Susceptibility of an Emetine-Resistant Mutant of *Entamoeba histolytica* to Multiple Drugs and to Channel Blockers

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Previously a cloned emetine-resistant mutant of the protozoal parasite *Entamoeba histolytica* was shown to overexpress a gene for an ameba homolog of the mammalian P-glycoprotein, a plasma membrane pump that removes hydrophobic drugs from multidrug-resistant tumor cells. Three sets of experiments were performed to better characterize the multidrug-resistant phenotype of the emetine-resistant ameba. First, the emetine resistance of the mutant ameba was reversed by concentrations of calcium and sodium channel blockers effective in reversing drug resistance by multidrug-resistant tumor cells, but it was reversed only in the presence of very high concentrations of the tricyclic antidepressants. Second, the mutant ameba showed cross-resistance to antiemic drugs used to treat luminal infection (iodoquinol and diloxanide) but were not cross-resistant to drugs used to treat invasive disease (chloroquine and metronidazole). Third, when amebae were loaded with radiolabeled emetine, the mutant parasites released the drug at ~1.6 times the rate of the wild-type organisms. We conclude that the emetine-resistant *E. histolytica* parasites have some but not all the features of the multidrug-resistant phenotype.

*Entamoeba histolytica* is a protozoal parasite that infects ~10% of the world's population, causing as many as 50 million cases of dysentery or liver abscesses and killing 100,000 persons per year (33, 38). Because poor countries cannot afford to make the changes in sanitary conditions that might eliminate the spread of amebic infection, amebiasis is controlled primarily by drug treatment of symptomatic persons (23, 38). Amebic infection restricted to the colonic lumen is treated with diloxanide fluorate and iodoquinol, whereas amebae that have invaded the colonic wall or the liver are treated with tissue-active agents such as metronidazole, emetine, and chloroquine (7, 23–25, 38). Although drug-resistant amebae are not as frequently described as are drug-resistant malaria parasites (4, 14, 15, 20, 22, 32, 35, 37), differences in drug susceptibilities among strains of amebae have been reported (6). There are also case reports of failed drug treatments (28, 34), which probably herald the development of drug resistance by ameba as a clinically important problem.

Tetracycline-resistant *Escherichia coli* cells actively expel the antibiotic via a 43-kDa inner membrane Tet protein, which has 12 membrane-spanning regions, is proton dependent, and is encoded by a plasmid (21). Proton-dependent pumps homologous to the Tet protein expel quinolone from *E. coli* (Bmr) and *Staphylococcus* spp. (NorA), methyleneo- mycin A from *Streptomyces* spp. (Mmr), and chloramphenicol from *Pseudomonas* spp. (CmlA [21]). In addition, bacteria expel heavy metals such as arsenic, antimony, cadmium, and zinc via a family of transport proteins, which use ATP hydrolysis to drive the pumps (21).

In contrast to bacterial systems, relatively little is known about drug efflux from eukaryotic cells. The best-studied eukaryotic system is the multidrug-resistant (MDR) tumor cell, which develops in vitro resistance to multiple hydrophobic anticancer drugs other than those used in the selection of the mutants (2, 9, 12, 17). MDR tumor cells have increased expression of a 170-kDa plasma membrane protein referred to as the P-glycoprotein or mdr gene product (8, 9, 12, 17, 31). The P-glycoprotein forms a channel through which some hydrophobic anticancer drugs are actively pumped out of MDR tumor cells in vitro (2, 12, 13, 30, 36). Therefore, the MDR tumor cells show a decreased accumulation of these drugs over time when compared with the drug-susceptible tumor cells; this is associated with an increased rate of efflux of the drugs by the tumor cell mutants (12, 13, 36). Calcium channel blockers, sodium channel blockers, and some tricyclic antidepressants inhibit P-glycoprotein function, so that in the presence of these drugs the MDR tumor cells become more susceptible to the hydrophobic chemotherapeutic drugs (12, 30, 36). Although mechanisms by which the channel blockers work is not known, some channel blockers have been shown to bind directly to the P-glycoproteins (12).

Overexpression of the P-glycoprotein pump may be associated with some types of drug resistance in eukaryotic protozoal parasites, including *Plasmodium*, *Giardia*, and *Leishmania* spp. (4, 5, 14, 15, 19, 22, 27, 35, 37). Chloroquine-resistant *P. falciparum* organisms actively expel radiolabeled drug; this efflux is inhibited by verapamil and by tricyclic antidepressants (4, 20, 22). Whether P-glycoprotein is involved in drug resistance is unclear, however, because the *P. falciparum mdr* gene was not associated with the chloroquine resistance phenotype in a cross of wild-type and chloroquine-resistant parasites (35). Decreased drug uptake has been found in metronidazole-resistant *Giardia* organisms, although the relationship to P-glycoprotein expression is not proven (5). An increased copy number of the P-glycoprotein gene in *Leishmania* spp. has been associated with vincristine resistance in one case (19) and has been found to be unrelated to methotrexate resistance in another case (27).

In summary, the importance of P-glycoprotein to parasite drug resistance remains controversial.

At present, the only model available to study mechanisms

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of drug resistance in *E. histolytica* is an emetine-resistant mutant clone C2 of the virulent HM-1:IISS strain, which was selected after mutagenesis with the alkylating agent ethyl methanesulfonate (26). Emetine, the oldest drug used to treat amebic infection, is a plant alkaloid that inhibits eukaryotic protein synthesis by blocking translocation of the peptidyl-tRNA from acceptor to donor site on the ribosome (1, 18, 25). The emetine-resistant *E. histolytica* mutant overexpresses mRNAs of one of four ameba P-glycoprotein genes that have been cloned and sequenced, although the copy number of this P-glycoprotein gene is not increased (2a, 10, 29). In addition, the emetine-resistant mutant clone C2 shows some characteristics of the MDR phenotype (3). Emetine resistance by the mutant amebae is reversed by the calcium channel blocker verapamil (3). Clone C2 is slightly cross-resistant to colchicine but is not cross-resistant to the anticancer drugs daunomycin and actinomycin D (3, 26).

Whether clone C2 is cross-resistant to other drugs used to treat amebic infection (iodoquinol, diloxanide, metronidazole, and chloroquine) is not known. The accumulation of radio labeled emetine by the mutant amebae is less than half that accumulated by the wild-type parent clone A parasites, although the rates of drug entry into and efflux from the parasites have not been measured (3).

The experiments here address three questions in an attempt to better characterize the MDR phenotype of the emetine-resistant mutant clone C2 amebae. First, can the emetine resistance of the clone C2 amebae be reversed by calcium blockers other than verapamil or by tricyclic antidepressants, which reverse the drug resistance of MDR tumor cells and malaria parasites (4, 12, 22, 30, 36)? Second, is the emetine-resistant mutant clone C2 cross-resistant to other drugs (iodoquinol, diloxanide, chloroquine, and metronidazole) frequently used to treat human amebiasis (7, 24, 25, 38)? Third, is the decreased accumulation of radio labeled emetine by the drug-resistant mutant clone C2 amebae with respect to that of the drug-susceptible wild-type clone A organisms associated with an increased efflux of the drug from the mutant parasites, as is the case for MDR tumor cells and chloroquine-resistant malaria parasites (3, 12, 13, 20, 36)?

**MATERIALS AND METHODS**

All drugs were from Sigma Co., St. Louis, Mo., except for diloxanide, which was generously donated by Boots Pharmaceuticals, Nottingham, England. Axenic *E. histolytica* trophozoites of strain HM-1:IISS were cultured in Diamond’s medium; the wild-type clone A was cultured without drug, and the mutant clone C2 was cultured with 25 µg of emetine per ml (11, 26). For drug testing, trophozoites in log phase growth were washed twice and incubated at 1,000 to 2,000 organisms per 200 µl of medium in duplicate wells in 96-well microtiter plates, which were kept at 37°C in a chamber containing a 2% oxygen atmosphere (7). To determine whether the microtiter plates would give similar results to those obtained when using sealed tubes (3), we incubated the mutant clone C2 and the wild-type clone A amebae in parallel wells with twofold dilutions of emetine beginning at 24 or 48 µg/ml. Control wells contained amebae incubated without emetine. After 3 days the plates were chilled to release parasites from the floor of the plates, and the parasites were fixed in 3% formalin and counted with a hemocytometer. To compare the growth of clone C2 amebae with that of clone A organisms in each experiment and in different repeats of the same experiment, the number of parasites at each drug concentration was expressed as a percentage of the number of parasites counted in control wells without drug. Standardized growth curves of clone C2 and clone A amebae were then plotted on the y axis versus the log of the concentration of emetine on the x axis. The emetine IC₅₀ (concentration of emetine that caused a 50% inhibition of growth) was determined for both the drug-resistant mutant clone C2 amebae and the drug-susceptible clone A amebae. In parallel wells, the emetine IC₅₀ for clone C2 amebae grown in the presence of 10 µg of verapamil per ml was determined (3).

Before attempting to reverse the emetine resistance of the mutant clone C2 amebae with the channel blockers or tricyclic antidepressants, we tested the susceptibility of the mutant amebae to each of these drugs in the absence of emetine. Clone C2 organisms were incubated with twofold dilutions of diltiazem, nifedipine, quinacrine, imipramine, or desipramine to determine the IC₅₀ of each drug. Concentrations well below the IC₅₀ of each drug were then added to clone C2 amebae in addition to emetine. For example, the IC₅₀ of diltiazem alone for clone C2 was found to be 100 µg/ml. In parallel rows, clone C2 parasites were exposed to twofold dilutions of emetine beginning at 24 or 48 µg/ml in the presence of 0, 3, 10, or 30 µg of diltiazem per ml. The concentration of diltiazem (and of the other drugs) that decreased the emetine IC₅₀ for the mutant clone C2 by a factor of 2 was then identified.

The relative susceptibilities of the emetine-resistant mutant clone C2 and the wild-type clone A for the drugs used to treat *E. histolytica* clinical infections were determined by incubating parasites in duplicate with twofold dilutions of iodoquinol, diloxanide, chloroquine, or metronidazole in microtiter wells (7, 25, 28, 38). After 3 days of growth, the parasites were fixed and counted. We tested for statistically significant differences between the standardized growth curves of clone C2 and clone A amebae by using a least-squares regression analysis to compare the linear portions of the curves.

To measure the efflux of radio labeled emetine, we incubated clone C2 parasites without emetine for 3 h. Then emetine-resistant clone C2 and wild-type clone A amebae, 10⁶ parasites per ml of Diamond’s medium, were incubated for 3 h at 37°C with 5 µg of emetine per ml including 10 µCi of [³¹⁵]H)emetine (Amersham, Chicago, Ill.). To test the effect of the calcium channel blocker verapamil on emetine efflux, we also incubated clone C2 amebae in parallel tubes containing radio labeled emetine and 10 µg of verapamil per ml. To obtain the zero time point before efflux, three 100-µl aliquots (three experiments on three different days) or six 100-µl aliquots (two experiments on two different days) of clone C2 and of clone A amebae were removed and placed in microtiter wells. The parasites were then applied to a glass filter and washed rigorously with a Titer Tech Cell Harvester (Skatron, Sterling, Va.). The rest of the organisms were pelleted in a microcentrifuge for 15 s, and the supernatant was removed and replaced with drug-free medium. After incubation in drug-free medium for 3, 6, and 12 min at 37°C, aliquots of clone C2 and A amebae were applied to filters, cut out, and counted with a beta-counter after addition of 2 ml of BetaMax (ICN, Cleveland, Ohio). Drug efflux results were log transformed to improve the normality of the efflux data distribution. Least-squares regression analysis was used to estimate the emetine efflux rates for the mutant clone C2 and wild-type clone A amebae and to determine whether differences in the efflux rates between the two types of amebae were statistically significant.
All experiments reported in this paper were performed at least three times.

RESULTS AND DISCUSSION

Drug testing in microtiter wells gives the same results as testing in 4-ml culture tubes. When parasites were grown in 4-ml tubes, the mutant clone C2 was more resistant to emetine than the wild-type clone A was; the calcium channel blocker verapamil reversed this resistance (3, 26). When amebae were grown in 200-μl microtiter wells under reduced oxygen pressure (7), the emetine IC₅₀ for clone C2 was >10 μg/ml, while the emetine IC₅₀ for clone A was ~2 μg/ml (Fig. 1). With the addition of 10 μg of verapamil per ml, the emetine IC₅₀ for clone C2 decreased by >10-fold to <1 μg/ml (Fig. 1). These results are consistent with those obtained by using the 4-ml tubes (3) and support the use of the much more labor- and materials-efficient microtiter well system (7).

Numerous ion channel blockers reverse the emetine resistance of the ameba mutant clone C2. Addition of the calcium channel blockers nifedipine and diltiazem or the sodium channel blocker quinidine decreased the emetine IC₅₀ for clone C2 (Fig. 1). The addition of nifedipine, diltiazem, or quinidine at 10 μg/ml resulted in a greater than 50% decrease in the emetine IC₅₀ for clone C2 (Fig. 1). These decreases in the emetine IC₅₀ for the mutant are unlikely to have been caused by a general toxicity of the calcium or sodium channel blockers, because the IC₅₀s of nifedipine, diltiazem, and quinidine alone were 90, 100, and 80 μg/ml, respectively (data not shown). The concentrations of the channel blockers that reversed the emetine-resistant phenotype of the amebae were similar to those that reverse the drug resistance of MDR tumor cells (12, 30, 36) and to those that reverse the chloroquine resistance of P. falciparum parasites (22). These results suggest but do not prove that a P-glycoprotein similar to that of the tumor cells is functioning in the drug-resistant ameba, although no particular mechanism for the inhibition by the channel blockers can be inferred (12).

Addition of 1 μg of the tricyclic antidepressant desipramine or imipramine per ml decreased the emetine IC₅₀ for clone C2 by at least 50% (Fig. 1). The IC₅₀s of desipramine and imipramine alone were each ~5 μg/ml (data not shown). The concentrations of the tricyclic antidepressants required to reverse emetine resistance in E. histolytica were much higher than those required to reverse chloroquine resistance in malaria parasites (<40 ng/ml of each tricyclic antidepressant [4]).

Emetine-resistant clone C2 parasites are more resistant than wild-type clone A amebae to iodoquinol and diloxanide but not to chloroquine and metronidazole. The susceptibilities of emetine-resistant mutant clone C2 and wild-type clone A amebae to drugs commonly used to treat amebiasis were compared (7, 24, 25, 38). Clone C2 amebae showed greater resistance than clone A parasites to iodoquinol (Fig. 2). The difference between the iodoquinol IC₅₀ for the mutant clone C2 (1.56 μg/ml; average of three experiments) and that of clone A (0.54 μg/ml) was statistically significant (P < 0.001). Similarly, the IC₅₀ of diloxanide (the active form of diloxanide furoate) for the clone C2 mutants (16.6 μg/ml) was increased (P < 0.001) compared with that for the wild-type clone A organisms (9.1 μg/ml) (Fig. 2). At the highest concentrations of both iodoquinol and diloxanide, amebae were alive but not growing (Fig. 2). In contrast, the highest concentrations of emetine, chloroquine, and metronidazole killed the amebae (Fig. 1 and 2).

The chloroquine IC₅₀s for the emetine-resistant mutant clone C2 amebae was slightly elevated compared with that for the wild-type clone A amebae, but the difference was not statistically significant (Fig. 2). The chloroquine IC₅₀s for the clone C2 mutants (166 μg/ml) and the wild-type clone A parasites (120 μg/ml) were both much greater than the chloroquine IC₅₀ for chloroquine-susceptible (~5 μg/ml) and chloroquine-resistant (~15 μg/ml) malaria parasites (20, 22). This result suggests that the amebae are relatively resistant to chloroquine, at least under the in vitro conditions used here.

The emetine-resistant clone C2 amebae were not more resistant than the susceptible wild-type clone A amebae to metronidazole, the drug used most frequently to treat amebiasis (Fig. 2) (6, 7, 23–25, 38). This may be because the
parasite P-glycoprotein is not involved in metronidazole resistance. Metronidazole-resistant *Trichomonas* spp. and anaerobic bacteria reduce and activate the drug less than do wild-type organisms (16, 24). On the other hand, metronidazole-resistant *Giardia* spp. selected in the laboratory take up less drug than susceptible parasites do, which is consistent with the action of a P-glycoprotein pump (5). The particular mdr (P-glycoprotein) gene amplified in the mutant clone C2 amebae, one of multiple mdr genes present in *E. histolytica* (10, 29), may not pump out metronidazole. Alternatively, metronidazole resistance may be associated with a particular mutation in the overexpressed ameba mdr gene, because single amino acid substitutions in the overexpressed P-glycoproteins of MDR tumor cells change both the efficacy and specificity of drug resistance (9, 17).

**Radiolabeled emetine is lost from drug-resistant clone C2 parasites faster than from susceptible clone A amebae.** The rate of emetine release by the drug-resistant clone C2 amebae was 1.6-fold higher than the rate of release by the wild-type clone A parasites, and this difference was statistically significant (P < 0.0001). Radiolabeled emetine associated with the drug-resistant mutant clone C2 parasites decreased by an average of 48% per min, whereas radiolabeled emetine associated with the drug-susceptible wild-type clone A decreased by an average of 30% per min, when the amebae were placed in drug-free medium. The rate of efflux by the mutant amebae is higher than that of the tumor cells (12) and is similar to that of chloroquine-resistant malaria parasites (20). The addition of 10 μg of verapamil per ml to clone C2 parasites slowed the emetine efflux so that there was no statistically significant difference from the efflux rate of the wild-type clone A. This result is consistent with results of the culture experiments that showed that the addition of verapamil to the mutant clone C2 parasites made them as susceptible to emetine as the wild-type clone A parasites were (Fig. 1). The results are also consistent with those from the malaria and tumor cell systems, in which rates of drug efflux from the resistant are greater than those from the susceptible organisms and drug efflux is inhibited by calcium channel blockers (12, 20, 22, 30, 36).

**Conclusions and significance.** The present experiments generally support the idea that the emetine-resistant clone C2 amebae actively expel hydrophobic drugs via a P-glycoprotein pump, as has been described for MDR tumor cells (12). First, calcium and sodium channel blockers that inhibit the function of mammalian P-glycoproteins (12, 30, 36) make clone C2 amebae more susceptible to emetine (3). Second, the emetine-resistant clone C2 amebae were cross-resistant to the hydrophobic antiamebic drugs iodoquinol and diloxanide, although they were not cross-resistant to the more polar drugs chloroquine and metronidazole. Third, resistant clone C2 organisms more quickly released radiolabeled emetine than did susceptible wild-type clone A parasites. Therefore, in this particular ameba mutant, the P-glycoprotein overexpression appears to be important for drug resistance. This is similar to the situation in vinblastine-resistant *Leishmania* spp. (19), but it may be different from that in chloroquine-resistant malaria parasites (35). It is remarkable that a single-celled ameba, which has a genome 1/20 the size of that of mammals and lacks mitochondria and enzymes that perform oxidative phosphorylation, contains a plasma membrane pump that functions in a way similar to that of the P-glycoprotein found in mammalian cells (12).

The significance of these results for understanding amebic drug resistance in the field and for treatment of amebiasis remains to be discovered. The cloned ameba P-glycoprotein genes may be used to make DNA or antibody probes for identifying P-glycoprotein overexpression in clinical isolates of amebae. New antiamebic drugs may be screened against the emetine-resistant mutant before they are tested against
amœbae that infect animals or humans. Different amœba mutants overexpressing one of the other P-glycoprotein genes present in the parasite (10) may be examined to determine whether their drug resistance profiles differ from that of the emetine-resistant mutant. Finally, it is possible that resistance of amœbae to some drugs occurs by efflux mechanisms similar to those previously described for bacteria (21).

ACKNOWLEDGMENTS

We heartily thank Aryeh Stein for his comments and advice concerning statistical analyses used here.

This work was supported by NIH grant AI 28395 and by the John D. and Catherine T. MacArthur Foundation. J.S. is an American Gastroenterology Association/Rorer Consumer Pharmaceuticals Research Scholar. A.B. was a summer undergraduate intern in the Division of Biological Sciences at the Harvard School of Public Health.

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