Role of P Glycoprotein in the Course and Treatment of *Encephalitozoon* Microsporidiosis

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Encephalitozoon microsporidia are obligate intracellular protozoan parasites that proliferate and differentiate within a parasitophorous vacuole inside host cells that are usually epithelial in nature. Isolates of the three species of the *Encephalitozoon* microsporidia, *E. cuniculi*, *E. hellem*, and *E. intestinalis*, were obtained from AIDS patients and cultured in green monkey (E6) kidney cells. Anti-P-glycoprotein (anti-Pgp) and anti-multidrug resistance-associated protein (anti-MRP) monoclonal antibodies were used to probe for multidrug resistance (MDR) pump epitopes and verapamil- or cyclosporin A- and probenecid-modulated intracellular calcein fluorescence were used to assess the expression of Pgp and MRP respectively in uninfected and infected cells. Pgp, but not MRP, was detected immunocytochemically and by verapamil- and cyclosporin A-potentiated intracellular fluorescence in both host cells and parasite developing stages. When an *in vitro* infection assay was employed, verapamil and cyclosporin A acted as chemosensitizing agents for the antiparasitic drug albendazole. These observations suggest that inhibiting host cell and perhaps parasite MDR pumps may increase the efficacy of antiparasitic agents in these and other microsporidia species.

Microsporidia are obligate intracellular protozoan parasites that constitute the phylum Microspora. Over 1,200 species have been identified, and to date at least 13 of these have been shown to infect humans (9, 21). The great majority of the reported clinical cases are in immunodeficient or immunosuppressed individuals. One agent, albendazole, has been found effective in the treatment of some forms of intestinal microsporidiosis and most cases of disseminated microsporidiosis (7, 21, 27), while the more toxic fumagillin has been used topically to treat ocular microsporidiosis (10, 21). To date there are no reports of the development of parasite resistance to either agent, but this may not be surprising given the difficulties with diagnosis and identification of parasite species, differing susceptibility of the various microsporidia species to the agents in question, and the relatively modest number of microsporidiosis cases reported.

The present study addresses the potential role of host cell and parasite multidrug resistance (MDR) pumps in microsporidiosis caused by three species of *Encephalitozoon* microsporidia, *E. hellem*, *E. intestinalis*, and *E. cuniculi*. These parasites proliferate and differentiate within a parasitophorous vacuole. This location within a parasitophorous vacuole means that the parasite is separated from the host's extracellular fluid by several membranes. These include the parasite plasma membrane and, in the case of the mature spore, the environmentally resistant spore coat, the parasitophorous vacuole membrane, and the host cell plasma membrane (9). An MDR pump in one or more of these membranes would significantly affect the concentration of any antiparasitic agent within the parasite.

In many protozoan parasites genes have been identified that

may be responsible for the expression of membrane pumps that produce MDR by lowering intracellular drug concentrations (32, 33). Such pumps are members of the superfamily of ATP binding cassette (ABC) pumps that are responsible for diverse cellular transport functions (1, 4, 32). In the present study we have attempted to determine what roles MDR pumps may have on the course and treatment of microsporidiosis. The study has (i) determined if infection with the microsporidia affects host cell clearance of calcein and calcein AM, transported by MDR-associated protein (MRP) and P-glycoprotein (Pgp) respectively; (ii) immunocytochemically probed for MRP and Pgp antigens in host cells and parasite stages; (iii) determined if chemosensitizers increase the efficacy of albendazole in the treatment of *in vitro* microsporidiosis; and (iv) determined if parasite stages express chemosensitizer-inhibitable ABC pumps.

MATERIALS AND METHODS

Parasite culture. *E. hellem*, *E. intestinalis*, and *E. cuniculi* isolates, originally obtained from AIDS patients, were cultured in green monkey kidney cells (E6) as described previously (35). All cultures were maintained in a CO₂ incubator at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, gentamicin (50 µg/ml), and amphotericin B (5 µg/ml).

Calcein loading and detection. Infected and uninfected cells were plated on 35-mm-diameter dishes with no. 1 coverslip bases 2 days prior to use. Dye loading was performed with cells maintained in growth medium. In experiments in which verapamil was used, this agent (final concentration, 10 µM) was added to the medium 45 min prior to the addition of calcein AM (final concentration, 2 µM) in dimethyl sulfoxide (DMSO) (final concentration, 0.01%). Fifteen minutes later the medium was removed and replaced with a 20 mM HEPES-buffered solution, pH 7.4, containing 135 mM NaCl, 5 mM KCl, 5 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM CaCl₂, 1.2 mM MgSO₄, and 1 mg each of glucose, bovine serum albumin (BSA), and ascorbic acid per ml. Ascorbic acid was used as an antiphotooxidation agent. The cells were imaged with an inverted confocal laser scanning microscope as described previously (23). Relative fluorescence was measured in cells by focusing on areas of cytoplasm that were away from the nucleus and free of vacuoles or areas of sequestered calcein.

The calcein extrusion by E6 cells was variable, presumably reflecting different

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degrees of plasma membrane Pgp expression. As a result, the calcein fluorescence of E6 cell cultures varied between cultures and between passages, depending on which clonal expansions of cells dominated the culture. To reduce the variance in relative fluorescence between treatment groups in an experiment, all cells used in a given experiment came from the same culture and passage number. Similarly, while the laser and gain settings were optimized for each experiment, they were kept constant throughout a given experiment.

In one experiment designed to determine the effect of Pgp and MRP inhibitors on calcein fluorescence, uninfected cells were plated as above and exposed to 10 μ M verapamil, 10 μ M cyclosporin A, or 100 μ M probenecid. The carrier for cyclosporin A was ethanol (final concentration, 0.1%). After 45 min in medium containing the transporter inhibitor, calcein AM was added to the medium for an additional 15 min as described above. Ethanol and DMSO carrier controls were carried out as appropriate. This carrier did not affect cell fluorescence at the concentrations used.

In order to determine if there was a calcein AM or calcein extrusion pump in the parasite, heavily infected cells were broken up by passing a cell suspension through a 26-gauge needle three times. The most abundant parasite stage, the mature spore, did not load with calcein, presumably due to its complex spore coat. Meronts and other single parasite stages were difficult to distinguish from vesiculated cell debris. However, chains of sporogonial stages were readily distinguished without the need for purification. Disrupted cells were therefore exposed to medium or medium containing one of the transporter inhibitors for 45 min and to calcein AM for an additional 15 min as above. The medium was then removed by centrifugation in a microcentrifuge and replaced with the HEPES-buffered solution as above. The cell suspension was then placed on the heated microscope stage, and the sporogonial stages were allowed to settle. Due to concerns that compounds such as polylysine might affect the membrane integrity of these small parasite stages (<2 μ m in width) the sporogonial chains were allowed to float freely. While there was some Brownian movement of these small parasite stages, because the chains averaged four cells at least one parasite cell was in focus in both the fluorescent and transmitted-light images at each observation.

Infection assay. A mixture of uninfected and *E. hellem*-infected cells was plated to confluence in wells of eight-well coverslip slides. The medium in the wells was then replaced daily for the next 3 days with medium containing albendazole alone in DMSO (final concentration, 0.01%), verapamil alone, cyclosporin A alone, albendazole and verapamil, or albendazole and cyclosporin A, each at a final concentration of 0.1 μ M. Control wells were given medium alone. All solutions contained the same final concentrations of the DMSO and ethanol carriers. After 3 days of exposure to these agents the medium was withdrawn, the monolayers were fixed with 10% neutral formalin and stained with Giemsa, and the number of infected and uninfected cells was counted to yield the percentage of infected cells in each well as described previously (14). The low concentration of agents was used because while 3 days of exposure to 1 μ M albendazole reduced infection by 30 to 60% in these cultures, the variance of this effect tended to obscure the potentiation effect of the verapamil and cyclosporin A. The low dose of verapamil was chosen because calcium channel blockers are known to inhibit spore germination (22), and we wished to avoid such a direct effect of verapamil on the spread of infection.

Immunocytochemistry. Infected and uninfected cells were plated onto eight-well chamber slides and 24 h later the medium was removed and the samples were fixed with acetone at -20°C for 10 min, followed by 10 min in 2% BSA in Tris-buffered saline (TBS) as a blocking agent. One of two anti-Pgp monoclonal antibodies or one anti-MRP monoclonal antibody was then used as a primary antibody. In one group of experiments mouse anti-Pgp clone F4 (Sigma Chemical Co., St. Louis, Mo.) was used diluted 1:40. The second anti-Pgp monoclonal antibody was clone G/1C obtained from Chemicon International Inc. (Temecula, Calif.), used at a concentration of 1:40. The anti-MRP monoclonal antibody was obtained from Alexis, Corp. (San Diego, Calif.), clone MRPM6, and was used at a dilution of 1:20. All the primary antibodies were diluted in 1% BSA in TBS and were incubated at 37°C for 1 h. After three TBS washes, the antibody binding sites were visualized by incubating samples in biotinylated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch Labs Inc., West Grove, Pa.), 1:300 in 1% BSA in TBS for 1 h at 37°C , followed by Streptavidin-Oregon Green 488 (Molecular Probes Inc., Eugene, Oreg.), 1:300 in TBS for 45 min at room temperature.

Statistical analyses. In experiments in which the means of several values were being compared, the data were first analyzed by a one-way analysis of variance followed by post hoc Tukey's protected *t* tests to determine the significance of differences between individual mean values. In experiments in which the levels of calcein fluorescence of sporogonial stages were compared when the parasites were treated with carriers and with verapamil or cyclosporin A, Wilcoxon two-

FIG. 1. Relative fluorescence of intracellular calcein in E6 cells exposed to 10 μ M verapamil (Verap), 10 μ M cyclosporin A (Cyclo A), or 100 μ M probenecid (Proben) for 1 h. *, significantly different from control ($P < 0.05$); error bar, standard error of the mean.

group rank tests were used to determine the significance of differences between means of replicate experiments.

RESULTS

Green monkey kidney cells were incubated with calcein AM, and their relative fluorescence was measured by confocal microscopy after removal of the probe from the medium. This fluorescence provided a measure of the intracellular concentration of the fluorescent calcein free acid which resulted from the removal of the acetoxymethyl groups from the calcein AM by cellular esterases. Calcein AM is extruded from cells by Pgp (1, 11), while MRP extrudes the free-acid form of this probe (11, 15). Verapamil and cyclosporin A were chosen as inhibitors of Pgp (5, 13), and probenecid was chosen as an inhibitor of MRP (13). Figure 1 illustrates the relative fluorescence of uninfected E6 cells and the effects of 10 μ M verapamil and cyclosporin A and 100 μ M probenecid on this fluorescence. The control values in this figure were measured in cells in carrier-free medium, as no carrier effect was observed on relative fluorescence. The two Pgp inhibitors significantly increased cell fluorescence, consistent with the inhibition of cell membrane Pgp extrusion of entering calcein AM, while probenecid had no significant effect on the cell fluorescence, indicating that these cells lacked MRP expression. Higher concentrations of probenecid (up to 1 mM) were used without significant effect on cell fluorescence.

Experiments were performed in which the calcein fluorescence was measured in uninfected and *Encephalitozoon*-infected E6 cells and in cells plated at the same time and treated in the same manner but with the additional exposure to 10 μ M verapamil for 60 min. Figure 2 illustrates the size of populations of uninfected and *E. intestinalis*-infected cells grouped by their relative fluorescence. These data are representative of multiple experiments performed using each of the three species of *Encephalitozoon* microsporidia. Typically $\geq 20\%$ more of the infected cells were found to exhibit a lower relative fluorescence than the uninfected cells (e.g., see Fig. 2a). There

FIG. 2. Relative fluorescence of intracellular calcein in uninfected and *E. intestinalis*-infected E6 cells. (a) Cells are grouped by relative fluorescence in increments of 10 units, and the percentage of the uninfected and infected cells falling within each fluorescence range is shown. (b) A similar experiment was performed with cells from the same culture that were treated with 10 μ M verapamil prior to exposure to calcein AM.

are at least three possible explanations for the observation that there was a larger subpopulation of infected cells with a lower relative fluorescence. The first is that the infected cells were losing their esterase activity or their membrane integrity and were therefore unable to generate or retain the intracellular fluorescent probe. The second explanation is that calcein-calcein AM extrusion pump activity is upregulated in infected cells. The third is that cells with high expression of Pgp were better able to accommodate the intracellular parasite and were thus more frequently selected for successful infection. The first explanation, that infected cells were deteriorating, was eliminated by the observation that the Pgp inhibitor, verapamil, increased the fluorescence of all cell populations and in many cases, such as that illustrated in Fig. 2b, it eliminated the difference between the number of uninfected and infected cells exhibiting the lower fluorescence.

Two anti-Pgp monoclonal antibodies and one anti-MPR monoclonal antibody were used to probe for epitopes on host cells and intracellular parasite stages. Both anti-Pgp antibodies recognized epitopes on the host cell plasma membrane and intracellular organelles (Fig. 3A and B), presumably the Golgi apparatus (26). They also recognized epitopes within the parasite. The numerous refractile spores that are visible in the transmitted light images (Fig. 3C and D) were not labeled. Antibody labeling of spores is difficult due to the chitinous and proteinaceous spore coat which appears to impair antibody penetration. However, both the merogonial parasite stages adjacent to the parasitophorous vacuole membrane and chains of sporogonial stages within the vacuole were labeled with the anti-Pgp antibodies, while the anti-MRP antibody failed to label epitopes in either the host cell or parasite stages (data not shown).

As the anti-Pgp antibodies recognized epitopes on both host cells and intracellular parasites while the anti-MRP antibody did not, and as both Pgp inhibitors, verapamil and cyclosporin A, increased host cell fluorescence while the MRP inhibitor, probenecid, did not, an infection assay was used to determine if the Pgp inhibitors could act as chemosensitizing agents in the treatment of an *in vitro* infection. The *E. hellem* strain used was selected because it yielded the most consistent and uniform infection. Table 1 summarizes the effects of using the antiparasitic agent, albendazole, in conjunction with either verapamil or cyclosporin A, at concentrations that alone had no effect on the number of cells infected with *E. hellem*. When either of the Pgp inhibitors were used in conjunction with albendazole there was a significant inhibition of infection, indicating that the verapamil and cyclosporin A were acting as chemosensitizers of albendazole (12).

Both anti-Pgp monoclonal antibodies recognized epitopes on the developing stages of *E. hellem*. To determine if such developing stages phenotypically expressed Pgp extrusion of calcein AM, the relative fluorescence of sporogonial stages was measured in cells exposed to calcein AM in the presence and absence of 10 μ M verapamil or cyclosporin A. The percentage difference in cell fluorescence when cells were exposed to the Pgp inhibitors when compared to the corresponding carrier control was determined. Exposure to verapamil or cyclosporin A significantly increased fluorescence ($45.5\% \pm 13.1\%$ and $31.8\% \pm 11.7\%$, respectively [mean $\pm$ standard error of the mean]) in these developing parasite stages as judged by the Wilcoxon signed-rank test ($P = 0.028$ and $P = 0.046$, respectively), suggesting that the parasite was able to extrude calcein AM by a Pgp-like pump. No statistically significant effect was found when 100 μ M probenecid was used in such experiments (data not given).

DISCUSSION

Many protozoan parasites have been found to possess MDR-like genes responsible for the expression of membrane proteins with homology to mammalian Pgp or MRP (6, 8, 17, 18, 28, 32, 33). The typical mammalian MDR membrane pump is made up of two homologous peptide halves, each with an intracellular ATP binding domain and six membrane-spanning domains (1). While Pgp and MRP pumps share similar ATP binding sites, their membrane spanning domains are signifi-

FIG. 3. Immunofluorescence of *E. hellem*-infected cells. (A and B) Confocal images of cells in which host cells and parasites were probed with a Sigma (A) and a Chemicon (B) anti-Pgp monoclonal antibody. (C and D) Transmitted-light images of the cells illustrated in panels A and B, respectively. Arrows indicate chains of parasite sporogonial stages, arrowheads indicate individual meronts, and N indicates a host cell nucleus. Bar = 2 μ m.

cantly different from one another. Protozoan parasite MDR-like membrane proteins are more variable in their structure than their mammalian counterparts. While some have the predicted Pgp structure (18), others have only one rather than two peptides (17), while others have two peptides but fewer than the total of 12 membrane spanning domains (32). Similarly, while mammalian Pgp and MRP pumps have been shown to play significant transport roles and are important in the development of MDR (1), it has not been possible to definitively

ascribe such roles to similar proteins in many of the protozoan parasites (6, 32, 33). There is some evidence to support the microsporidia resembling fungi more than protozoa (19). ABC proteins have been extensively studied in yeasts, where they have been found to be involved in pheromone secretion in sexual reproduction, mitochondrial function, and stress response to cellular detoxification, as well as drug resistance (3). In *Saccharomyces cerevisiae* a pleiotropic drug resistance network of genes has been identified that is comprised of tran-

TABLE 1. Effect of 3 days of drug treatment on the percentage of E6 cells infected with *E. hellem*

Treatment ^a	Mean % cells infected $\pm$ SEM	Significance of effect ^b
Carrier	41.7 $\pm$ 1.1	
Verapamil	42.9 $\pm$ 0.8	NS
Cyclosporin A	42.8 $\pm$ 1.0	NS
Albendazole	39.8 $\pm$ 1.0	NS
Albendazole + verapamil	25.1 $\pm$ 1.6	Different from carrier ($P < 0.05$) Different from albendazole ($P < 0.05$)
Albendazole + cyclosporin A	30.4 $\pm$ 1.6	Different from carrier ($P < 0.05$) Different from albendazole ($P < 0.05$)

^a All agents were used at a concentration of 0.1 μ M.

^b Significance tested by one-way analysis of variance and Tukey's protected *t* test. NS, not significant.

scriptional regulators that are responsible for the expression of multiple ABC transporters involved in the development of drug resistance (20).

The present study points to *Encephalitozoon* microsporidia possessing epitopes that are identified by monoclonal antibodies against mammalian Pgp but not by a monoclonal antibody against mammalian MRP. This study also suggests that verapamil and cyclosporin A, two agents that inhibit Pgp pump activity, inhibit *Encephalitozoon* Pgp-like extrusion of calcein AM. It is tempting to suggest that a Pgp-like pump in this microsporidian genus may extrude chemotherapeutic agents and, if overexpressed, may contribute to the development of drug-resistant strains. However similar early observations were made with *Plasmodium falciparum* when verapamil was shown to reverse chloroquine resistance (25) and the *pfmdrl* gene was observed to be amplified in some chloroquine-resistant strains of the parasite (36). However, it is more than a decade since these early observations, and the relationship between the *pfmdrl* gene product, Pgh1, and drug resistance has not yet been fully elucidated, although it is known that mutations in *pfmdrl* can reduce parasite chloroquine accumulation and impart resistance (4). The expression of such resistance may involve mutations in additional genes, however (29). Similarly in *Entamoeba histolytica*, multiple Pgps may be involved in the expression of emetine resistance (8).

We observed that verapamil and cyclosporin A act as chemosensitizing agents with albendazole to reduce *Encephalitozoon* infection in E6 cells in culture. These agents have differing mechanisms of action when inhibiting Pgp pumps (16, 31). This suggests that the albendazole was extruded from either the parasite, parasitophorous vacuole, or host cell by a Pgp pump and that the inhibitor or chemosensitizer increased the intracellular concentration of this agent. Based on the calcein fluorescence studies summarized in Fig. 1 and the immunocytochemical demonstration of Pgp epitopes in the host cell, it seems likely that the major site of action of these chemosensitizers is the host cell plasma membrane. The resolution in the immunocytochemical study did not distinguish between anti-Pgp epitopes on the parasitophorous vacuole membrane and those on adjacent host cell organelles and parasite developing stages. As the *Encephalitozoon* parasitophorous vacuole membrane appears to be highly permeable (23), it seems unlikely that this will be a site of effective drug extrusion, although it is

possible that a parasitophorous vacuole rather than the parasite itself is the site of a drug efflux pump. There appears to be a MRP-like pump at the interface between the *Cryptosporidium parvum* parasitophorous vacuole and the host cell cytoplasm in the area of the feeder organelle (28). The parasite plasma membrane would be expected to be less accessible to chemosensitizer, particularly verapamil, at an effective concentration. Epithelial cells are a target for microsporidian infection, and they are known to possess MDR pumps, often polarized on a given membrane (2, 16). Thus, the use of chemosensitizers with agents such as albendazole may be an effective mechanism of treating unresponsive microsporidiosis in such cells. Without knowing the relationships of MDR pumps in the membranes of multicompartments systems such as *Encephalitozoon*-infected cells, it is difficult to predict the effect of inhibiting or changing the expression of these pumps on the efficacy of a particular antiparasitic agent. For example, in the case of *P. falciparum*, Pgh1 in the parasite food vacuole membrane increases chloroquine accumulation and drug susceptibility, while its expression in the parasite plasma membrane reduces susceptibility and increases resistance to aminoalcohols (4).

The observation that at least 20% more cells infected with microsporidia were found to fall into low calcein fluorescence categories than uninfected cells and that the fluorescence of all cells, infected and uninfected, was increased by verapamil suggests either that infection modified the host cell Pgp expression or that those cells that overexpressed Pgp accommodated the parasite infection more readily. There is evidence for both of these alternatives with intracellular parasite infections. *Toxoplasma gondii* infection of cells expressing a Pgp pump inhibits extrusion of the probe, rhodamine-123 (34), apparently due to decreased expression of host cell membrane Pgp. Also inhibition of the Pgp pump of this parasite with a cyclosporin A analogue that only inhibits the Pgp but does not bind cyclophilins reduced parasite growth both in vivo and in vitro (30). The latter observation suggests that Pgp-like pumps may serve parasite metabolic needs and support parasite growth and differentiation.

The present study indicates that host cells expressing a Pgp pump protect intracellular stages of *Encephalitozoon* microsporidia from agents such as albendazole, probably by limiting the intracellular concentration of the drug. In addition, the parasite developing stages also have a Pgp pump which may further contribute to protecting the parasite from albendazole. A chemosensitizer inhibiting these pumps would therefore be expected to increase the effectiveness of this antiparasitic agent.

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