johnson et al. 1998), and the development of whorls of endoplasmic reticulum-derived membranes (Shaw et al. 2000).

The molecular and biochemical characterization of *Entamoeba* proteasome is limited. The presence of 20S proteasomes having a three-hydrolytic-activity complex, and another large (11S) complex which is distinct from proteasomes, has been reported in *E. histolytica* (Scholze et al. 1996). In addition, the purified *E. invadens* proteasomes displayed the enzymatic activities, overall size, and subunit structures typical of proteasomes characterized from other cell types (Gonzalez et al. 1997, 1999). There are, however, differences in the relative proteolytic activities between mammalian and protozoan parasite proteasomes: mammalian proteasomes have a higher chymotrypsin-like activity, whereas the proteasomes of *T. brucei* (Hua et al. 1996), *T. cruzi* (Gonzalez et al. 1996), and *Entamoeba* (Gonzalez et al. 1997, 1999), have higher trypsin-like, moderate peptidyl-glutamyl peptide hydrolyzing, and low chymotrypsin-like activities. Further exploitation of the differences between the parasite and host proteasomes could allow those of parasites to act as new targets for chemotherapy.

In summary, our results show that proteasome inhibitors block both growth and encystation but have little effect on the excystation of *Entamoeba*, suggesting that protein degradation by proteasomes contributes to both the growth and encystation of *Entamoeba*, but not to the excystation.

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ORIGINAL PAPER

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Culture, isolation and propagation of *Babesia caballi* from naturally infected horses

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Abstract Thirteen blood samples of horses from South Africa, five of which were seropositive for *Babesia caballi* and eight for both *B. caballi* and *Theileria equi*, were subjected to in vitro culture to identify carrier animals. None of the animals had a detectable parasitaemia on Giemsa-stained blood smears before culture initiation. Cultures were initiated in L-cysteine-enriched medium, either in an oxygen-reduced gas mixture or in a 5% CO_2-in-air atmosphere. All five animals seropositive for *B. caballi* were identified as carrier animals using an oxygen-reduced atmosphere, whereas only four samples became culture positive under normal atmospheric conditions. Among the eight samples seropositive for both *B. caballi* and *T. equi*, two were identified as carriers for both. The remaining six samples were identified as carrying only *T. equi*.

Introduction

Equine piroplasmosis is a tick-borne disease of equidae (horses, mules, donkeys and zebras) caused by two intraerythrocytic protozoan parasites, *Babesia caballi* and *Theileria equi*. The latter, previously known as *Babesia equi*, multiplies in lymphoid cells before invading erythrocytes (Schein et al. 1981) and has therefore recently been reclassified as a *Theileria* species (Mehlhorn and Schein 1998).

Equine piroplasmosis is widespread in South Africa. A serological survey revealed that, of 6,350 serum samples collected from all over South Africa, nearly 80% were positive for *T. equi* and approximately 50% were positive for *B. caballi* (De Waal 1995). Despite this, during culture diagnostic procedures designed to detect *T. equi* parasites in carrier animals (Zweygarth et al. 1997, 1999), *B. caballi* was only incidentally demonstrated. A definite diagnosis of equine piroplasmosis relies on the demonstration of parasites in a stained blood smear; a demonstrable parasitaemia is rarely observed. In vitro culture techniques may therefore be considered for diagnosis, either alone or to supplement other procedures such as serology or DNA-based methods. In the case of *B. caballi* the carrier status of four out of nine horses previously experimentally infected has been successfully confirmed by culture (Holman et al. 1993).

The aim of this study was to demonstrate the presence of, and to isolate, *B. caballi* parasites found in naturally infected horses by using a culture technique originally developed for the in vitro diagnosis of *T. equi*.

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Materials and methods

Blood samples

Blood samples were collected from horses at the National Yearlings Sale in March 2000. Serum was prepared and examined for anti-*B. caballi* and anti-*T. equi* antibodies using the indirect fluorescent antibody (IFA) test as described for *B. caballi* by Madden and Holbrook (1968). A titre of ≥1:80 was considered to be positive, and 13 out of 515 samples were found to be positive for *B. caballi*, and of these 13, 8 were also positive for *T. equi*.

Blood samples were collected from the *B. caballi* seropositive horses by venipuncture into sterile vacuum tubes containing EDTA as anticoagulant (Vacutainer, Becton Dickinson, Meylan, France). The samples were brought to the Onderstepoort Veterinary Institute (OVI) on ice in a polystyrene container and processed as below. Thin blood smears, prepared from all blood samples, were stained with Giemsa stain and examined microscopically. No parasites were detected during these examinations. None of the horses that were serologically positive for *B. caballi* had a history of previous treatment with antiprotozoal agents.

Culture medium

A modified HL-1 medium (BioWhittaker, Walkersville, Md.) as described recently for the culture initiation of *T. equi* (*B. equi*)

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INHIBITION OF EXCYSTATION AND METACYSTIC DEVELOPMENT OF *ENTAMOEBA INVADENS* BY THE DINITROANILINE HERBICIDE ORYZALIN

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ABSTRACT: The effect of oryzalin on excystation and metacystic development of *Entamoeba invadens* strain IP-1 was examined by transfer of cysts to a growth medium containing the drug. Excystation, which was assessed by counting the number of metacystic amoebae after induction of excystation, was inhibited by oryzalin in a concentration-dependent manner. Metacystic development, which was determined by the number of nuclei in metacystic amoebae, was also inhibited by oryzalin because the percentage of 4-nucleate amoebae at day 1 remained unchanged at day 3. The addition of oryzalin after the induction of excystation decreased the number of metacystic amoebae, compared with control cultures. When cysts were incubated for 1 day in growth medium plus oryzalin, little increase in the number of metacystic amoebae was observed after removal of the drug. Excystation and metacystic development were further inhibited when the cysts were incubated for 30 min in encystation medium containing oryzalin before transfer to growth medium with the drug. When cysts were incubated for 30 min in encystation medium before transfer to growth medium without the drug, metacystic amoebae decreased in number. Pretreatment of cysts with oryzalin for 30 min in phosphate-buffered saline markedly reduced viability and prevented excystation in growth medium with or without the drug. The results indicate that oryzalin inhibits excystation and metacystic development of *E. invadens*, suggesting that it may be an inhibitor of *Entamoeba* infection.

Oryzalin, a dinitroaniline preemergent herbicide, binds to tubulin and inhibits the dynamics of microtubule assembly and disassembly in plants (Bajer and Moré-Bajer, 1986; Morejohn et al., 1987). In recent years, evidence has accumulated that the dinitroanilines, including oryzalin and trifluralin, are effective against certain parasitic protozoa, e.g., *Leishmania* species (Chan and Fong, 1990; Chan et al., 1991, 1993), *Trypanosoma brucei* (Chan et al., 1993), *Plasmodium falciparum* (Nath et al., 1992), *Toxoplasma gondii* (Stokkermans et al., 1996), and *Cryptosporidium parvum* (Arrowood et al., 1996; Armson et al., 1999). We have demonstrated that these dinitroanilines inhibit the growth of *Entamoeba histolytica* and *E. invadens*. Oryzalin was more potent than trifluralin, and *E. invadens* was more resistant to these drugs than *E. histolytica* (Makioka et al., 2000a). The results also indicated that values of concentration for 50% inhibition (IC_{50}) of these drugs for *Entamoeba* spp. were higher than those for other parasitic protozoa. We also demonstrated that in vitro encystation of *E. invadens* is inhibited by oryzalin (Makioka et al., 2000b).

Excystation and metacystic development of *Entamoeba* sp. are necessary for infection. Although these processes were described previously for *E. histolytica* (Dobell, 1928; Cleveland and Sanders, 1930), little is known about the mechanisms involved. Knowledge of excystation and encystation are limited because no axenic encystation medium is available for this parasite (López-Romero and Villagómez-Castro, 1993). Studies have been carried out on axenic in vitro encystation of *E. invadens*, a reptilian parasite, because of its close similarity with *E. histolytica* in its morphology and life cycle (McConnachie, 1969). Because excystation and metacystic development of *E. invadens*, even regarding minute details, are entirely in agreement with those for *E. histolytica* (Geiman and Ratcliffe, 1936), in vitro excystation of *E. invadens* may also become a useful model for excystation of the human parasite.

The process of excystation includes a loosening and separation of the protoplasm from the cyst wall and escape of the whole organism through a minute pore in the cyst wall. Metacystic development is the process by which one 4-nucleate amoeba develops into 8, 1-nucleate amoebulae, which grow to become trophozoites (Dobell, 1928; Cleveland and Sanders, 1930; Geiman and Ratcliffe, 1936). Because nuclear and cytoplasmic division do not always occur simultaneously, many combinations of cystic and metacystic nuclei occur before the 4-nucleate amoeba produces 8 amoebulae (Dobell, 1928; Cleveland and Sanders, 1930; Geiman and Ratcliffe, 1936). Excystation of *E. invadens* can be induced in vitro by transfer of cysts from an encystation medium to a growth medium (McConnachie, 1955; Rengpien and Bailey, 1975; Garcia-Zapien et al., 1995). Using this in vitro excystation system for *E. invadens*, we recently demonstrated that the actin-modifying drugs latrunculin A and jasplakinolide inhibited excystation and metacystic development, whereas cytochalasin D unexpectedly enhanced these processes (Makioka et al., 2001). We report here that the antimicrotubule drug oryzalin inhibits excystation and metacystic development of *E. invadens*.

MATERIALS AND METHODS

Trophozoites of the IP-1 strain of *E. invadens* were cultured in the axenic growth medium BI-S-33 (Diamond et al., 1978) at 26 C. To obtain cysts, trophozoites (5×10^5 cells/ml) were transferred to an encystation medium called 47% LG (LG is BI without glucose; Sanchez et al., 1994). After 3 days, the cells were harvested and treated with 0.05% sarkosyl (Sigma Chemical Co., St. Louis, Missouri) to destroy trophozoites (Sanchez et al., 1994). The remaining cysts were washed with phosphate-buffered saline (PBS) and counted. Excystation was induced by transferring these cysts to the growth medium. Viability of cysts was determined by trypan blue dye exclusion. For experiments on the effect of oryzalin on excystation and metacystic development of *E. invadens*, duplicate cultures of 5×10^5 cysts/ml were incubated with oryzalin (5–200 μM) for 3 days. Metacystic amoebae and cysts were counted daily, and viability was determined by trypan blue dye exclusion. Oryzalin (3, 5-dinitro-N^3, N^3-dipropylsulfanilamide) was purchased from Accustandard, Inc., New Haven, Connecticut. It was stored as a 100-mM stock solution in dimethyl sulfoxide (DMSO). Control cultures received the same volume of DMSO.

For estimation of metacystic development of *E. invadens*, duplicate cultures (5×10^5 cysts/ml) with and without 100 μM oryzalin were incubated for 3 days. Cells were harvested at days 1 and 3 and stained

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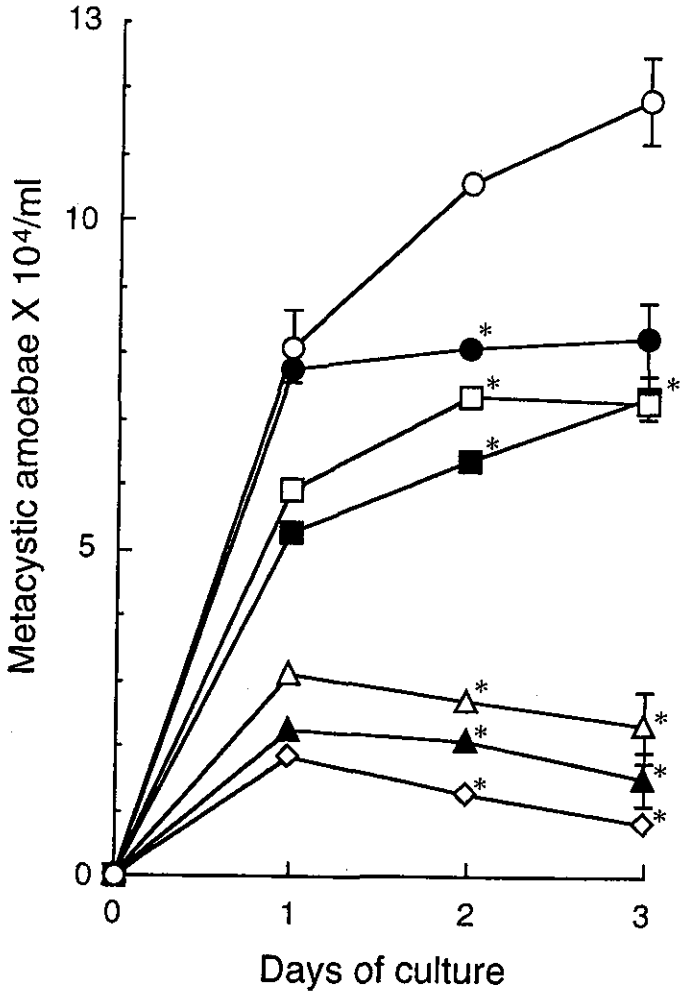

FIGURE 1. Effect of oryzalin on the number of metacystic amoebae of *Entamoeba invadens*. The cysts were transferred to the growth medium containing various concentrations of oryzalin. Mean numbers ± SE of the metacystic amoebae for duplicate cultures are plotted (* $P < 0.05$). Concentrations are shown by open circles (0 μM), solid circles (5 μM), open squares (10 μM), solid squares (25 μM), open triangles (50 μM), solid triangles (100 μM), and open diamonds (200 μM).

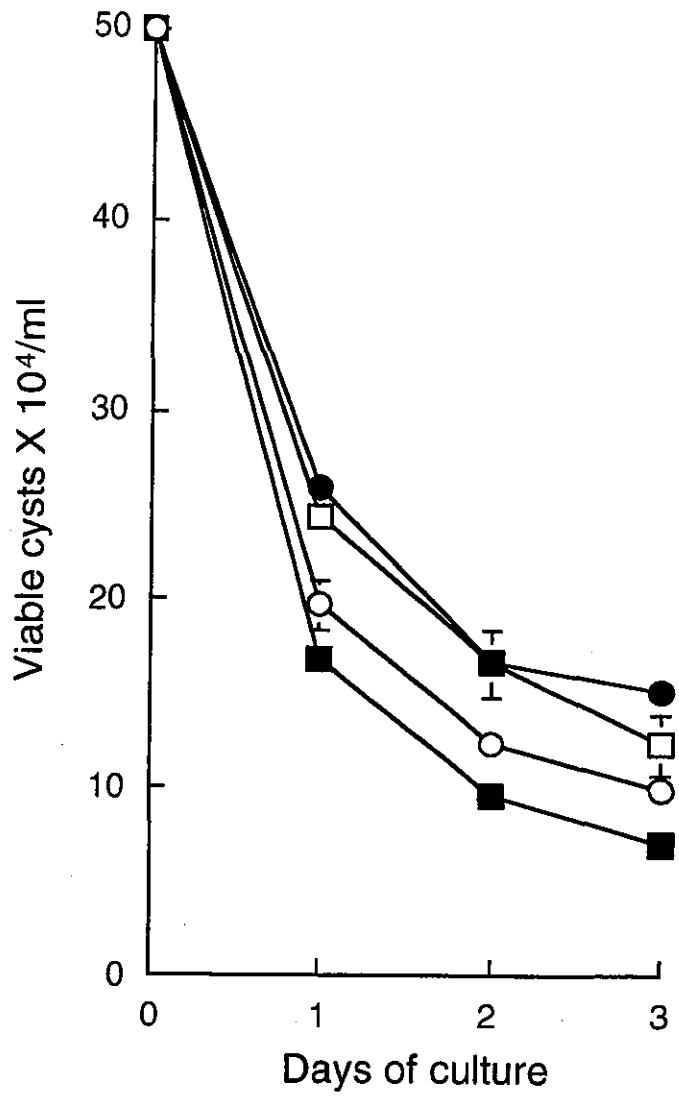

FIGURE 2. Effect of oryzalin on cyst viability of *Entamoeba invadens*. Mean numbers ± SE of viable cysts in duplicate cultures are plotted. The experimental conditions are the same as those for Figure 1. The concentrations are shown by open circles (0 μM), solid circles (50 μM), open squares (100 μM), and solid squares (200 μM).

with modified Kohn. The number of nuclei per amoeba was determined by counting at least 100 amoebae twice.

For experiments on the effect of oryzalin on excystation and metacystic development after induction of excystation, duplicate cultures (5×10^5 cysts/ml) were incubated for 1 day; then oryzalin was added to 1 culture at a concentration of 100 μM. The cultures were incubated for an additional 2 days, and the metacystic amoebae were counted.

For experiments on the reversibility of oryzalin, duplicate cultures containing 100 μM oryzalin were incubated for 1 day. Cells were then centrifuged at 400 *g* for 5 min after chilling on ice, and the spent medium was removed. Cells were washed twice with growth medium and then resuspended in fresh growth medium. No replacement of the medium was carried out in the control cultures. The cultures were incubated for an additional 2 days, and the metacystic amoebae were counted.

For evaluation of the effect of pretreatment with oryzalin on excystation and metacystic development, cysts were first incubated for 30 min in encystation medium or PBS with or without 100 μM oryzalin and then transferred to growth medium with or without the same drug concentration. The cultures were incubated for another 3 days, and the metacystic amoebae and cysts were counted.

All experiments were performed at least 3 times, and similar results were obtained in each replicate. Therefore, representative data from duplicate cultures in single experiments are shown in the results.

RESULTS

Effect of oryzalin on excystation

The number of metacystic amoebae at day 1 of incubation with 5–200 μM of oryzalin was reduced in a concentration-dependent manner, compared with controls (Fig. 1). Amoebae cultured without the drug increased in number from day 1 to day 3. In contrast, no increase in the number of metacystic amoebae occurred from day 1 to day 3 in cultures exposed to ≥ 50 μM of oryzalin. An IC_{50} value (concentration for 50% inhibition) for oryzalin was estimated at 30 μM from these data.

Effect of oryzalin on cyst viability in growth medium

As shown in Figure 2, the number of viable cysts in cultures containing 50 or 100 μM of oryzalin was higher than in cultures

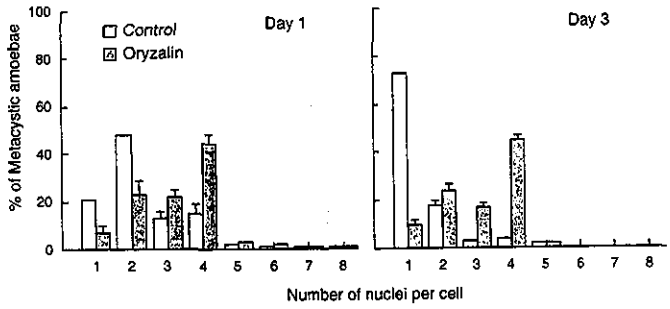

FIGURE 3. Effect of oryzalin on metacystic development of *Entamoeba invadens*. The cysts were transferred to growth medium with or without 100 μM oryzalin. The numbers of nuclei per metacystic amoeba stained with modified Kohn at day 1 and day 3 of incubation were counted, and the percentage of amoebae was determined.

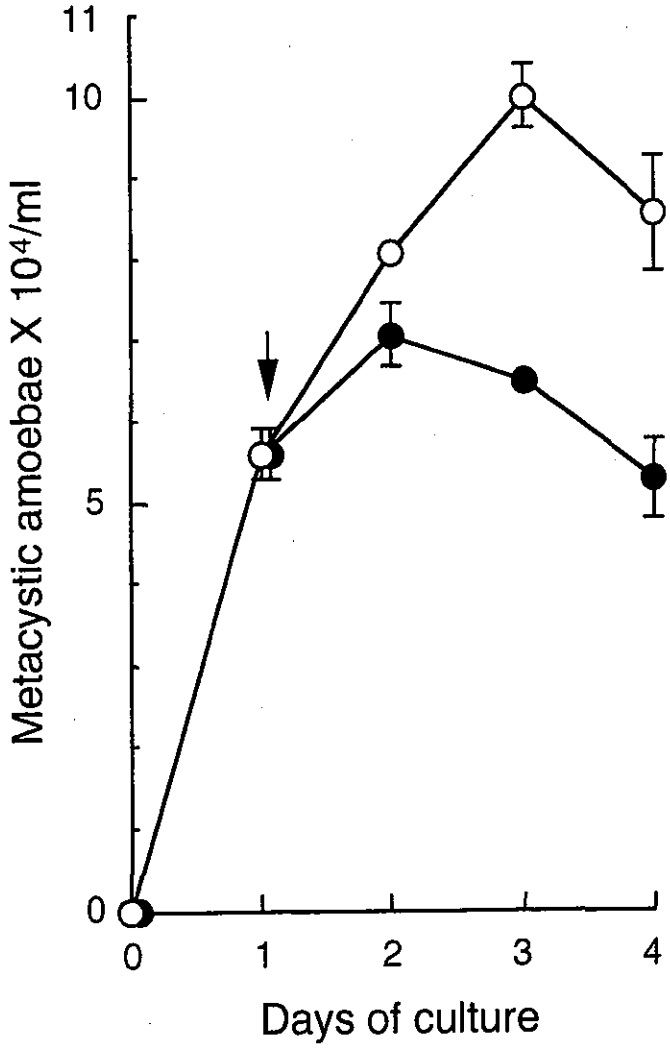

FIGURE 4. Effect of oryzalin on excystation and metacystic development of *Entamoeba invadens* after induction of excystation. After 1 day of incubation in growth medium (arrow), oryzalin was added to the cultures at a concentration of 100 μM. The cultures were incubated for an additional 2 days, and the metacystic amoebae were counted. Mean numbers ± SE of metacystic amoebae in duplicate cultures are plotted. Oryzalin cultures (solid circles); control cultures (open circles).

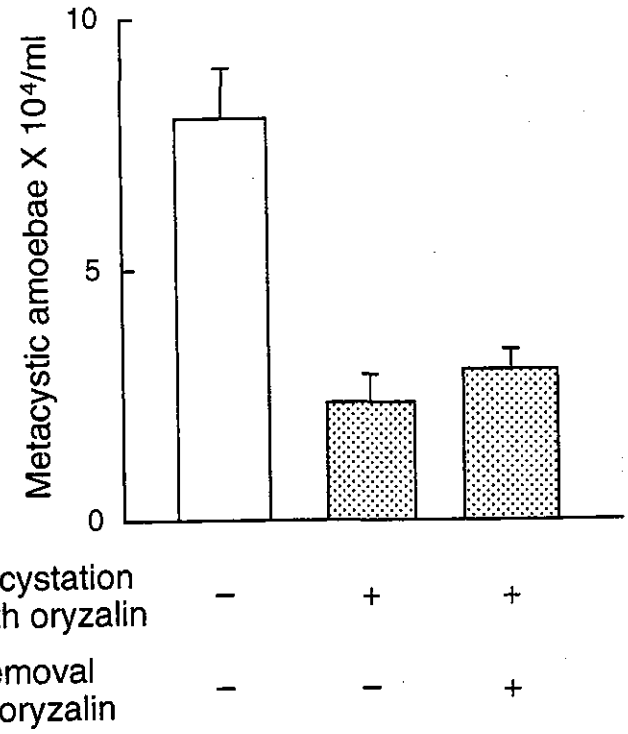

FIGURE 5. Effect of the removal of oryzalin on excystation and metacystic development of *Entamoeba invadens*. After exposure of the cysts to 100 μM oryzalin in the growth medium for 1 day, the drug was removed by replacement of the medium with the drug-free growth medium and the cultures were further incubated for 3 days. Mean numbers + SE of metacystic amoebae in duplicate cultures are plotted.

not exposed to the drug during incubation. Oryzalin at 200 μM slightly reduced the number of viable cysts, compared with the control culture.

Effect of oryzalin on metacystic development

As shown in Figure 3, 15% of metacystic amoebae were 4-nucleate and 82% of those were 1- to 3-nucleate at day 1 of incubation in cultures minus oryzalin, whereas 44% of amoebae were 4-nucleate and 52% of those were 1- to 3-nucleate in cultures containing 100 μM oryzalin, suggesting slower development of metacystic amoebae in the presence of oryzalin. At day 3, only 4% of amoebae were 4-nucleate and the percentage of 1-nucleate amoebae reached to 73% in controls, whereas 45% of amoebae were still 4-nucleate and only 10% were 1-nucleate in cultures with oryzalin.

Effect of oryzalin on the number of metacystic *amoebae* after induction of excystation

To examine the effect of oryzalin on excystation and metacystic development after induction, oryzalin (100 μM) was added to cultures at day 1 of excystation and metacystic amoebae counted 1 and 2 days later. As shown in Figure 4, metacystic amoebae were decreased in number at 1 and 2 days after the addition of oryzalin compared with those in cultures without the drug.

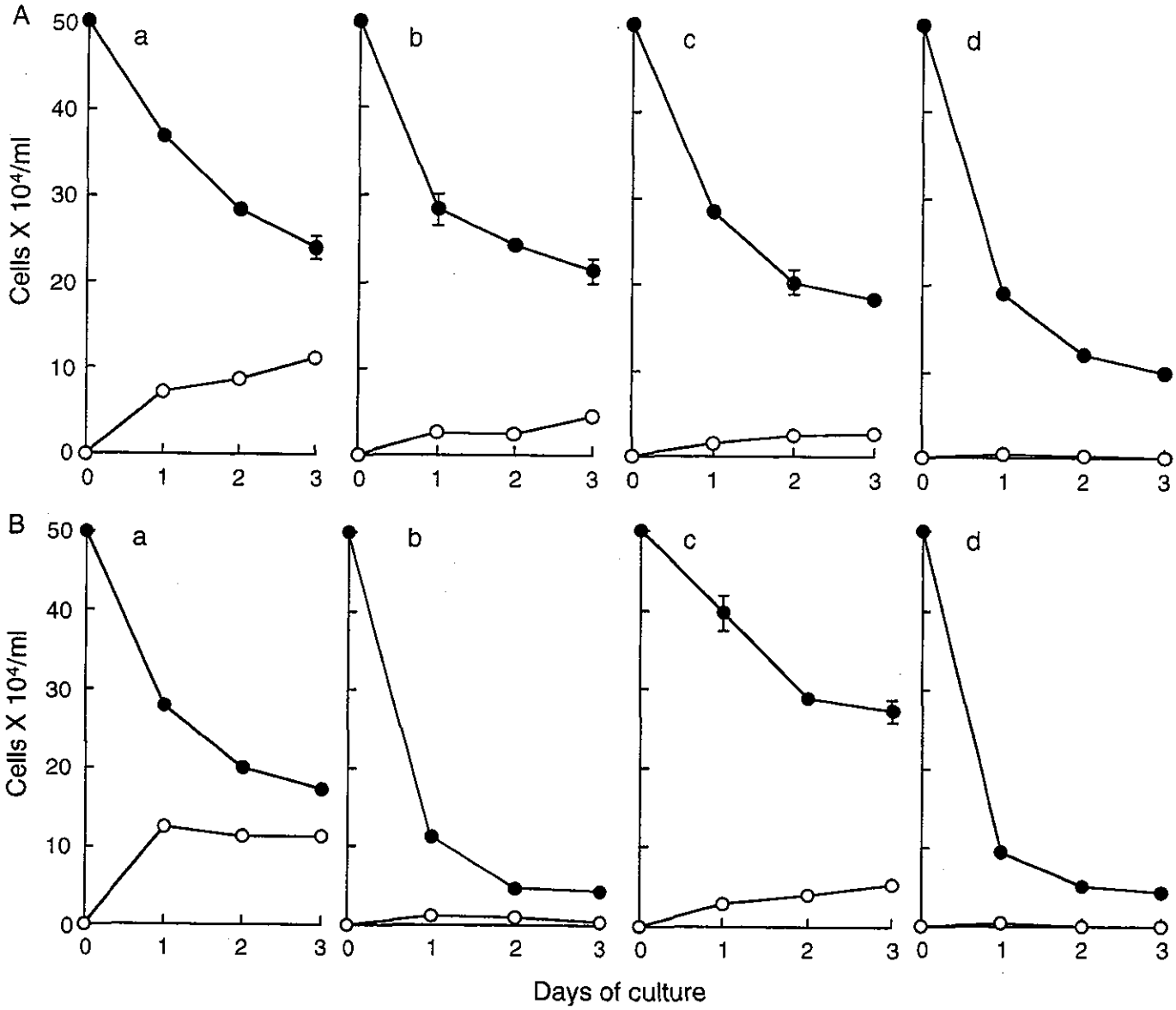

FIGURE 6. Effect of pretreatment with oryzalin on excystation and metacystic development of *Entamoeba invadens*. The cysts were incubated for 30 min in the encystation medium (**A**) or in PBS (**B**) with or without 100 μM oryzalin before transfer to growth medium with or without the drug. Mean numbers ± SE of metacystic amoebae (open circles) and cysts (solid circles) for duplicate cultures are plotted. Pretreatment without oryzalin and culture without the drug (a); pretreatment with the drug and culture without the drug (b); pretreatment without the drug and culture with the drug (c); pretreatment with the drug and culture with the drug (d).

Reversibility of the effect of oryzalin on the number of metacystic amoebae

To determine whether the inhibitory effect of oryzalin on excystation was reversible, spent medium containing 100 μM oryzalin for 1 day of incubation was replaced with drug-free growth medium. After removal of the drug, little increase in the number of metacystic amoebae occurred (Fig. 5).

Effect of pretreatment with oryzalin on the number of metacystic amoebae and cysts

Pretreatment of cysts with 100 μM oryzalin in encystation medium before transfer to the growth medium containing the drug reduced the number of metacystic amoebae during incubation as compared with that without the pretreatment (Fig. 6A). When cysts pretreated with oryzalin in encystation medium were incubated in growth medium without the drug, the number of metacystic amoebae was lower than that observed without the pretreatment (Fig. 6A). When cysts were incubated in PBS without oryzalin before transfer to growth medium without the drug, the number of metacystic amoebae was similar to that in cultures pretreated with oryzalin in encystation medium (Fig. 6B). When cysts were pretreated with oryzalin in PBS before transfer to growth medium with or without the drug, no metacystic amoebae appeared and few viable cysts remained (Fig. 6B).

DISCUSSION

The results clearly indicate that oryzalin inhibits excystation and metacystic development of *E. invadens*, with the 2 different actions of oryzalin being a static effect at concentrations at 5–25 μM and a toxic or cidal effect at concentrations of 50 μM and higher. The inhibitory effect of oryzalin on excystation is not caused by its toxic effect on cysts because the number of viable cysts in cultures containing 50 or 100 μM oryzalin was higher than in control cultures. The process of excystation includes a loosening of the amoeba from the cyst wall and escape from the cyst through a small pore in the wall, but it does not include nuclear division. Because microtubules are seen only during nuclear division in *Entamoeba* sp. (Orozco et al., 1988), the question of how oryzalin inhibits excystation remains unresolved. Although the primary mode of oryzalin action is inhibition of microtubule formation, it also inhibits intracellular free Ca^{2+} signaling in mammalian cells and has antitumor activity in animals (Powis et al., 1997). We have recently found that depletion of extracellular Ca^{2+} and inhibition of intracellular Ca^{2+} flux block excystation of *E. invadens* (Makioka et al., 2002). It is possible that oryzalin blocks the excystation through inhibition of Ca^{2+} signaling. After excystation, the metacystic amoebae with 4 nuclei grow rapidly and divide to form 8 amoebulae (Dobell, 1928; Cleveland and Sanders, 1930; Geiman and Ratcliffe, 1936). This process includes nuclear division, a target of oryzalin action. The percentage of amoebae with 4 nuclei in cultures containing oryzalin was higher than that in controls, indicating that nuclear division of the excysted amoebae with 4 nuclei was inhibited by oryzalin. In this regard, previous observations indicated an accumulation of *E. invadens* trophozoites arrested in the mitotic phase in cultures containing oryzalin (Makioka et al., 2000b). No studies on molecular and biochemical changes that accompany development from metacystic amoebae to trophozoites have been published, and the biological significance of this process remains unresolved. For these studies, it is necessary to separate metacystic amoebae from cysts. An effort has been made to separate metacystic amoebae from cysts by centrifugation through discontinuous Percoll gradients, which is a useful method for separation of cysts from trophozoites of *E. invadens* (Avron et al., 1983), but this was unsuccessful. This suggests that there is a difference in density between metacystic amoebae and trophozoites.

Previous studies demonstrated that oryzalin inhibits growth and encystation of *E. invadens*, although concentrations ≥200 μM were needed to observe any effect (Makioka et al., 2000a, 2000b). We now report that oryzalin at concentrations ≥50 μM inhibits excystation and metacystic development of *E. invadens*. Thus, excystation and metacystic development of *E. invadens* are more sensitive to oryzalin than growth and encystation. Little recovery of excystation and metacystic development occurred even after removal of oryzalin, suggesting that once cysts were exposed for 1 day with oryzalin in the growth medium they lost the ability to excyst. Similarly the irreversible effects of oryzalin were demonstrated in a previous study on encystation of *E. invadens* (Makioka et al., 2000b). The results of pretreatment with oryzalin in the encystation medium indicate that it contributes to further inhibition of excystation in growth medium with the drug and that the oryzalin effect still remains, although to a lesser extent, in growth medium in the absence of the drug. Because pretreatment of cysts with oryzalin in PBS prevented the appearance of metacystic amoebae and caused a marked decrease in the number of viable cysts, it is concluded that oryzalin in PBS has a cystocidal effect.

In summary, oryzalin is effective in blocking excystation and metacystic development of *E. invadens*, suggesting that it could become a useful inhibitor of *Entamoeba* infection.

ACKNOWLEDGMENTS

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Identification and Characterization of Two Isoenzymes of Methionine γ-Lyase from *Entamoeba histolytica*

A KEY ENZYME OF SULFUR-AMINO ACID DEGRADATION IN AN ANAEROBIC PARASITIC PROTIST THAT LACKS FORWARD AND REVERSE TRANS-SULFURATION PATHWAYS*

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To better understand the metabolism of sulfur-containing amino acids, which likely plays a key role in a variety of cell functions, in *Entamoeba histolytica*, we searched the genome data base for genes encoding putative orthologs of enzymes known to be involved in the metabolism. The search revealed that *E. histolytica* possesses only incomplete cysteine-methionine conversion pathways in both directions. Instead, this parasite possesses genes encoding two isoenzymes of methionine γ-lyase (EC 4.4.1.11, EhMGL1/2), which has been implicated in the degradation of sulfur-containing amino acids. The two amebic MGL isoenzymes, showing 69% identity to each other, encode 389- and 392-amino acid polypeptides with predicted molecular masses of 42.3 and 42.7 kDa and pIs of 6.01 and 6.63, respectively. Amino acid comparison and phylogenetic analysis suggested that these amebic MGLs are likely to have been horizontally transferred from the Archaea, whereas an MGL from another anaerobic protist *Trichomonas vaginalis* has MGL isotypes that share a common ancestor with bacteria. Enzymological and immunoblot analyses of the partially purified native amebic MGL confirmed that both of the MGL isotypes are expressed in a comparable amount predominantly in the cytosol and form a homotetramer. Recombinant EhMGL1 and 2 proteins catalyzed degradation of L-methionine, DL-homocysteine, L-cysteine, and *O*-acetyl-L-serine to form α-keto acid, ammonia, and hydrogen sulfide or methanethiol, whereas activity toward cystathionine was negligible. These two isoenzymes showed notable differences in substrate specificity and pH optimum. In addition, we showed that EhMGL is an ideal target for the development of new chemotherapeutic agents against amebiasis by demonstrating an amebicidal effect of the methionine analog trifluoromethionine on trophozoites in culture (IC_{50} 18 μM) and that this effect of trifluoromethionine was completely abolished by the addition of the MGL-specific inhibitor DL-propargylglycine.

Entamoeba histolytica is a causative agent of amebiasis, which annually affects an estimated 48 million people and results in 70,000 deaths (1). The most common clinical presentation of amebiasis is amebic dysentery and colitis; extraintestinal abscesses, *i.e.* hepatic, pulmonary, and cerebral, however, are also common and often lethal. This microaerophilic anaerobe has been considered to be a unique eukaryotic organism because it apparently lacks organelles typical of eukaryotic organisms such as mitochondria, the rough endoplasmic reticulum, and the Golgi apparatus (2). However, a recent demonstration of genes encoding mitochondrial proteins, *i.e.* cpn60 and pyridine nucleotide transhydrogenase (3), together with electron micrographic demonstration of the rough endoplasmic reticulum and the Golgi apparatus (4), suggested the presence of a residual organelle of mitochondria (called crypton or mitosome) (5, 54) and also indicated that this group of parasitic protists possess a unique organelle organization. This parasite also reveals numerous unusual aspects in its metabolism (6), highlighted by the lack of the tricarboxylic acid cycle (7) and glutathione metabolism (8). In addition, recent studies suggesting the horizontal transfer of genes encoding a variety of fermentation enzymes from bacteria (9), and genes encoding malic enzyme and acetyl-CoA synthase from the Archaea (10) have placed this protozoan organism at a unique position in eukaryotic evolution.

One of these unique metabolic pathways found in this parasite is the biosynthetic and degradative pathway of sulfur-containing amino acids, especially cysteine, which has been demonstrated to be essential for the growth and various cellular activities of amoebae (11, 12). Sulfur-containing amino acid metabolism varies among organisms (Fig. 1, also reviewed in Ref. 13). In mammals, cysteine is produced solely from incorporated methionine and serine via *S*-adenosylmethionine, homocysteine, and cystathionine in a pathway called the reverse trans-sulfuration pathway. In contrast, plants, fungi, and some bacteria have a so-called sulfur assimilation pathway to fix inorganic sulfur onto a serine derivative (*O*-acetylserine,

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB094499 and AB094500.

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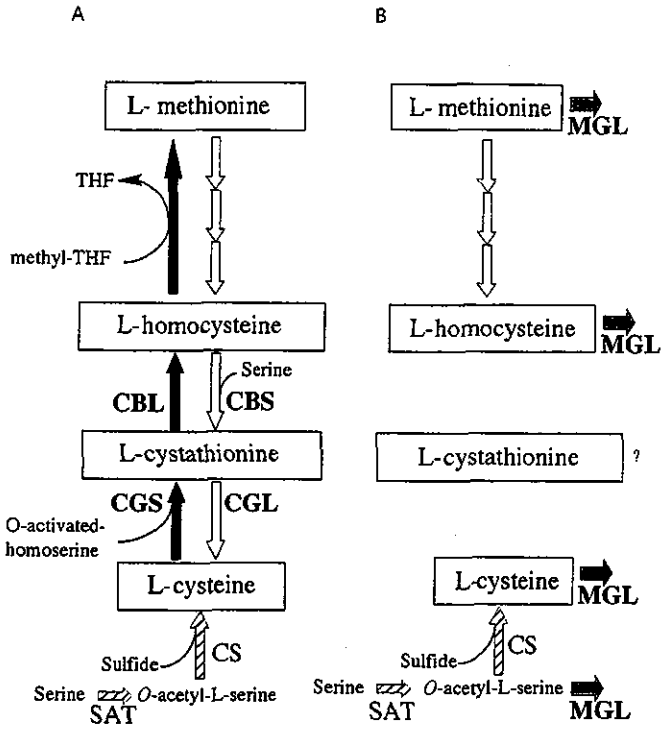

FIG. 1. **A schematic representation of sulfur-containing amino acid metabolism in general (*A*) and in *Entamoeba* (*B*).** Only enzymes that belong to the γ-subfamily of PLP-dependent enzymes are shown (in *bold*), together with CS and CBS, which belong to the β-family, and SAT. Biochemical steps involved in both forward and reverse trans-sulfuration reactions are indicated by *filled* and *open arrows*, respectively. Genes encoding the first three steps of the reverse trans-sulfuration pathway have been identified in the *E. histolytica* genome data base (data not shown). *Gray arrows* indicate reactions catalyzed by MGLs and *hatched arrows* indicate sulfur assimilatory steps we previously reported (14, 15).

OAS)[1] to synthesize cysteine. These organisms are also capable of converting cysteine into methionine via a trans-sulfuration sequence in the opposite orientation (also called the methionine biosynthetic pathway). We previously demonstrated that *E. histolytica* possesses the sulfur assimilatory cysteine biosynthetic pathway, and is capable of producing cysteine *de novo* (14, 15). We have also demonstrated (15) that major enzymes in this pathway, serine acetyltransferase (SAT) and cysteine synthase (CS), play a central role in the control of the intracellular cysteine concentrations, and in the antioxidative stress defense mechanism of this gluthathione-lacking parasite (8).

One important question remaining about the sulfur-containing amino acid metabolism in this parasite, and also in anaerobic protists in general, is how these parasites degrade toxic sulfur-containing amino acids since they possess apparently incomplete trans-sulfuration pathways in both the forward and reverse orientation (data not shown, see the present study). Thus, in order to better understand the metabolism, particularly degradation, of these sulfur-containing amino acids in *E. histolytica*, we attempted to isolate other essential genes encoding proteins involved in sulfur amino acid metabolism.

[1] The abbreviations used are: OAS, *O*-acetylserine; TFMET, trifluoromethionine; TIGR, The Institute for Genomic Research; SAT, serine acetyltransferase; CS, cysteine synthase; MGL, methionine γ-lyase; PPG, DL-propargylglycine; PLP, pyridoxal 5′-phosphate; CGL, cystathionine γ-lyase; CGS, cystathionine γ-synthase; CBL, cystathionine β-lyase; CBS, cystathionine β-synthase; ORF, open reading frame; GST, glutathione *S*-transferase; rEhMGL, recombinant EhMGL; NJ, Neighbor-Joining; MP, maximum parsimony; ML, maximum likelihood; MOPS, 4-morpholinepropanesulfonic acid; MES, 4-morpholineethanesulfonic acid.

We identified and characterized two isotypes of the unique enzyme, methionine γ-lyase (MGL; EC 4.4.1.11) and their encoding genes, which, we propose, function in the degradation of sulfur amino acids in this parasite. We show a line of evidence suggesting that the MGL genes and their proteins were likely derived from the Archaea by horizontal transfer as shown for other metabolic enzymes in this parasite (10). In addition, we also demonstrate that the methionine analog trifluoromethionine (TFMET) has a cytotoxic effect on amebic trophozoites that is abolished by a specific inhibitor of MGL, indicating that MGL is exploitable as an attractive target for the development of new amebicidal compounds.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—L-methionine, L-cysteine, DL-homocysteine, OAS, *O*-succinyl-L-homocysteine, *O*-acetyl-L-homoserine, DL-propargylglycine (PPG), 3-methyl-2-benzothiazolinone hydrazon hydrochloride, trichloroacetic acid, pyridoxal 5′-phosphate (PLP), and other chemicals were commercial products of the highest purity available unless otherwise stated. TFMET was a gift from Dr. Cyrus J. Bacchi (Haskins Laboratories and Department of Biology, Pace University, New York).

Microorganisms and Cultivation—Trophozoites of *E. histolytica* strain HM-1:IMSS cl-6 (16) were maintained axenically in Diamond's BI-S-33 medium (11) at 35.5 °C. Trophozoites were harvested at the late-logarithmic growth phase 2–3 days after inoculation of one-twelfth to one-sixth of a total culture volume. After the cultures were chilled on ice for 5 min, trophozoites were collected by centrifugation at 500 × *g* for 10 min at 4 °C and washed twice with ice-cold phosphate-buffered saline, pH 7.4. Cell pellets were stored at −80 °C until use.

Search of the Genome Data Base of E. histolytica—The *E. histolytica* genome data base at the Institute for Genomic Research (TIGR, *//www.tigr.org/tdb/*) was searched using the TBLASTN algorithm with protein sequences corresponding to the PLP-attachment site of cysteine- and methionine-metabolizing enzymes (PROSITE access number PS00868). This motif is conserved among the γ−subfamily (α-family) of PLP enzymes (for the classification of PLP enzymes used in this study, see Ref. 17), i.e. cystathionine γ-lyase (CGL), cystathionine γ-synthase (CGS), and cystathionine β-lyase (CBL) from a variety of organisms. We also searched for amebic orthologs that belong to the β-family of PLP enzymes using the PLP-attachment site from CS of *E. histolytica* and cystathionine β-synthase (CBS) from yeast and mammals.

Cloning of E. histolytica MGL1 and MGL2 and Production of their Recombinant Proteins—Based on nucleotide sequences of the protein-encoding region of the two putative amebic MGL genes (*EhMGL1* and *EhMGL2*), two sets of primers, shown below, were designed to amplify the open reading frames (ORF) of *EhMGL1* and to construct plasmids to produce glutathione *S*-transferase (GST)-EhMGL fusion proteins. The two sense and two antisense primers contained the *Sma*I restriction site (underlined) either prior to the translation initiation site or next to the stop codon (*bold*), respectively. The primers used are: *EhMGL1* (sense), 5′-CAT<u>CCCGGG</u>**GATG**ACTGCTCAAGATATTACTACTAC-T-3′ (37-mer); *EhMGL1* (antisense), 5′-TAG<u>CCCGGG</u>**ATTA**CCAAAG-CTCTAATGCTTGTTTTAA-3′ (37-mer); *EhMGL2* (sense), 5′-CAT<u>CCC-GGG</u>**TATG**TCTCAATTGAAGGATTTACAAACA-3′ (37-mer); *EhMGL2* (antisense), 5′-TAG<u>CCCGGG</u>**ATTA**GCATTGTTCAAGAGCTTGTTTT-AA-3′ (37-mer).

The cDNA library of *E. histolytica* trophozoites constructed in a lambda phage (14) was used as the template for polymerase chain reaction (PCR) using the following parameters. An initial step for denaturation and *rTaq* (Takara Bio Inc., Shiga, Japan) activation at 94 °C for 15 min was followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 30 s, and extension at 72 °C for 1 min. A final step at 72 °C for 10 min was used to complete the extension. Approximately 1.1-kb PCR fragments were obtained and cloned into the *Sma*I site of a pGEX-6P-1 expression vector (Amersham Biosciences K.K., Tokyo, Japan). The final constructs were designated as pGEX6P1/MGL1 and pGEX6P1/MGL2, respectively.

Nucleotide sequences were confirmed by using appropriate synthetic sequencing primers, a BigDye Terminator Cycle Sequencing Ready Reaction Kit, and an ABI PRISM 310 genetic analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan), according to the manufacturer's protocol. To express the recombinant proteins in *Escherichia coli*, pGEX6P1/MGL1 and pGEX6P1/MGL2 were introduced into *BL21 (DE3)* (Novagen Inc., Madison, WI) host cells. Expression of the GST-MGL1 and GST-MGL2 fusion proteins was induced with 1 mM isopro-

pyl-β-thiogalactoside at 18 °C for 20 h. The fusion proteins were purified using a glutathione-Sepharose 4B column (Amersham Biosciences) according to the manufacturer's instructions. The recombinant EhMGL1/2 (rEhMGL1/2) were obtained by digestion of these fusion proteins with PreScission Protease (Amersham Biosciences) in the column, followed by elution from the column and dialysis at 4 °C with 100 mM sodium phosphate buffer, pH 6.8, containing 0.02 mM PLP.

The final purified recombinant EhMGL (rEhMGL) proteins were presumed to contain 10 additional amino acids (GPLGSPEFPG) at the N terminus. The purified enzymes were stored at −80 °C with 30–50% dimethyl sulfoxide until use. No decrease in enzyme activity was observed under these conditions for at least 3 months. Protein concentrations were determined by Coomassie Brilliant Blue assay (Nacalai Tesque, Inc., Kyoto, Japan) with bovine serum albumin as the standard.

Amino Acid Alignments and Phylogenetic Analyses—The sequences of MGL and other members of the γ-subfamily of PLP enzymes showing similarities to the amino acid sequences of EhMGL were obtained from the National Center for Biotechnology Information (NCBI, //www.ncbi.nih.gov/) by using the BLASTP algorithm. The alignment and phylogenetic analyses were performed with ClustalW version 1.81 (18) using the Neighbor-Joining (NJ) method with the Blosum matrix. An unrooted NJ tree composed of the amino acid sequences of 13 MGLs and 10 other members of the γ-subfamily of PLP enzymes from various organisms with two EhCSs (β-family of PLP enzymes) as the outgroup was drawn by Tree View ver.1.6.0 (19). Branch lengths and bootstrap values (1000 replicates) were derived from the NJ analysis. Phylogenetic analyses by the maximum parsimony method (MP) and maximum likelihood method (ML) were also conducted using PROTPARS (PHYLIP version 3.57c, Ref. 20) and ProtML (MOLPHY version 2.3, Ref. 21), respectively.

Subcellular Fractionation of the Crude Extract—The lysate of ~3 × 10^6 *E. histolytica* trophozoites was prepared by two cycles of freezing and thawing in 1 ml of cell lysis buffer: 100 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, 0.02 mM PLP, 1 mM dithiothreitol, and a protease inhibitor mixture (Complete Mini EDTA-free, Roche Applied Science, Tokyo, Japan), and 1 μg/ml of *N*-(3-carboxyoxirane-2-carbonyl)-leucyl-amino(4-guanido)butane (E-64, Sigma). The whole lysate was then centrifuged at 14,000 × *g* in a microcentrifuge tube for 20 min at 4 °C to separate the supernatant (soluble cytosolic fraction) and the pellet (debris, membrane, and nuclear fraction).

Anion Exchange Chromatography of the Native Form MGLs—A supernatant fraction obtained from 2 g (wet weight) of the trophozoite pellet, as described above, was filtered with a 0.45-μm-pore mixed cellulose membrane (Millex-HA, Millipore Corporation, Bedford, MA). The sample buffer was exchanged with buffer A (20 mM Tris-HCl, pH 8.0, 0.02 mM PLP, 1 mM dithiothreitol, 1 mM EDTA, and 0.1 μg/ml of E-64) by using a Centricon Plus-20 (Millipore). A 20-ml sample containing ~100 mg of total protein was loaded on a DEAE-Toyopearl HW-650S column (7.5 × 1.6 cm, 15-ml bed volume, Tosoh, Tokyo, Japan) that was previously equilibrated with buffer A. The column was further washed with ~50 ml of buffer A until the A_{280} dropped below 0.1. The bound proteins were then eluted with a 50-ml linear potassium chloride gradient (0–0.5 M) in buffer A at a flow rate of 0.8 ml/min. All 0.8-ml fractions were concentrated to 0.2 ml with a Centricon YM-10 (Millipore). All procedures were performed at 4 °C. The amount of MGL in each fraction was assessed using the hydrogen sulfide assay and immunoblotting as described below.

Size Exclusion Chromatography of Recombinant and Native EhMGLs—To estimate the molecular mass of the recombinant and native EhMGLs, gel filtration chromatography was performed. Approximately 500 μg of recombinant and 100 μg of partially purified native EhMGL were dialyzed against buffer B (20 mM Tris-HCl, pH 8.0, 0.02 mM PLP, and 0.2 M KCl) and concentrated to 1 ml with the Centricon Plus-20. The concentrated samples were then applied to a column of Toyopearl HW-65S (70 × 1.6 cm, 140-ml bed volume, Tosoh) preequilibrated with buffer B. The recombinant and native MGLs were eluted with buffer B at a flow rate of 0.8 ml/min. The peaks were detected by measuring absorbance at A_{280} (recombinant MGLs) and immunoblotting (native MGLs). The same column was calibrated with blue dextran (2000 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa) (Amersham Biosciences).

Immunoblot Anaylsis—Polyclonal antisera against recombinant EhMGL1 and 2 were raised in rabbits by Sigma-Genosys (Hokkaido, Japan). Immunoblot analysis was carried out using a polyvinylidene difluoride (PVDF) membrane as described in (22). The blot membrane was visualized by using alkaline phosphatase conjugate-coupled secondary antibody with NBT/BCIP solution (Roche Applied Science) according to the manufacturer's protocol.

Two-dimensional Polyacrylamide Gel Electrophoresis—First-dimensional electrofocusing of two-dimensional PAGE was performed using Immobiline Drystrip, pH 3–10 NL, 7 cm and IPG Buffer pH 3–10 NL (Amersham Biosciences) according to the manufacturer's protocol. Second dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed on 12% SDS-PAGE gel using prestained SDS-PAGE standards, Broad Range (Bio-Rad Laboratories, Inc., Tokyo, Japan), as a molecular marker.

Enzyme Assays and Kinetic Calculations—The enzymatic activitiy of MGL was monitored by measuring the production of α-keto acid, ammonia, and hydrogen sulfide or methanethiol. The standard MGL reaction was performed in 200 μl of 100 mM sodium phosphate buffer, pH 6.8, a reaction mixture containing 0.02 mM PLP, and 0.1–10 mM of each substrate with appropriate amounts of each enzyme.

The α-keto acid assay was performed as described (23). The MGL reaction was terminated by adding 25 μl of 50% trichloroacetic acid. After the proteins were precipitated by centrifugation at 14,000 × *g* for 5 min at 4 °C, 100 μl of the supernatant was mixed with 200 μl of 0.5 M sodium acetate buffer, pH 5.0, and 80 μl of 0.1% of 3-methyl-2-benzothiazolinone hydrazon hydrochloride, and then incubated at 50 °C for 30 min. After the mixture had cooled to room temperature, absorbance at A_{320} was measured. Pyruvic acid and 2-α-butyric acid were used as standards.

For the detection of ammonia, the nitrogen assay (24) was used. A 50-μl sample of the supernatant, as for the α-keto acid assay, was mixed with 50 μl of Nessler's reagent (Nakalai) and 75 μl of 2 N sodium hydroxide, and then incubated at 25 °C for 15 min. Absorbance at A_{440} was measured. Ammonium sulfate was used as a standard.

The hydrogen sulfide assay was performed as described (25–27). Briefly, the MGL reaction was terminated by adding 20 μl of 20 mM *N,N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 N HCl and 20 μl of 30 mM $FeCl_3$ in 1.2 N HCl. After further incubation in the dark for 20 min, the proteins were precipitated by centrifugation at 14,000 × *g* for 5 min at 4 °C, and then the absorption at OD_{650} of the supernatant was measured to quantitate the formed methylene blue. Na_2S was used as a standard.

The methanethiol assay was performed as described (28) using 5,5′-dithio-bis-(2-nitrobenzoic acid). One-hundred microliters of the sample supernatant were mixed with 1 μl of 100 mM 5,5′-dithio-bis-(2-nitrobenzoic acid) in ethanol, and after 2 min incubation at room temperature, absorbance at A_{412} was measured. L-cysteine was used as a standard.

The cysteine and cystathionine assay was performed as described (29). The ninhydrin reagent was prepared by dissolving 1 g of ninhydrin in 100 ml of glacial acetic acid and adding 33 ml of glacial phosphoric acid. For the determination of cystathionine, 0.2 ml of cystathionine-containing solution was mixed with 0.33 ml of the ninhydrin reagent and boiled at 100 °C for 5 min. The solution was then cooled on ice for 2 min and at room temperature for a further 10 min. Absorbance at A_{455} was measured. Cysteine concentrations were determined using the same protocol except for the measurement of absorbance at A_{560}.

Kinetic parameters were estimated with Lineweaver-Burk plots using Sigma Plot 2000 software (SPSS Inc., Chicago, IL) with the Enzyme Kinetics module (version 6.0, Hulinks, Inc., Tokyo, Japan).

Assay of the Inhibition of rEhMGL by DL-Propargylglycine—An α-keto acid assay (described above) with L-methionine as the substrate was performed to evaluate the inhibitory effects of DL-propargylglycine (PPG) on the activity of rEhMGLs. rEhMGL (5 μg) was preincubated with various concentrations of PPG in the standard MGL reaction mixtures (described above) in the absence of L-methionine at 36 °C. The preincubation time was 5 min for kinetic analyses and 1 to 60 min to characterize the slow binding of this inhibitor. After preincubation, the reaction was initiated by adding an appropriate amount of L-methionine to the reaction mixture.

In Vitro Assessment of Amebicidal Reagents—To assess the amebicidal effect of TFMET, the trophozoites were cultured in the BI-S-33 medium containing various concentrations of TFMET or metronidazole, the therapeutic compound commonly used for amebiasis, as a control. After cultivation at 35.5 °C for 18 h, cell survival was assessed with the cell proliferation reagent WST-1 (Roche Applied Science). Briefly, the trophozoites were seeded on 96-well microtiter plates in 200 μl of BI-S-33 medium at a density of 2 × 10^4 cells per well (1 × 10^5 cells/ml), and the lid was completely sealed with a sterilized adhesive silicon sheet (Corning, New York). After these plates were further incubated at 35.5 °C for 18 h, 20 μl of WST-1 reagent was added to each well and the incubation was continued for 2 more hours. The optical density at A_{445} was measured with that at A_{595} as a reference using a microplate reader (Model 550, Bio-Rad, Tokyo, Japan). The initial density and incubation